Review

Wnt signaling in adult intestinal stem cells and cancer

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Signaling initiated by secreted glycoproteins of the Wnt family regulates many aspects of embryonic development and is involved in homeostasis of adult tissues. In the gastrointestinal (GI) tract the Wnt pathway maintains the self-renewal capacity of epithelial stem cells. The stem cell attributes are conferred by mutual interactions of the stem cell with its local microenvironment, the stem cell niche. The niche ensures that the threshold of Wnt signaling in the stem cell is kept in physiological range. In addition, the Wnt pathway involves various feedback loops that balance the opposing processes of cell proliferation and differentiation. Today, we have compelling evidence that mutations causing aberrant activation of the Wnt pathway promote expansion of undifferentiated progenitors and lead to cancer. The review summarizes recent advances in characterization of adult epithelial stem cells in the gut. We mainly focus on discoveries related to molecular mechanisms regulating the output of the Wnt pathway. Moreover, we present novel experimental approaches utilized to investigate the epithelial cell signaling circuitry in vivo and in vitro. Pivotal aspects of tissue homeostasis are often deduced from studies of tumor cells; therefore, we also discuss some latest results gleaned from the deep genome sequencing studies of human carcinomas of the colon and rectum.

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1. Introduction

Extracellular Wnt proteins act in metazoans as morphogens to regulate diverse processes throughout embryonic development, such as cell proliferation, differentiation, cell migration and cell polarity. In adulthood, Wnt signaling is essential for the maintenance of somatic stem cell and committed progenitor cell compartments. The pathway is also involved in tissue regenerative processes following injury [1,2]. Overall, there are at least three distinct “branches” of Wnt signaling (reviewed in [3]). The best studied so called “canonical” pathway depends on β-catenin as its key effector (Fig. 1). Besides its engagement in cadherin-based adherens junctions [4], β-catenin associates with DNA-binding
proteins of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family (further referred to as TCFs) to modulate expression of context-specific target genes. In the absence of the Wnt stimulus, cytosolic β-catenin is marked for degradation by a protein complex that includes serine/threonine kinases casein kinase 1 alpha (CK1ε) and glycogen synthase kinase 3 (GSK3) [5]. Scaffolding of the kinases and β-catenin is mediated by axis inhibition protein (Axin) [6] and adenomatous polyposis coli (APC) tumor suppressors [7]. Ultimately, N-terminally phosphorylated β-catenin is ubiquitinated by F-box-containing beta-transducin repeat containing (β-TrCP) E3 ubiquitin protein ligase and subsequently destroyed by the proteasome [8,9]. In unstimulated cells the TCF proteins are associated with transcriptional repressors of the groucho/transducin-like enhancer of split (TLE) family and block expression of Wnt-responsive genes [10]. Wnt molecules bind to a receptor complex composed of a seven-transmembrane receptor of the Frizzled family and co-receptor low density lipoprotein receptor-related protein (LRP) [11]. The ligand–receptor engagement triggers a cascade of events that include phosphorylation of the adaptor protein Disheveled (Dvl) by CK1ε [12]. In addition, the intracellular portion of LRP is phosphorylated by CK1γ and GSK3, and the LRP–Axin complex is subsequently formed [13]. Simultaneously, the phosphorylated amino acid residues in Axin (these modifications are catalyzed by GSK3) are removed by protein phosphatase 1 (PP1) [14] or PP2A [15]. The dephosphorylated protein constitutes “closed” conformation that is unable to interact with β-catenin and, consequently, β-catenin phosphorylation is inhibited. In an alternative model proposed by Li and colleagues, the β-catenin destruction complex remains intact in the Wnt signal receiving cell; however, active signaling suppresses β-catenin ubiquitination, which leads to the saturation of the complex with phosphorylated β-catenin [16]. In any case, β-catenin accumulates in the cell cytoplasm and nucleus, where it displaces the groucho/TLE proteins from TCFs. Beta-catenin contains a transactivation domain, and TCF–β-catenin complexes thus act as bipartite transcriptional activators of specific target genes such as c-myc [17], cyclin D1 [18,19], CD44 [20] and Axin2 [21]—for a comprehensive list of Wnt target genes, please refer to the Wnt homepage www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes.

