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NOTCH Signaling and ATOH1 in Colorectal Cancers

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Abstract

The Notch receptor signaling pathway regulates expression of the basic helix-loop-helix transcription factor ATOH1 (Math1/Hath1) to determine cell fate in the intestine. In differentiating intestinal stem cells, high levels of Notch activity specify absorptive enterocyte/colonocyte differentiation, whereas high ATOH1 activity specifies secretory (goblet, enteroendocrine, and Paneth) cell differentiation. In colorectal cancer, ATOH1 is a tumor suppressor that is silenced in most tumors, while Notch is oncogenic and often highly active in human tumors. In other gastrointestinal malignancies with features of intestinal metaplasia, such as esophageal and gastric cancers, the Notch-ATOH1 pathway becomes activated. In cancers and preneoplastic tissues that retain the ability to activate ATOH1, therapeutic targeting of this pathway can be achieved by inhibiting Notch activity (with Notch-targeting antibodies or small-molecule inhibitors of γ -secretase). Thus, targeting the Notch-ATOH1 pathway represents a novel approach to differentiation therapy in gastrointestinal cancers.

Keywords

Notch; Delta-like; Jagged; Atoh1; Math1; Hath1; γ -secretase; Intestine; Colon; Crypts of Lieberkühn; Stem cell; Cancer; Differentiation

Introduction

Molecular pathways that regulate development and homeostasis of the adult intestinal epithelium are frequently misregulated in intestinal cancers and represent potential targets for new therapeutics. The Notch-Atoh1 signaling pathway is key to determining self-renewal versus differentiation of intestinal stem cells. The Notch-Atoh1 pathway has emerged as an important regulator of colorectal cancer (CRC) and thus represents a potential therapeutic target that could prove valuable in our fight against CRC. Here we will review the current model of cell fate determination that is controlled by the Notch-Atoh1 pathway, contribution of this pathway to gastrointestinal cancers, and its impact on therapeutic approaches that utilize Notch as a molecular target.

The Notch-Atoh1 Pathway in Normal Intestinal Development and Differentiation

The intestinal epithelium is a dynamic self-renewing tissue that consists of multiple cell lineages that are actively and constantly renewed throughout the life of the organism. The crypts of Lieberkühn are the proliferative compartment of the intestine with stem cells residing near the base of colonic crypts. The stem cells give rise to rapidly proliferating transit-amplifying cells that differentiate into three main types of cells: colonocytes (enterocytes in the small intestine), goblet, and enteroendocrine cells. Paneth cells constitute the fourth major cell type that reside adjacent to stem cells at the base of small intestinal crypts. These major cell types are classified either as absorptive (colonocytes or enterocytes) or secretory (goblet, Paneth, and enteroendocrine cells), reflecting distinct genetic programs underlying differentiation of each class.

Notch Signaling is Essential for Making the Lineage Fate Decisions for Developing Epithelial Cells to Become Either an Absorptive or a Secretory Cell Notch proteins (Notch1, 2, 3, and 4) are type I transmembrane receptors which require cleavage for their activation. When bound by ligands (Delta-like 1, 2, 3 and Jagged 1 and 2 proteins) on adjacent cells, the Notch receptors undergo cleavage at the extracellular and intracellular faces of the plasma membrane to release the Notch intracellular domain (NICD) [1]. Cleavage of Notch occurs first on the extracellular side by the disintegrin and metalloproteinases ADAM10/ADAM17, which allows subsequent cleavage at the intracellular side by the γ -secretase complex to release NICD. NICD subsequently translocates to the nucleus, where it displaces transcriptional repressors and recruits transcriptional activators to convert the CSL (*RBPJ*) complex from transcriptional repression to activation (Fig. 1a). In the adult intestine, Notch1 and Notch2 are expressed in the crypt epithelium, and ligands Dll1, Dll4, and Jag1 are expressed in scattered cells within the crypts [2]. Studies of transgenic animals expressing NICD in the intestinal epithelium found altered intestinal secretory cell production [3, 4]. Conversely, genetic models that selectively delete Notch1 and Notch2 or its DNA-binding partner, CSL (*Rbpj*), in the intestinal epithelium of mice, produce extra intestinal secretory cells [5, 6, 7]. Similar effects were observed in transgenic mice in which Notch activity was modified by other genetic manipulations. Loss of Notch activity and conversion of progenitors to secretory cells was observed in animals with defective glycosylation of Notch, caused either by mutation of the fucosyl transferase POFUT1 or by mutation of the enzyme responsible for fucose synthesis (FX), or by mutation of Mindbomb1, a protein essential for endocytosis of Notch ligands and efficient Notch activation [8–10]. Importantly, conversion of progenitors to secretory cells was also observed when animals are treated with small-molecule inhibitors of γ -secretase (GSIs), which prevent ligand-induced cleavage of Notch and production of NICD (Fig. 1a) [11–13]. Altered cellular differentiation was also shown in zebrafish and fruit flies with abnormal Notch signaling [14, 15]. Thus, Notch activity has been shown to promote differentiation of intestinal absorptive cells and to block differentiation of intestinal secretory cells.

