Transition From Colitis to Cancer: High Wnt Activity Sustains The Tumor-Initiating Potential Of Colon Cancer Stem Cell Precursors

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Abstract

Ulcerative colitis (UC) increases the risk of colorectal cancer (CRC), but the mechanisms involved in colitis-to-cancer transition (CCT) are not well understood. CCT may involve an inflammation-dysplasia-carcinoma progression sequence compared to the better characterized adenoma-carcinoma progression sequence associated with sporadic CRC. One common thread may be activating mutations in components of the Wnt/β-catenin signaling pathway, which occur commonly as early events in sporadic CRC. To examine this hypothesis, we evaluated possible associations between Wnt/β-catenin signaling and CCT based on the cancer stem cell (CSC) model. Wnt/β-catenin immunostaining indicated that UC patients have a level of Wnt-pathway-active cells that is intermediate between normal colon and CRC. These UC cells exhibiting activation of the Wnt pathway constituted a major subpopulation (52%±7.21) of the colonic epithelial cells positive for aldehyde dehydrogenase (ALDH), a putative marker of precursor colon CSC (pCCSC). We further fractionated this subpopulation of pCCSC using a Wnt pathway reporter assay. Over successive passages, pCCSCs with the highest Wnt activity exhibited higher clonogenic and tumorigenic potential than pCCSCs with the lowest Wnt activity, thereby establishing the key role of Wnt activity in driving CSC-like properties in these cells. Notably, 5/20 single cell injections of high-Wnt pCCSC resulted in tumor formation, suggesting a correlation with CCT. Attenuation of Wnt/β-catenin in high-Wnt pCCSC by shRNA-mediated downregulation or pharmacological inhibition significantly reduced tumor growth rates. Overall, the results of our study indicates (i) that early activation of Wnt/β-catenin signaling is critical for CCT, and (ii) that high levels of Wnt/β-catenin signaling can further demarcate ALDH+ tumor-initiating cells in the non-dysplastic epithelium of UC patients. As such, our findings offer
plausible diagnostic markers and therapeutic target in the Wnt signaling pathway for early intervention in CCT.

**Introduction**

Chronic inflammation is associated with many cancers (1). For example, patients with ulcerative colitis, a chronic inflammatory disease of the large bowel, are at substantially increased risk of developing a form of colorectal cancer (CRC) known as colitis-associated cancer (CAC) (2, 3). Moreover, few tools are available for detecting the colitis-to-cancer transition that allows early diagnoses at more treatable stages. Although CAC is thought to involve mutational events, epigenetic modifications and influences from the microenvironment, the pathogenesis of CAC is unclear.

A gene frequently found to be mutated early in the adenoma-to-carcinoma sequence in sporadic CRC is *Adenomatous Polyposis Coli (APC)*, a tumor suppressor, which is a negative regulator of Wnt (wingless)/β-catenin signaling (4, 5). APC is also found to be mutated at the germline level in familial adenomatous polyposis (FAP), an autosomal dominant colon disorder, which promotes the development of CRC (6). APC is an important component of the destruction complex in the Wnt/β-catenin signaling pathway, which includes glycogen synthase kinase 3β (GSK3β), axin 2, casein kinase 1α (CK1α) and β-catenin.

In the absence of Wnt ligand, β-catenin is located primarily at the cell membrane, along with a small, dynamic cytoplasmic pool, which is targeted for proteosomal degradation when bound to the destruction complex. In the presence of Wnt ligands, the destruction complex
undergoes dissociation, resulting in the stabilization and accumulation of free β-catenin in the cytoplasm, and subsequent translocation into the nucleus. Following nuclear translocation, β-catenin displaces transcriptional co-repressors, allowing direct binding to the transcription factor, T-cell factor (TCF)/Lymphocyte enhancer factor (LEF) and subsequent transcription of Wnt-target genes including axin2, LGR5, c-Myc, and cyclin D1 (4). Indeed, activating mutations in any one of the components of the Wnt/β-catenin signaling cascade results in constitutive activation of this pathway as observed in cases of sporadic CRC and FAP (4). Furthermore, high Wnt/β-catenin signaling in sporadic CRC cells has been demonstrated to mark the cancer stem cell (CSC) compartment (7). Other roles for Wnt/β-catenin signaling in the intestinal tract include maintenance of adult crypt structure and proliferation of intestinal epithelial progenitor cells (5, 8).

The contribution of Wnt/β-catenin signaling to the colitis-to-cancer transition is poorly understood. Initial mutational studies suggested that activation of Wnt/β-catenin signaling in the colitis-to-cancer transition is much less frequent than in sporadic CRC, and occurs ‘later’ in the pathogenic cascade (9). As discussed below, our findings suggest that Wnt/β-catenin signaling may occur earlier and be more prevalent than previously thought. We and others reported that sporadic CRC is initiated by a rare population of crypt cells called colon cancer stem cells (CCSCs) (10-13). We showed that high aldehyde dehydrogenase (ALDH) expression marked the CCSCs and enriched the tumor-initiating cell (TIC) population (10). We recently identified a similar ALDHhigh population in the normal-appearing, non-dysplastic colonic epithelium of UC patients (14). These results suggested for the first time that CAC might have a CSC origin. Because they have the capacity to initiate the colitis-dysplasia-cancer transition, we refer to these
cells as precursor-CCSCs (pCCSCs). pCCSCs can be propagated in vivo as tumor xenografts and in vitro as non-adherent spheres. The success rate of generating spheres from ALDH\textsuperscript{high} cells derived from non-dysplastic colitic colon is low, 5-13% (14) and similar to the incidence of CAC in the UC population, ~2-19% (2).