The β-catenin-independent, ‘noncanonical’ Wnt signaling cascades utilize distinct signaling mechanisms to relay the signal from the Wnt receptor complex. The planar cell polarity (PCP) pathway activates small GTPases ras-related C3 botulinum toxin substrate 1 (Rac1) and ras homolog gene family member A (RhoA). The pathway also includes protein kinases rho-associated, coiled-coil-containing protein kinase (ROCK) and c-Jun N-terminal kinase (JNK) that in turn induce cytoskeletal remodeling or elicit a transcriptional response, respectively [22]. PCP signaling is implicated in the establishment of cell polarity and cell migration [23,24]. The second relatively well-characterized noncanonical pathway, Wnt/Ca2+ signaling, stimulates phospholipase C (PLC) through the action of heterotrimeric G proteins. The resulting mobilization of intracellular Ca2+ activates Ca2+-dependent effectors that include calcium calmodulin mediated kinase II (CAMKII), protein kinase C (PKC) and calcineurin (reviewed in [25]). The Wnt/Ca2+ signaling branch is implicated in inflammation and promotion of cancer [26]. Wnt ligands can also engage tyrosine kinase-like orphan receptor 2 (ROR2) [27,28] and receptor-like tyrosine kinase (RYK) [29] as receptors; however, there is only little insight into these alternative Wnt signaling pathways. Importantly, despite the general consensus on the key role of β-catenin-independent Wnt signaling in development and cancer, the precise molecular mechanisms of the noncanonical pathways remain mostly unknown. This is mainly due to the fact that in contrast to Wnt/β-catenin signaling, the field of the noncanonical pathways suffers from the lack of suitable reagents and robust functional assays.
The mammalian genome encodes 19 Wnt ligands and 10 Frizzled receptors. It is presumed that the combinatorial complexity among individual ligands and their cognate receptors (and co-receptors) dictates the particular type of the downstream pathway. Some Wnts (including Wnt1 and Wnt3a) preferentially trigger canonical signaling, other such as Wnt5a and Wnt11 initiate the β-catenin-independent pathways (reviewed in [30]. Notably, the PCP and Wnt/ROR pathways can antagonize the canonical Wnt cascade; this indicates possible crosstalk among various types of Wnt signaling [31,32]. The output of Wnt signaling is regulated at virtually all cellular levels. Since detailed description of the Wnt signal relay and modulation goes beyond the scope of this review, we refer to some excellent recent review articles for additional information [25,33–37]. Here, we will briefly summarize some extracellular and membrane activators or inhibitors of Wnt signaling, as these molecules regulate the interaction between the signal-receiving cell and its tissue microenvironment. Secreted polypeptides of the Dickkopf (Dkk) family [38–40], sclerostin (Sost) [41,42] and Wnt modulator in surface ectoderm (WISE); also known as sclerostin domain containing 1 (Sostdc1)] [43] interfere with canonical signaling by binding to the LRP co-receptor. In contrast, two distinct classes of secreted Wnt pathway agonists, Norrin [44] and R-spondins (Rspos) [45,46], augment the Wnt activity. Independently of Wnts, Norrin potentiates β-catenin signaling by direct binding to Dkk3, thereby promoting its oligomerization [47]. Rspos form a complex with their receptor leucine-rich G protein coupled receptor 4/5/6 (LRP4/5/6) [48–50]. Rspos also associates with zinc and ring finger 3 (Znfr3) and ring finger 43 (Rnf43) transmembrane ubiquitin ligases that were identified as negative regulators of Wnt signaling. These related ligases decrease the stability of Frizzled through ubiquitin-mediated degradation. Rspos binding promotes removal of Znfr3 from the plasma membrane, and consequently the levels of Frizzled (and also LRP) are increased, which leads to the enhanced Wnt response [51,52]. In an analogous way, the LRP6 co-receptor is endocytosed upon its binding to Dkk1 and transmembrane protein Kremen; this, however, leads to downregulation of Wnt signaling [53].

2. Cell renewal in the GI tract

The architecture of the small intestine and colon is designed to maximize the surface of the organ to resorb nutrients and water from food. In both anatomical segments of the GI tract, the single-cell epithelial sheet penetrates into the underlying connective tissue of lamina propria to form tubular glands called crypts. In addition, luminal protrusions of the mucosa termed villi are present in the small intestine to further enhance the surface area.

The epithelial lining represents one of the most intensively self-replenishing organs in mammals. With a rate of entire renewal every 3–5 days along the crypt-villus axis [54], this dynamic and organized cell turnover represents an attractive paradigm for tissue maintenance studies (reviewed in [55]). The homeostasis is sustained by crypt resident multipotent intestinal stem cells (ISCs). They give rise to a pool of highly proliferative progenitors called transit-amplifying (TA) cells. These cells undergo several rounds of cell divisions and commence differentiation towards all intestinal lineages as they migrate upwards the crypt length to the crypt orifice (Fig. 2). The villi receive fully mature cells that fulfill the digestion- and resorption-associated functions of the tissue. While absorptive enterocytes participate in transport processes, goblet cells secrete mucus to lubricate the mucosal surface. Moreover, rare peptide hormone-releasing enteroendocrine cells are found scattered among the cells of major epithelial cell lineages. The ISCs of the crypt also generate additional minor mucosal populations, such as M cells that transport antigens from the intestinal lumen to underlying Peyer’s patches [56] and tuft cells, rare cells producing opioids and enzymes involved in prostaglandin synthesis [57]. The continuous proliferation of crypt cells is counterbalanced by cell shedding at the tip of the villus. Paneth cells of the small intestine represent an exception to this scheme. These bacteriostatic compound-producing cells do not follow the migration pattern but descend towards the crypt base, where they persist for 6–8 weeks [58]. Analogous to the Paneth cells, a subset of enteroendocrine cells have been observed to drift towards the bottom part of the crypt. Remarkably, these cells are hallmarked by co-expression of both stem cell and mature endocrine markers [59]. The situation in the colon reminds the small intestine; however, its surface does not contain the protruding villi, and the Paneth cells are not present in the colonic crypts.

2.1. ISCs

To date, two populations of putative ISCs have been identified in the small intestine epithelium; the populations occupy distinct but neighboring locations in the crypt. Crypt base columnar (CBC) ISCs are slender, actively dividing cells that are interspersed among the Paneth cells at the most bottom part of the crypt (Fig. 2). CBC cells can be visualized using their unique marker Lgr5. Barker and colleagues produced a knock-in allele into the Lgr5 locus by inserting the EGFP-IRESCreERT2 expression cassette downstream of the translation-initiation codon of the gene [60]. The mouse (designated Lgr5-EGFP-IRESCreERT2) allowed direct visualization of Lgr5-positive cells in tissues by means of EGFP detection. Moreover, upon crossing to Rosa26lacZ reporter (Rosa26R) mice and activation of Cre enzyme by tamoxifen the allele enabled long-term lineage tracing of the progeny of CBC cells. The “stemness” of CBC cells, i.e. longevity and potency to produce all intestinal cell lineages, was also confirmed by regeneration of the damaged colonic epithelium from single Lgr5+ cells [61]. Moreover, expression profiling of Lgr5-EGFP-IRESCreERT2 cells (fractionated according to the EGFP production) unraveled the gene signature of Lgr5+ CBC cells
by pulse-chase labeling using transgenic mice expressing histone 2B mark a unique cell population within the crypt [62,74]. Moreover, no broadly expressed throughout the crypt compartment and do not the identity of musashi RNA-binding protein 1 that some proposed CBC cell markers such as that express the Notch ligand delta-like 1 (Dll1) [80] or dedifferentiated damage. These include short-lived progenitors of all secretory lineages to revert to stem-like cells to replenish the crypt upon extensive tissue Paneth cells [75]. Very recently, Buczacki and colleagues re-examined cells and these genes display expression along the crypt axis [74].