Notch Selects Hes1 Versus Atoh1 in the Intestinal Epithelium *Hes1* (hairy and enhancer of split 1, a basic helix-loop-helix transcriptional repressor), a key target of Notch transcriptional activity, is important for differentiation of the intestinal absorptive cells. *Hes1*-null mice develop too many goblet, Paneth, and enteroendocrine cells, at the expense of absorptive enterocytes [16, 17]. *Atoh1* (atonal homolog 1, also called Math1 or HATH1, a basic helix-loop-helix transcriptional activator) is transcriptionally repressed by *Hes1*, and plays a reciprocal role to positively promote secretory lineage differentiation (Fig. 1a). *Atoh1*-null mice fail to form any secretory cell types and mice die shortly after birth [18]. Intestine-specific *Atoh1* knockout mice, which used the mosaic *Fabp-cre* strain to produce a crypt-by-crypt mosaic of *Atoh1*-null and adjacent wild-type tissue, demonstrated that *Atoh1*

is essential for intestinal secretory cell commitment in the adult intestine, with epithelial progenitors undergoing a secretory-to-absorptive fate switch [19]. Conversely, transgenic *Atoh1* overexpression converts progenitor cells into the secretory fate [20]. Together, these studies show that *Atoh1* and Notch play opposing roles in promoting secretory versus absorptive intestinal epithelial cell types. Thus, the relative activity of Notch versus *Atoh1* determines whether an intestinal progenitor adopts an absorptive (Notch active) or secretory (*Atoh1* active) fate (Fig. 1a).

Mechanisms by which Notch and ATOH1 transcriptional activity are reciprocally regulated remain an area of investigation. Notch receptors are active in epithelial progenitor cells including the intestinal stem cells, which require Notch activity for their maintenance [6, 7, 21]. Delta-like ligands Dll1 and Dll4 are expressed by goblet and Paneth cells, likely under the control of ATOH1 [22, 23•, 24], whereas the expression pattern of Jagged ligands Jag1 and Jag2 is less clear, but is likely regulated by the Wnt/ β -catenin pathway [2, 22, 25, 26•, 27]. Thus, in the intestinal crypt, the level of Notch ligand exposure of intestinal stem and progenitor cells is likely to be critical for determining stem cell renewal or differentiation into absorptive or secretory lineage cells. However, in the absence of ATOH1, Notch activity is dispensable for stem cell renewal, proliferation, and absorptive cell differentiation [23•], suggesting that the key role for Notch is to regulate expression of ATOH1 and consequently cell fate.

The Wnt/ β -catenin pathway is also involved in regulating ATOH1 expression. GSK3- β can phosphorylate ATOH1 and target it for ubiquitination and degradation by the proteasome (Fig. 1b) [28, 29•]. This targeted degradation appears reciprocal to the involvement of GSK3- β in the Wnt/ β -catenin pathway. When the Wnt pathway is inactive, β -catenin is phosphorylated by GSK3- β and targeted for degradation by the Axin/APC complex (Fig. 1a). In this context, ATOH1 escapes GSK3- β phosphorylation and is spared from degradation [29•, 30]. Upon Wnt/ β -catenin pathway stimulation, the APC/Axin complex is disrupted and β -catenin escapes phosphorylation and degradation; in this context, GSK3- β phosphorylates and targets ATOH1 for degradation (Fig. 1b). Several genes have been identified as transcriptional targets of both active Notch and β -catenin, including *Hes1* (Fig. 1b), which in turn transcriptionally represses ATOH1 [26•, 30]. Together, the Notch and Wnt pathways use several mechanisms to regulate ATOH1 expression to control cell fate determination within the intestinal epithelium. A more complete understanding of how crosstalk between the Notch and Wnt pathways impacts intestinal homeostasis as well as tumorigenesis requires further investigation.

