ATOH1 is a master transcription factor for the secretory lineage differentiation of intestinal epithelial cells (IECs). However, the comprehensive contribution of ATOH1+ secretory lineage cells to the homeostasis, repair, and tumorigenesis of the intestinal epithelium remains uncertain. Through our ATOH1+ cell-lineage tracing, we show here that a definite number of ATOH1+ IECs retain stem cell properties and can form ATOH1+ IEC-derived clonal ribbons (ATOH1+ICRs) under completely homeostatic conditions. Interestingly, colonic ATOH1+ IECs to maintaining the stem cell population under both homeostatic and pathologic conditions and further illustrate the high plasticity of the crypt-intrinsic stem cell hierarchy.

INTRODUCTION

The intestinal epithelium is maintained by intestinal stem cells (ISCs), which reside at the bottom of the crypt (Okamoto and Watanabe, 2016). ISCs are identified by the expression of specific genes, such as leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) (Barker et al., 2007). The differentiation of intestinal epithelial cells (IECs) predominantly proceeds in a unidirectional manner, starting from the ISCs to the terminally differentiated cells. In the earliest phase of this process, progenitor cells are committed either to a secretory or absorptive lineage based on the expression of key transcription factors such as atonal homolog-1 (ATOH1) or hairy and enhancer of split-1 (HES1) (Jensen et al., 2000; Yang et al., 2001).

However, a recent study showed that small-intestinal IECs committed to the absorptive lineage can exhibit ISC-oriented de-differentiation upon severe epithelial damage (Tetteh et al., 2016). Other studies have further highlighted the plasticity and ISC-oriented de-differentiation of secretory lineage-committed IECs in the small intestine, which are identified by the expression of NGN3 (Schonhoff et al., 2004), Delta-like 1 (DLL1) (van Es et al., 2012), and doublecortin-like kinase 1 (DCLK1) (Westphalen et al., 2014). ATOH1+ label-retaining cells (LRCs) reside at the +4 position and serve as secretory progenitor cells, but they can also exhibit ISC properties upon severe tissue injury (Buczacki et al., 2013). These studies indicated that a collective proportion of ATOH1+ secretory lineage-committed IECs retain their potential to revert to ISCs and can exhibit ISC-specific properties upon severe tissue injury to compensate for the massive loss of genuine ISCs (Mills and Sansom, 2015). Consistent with this finding, a recent single-cell analysis of LGR5+ IECs identified the rare presence of ATOH1+LGR5+ double-positive cells in normal small-intestinal crypts (Kim et al., 2016). However, the comprehensive and dynamic contributions of these ATOH1+ potential ISCs under normal and pathologic conditions have yet to be described. In addition, the key factors that can recruit a specific subset of ATOH1+ IECs back to the ISC pool upon severe epithelial damage remain mostly uncertain.

In intestinal tumorigenesis, several studies have shown that LGR5+ ISCs are the cells of origin of sporadic adenomatosus polyposis coli (Apc)-deficient tumors (bottom-up model) (Barker et al., 2009). Other studies have shown that villus cells can initiate tumor development through overexpression of GREM1 or constitutive activation of the nuclear factor kappa-B (NF-κB) pathway (Davis et al., 2015; Schwitalla et al., 2013). These studies raise the possibility that de-differentiation of lineage-committed IECs may constitute another pathway of intestinal
Figure 1. Establishment of ATOH1* Cell-Lineage Tracing

(A) Schematic representation of the alleles used to establish the Atoh1tdTomato mice.

(B) Co-staining of ATOH1 (green) and tdTomato (red) in small-intestinal and colonic tissues. Atoh1tdTomato mice were administered RU486 in either a single dose (single dose) or for 5 consecutive days (five-dose) and were then analyzed on the day following the final treatment. Note that all of the tdTomato+ IECs co-expressed ATOH1.

