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REVIEWS

Review

Wnt Signaling in 3D: Recent Advances in the Applications of Intestinal Organoids

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Intestinal organoids grown from adult stem cells have emerged as prototype 3D organotypic models for studying tissue renewal and homeostasis. Owing to their strict dependence on Wnt signaling, intestinal organoids offer an unprecedented opportunity to examine Wnt pathway regulation in normal physiology and cancer. We review how alterations in growth factor dependency and organoid morphology can be exploited to identify Wnt signaling mechanisms, characterize mutated pathway components, and predict responses of patient-derived tumors to targeted therapy. We discuss current deficits in the understanding of genotype–phenotype relationships that are to be considered when interpreting mutation-induced changes in organoid morphology.

3D Organoids: Then and Now

The first successful protocol for long-term *in vitro* propagation of 3D epithelial tissue derived from individual intestinal stem cells (ISCs) was established by Clevers and colleagues in 2009 [1]. These so called 'organoids' or 'mini-guts' consisted of 3D self-organizing structures starting from a single *Lgr5*$^{+}$ mouse ISC, and fully recapitulated the crypt–villus architecture and physiology of the intestinal epithelium (Box 1) [1]. This breakthrough was propelled by a decade of *in vivo* studies that unraveled the specific growth factor requirements for homeostatic maintenance of ISCs and intestinal **tissue renewal** (see Glossary) (Box 2) [2]. In particular, the essential growth factor cocktail required for the *in vitro* propagation and differentiation of ISCs consisted of the Wnt pathway agonist R-spondin (Rspo), epidermal growth factor (EGF), and the BMP inhibitor Noggin [1].

Building on the first protocols for growing intestinal organoids, it has now become possible to establish 3D culture systems for a multitude of tissues: colon [3,4], liver [5,6], pancreas [7,8], stomach [9,10], esophagus [11], fallopian tube [12], prostate [13,14], taste buds [15], and salivary glands and tongue [16]. Although the growth factor requirements of these cultures differ and depend on the tissue of origin, the requirement for Wnt, Rspo, or a combination of both represents a shared feature of the majority of organoid types. Thus, organoid cultures can be exploited to investigate general as well as tissue-specific mechanisms by which Wnt-mediated signaling controls tissue renewal by regulating stem and progenitor cell activities as well as cell fate specification. Moreover, in conjunction with recent advances in CRISPR/Cas9 applications, organoids offer excellent opportunities for investigating mutation-induced disease mechanisms and linking these findings to therapeutic sensitivity/vulnerabilities. Lastly, organoids represent an excellent model for translational and regenerative studies, given their ability to reconstitute epithelial tissues *in vivo* [17]. For instance, wild-type murine and human colonic organoids were shown to engraft long-term into the murine mucosa following orthotopic transplantation [17,18].

It should be noted that organoids can also be established by stepwise differentiation from cultures of induced pluripotent stem cells (iPSCs) [19,20]. In this review, however, we will specifically focus on **adult stem cell**-derived organoids.

Illustrated by key examples, various approaches, and genotype–phenotype relationships, we review how adult stem cell- and patient-derived 3D organoid cultures can be employed to gain insight into the regulatory mechanisms of Wnt signaling in physiologically meaningful contexts. We summarize how alterations in organoid morphology, niche factor dependency, and gene expression profiles are useful to determine the impact of disease-linked mutations on the complex networks of negative and positive feedback in Wnt signaling regulation. In addition, we discuss similarities and differences between mouse and human small intestinal and colon organoid cultures, as well as current limitations that should be considered when applying these organoid systems for disease modeling.

Highlights

Using organoid technology, adult epithelial stem cells can be expanded *in vitro* to generate self-organizing 3D epithelial structures that closely recapitulate the architecture and cellular composition of the tissue of origin.

Because of their strict dependence on Wnt ligands for survival and growth, intestinal organoids have become the tool of choice for *in vitro* studies aimed at revealing mechanistic aspects of Wnt signaling.

Adult stem cell- and patient-derived intestinal organoids can serve as a platform for investigating mutation-induced disease mechanisms. Insights into how specific mutations alter Wnt activity are becoming a key determinant for tailored therapeutic approaches.

Given the rapid expansion of the organoid field and its applications, there is an increasing need for a standardized method to interpret organoid genotype–phenotype relationships.

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Box 1. The Architecture of the Small Intestinal Epithelium

The small intestine is the most rapidly self-renewing tissue in adult mammals, and has a turnover of 3–5 days in mice [93]. Its architecture is simple and consists of a single epithelial layer that regularly alternates in crypt and villus units (Figure I). The intestinal invaginations, or crypts, harbor the 'stem cell zone' where *Lgr5*$^{+}$ stem cells reside and divide to give rise to transit-amplifying progenitor cells. These daughter cells then migrate upwards towards the villi, which are digit-like protrusions that flank the crypts. Villi constitute the 'differentiated domain' of the small intestine as cells differentiate during their migration along the crypt–villus axis. The main differentiated epithelial cell types of the intestine are: enterocytes, goblet cells, tuft cells, enteroendocrine cells, and Paneth cells (Figure I). These belong to two lineages, absorptive and secretory, and have specialized tasks that underlie the dual function of the organ as a permeable mucosa for nutrient absorption and as a protective barrier against external agents [94]. At the end of their lifespan, intestinal epithelial cells undergo apoptosis when they reach the tip of the villus and are shed into the intestinal lumen [95].

