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Review

Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision

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Continuous renewal of the intestinal epithelium requires coordinated regulation to maintain the balance between proliferation and differentiation of the epithelial stem cells and immature progenitor cells. Canonical Wnt signaling has long been regarded as the signaling pathway playing a central role in this epithelial cell fate determination; however, recent studies have shown that Notch signaling is also indispensable for this process. Here, we review the current concepts of how the Wnt and Notch pathways control intestinal epithelial cell fate decisions, particularly focusing on their crosstalk at both tissue and cellular levels. As several features are shared between stem cell renewal and cancer cell renewal, comprehensive understanding of how the Wnt and Notch signaling pathways cooperate and integrate in the gut epithelium has significant implications for the development of novel therapeutic modalities for intestinal neoplasia.

Key words: intestinal epithelial cell, Wnt, Notch, bHLH transcription factor, Hath1

Introduction

A variety of intestinal epithelial functions, such as digestion and absorption of nutrients and protective functions against luminal pathogens, are supported by epithelial cells of multiple lineages that are organized into crypt–villus units along the vertical axis. The small intestinal epithelium comprises differentiated cells of four principal lineages (absorptive enterocytes and three secretory lineages consisting of goblet, enteroendocrine, and Paneth cells), whereas the colonic epithelium consists mostly of absorptive enterocytes and goblet cells. The homeostasis of this multicellular tissue organization is maintained through the presence of stem cells residing near the base of crypts.[1–4] The stem cells are able to self-renew throughout life and generate several types of more committed precursor cells that actively divide within the proliferative compartment spatially confined to the lower part of the crypt. After several rounds of cell division, these transit-amplifying precursor cells differentiate into one of the mature cell lineages, migrate upward to the top of the vertical axis, and are exfoliated into the lumen, whereas Paneth cells migrate down to the crypt base.

The stem cell-based tissue renewal in the intestine requires coordinated regulation to maintain the balance between proliferation and differentiation. From the viewpoint of structural organization of an entire crypt–villus unit, the term "proliferation" refers to the process by which precursor cells replicate so as to expand the cellular pool to ensure the cell number in individual units, whereas "differentiation" refers to the process by which the transit-amplifying precursor cells give rise to functionally mature cells of a particular lineage. Even under normal conditions, the finely tuned equilibrium between proliferation and differentiation is critically important to maintain the appropriate size, shape, and function of the epithelium. In addition, the regulation to maintain this balance is highly flexible and dynamic, because the proliferation of precursor cells is accelerated to renew more than the steady-state number of cells in response to epithelial injuries.[5,6] This clearly indicates that certain signaling mediators exist to sense the alteration of the tissue environment and instruct epithelial progenitor cells to appropriately proliferate or differentiate. In addition, such coordinated maintenance of the epithelial homeostasis must be strictly linked to the cell fate decision at the single cell level. "Cellular" proliferation requires functional cooperation of specific machineries to execute precise programs

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for the progression through the cell cycle, whereas the differentiation process, which induces a novel program of gene expression for a specialized phenotype, should be accompanied by cell cycle arrest. This indicates that the decision whether to proliferate or to differentiate must be carefully determined during each cell cycle of individual cells, and that the mechanism regulating this determination might be the direct target of the extracellular signaling mediators.

One of the important signaling systems regulating the intestinal epithelial homeostasis is the canonical Wnt pathway. Since it was found that a large number of hereditary and sporadic cases of colorectal carcinoma are associated with mutations of the tumor suppressor adenomatous polyposis coli (*APC*) gene, a number of studies have established the canonical Wnt pathway as a key regulator of proliferation of the stem cells as well as of the transit-amplifying cells in the intestinal crypts.[7] More recently, however, a fascinating picture has emerged with regard to the fundamental roles of a family of bHLH (basic helix-loop-helix) transcription factors and its upstream Notch signaling in regulating the differentiation and cell-type specification of intestinal epithelial cells. Therefore, it is becoming apparent that intestinal homeostasis is finely controlled by the integration of multiple signaling pathways, such as Wnt and Notch signaling, and recent studies have begun to answer questions about how these multiple signals interact and collaborate with one another. In this article, we review the recent advances in our understanding of how the homeostasis of the gut epithelium is controlled, particularly focusing on the crosstalk between the Wnt and Notch pathways and the integrative signaling mechanisms at both tissue and cellular levels.