Our findings to date indicate that pCCSCs are a valuable experimental model with which to interrogate the colitis-to-cancer transition, especially when considering early, pre-neoplastic events (14). In particular, use of this model could pave a path for the development of methods that could aid early disease diagnosis and targeted drug therapy, which together may prevent the progression from colitis to cancer. Although pCCSCs might be involved in the colitis-to-cancer transition (14), mechanisms underlying this transformation are unknown. Because initiation of sporadic CRC has been associated with activating mutations in the Wnt/\beta-catenin signaling pathway (15, 16), and CCSCs exhibit high Wnt/\beta-catenin signaling (7), we re-examined the role of Wnt/\beta-catenin signaling in the colitis-to-cancer transition. We proposed the existence of a Wnt/\beta-catenin -dependent CSC hierarchy that is operative ‘early’ in the pathogenesis of CAC. We demonstrate that high Wnt/\beta-catenin signaling enriches tumor initiation activity from pCCSCs to such a degree that recapitulation of the adenocarcinoma phenotype is possible from a single cell.
Materials and Methods

Human Subjects

Tissues from colitis patients and colon cancer patients were retrieved under pathologic supervision with Institutional Review Board approvals at the University of Florida and the University of Michigan. Normal colon tissues were obtained from a local organ procurement organization (Life Quest; Gainesville, Florida).

Animals

Inbred NOD-SCID mice (5-6 weeks old) were used. Mice were maintained under pathogen-free conditions. Experiments were approved by the University of Florida Institutional Animal Care Committee.

Cell Culture

ALDH\textsuperscript{high} sphere isolates were obtained from UC and CRC patients. The former are pCCSC; the latter are CCSC. The sphere isolates used in the study are CT-1, CT-2 and CA. CT-1 and CT-2 are two colitis sphere isolates (pCCSCs) obtained from two different colitic patients, CT-1 suffered from colitis for 8 years and CT-2 for 3 years. CA is a cancer sphere isolate (CCSC) obtained from a sporadic CRC patient. Isolated cells were cultured in serum–free media as previously described by Carpentino et al (14). Serum-free media is referred to as defined media.
**In vitro limiting dilution assay (clonogenic potential)**

Cells with high or low eGFP intensities were deposited at 1, 2, 3, 4, 6, 8, 10, 12, 16, 18, 20 and 24 cells per well of 96-well, ultra-low adhesion plates (Corning, NY) containing defined media. For each cell density, 8 wells were plated. Clonal frequency and statistical significance were evaluated with the Extreme Limiting Dilution Analysis (ELDA) 'limdil' function (7). This assay was carried out with cells obtained from Wnt\textsuperscript{high}-pCCSCs and Wnt\textsuperscript{high}-CCSCs and from ESA\textsuperscript{+/+}/H2K\textsuperscript{d-} cells derived from dissociated tumor xenografts.

**In vivo limiting dilution assay (tumorigenic potential), single cell xenograft tumors and serial passages**

For primary tumor xenografts, 10, 100 and 1000 Wnt\textsuperscript{high}-pCCSCs and Wnt\textsuperscript{high}-CCSCs (sphere isolates) with the 2\% lowest and the 2\% highest eGFP expression levels, corresponding to Wnt\textsuperscript{high} and Wnt\textsuperscript{low} cells, were deposited by FACS into a 96-well plate containing defined medium admixed with Matrigel at a 1:1 ratio such that the total volume was 100\(\mu\text{l}\) and injected as previously described (10). For secondary tumors, the ALDH\textsuperscript{high}-Wnt\textsuperscript{high} and ALDH\textsuperscript{high}-Wnt\textsuperscript{low} primary tumor xenografts were dissociated and the 10, 100, and 1,000 ESA\textsuperscript{+/+}/H2K\textsuperscript{d-} cells with the 10\% highest and 10\% lowest eGFP expression levels (Supplementary Fig. S2A) – corresponding to Wnt\textsuperscript{high} and Wnt\textsuperscript{low} – were injected as described above (Supplementary Fig. S2B). Single cell injections were carried out as described for the secondary tumors for both Wnt\textsuperscript{high} and Wnt\textsuperscript{low} cells enriched from primary tumor xenografts. Likewise, single cell ALDH\textsuperscript{high} secondary tumors were generated. Tertiary tumors were generated with 10, 100 and 1000 ESA\textsuperscript{+/+}/H2K\textsuperscript{d-} cells with the 10\% highest and lowest eGFP intensities enriched from Wnt\textsuperscript{high} secondary tumors (S2B). The
mice were sacrificed either after twelve weeks post tumor injection or if the tumor size reached
1 cm x 1 cm. The tumors were measured twice a week using digital calipers.