The Role of Notch in Colorectal Cancer

Notch pathway activation is known to be procarcinogenic in many organ systems [31]. Increasing evidence supports the involvement of Notch signaling in colorectal carcinogenesis. *NOTCH1* and *NOTCH2*, as well as *JAGGED* ligands and *HES1*, are expressed in human colon adenocarcinomas and colorectal cancer cell lines [25, 30, 32, 33, 34•]. Active Notch and its transcriptional target *Hes1* are also enriched in adenomas from *APC* mutant mice [6, 7, 25, 30, 34•]. Overexpression of NICD in *APC* mutant mice enhanced tumorigenesis [34•], whereas deletion of one allele of *Jag1* reduced tumor formation [26•]. The mechanism by which Notch is activated in colon cancer remains undefined. However, the E3 ubiquitin ligase responsible for targeting NICD for degradation, FBW7, is frequently mutated in colon cancer [35]; mutation of *Fbw7* enhanced tumor formation in mice, likely via a Notch-dependent mechanism (Fig. 1a and b) [36]. Given the ascribed gatekeeper role for Notch signaling in proliferation and differentiation of the intestinal epithelium, a contributory role for Notch signaling in colorectal tumorigenesis is likely.

Additional evidence supporting a potential role for Notch signaling involvement in intestinal tumorigenesis came from the treatment of *APC^{min/+}* mice with γ -secretase inhibitors (GSIs). This treatment resulted in reduced proliferation, *Atoh1* overexpression, and differentiation of some adenoma cells to nonproliferating goblet cells [6]. More recently, GSI treatment was shown to quantitatively shrink adenomas in *APC^{min/+}* mice [37]. Experiments in CRC cell lines showed a minimal effect of GSI alone in general; however, a subset of cell lines that can potentially respond to GSI treatment were also identified (discussed further below). Nonetheless, a significant proapoptotic effect of GSI on CRC cell lines was observed when given alone [33] or in combination with cytotoxic drugs such as taxanes or platinum compounds [38, 39]. In addition to GSIs, antibody-mediated inhibition of NOTCH1 blocked growth of CRC cells with less effect on normal intestinal differentiation [40]. Thus, Notch signaling represents a potential target for antitumor therapy of colorectal cancer.

The Role of *Atoh1* in Colorectal Cancer

Regulation of *Atoh1* by Notch signaling is well described in intestinal development and adult epithelial homeostasis. *Atoh1* expression opposes Notch function and commits cells to differentiate into secretory cells. In accordance with the contributory role of Notch in tumorigenesis, a definitive tumor suppressor function has been described for ATOH1 in CRCs [28, 41•, 42]. Analysis in human colon tumor cell lines and primary tumors showed a significant decrease of *ATOH1* expression in approximately 70% of CRCs, with significantly lower expression in carcinomas compared to adenomas [28, 41•]. Examining the mechanism of *ATOH1* silencing with gene copy number analysis demonstrated at least one deletion in approximately 50% of tumors, but sequencing did not identify point mutations. Additional DNA methylation analysis of the CpG island encompassing the *ATOH1* transcription start site and coding region showed *ATOH1* CpG methylation in approximately 70% of tumors (Fig. 1b) [41•]. These data showed that *ATOH1* is frequently silenced in CRC by both genetic and epigenetic mechanisms.

