GASTROENTEROLOGY 2010;139:918–928

Atonal Homolog 1 Is Required for Growth and Differentiation Effects of Notch/γ-Secretase Inhibitors on Normal and Cancerous Intestinal Epithelial Cells

AVEDIS KAZANJIAN,* TAEKO NOAH,* DOUGLAS BROWN,‡ JARRED BURKART,§ and NOAH F. SHROYER*,‡,‖

*Division of Gastroenterology, Hepatology, and Nutrition, §Summer Undergraduate Research Fellowship Program, ‖Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; ‡University of Cincinnati, College of Medicine, Cincinnati, Ohio

BACKGROUND & AIMS: The atonal homolog 1 (Atoh1) transcription factor is required for intestinal secretory (goblet, Paneth, enteroendocrine) cell differentiation. Notch/γ-secretase inhibitors (GSIs) block proliferation and induce secretory cell differentiation in the intestine. We used genetic analyses of mice to determine whether Atoh1 mediates the effects of GSIs in normal and cancerous intestinal epithelia. **METHODS:** We studied mice with intestine-specific disruption of *Atoh1* (*Atoh1*$^{\Delta intestine}$), the adenomatosis polyposis coli (*APC*)^{min} mutation, both mutations (*Atoh1*$^{\Delta intestine}$; *APC*^{min}), or littermate controls; mice were given GSI or vehicle. Colorectal cancer (CRC) cell lines were treated with GSI or vehicle and with small hairpin RNAs to reduce *ATOH1*. Differentiation and homeostasis were assessed by protein, RNA, and histologic analyses. **RESULTS:** GSIs failed to induce secretory cell differentiation or apoptosis or decrease proliferation of *Atoh1-null* progenitor cells, compared with wild-type cells. Exposure of *APC*^{min} adenomas to GSIs decreased proliferation and increased secretory cell numbers in an Atoh1-dependent manner. In CRC cells treated with GSI, ATOH1 levels were correlated inversely with proliferation. ATOH1 was required for secretory cell gene expression in cell lines and in mice. **CONCLUSIONS: ATOH1 is required for all effects of GSIs in intestinal crypts and adenomas; Notch has no unique function in intestinal progenitors and cancer cells other than to regulate ATOH1 expression. Reducing ATOH1 activity might mitigate intestinal toxicity from systemic GSI therapy for nonintestinal diseases. Among gastrointestinal malignancies, ATOH1 mediates the effects of GSIs, so ATOH1 expression levels might predict responses to these inhibitors. We propose that only the subset of CRCs that retain ATOH1 expression will respond to GSIs.**

Keywords: Biomarkers; Cell Fate Specification; Basic Helix-Loop-Helix Transcription Factor; Notch Intracellular Domain.

BASIC–ALIMENTARY TRACT

Molecular pathways that regulate normal development and homeostasis are frequently misregulated in cancers and represent potential targets for clinical intervention. The Notch signaling pathway is key to determining self-renewal versus differentiation of intestinal stem cells. The importance of Notch signaling in colorectal cancer (CRC) tumorigenesis only recently has been recognized, suggesting that this pathway is a target for new CRC therapeutics.[1]

The intestinal epithelium is composed of 4 main cell types: absorptive enterocytes and 3 secretory cell types: Paneth, enteroendocrine, and goblet cells. Notch signaling controls the fate of intestinal progenitors by differentially regulating 2 opposing basic helix-loop-helix transcription factors, *hairy/enhancer of split 1* (*HES1*, also called *HRY*)[2] and *atonal homolog 1* (*ATOH1*, also called *Math1* or *HATH1*).[3] Progenitors with low levels of Notch express high levels of *ATOH1* and commit to a secretory cell fate, whereas progenitors with high levels of active Notch express *HES1*, which in turn repress *ATOH1*, and become absorptive enterocytes.[1,4–9] This model of alternate fate selection is based on regulation of *Notch*, *atonal*, and *enhancer-of-split* genes via lateral inhibition in *Drosophila melanogaster*.[10,11] Thus, ATOH1 is thought to be a critical gatekeeper for the program of Notch-directed differentiation of intestinal stem cells.

Recent studies have reported loss of *ATOH1* expression in human CRCs.[12] We recently confirmed that approximately 80% of human CRCs silence *ATOH1*, and showed that the mechanism includes both genetic and epigenetic silencing.[13] Moreover, we showed that *Atoh1* mutant mice (*Atoh1*$^{\Delta intestine}$) are highly susceptible to tumor formation using both azoxymethane and adenomatosis polyposis coli (*APC*)$^{min/+}$ mouse models of CRC.[13] Taken together, these results suggest that *ATOH1* may be the key target of

Abbreviations used in this paper: APC, adenomatosis polyposis coli; ATOH1, atonal homolog 1; BrdU, bromodeoxyuridine; CRC, colorectal cancer; DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DLL1, delta like 1; DMSO, dimethyl sulfoxide; GSI, γ-secretase inhibitor; HES1, hairy/enhancer of split 1; MUC2, MUCIN2; RT-PCR, reverse-transcription PCR; shRNA, short hairpin RNA; SPDEF, SAM pointed domain containing ETS transcription factor; WT, wild type.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.05.081

the Notch pathway regulating differentiation and proliferation within CRCs.