**In vivo indomethacin treatment and β-catenin knockdown tumors**

One hundred Wnt\textsuperscript{high}\textsuperscript{−}pCCSCs and Wnt\textsuperscript{high}\textsuperscript{−}CCSCs (sphere isolates) with the 2% lowest and highest eGFP intensities (corresponding to Wnt\textsuperscript{high} and Wnt\textsuperscript{low} cells), admixed with Matrigel at 1:1 ratio in a total volume of 100 μl, were injected subcutaneously into the hind flanks of NOD-SCID mice. At day 4 post-injection, the mice were injected with 2.5-mg/kg indomethacin (Calbiochem, Billerica, MA) or DMSO (control) i.p. every 12 hours for 6 weeks. For details of β-catenin knockdown refer to the supplementary methods, available online. For β-catenin knockdown tumors, 100 cells each of scrambled (Sc) [control], #1 and #2 shRNA transduced Wnt\textsuperscript{high}\textsuperscript{−}pCCSCs and Wnt\textsuperscript{high}\textsuperscript{−}CCSCs were prepared for injection as mentioned above. Tumor size was measured twice a week for 6 weeks using calipers.

**Statistical analysis**

Data are presented as means ± standard error as indicated in the figure legends. Statistical significance was defined as p ≤ 0.05 determined by an unpaired Student’s t-test or a one-way analysis of variance. To compare tumor growth rates, a mixed linear model was used with tumor volume as the response variable and with time and group as explanatory variables. We included subject (mouse) as a random effect, and we assumed a compound symmetric covariance structure.
Results

Increased activation of Wnt/β-catenin signaling in ALDH+ pCCSCs in colitis

To determine the functional importance of Wnt/β-catenin signaling in the colitis-to-cancer transition, we did immunohistochemistry to detect the presence of nuclear/cytoplasmic β-catenin and ALDH in colonic tissues from healthy controls, UC patients, and sporadic CRC patients. Patient characteristics for these tissues are in Supplementary Table 1. Active Wnt/β-catenin signaling was detected by nuclear/cytoplasmic β-catenin staining (Supplementary Fig. S1A). There were significantly more Wnt/β-catenin pathway active cells in colitis (2.5 fold increase) and CRC (4.5 fold increase) than in normal colon (Supplementary Fig. S1B). Similar to nuclear/cytoplasmic β-catenin, the percentage of ALDH+ cells in colitis samples was in between normal colon and colon cancer samples (Fig. 1A and B) (14). This suggests that both Wnt/β-catenin signaling activity and ALDH expression in normal, colitic and CRC colon tissue samples may parallel the normal-to-colitis-to-CRC transition. The co-immunostaining results showed an expansion of the Wnt-active/ALDH+ tumor-initiating cell (TIC) population from normal-to-colitis and colitis-to-CRC (Fig. 1A). Indeed, about 52% of the ALDH+ cells in colitis were Wnt-active, indicating that Wnt-active cells represent a major subpopulation of pCCSCs (Fig. 1B). Our immunostaining data thus suggest an ‘early’ role for activation of Wnt/β-catenin signaling in the colitis-to-cancer transition.

Generation and validation of a reporter construct specific for Wnt/β-catenin signaling

To ascertain the functional importance of Wnt/β-catenin signaling activity in pCCSCs during the colitis-to-cancer transition, we used a lentiviral dual fusion Wnt reporter, TTLG
(consisting of 6x TCF/LEF binding sites and a minimal thymidine kinase promoter regulating firefly luciferase and eGFP reporter genes) (Fig. 2A). TTLG was transduced into pCCSCs (CT-1 and CT-2) as well as into CCSCs (CA) derived, respectively, from UC and sporadic CRC patients. CCSCs were operationally defined as ALDH\textsuperscript{high} cells derived from CRC tissues that have demonstrated the ability to undergo serial passaging through immunocompromised mice with limited cell numbers while retaining the ability to recapitulate the primary tumor (10). CCSCs served as a control for pCCSCs throughout the study, since CCSCs with high Wnt/\(\beta\)-catenin signaling have been demonstrated to display cancer stem cell properties (7). pCCSCs were operationally defined as ALDH\textsuperscript{high} cells derived from colitic colon that (i) were isolated from nondysplastic UC colon, (ii) could be serially passaged through immunocompromised mice, and (iii) developed, first an anaplastic phenotype, and on serial passage transformed into a poorly differentiated adenocarcinoma (14). TTLG specificity was verified by comparing the expression of Wnt target genes (Fig. 2B, Supplementary Fig. S3A), in TTLG-eGFP\textsuperscript{high} and TTLG-eGFP\textsuperscript{low} populations of pCCSCs and CCSCs. These correspond, respectively, to Wnt\textsuperscript{high} and Wnt\textsuperscript{low} cell populations. TTLG specificity was confirmed by nuclear/cytoplasmic active \(\beta\)-catenin (ABC) staining, which indicated active Wnt/\(\beta\)-catenin signaling (Fig. 2C, Supplementary Fig. S3B). Wnt\textsuperscript{high}-pCCSCs were distinguishable from CCSCs based on differences in Wnt target gene expression profiles (Fig. 3B). Collectively, our findings not only confirm that TTLG is a valid Wnt/\(\beta\)-catenin signaling reporter, but also supports the findings of immunochemical analysis demonstrating that active Wnt/\(\beta\)-catenin signaling demarcates a major subpopulation of pCCSCs.
Wnt<sup>high</sup>-pCCSCs exhibit CCSC properties while Wnt<sup>low</sup>-pCCSCs correspond to a non-self-renewing progenitor population

