Prevention and Epidemiology

**NOTCH Signaling Is Required for Formation and Self-Renewal of Tumor-Initiating Cells and for Repression of Secretory Cell Differentiation in Colon Cancer**

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**Abstract**

*NOTCH* signaling is critical for specifying the intestinal epithelial cell lineage and for initiating colorectal adenomas and colorectal cancers (CRC). Based on evidence that *NOTCH* is important for the maintenance and self-renewal of cancer-initiating cells in other malignancies, we studied the role of *NOTCH* signaling in colon cancer–initiating cells (CCIC). Tumors formed by CCICs maintain many properties of the primary CRCs from which they were derived, such as glandular organization, cell polarity, gap junctions, and expression of characteristic CRC molecular markers. Furthermore, CCICs have the property of self-renewal. In this study, we show that *NOTCH* signaling is 10- to 30-fold higher in CCIC compared with widely used colon cancer cell lines. Using small-molecule inhibition and short hairpin RNA knockdown, we show that *NOTCH* prevents CCIC apoptosis through repression of cell cycle kinase inhibitor p27 and transcription factor *ATOH1*. *NOTCH* is also critical to intrinsic maintenance of CCIC self-renewal and the repression of secretory cell lineage differentiation genes such as *MUC2*. Our findings describe a novel human cell system to study *NOTCH* signaling in CRC tumor initiation and suggest that inhibition of *NOTCH* signaling may improve CRC chemoprevention and chemotherapy. *Cancer Res;* 70(4); 1469–78. ©2010 AACR.

**Introduction**

Colorectal cancer (CRC) is the second leading cause of U.S. cancer death (1). For metastatic CRC, the 5-year survival rate is ∼10% (1). A mechanistic understanding of CRC initiation, recurrence, and metastasis is therefore an important goal. Several studies have shown that *WNT* and *NOTCH* pathways help maintain intestinal homeostasis, regulate cell fate decisions, and play important roles in CRC tumorigenesis and progression (2–8). The majority of CRC tumors have increased *WNT* signaling (9). In normal intestinal homeostasis, *WNT* signaling stimulates intestinal stem and progenitor cell proliferation but paradoxically also causes terminal differentiation into Paneth cells. One might therefore expect that CRC cells would attempt to differentiate terminally into Paneth cells. However, other signaling pathways active in CRC prevent terminal differentiation and maintain self-renewal capacity. A candidate is the *NOTCH* signaling pathway.

The role of *NOTCH* signaling in CRC is less well characterized than *WNT*. *NOTCH* signaling is triggered through the binding of a ligand on the membrane of one cell (Delta/Delta-like/Jagged/Serrate) to a receptor (*NOTCH1/*NOTCH2/*NOTCH3/*NOTCH4) on the membrane of the contacting cell. This causes proteolytic cleavage of *NOTCH* receptors to release the cytoplasmic tail of *NOTCH* (NICD; ref. 10). NICD translocates to the nucleus and associates with CSL transcription factors (CBF1/RBPJκ/Suppressor of Hairless/Lag-1) and coactivator Mastermind to turn on transcription of target genes (11). The best-characterized targets of *NOTCH* are hairy/enhancer of split (*HES*) family of transcription factors, particularly *HES1* in the intestine (12, 13). In normal mouse intestine, inhibition of *NOTCH* signaling results in exit from the proliferative compartment and differentiation into postmitotic goblet cells (7). Similar results are seen in knockouts of other critical *NOTCH* signal transduction components, including *Hes1*, *Rbpjκ*, *Notch 1* and 2 receptors, and *Pofut1* knockouts for normal intestine (6–8, 12, 14). *Apc* mutant intestinal adenoma cells, which have elevated *WNT* signaling, also respond to *NOTCH* signaling inhibition by terminal differentiation into goblet cells, accompanied by cell cycle arrest and/or apoptosis (7, 15). Therefore, suppression of *NOTCH* signaling may be a powerful mechanism for directing both normal intestinal enterocyte progenitors and *Apc* mutant intestinal cancer cells to differentiate down a secretory lineage.