Canonical Wnt signal and intestinal epithelial proliferation

Wnt proteins are secreted glycoproteins, of which there are about 20 in mammals. The classical view of this pathway is that, upon binding to their receptors, Wnt proteins induce intracellular inactivation of glycogen synthase kinase3β (GSK3β), a component of the so-called destruction complex, which also contains APC and axin. The resultant dephosphorylation and stabilization of β-catenin, a substrate of GSK3β, lead to the nuclear translocation of β-catenin and activation of target genes by the complex consisting of β-catenin and the TCF family of transcription factors.[8]

A large body of evidence shows that activation of the canonical Wnt pathway is essential to maintain the crypt cell population in a proliferative state. The nuclear accumulation of β-catenin is preferentially observed in cells located at the base of crypts and decreases as cells move toward the top of the crypts.[9] This clearly indicates that the transit-amplifying progenitors are under the influence of the Wnt signal, whereas terminally differentiated cells, other than Paneth cells, are in areas where the canonical Wnt signal is inactive. Consistently, it has been shown that in β-catenin or TCF4 knockout mice, or when Wnt signaling is inactivated by overexpression of its diffusible inhibitor Dkk1, there is a significant loss of proliferative epithelial cells.[10–13] Conversely, when the Wnt pathway is overactivated by mutations in *APC* or *β-catenin*, many of the epithelial cells enter into the proliferative state and display a failure of the differentiation programs in these cells.[14–16]

These in vivo data suggest that Wnt signaling is directly linked to the promotion of cellular proliferation and, more specifically, the regulation of progression through cell cycle. In this regard, previous papers pointed to the downregulation of $p21^{CIP1/WAF1}$, a cyclin-dependent kinase inhibitor (CKI), as an important mechanism that might mediate Wnt-dependent growth promotion. A microarray analysis showed that $p21^{CIP1/WAF1}$ was one of the genes whose expression was increased by inhibition of Wnt signaling in human colorectal cancer-derived LS174T cells.[9] In an experimental system employing doxycycline-inducible expression of dominant-negative (dn) TCFs, the inhibition of Wnt signaling caused the cell cycle arrest at the G1 phase, and this growth inhibition was mediated by downmodulation of $p21^{CIP1/WAF1}$ expression levels. Furthermore, the TCF-4 target gene *c-Myc*[17] has been shown to play a central role in Wnt-mediated repression of $p21^{CIP1/WAF1}$ expression at the transcriptional level through its direct binding to the $p21^{CIP1/WAF1}$ gene promoter.[9] These data suggest that *c-Myc* gene induction and the subsequent repression of $p21^{CIP1/WAF1}$ by c-Myc might be the intracellular mechanism by which Wnt signaling regulates the G1/S transition and cell cycle progression.

Indeed, such a signaling cascade has been shown to be, at least in certain contexts, functional in vivo, because abnormal features of proliferation/differentiation in the adult murine intestine, which occur with the single deletion of *APC*, are mostly rescued when the *c-Myc* gene is simultaneously deleted.[18] Furthermore, this restoration of the morphologically normal phenotype in double mutant mice for *APC* and *c-Myc* is accompanied by restoration of *p21* expression within the crypts, further suggesting the involvement of $p21^{CIP1/WAF1}$ in the Wnt–c-Myc pathway-mediated growth control of progenitor cells. However, it remains unclear whether this p21-mediated mechanism also plays a role in maintaining epithelial homeostasis under the physiological conditions, since no obvious G1 arrest and no induction of $p21^{CIP1/WAF1}$ are observed when *c-Myc* alone is conditionally deleted.[19,20] This raises the possibility that the Wnt–

c-Myc–p21 axis of signaling is important for crypt cell regulation only in the context of aberrantly activated Wnt signaling and is dispensable for normal intestinal epithelium homeostasis, although further studies are required to clarify this issue.

Nevertheless, the proposed role of the Wnt–c-Myc–p21 axis of signaling still seems interesting in terms of its function as an intracellular molecular switch between proliferation and differentiation. It has been shown that, in LS174T cells, conditional expression of $p21^{CIP1/WAF1}$ alone is able not only to induce G1 arrest but also to allow cells to differentiate, as assessed by the upregulation of several marker genes.[9] This finding is con-sistent with the previous findings that CaCo2 cells, generally used as a model for the spontaneous differentiation of intestinal epithelial cells, also involve the upregulation of $p21^{CIP1/WAF1}$ when they become confluent and differentiate.[21] These data suggest that, at least in certain circumstances, the cell fate choice between proliferation and differentiation is regulated by modulation of the expression of $p21^{CIP1/WAF1}$ via the direct induction of c-Myc by Wnt signaling.

