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Review

Wnt signaling in gut organogenesis

Michael P. Verzi and Ramesh A. Shivdasani*

Department of Medical Oncology; Dana-Farber Cancer Institute; and Department of Medicine; Harvard Medical School; Boston, Massachusetts, USA

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Wnt signaling regulates some aspect of development of nearly all endoderm-derived organs and Wnts mediate both differentiation and proliferation at different steps during visceral organogenesis. Wnt2b induces liver formation in zebrafish [1] and may combine with other inducers, Fibroblast Growth Factors 1 & 4 and Bone Morphogenetic Protein 4, to specify the mammalian liver.[2-5] Later in development, Wnts are critical for liver expansion and, finally, for terminal hepatocyte differentiation,[6-12] as reviewed elsewhere in this issue (Monga). Likewise, in the pancreas, Wnts drive proliferation of exocrine and endocrine cells[13,14] and promote acinar cell differentiation,[13,15] as reviewed in the chapter by Murtaugh. Here we examine the intricate involvement of Wnt signaling in growth and differentiation of the digestive tract.

Palmitoylated Wnt glycoproteins bind to the ectodomains of a receptor complex between Low-density lipoprotein receptor-related LRP5/6 and seven-pass transmembrane Frizzled proteins.[16,17] Ligand binding triggers an intracellular signaling cascade that inactivates the tumor suppressor APC, an essential component of a protein complex that, in the absence of Wnt signals, phosphorylates the cytoplasmic pool of β-catenin and targets it for destruction. Inactivation of the degradation complex results in accumulation of β-catenin, which enters the nucleus and trans-activates gene expression through sequence-specific DNA-binding transcription factors of the Tcf/LEF family. Simply put, canonical Wnt signaling thus alters the transcriptional state of Tcf/LEF target genes. Although this simplified view ignores the complex regulation of the canonical pathway and known roles for Wnt proteins in planar cell polarity and calcium-dependent signaling, it is a useful starting point to consider canonical Wnt functions in organogenesis. Other contributions in this volume describe molecular features of Wnt signaling in greater detail (Habas). We focus on the role of Wnt signaling in development of the intestine and stomach, with emphasis on recent advances.

The stomach and intestine arise from a contiguous tube, with the luminal lining (mucosa) derived from embryonic endoderm and sub-mucosal cells derived from the splanchnic mesoderm. Early in development, the endoderm and surrounding mesenchyme in the stomach and intestine are morphologically indistinguishable.[18] Undifferentiated gut-specific endoderm is subsequently patterned to generate distinct segments along its anterior-posterior (A-P) axis, largely on the basis of positional information encoded in the underlying mesenchyme.[18,19] The small intestine mucosa extends characteristic finger-like luminal projections or villi, structures that are maintained throughout life by continuous regeneration of stem and progenitor cells found in sub-mucosal invaginations known as the crypts of Lieberkühn. A repeating structure of villi and intervening crypts runs the full length of the small bowel; organization of the colon (large intestine) is substantially similar, except that villi are replaced by flat cuffs. The role of canonical Wnt signaling in adult crypt-villus homeostasis is well established[20] and represents the most intensively studied function of canonical Wnt signaling in the digestive tract.

Colorectal cancer, a common disease, results from constitutive activation of the canonical Wnt signaling pathway, which drives epithelial proliferation and differentiation in normal intestinal crypts.[21-23] Understanding how Wnt signaling relationships are established during gut development is therefore especially important in the intestine and will advance appreciation of oncogenic mechanisms.

Intestine

Intestinal epithelium contains four (small intestine) or three (colon) cell lineages that serve distinct functions. At the base of each small intestine (but not colonic) crypt are a handful of Paneth cells, which secrete anti-microbial defensin peptides.[24,25] Also at or near the crypt base are the intestinal stem cells, which give rise to all daughter epithelial lineages.[26,27] The stem cells' immediate progeny, a transit amplifying population, reside above the Paneth and stem cells and proliferate rapidly within the crypt microenvironment before their progeny migrate upward to populate small bowel villi or colonic cuffs. As cells move upward they exit the cell cycle and differentiate into enterocytes, goblet cells and enteroendocrine cells, which function to absorb nutrients, produce mucus and secrete hormones, respectively. In less than one week, cells transition from proliferating progenitors to differentiated descendants and die as they complete their migration toward the villus tip. The crypt-villus border demarcates a boundary between proliferating cells that are in the course of lineage commitment and differentiated, post-mitotic cells. The balance between these diverse populations is critical for maintaining epithelial homeostasis and bowel function.

Crypt cell turnover. Genetic studies in mice implicate the canonical Wnt pathway in virtually every known crypt function.