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tumorigenesis (top-down model). Accordingly, inflammatory bowel disease (IBD) patients develop colitis-associated cancers (CACs) via a pathway distinct from that of sporadic colon cancers. Established CACs are abundant in Atoh1+ cells and, thus, retain features of secretory lineage-committed cells, such as mucin production (Park et al., 2006). The robust expression of ATOH1 in CACs may be supported by environmental tumor necrosis factor alpha (TNF-α) and contributes to maintaining their highly malignant properties (Fukushima et al., 2015). However, the potential contribution of ATOH1+ secretory lineage-committed IECs as the origin of CAC tumor stem cells has yet to be described.

In this study, using the ATOH1+ cell-lineage-tracing model, we showed that colonic ATOH1+ IECs could give rise to functional ISC-like cells under both homeostatic and pathologic conditions. The results highlight the unexpectedly broad contribution of ATOH1+ IEC-derived ISCs to the maintenance, regeneration, and progression of colitis-associated tumorigenesis in the colonic epithelium.

RESULTS

Lineage Tracing of ATOH1+ IECs Labels Secretory Lineage IECs

To elucidate the dynamic contribution of ATOH1+ IECs to the maintenance of the intestinal epithelium, we planned a lineage-tracing experiment. Here, we crossed Atoh1Cre-PGR; Rosa26-LSL-tdTomato mice to generate Atoh1Cre-PGR; Rosa26-LSL-tdTomato mice (Atoh1tdTomato mice, Figure 1A). In these mice, the effect of haploinsufficiency due to the knockin allele could not be observed, as confirmed through the analysis of Atoh1 mRNA and protein expression in the small intestine and colon (Figures S1A–S1C). To optimize the RU486-mediated tdTomato labeling of ATOH1+ IECs, we compared the labeling efficiency between a single dose of RU486 and the injection of RU486 for 5 consecutive days. Both protocols successfully labeled ATOH1+ IECs in the crypts of the small intestine and colon (Figure 1B). The 5-dose protocol resulted in a higher labeling efficiency (Figure 1C) and was therefore employed in the majority of the following experiments.

The analysis performed 24 hr after a single dose of RU486 showed that all secretory lineage IECs and some Ki-67+ IECs were initially labeled by tdTomato (Figures 1D and 1E). Conversely, all of the tdTomato+ IECs were completely negative for HES1 (Figure S1D) and for other absorptive lineage markers (Figure S1E). To further confirm the labeling of mitotic IECs, the uptake of 5-ethyl-2′-deoxyuridine (EdU) was examined in ATOH1+ IECs. Using CD24 as a marker for lower crypt IECs (Figure 1F) (Sato et al., 2012), we found that 4.7% of the CD24high/mid tdTomato+ IECs were also positive for EdU (Figure 1G).

These results collectively confirmed that our ATOH1+ IEC lineage-tracing system initially labeled both postmitotic and mitotic secretory lineage-committed IECs in a highly specific manner.

**Atoh1+IECs that Retain an ISC-like Phenotype Exist within Normal Intestinal Crypts**

LGR5+ ISCs are located at the bottom of the crypt between Paneth cells (Barker et al., 2007). To determine whether any LGR5+ ISCs were labeled by our lineage-tracing system, we crossed our Atoh1tdTomato mice with Lgr5-EGFP-ires-creERT2 mice to generate Lgr5-EGFP-ires-creERT2; Atoh1Cre-PGR+; Rosa26-LSL-tdTomato mouse (Lgr5EGFP; Atoh1tdTomato). The induction of Atoh1Cre-PGR allele-dependent tdTomato labeling in Lgr5EGFP; Atoh1tdTomato mice showed that the tdTomato+ IECs were clearly distinct from LGR5+ ISCs (Figure 2A). However, flow cytometric analysis of ATOH1+ IECs revealed a rare population of LGR5-EGFP+ ATOH1+ double-positive IECs in the small intestine of Lgr5EGFP; Atoh1tdTomato mice (Figure 2B). Consistently, RNAscope in situ hybridization (RNAscope ISH) clearly exhibited Lgr5+/Atoh1+ double-positive cells, most frequently at the +4 position or +3 position of the small intestine and colon, respectively (Figure 2C). The integrity of our RNAscope analysis was validated by using positive control tissues (Figures S2A and S2B). Also, tdTomato+ crypt cells co-expressing Ascl2 were found in both regions (Figure 2D).