To determine the functional significance of Wnt/β-catenin signaling in pCCSCs, we subjected ALDH<sup>high</sup>-Wnt<sup>high</sup> and ALDH<sup>high</sup>-Wnt<sup>low</sup> sphere cells (pCCSCs and CCSCs) (Supplementary Fig. S2A) to <i>in vitro</i> clonogenic assays (limiting dilution assays [LDA]) and <i>in vivo</i> tumorigenic assays under limiting dilution conditions (Supplementary Fig. S2B). Tumors so obtained are designated as primary xenograft tumors. However, there was no significant difference in the overall frequency of clonogenicity (Fig. 3A-C) or rate of tumor formation (Supplementary Table 2) for the ALDH<sup>high</sup>-Wnt<sup>high</sup> vs ALDH<sup>high</sup>-Wnt<sup>low</sup> primary xenograft tumors. These results may be attributed to a starting cell population of ALDH<sup>high</sup>-pCCSCs and CCSCs that are already enriched for progenitor and stem cells (14). As our study involves enrichment of the TIC fraction, the purity of the cell populations may not be absolute. This was confirmed by eGFP expression studies: FACS analysis revealed heterogeneity of eGFP expression in ALDH<sup>high</sup>-Wnt<sup>low</sup> and ALDH<sup>high</sup>-Wnt<sup>high</sup> primary xenograft tumors (Supplementary Fig. S4A) where a portion of cells in the ALDH<sup>high</sup>-Wnt<sup>low</sup> primary xenograft tumor had increased eGFP expression corresponding to high Wnt-activity. However, the percentage of Wnt-active cells was significantly greater in ALDH<sup>high</sup>-Wnt<sup>high</sup> than in ALDH<sup>high</sup>-Wnt<sup>low</sup> primary xenograft tumors (Supplementary Fig. S4B-C). In addition, the resulting ALDH<sup>high</sup>-Wnt<sup>low</sup> primary xenograft tumors phenocopied the histological appearance of ALDH<sup>high</sup>-Wnt<sup>high</sup> primary xenograft tumors (Fig. 3D) demonstrating a poorly differentiated adenocarcinoma phenotype. Since tumor initiation is a property of both progenitor cells and stem cells (SC), we serially passaged these primary xenograft tumors based on two extreme levels of Wnt-activity to further...
distinguish these two populations. Similar to CSCs, CCSCs retain the ability to both initiate tumors on serial passaging and to undergo self-renewal. Wnt/β-catenin signaling has been implicated in the self-renewal of adult colon SCs and CCSCs (17, 18). To demonstrate this phenomenon and also to enrich for stem-like cells in pCCSCs, secondary and tertiary tumors were generated from the 10% highest and lowest fluorescent cells derived from primary ALDH$^{\text{high}}$-Wnt$^{\text{high}}$ xenograft tumor (S1A). Simultaneously, an in vitro LDA was performed to determine clonogenicity (Supplementary Fig. S2B). Clonal frequency correlated with tumor-forming potential wherein ALDH$^{\text{high}}$-Wnt$^{\text{high}}$ tumor xenograft cells formed tumors at a greater frequency than ALDH$^{\text{high}}$-Wnt$^{\text{low}}$ tumor xenograft cells. ALDH$^{\text{high}}$-Wnt$^{\text{low}}$ xenograft cells largely failed to grow with subsequent passages (9 success in 69 attempts) (Fig. 4A-F and Table 1). Also, tumors derived from ALDH$^{\text{high}}$-Wnt$^{\text{high}}$ sphere and tumor xenograft cells in subsequent passages grew at a faster rate following injections of 10 cells (Supplementary Fig. S5). Similar results were obtained with secondary tumors derived from primary ALDH$^{\text{high}}$-Wnt$^{\text{low}}$ xenograft tumors (Supplementary Fig. 6A-C). These data indicate that pCCSCs generate self-renewing Wnt$^{\text{high}}$ cells. Thus, high Wnt-activity associates with sustained tumor-initiation and self-renewal.