A pool of slowly cycling stem cells has been proposed to co-inhabit the intestinal crypt. Based on predominant location at the “+ 4” position from the crypt base, several markers were identified in this stem cell population. These include polycomb protein B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) [67], telomerase reverse transcriptase (Tert) [68], homeobox-only protein (Hopx) [69], and leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) [70,71]. Several studies have described mobilization of otherwise quiescent Bmi1+ stem cells upon radiation-induced injury [72] or diphtheria toxin-mediated elimination of Lgr5+ cells engineered to produce the diphtheria toxin receptor [73]. Recent effort significantly contributed to elucidation of the identity of “reserve” stem cells. Multicolor mRNA fluorescence in situ hybridization (FISH) assays and expression profiling revealed that prospective markers of quiescent stem cells, Bmi1, Hopx and Tert are broadly expressed throughout the crypt compartment and do not mark a unique cell population within the crypt [62,74]. Moreover, no other intestinal cryptal cells but Paneth cells retained labels as revealed by pulse-chase labeling using transgenic mice expressing histone 2B-green fluorescent protein (H2B-GFP) [75] or by multi-isotope imaging mass spectrometry (MIMS) [76]. Of note, single-molecule FISH showed that some proposed CBC cell markers such as CD133/prominin 1 [77,78] and musashi RNA-binding protein 1 (Msi1) [79] are not restricted to CBC cells and these genes display expression along the crypt axis [74].

Interestingly, two progenitor populations have been demonstrated to revert to stem-like cells to replenish the crypt upon extensive tissue damage. These include short-lived progenitors of all secretory lineages that express the Notch ligand delta-like 1 [Dll1] [80] or dedifferentiated Paneth cells [75]. Very recently, Buczacki and colleagues re-examined the nature of quiescent stem cells in the crypt [81]. The authors devised a new strategy for labeling all crypt cells except the Paneth cells by transient expression of H2B-yellow fluorescent protein (H2B-YFP). Several days after the labeling all label-retaining cells (LRCs) were detected in the lower part of the crypt. These LRCs co-expressed the CBC stem cell marker Lgr5 as well as markers of the Paneth and enteroendocrine cell lineages [e.g. matrix metalloproteinase 7 (Mmp7) and chromogranin A (Chga), respectively]. Moreover, after a longer period following initial labeling, LRCs without any division converted to mature Paneth and enteroendocrine cells. Intriguingly, isolated LRCs possess multi-lineage growth potential and form so-called organoids in culture (see further).

To test whether LRCs display stem cell-like properties in vivo, Buczacki and colleagues developed a novel dimerizable Cre recombinase methodology to mark LRCs. In healthy mice, the marked cells did not divide; however, upon tissue injury LRCs proliferated extensively and produced clones comprising all epithelial cell types. These findings possibly unify theories about the identity of intestinal stem cells. Actively cycling Lgr5+ CBC cells generate all epithelial cell lineages. The cells also produce a population of non-dividing LRCs that differentiate into Paneth and enteroendocrine cells. These precursor cells express markers for the proposed “+ 4” stem cells and can function as quiescent stem cells activated when the tissue is damaged. In conclusion, crypt cells display substantial plasticity and the stemness might be extrinsically imposed on cells occupying the respective location within the crypt.

2.2. Wnt signaling represents the principal force behind intestinal epithelial homeostasis

Numerous studies conducted both in vivo and in vitro have firmly established the role of Wnt signaling in the preservation of stem cell proliferation and pluripotency (reviewed in [55]). Genetic disruption of the pathway’s ultimate effectors Tcf4 (official symbol Tcf7l2) [82,83] or β-catenin (Ctnmb1) [84,85] are associated with demise of the intestinal crypts. Similar collapse of the intestinal architecture occurs when the Wnt cascade is counteracted through ectopic expression of the secreted Wnt antagonist Dkk1 [86,87]. Conversely, aberrant activation of the Wnt pathway increases Lgr5+ stem cell numbers as observed upon injection of Wnt agonist Rspo1 into mice [46]. The robust proliferation in the crypt is facilitated by direct repression of cell cycle inhibitor p21Cip1/Waf1 by the Tcf4 target gene c-myc [88]. Expectedly, the crypt progenitors are depleted by G1 arrest and concomitant differentiation upon c-myc ablation [89]. Strikingly, in the colon the Tcf4 factor acts in an opposing manner to restrict the expansion of the colonic epithelium [90].

The identification of ISCs was directly related to the analysis of TCF-β-catenin-responsive genes in human tumor cells. The results of this analysis were subsequently confirmed in mouse intestinal tissue [88]. The expression of stem cell markers Ascl2, Ephb2, Bmi1, Lgr5 and Tert is governed by the canonical Wnt pathway [88,91–95]. Moreover, the strength of the Wnt cascade is restrained in CBC cells by the pathway negative feedback-loop mechanisms that involve upregulation of the β-catenin negative regulator Axin2 [21] and expression of Frizzled-specific ubiquitin ligases Rnf43 and Znf3 [51,52]. The simultaneous conditional ablation of both Rnf43 and Znf3 in the mouse intestine induces expansion of the proliferative zone of the crypts that eventually leads to formation of intestinal adenomas [52]. In contrast, the genetic inactivation of Lgr5 in the intestine results in no obvious phenotype. However, deletion of the homologous Lgr4 gene has damaging effects on crypt stem cells and Lgr4/5 double-deficiency leads to demise of intestinal crypts [49]. Since Lgr4 functions equivalently as the Rspo receptor, the observed phenotypes might be explained by functional redundancy of Lgr4 and Lgr5 [49,50,96]. Finally, another Wnt signaling target gene Troy produces a transmembrane protein that suppresses Wnt/Rspo signaling via its association with Lgr5 [64].