CD24 is commonly expressed by small-intestinal and colonic crypt IECs (Figure 1F) and therefore used to identify and isolate LGR5+ ISCs or Paneth cells (von Furstenberg et al., 2011). Our analysis of the CD24high, CD24mid, and
Figure 2. ATOH1+ IECs Include a Cell Population that Retains the Expression of Stem Cell-Specific Genes

(A) Co-staining of Lgr5-EGFP (green) and tdTomato (red) in the small-intestinal and colonic crypts of Lgr5EGFP Atoh1tdTomato mice on the day following the completion of the five-dose RU486 treatment.

(B) Representative flow plots of the small-intestinal IECs recovered from the Lgr5EGFP Atoh1tdTomato mice on the day following the completion of the five-dose RU486 treatment.

(C) RNAscope in situ hybridization (RNAscope ISH) for Lgr5 (green) and Atoh1 (red) in the small-intestinal and colonic crypts of wild-type mice. The white dotted line shows the cell margin of a Lgr5+ Atoh1+ double-positive cell. Position number of Lgr5+ Atoh1+ double-positive cells (n = 30 of three independent experiments for each analysis) within a crypt was determined based on its relative position from the bottom of the crypt. Images of the colon are re-used in Figure 4C.

(legend continued on next page)
CD24<sub>low</sub> fraction of small-intestinal IECs confirmed that the CD24<sub>high</sub> and CD24<sub>mid</sub> population includes LGR5<sup>+</sup> ISCs, Paneth cells, and enteroendocrine cells (Figure 2E). In contrast, the CD24<sub>low</sub> population did not include these cell populations. Therefore, the combined population of CD24<sub>high</sub> and CD24<sub>mid</sub> small-intestinal IECs (CD24<sub>high/mid</sub>) was collected from the Atoh1<sub>tdTomato</sub> mice on the day after the administration of a single dose of RU486 and sorted into tdTomato<sup>+</sup> and tdTomato<sup>-</sup> cell fractions (Figure 2F). qRT-PCR analysis of these cell populations showed that gene markers for secretory cell progenitors (Dll1) and genes recently identified as markers of LRCs (Nfatc3, Nfat5) (Buczacki et al., 2013) were highly expressed in the CD24<sub>high/mid</sub> tdTomato<sup>+</sup> IECs (Figure 2G). Exceptionally, CD82 showed highest expression in CD24<sub>high/mid</sub> tdTomato<sup>-</sup> IECs, indicating its preferential expression in the Atoh1<sup>+</sup> IEC population. In addition, ISC markers such as Lgr5, Olfm4, and Ascl2 presented the highest expression in the CD24<sub>high/mid</sub> tdTomato<sup>-</sup> population (Figure 2H). However, expression of these genes was also detected in the CD24<sub>high/mid</sub> tdTomato<sup>+</sup> IEC population, although at a much lower level. These results sufficiently indicated that ATOH1<sup>+</sup> IECs in the normal intestinal epithelium consist of a heterogeneous cell population, including secretory lineage progenitor cells, and a small population of IECs retaining ISC-specific gene expression.