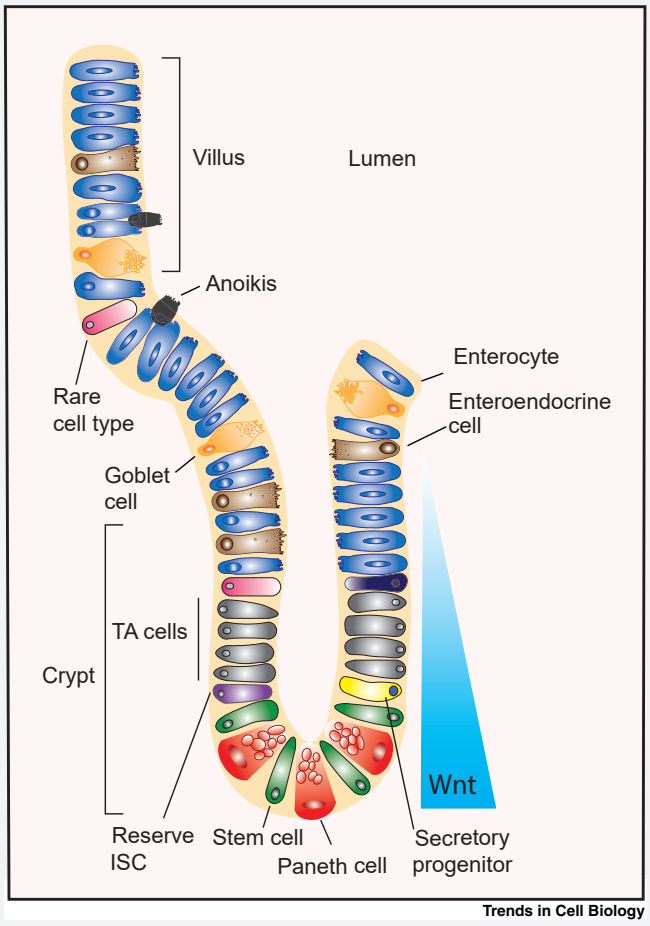

Figure I. Organization of the Small Intestinal Epithelium.

Rapidly dividing Lgr5-positive crypt base columnar intestinal stem cells (ISCs, green) reside at the bottom of the crypt intercalated with Paneth cells (red). The ISCs give rise to transit-amplifying (TA) progeny cells (grey) and secretory progenitor cells (yellow). By continuously and rapidly proliferating, TA cells occupy most of the intestinal crypt and are responsible for generating terminally differentiated enterocytes (sky-blue). Because the Wnt pathway is one of the main drivers of stem cell proliferation and maintenance, its activity levels are the highest at the bottom of the crypt and decrease towards the villus domain, creating a Wnt gradient (light-blue triangle). Secretory progenitors differentiate into Paneth, goblet, or enteroendocrine cells (red, orange, and brown, respectively). Reserve ISCs (purple) primarily reside in a quiescent state (G0 phase) and are capable of generating all intestinal cell types in case of injury. Rare intestinal cell types are represented in pink and dark blue.

Glossary

Adult stem cells: multipotent undifferentiated cells that are found throughout the adult body and that retain the ability to self-renew and give rise to all cell types of a specific tissue, which ensures replacement of dying cells and regeneration of damaged tissues.
CHIR-99021 (CHIR): an inhibitor of GSK3β kinase, an essential component of the β-catenin destruction complex.
Porcupine (Porc): an endoplasmic reticulum (ER)-resident enzyme that modifies Wnt ligands by attaching palmitoleate, a modification essential for their secretion.
R/Z DKO: double-knockout mutant of the Wnt negative regulator *Rnf43* and its homolog *Znrf3*.
Stem cell niche: the microenvironment where adult stem cells reside and receive multiple stimuli from specialized neighboring cells aimed at supporting their growth and maintaining their stemness. Perturbations of such stimuli influence stem cell behavior by promoting either their quiescence, proliferation, or differentiation.
Tankyrases: poly-ADP-ribosyltransferases that activate the Wnt signaling pathway by mediating poly-ADP-ribosylation of AXIN, a step required for its subsequent degradation. Inhibitors of these enzymes have been developed to reduce Wnt activity levels.
Tissue renewal: the process by which somatic cells at the end of their lifespan are replaced by new cells generated by the adult stem cell pool within the tissue. The rate of renewal is tissue-specific and depends on the rate of stem cell division.

Box 2. The Intestinal Niche and Its Signaling Pathways

Intestinal homeostasis is dependent upon the maintenance and tight regulation of the stem cell compartment at the bottom of the crypt, which not only harbors *Lgr5*$^{+}$ stem cells but also a type of secretory cells termed Paneth cells (Figure I). Paneth cells are the only terminally differentiated cells that reside at the bottom of intestinal crypts, tightly intermingled with *Lgr5*$^{+}$ stem cells [95].

Paneth cells constitute an intestinal niche for *Lgr5*$^{+}$ stem cells because they produce several ligands and growth factors that are essential for intestinal stem cells (ISCs), such as epidermal growth factor (EGF), Notch ligands, and Wnt3. These factors reinforce stem cell identity and cooperate in driving stem cell proliferation [96]. Although Paneth cells comprise an important component of the intestinal niche, there is a degree of redundancy in the supply of key growth factors *in vivo*. For instance, subepithelial mesenchymal cells that surround crypts secrete additional niche factors, such as Wnt ligands and Wnt agonists, R-spondins, that are vital for stem cell maintenance [89]. The activity of all niche cells together ensures the establishment of a Wnt gradient that peaks at the bottom of the crypt, where it drives homeostatic self-renewal and proliferation of ISCs, and that progressively diminishes towards the upper parts of the crypt, to allow cellular differentiation (Figure I) [45]. Lastly, mesenchymal cells surrounding the crypt bottoms also secrete factors that antagonize BMP signaling to allow crypt formation and prevent BMP-mediated initiation of cellular differentiation [97].