Notch signaling and cell-type specification in intestinal epithelium

Recent studies have revealed that Notch signaling controls selective cell fate decisions and subsequent differentiation in the intestine. Notch receptors and their ligands are transmembrane proteins that mediate cell–cell communication. Upon binding to their ligands, Notch proteins are cleaved by γ-secretase, and, in turn, the Notch intracellular domain (NICD) translocates to the nucleus, where it activates transcription of genes that are targeted by the nuclear NICD/RBP-J complex.[22,23] Several components of the Notch pathway are expressed in adult intestinal crypt cells, suggesting a role for Notch signaling in gene expression programs in immature proliferating compartment cells.[24,25]

The first evidence that Notch signaling plays a role in cell-type specification in the intestine was reported in *Hes1* (hairy and enhancer of split 1) knockout mice.[26] *Hes1* is a bHLH-type transcriptional repressor[27] whose expression is transcriptionally activated by the Notch signal.[28] The deletion of the *Hes1* gene resulted in the generation of excessive numbers of goblet cells, enteroendocrine cells, and Paneth cells, all of which possess a secretory character.[26] Subsequently, it was shown that the gene encoding another bHLH factor, *Math1* (mouse atonal homolog1), one of the genes repressed by Hes1, is required for the differentiation into the three secretory lineages, because the intestinal epithelium of *Math1*-mutant mice is populated only by absorptive cells.[29] These data suggest that the choice between the absorptive or secretory fate might be the first decision made by each progenitor cell, and that Hes1 and Math1 play opposite roles in this decision making. A number of studies have since revealed how differentiation into each secretory lineage is regulated in the gut epithelium. For example, *Ngn3* (neurogenin3), which also encodes a bHLH-type transcription factor, is indispensable for the cell fate decision in some later steps than that regulated by Hes1/Math1, since $Ngn3^{-/-}$ mice lack enteroendocrine cells, while the other three cell lineages remain unaffected.[30] Likewise, it has been shown that the choice of lineage commitment among the Math1-specified secretory cell types is controlled by several other transcriptional regulators such as NeuroD[31], KLF4 (Kruppel-like factor 4),[32] or Gfi1.[33] These studies are of considerable importance for understanding the stepwise regulation of the differentiation into each lineage of epithelial cells and for clarifying the molecular hierarchy during this process; however, this issue is beyond the scope of this article and is reviewed elsewhere.[34]

Signaling crosstalk between Wnt and Notch pathways for balancing intestinal proliferation/differentiation

It should be emphasized that Math1-deleted mice show not only the loss of terminally differentiated secretory cells but also an increase in the population of secretory progenitors in the proliferative compartment.[29] Thus, the function of Math1 might be coupled not merely with the alteration of cellular phenotypes but also with the cell fate determination between proliferation and differentiation of the transit-amplifying cells.

Such a role of Math1 in the cell fate decision has been further supported by several studies in which the more extreme effects of upstream Notch signaling on gut homeostasis were revealed. Inactivating Notch signaling by conditional targeting of *RBP-J* leads to expansion of the goblet cell population, together with the significant loss of proliferative compartment cells.[35] Conversely, overactivation of Notch signaling by the conditional expression of NICD in the intestinal epithelium results in inhibition of all secretory lineages, together with the amplification of the intestinal progenitor pool.[36] These results clearly suggest that Notch signaling is required for maintaining crypt cells in a proliferative state, at least in part, through its negative regulation of Math1. Intriguingly, the effects of overexpressed NICD on cellular proliferation are discernible only in the intervillus regions, where Wnt signaling is physiologically active.[36] Conversely, enhanced Notch signaling activity is observed in the multiple intestinal neoplasia (APC^{min}) mice, which display hyperactivation of the Wnt signal because of a mutation of the *APC*