*Correspondence to: Ramesh A. Shivdasani; Dana-Farber Cancer Institute; 44 Binney Street; Boston, Massachusetts 02115 USA; Tel.: (617).632.5746; Fax: (617).582.8490; Email: ramesh_shivdasani@dfci.harvard.edu

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Homozygous deletion of *Tcf4*, a key transcriptional effector of Wnt signaling, compromises fetal and neonatal intervillus cells, the developmental forerunners of the crypts of Lieberkühn. These cells proliferate poorly and fail to feed the villus compartment, leading to death of animals from presumed absorptive dysfunction; expression of transcripts that mark normal crypts and intervillus regions is markedly reduced.[28] These results are commonly regarded as proof of an essential role for canonical Wnt signaling in establishing the proliferative intervillus niche but they may equally reflect other, Wnt-independent functions for Tcf4. Stomach development seems unaffected in *Tcf4*$^{-/-}$ mice, which is not surprising as other evidence for a Wnt requirement in stomach epithelium is sparse. Nor is the Tcf4 requirement manifested equally along the intestine; the distal colon is spared. Because Wnt signaling seems to be essential for all intestinal crypts, such sparing may be attributed to redundancy among Tcf/LEF proteins. However, double-mutant *Tcf3*$^{-/-}$*;Tcf4*$^{-/-}$ and *Tcf4*$^{-/-}$*;Lef1*$^{-/-}$ embryos die of other developmental defects before they are old enough to examine the question of redundancy in gut epithelium.

As an alternative to examining Wnt-pathway function by gene targeting, two groups expressed the extracellular Wnt antagonist Dickkopf1 (Dkk1) in adult transgenic mice. This manipulation caused rapid loss of crypts and demonstrates that Wnt signaling is required not only to form crypts but also to maintain them in adults.[29,30] Similarly, acute genetic ablation of *APC*, a negative regulator of canonical Wnt signaling, in adult mice induces crypt properties in villous epithelial cells, including proliferation and expression of crypt markers.[31,32] The secreted protein R-spondin1 potentiates canonical Wnt signaling, partially independent of the Lrp5/6 co-receptor; injection of recombinant R-spondin1 in adult mice results in rapid and reversible crypt hyperproliferation.[33] In summary, loss of Wnt signals is associated with loss of crypt regenerative capacity and aberrant activation of the pathway leads to uncontrolled growth. The sum of these studies strongly implicates the Wnt pathway in gut epithelial progenitor cell properties, including a proliferative role that echoes Wnt function in the liver and pancreas, and reveals that Wnt signaling in intestinal crypts is tightly controlled.

Differentiation of secretory cell lineages. In addition to supporting progenitor cell expansion, the canonical Wnt pathway is required for proper differentiation and localization of secretory cell lineages. From their normal location at the crypt base, one infers that Paneth cells encounter especially high Wnt concentrations; indeed, Wnt activity in adult intestine is most easily detected in Paneth cells, both by immunostaining for nuclear β-catenin and reporter gene activity in the TOP-GAL mouse line.[34-36] Inactivation of the Wnt pathway in each of the experimental mouse models summarized above results in loss of Paneth cell markers,[36-38] whereas pathway activation increases Paneth cell numbers dramatically.[31,32] In mice with activating Wnt mutations, Paneth cells also lose crypt-restricted localization and frequently come to lie along small bowel villi. Similarly, disruption of the Wnt receptor gene *Frizzled5* mis-localizes Paneth cells high along intestinal villi.[36] These observations are explained in large part by Wnt regulation of the intercellular EphB/ephrin-B signaling system for attraction or repulsion between cells. Wnt-dependent EphB/Ephrin-B signaling seems to be responsible for partitioning differentiated epithelial cells and promoting cryptward migration of the Paneth lineage.[39,40] The proximal promoters of several Paneth cell-specific α-defensin genes contain conserved Tcf/LEF binding motifs. Control of gene transcription through these *cis*-elements is another aspect of Wnt function[36] and likely represents a portion of the specific Wnt-dependent transcriptional program in Paneth cells.

The role of Wnt in differentiation of enteroendocrine and goblet cells is appreciated with lesser clarity. *Tcf4*$^{-/-}$ mice carry very few intestinal goblet cells and no enteroendocrine cells; they die before Paneth cells can be recognized morphologically[28] but lack Paneth-cell gene products that are readily detected in control littermates.[36] Inactivation of Wnt signaling through a *DKK1* transgene resulted in surprising loss of all intestinal secretory cell lineages but not of absorptive enterocytes; these results hint at an essential role for Wnt in a primitive secretory progenitor or separately in each mature secretory cell type.[29] In Wnt-activating APC mutants, Paneth cell numbers are increased, however this may be at the expense of goblet and enteroendocrine cells, as a dramatic decrease of these cell lineages is observed.[31,32] Taken together, these studies suggest that Wnt signaling may be necessary to establish the secretory lineages but sustained, high-level Wnt activity is especially relevant in Paneth cells. Other observations suggest that the role of the Wnt pathway in intestinal cell differentiation may be more complex: R-spondin, which activates Wnt signaling and induces crypt hyperproliferation, does not affect differentiation or localization of mature cells.[33]