High Wnt-Activity Confers More Efficient CSC Activity to pCCSCs (ALDH$^{\text{high}}$ sphere cells)

To test whether high Wnt-activity confers an additional level of enrichment to the already existing pCCSC marker ALDH$^{\text{high}}$, single cell injections of ALDH$^{\text{high}}$ cells enriched from ALDH$^{\text{high}}$ primary tumor xenografts and Wnt$^{\text{high}}$ and Wnt$^{\text{low}}$ cells enriched from ALDH$^{\text{high}}$-Wnt$^{\text{high}}$ primary tumor xenografts were performed. We had a 25% tumor formation success rate
(5 of 20 injections resulted in a tumor) from single ALDH\textsuperscript{high}-Wnt\textsuperscript{high} tumor cell, whereas none of the ALDH\textsuperscript{high} or the ALDH\textsuperscript{high}-Wnt\textsuperscript{low} single tumor cells developed into a palpable mass (Fig. 5A and Table 1). The resulting ALDH\textsuperscript{high}-Wnt\textsuperscript{high} tumor displayed histological characteristics of a well-differentiated adenocarcinoma in contrast to the poorly differentiated adenocarcinoma phenotype of primary ALDH\textsuperscript{high} tumor xenograft and Wnt\textsuperscript{high} tumor xenograft at the primary stage (Fig. 5B and 3D). Furthermore, besides the differential \(\beta\)-catenin expression, the tumor revealed rare cells with expression of the goblet cell marker Muc2, thus confirming tumor heterogeneity (Fig. 5C). These results confirm the greater level of CCSC enrichment bestowed by high Wnt-activity on ALDH\textsuperscript{high} cells.

Inhibition of sustained Wnt-activity in CT-2 Wnt\textsuperscript{high}-pCCSCs (ALDH\textsuperscript{high} sphere cells) reduces tumor growth rates

To ascertain if high levels of sustained Wnt-activity are necessary for tumor-initiation and growth in CAC, we used an RNA interference approach in which we inhibited \(\beta\)-catenin using two shRNAs, \#1 and \#2 (19). A scrambled (Sc) shRNA was used as a control. Following lentiviral transduction and drug selection, the efficacy of the knockdown was confirmed by FACS. eGFP levels as a measure active Wnt/\(\beta\)-catenin signaling was reduced for both shRNA, with shRNA \#1 showing the greatest reduction, compared the Sc shRNA for Wnt\textsuperscript{high}-pCCSCs (Supplementary Fig. S7A). These results were confirmed by western blotting analysis to detect \(\beta\)-catenin protein (Fig. 6A). \textit{In vivo} studies were done with shRNA \#2 transduced into the Wnt\textsuperscript{high}-pCCSC cell population. Tumors derived from shRNA \#2 transduced Wnt\textsuperscript{high}-pCCSCs and -CCSCs grew at a significantly slower rate compared to Sc transduced Wnt\textsuperscript{high}-pCCSC and -
CCSC derived tumors (Fig. 6B, Supplementary Fig. S7B). Furthermore, we used a non-steroidal anti-inflammatory drug (NSAID), indomethacin, to attenuate Wnt/β-catenin signaling in Wnt\textsuperscript{high}-pCCSCs by suppressing β-catenin expression (20, 21). Wnt\textsuperscript{high}-pCCSC tumors in vehicle-treated mice grew significantly faster than in indomethacin treated animals. The decreased Wnt-activity in tumors from mice treated with indomethacin was confirmed by immunostaining for β-catenin (Fig. 6C). This suggests a definitive role for high-level Wnt signaling in determining the rate at which colitis progresses towards CAC. Thus, therapeutic targeting of Wnt/β-catenin signaling may abrogate the progression of colitis to cancer.

**Discussion**

In the present study, we demonstrated early activation of Wnt/β-catenin signaling in the colitis-to-cancer transition based on immunohistochemical analysis as well as on *in vitro* and *in vivo* functional assays. Our initial immunohistochemical studies demonstrated that colitic epithelial cells may exhibit tumor-initiating activity based on their expression of ALDH along with an elevated co-expression of nuclear/cytoplasmic β-catenin (Fig. 1A and B). We confirmed an association of high levels of Wnt/β-catenin signaling with self-renewal, sustained tumor-initiation and tumor heterogeneity, the three properties of CSCs. Moreover, we showed the importance of high Wnt activity as an additional enrichment marker of pCCSCs within the ALDH\textsuperscript{high} population that promotes the progression from colitis to cancer.
Initial reports suggested that the Wnt/β-catenin signaling was activated late during disease pathogenesis (22-24). However, more recent studies reported early Wnt/β-catenin pathway activation (25, 26) similar to that observed in colorectal cancer (27). Moreover, the conclusions of the initial reports were mainly based on mutational analysis of Wnt signaling pathway components, while recent findings (including our own) suggesting early Wnt/β-catenin pathway activation are based on immunohistochemical data. To further examine this finding, we generated a Wnt/β-catenin reporter, which was validated by immunostaining to detect active β-catenin and real-time qPCR for representative Wnt/β-catenin pathway target genes. We observed a consistently higher expression level of all Wnt/β-catenin pathway target genes except for c-Myc in CCSCs, which was consistent with the results of Vermeulen et al (7). We speculate that the decrease in expression of some Wnt/β-catenin pathway target genes in the Wnt\textsuperscript{high} population (Fig. 2B, S3A) could be a result of CpG island methylation as reported by de Sousa E Melo et al in CRC (28).