**Atoh1<sup>+</sup> IECs Constitutively Exhibit ISC Properties under Homeostatic Conditions**

To identify the possible contribution of ATOH1<sup>+</sup> IECs as potential ISCs, the dynamic changes in the tdTomato<sup>+</sup> IEC distribution were traced for up to 20 days. On day 6, tdTomato<sup>+</sup> IECs were found both in the crypts and in the villi, showing mostly a scattered distribution pattern (Figure 3A). At a later phase, the overall number of tdTomato<sup>+</sup> IECs showed a clear decrease, indicating that most of these cells exhibit only a short lifetime. However, we found clusters of tdTomato<sup>+</sup> IECs in both the small intestine and the colon on day 13 and day 20 (Figure 3A). These tdTomato<sup>+</sup> IEC clusters clearly formed a continuous array of IECs along the crypt-villus axis, indicating that they represent clones of IECs arising from a common ATOH1<sup>+</sup> IEC-derived ISC origin. Thus, we would like to designate these clusters of IECs as ATOH1<sup>+</sup> IEC-derived clonal ribbons (ATOH1<sup>+</sup>ICRs), representing crypts dominated by ATOH1<sup>+</sup> IEC-derived ISCs. The incidence of ATOH1<sup>+</sup> ICR formation was higher in the colon than in the small intestine, reaching up to 1.0 ribbon per 1,000 crypts (Figure 3B). No ATOH1<sup>+</sup> ICR formation was found by RU486 treatment in mice carrying Rosa26-LSL-tdTomato allele alone (Nut<sub>tdTomato</sub>) or by vehicle-alone treatment in Atoh1<sub>tdTomato</sub> mice. Compared with the previous lineage tracing of small-intestinal DLL1<sup>+</sup> IECs (van Es et al., 2012) or DCLK1<sup>+</sup> IECs (Westphalen et al., 2014), colonic ATOH1<sup>+</sup> IECs formed clonal ribbons at a frequency that was at least 10-fold higher under homeostatic conditions. Such a regional difference may simply represent the preferential labeling efficiency of our system or may represent the difference in the population size of ATOH1<sup>+</sup> IECs that retain ISC-like properties. Also, as we did not use a multi-color reporter, it remains possible that the ATOH1<sup>+</sup> ICRs observed in vivo may not necessarily represent a single-clone origin but may be composed of multi-cell origin.

These ATOH1<sup>+</sup>ICRs contained all lineages of differentiated IECs and Ki-67<sup>+</sup> IECs (Figures 3C and 3D), thereby indicating the possibility that the resident ATOH1<sup>+</sup> IECs had acquired genuine ISC-specific function and thus took over the whole crypt-villus unit.

Accordingly, analysis of the ATOH1<sup>+</sup>ICRs in Lgr5<sup>EGFP</sup>Atoh1<sub>tdTomato</sub> mice showed the clear existence of LGR5-EGFP<sup>+</sup>tdTomato<sup>+</sup> double-positive IECs in ATOH1<sup>+</sup>ICRs (Figure 3E). To further verify the acquisition of ISC properties by ATOH1<sup>+</sup> IECs, we employed the organoid culture system. Small-intestinal or colonic tissues from Atoh1<sub>tdTomato</sub> mice were subjected to organoid culture at 20 days after RU486 induction. Among the established organoids, we found organoids that completely consisted of tdTomato<sup>+</sup> IECs (Figure 3F). These tdTomato<sup>+</sup> organoids could be re-organized across passages. The efficiency of forming these tdTomato<sup>+</sup> organoids was highly consistent with the time-dependent appearance of ATOH1<sup>+</sup>ICRs in vivo (Figure 3G). Induction of ATOH1<sup>+</sup>ICR formation appeared to be driven by an IEC-intrinsic mechanism, as
A. Day 6, Day 13, Day 20 images of small intestine and colon showing cellular structures.