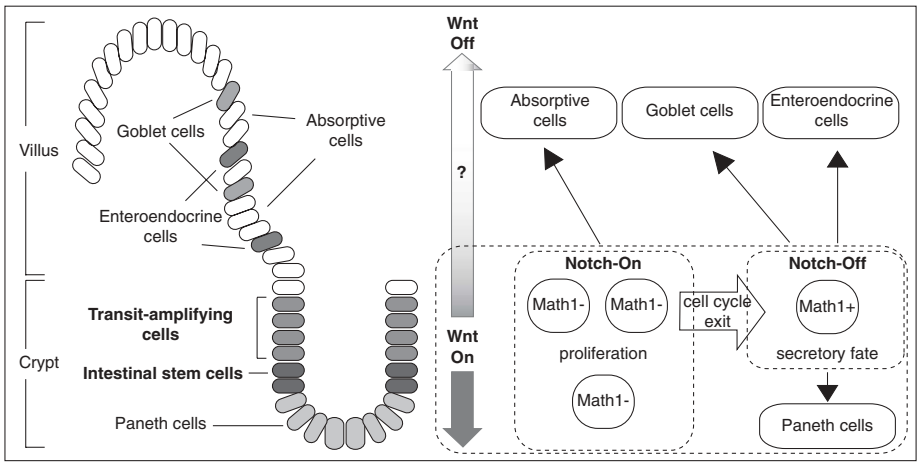

Fig. 1. The role for Wnt and Notch pathways in intestinal epithelial proliferation and differentiation. Organization of the intestinal epithelium within a crypt–villus unit is depicted on the left. The common pluripotent stem cells can give rise to four lineages of terminally differentiated cells; one is absorptive and the other three (goblet, enteroendocrine, and Paneth cells) have secretory phenotypes. The stem cells reside at position 4–5 from the crypt base, where the Paneth cells are located. Transit-amplifying cells produced from the stem cells actively divide within a crypt region, ensuring the number of cells in each crypt. These progenitors migrate upward as they differentiate into absorptive cells, goblet cells, and enteroendocrine cells, whereas they move downward as they become Paneth cells. Wnt signaling plays an essential role in the proliferation of both stem cells and transit-amplifying cells. The nuclear accumulation of β-catenin is preferentially observed in cells located at the base of crypts and decreases in a gradient as cells move upward, suggesting that Wnt proteins are abundant in the lower part of the crypt. However, the precise border or the area where the Wnt signaling in each progenitor or its offspring becomes inactivated remains unclear. Within the proliferative compartment, the choice between proliferation and commitment to a secretory lineage is made in each progenitor cell, involving the bHLH transcription factor Math1, which is negatively regulated by Notch signaling. Notch-activated cells in the Wnt-activated population keep proliferating, and are thought to give rise to enterocytes, whereas inactivation of Notch in this population leads to commitment to a fate in a prospective secretory lineage via a cell cycle exit and Math1 expression

gene,[14] suggesting that the Wnt signal is mechanistically epistatic to the Notch signal.[35]

Together with the established role of Wnt signaling in regulating crypt epithelial progenitors, these collective findings suggest the following model.[37] The multipotent progenitors require both Wnt and Notch signals to be activated for fulfilling continuous proliferation without differentiation. Once some cells in this Wnt- and Notch-activated population escape from the Notch signal, they stop proliferating and are directed toward the secretory fate, in parallel with the acquisition of the Math1 function. Even when one considers that terminally differentiated secretory cells eventually reside in areas where the Wnt signal is not active, it is likely that they need to be under the influence of Wnt signaling for some time before terminal differentiation, since all classes of secretory cells are lost when Wnt signaling is completely blocked.[11] Meanwhile, if cells in this Wnt- and Notch-active population lose the Wnt signal, for example, because of their positional changes along the vertical axis, they differentiate as absorptive cells. Although there still remain many things to be clarified, the model described here depicts the current understanding of how the Wnt and Notch pathways cooperate to control intestinal epithelial homeostasis (Fig. 1).

Intracellular mechanisms supporting the crosstalk between the Wnt and Notch pathways

What are the mechanisms that link such integrative but complicated signaling by Wnt and Notch to the cell fate decision of individual cells? One possible explanation is that Notch signaling or its downstream component, Math1, is directly involved in the cell fate decision, and the Wnt signal is simply modulating or enhancing the Notch signal. In this context, Leow et al.[38] have shown that overexpression of Hath1, the human homolog of Math1, in an aggressive colon cancer cell line, HT29, inhibits its proliferation both in vitro and in vivo. Expression of CKI $p27^{Kip1}$ as well as the differentiation marker gene *MUC2* is upregulated together with this growth inhibition by Hath1, whereas that of the cell cycle regulator cyclin D1 is downregulated.[38] Importantly, it was shown that Hath1 mRNA expression is repressed by constitutively activated Wnt signaling in colon cancer cells. These data indicate the presence of hierarchical regulation between Wnt and Notch pathways in intestinal epithelial cells at the single cell level. It would be of considerable importance to extend their study and address the questions as to what the molecular mechanism is by which Wnt signaling leads to the

repression of Hath1 mRNA, and how the Hath1-dependent alteration of a transcriptional program results in the changes in expression levels of cell cycle regulators.