*NOTCH* signaling plays an important role in intestinal tumor initiation but not progression in mice (15). Transgenic expression of NICD in the intestine leads to expansion of enterocyte progenitor cells (6) and increases the number of...
adenomas in ApcMin mice (15). In addition, inhibition of NOTCH by Jag1 deletion decreases adenoma initiation in ApcMin mice (16). In human CRCs, NOTCH signaling is high in adenomas and early-stage CRCs (16, 17) but low in advanced, later-stage, and metastatic CRCs (15). The molecular mechanisms that cause NOTCH signaling to be important for early-stage CRC initiation are not understood, and dramatically fewer mechanistic studies of NOTCH signaling in human CRC cell lines have been performed. Pharmacologic and small interfering RNA–mediated NOTCH signaling inhibition in colon cancer cell lines in vitro enhances sensitivity to cytotoxic chemotherapy. However, in the absence of cytotoxic chemotherapy, endogenous NOTCH signaling levels are present but generally low (18–20). In summary, although NOTCH signaling seems to be very important in adenoma formation and CRC tumorigenesis, the relatively low endogenous signaling levels in many commonly used CRC cell lines have limited mechanistic studies of NOTCH signaling in human CRC cells that could provide important insights into better ways to improve CRC chemoprevention and chemotherapy.

Colon cancer-initiating cells (CCIC; refs. 21–24) maintain biological similarity to primary human CRCs. Like most primary CRCs and their metastases, CCIC can maintain an organized glandular structure, with preserved cell polarity, gap junctions, and expression of differentiation markers.
typical for CRC, such as cytokeratin 20 (CK20) or carcinoembryonic antigen (CEA), which are often not highly expressed in commonly used CRC cell lines typically used for in vitro studies (21, 23, 24). CCICs have the property of self-renewal (25) and can form new tumors after serial transplantation (21, 23, 24). Because previous studies have shown that NOTCH signaling plays a role in T-cell leukemia (26) and breast (27), brain (28–30), and pancreatic (31) cancer CIC, we studied NOTCH signaling in CCIC. Here, we show that NOTCH signaling is markedly higher in CCIC compared with commonly used CRC cell lines and plays important roles in the intrinsic maintenance of CCIC viability, tumor formation, self-renewal, and the alternate expression of enterocyte or secretory intestinal cell lineage differentiation markers. These data describe a novel human CRC cell system to study NOTCH signaling in CRC tumor initiation, and they suggest that inhibition of NOTCH signaling is likely to be an important mechanism to improve CRC chemoprevention and chemotherapy.

Materials and Methods

**Isolation and culture of CCIC.** Human colon cancer samples were obtained from colon cancer resections and liver metastasis under protocols approved by the University of California-Irvine Institutional Review Board. Fresh CRC tissue was extensively washed with PBS, minced, and incubated at 37°C with collagenase. Cells were then strained through 40-μm filter and used for in vitro culture or xenograft. CCIC cultures were derived essentially as described previously (22) and cultured as spheres in ultralow-attachment flasks in DMEM/F12 containing nonessential amino acids, antibiotic-antimycotic, N2 supplement (Invitrogen), B27 supplement (Invitrogen), heparin (4 μg/mL; Sigma), epidermal growth factor (40 ng/mL), and basic fibroblast growth factor (20 ng/mL) at 37°C and 5% CO2. For xenograft assays, primary tumor cells or CCICs were injected in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice s.c. as a 1:1 mixture of Matrigel and CCIC medium. For DAPT treatment, cells were treated with 10 μmol/L DAPT (EMD Biosciences) or DMSO for 48 h and used for cell cycle analysis and Western blotting. For three-dimensional culture of CCIC, single CCICs were mixed with 2.5 mg/mL collagen (BD Biosciences) and 7.5% Matrigel (BD Biosciences) and plated in Transwell dishes overlaid with medium.

**Culture of colon cancer cell lines.** HCT116, DLD1, HT29, and SW480 were purchased from the American Type Culture Collection and cultured according to recommended medium conditions at 37°C and 5% CO2.