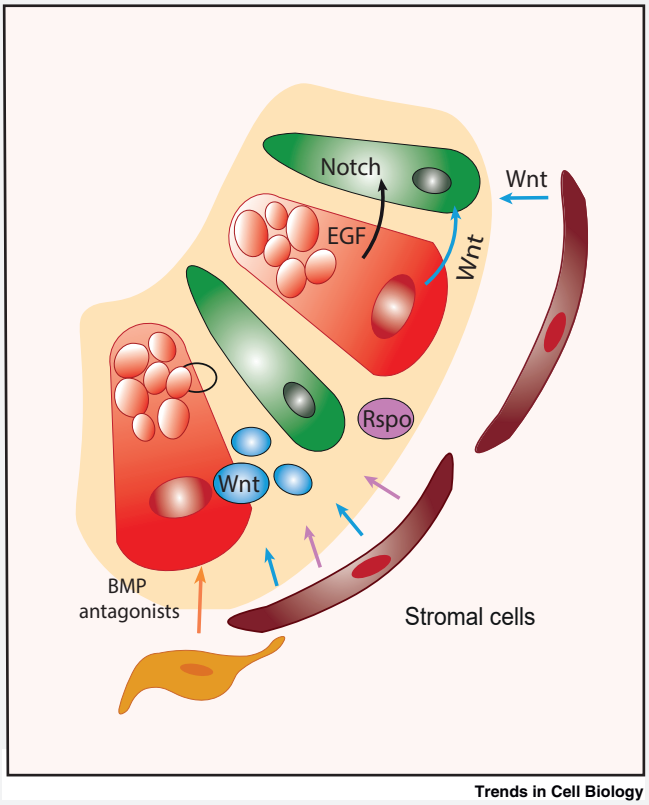

Figure I. The Small Intestinal Stem Cell (ISC) Niche.
The intestinal stem cell niche is composed of Paneth cells (in red) that provide ISCs (green) with the essential growth factors, such as Wnt ligands, epidermal growth factor (EGF), and Notch ligands, and mesenchymal cells (burgundy) that provide additional Wnt ligands and Rspo. BMP ligands that antagonize stemness maintenance are neutralized at the bottom of the crypt by BMP antagonists secreted by surrounding mesenchymal cells (orange).

Wnt Signaling in Tissue Renewal and Cancer

In humans and mice, 19 Wnt genes encode the evolutionarily conserved secreted Wnt lipoglycoproteins that control a variety of cellular processes, including stem cell maintenance, proliferation, migration, and polarity. Wnt proteins interact with members of the Frizzled (FZD) family as well as a diverse set of

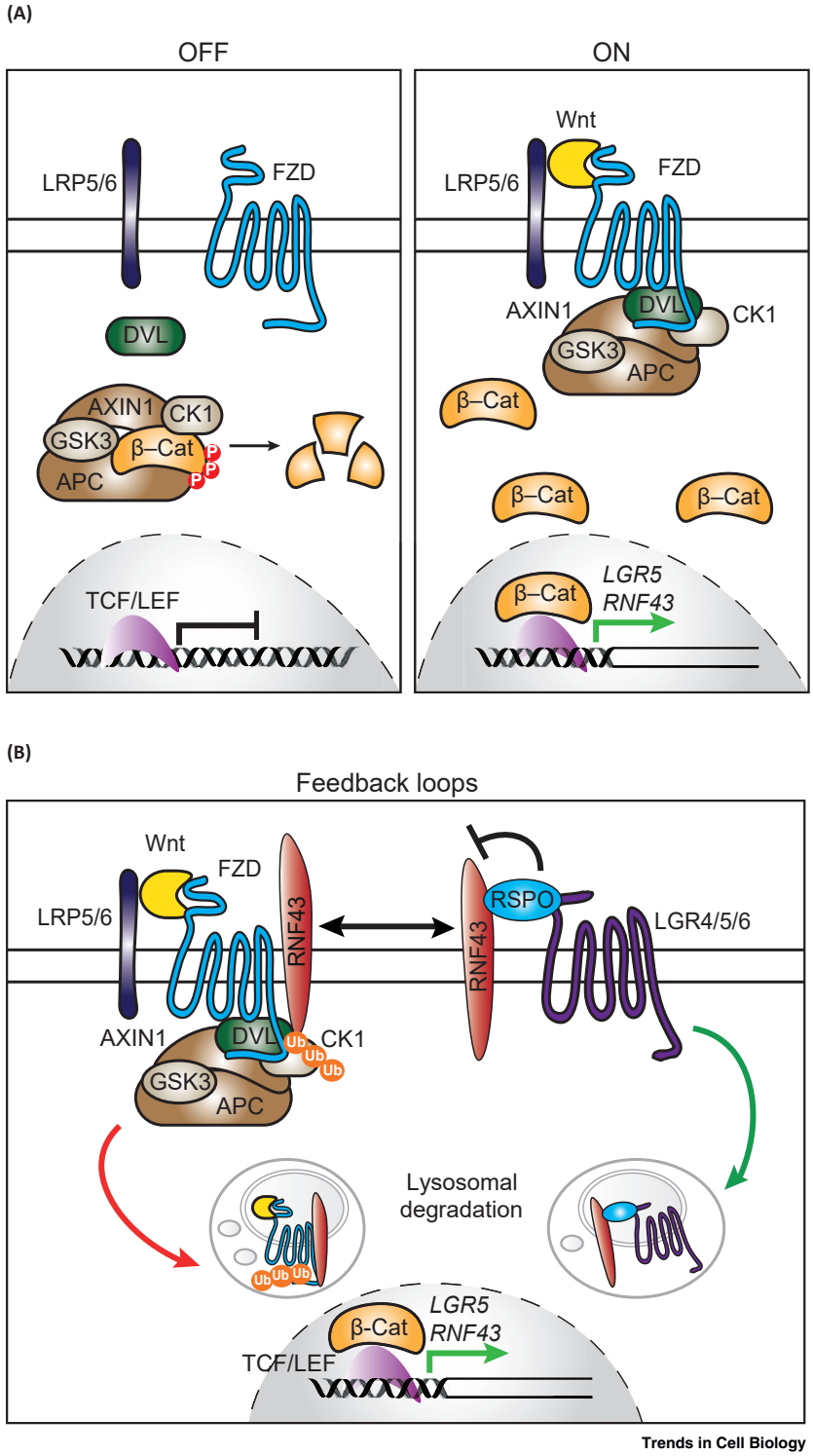

Figure 1. The Wnt/β-Catenin Signaling Pathway and Its Regulatory Loops.

(A) (Left) In the absence of Wnt ligands, β-catenin is continuously degraded by the destruction complex that is composed of two scaffold proteins, APC and AXIN, and two kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1).

(Figure legend continued at the bottom of the next page.)

coreceptors at the surface of recipient cells, and the combined ligand–receptor interactions determine downstream signaling responses [21,22]. Wnt-induced signaling pathways are classified into two main branches that are referred to as β-catenin-dependent and β-catenin-independent pathways [23]. In this review we will mainly focus on the Wnt/β-catenin pathway that comprises a major driving force for epithelial tissue renewal and is a frequent target for misregulation in cancer [24,25].