Another possible mechanism is that the Wnt and Notch signals might converge in a certain cell cycle control step. In this context, by using several human nonintestinal cell lines, Notch has been shown to induce degradation of CKIs such as $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ through RBP-J-mediated transcriptional activation of *SKP2* (S-phase kinase-associated protein 2), the F-box subunit of the ubiquitin-ligase complex SCF^{skp2}.[39] Considering the possible involvement of the Wnt–Myc-dependent downregulation of $p21^{CIP1/WAF1}$ in cell cycle regulation as discussed earlier in this article, this observation is quite interesting, since it can be postulated that Wnt and Notch signaling might modulate $p21^{CIP1/WAF1}$ function at the transcriptional and posttranslational levels, respectively, and that this dual regulation of $p21^{CIP1/WAF1}$ might control the G1/S transition in each progenitor cell. It is known that the mode of cell cycle regulation by Notch signaling is highly cell type- or cellular context-dependent, and, therefore, it would be of importance to investigate such effects of Notch signaling on cell cycle regulators in the context of intestinal epithelial cells or tissues, particularly in association with Wnt signaling activity.

In addition to these possible interplays, we have recently reported another level of crosstalk between the Wnt and Notch pathways. By using several human colon cancer cell lines, we found that the Hath1 protein is targeted by proteasome-mediated degradation via its phosphorylation by GSK3β in a tissue-specific manner.[40] Importantly, Hath1 is degraded only when the Wnt signal is active and β-catenin escapes from its GSK3β-mediated phosphorylation and proteolysis. On the other hand, once the Wnt signal is inactivated, Hath1 becomes stabilized and exerts its function as a transcriptional activator, while β-catenin is destabilized by the well-characterized mechanism. These data suggest that Wnt signaling directly regulates the balance between the proliferation and differentiation programs of a cell, taking advantage of GSK3β as a molecular switch between β-catenin and Hath1 functions. Indeed, it was shown that Hath1 expression is repressed at the protein level in some human colon cancer tissues in which the Wnt signal is activated, strongly suggesting that this mechanism might be involved in growth promotion as well as in the maintenance of the undifferentiated state of the cancer cells.[40] Meanwhile, given that the inactivation of Notch signaling even under Wnt-active conditions results in Math1 protein expression and the differentiation of some progenitors into secretory lineages,[35] the Wnt–GSK3β-mediated mechanism of Hath1 proteolysis might seem not to conform to the simplified model described before. However, it can be speculated that this mechanism might act to ensure that Hath1 proteins are fully activated in the transit-amplifying cells as they differentiate and move out of the Wnt-active area, guiding these cells into the following steps of terminal differentiation in vivo. Identification of the precise mechanism by which Hath1 is destabilized by Wnt signaling, or how this Wnt–GSK3β-dependent Hath1 proteolysis is involved in the maintenance of gut homeostasis in vivo, might help us understand the multistep interaction and collaboration between Wnt and Notch signaling in the intestinal epithelium.

Conclusion

Recent works have established the essential functions of the Wnt and Notch pathways in regulating intestinal epithelial homeostasis; however, growing evidence has indicated that the cell fate determination and lineage commitment of intestinal epithelial cells are not governed by a simple rule, but by the complicated and multilayered interaction between these pathways. Moreover, the intracellular mechanism by which the Wnt and Notch pathways function together to control proliferation or differentiation of individual cells remains to be clarified. We are just beginning to understand the complex interplay between these signaling pathways, and future studies will reveal in more detail the mechanisms by which their crosstalk contributes not only to the physiological process of self-renewal but also to the regenerating process after injuries to the gut epithelium. Furthermore, as significant similarities are likely to exist between the behavior of intestinal epithelial progenitor cells and that of cancer progenitor cells, advances in this field will also provide significant insight into how the Wnt and Notch pathways coordinately drive oncogenic potential and what might be novel targets for improved treatments for intestinal cancers.

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