**Lentiviral constructs and infection.** The lentiviral vectors pLKO.1-puro, pLKO.1-scrambled-shRNA (Addgene), and pLKO.1-shRNA that targets RBPJκ (TRCN0000016203, Sigma) were transfected into 293T cells along with pCMV and pMP2G. Lentiviral RBPJκ/NOTCH reporter was purchased as a prepackaged lentivirus from SA Biosciences. CCICs and colon cancer cell lines were infected with lentivirus at a multiplicity of infection of 50 in the presence of polybrene (1 μg/mL) and selected in puromycin according to the manufacturer’s protocol and similar to previous studies (32, 33). Concentrations for puromycin selection were determined by plotting a kill curve. Mock-infected cells did not survive puromycin selection. Stable cell lines were then used for fluorescence-activated cell sorting (FACS) analysis to assay for NOTCH reporter activity.

**Flow cytometry.** Flow cytometry was performed on single CCIC with CD133 antibody (Miltenyi Biotec), CD44 (BDPharmingen), and ESA (Biomedia). Enzyme assay for ALDH1 was performed using the Aldeflour kit (Stem Cell Technologies). For reporter assays, stable CCICs with reporter were analyzed for green fluorescent protein (GFP) expression. For cell cycle analysis, CCICs were fixed and stained with propidium iodide and then analyzed on a Becton Dickinson flow cytometer.

**Immunohistochemistry.** Immunohistochemistry and immunofluorescence were performed on paraffin-embedded sections from xenografts and three-dimensional cultures as previously described (34). β-Catenin immunocytochemistry was performed on four CCIC lines to evaluate subcellular

![Figure 2](http://www.aacrjournals.org)
localization. Antibodies are listed in Supplementary Materials and Methods.

**Gene expression profiling.** Expression profiling was carried out using total RNA from two CCIC lines in quadruplicates on Affymetrix GeneChip Human Exon 1.0 ST Array. The Cyber-T program was used to determine statistically significant, differentially expressed genes compared with normal colon data sets (35). Differentially regulated genes were analyzed by Ingenuity Pathway Analysis. Expression data were confirmed by Taqman in four CCIC lines (including the two that were profiled).

**Protein isolation and Western blotting.** Protein isolation and Western blot analysis were carried out as previously described (34). Antibodies are listed in Supplementary Materials and Methods.

**RNA isolation and quantitative reverse transcription-PCR.** Total RNA was extracted using Qiagen RNeasy kit and reverse transcribed, and gene expression was quantified on ABI PRISM HT7900. Expression levels are an average of three independent experiments in two CCIC lines. All expression levels are normalized to GAPDH and RPLPO.

**Results**

**CCICs express CRC markers and retain tumor initiation and self-renewal capacity in long-term culture.** To study the role of NOTCH and other critical signal transduction pathways for human CRCs, we derived CCIC from colon cancers resected from 10 patients (Supplementary Table S1). In agreement with other groups, we found that CCIC can form xenografts in NOD/SCID mice that maintain the histopathology of primary human CRCs (21, 23, 24, 36). CK20 and CEA are CRC peptide markers commonly used in routine anatomic pathology studies of human CRC patient tumors. Xenografts derived from injection of CCIC in NOD/SCID mice are CK20 and CEA positive and CK7 negative (matching the marker pattern most commonly seen in patient CRC specimens; Fig. 1A). At the same time, commonly used CRC cell lines that form tumors in xenograft assays do not recapitulate the glandular architecture, maintain cell polarity, or express the immunohistochemical markers for CRC typically used to confirm colonic origin in primary and metastatic CRC specimens (Supplementary Fig. S1A).

CD133, CD44, ESA, and ALDH1 have previously been shown to be important CCIC markers (21, 23, 24, 36). As expected, FACS analysis showed that the CCICs we derived also express CD133, CD44, ESA, and ALDH1 (Fig. 1B). To evaluate CCIC capacity for self-renewal, we injected CCIC spheres in the flank of NOD/SCID mice at regular intervals to assay its tumor-forming ability. As expected, the CCICs we derived faithfully maintain the histology and marker expression in xenograft assays over multiple passages (Fig. 1C). To date, we have tested up to six passages of CCIC lines that have maintained self-renewal capacity for more than 1 year.
Consistent with previous studies (37), single CCIC cells can form carcinomatous glands in collagen three-dimensional culture in vitro. Like the xenografts, the cells in these colonies maintain architectural features typical of differentiated CRC, including the formation of gland lumens and the expression of typical CK7+/CK20+ cytokeratin pattern seen in bona fide primary CRC (Fig. 1D). Some of the cells also have nuclear β-catenin staining indicative of elevated WNT signaling seen in CRCs (Fig. 1D). In contrast, commonly found CRC cell lines in three-dimensional culture (Supplementary Fig. S1B) form solid nests of unpolarized cells that lack signs of glandular organization and other features of colon differentiation.