While these studies highlight the many facets of Wnt function in intestinal homeostasis, they also point to our limited understanding of how input from a single signaling pathway is interpreted to mediate diverse outcomes. Defining the basis for the different outcomes of Wnt activity—progenitor cell proliferation, lineage decisions, differentiation and cell migration—thus remains a major unsolved problem. Illustrating one example, disruption of the Wnt target gene Sox9 affects Paneth and goblet cell differentiation but not localization or differentiation of enteroendocrine cells.[37] Overlapping sets of critical Wnt target genes must similarly mediate distinct functions in gut epithelium and also respond to the other signaling pathways that influence mucosal function, including Notch.[41-43] In *Math1*$^{-/-}$ mice, for example, secretory cell differentiation is compromised in a fashion reminiscent of genetic models with Wnt inactivation;[44-46] these results suggest that interactions between the Wnt and Notch pathways could determine secretory cell fates. Although attention is now shifting toward investigation of the molecular basis of individual Wnt effects, few themes have emerged to allow general conclusions.

Source and identity of intestinal Wnts. In light of the subjacent mesenchyme's requirement in gut epithelial viability and function,[18] one might assume that this tissue is the source of Wnt signals for crypt progenitors. However, detailed investigation has failed to identify Wnt-expressing cells among the loosely arranged fibroblasts that make up most of the mesenchyme or the myofibroblast network apposed to the basement membrane surrounding the intestinal crypts. By contrast, several Wnt mRNAs are identified within crypt epithelial cells, including Wnt3, Wnt6 and Wnt9b,[47] which hints at the possibility of autocrine or paracrine Wnt regulation of intestinal crypt functions.

In both mouse and chick, components of the canonical Wnt pathway are expressed in complex spatial and temporal patterns along the anterior-posterior axis of the embryonic gut, suggesting the possibility of roles in patterning and organogenesis;[48-50] such

functions remain uncertain. These comprehensive in situ hybridization analyses over multiple developmental stages revealed that Wnts and other pathway components are often restricted in expression in correspondence with distinct morphologic regions in the gut. For example, Frizzleds 4, 7 and 8 all contain an expression boundary within the developing chick cecum, suggesting a role in development of this structure.[49,50] Future studies identifying the functions of these and other Wnt pathway components during gut organogenesis may clarify the reasons for complex expression patterns but functional analysis in mice is likely to be confounded by genetic redundancies.

Wnt function in villus morphogenesis. Before the fetal small intestine generates its hallmark villi, the prospective gut mucosa is a simple, pseudostratified epithelium. Following rapid morphological changes that occur around the 14th day of gestation in mice, the earliest villus epithelial cells and intervening crypt precursors are distinguished from each other by expression of maturation and progenitor markers, respectively. Most studies we have summarized thus far were conducted in adult mice, and from their results one might infer that Wnt signaling shapes the earliest intestinal crypts. Surprisingly, the evidence for canonical Wnt activity does not appear in the developing intestine until about E16.5, as determined in TOP-GAL reporter mice or by nuclear localization of β-catenin.[35] An additional surprise is that early Wnt reporter activity does not localize in the intervillus space, where the proliferating forerunners of intestinal crypts reside, but rather in maturing epithelial cells lining the earliest villi.[35] In mice, intervillus regions begin to invade the sub-mucosa to form crypts well after birth, and canonical Wnt activity shifts from the villi to the intervillus regions about three days after birth. Thus, Wnt signaling may play a crucial role in establishing the crypts of Lieberkühn but appears to be inactive during villus morphogenesis even though there is clear anatomic separation of proliferating (intervillus) and differentiated (villus) cells. Despite absence of Wnt reporter activity, the genetic requirement for Tcf4 is manifested at the level of intervillus cell proliferation and early villus morphogenesis is intact in *Tcf4*$^{-/-}$ intestines.[28] One possibility is that fetal intervillus Wnt activity is below the limits of detection, but this does not explain its presence in maturing villus cells; alternatively, the phenotype of *Tcf4*$^{-/-}$ mice may reflect a function other than transduction of the canonical Wnt signal. Identification of genes that Wnts regulate during gut organogenesis may help resolve these questions.