Forced expression of *ATOH1* in the CRC cell line HT-29 inhibited proliferation and growth in soft agar and xenografts [28]. Further evidence of the antiproliferative tumor suppressor function of *Atoh1* was determined in animal models. Intestine-specific deletion of *Atoh1* in mice (*Atoh1^{Δintestine}*) was used in several mouse models of CRC. Treating *Atoh1^{Δintestine}* mice with azoxymethane, a chemical carcinogen that induces colonic tumors, showed a significant enhancement of tumorigenesis in *Atoh1^{Δintestine}* mice compared to wild-type littermates. Similar analyses showed a significantly greater tumor burden in the colons of *Atoh1^{Δintestine}; APC^{min/+}* double-mutant mice compared to *APC^{min/+}* mice [41•]. Enhanced tumor formation was also observed upon acute deletion of *Atoh1* and *APC*, supporting the concept that silencing of *Atoh1* augments early tumor formation [30]. Increased colitis-associated tumorigenesis was also observed in *Atoh1^{Δintestine}* mice (our unpublished data). Together, these data firmly establish *Atoh1* as a tumor suppressor, and also provide a robust colonic tumorigenesis model in mice.

The relationship between *Atoh1* and Notch in colon tumors was illuminated by GSI studies. Initially, GSI treatment of *APC^{min/+}* adenomas showed reduced proliferation, increased *Atoh1* expression, and differentiation of cells into postmitotic goblet cells [6]. In fact, re-expression of *Atoh1* in adenomas is the key molecular event underlying the effect of γ -secretase inhibitors on tumors. The role of *Atoh1* in response to GSIs in tumorigenesis was determined in *Atoh1^{Δintestine}; APC^{min}* double-mutant mice compared to *APC^{min/+}* neoplasms. Tumors lacking *Atoh1* showed no response to GSI treatment, with no reduction in proliferation nor differentiation into secretory goblet cells [23•]. These data indicate that a diametric relationship between Notch and *Atoh1* occurs both in normal differentiation and in tumorigenesis, where Notch is oncogenic and *Atoh1* is tumor suppressive.

Consistent with these findings in mouse models of colon cancer, the ability of CRC cell lines to respond to GSI-induced Notch inhibition was Atoh1 dependent. CRC cells such as LS174T and HT29 Cl16E, which retained the ability to upregulate ATOH1, respond to GSI treatment with a significant decrease in proliferation and upregulation of secretory (goblet) cell markers [23•, 25]. However, CRC cells that have lost the ability to express ATOH1, such as by deletion or methylation (as discussed above for human colon tumors), show no effect of GSI treatment on proliferation or differentiation [23•]. Loss of APC has been associated with degradation of ATOH1, suggesting another level of regulation of ATOH1 in CRCs. Restoring ATOH1 in the context of CRC cells with mutant APC blocked xenograft growth [28]. These findings suggest that patients whose tumors have silenced ATOH1 may be refractory to Notch or γ -secretase inhibitor therapy, whereas patients whose tumors retain ATOH1 expression are the best candidates for such treatments.

Finally, in contrast to the tumor suppressor function described for ATOH1 in most colon adenocarcinomas, ATOH1 might have a different role in colorectal cancers that retain prominent secretory cell components, such as mucinous adenocarcinomas, signet ring carcinomas, and intestinal neuroendocrine tumors [42, 43]. In these tumors, as well as in adenomatous tumors that have silenced ATOH1, identification of the transcriptional targets of ATOH1 which can enforce cell cycle arrest may provide candidates for new cancer therapies. One such target, SPDEF, was shown to efficiently halt proliferation and induce goblet cell differentiation in normal intestinal epithelium [44]. Determining whether SPDEF can function as a tumor suppressor in vivo, and how it could be activated in human cancers, is an area of current investigation.

The Notch-Atoh1 Pathway in Other Gastrointestinal Cancers

The Notch-Atoh1 pathway has also been described in gastric tumor cells. Notch receptors (1, 2, and 3) and the downstream target Hes1 have been shown to be expressed on normal gastric mucosa, whereas Atoh1 expression is undetectable [45–47]. Similarly, gastric cancer cell lines retain their Notch receptors (1, 2, and 3) and Hes1 expression. However, in contrast to the normal mucosa, ATOH1 is expressed in some of these cell lines and its expression correlates with intestinal mucin production (intestinal metaplasia) [46, 47]. In vitro analysis showed that ATOH1 transcriptionally regulates mucin genes such as MUC6 and MUC5AC, similar to ATOH1 regulation of MUC2 described in CRC cells [28, 47]. Moreover, ATOH1 expression was observed both in mouse and human metaplastic mucosa [46].