In unstimulated cells, the transcriptional coactivator β-catenin is sequestered and marked for degradation by a large cytoplasmic 'destruction complex' composed of the scaffold proteins adenomatosis polyposis coli (APC) and AXIN, as well as the kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) [26] (Figure 1A). Upon Wnt-mediated activation of FZD and its coreceptor LRP5/6 [27,28], key components of the destruction complex are recruited to the plasma membrane, allowing cytosolic accumulation of β-catenin and its translocation to the nucleus [29] (Figure 1A). Subsequently, nuclear β-catenin associates with transcription factors of the TCF/LEF (T cell factor/lymphoid enhancer-binding factor) family to drive the expression of Wnt target genes [30,31] (Figure 1A).

During tissue homeostasis, precise tuning of Wnt signaling strength is of key importance for the execution of the different Wnt-controlled programs that are required for adult stem cell maintenance, proliferation, and cell fate specification [32]. In ISCs, Wnt signaling is crucially balanced by the membrane-bound E3 ligases RNF43 (RING finger protein 43) and ZNRF3 (zinc and RING finger 3) that promote ubiquitin-dependent endocytosis and lysosomal degradation of FZD receptors [33,34]. This negative feedback loop attenuates Wnt signaling activity and prevents stem cell zone expansion and tumor growth (Figure 1B). The activity of RNF43 and ZNRF3 is counterbalanced by Rspo, a Wnt-potentiating factor that is secreted by the **stem cell niche** [35–37]. A complex formed by Rspo and the LGR4/5/6 (leucine-rich repeat-containing G protein-coupled receptors 4–6) receptors binds and removes RNF43/ZNRF3 from the stem cell surface to cause accumulation of FZD receptors and an enhanced state of Wnt responsiveness that is required for the maintenance of stem cell populations [35,38] (Figure 1B).

Mutation-induced activation of Wnt/β-catenin signaling and concomitant deregulation of Wnt target gene expression is widely associated with human cancer [39]. A main consequence of inappropriate Wnt signaling is the conferral of an undifferentiated state that promotes tumor growth, metastatic spread, and therapy resistance [40–42]. Downstream Wnt pathway activation by mutations in core destruction complex components APC, axin, and β -catenin are well-documented cancer-promoting events [39]. More recently, deregulation of Wnt receptor activity through mutations in the RNF43/LGR5/Rspo axis have emerged as a major cancer driver event in a broad range of tumor types [39]. Mechanistic insights into how specific mutations alter Wnt signaling are becoming a key determinant for tailored therapeutic approaches [43,44], reinforcing the importance of unraveling details of Wnt pathway functioning in adult tissues and cancer.

Mouse- and Human-Derived Intestinal Organoids: Similarities and Differences between Wnt-Associated Genotype–Phenotype Relationships

Organoids established from the mouse small intestinal epithelium display remarkable similarity to the structural organization and morphology of their organ of origin. These organoids are characterized by

The main function of the destruction complex is to capture and phosphorylate β-catenin, marking it for ubiquitination and subsequent proteasomal degradation. (Right) Wnt proteins bind to their cognate receptor Frizzled (FZD) and the coreceptor LRP5/6 to promote the formation of a multicomponent receptor complex. The cytosolic protein Dishevelled (DVL) is recruited to the plasma membrane by the receptor complex where it triggers further recruitment of destruction complex components. As a consequence, β-catenin accumulates in the cytoplasm of Wnt-activated cells and translocates to the nucleus where it promotes the transcription of Wnt target genes. (B) Regulatory feedback loops initiated by the Wnt target genes *RNF43* and *LGR5*. RNF43 ubiquitinates FZD and marks it for lysosomal degradation (red arrow). This leads to attenuation of Wnt signaling. R-spondin (Rspo) proteins bind to their cognate receptors LGR4/5/6 and to the extracellular domain of RNF43. By driving internalization of the Rspo/RNF43/LGR trimeric complex, LGRs act in concert with Rspo to mediate RNF43 clearance from the plasma membrane, thereby stabilizing FZD receptors and potentiating Wnt signaling (green arrow). Abbreviations: β-cat, β-catenin; P, phosphorylation; TCF/LEF, T cell factor/lymphoid enhancer-binding factor; Ub, ubiquitin.

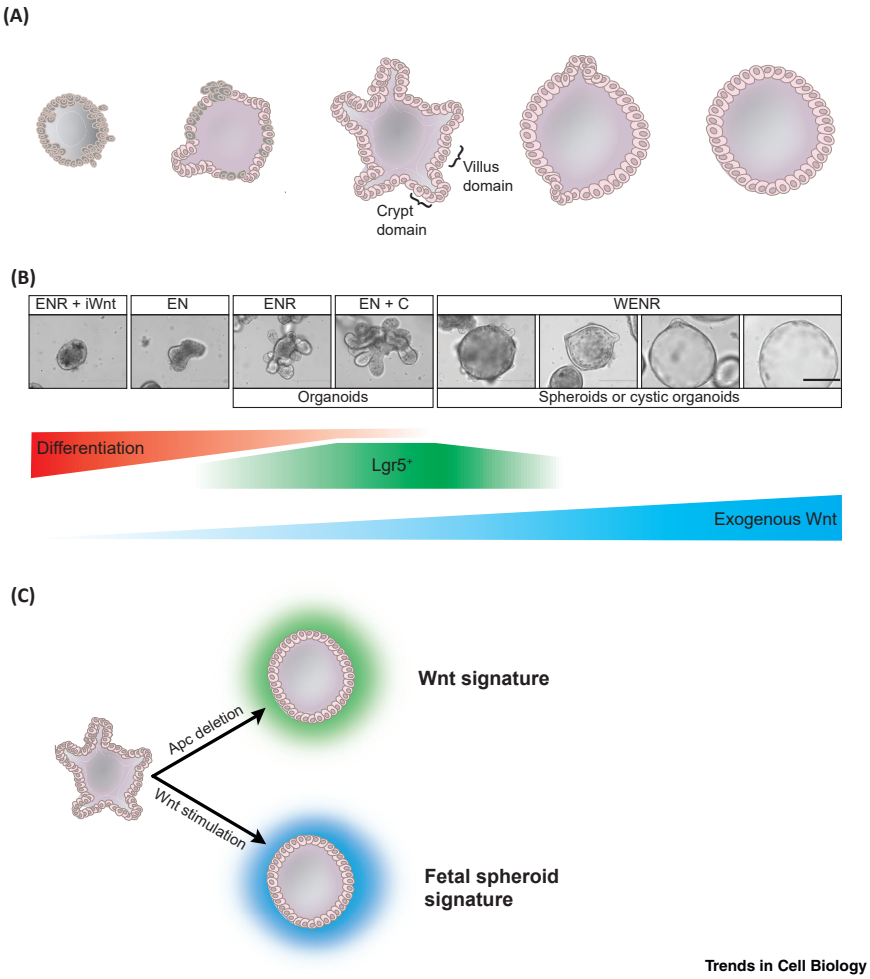

Figure 2. A Guide to Wnt-Related Phenotypes in Mouse Intestinal Organoids.