The noncanonical Wnt pathway activated by Wnt5a is implicated in regenerative processes of the GI tract. Although Wnt5a is crucial for embryonic gut development, it is dispensable in homeostasis of postnatal intestinal tissue [97]. However, in case of mucosal woundening Wnt5a augments transforming growth factor β (TGF-β) signaling to restrict epithelial proliferation and promote restoration of the proper tissue architecture [98]. Surprisingly, these outcomes are achieved through the induction of cell cycle inhibitors such as p15INK4B [98] and do not comprehend inhibition of canonical Wnt signaling as previously assumed [99,100].

2.3. The ISC niche

The stem cell permissive environment is constituted by neighboring Paneth cells [101] and subepithelial myofibroblasts that tightly line the crypt base basal lamina [102]. This close association facilitates direct supply of ICSs with essential pro-proliferative factors that include Paneth-derived Dlls, epidermal growth factor (EGF), Wnt3 and mesenchyme-secreted Wnt2b [101,103]. A subpopulation of c-Kit+ steel factor receptor (also designated CD117)-positive goblet cells of the colonic crypt substitutes for the Paneth cells in the caudal regions of the gut. These secretory cells produce Dll1/4 and EGF cues much like their Paneth equivalents [104]; however, they are devoid of the Wnt production [103]. Paneth cells and myofibroblast-derived niches likely
act redundantly since neither complete ablation of the Paneth cells nor the epithelium-restricted conditional inactivation of Wnt3 coincide with depletion of ISCs [103].

The Wnt signaling cascade is tightly controlled to prevent (over)proliferation of ISCs (reviewed in [105]). The expression of the Wnt cues displays a diminishing slope along the crypt-villus axis [106]. In addition, the Wnt proteins are locally attenuated at the +4 position by production of the Wnt antagonists secreted Frizzled-related proteins (sFRPs) [106]. Furthermore, the Wnt pathway output is modulated by co-operative activity of the Hedgehog and bone morphogenic protein (BMP) cascades [107]. In more detail, as the progenitor cells further decline from the crypt base, the Hedgehog–induced, mesenchyme-to-epithelium BMP signaling promotes differentiation while restraining proliferation [108]. Importantly, in the colon ISCs are locally protected from the pro-differentiation BMP signals by secreted stroma-derived proliferation [108]. In concordance with this concept, inappropriate activation of Hedgehog signaling results in BMP-mediated depletion of the progenitor compartment [107]. Conversely, shortage of the Hedgehog [109,110] and/or BMP signals [108,111] is associated with impaired Wnt control and results in cancer development. Additionally, the mitogenic activity of Paneth cell-derived EGF is counterbalanced by expression of pan-EGFR inhibitor Lrig1 [70,71]. Accordingly, the genetic inactivation of Lrig1 results in unrestricted precursor expansion predisposing to onset of intestinal cancer [70,71].

The binary cell fate decisions between secretory versus absorptive cell lineages are governed by the synergism between the Wnt and Notch pathways (reviewed in [63]). Wnt signaling controls high crypt-specific expression of EphB2 and EphB3 in CBCs or Paneth cells, respectively, and establishes a gradient of repressive EphB2/3-eprhin B1 interaction that drives proper cell positioning along the crypt–villus axis [112]. Moreover, the Wnt signals are required for commitment, maturation and location of the Paneth cells [103,113–115]. Inactivation of Wnt signaling target gene SRY-box containing gene 9 (Sox9) results in the loss of Paneth cells [116,117]. The Notch1 and Notch2 receptors—produced on CBC cells—engage with their DIl1 and DIl4 ligands presented by the Paneth cells [118,119]. The proliferation of the Notch signal–receiving cell is then promoted by Notch–responsive gene hairy and enhancer of split 1 (Hes1) that transcriptionally represses cyclin-dependent kinase inhibitors p27Kip1 and p57Kip2 [120]. Consistently, the DIl1/D4 compound mutant mice display premature differentiation of intestinal stem cells [119]. However, as mentioned previously, since stem cells retain their clonogenic capacity in the absence of the Paneth cells, the lateral Notch signals might be dispensable. Collectively, the homeostasis of the intestinal epithelia is governed by an interconnected circuitry of developmental signaling pathways that balance the opposing processes of cell proliferation and differentiation.

### 3. Experimental approaches to studying the gut epithelium in vitro

Until recently, the study of the bowel physiology has been restricted to genetically manipulated model organisms or gut explants transplanted into immunocompromised or syngeneic hosts [121]. In living animals the organ is hardly accessible for direct experimental manipulation or consecutive examinations. Many attempts were made to produce cultures mimicking growth and differentiation of the intestinal tissue, but the experiments were for a long time unsuccessful. The main obstacle was the reduced viability of epithelial cells, which without the support from the basement membrane and stroma underwent apoptosis [122,123]. Lately developed three-dimensional (3D) culture systems preserved the tissues architecture and cellular differentiation; however, these systems were restricted to embryonic gut tissue and explanted cells displayed limited viability [124,125].