**Elevated WNT and NOTCH signaling in CCIC as compared with normal colon tissue.** The molecular mechanisms that sustain CCIC are poorly characterized. To explore critical biological pathways regulating CCIC, we generated gene expression profiles using Affymetrix whole-genome exon arrays and compared them with normal colon profiles. The differentially expressed genes were then used in Ingenuity Pathway Analysis to identify functional categories and signaling pathways that are enriched. Because of relevance to CRC and other CIC types, we focused on WNT and NOTCH signaling. Similar to previous studies showing high levels of WNT signaling in commonly used CRC cell lines, WNT targets such as MYC, CD44, CTNNB1, LEF1, TLE4, and CD44 are all expressed at significantly higher levels in CCIC compared with normal colon (Supplementary Table S2). These data are also consistent with the presence of nuclear β-catenin protein seen in CCIC in vitro cultures, which is seen in approximately 50% to 90% of CCIC. Similarly, canonical NOTCH target genes, such as HES1, HES4, and HES6, and signaling components, such as JAG1, JAG2, and NOTCH1, were all significantly higher in CCIC (Supplementary Table S2). To validate the array data for NOTCH components and targets, we performed quantitative PCR analysis in CCIC derived from nine CCIC lines. In the CCIC lines tested, the canonical NOTCH target gene HES1 and pathway components JAG1 and NOTCH1 are significantly higher compared with normal colon epithelium (Fig. 2). In summary, CCICs have elevated levels of WNT and NOTCH target genes and signaling components compared with normal colon tissue.

**CCICs have active NOTCH signaling.** Because our gene expression analyses were exploratory and normal colon tissue was not cultured in the same way as CCIC (which could confound data interpretation), we used NOTCH reporter constructs to test directly whether CCICs have active NOTCH signaling. We infected CCIC and commonly used CRC cell lines with a lentiviral NOTCH GFP reporter construct for direct comparison. FACS analysis of CCIC showed high levels of NOTCH activity. Even in CCIC lines that are far from the highest in endogenous NOTCH target gene expression (such as CCIC-1, second bar series in Fig. 2C), >33% of the cells have active NOTCH signaling as indicated by high GFP expression (Fig. 3A). However, commonly used colon cancer cell lines have little (SW480) to no active NOTCH signaling (HCT116, DLD1, and Colo205; all <3% of cells; Supplementary Fig. S2). In summary, CCICs have approximately 10- to 30-fold more cells with high levels of NOTCH signaling than commonly used colon cancer cell lines. Similar results were seen with studies using a NOTCH signaling luciferase reporter gene (data not shown).

γ-Secretase is a critical component of NOTCH signal transduction at the cell membrane. DAPT is a γ-secretase inhibitor that inhibits NOTCH signaling by blocking the γ-secretase complex responsible for the S3 cleavage to release the NICD domain (38). To further validate that CCICs have elevated NOTCH signaling, we transiently treated CCIC with DAPT. Consistent with inhibition of NOTCH signaling, DAPT caused a ≥50% reduction in the number of GFP+ CCIC (Fig. 3A). To investigate if active NOTCH signaling is present in CCIC in three-dimensional culture, we performed immunofluorescence for NICD with an antibody that recognizes NICD. NICD staining is seen in the nucleus of CCIC in three-dimensional in vitro cultures, indicating active NOTCH signaling (Fig. 3B).