(A) Schematic representation of the effect of increasing Wnt concentration on organoid viability and morphology. Complete absence of Wnt is not compatible with organoid survival, whereas a gradual increase in Wnt concentration first induces bud formation and then promotes organoids to acquire a cystic morphology. Dead cells are depicted in grey, live cells in pink. (B) Bright field microscopy of small intestinal mouse organoids under different culture conditions (C) Schematic representation of the morphology switch from a crypt–villus structure to a cystic structure upon *Apc* deletion or exogenous Wnt stimulation in mouse small intestinal organoids. *Apc*-depleted organoids show a Wnt-signature with upregulation of *Lgr5* (green circle), whereas Wnt-stimulated organoids upregulate a signature typical of fetal intestinal spheroids (blue circle). Abbreviations: C, CHIR-99021; EN, EGF/Noggin; ENR + iWnt, EGF/Noggin/Rspo plus inhibition of Wnt ligand secretion; WENR: exogenous Wnt plus ENR. Scale bar, 200 μm.

budding structures that harbor *Lgr5*$^{+}$ stem cells at their base, representing intestinal crypts, interposed by domains carrying all differentiated cell types, representing villi (Figure 2A) [1]. Such architectural organization is the result of well-coordinated crosstalk among multiple signaling pathways and requires the formation of spatially defined growth factor gradients along the crypt–villus axis. Supplementation of intestinal organoids with Rspo is essential for the formation of a Wnt gradient that peaks at the base of crypts to promote proliferation of cells within the stem cell zone as well as proper cell positioning [45,46]. Together, these events ultimately drive bud (crypt) formation *in vitro* [47] and are concomitant with the stochastic appearance of specialized nondividing secretory niche cells (Paneth cells) in the crypt, which represents a first 'symmetry-breaking' event during organoid formation [47,48].

Although murine small intestinal organoids self-organize and display budding structures when supplemented with EGF/Noggin/Rspo (ENR) medium [1] (Figure 2A,B), the addition of exogenous Wnt3a has proved to be essential for the establishment and propagation of murine colon organoids [3]. In fact, colon organoids derived from *Lgr5–GFP*-expressing mice entirely lost GFP expression when cultured for 3 days in ENR medium and, at the same time, displayed increased expression of differentiated epithelial cell markers [3]. These findings argue that secretory niche cells present in colon crypts do not produce sufficient amounts of Wnt to support $Lgr5^+$ stem cell expansion *in vitro*. In line with these observations, $Reg4^+$ deep secretory cells, that were identified as the colonic equivalent of small intestinal Paneth cells, only express low levels of Wnt3 [49]. Hence, the expansion of colonic stem cells *in vitro* depends on exogenous Wnt supplementation [3].

Supplementation of murine small intestinal organoids with exogenous Wnt3a brings about a dramatic shape change in which organoids convert from a budded structure to a cystic morphology [1] (Figure 2A,B). A similar morphological change of organoids is observed upon genetic inactivation of *APC*, a frequent driver event in colorectal cancer (CRC) [50,51]. Strikingly, although both Wnt supplementation and *APC* deletion drive Wnt pathway activation and mediate cystic organoid growth, the underlying gene programs appear to be different [52]. This is illustrated by the observation that *APC* deletion induces a classical Wnt target gene signature, characterized by increased expression of *Lgr5*, whereas Wnt3a supplementation induces an *Lgr5*-independent gene signature that resembles fetal spheroids [53] (Figure 2C). Such spheroids, derived from murine fetal epithelium, are mainly composed of proliferating cells that lack the transcriptional signatures of differentiated cell types found in the adult intestine [54]. Conversely, it was shown that stimulation of fetal spheroids with Wnt3a-conditioned medium can induce their maturation *in vitro* and the appearance of budding structures [55,56]. Together, these findings indicate that the consequences of Wnt treatment of organoids may depend on their developmental state.

Recently, adult intestinal tissues were shown to reactivate aspects of fetal development as a mode of tissue repair after injury [57]. Thus, Wnt treatment of organoids might impose a regenerative state upon the intestinal epithelium that is reminiscent of inflammation-induced wound repair. Indeed, both Wnt and Rspo production are increased in inflamed colon tissue to drive epithelial cell proliferation, crypt fission, and ultimately tissue regeneration [58,59]. It should be noted that Wnt-independent mechanisms also play a role in the regenerating epithelium, as shown by Miyoshi and colleagues, who observed a dose-dependent effect of prostaglandin E2 on spheroid size [60].

In general, different Wnt-activating stimuli induce differential responses and morphological changes in organoids. Supplementation with increasing concentrations of the Wnt-potentiating niche factor Rspo drives increased expression of both Wnt target genes and intestinal stem cell markers such as *AXIN2* and *LGR5*, while a budding organoid shape with crypt and villus domains is maintained [45]. Although Rspo is supplied to the overall culture medium, its effects remain highly localized owing to the restricted expression of Wnt and its target genes *LGR5* and *RNF43* at the bottom of crypts [33]. By contrast, treatment with the GSK3β inhibitor **CHIR-99021 (CHIR)**, which ubiquitously and strongly activates β-catenin-mediated transcription, induces discordant phenotypic alterations in mouse small intestinal organoids, including cystic or budding shapes or mixed populations [33,45,61–63] (Figure 2B). Of note, CHIR treatment, in combination with histone deacetylase inactivation via valproic acid, comprises an effective procedure for the enrichment of $Lgr5^+$ cells in intestinal organoids [62].