Similar to neoplasias of the stomach, the Notch-ATOH1 pathway has also been described in the pathogenesis of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) [48, 49]. Reflux of bile acids induces intestinal metaplasia characterized by goblet cell production—a hallmark of BE, which is the premalignant lesion of EAC. GSI treatment of EAC cell lines, as well as a rat model of reflux-induced BE, achieves the same effects via the Notch-Atoh1 pathway as it does in the intestine, namely forced differentiation into postmitotic goblet cells and loss of proliferation [48, 49, 50••]. Although the role of ATOH1 in these lesions has not been well characterized and no specific role in tumorigenesis has been described, we suggest that GSI treatment of gastric and esophageal intestinal metaplasias or cancers which can express ATOH1 will result in forced differentiation and loss of proliferation. Thus, targeting the Notch-ATOH1 pathway represents a novel approach to differentiation therapy in GI cancers.

Conclusions

During differentiation of the intestinal epithelium, the relative activity of Notch versus ATOH1 determines whether an intestinal progenitor adopts an absorptive (Notch active) or secretory (ATOH1 active) fate. In CRC, several mechanisms work to skew the normal Notch-ATOH1 balance to favor Notch activation. These mechanisms include silencing of ATOH1, stabilization of Notch, and activation of Notch ligands (eg, Jagged1). Gain of Notch activity and/or loss of ATOH1 activity enhances tumorigenesis; thus, Notch functions as an oncogene whereas ATOH1 functions as a tumor suppressor in CRC. However, the specific mechanisms by which Notch and ATOH1 activities function in cancer remain unknown. The Notch-ATOH1 pathway is active in other GI malignancies with features of intestinal metaplasia (eg, esophageal adenocarcinoma and gastric carcinoma). Therapeutic targeting of the Notch-ATOH1 pathway can be achieved by inhibiting Notch activation with γ -secretase inhibitors or Notch-directed antibodies. However, this approach is dependent on ATOH1 expression and is therefore likely to benefit a subset of patients with GI malignancies.

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Fig. 1.

Schematic representation of the role of NOTCH and ATOH1 in intestinal differentiation and carcinogenesis. **a** Notch signaling is activated upon engagement of the Notch receptor by its ligand (delta/serrate/lag-2 ligands; DSL) on an adjacent cell. Upon ligand binding, the Notch receptor is sequentially cleaved by ADAM 10/17 and the γ -secretase complex to release the Notch intracellular domain (NICD). NICD translocates into the nucleus where it complexes with CSL to activate transcription of gene targets. HES1 (hairly/enhancer of split), a major target of NICD signaling, acts as a transcriptional repressor of atonal homolog 1 (ATOH1). Cells that activate Notch/Hes1 and suppress ATOH1 adopt the absorptive fate. ATOH1 up-regulates secretory cell gene transcription such as mucin 2 (MUC2) and specifies secretory cell (goblet, enteroendocrine, and Paneth cell) fate. Thus, cells with lower levels of NICD and higher levels of ATOH1 adopt the secretory fate. Ubiquitination of NICD by Fbw7 (F-box and WD repeat domain containing 7) targets NICD for proteasomal degradation and may be one mechanism by which secretory fate is selected. In differentiating epithelial cells, β -catenin forms a complex with adenomatous polyposis coli (APC) and GSK3- β kinase, which phosphorylates β -catenin and targets it for degradation. **b** A hallmark of colorectal tumorigenesis is the constitutive activation of Wnt/ β -catenin signaling, typically by inactivation of the tumor suppressor gene APC. APC inactivation allows β -catenin nuclear accumulation and transcription of targets such as cMYC, CyclinD1, and HES1. Notch is frequently activated during colorectal tumorigenesis, resulting in transcriptional activation of target genes, several of which are co-regulated by β -catenin such as cMYC, CyclinD1, and HES1. HES1 acts as a transcriptional repressor of cell cycle inhibitors such as p27 and p57. Fbw7 functions as a tumor suppressor and is commonly inactivated in colorectal cancers, thus disrupting its ability to degrade NICD. ATOH1 functions as a tumor suppressor in colorectal cancer and is frequently silenced by CpG island methylation (Me-CpG) and/or deletion. In tumors with APC inactivation, GSK3 kinases are available to phosphorylate ATOH1 and target it for degradation, providing another mechanism for ATOH1 silencing in colorectal cancer.