**γ-Secretase inhibitors induce goblet cell lineage markers and cause apoptosis in CCICs.** Because NOTCH signaling is active in CCIC, we tested whether NOTCH is required for CCIC tumor formation. CCICs treated with DAPT had no NICD detectable by Western blot analysis (Fig. 4A, right). Similarly, DAPT-treated CCICs downregulated NOTCH targets HES1 and HES5 (Fig. 4A, left). In the mouse intestine, Hes1 represses Atoh1, a bHLH transcription factor that

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**Figure 4.** DAPT blocks NOTCH signaling in CCIC and causes expression of goblet cell markers. A, left, expression of HES1 and HES5 in CCIC decreases on treatment with DAPT; right, DAPT treatment inhibits cleavage of NOTCH by γ-secretase, as NICD is not detected by immunoblotting in CCIC. B, DAPT relieves repression caused by expression of HES1 on MATH1, MUC2, and CDK inhibitor p27. C, MUC2+ cells is increased by 2.5-fold on transient treatment with DAPT. Counted MUC2+ cells in 100 colonies in five independent experiments.
promotes intestinal stem cell differentiation toward secretory lineages (39). DAPT treatment of CCIC increases expression of ATOH1 and expression of goblet cell marker MUC2 (40, 41), likely caused by lower HES1 levels (Fig. 4B).

In addition, in the mouse intestine, Hes1 represses transcription of cyclin-dependent kinase (CDK) inhibitors p27/CDKN1B and p57/CDKN1C and arrests intestinal progenitor cells that differentiate down the goblet cell lineage (8). Quantitative PCR analysis of human CCIC shows that NOTCH pathway inhibition with DAPT relieves repression of p27 but not p57 (Fig. 4B; data not shown). In addition, we treated CCIC that had been cultured in three-dimensional collagen matrix for 7 to 10 days and had already formed adenocarcinomatous glands. Consistent with studies of NOTCH inhibitors in Apc mice where NOTCH inhibition causes Apc mutant intestinal adenoma cells to differentiate terminally into postmitotic goblet cells (7, 15), DAPT increased the number of MUC2-immunoreactive cells in previously formed CCIC glands (Fig. 4C; Supplementary Fig. S3).

Next, we examined if NOTCH signaling is required at earlier stage for CCIC to initiate three-dimensional culture tumor formation. Single CCICs plated in three-dimensional culture and treated with DAPT could no longer form adenocarcinomatous glands but only disorganized CCIC clusters that were MUC2 negative and had no self-renewal capacity (Fig. 5A). In contrast, DAPT did not affect proliferation and colony formation in HCT116 cells, which have low levels of NOTCH signaling (Supplementary Fig. S4). To test if NOTCH inhibition causes CCIC to undergo apoptosis, we treated CCIC in nonadherent cultures with DAPT and performed cell cycle analysis. FACS analysis showed an increase in the sub-G1 population, indicating that CCICs were undergoing apoptosis (Fig. 5B). To further validate whether DAPT induces CCIC apoptosis, we performed Western blots for cleaved caspase-3. DAPT increased cleaved caspase-3 in CCIC, consistent with a triggered apoptosis response (Fig. 5C). In summary, human CCICs in both nonadherent and three-dimensional culture have active NOTCH signaling and express markers of NOTCH signaling such as nuclear NICD, HES1, and HES5. NOTCH signaling is maintained in CCIC adenocarcinomatous glands in three-dimensional culture and express CRC markers such as CK20 and CEA. Treatment with the NOTCH inhibitor DAPT induces CCIC apoptosis and eliminates the CCIC capacity for self-renewal and formation of new tumors.

**Knockdown of RBPJκ leads to expression of goblet cell markers and a decrease in enterocyte markers and tumor foci.** DAPT inhibits γ-secretase, which acts on NOTCH as well as other signal transduction pathways in the cell (42, 43). To test whether inhibition of NOTCH signaling...
specifically causes CCIC to express goblet cell differentiation markers and initiate apoptosis, we transduced CCICs with a lentivirus expressing short hairpin RNA (shRNA) for the critical NOTCH effector RBPJκ/CBF1/CSL. Quantitative PCR and Western blot analysis confirmed efficient knockdown of RBPJκ (Fig. 6A, left and middle). Consistent with NOTCH inhibition, RBPJκ knockdown decreased RBPJκ/NOTCH target gene HES1 (Fig. 6A, left). Quantitative PCR analysis showed that RBPJκ knockdown led to increased expression of ATOH1 and the goblet cell marker MUC2 (Fig. 6A, right). CK20, a marker of the enterocyte lineage, decreases consistent with the effect of inhibiting NOTCH signaling (Fig. 6A, right). In addition, as seen with DAPT treatment, RBPJκ shRNA knockdown relieved repression of the cell cycle inhibitor p27 but not p57 (Fig. 6A, right). In summary, the effect of RBPJκ knockdown on the expression of target genes in CCIC is essentially the same as blocking NOTCH signaling pharmacologically with DAPT. Likewise, effects on colon cell marker expression are consistent with changes induced by NOTCH pathway inhibition in the mouse intestine.