Stomach

The role of Wnt signaling in stomach development seems to be very different from that in the intestine, and whereas adult intestinal crypts depend on Wnts for myriad functions, gastric gland units, which are operationally similar, do not display a similar dependence. Nor do gastric cancers share a link to the canonical Wnt pathway.[51,52]

Mouse embryonic stomach endoderm closely resembles its intestinal counterpart until approximately gestational day 12; modulation of Wnt signaling after this stage appears to be critical for stomach differentiation. An insight into how stomach mesenchyme specifies its overlying epithelium came through the study of mice and cells lacking the homeodomain transcription factor Barx1, which is specifically and highly expressed in embryonic stomach mesenchyme. Barx1$^{-/-}$ embryos develop a rudimentary stomach that is notable for ectopic presence of intestinal epithelium in the distal segment, indicating a homeotic transformation.[53,54] In recombinant cultures of embryonic tissue, normal stomach mesenchyme induces stomach epithelial marker expression in overlying endoderm, whereas mesenchyme depleted of Barx1 induces intestinal epithelial genes instead. Findings in both cultured tissue and mice hence reveal Barx1 as a pivotal factor in mesenchymal specification of stomach epithelium; furthermore, Barx1 imparts proper morphology and size to the developing stomach. A search for the molecules that might mediate mesenchymal induction of stomach epithelium uncovered two prime candidates, Sfrp1 and Sfrp2, soluble extracellular Wnt antagonists. Expression of these factors requires Barx1 and exogenous Sfrp restores the ability of Barx1-deficient mesenchyme to induce stomach-specific epithelium in vitro.[53]

These observations strongly suggest an inhibitory role for Wnt signaling in stomach organogenesis and for Barx1 in suppressing that signal to enable stomach epithelial specification; Wnt function in stomach development is hence distinct from that in the intestine. In the TOP-GAL reporter mouse strain,[34] which expresses β-galactosidase (LacZ) in sites of canonical Wnt activity, a strong signal appears in normal mouse stomach endoderm by the 9th gestational day, begins to decay soon thereafter, and is no longer detectable in stomach mucosa at E16.5, when the Wnt pathway is first activated in intestinal villi. The purpose for Wnt signaling in the prospective stomach is unclear, but it is confined to the endoderm in a domain that corresponds very closely to the restricted expression of Barx1 in the underlying mesenchyme. Consistent with the idea that Barx1 acts to suppress Wnt signaling in adjacent endoderm, Wnt activity is protracted in Barx1-null stomach, where it persists at least until birth.[53,54] Ectopic, Barx1-independent activation of the Wnt pathway using a floxed, activating β-catenin allele[55] produces a stomach phenotype that resembles the Barx1-null organ, including ectopic expression of intestinal markers.[54] This result independently validates the inhibitory role of Wnt signaling in stomach development.

Reduction or loss of Sfrp expression in the Barx1$^{-/-}$ stomach implies that Barx1 antagonizes Wnt signaling at least in part through Sfrp genes. However, it is unclear if these are direct transcriptional targets for Barx1 regulation or which Wnt ligands are relevant in stomach organogenesis. As in the intestine, which expresses multiple Wnt mRNAs,[47,49,50] Heller and colleagues demonstrated the presence of several Wnt transcripts in various zones in fetal foregut mesenchyme.[56] They also forced expression of Wnt1 and Wnt5a under control of the *Pdx1* promoter, which is active in the distal stomach and proximal duodenum.[57] Wnt1 overexpression extended stomach-type differentiation into the proximal intestine, which represents a phenotype opposite to that of *Barx1*$^{-/-}$ mice, and in some cases the distal stomach was duplicated; the pyloric sphincter failed to develop.[56] Wnt5a overexpression reduced stomach size without affecting shape or histology. Wnt5a is also found in chick embryonic stomach mesenchyme during mucosal differentiation and its overexpression seems to promote activation of stomach-specific epithelial genes.[58] Because these studies rely on gene overexpression and occur outside of a physiologic context, their connection with normal development may be limited. The role and gene targets of Wnt activity in the stomach before epithelial specification are unknown.

Observations in various experimental models raise the intriguing possibility that repression of Wnt signaling is a general requirement

for organogenesis in foregut derivatives. Forced Wnt activation induces intestinal differentiation in prospective mouse lung[59] and stomach.[54] In early Xenopus embryos, Wnt/β-catenin activity also needs to be repressed in anterior endoderm to permit liver and pancreas development. High endogenous β-catenin activity in posterior endoderm promotes intestinal differentiation and represses foregut fates; inhibition of β-catenin activity in this region induces premature expression of early liver and pancreas markers.[60] One simplistic possibility, observed in different organs and multiple organisms, is that Wnt signaling in endodermal derivatives leads to intestinal differentiation as the default[53,59,60] and that distinct mechanisms operate in different regions of the digestive tract to suppress Wnt activity and allow alternative tissue fates.

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