Apart from colitis, cross-talk between Wnt/β-catenin signaling and other pathways (such as Transforming growth factor-β (TGF-β)/Bone morphogenic protein (BMP), Hedgehog (Hh), Notch, and mitogen-activated protein kinase (MAPK)) have been reported during development, adult homeostasis, SC maintenance and in other diseases (29-33). For example, in murine models of colitis, Lee et al., reported activation of Wnt/β-catenin signaling in development of dysplasia,
which was mediated through the PI3K/PTEN cascade (34). Based on these findings, we speculate that ‘early’ activation of Wnt/β-catenin pathway in the colitis-to-cancer transition could be the result of pathway cross-talk rather than mutational inactivation of pathway components.

For the first time in UC, our study highlighted two distinct functional TIC populations in pCCSCs based on serial passaging. The two populations may be analogous to long-term TIC (LT-TIC) and tumor transit amplifying cells (T-TAC) as described by Dieter et al. (35, 36). LT-TICs are CSCs that are characterized by sustained tumorigenicity as evidenced by in vivo self-renewal and recapitulation of tumor heterogeneity, while the T-TAC may be the non-self-renewing progenitor population (35, 36). Previously, we showed that the minimum number of colitis-derived ALDHhigh cells (pCCSCs) that initiated tumor formation in mice was 50 (14). In the present study, we compared the tumorigenic potential of ALDHhigh and ALDHhigh-Wnthigh cells at the single cell level and demonstrated that a single ALDHhigh-Wnthigh tumor cell was sufficient to initiate tumor formation and satisfied all three criteria for CSCs; indeed, the tumor initiating function was enriched by greater than 10-fold. The successful single cell injections suggest that the ALDHhigh-Wnthigh cells reside at the apex of the CSC hierarchy (Supplementary Fig. S8). In this study, samples consisted of 2 colitis sphere isolates (pCCSCs) obtained from two individual colitic patients. This small sample size is due to the low percentage of colitic colons harboring pCCSCs, and attests to the difficulty of propagating these cells both in vivo and in vitro, which reflects the infrequently occurring transitions of colitis-to-cancer. In contrast, using the exact same methodology, our ability to propagate frank sporadic colon cancer as a tumor was nearly 90%, with a frequency of in vitro sphere formation of 50-60%.
Progenitor cells are incapable of self-renewal and only contribute to tumor formation in primary mice, i.e., they fail to generate tumors on subsequent passages (35, 36). Failure of Wnt\textsuperscript{low} cells to consistently generate tumors following \textit{in vivo} serial passaging suggested that this cell population is dominated by progenitor cells. The equivalent tumorigenic potential between Wnt\textsuperscript{high} and Wnt\textsuperscript{low}-pCCSC and -CCSC populations during primary xenograft transplantation is consistent with the findings of David et al for CRC (37). However, in contrast to our study, those authors reported no correlation between the level of Wnt/β-catenin signaling and tumorigenicity. This discrepancy could possibly be explained by differences in the starting populations and enrichment techniques. Moreover, unlike our findings, their conclusions were based on primary tumor xenograft studies. We attribute the equivalent clonogenic and tumorigenic potential of Wnt\textsuperscript{high} and Wnt\textsuperscript{low} cell populations to the presence of both CSC and progenitor cells in the ALDH\textsuperscript{high} starting population, which was confirmed by serial transplantations of the Wnt\textsuperscript{high} and Wnt\textsuperscript{low} primary tumor xenografts. The ability of the control Wnt\textsuperscript{high}-CCSCs to propagate with serial \textit{in vivo} passages are in agreement with the results of Vermeulen et al., for sporadic CRCs, wherein they reported that Wnt\textsuperscript{high}-CCSCs are capable of self-renewal (7).

We also tested whether high Wnt-activity could be used as a therapeutic target to mitigate the colitis-to-cancer transition. To this end, we knocked down β-catenin levels using an RNAi strategy to reduce Wnt-activity. Knockdown by shRNA #1 was so effective that the transduced cells did not survive more than a week in culture, likely owing to role of Wnt/β-catenin pathway in survival (38). We observed a reduction in tumorigenicity of Wnt\textsuperscript{high}-pCCSCs, following β-catenin knockdown (shRNA #2; Fig. 6B), which was further confirmed by pharmacological
inhibition of β-catenin using indomethacin, an NSAID. Previously, indomethacin was used as pharmacotherapy for treating UC. Though this drug is no longer used to treat UC (39-41), our study indicates the potential benefits of Wnt inhibition using pharmacological agents in attenuating the colitis-to-cancer transition.