To analyze if RBPJκ knockdown affects CCIC tumor focus formation in three-dimensional culture, we plated cells transduced with either shRNA for RBPJκ or a scrambled control and selected for puromycin-resistant colonies. Cells expressing shRNA against RBPJκ-infected cells were unable to form colonies as compared with control (Fig. 6B). To test whether RBPJκ knockdown induced apoptosis in CCIC, we performed cell cycle analysis. Cell cycle analysis revealed that, up to 4 days after infection, the scrambled and RBPJκ knockdown populations had similar FACS profiles (Fig. 6C). However,
after 4 days, a sub-G1 peak starts appearing only in the RBPs knockout population, consistent with apoptosis. At 8 days after infection, 60% of RBPs knockout cells undergo apoptosis, and by 15 days, this number increases to 90% (Fig. 6C). Cleaved caspase-3 also increases post-RBPJ knockdown, confirming that the cells are apoptotic (Fig. 6D). In summary, these data are consistent with a requirement for NOTCH signaling for CCIC survival (due to suppression of apoptosis), proliferation, and subsequent tumor formation and self-renewal.

Discussion

Previous work has shown that NOTCH signaling is highly active in cancer-initiating cells in human T-cell leukemia, gliomas, medulloblastoma, and pancreatic cancers (26, 28, 29, 31). Here, we show that NOTCH signaling is also highly active in human CCIC. NOTCH signaling is significantly higher than commonly used CRC cell lines, with an approximately 10- to 30-fold increase in the number of cells with active NOTCH signaling (Fig. 3; Supplementary Fig. S2). NOTCH therefore plays an important role in the intrinsic maintenance of CCIC viability, tumor formation, self-renewal, and the alternate expression of enterocyte (HES1, HES5, CK20, and CEA) or secretory cell lineage (MUC2 and ATOH1) differentiation markers (Figs. 4 and 6; Supplementary Fig. S3).

In serum-free nonadherent culture conditions, where CCICs have minimal expression of intestinal enterocyte/secretory lineage differentiation marker genes (44) and are in a more "stem cell/early progenitor-like" state, NOTCH signaling is highly active and suppresses both CCIC apoptosis and cell cycle arrest (Figs. 4 and 6). Inhibition of NOTCH signaling activates the intrinsic apoptosis pathway, causing cleavage of caspase-3 (Figs. 5 and 6). Additionally, repression of NOTCH signaling inhibits CK20 expression and increases expression of ATOH1 (which causes secretory lineage-committed intestinal cells to exit the cell cycle) and the CDK inhibitor p27/CDKN1B, which causes cell cycle arrest. Repression of NOTCH does not affect p57/CDKN1C expression. In normal mouse intestine, NOTCH inhibition causes derepression of Atoh1, p27, and p57. In this context, individual p27 and p57 knockouts have little effect and knockout of both p27 and p57 are required for the conversion of enterocyte progenitors into postmitotic goblet cells (8). Therefore, the effect of NOTCH inhibition to arrest human CCIC but not affect p57 levels seems to reflect a lack of redundancy between p27 and p57 or a different mechanistic requirement for cell cycle inhibitors than that used in normal mouse intestine. Future experiments to study NOTCH inhibition in cultured normal human intestinal stem cells and Apc<sup>−/−</sup>:p27<sup>−/−</sup>:p57<sup>−/−</sup> intestinal tumors may help to resolve whether this difference is due to species (human versus mouse) or cell type (CCIC versus normal intestinal stem cells).