Whereas mouse small intestinal organoids give rise to all differentiated cell types of their *in vivo* counterparts, human small intestinal and colon organoids display a relatively undifferentiated profile as a result of the growth conditions required for their long-term propagation [1,3]. For instance, supplementation with both p38 MAPK inhibitor and exogenous Wnt was reported to be indispensable for human cultures. Removal of exogenous Wnt uniformly induces enterocyte differentiation of human colon organoids [3]. To achieve a higher degree of cellular diversity, Fujii and colleagues recently developed refined culture conditions for human colon organoids in which they replaced the p38 inhibitor with other key intestinal growth factors of the intestinal tract *in vivo* – insulin-like

growth factor 1 (IGF-1) and fibroblast growth factor 2 (FGF-2). The addition of these growth factors promotes the specification of differentiated cell types and the acquisition of a budding morphology, indicating that optimized growth conditions allow improved representation of *in vivo* tissue physiology [64].

In summary, these findings indicate that distinct organoid morphologies do not necessarily correlate with an explicit molecular profile. Discrepancies in the expression of Wnt target genes and stem cell markers upon treatment with different Wnt stimuli may be due to treatment duration, lack of regulated signaling strength, or defects in gradient formation, thereby preventing the specification of particular cell lineages. As a general consensus, budding organoids most closely resemble the homeostatic state of the intestinal epithelium because of their highly localized Wnt activity within the stem cell niche and the formation of a Wnt gradient along the crypt–villus axis.

Modeling and Evaluating Downstream Wnt Pathway Cancer Mutations in Intestinal Organoids

Over recent years, organoids have come of age as innovative cancer models [65]. The introduction of cancer mutations into healthy organoids using CRISPR/Cas9-mediated genome editing faithfully mimics cancer onset and progression as revealed by xenotransplantation experiments [66,67]. Furthermore, patient-derived organoids, that can be established from both tumor biopsies and neighboring normal tissue, encompass advantageous resources of great utility for fundamental and translational research. Multiple studies have revealed that patient-derived tumor organoids retain organ site-specific histological and genetic traits, and can exhibit metastatic behavior when transplanted into immunocompromised mice [67,68]. The possibility of building living patient-derived organoid biobanks that represent all known cancer subtypes offers great potential for understanding cancer genomics, drug responses, and for performing drug-sensitivity screens for the development of personalized cancer treatments [69–72].

The ability of cancer cells to acquire self-renewing properties independently of stem cell niche-derived signals has emerged as an essential prerequisite for tumor progression and metastasis by allowing cells to maintain an undifferentiated state and grow in a hostile environment. Cancer mutation-induced niche-independent growth can be assessed by withdrawal of essential factors such as Wnt and Rspo from the organoid culture medium. This is widely exemplified by mutations that impair the activity of the Wnt pathway destruction complex. For instance, organoids derived from APC-mutant tumors of *Lgr5–GFP–ires–CreERT2* × *Apc*$^{fl/fl}$ mice displayed Rspo-independent growth and a cystic morphology [3] (Figures 2 and 3). In later studies, two independent groups engineered patient-derived organoids from healthy colon tissue with mutations that mediate CRC transformation and metastasis, including inactivating mutations in *APC*, *TP53*, and *SMAD4*, and activating mutations in *KRAS* and *PIK3CA* [66,73]. Both studies confirmed that *APC* loss is essential for metastatic potential and allows organoids to grow in the absence of both Wnt and Rspo with concomitant acquisition of a cystic morphology (Table 1). Of note, *APC*-mutant organoids displayed increased expression of the Wnt target gene *AXIN2* when Wnt and Rspo were omitted, suggesting that withdrawal of these two factors exacerbates the contribution of *APC* loss to aberrant Wnt pathway activation [69]. Similarly, *Apc* knockout in small intestinal mouse organoids also induced upregulation of Wnt target genes, including *Rnf43*. Accordingly, *Apc*-knockout organoids fail to retain Wnt3 ligands at the cell surface owing to sustained Rnf43-mediated degradation of the Fzd receptor [45]. These observations highlight how organoids have contributed to an increased understanding of how *Apc* loss impacts on epithelial homeostasis and have fueled the concept that cancer cells grow independently of their niche.

To distinguish the effects of *APC* loss from those of receptor-mediated signaling, and to understand the difference between oncogenic and physiological Wnt responses, Michels *et al.* compared the transcriptome and proteome of *APC*-ablated human colon organoids to wild-type organoids stimulated with Wnt [52]. This approach revealed characteristic Wnt-receptor and *APC*-knockout signatures and, moreover, pointed to an association between the Wnt-mediated signature and the

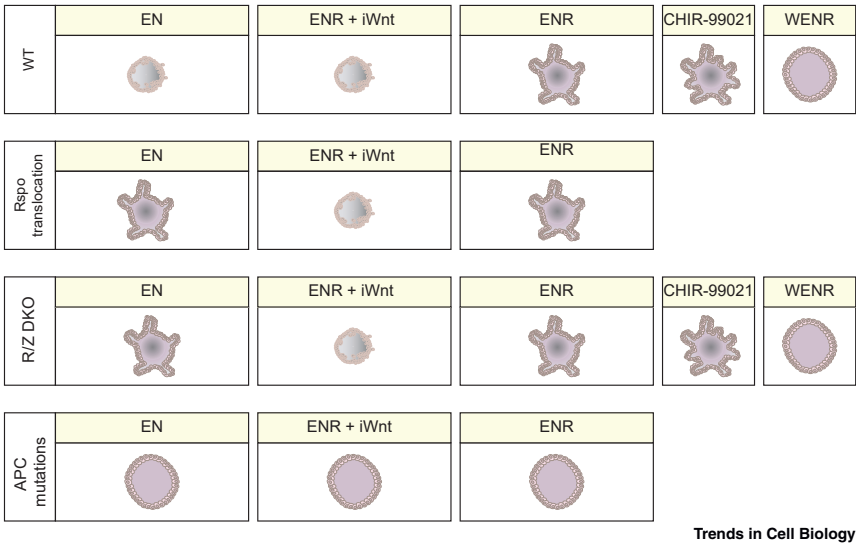

Figure 3. Schematic Overview of Different Organoid Morphologies Associated with Wnt Pathway Alterations in Small Intestinal Mouse Organoids.