Currently, there exists no direct evidence of a genetic cause for the increased risk of CRC in patients with UC. However, several molecular consequences such as generation of reactive oxygen species, microsatellite instability, telomere shortening and chromosomal instability have been attributed to inflammation-driven genomic stress that leads to CRC (42). Though these studies have shed light on our understanding of inflammation-associated carcinogenesis, these markers lack sensitivity or specificity to be used as reliable biomarkers with which to assess the risk of CRC in patients with UC (42-46). Here, we not only identify the pCCSCs in UC colons, but also, we demonstrate that high Wnt/β-catenin signaling activity could be one of the mechanisms that drives the colitis-to-cancer transition. While ALDH may be a more inclusive marker for pCCSCs and CCSCs, the use of ALDH\textsuperscript{high}-Wnt\textsuperscript{high} as a marker panel may provide a more specific method of screening for pCCSCs in patients with colitis and indicative of an increased risk of malignant transformation in UC patients. Those chronic UC patients bearing an epithelial phenotype exhibiting high Wnt activation might be best served by a prophylactic colectomy. Additional clinical studies are warranted to rigorously demonstrate such a correlation.

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References


Table 1. Tumorigenic and self-renewal potential of Wnt<sup>high</sup> vs Wnt<sup>low</sup> cells derived from pCCSCs and CCSCs

<table>
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<td></td>
<td>1000</td>
</tr>
<tr>
<td>CT-1</td>
<td>Secondary Tumor (2&lt;sup&gt;o&lt;/sup&gt;)</td>
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<td>CT-2</td>
<td>Secondary Tumor (2&lt;sup&gt;o&lt;/sup&gt;)</td>
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<td>5/6</td>
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<td>Wnt&lt;sub&gt;low&lt;/sub&gt; (1&lt;sup&gt;o&lt;/sup&gt;)</td>
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<td>ALDH&lt;sub&gt;high&lt;/sub&gt; (1&lt;sup&gt;o&lt;/sup&gt;)</td>
<td>ND</td>
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<td>Tertiary Tumor (3&lt;sup&gt;o&lt;/sup&gt;)</td>
<td>Wnt&lt;sub&gt;high&lt;/sub&gt; (2&lt;sup&gt;o&lt;/sup&gt;)</td>
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<tr>
<td>CA</td>
<td>Secondary Tumor (2&lt;sup&gt;o&lt;/sup&gt;)</td>
<td>Wnt&lt;sub&gt;high&lt;/sub&gt; (1&lt;sup&gt;o&lt;/sup&gt;)</td>
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<td>Tertiary Tumor (3&lt;sup&gt;o&lt;/sup&gt;)</td>
<td>Wnt&lt;sub&gt;high&lt;/sub&gt; (2&lt;sup&gt;o&lt;/sup&gt;)</td>
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Enriched cell subsets obtained from pCCSCs (CT-1 and CT-2) and CCSCs (CA) were injected into the flanks of NOD-SCID mice as indicated. Ratios show the number of tumors after twelve weeks at the given number of cells injected (numerator) and number of mice (denominator). In column 3: "1<sup>o</sup>" indicates cell subsets enriched from primary tumor xenografts; "2<sup>o</sup>" indicates cell subsets derived from the secondary tumors. ND indicates not determined.
Figure 1. Expression of Wnt/β-catenin activity in ALDH+ cells from normal colon, colitic colon and CRC colon. A, Immunohistochemical co-localization (white arrows) of ALDH+ cells (green) and nuclear (blue)/cytoplasmic β-catenin expression (red). β-catenin expression limited to membranes indicates low/no Wnt activity. Nuclear/cytoplasmic β-catenin indicates active Wnt/β-catenin signaling. B, ALDH+ and β-catenin expression represented as a percentage of crypt epithelial cells. Scale bar: 10µm. Bars represent mean±SEM (normal colon; n=3; colitis: n=5; CRC: n=4; 4,000-6,000 epithelial cells were counted per condition), *p<0.05.

Figure 2. Validation of the dual fusion Wnt/β-catenin reporter. A, Schematic of the dual fusion Wnt reporter, TTLG (T-cell factor/lymphocyte enhancer factor binding site, thymidine kinase minimal promoter, firefly luciferase and enhanced Green Fluorescent Protein). B, Real-time qPCR of Wnt/β-catenin pathway target genes was done in triplicate for the 2% highest and lowest eGFP expressing fractions within TTLG-transduced pCCSCs (CT-2) and CCSCs (CA). Bar heights indicate the log₂ fold change in expression of 4 genes by TTLG-eGFPhigh (Wnthigh) and TTLG-eGFPlow (Wntlow) fractions. C, TTLG-eGFP fractions (2% highest and lowest) of the CT-2 and CA sphere isolates immunostained for activated β-catenin (ABC) localized to nucleus/cytoplasm. Scale bar: 25µm. ABC: red; Nucleus: blue; Merged stains: pink. Error bars denote mean±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3. ALDHhigh-Wnthigh and ALDHhigh-Wntlow primary tumor xenografts (1o) are equipotent. A, CT-1 pCCSCs, B, CT-2 pCCSCs and C, CA CCSCs were plated for limiting dilution assays. The y-axis indicates the clonogenic potential, represented as the minimum number of cells required to form a single sphere (error bars: mean±SEM with 95% Confidence Intervals, N=8). D, Histology of ALDHhigh-Wntlow - and ALDHhigh-Wnthigh -derived primary tumor xenografts showing a poorly differentiated adenocarcinoma phenotype with occasional lumens (arrows). Scale bar: 50µm. Bars indicate mean±SEM. 1: cell subsets derived from ALDHhigh dissociated sphere isolates.