In three-dimensional solid-phase culture conditions, individual CCICs form tumor foci. Under these conditions, CCICs express differentiated enterocyte lineage markers such as HES1 and CK20. In this system, endogenous NOTCH signaling suppresses goblet cell differentiation markers and promotes proliferation. NOTCH inhibition decreases the number of CCICs expressing CK20 and simultaneously increases CCICs expressing secretory lineage markers such as MUC2. Overall, our data in CCIC are consistent with mouse studies showing that blocking NOTCH in the context of overactive WNT signaling can cause mouse intestinal cancer cells expressing enterocyte lineage markers to transdifferentiate into postmitotic goblet cells and reduce tumor growth (7, 16). Functionally, NOTCH inhibition causes CCIC to initiate apoptosis and to lose the capacity to form tumors and the property of self-renewal. An interesting aspect of CCIC with NOTCH inhibition by either RBPs knockout or DAPT treatment in three-dimensional culture is that the cells expressing secretory differentiation markers, such as MUC2, are always seen individually surrounded by CK20<sup>+</sup> cells and not adjacent to one another (Fig. 4C; data not shown). In multiple metazoan developmental systems ranging from Drosophila to mice, NOTCH signaling is thought to be mediated by ligand-receptor cell-cell interactions, whereby a cell that is beginning to differentiate prevents its neighbors from differentiating in the same way at the same time by "lateral inhibition." Therefore, future studies of CCICs in three-dimensional culture may help our understanding of the mechanistic roles of NOTCH signal transduction components in the process of lateral inhibition of differentiation, with particular focus on the intestinal cell lineages and gland formation.

In the mouse, overexpression of NOTCH signaling alone does not cause intestinal tumor formation. However, activation of both WNT and NOTCH pathways, such as in Apc<sup>−/−</sup> mouse intestine, increases the number of adenomas that initiate compared with WNT activation alone. In the context of inhibited WNT signaling (such as Tcf4<sup>−/−</sup> mice), NOTCH activation is not sufficient to initiate tumors (15). Human CCICs have high levels of both WNT and NOTCH signaling. Similar to the studies in mice and commonly used CRC cell lines, blocking WNT activity in CCIC (such as by expression of the WNT inhibitor DKK1) significantly inhibits the number and size of CCIC tumors that form (Supplementary Fig. S5). In the context of WNT inhibition, however, CCIC viability and proliferation is sufficiently impaired that we cannot readily study the effect of combined WNT/NOTCH inhibition on expression of enterocyte or secretory lineage differentiation markers because of extensive cell cycle arrest and apoptosis. In summary, consistent with mouse and commonly used human CRC cell line studies, our data in CCIC indicate that high NOTCH activity is insufficient to allow CCIC to overcome WNT inhibition.

Overall, our results in human CCIC are largely consistent with the mouse phenotypes observed when NOTCH signaling is blocked (7, 8). CCICs maintain the capacity to express differentiation markers of multiple lineages and depend on self-renewal pathways such as NOTCH. It is possible that in human colon, active NOTCH signaling is required in the initial stages of tumor formation. In the absence of NOTCH signaling, a colon enterocyte progenitor cell with active WNT signaling would undergo terminal differentiation and not give rise to an adenoma, similar to mouse model studies (15).
Epidemiologic, preclinical, and clinical studies show that nonsteroidal anti-inflammatory drugs (NSAID), such as sulindac, are effective in decreasing the number of colon adenomas that initiate in patients (45–48). Two direct targets of NSAIDs are of cyclooxygenase (COX) I and COX2 enzymes. However, other targets of sulindac may also play a role in inhibition of CRC. For example, a recent study showed that amide derivatives of sulindac that do not inhibit COX1 or COX2 can inhibit CRC cell line growth (49). In parallel, clinical trials of sulindac sulfide and other NSAIDs are also being performed to block the formation of β-amyloid plaques in Alzheimer disease patients (50). The proposed mechanism of action for NSAIDs to inhibit β-amyloid plaque formation is through inhibition of the γ-secretase complex (50). In cell culture and mice, sulindac inhibits cleavage of NOTCH receptors (50). Therefore, it is tempting to speculate that (in addition to COX1/2 inhibition) an important mechanism of NSAID colon adenoma chemoprevention activity is the inhibition of the γ-secretase complex and NOTCH signaling in CCICs as they arise in the colon. In the future, it will be important to conduct correlative studies of NOTCH signaling inhibition in NSAID colon adenoma chemoprevention trials to address this question.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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