B. Graph showing the number of tdTomato+ crypts (ATOH1+ ICR) per 100 crypts over time.

C. Images of MUC2, CHGA, DCLK1, villin, and lysozyme in small intestine and colon.

D. Ki-67/tdTomato images for small intestine and colon.

E. Merge, Lgr5-EGFP, and tdTomato images for small intestine and colon.

F. Images showing Lgr5+ cells at Day 1, Day 3, Day 6, and post-passage.

G. Bar graph showing the formation rate of tdTomato+ organoids (ATOH1+ ICR) over time.

H. Scatter plot showing the distribution of EGFP vs. tdTomato at Day 20.

I. NES: NaN, FDR q-value: 1.0, p-value: NaN

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tdTomato+ organoids could be formed through RU486-mediated induction in vitro (Figure S3A) and maintained beyond passing (Figure S3B). However, the efficiency of forming tdTomato+ organoids in vitro was lower in the organoids of Atoh1tdTomato+ mice than in those of Lgr5-EGFP-IRESCreERT2; Rosa26-LSL-tdTomato+ mice (Lgr5tdTomato mice) (Figures S3C and S3D). In addition, tdTomato+ organoids could be re-organized from a single tdTomato+ cell, also at a lower frequency compared with LGR5high cells (Figures S3E and S3F). Those single-cell-derived tdTomato+ organoids could be re-organized beyond passing (Figure S3G) and give rise to both secretory and absorptive cells (Figure S3H). These results indicate the acquirement of multi-potency by ATOH1+ IECs in vitro.

To further identify the properties of ATOH1+ IEC-derived ISCs, LGR5-EGFP+tdTomato+ double-positive ISCs were collected from Lgr5-EGFP-tdTomato mice on day 20 (Figure 3H), and global gene expression in these cells was compared with LGR5-EGFP+ single-positive ISCs. Gene set enrichment analysis (GSEA) showed that the expression of stem cell-signature genes in LGR5-EGFP+tdTomato+ double-positive ISCs was comparable with that in LGR5-EGFP+ single-positive ISCs (Figure 3I). These results confirmed that a specific population of ATOH1+ IECs is constitutively re-directed to exhibit genuine ISC-specific properties and participate in the maintenance of the normal intestinal epithelium.

Colonic Inflammation Promotes the Generation of ATOH1+ IEC-Derived Clonal Ribbons

Following epithelial injury, various signaling pathways are activated within the intestinal epithelium to aid in optimization of the regeneration program (Okamoto et al., 2009). In the present study, only a minimal increase in the number of small-intestinal ATOH1+ICRs was observed following irradiation-induced damage (control group, 0.20 ribbons per 1,000 crypts; irradiation group, 0.63 ribbons per 1,000 crypts; data not shown). Thus, we examined whether the recruitment of colonic ATOH1+ IECs to the ISC pool and the subsequent formation of ATOH1+ICRs could be enhanced in a dextran sodium sulfate (DSS) colitis model. A significant reduction in body weight in the DSS-colitis mice indicated the successful induction of colitis (Figure S4A). At day 2, we found only a small number of tdTomato+ IECs in the rectum of DSS-colitis mice (Figure S4B). However, at day 6, we found clear increase in the number of tdTomato+ IECs in DSS-colitis mice (Figure 4B). At this time period, LGR5+ATOH1+ double-positive cells were not clearly identified in the rectal crypts of DSS-colitis mice (Figure S4A). Importantly, ATOH1+ICRs appeared in the rectum of DSS-colitis mice as early as day 6, a time period when they were never observed in the control mice. Some of the massive tdTomato+ area in the DSS-colitis mice at day 13 represented a cluster of tdTomato+ IECs extending from the ulcer-edge crypts, which covered the surface of the adjacent wound (Figure 4D). This observation indicated that ATOH1+ IEC progenies actively contribute to the early phase of tissue repair by forming the wound-associated...
Figure 4. Formation of ATOH1+ IEC-Derived Clonal Ribbon Is Significantly Promoted during Regeneration from Colitis-Induced Epithelial Damage