Recently, Ootani and colleagues established a long-term 3D culture system to grow explants of the mouse small or large intestine. The tissue is propagated as expanding intestinal spheres in collagen using an air–liquid interface setting [126]. Moreover, Sato and co-workers developed a straightforward methodology for continuous cultures of primary intestinal epithelium. This approach allows generation of sphere-like “organoids” from resected mouse crypts or most interestingly, from single Lgr5+ [127] or EphB2BMP3 [128] small intestinal or colonic cells, respectively. These “mini-guts” fully resemble the intestinal architecture with crypt- and villus-like cell compartments that enclose to form a central lumen lined by differentiated cells (reviewed in [129]) (Fig. 3). Isolated cells are grown in matrigel, which simulates the native embedding of the glands. The Wnt agonist Rspo, BMP-inhibitor Noggin and EGF constitute the essential culture supplements to reproduce the stem cell niche stimuli. In the crypt-like entities “budding” from the organoid surface, the Wnt cues are provided by the stem cell-neighborhing Paneth cells since conditional excision of Wnt3 halts organoid proliferation [103]. Consistently, growth in Wnt3+/− small intestinal organoids is restored upon Wnt3a administration or in co-culture with feeder cells derived from the gut mesenchyme. In contrast, the colon-derived organoids strictly rely on exogenous Wnt3a. The disparity in the Wnt dependency of small intestinal versus colonic cultures underlines the already mentioned fact that in the colonic crypts the Wnt signal is mainly provided by myofibroblasts [103,104]. Interestingly, human biopsy-derived colonic organoids require additional supplements for their survival. Addition of prostaglandin E2 blocks anoikis and potentiates growth factor signaling [128]. Moreover, nicotinamide and small molecule inhibitors of activin A receptor, type Il-like kinase 4 (Alk4) and p38 kinase significantly contribute to the long-term maintenance of proliferating organoids [130].

The ex vivo intestinal culture systems represent an unprecedented tool for therapeutic applications. Indeed, organoids can successfully engraft and regenerate chemically damaged mucosa when introduced into the recipient colon [61]. Interestingly, in the presence of exogenous Wnt3a, mouse small intestine organoids change their crypt–villus architecture and form rounded cysts lacking differentiated cell types. Similar “spheroids” can be derived from mouse or human tumors displaying hyperactive Wnt signaling [101,127]. Very recently, Dekkers and colleagues generated intestinal organoids from the biopsies of patients suffering from cystic fibrosis, a disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Strikingly, forskolin, the adenylate cyclase activator, induced rapid swelling of organoids derived from control healthy individuals. However, this effect—corresponding quantitatively to forskolin-induced anion currents—was strongly reduced in CFTR-deficient organoids. In addition, the behavior was phenocopied when wild-type organoids were cultured with CFTR-specific inhibitors. Even more interestingly, in the mouse model of cystic fibrosis the in vitro function (i.e. forskolin-induced swelling) of the CFTR mutant protein was recovered by CFTR function–restoring drugs or by lowering temperature [131]. Taken together, the intestinal organoids can be utilized not only to investigate the crucial aspects of gastrointestinal biology, but their usage could...
facilitate functional studies, diagnosis and personalized treatment of various disorders including cancer.

4. Tumor formation in the intestine

Deregulation of Wnt signaling is associated with onset of cancer, most notably carcinoma of the colon and rectum (reviewed in [132]). It is presumed that in colorectal tumors the first oncogenic mutation provides selective advantage to the epithelial cell that multiplies and generates a tiny clump of cells called microadenoma. In the majority of sporadic colorectal tumors these “initiatory” mutations frequently occur in the APC gene. Subsequent mutations in other genes, such as TP53 (encodes p53 protein), phosphatase and tensin homolog (PTEN), mothers against decapentaplegic homolog 4 (SMAD4), Kirsten rat sarcoma viral oncogene homolog (KRAS), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PI3KCA) are followed by clonal expansion of transformed cells [133,134]. The process generates a malignant tumor that can invade through the basement membrane and eventually spread to distant organs [135].

Germinal mutations of the APC gene underlie a hereditary neoplastic syndrome, the Familial Adenomatous Polyposis (FAP) [136,137]. The FAP-affected individuals carry one defective copy of the APC gene and develop hundreds of benign colonic APC-deficient lesions termed polyps. The polyps progress towards malignancy through ordered histopathological stages that include high-grade dysplasia, adenoma and invasive adenocarcinoma [138]. As in the case of sporadic cancer this stepwise evolvement is driven by successive accumulation of additional mutations [139]. Hyperactive Wnt signaling might also result from mutational inactivation of the AXIN1 [140] or AXIN2 [141] genes. Finally, approximately 5% of colorectal carcinomas (CRCs) contain mutations that compromise the N-terminal regulatory amino acids of β-CATENIN [142]. In either case, stabilized β-CATENIN mediates inappropriate transcriptional activation of the TCF-β-CATENIN target genes, thus driving pathological transformation of the gut epithelium [143,144]. The majority of changes in the APC gene are frame-shift or nonsense mutations (reviewed in [145]) that result in production of truncated polypeptides retaining some residual capacity to reduce β-catenin-dependent transcription. The capacity differs according to the extent of the APC truncation with longer proteins being more effective than the shorter APC variants [146–148]. Interestingly, in FAP-affected individuals various clinical manifestations of the disease correlate with the extent of the APC truncation (reviewed in [149]). In agreement with these observations two recent studies indicated that individual anatomical segments of the GI tract are differentially sensitive to aberrant Wnt signaling. Lesions displaying excessive Wnt signaling are mainly located in the distal colon, whereas moderate perturbations of the Wnt pathway favor tumor development in the upper GI and right colon [150,151].

Both hereditary and sporadic forms of bowel cancer have been recapitulated in experimental mouse models (reviewed in [152]). Multiple intestinal neoplasia (Min) mice harbor a nonsense mutation in the coding region of the APC gene [153,154]. Similarly, to the FAP individuals these mice develop numerous polyps; however, the tumor burden is mainly located in the small intestine. Notably, the incidence and distribution of gut tumors greatly vary in individual APC-deficient mouse strains [155] (reviewed in [156]). As in the FAP patients the severity of polyposis correlates with an “optimal” level of aberrant Wnt signaling. Mice expressing mutated Apc protein that partially retains its ability to inhibit Wnt signaling show earlier-onset and larger and more numerous and dysplastic lesions [157]. These data support the previous notion that a specific dosage of Wnt/β-catenin signaling, rather than constitutive activation of the pathway, is essential for tumor formation [150,158].