Gamma-secretase inhibitors (GSIs) are small molecules first developed for their ability to inhibit processing of the Alzheimer's related β-amyloid peptide from the amyloid precursor protein.[14] Subsequently, these drugs were shown also to inhibit ligand-dependent Notch cleavage and activation.[15] More recently, Notch-sparing GSIs have been developed that selectively inhibit amyloid precursor protein processing but have less effect on Notch processing, and thus avoid potential side effects on Notch-regulated organ systems such as the intestine.[16] In contrast, nonselective GSIs cause a dose-dependent conversion of small intestinal and colonic progenitors to the secretory cell fate, accompanied by activation of *Atoh1* and down-regulation of *Hes1* expression in intestinal crypt progenitors.[1,17–19] In addition, treatment of $APC^{min/+}$ mice with GSIs resulted in reduced proliferation, *Atoh1* overexpression, and differentiation of some adenoma cells to nonproliferating goblet cells.[1] More recently, GSI treatment was shown to quantitatively shrink adenomas in $APC^{min/+}$ mice.[20] Experiments in CRC cell lines showed a minimal effect of GSI alone, but a pro-apoptotic effect when given in combination with cytotoxic drugs such as taxanes or platinum compounds.[21–23] In addition to these effects, GSIs have been proposed as chemotherapeutic agents in Barrett's esophagus, gastric cancer, and several non-GI cancers.[23–25] Thus, Notch-targeted GSIs are a promising class of small molecules for treatment of gastrointestinal neoplasias. However, in these studies the role of ATOH1 in mediating these effects of Notch inhibitors was not examined. Here we test the hypothesis that GSI-mediated Notch inhibition critically requires ATOH1, in normal and cancer cells, for growth arrest and differentiation into secretory cells.

Materials and Methods

Complete materials and methods are available in the Supplementary Materials and Methods section.

Mice and Treatments

Wild type (WT), $Atoh1^{\Delta intestine}$, APC^{min}, and APC^{min}; $Atoh1^{\Delta intestine}$ mice were treated either with vehicle or GSI-20 (EMD, Brookfield, WI) at 10 μmol/L/kg once (1 × GSI) or twice (2 × GSI) a day for 5 days.

Immunohistochemistry

Sections were stained for bromodeoxyuridine (BrdU; Developmental Studies Hybridoma Bank, Iowa City, IA), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), lysozyme (Invitrogen, Carlsbad, CA), and chromogranin A (ImmunoStar, Inc, Hudson, WI). Two-way analysis of variance (ANOVA) and Bonferroni post hoc analysis were used to measure significance (for BrdU and cleaved caspase-3 analysis).

Cell Culture and Treatments

HCT116, HT29, LOVO, LS174T, RKO, and SW480 were treated with dimethyl sulfoxide (DMSO) or 5 μmol/L N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sigma, St Louis, MO) for 4 days. Nontargeting and ATOH1–short hairpin RNA (shRNA) constructs (Sigma) packaged into lentivirus were used to infect cells before treatment. The Cell Counting-8 Kit (Dojindo, Rockville, MD) and BrdU cell proliferation kit (Millipore, Billercia, MA) were used to measure proliferation.

Immunoblotting

CRC cell and intestinal protein lysates were used for immunoblotting. The following antibodies were used: mouse immunoglobulin (Ig)M anti-actin (Developmental Studies Hybridoma Bank), rabbit polyclonal anti-HES1 (Dr Nadean Brown, Cincinnati Children's Hospital), rabbit polyclonal anti-cleaved Notch 1 (Val1744; Cell Signaling Technology), mouse monoclonal anti-Notch1 (mN1A; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-p27[Kip1] (BD Transduction Laboratories, San Jose, CA), rabbit polyclonal anti-trefoil factor 3 (Dr Daniel Podolsky, University of Texas), and rabbit polyclonal anti–poly-adenosine diphosphate (ADP) ribose polymerase (Cell Signaling Technology).

Quantitative Reverse-Transcription Polymerase Chain Reaction

CRC cells treated with DMSO or DAPT (and shRNA) were harvested and used for RNA purification (Qiagen, Valencia, CA), DNase digestion, complementary DNA (cDNA) synthesis (Invitrogen), and SYBR Green-based quantitative reverse-transcription polymerase chain reaction (RT-PCR) (Agilent Technologies, Stratagene, Santa Clara, CA). A similar procedure was used for quantitative RT-PCR from intestinal tissues treated with vehicle or GSI (GSI-20, 10 μmol/L/kg once a day for 5 days). The primers for the quantitative RT-PCR are listed in Supplementary Table 1 and Supplementary Table 2. A 2-tailed Student *t* test and 2-way ANOVA with Bonferroni post hoc test were used for bivariate analysis.

Results

Atoh1 *Is Required for the Effects of Notch Inhibition on Differentiation in the Intestine*

To genetically determine the role of Atoh1 in mediating the effects of Notch inhibition in the intestine, we treated *WT* and $Atoh1^{\Delta intestine}$ mice with vehicle or GSI. GSI treatment effectively inhibited Notch activation, as determined by a significant decrease in Notch intracellular domain and its target HES1 (Supplementary Figures 1 and 2). $Atoh1^{\Delta intestine}$ mice are mosaic, such that in the distal ileum and colon they harbor approximately 75% *Atoh1-null* crypts that lack all secretory cells, with