Formation of ATOH1+ IEC-derived clonal ribbon (ATOH1+ICR) was compared between the DSS-colitis mice (DSS) and control mice (Control). (A) Experimental design for the lineage-tracing analysis of Atoh1+ IECs in DSS-colitis-induced Atoh1tdTomato mice. (B) Labeling of ATOH1+ IECs-derived cells by tdTomato (red) at day 6 in the colon of DSS-colitis mice and control mice. (legend continued on next page)
epithelium (Seno et al., 2009). Consequently, we found an increase in the number of ATOH1+ICRs in the rectum of DSS-colitis mice at a later phase (day 20, Figures 4E and 4F). Such an increase in the number of ATOH1+ICR was never found by pre-labeling ATOH1+ cells before DSS treatment (Figures S4G and S4H), thus indicating that colitis-induced ATOH1+ICR formation is initiated by ATOH1+ IEC-derived cells that are less sensitive to DSS-induced damage, or ATOH1+ cells that have newly appeared during the post-DSS labeling period.

Collectively, we found that generation of ATOH1+ICRs was significantly promoted at the earliest phase of the tissue repair process in DSS-colitis mice, and thereby partly contributed to the regeneration of colitis-associated wounds.

**Inflammatory Cytokines and Bacterial Components Coordinately Promote the Formation of ATOH1+ICRs**

To obtain insight into the mechanism underlying the promotion of ATOH1+ICR formation in the colitic environment, the tdTomato+ IEC population in the rectum of DSS-colitis-induced Atoh1tdTomato mice was collected (Figure 5A) and subjected to microarray analysis. MA plot analysis identifiedDll1 as one of the highly upregulated genes in the tdTomato+ IEC population of the DSS-colitis mice (Figure 5B). Consistently, a clear increase in the number of DLL1+tdTomato+ double-positive IECs was confirmed in the rectal tissue of the DSS-colitis mice (Figure 5C).

Pathway analysis of the microarray data additionally revealed that gene sets representing the general inflammatory response were most significantly upregulated. Furthermore, we found that the response to interleukin (IL)-1 and TNF-α was significantly upregulated in the ATOH1+ IECs of the DSS-colitis mice (Figure 5A). The NF-κB pathway represents the major downstream signaling pathway shared by TNF-α and IL-1β (Mercuso and Manning, 1999). A previous study highlighted the importance of constitutive NF-κB pathway activation in the dedifferentiation of mouse small-intestinal villus IECs (Schwitalla et al., 2013). Our GSEA consistently revealed that the NF-κB pathway gene set was significantly upregulated in the tdTomato+ IECs of the DSS-colitis mice (Figure 5D), as were genes related to stem cell maintenance (Figure 5E). Thus, in DSS colitis, ATOH1+ IEC-derived cells appeared to gain stem cell-like gene expression under an increased activation level of the NF-κB pathway.

To validate the contribution of NF-κB signaling to ATOH1+ICR formation, we performed in vitro analyses using organoid culture. Colonic organoids from Atoh1tdTomato mice were cultured under DBZ-induced differentiation conditions, which clearly promoted commitment to the ATOH1+ secretory cell lineage, and also promoted downregulation of ISC-specific genes (Figure S5B). To induce activation of the NF-κB pathway, two representative bacterial components, such as lipopolysaccharide (LPS) and flagellin, were employed in addition to TNF-α and IL-1β. The combined addition of these ligands (TILF cocktail) clearly upregulated the expression of IL-8 in DBZ-treated colonic organoids (Figure S5C) and induced the degradation of IkBα (Figure S5D), which confirmed the activation of the NF-κB pathway in secretory cell-committed organoids. Using these secretory cell-committed organoids, we proved that addition of the TILF cocktail could induce the formation of colonic ATOH1+ICRs in vitro (Figures 5F and 5G). This in vitro formation of ATOH1+ICRs in secretory cell-committed organoids clearly required the addition of all ligands (Figure 5H). Specific blockade of the NF-κB pathway using small-molecule compounds (Figure S5E) consistently inhibited the formation of ATOH1+ICRs in a dose-dependent manner (Figures 5I and 5J). Thus, we found that the NF-κB pathway functions as a key pathway in promoting the exhibition of ISC properties by ATOH1+ IEC-derived cells. Accordingly, the addition of TILF ligands clearly upregulated the expression of ISC markers Ascl2 and Smoc2, in addition to Dll1 (Figure 5I), suggesting that the expression of these genes may play a key role in the inflammation-induced formation of ATOH1+ICRs. Such a TILF-cocktail-induced upregulation of ISC markers was never observed in organoids treated without DBZ (Figure S5G). However, the expression level of Lgr5 remained unchanged both in DBZ-treated and untreated organoids (Figures S1 and S5G), possibly due to its low expression level in early reprogrammed ISCs.