Inactivation of Apc throughout the mouse intestinal epithelium using conditional (cKO) alleles of the gene instantly promotes cellular proliferation while impairing differentiation. Affected cells thereby acquire an aberrant “crypt-like” phenotype resulting in massive expansion of the stem cell compartment [159–161]. The growth of Apc-deficient tumors depends on the activity of the Wnt pathway target gene c-myc since deletion of the c-myc gene rescues the deleterious changes observed upon Apc loss [162,163]. The cancer-initiating event, i.e. the (hyper)activation of the Wnt pathway, has to occur within the stem cell pool to be successfully propagated [164]. This was demonstrated by targeted Apc deletion in Lgr5+ CBC cells [161] or by ectopic expression of stabilized β-catenin in Bmi1+ [67] or CD133+ [78] cells. In contrast, the loss of Apc in short-lived progenitors gives rise to microadenoma that fails to transform to malignancy [161]. This “bottom-up” concept was challenged by recent findings indicating that Lgr5-negative enterocytes on the villus can de-differentiate and re-express stem cell markers including Ascl2, Lgr5, Rnf43, and Troy. The de-differentiation can be achieved by simultaneous perturbation of the Wnt and NF-κB pathways. The synergy between NF-κB and Wnt signaling is attributed to the interaction of the NF-κB component v-rel reticuloendotheliosis viral oncogene homolog A (RelA)/p65 with β-catenin. RelA-mediated recruitment of the activatory CREB-binding protein (CBP) to TCF-β-catenin complexes enhances the Wnt signaling output [165]. These results supported the “top down” concept of adenoma formation. Although the view that tumor-initiating cells originate in the stem cell compartment is still prevailing, the two models of intestinal carcinogenesis are possibly not mutually exclusive. Interestingly, Myant and colleagues published a report on the critical role of NF-κB signaling and reactive oxygen species (ROS) in tumorigenesis upon Apc loss. Apc-deficient cells display activated Ral1 that drives production of ROS and stimulates NF-κB signaling. This leads to the stem and progenitor cell compartment hyperpolarization and promotes cellular transformation [166]. These findings provided the mechanistic explanation why inflammatory signaling might increase the risk of colorectal cancer.

Established intestinal tumors are composed of heterogeneous cell populations, but they preserve a cellular hierarchy that is reminiscent of the normal crypt architecture. Consistently, a minor subset of cells display features shared with somatic stem cells such as self-renewal and multipotency. These cells termed cancer stem cells (CSCs) sustain progressive growth of the lesion and retain the ability to initiate tumor when engrafted to the recipient animal [167–170]. Elevated Wnt signaling is a hallmark of intestinal CSCs, and thereby they are highly enriched in mRNA encoding CBC-specific genes including Ascl2, EphB2 and Lgr5 [66,128,169,171]. Recently, the doublecortin-like kinase 1 (Dclk1) has been identified to exquisitely earmark the transformed stem cell population. Accordingly, specific ablation of Dclk1+ cells prevented growth of polyps spontaneously developed in Apc+/−/Min mice [172].

The tumor cell interacts with its microenvironment and the environmental cues influence and promote various steps of tumor development. Tumor-associated stroma includes a wide variety of cell types including bone-marrow derived immune cells, cancer-associated fibroblasts and myofibroblasts, mesenchymal stem and endothelial cells [173,174] (reviewed in [175]). Moreover, in mouse adenomas located in the proximal part of the gut the CSC niche is generated by metaplastic Paneth cells [52]. A similar arrangement was found in colon adenomas in which prospective CSCs are possibly supported by Paneth cell-like deep crypt secretory cells [171]. In humans CRC tumor-resident myofibroblasts produce hepatocyte growth factor (HGF), which locally enhances Wnt signaling activity in neighboring cells [169]. This observation plausibly explains the well-known “β-catenin paradox” postulating that only a fraction of cells within the APC-deficient tumor harbor nuclear β-catenin protein [176,177]. Similarly, in a large proportion of adenocarcinomas EphB2+high CSCs reside closer to the stroma, whereas transformed cells with low levels of EphB2 are positioned in the luminal part of the tumor [66]. Of note, several studies documented that β-catenin nuclear translocation requires—besides APC deficiency—input from diverse kinases including AKT [178], RAF1 and JNK2 [179,180], protein kinase A (PKA) [181] and P21-activated kinase 1 (PAK1) [182].
Importantly, in human CRC differentiated tumor cells can revert to CSC phenotype by effect of factors secreted from stromal myofibroblasts [169]. In summary, the CSC studies support the idea that cancer cells display much more plasticity than previously anticipated. The stemness can be elicited not only by particular changes in the genome of transformed cells (as documented in the mouse model of concurrent Wnt and NF-κB pathway activation), but also by extrinsic factors originating from the tumor microenvironment.