Wild-type (WT) mouse small intestinal organoids are compared with organoid lines that carry various mutated Wnt pathway components. For each organoid line, morphological alterations under different culture conditions are shown. Abbreviations: CHIR-99021, GSK3β inhibitor; EN, EGF/Noggin, ENR: EGF/Noggin/Rspo; iWnt, inhibition of Wnt ligand secretion; R/Z DKO, *Rnf43* and *Znrf3* double-knockout; WENR, Wnt/ENR.

aggressive CRC subtype CMS4 that is characterized by poor prognosis [52]. Although this study represents an excellent starting point for detailed characterization of Wnt responses in CRC, the next challenge will be to unveil the underlying molecular mechanisms.

In addition to *APC* loss, organoid models have provided a sensitive means to profile cancer-derived genetic lesions that give rise to truncated APC proteins of various lengths. APC tumor-suppressor activity largely resides in the C-terminus of the protein that contains multiple repeat elements for interactions with other components of the β-catenin destruction complex [29]. How partial or complete loss of APC tumor-suppressor function links to different levels of Wnt pathway activation and susceptibility to anti-Wnt-based cancer therapy was investigated using mouse small intestinal organoids [74,75]. Incremental *Apc* truncations were introduced using CRISPR/Cas9, and the resulting organoid lines were evaluated for morphology, growth factor-independent growth, and sensitivity to treatment with inhibitors of **Porcupine (Porc)**, the acyltransferase that performs an essential lipidating step in Wnt secretion [76]. Whereas all *Apc*-mutant organoids adopted a cystic morphology, the lines that retained a specific C-terminal region carrying the 'β-catenin inhibitory domain' remained sensitive to Porc inhibition and Rspo withdrawal [74], thus proving the existence of an Apc threshold for pathological levels of Wnt activation. Analogously, it was shown that organoids bearing early truncations in *Apc* that affected its binding to Axin1 and β-catenin were not sensitive to **Tankyrase** inhibition [75].

The ability of organoids to display Wnt/Rspo-independent growth strongly correlates with downstream Wnt pathway defects. This is illustrated by a report of Fujii *et al.*, who established a biobank of 55 CRC tumor organoid lines comprising common and rare cancer subtypes, as well as 41 matching normal colorectal organoids [70]. The largest fraction of CRC-derived organoids displayed combined Wnt and Rspo independency, which corresponded with the occurrence of mutations in *APC*, *CTNNB1*, or *TCF7L2* (Table 1). By contrast, three organoid lines derived from rare CRC subtypes, classified as mucinous adenocarcinoma and neuroendocrine carcinoma, displayed Wnt-dependent growth which correlated with a lack of known Wnt pathway mutations [70] (Table 1). This study reveals the usefulness of organoids for the identification of tumor subsets that show Wnt-dependent growth

Table 1. Linkage of Wnt and Rspo Niche Factor Independence and Wnt Pathway Driver Mutations in Intestinal Organoids

Tissue of origin	Mutations in Wnt pathway components	Wnt independency	Rspo independency	Refs
Large intestine	*WT*	No	No	[3,4]
Large intestine	*APC*	Yes	Yes	[66,73]
Adenoma/CRC	*APC*	Yes	Yes	[70]
Adenoma/CRC	*CTNNB1*	Yes	Yes	[70]
Adenoma/CRC	*TCF7L2*	Yes	Yes	[70]
Mucinous adenocarcinoma	*WT*	No	No	[70]
Neuroendocrine carcinoma	*WT*	No	No	[70]
Sessile serrated adenomas	*RNF43*	No	Partially	[81]
Adenoma/CRC	*RNF43*	No	No	[70]

and thus display a druggable vulnerability that can be exploited for therapies aimed at Wnt or Wnt receptor inhibition [43,69].

In addition to APC, the contribution to intestinal stem cell maintenance of several other members of the destruction complex was investigated by loss-of-function experiments in mouse intestinal organoids. For instance, combined deletion of the homologs *Axin1/2* or *Gsk3α/β* induced a cystic morphology which phenocopied *Apc* loss [77], whereas depletion of *CK1ε/δ* kinases mediated arrested organoid growth [78] (Figure 3). Furthermore, expression of a stabilized form of β-catenin resulted in organoids that displayed cystic morphology and were capable of surviving without Rspo supplementation [79] (Figure 3). Thus, downstream Wnt pathway activation in cancer generally mediates the acquisition of a Wnt/Rspo-independent growth state and a cystic phenotype in mouse intestinal organoids.

Taken together, genetically modified as well as patient-derived intestinal 3D cultures offer an unprecedented opportunity to position the effects of cancer mutations within the Wnt/β-catenin pathway, deepen our understanding of how pathway regulation correlates with cell fate specification, and link different modes and levels of Wnt activity to specific phenotypes.

Modeling and Analyzing Defective Wnt Receptor Signaling in Intestinal Organoids

Over recent years, deregulated Wnt receptor activity has emerged as an alternative major pathway for cancer development. The underlying mutational routes involve either inactivating mutations in *RNF43* and *ZNRF3* or overexpression of *RSPO*, both of which drive FZD overexpression and, consequently, a Wnt-hypersensitive growth state. The tumor-suppressor roles of RNF43 and ZNRF3 were first evaluated *in vivo*, in the mouse intestine. Although single deletions of *Rnf43* or *Znrf3* did not yield an evident phenotype, combined ablation of *Rnf43*/*Znrf3* (double-knockout, **R/Z DKO**) led to expansion of Paneth and *Lgr5*$^{+}$ cells, nuclear accumulation of β-catenin, and adenoma formation [33]. The underlying molecular mechanism was elucidated by the use of small intestinal organoids in combination with small-molecule inhibitors. First, RNF43 activity was positioned upstream of the destruction complex by the observation that organoid death induced by RNF43 overexpression was rescued by treatment with the GSK3β inhibitor CHIR, but not by supplementation with Wnt3a. Next, R/Z DKO organoids were found to grow independently of Rspo while they remained dependent on Wnt, as shown by their sensitivity to Porc inhibitors (Figure 3) [44]. Together, these findings substantiated a model in which R/Z depletion drives a state of Wnt hypersensitivity, mimicking the molecular effects of Rspo [33]. By contrast to downstream Wnt pathway activating mutations, R/Z DKO mouse small

intestinal organoids maintain a budding morphology, reflecting the highly localized supply of Wnts by Paneth cells and the ability to form a Wnt gradient along the crypt–villus axis (Figure 3).