To validate the ISC property of TILF-treated Atoh1+IEC-derived cells in vitro, we tested whether TILF-treated Atoh1tdTomato mice organoids could newly reconstruct tdTomato+ organoids and expand their progeny beyond passaging. As expected, TILF-treated organoids could reconstitute secretory cell-committed organoids beyond passaging from...
single isolated cells (Figure 5K). Also, an increased number of tdTomato+ organoids was re-constructed from single isolated IECs that were recovered from the DSS-colitis mice (Figures 5L and 5M), indicating enrichment of cells retaining ISC-like properties.

**ATOH1+ IECs Comprise Colitis-Associated Tumors and Acquire Tumor Stem Cell Properties**

CAC develops through a pathway distinct from that of sporadic colon cancer (Feagins et al., 2009; Thorsteinsdottir et al., 2011) and shows pathologic features such as high ATOH1 expression (Kano et al., 2013; Park et al., 2006). However, the precise role of ATOH1+ IECs in CACs remains uncertain. We have previously shown that TNF-α mediated activation of the NF-κB pathway in IECs plays an indispensable role in azoxymethane (AOM)-DSS tumor formation in mice (Onizawa et al., 2009). Hence, we asked whether the tumors of AOM-DSS model mice (Parang et al., 2016) harbor ATOH1+ tumor cells and exhibit features of CACs (Figure 6A). It was found that AOM-DSS tumors contained a substantial number of ATOH1+ tumor cells and expressed ATOH1 at a high level (Figures S6A–S6C). Furthermore, increased production of mucin was observed in these tumors (Figure S6D), suggesting that they faithfully phenocopy CACs.

At the early stage of AOM-DSS tumor labeling in Atoh1tdTomato mice, only a few isolated tdTomato+ tumor cells were found (day 56, Figure 6B). However, clear expansion of the tdTomato+ area within the tumor was observed after day 70, which represented a continuous cluster of tdTomato+ tumor cells (Figures 6C and 6D). The ratio of these tumor ATOH1+ICRs among tumor-forming ducts did not increase during the tumor development (Figure 6D), but they clearly included Ki-67+ cells and ATOH1+ cells and showed nuclear translocation of β-catenin (Figure 6E). In contrast, they lacked expression of secretory cell markers other than MUC2 (Figure S6E). Importantly, some of the tdTomato+ cells co-expressed tumor stem cell markers such as CD44 or CD133 (Zeilstra et al., 2008; Zhu et al., 2009) (Figure S6F). Moreover, Lgr5+ cells were present in the tdTomato+ tumor clonal ribbons, as shown by the RNAscope ISH analysis (Figure 6F). Consistent with this finding, LGR5-EGFP+tdTomato+ double-positive tumor cells were found in the AOM-DSS tumors of the Lgr5EGFP;Atoh1tdTomato mice, in addition to LGR5-EGFP+ single-positive tumor cells (Figure 6G). Taken together, these results collectively indicated that ATOH1+ IECs contributed to the development of AOM-DSS tumors by acquiring tumor stem cell properties. However, the existence of LGR5-EGFP+ single-positive tumor cells...
Figure 6. ATOH1+ IECs Contribute to the Formation of AOM-DSS-Induced Colitic Tumors and Acquire the Properties of Tumor Stem Cells

(A) Experimental design combining the AOM-DSS tumor model and ATOH1+ IEC lineage tracing.