The outcome of Wnt signaling is fine-tuned by intriguing interplay between the Wnt and Hippo pathways (reviewed in [183]). Hippo signaling is an evolutionarily conserved mechanism involved in the control of organ size during development and regeneration (reviewed in [184,185]). The growth-limiting and differentiation-promoting signal is mediated by serine/threonine kinases STE20-like protein kinase 1 (MST1; alternative name STK4) and related MST2 (STK3), which are homologous to Dro sophila Hippo. The kinases form an essential part of a protein complex that phosphorylates and inhibits Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). YAP/TAZ associate with different transcription factors including the TEA domain/Transcription Enhancer Factor (TEAD) family and activate genes regulating tissue growth and cellular viability. Wnt signaling augments the levels of TAZ, which is otherwise degraded by the β-catenin destruction complex. In detail, β-catenin interacts with TAZ and promotes its β-TrCP-mediated ubiquitination and degradation. Consequently, a significant fraction of genes regulated by the Wnt pathway depend on TAZ/TEAD. Furthermore, TAZ activates a considerable portion of genes in APC-deficient CRC cells [186]. The other Hippo executor YAP1 interacts with β-catenin and together with the transcription factor T-box protein 5 (TBX5) they form a tripartite complex. The complex drives transcription of antiapoptotic genes such as BCL2-like 1 (BCL2L1) and Bcl2 related antiapoptosis protein containing 5 (BIRC5) in cancer cells. YES1, Src-like kinase, is essential for the formation of the YAP1–β-catenin-TBX5 complex on the target promoters. Hence, YES1 inhibition by cancer drug dasatinib suppresses growth of tumors dependent on the β-catenin transcription activity [187]. Recently, Jeong and colleagues discovered a novel mechanism controlling cellular abundance of the Ras protein, which similarly to TAZ degradation involves the β-catenin destruction complex. Ras is subjected to direct phosphorylation by GSK3β that primes it for β-TrCP recognition and subsequent destruction in the proteasome. Moreover, a positive correlation between Ras stabilization, aberrant Wnt signaling and tumorigenesis was detected in mouse models of intestinal cancer and in human colorectal adenomas and carcinomas [188]. In conclusion, mutational events compromising the function of the β-catenin destruction complex might underline the oncogenic activity of multiple signaling pathways leading to cellular transformation and tumor formation.

5. High-throughput studies of human colorectal cancer genome

Human cancer is a genetic disease caused by mutations in tumor suppressor genes and oncogenes. Solid tumors evolve over time from benign to malignant lesions by acquiring genetic alterations that drive tumor progression (reviewed in [133]). The tumor-specific mutations give clues as to which pathways and cellular processes underlie tumor initiation and progression. In concordance with these findings, several agents that combat tumors by interfering with aberrant Wnt signaling were developed in recent years. Growth of Wnt-driven cancers can be suppressed by Frizzled-10 [195] or LRP6-specific (“blocking”) [196] antibodies or by treatment with soluble Frizzled acting as a Wnt “decoy” receptor [197]. Importantly, small molecule inhibitor LGK-874, which specifically analyzed numerous human colon and rectal samples using several state-of-the-art molecular biology approaches. Massive parallel “next-generation” sequencing was applied to analyze the exome, transcriptome and to detect gene copy-number alterations in 11 [192], 276 [134] and 72 [193] tumors and normal pairs. Moreover, single-nucleotide polymorphisms (SNP), microsatellite instability and promoter methylation status were evaluated in the majority of the specimens. This integrative molecular characterization yielded an unprecedented insight into the pathobiology of colorectal cancer. Cancer specimens with microsatellite instability (MSI) contained up to several thousands of non-synonymous mutations affecting protein-coding genes on average; the mutation rate of microsatellite-stable (MSS) CRCs was substantially lower—these tumors contained approximately dozens of non-synonymous alterations. The majority of these changes were “passenger” mutations with no effect on tumor initiation or progression. However, each CRC irrespective of the microsatellite stability status contained three to six “driver” mutations that conferred a selective growth advantage to the cell and promoted tumorigenesis (reviewed in [194]). Changes altering the genes involved in the Wnt signaling pathway were found in more than 90% of all tumors, including inactivation in APC or activating mutations in β-CATENIN (CTNNB1) in 80% of cases. In contrast to MSS neoplasia, MSI tumors display lower mutation rate of APC whilst showing enhanced numbers of alterations targeting other Wnt cascade negative regulators. Mutations and/or deletions were found in AXIN2, SORCS9, as well as in the DKK family members and F-box and WD repeat domain containing 7 (FBXW7) gene (FBXW7 encodes a subunit of the β-catenin destruction complex), TCF4 (TCF7L2) was mutated in 31% of MSI and in 12% of MSS cancers. Moreover, the TCF4 locus was deleted in a subset of the examined cases [134]. In approximately 3% of CRCs an intrachromosomal deletion between TCF4 and adjacent vesicle transport through interaction with t-SNAREs 1A (VTI1A) was observed [134,192]. In three separate cases the 3′ coding part of TCF3 (TCF7L1) on chromosome 2 was fused to the chromosome 11 region containing the neuron navigator 2 (NAV2) gene on chromosome 11 [134]. Both types of rearrangements are “in-frame”, thus producing fusion polypeptides that lack the β-catenin binding domain of the TCF partner. Bass and colleagues reported that the VTI1A-TCF4 protein promotes anchorage-independent growth of cancer cells [192]; however, how the fusion molecules contribute to tumorigenesis remains elusive. In addition, Sehgah and colleagues identified additional rearrangements involving the RSPO family members in 10% of colon tumors. The detected alterations generate fusion of either RSPO2 or the eukaryotic translation initiation factor 3, subunit E (EIF3E) gene or RSPO3 and the protein tyrosine phosphatase, receptor type K (PTPRK) gene [193]. The RSPO2–EIF3E fusion produces functional (i.e. translated) transcript of RSPO2 driven by the EIF3E promoter. The RSPO3–PTPRK rearrangements represent in-frame fusions that preserve RSPO3-coding exons except for the signal peptide sequence, which is replaced by the secretion signal of PTPRK. In minor cases RSPO3 inversions were found placing the gene in the proximity of PTPRK. Interestingly, the expression of RSPO2 and RSPO3 in tumors containing the rearrangements was elevated compared to samples without the fusions. Since all RSPO/3 fusion proteins retained biological activity and occurred in a mutually exclusive manner with the APC mutations, they likely represent oncogenic drivers that circumvent intact APC [193]. Intriguingly, many tumors that harbor APC mutations contain additional alterations in gene(s) encoding the Wnt pathway components; moreover, a fraction of MSS and MSI tumors exhibit marked overexpression of the Wnt receptor Frizzled 10 (FZD10). This implies that multiple changes affecting Wnt signaling confer selective advantage to the transformed cell. In concordance with these findings, several agents that combat tumors by interfering with aberrant Wnt signaling were developed in recent years. Growth of Wnt-driven cancers can be suppressed by Frizzled-10 [195] or LRP6-specific (“blocking”) [196] antibodies or by treatment with soluble Frizzled acting as a Wnt “decoy” receptor [197]. Importantly, small molecule inhibitor LGK-874, which specifically
interferes with the catalytic activity of acyltransferase Porcn and abrogates posttranslational processing of Wnt ligands [198], entered the phase I clinical trial http://clinicaltrials.gov/show/NCT01351103 [199]. Therapeutic intervention in tumors containing defects in the Wnt pathway components downstream of the receptor complex might include inhibitors of tankyrase, enzyme that regulates stability of \( \beta \)-catenin destruction complex through AXIN2 by poly(ADP-ribose)ylation [198,200]. Tankyrase inhibitors stabilize Axin and promote \( \beta \)-catenin degradation even in tumor cells containing mutated APC [201,202]; such inhibitors thus represent particularly promising pharmaceutical reagents. Finally, the interaction of \( \beta \)-catenin with its transcriptional partners TCF4 [203,204] or BCL9 [205] can be disrupted via tailored peptides. In summary, high-throughput analyses of tumor specimens have supported the previous assumption that effective drugging of the aberrant Wnt pathway might be essential for cancer treatment (reviewed in [206,207]).