The results of these studies predict that deletion of both *RNF43* and *ZNRF3* are required to obtain full Rspo independency. Indeed, inactivating *RNF43* mutations are frequently accompanied by silencing of *ZNRF3* [80], whereas single *RNF43*-mutated CRC organoids failed to survive upon withdrawal of Rspo [81]. Titration of Rspo, however, showed that *RNF43*-mutated patient-derived colon organoids from sessile serrated adenomas display reduced dependence, but not full independence, on Rspo supplementation, indicating that single RNF43 mutations might confer a partial phenotype [71] (Table 1).

RNF43 mutations have emerged as a frequent event in various cancer types [82–84]. The ability to grow patient-derived tumor organoid cultures has opened up the possibility to examine the sensitivity of individual tumors for treatment with Wnt blocking agents such as Porc inhibitors *in vitro* [69]. Within a living biobank of 22 CRC organoids, an RNF43-mutant organoid line indeed displayed sensitivity to Porc inhibition [69], indicating the potential suitability of this approach for the prediction of therapeutic responses.

Aberrant, translocation-mediated expression of *RSPO2* and *RSPO3* represents an alternative mechanism that endows CRC cells with niche-independent growth properties [85]. These genomic alterations appear to be mutually exclusive with mutations in other Wnt pathway components, confirming their independent cancer-driver effects. Excessive Rspo production causes increased removal of RNF43 and ZNRF3 from the cell surface and, as a consequence, accumulation of FZD [86]. Thus, Rspo overproduction drives a Wnt-hypersensitive state similar to *R/Z* loss. This assumption was verified by the CRISPR/Cas9-mediated introduction of *RSPO* fusion genes into intestinal organoids. As expected, these organoids grew independently of exogenous Rspo but were sensitive to Porc inhibition, confirming the role of these translocations in driving upstream Wnt pathway activation [87] (Figure 3).

In summary, examination of patient-derived organoids for growth dependencies and for sensitivity to Wnt pathway small-molecule inhibitors allows faithful mapping of Wnt pathway defects and offers a robust indication of treatment opportunities in cancer. However, when employing organoids in preclinical studies it should be considered that they only model the epithelial compartment of the intestine. Redundant Wnt-secreting populations have been recently identified in the subepithelial stroma, and these could be responsible for a different response to Porc inhibitors *in vivo* [88,89]. In this regard, the multiple efforts that are being made to enhance organoid complexity by coculturing different cell types together with epithelial cells will certainly improve the power of 3D *in vitro* models [88].

Concluding Remarks

The primary role of Wnt signaling in regulating stem cell homeostasis and cell fate decisions is a well-established concept. In recent years, the stem cell field has been revolutionized by the advent of organoid technology. In fact, it is now possible to grow adult stem cells from most epithelial tissues in a dish to obtain 3D self-organizing structures that also contain differentiated cells. One evident commonality is that, regardless of their tissue of origin, adult stem cell-derived organoids depend on the canonical Wnt signaling pathway for their propagation, maintenance of stemness, and the generation of terminally differentiated, specialized cells. We have reviewed several examples by which adult stem cell-derived intestinal organoid cultures are exploited to study Wnt/β-catenin signaling and its regulation during tissue homeostasis and in cancer. In addition, we have described how patient-derived organoids are employed to position cancer growth-linked Wnt pathway defects and to predict tumor sensitivity to Wnt-targeted therapies.

Despite these advantages, intestinal organoids still present several aspects that need to be clarified to improve their applications. For instance, the biological meaning of cystic growth instead of a budding morphology, and the precise cellular composition and function of such structures, require further investigation. We anticipate that single-cell sequencing technologies will help to characterize and understand the molecular consequences of specific Wnt pathway mutations or treatment with

exogenous Wnt or small-molecule inhibitors (see Outstanding Questions). In any case, these findings indicate that genotype–phenotype relationships need to be carefully approached and require substantiation with molecular profiling.

Another potential issue concerns the standardization of organoid cultures. A confounding factor might be the presence of serum components and other secreted factors in Wnt- and Rspo-conditioned media. Such unknown substances might be a source of variability in organoid cultures in different laboratories and might affect physiological cellular activities and morphology. Recently, several efforts were made to improve and standardize organoid growth conditions. For instance, Wnt3a proteins could be copurified in a complex with afamin [90]. The afamin–Wnt complex was shown to be biologically active and intestinal organoids could be propagated by using this alternative Wnt source [90]. In addition, Wnt3a-packaged liposomes were shown to support the growth of human organoids from both healthy and diseased intestine and liver [91], and surrogate Wnt agonists were developed and shown to support the growth of liver, pancreas, and stomach organoids [92]. These examples illustrate that, over time, the culturing of organoids will likely move towards more standardized culture conditions.

In conclusion, adult stem cell-derived organoids represent an extraordinary tool for the quantitative and qualitative characterization of Wnt signaling in a physiologically relevant environment. With the possibility of building 3D tumor models, organoids hold great promise for the in-depth investigation of carcinogenic mechanisms and they are already widely exploited in drug testing.

Outstanding Questions

What is the molecular basis of the differential organoid phenotypes following interference (mutations, small-molecule inhibitors) with downstream Wnt pathway destruction complex activity?

What are the similarities and differences between the molecular mechanisms and transcriptional programs underlying Wnt-driven tissue renewal versus Wnt-driven tissue repair? What markers can be used reliably to distinguish between these phenotypes?

How do partial niche independency phenotypes contribute to cancer growth?

How can culture conditions of human intestinal organoids (small intestine and colon) be optimized to allow standardized and accurate representation of the range of cellular subtypes that populate the adult intestine?

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