Figure 4. Clonogenic potential of primary and secondary tumor-derived ALDHhigh-Wnthigh and ALDHhigh-Wntlow cells. Cell fractions obtained from A and B, CT-1 pCCSCs, C and D, CT-2 pCCSCs and E and F, CA CCSCs-derived primary (A, C and E) and secondary (B, D and F) tumors were plated for limiting dilution assays. The y-axis indicates clonogenic potential, which is represented as the minimum number of cells required to form a single sphere (error bars: mean±SEM with 95% Confidence Intervals, **p<0.001, ***p<0.0001, N=8). 1o: cell subsets enriched from primary tumor xenografts; 2o: cell subsets enriched from secondary tumors.

Figure 5. ALDHhigh-Wnthigh single cell tumor study demonstrates CCSC properties. A, Tumor derived from primary ALDHhigh-Wnthigh CT-2 tumor xenografts. Left upper panel (bright field) shows a representative eGFP-expressing (left lower panel) single cell used to generate a tumor (right panel). B, Histology of a single, Wnthigh-cell-derived tumor (left) versus an ALDHhigh primary tumor xenograft (from 500 cells, right). Histology reveals a differentiated adenocarcinoma with discrete tubules (left). A poorly differentiated phenotype with only occasional lumens resulted from the ALDHhigh sphere cell injection (500 cells, right). Scale bar: 50µm. C, Differential expression of β-catenin (left) and Muc 2 (right) from a tumor-derived from
a single Wnt$^{\text{high}}$ cell. Rare Muc2 immunostained cells signifying focal intestinal differentiation. Scale bar: 25μm.

**Figure 6.** Inhibition of high Wnt/β-catenin signaling in CT-2 pCCSCs attenuates cancer progression. A, Immunoblotting for β-catenin reveals successful inhibition by shRNA. Two different shRNA, #1 and #2, show decreased expression of β-catenin. The graph below displays the relative density of the β-catenin protein levels normalized to β-actin. B, The graph depicts a decreased tumor growth rate in tumors derived from shRNA #2 vs. scrambled (Sc) shRNA transduced ALDH$^{\text{high}}$-Wnt$^{\text{high}}$ sphere cells, N=5. C, Tumor latency curves of indomethacin-treated and DMSO-treated (control) ALDH$^{\text{high}}$-Wnt$^{\text{high}}$ primary tumor xenografts generated by an injection of 100 cells, N=4. Bars indicate mean±SEM. **p<0.001, ***p<0.0001. D, Immunohistochemistry for β-catenin expression. Decreased expression of β-catenin was detected in tumors of indomethacin-treated vs. DMSO-treated (control) mice. Scale bar: 25μm.
Figure 1:
Figure 2:
Figure 4.

A. CT-1 Primary tumor

CT-1 Secondary tumor

> than 800

> than 2000

1 in every

Wnt\text{low} (I^0) \quad Wnt^{high} (I^0)

1 in every

Wnt^{low} (2^0) \quad Wnt^{high} (2^0)

B. CT-2 Primary tumor

CT-2 Secondary tumor

> than 800

> than 800

1 in every

Wnt^{low} (I^0) \quad Wnt^{high} (I^0)

1 in every

Wnt^{low} (2^0) \quad Wnt^{high} (2^0)

C. CA Primary tumor

CA Secondary tumor

> than 2000

> than 2000

1 in every

Wnt^{low} (I^0) \quad Wnt^{high} (I^0)

1 in every

Wnt^{low} (2^0) \quad Wnt^{high} (2^0)
Figure 5:

A

B

C

β-Catenin

Muc-2
Figure 6:

A

\[
\begin{array}{ccc}
\text{Sc} & \#1 & \#2 \\
\beta\text{-Catenin} & \text{Image} & \text{Image} \\
\beta\text{-Actin} & \text{Image} & \text{Image} \\
\end{array}
\]

B

\[
\text{Tumor volume (mm}^3\text{)} \quad \text{Sc} \quad \#2 \\
\text{p<0.0001} \\
\begin{array}{c}
0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \\
\text{Weeks} \\
\end{array}
\]

C

\[
\begin{array}{c}
\text{Tumor volume (mm}^3\text{)} \\
\text{Indomethacin} \quad \text{Indomethacin} \\
\text{p<0.005} \\
\begin{array}{c}
0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \\
\text{Weeks} \\
\end{array}
\]

D

\[
\begin{array}{c}
\text{Indo} \\
\text{DMSO} \\
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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Transition From Colitis to Cancer: High Wnt Activity Sustains The Tumor-Initiating Potential Of Colon Cancer Stem Cell Precursors


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