6. Concluding remarks

In this review, we summarized our understanding of the unique properties and activity of intestinal stem cells. The state-of-the-art evidence is changing the concept of co-existence of two distinct stem-cell populations within the crypt. Instead, the crypt cells display considerable plasticity that can reverse the cell hierarchy. In either case, the homeostatic self-renewal of the gut epithelium is driven by the signaling activity of secreted Wnt proteins. Moreover, the Wnt cues are required to reestablish the normal crypt architecture following mucosal injury. Perturbations within the Wnt pathway initiate tumor development and malignant progression of intestinal neoplasia. In recent years, integrative studies including systematic sequencing of large numbers of colorectal tumors generated an unprecedented insight into the molecular and malignant progression of intestinal neoplasia. In recent years, integrative studies including systematic sequencing of large numbers of colorectal tumors generated an unprecedented insight into the molecular basis of colorectal cancer. These studies underpin the role of the Wnt pathway in cancerogenesis. Finally, these studies provided the rationale for design and production of anti-tumor agents combating cancer by interfering with aberrant Wnt signaling.

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References

[1] FJ. Secretro, LH. Hoegner, Jj. Westendorf, Curr. Osteoporos. Rep. 7 (2009) 64–69.
[2] DL. Zhang, Lj. Gu, L. Liu, C. Yang, W. Sun, Z. Li, Ck. Sung, Biochem, Biophys. Res. Commun. 378 (2009) 149–151.
[3] R. Nusse, Cold Spring Harb. Perspect. Biol. (2012) 4.
[4] M. Ozawa, H. Baribault, R. Kemler, EMBO J. (1989) 1711–1717.
[5] E. Siegried, EL. Wilder, N. Perrimon, Nature 367 (1994) 76–80.
[6] S. Ikeda, S. Kishida, H. Yamamoto, H. Murai, S. Koyama, A. Kikuchi, EMBO J. 17 (1998) 6529–6538.
[7] B. Rubinfeld, B. Souza, I. Albert, O. Muller, SJ. Chamberlain, F.R. Masiarz, S. Munemitsu, P. Polakis, Science 262 (1993) 1731–1734.
[8] H. Abele, A. Bauer, J. Stappert, A. Kupret, R. Kemler, EMBO J. 16 (1997) 3797–3804.
[9] M. Kitagawa, S. Hatakeyama, M. Shireine, M.Matsumoto, N. Iishiha, K. Hattori, I. Nakamichi, A. Kikuchi, K. Nakayama, EMBO J. 18 (1999) 2401–2410.
[10] J. Roose, H. Clevers, Biochim. Biophys. Acta 1424 (1999) M23–M37.
[11] L. Schweizer, H. Harniss, BMC Cell Biol. 4 (2003) 4.
[12] O. Bernatou, RS. Ganj, JP. Dijkstraeus, P. Konig, J. Ceverkova, T. Polonio, P. Krejci, G. Schulte, V. Bryja, J. Biol. Chem. 286 (2011) 10366–10410.
[13] J. Bile, Y.L. Huang, G. Davidson, T. Zimmermann, C.M. Cruca, M. Bienen, C. Niehrs, Science 306 (2011) 1619–1622.
[14] W. Luo, A. Peterson, BA. Garcia, G. Goobs, B. Kofahl, R. Heinrich, J. Shahinowitz, D.F. Hunt, H. Yost, D.M. Vimboer, EMBO J. 26 (2007) 1511–1521.
[15] S.E. Kim, H. Huang, M. Zhao, X. Zhang, A. Zhang, M.V. Semonov, B.T. MacDonald, J. Garcia Abreu, L. Peng, X. He, Science 340 (2013) 867–870.
[16] V.S. Li, SS. Ng, P.J. Roersma, T.Y. Low, W.R. Karthaus, JP. Gerlach, S. Mohammed, AJ. Heck, MM. Maurice, T. Mahmoudi, H. Clevers, Cell 149 (2012) 1245–1256.
[17] T.C. He, A.B. Sparks, C. Rago, H. Hermeking, L. Zawel, LT. da Costa, P.J. Morin, B. Vogelstein, K.W. Kizziher, Science 281 (1998) 1509–1512.
[18] M. Shittman, J. Zhurinsky, I. Simcha, C. Albanese, M. D’Amico, R. Pestell, AJ. Heck, MM. Maurice, T. Mahmoudi, H. Clevers, Cell 149 (2012) 1245–1256.
[19] L. Schweizer, H. Varmus, BMC Cell Biol. 4 (2003) 4.