(B) H&E staining and fluorescence images of a rectal tumor tissue section on day 56. A few isolated tdTomato+ cells (red) were found within the tumor tissue.

(C) Stereoscopic view (upper panels) and tissue section (lower panels) of a massive rectal tumor (black and white arrowheads) on day 70.

(legend continued on next page)
indicated that these AOM-DSS tumors were mosaic and potentially consisted of a heterogeneous population of ATOH1+ and ATOH1− tumor stem cells.

As expected, we observed that both tdTomato+ and tdTomato− AOM-DSS tumor cell organoids could be established in vitro (Figure 6H). The tdTomato+ AOM-DSS tumor organoids consisted solely of tdTomato+ cells and shared the Wnt-independent growth ability of the tdTomato− tumor organoids. Furthermore, they could be maintained across several passages and form tumors via subcutaneous transplantation into nude mice in a dose-dependent manner (Figure 6I). The re-produced tumors of the nude mice consisted of both tdTomato+ and tdTomato− proliferating cells (Figure 6J), which conserved the mosaic features of the original AOM-DSS tumor. These results collectively indicated that AOM-DSS tumors harbored ATOH1+ IEC-derived tumor stem cells, in addition to tumor stem cells of other origins. Thus, colitis-associated tumorigenesis may represent another pathologic condition in which the acquisition of stem cell-like properties by ATOH1+ IECs can be promoted.

**DISCUSSION**

In our lineage-tracing model, the frequency of forming colonic ATOH1+ICRs reached 250–300 ribbons per mouse under homeostatic conditions and 1,500–1,800 ribbons per mouse in DSS colitis, which certainly exceeded previous models (Tetteh et al., 2016; van Es et al., 2012; Westphalen et al., 2014). Thus, our results highlight the contribution of ATOH1+ IECs in the maintenance of the ISC pool, particularly in the colon. We also succeeded in clearly visualizing the dramatic and time-dependent increase in the frequency of colonic ATOH1+ICR formation in the DSS-colitis model. This result underpins the previous report of DCLK1+ IECs, which indicated the increased ISC conversion of colonic DCLK1+ IECs in DSS-colitis mice but did not successfully identify increased formation of DCLK1+ IEC-derived clonal ribbons (Westphalen et al., 2014).

Previous studies have identified at least three subpopulations of small-intestinal ATOH1+ IECs that may retain their potential to undergo conversion back to ISCs: ATOH1+LGR5− double-positive IECs at the +1 to +3 position (Kim et al., 2016), ATOH1+LRCs at the +4 position (Buczacki et al., 2013), and ATOH1+DLL1+ IECs at the +5 position (van Es et al., 2012). However, the origin of cells that can form clonal ATOH1+ICRs may be heterogeneous and may differ from the small-intestinal counterparts. For example, the existence of colonic LRCs is not well confirmed, and small-intestinal LRCs have never been shown to form clonal ribbons under homeostatic conditions (Kim et al., 2016). The existence of ATOH1+LGR5+ double-positive IECs in the normal or colitic colon has never been identified in previous reports. In our current analysis, we successfully identified a rare population of ATOH1+LGR5+ double-positive cells both in the small intestine and colon (Figure 2C). However, most of these ATOH1+LGR5+ double-positive cells appeared to be lost under induction of DSS colitis (Figure 4C). Therefore, ATOH1+LGR5+ double-positive cells may be the dominant origin of ATOH1+ICRs under homeostatic conditions but may be less involved in ATOH1+ICR formation under DSS colitis.

We have previously shown that DLL1+ IECs reside at the lowest part of the crypts, both in the small intestine and in...
the colon (Shimizu et al., 2014). Furthermore, an increase of DLL1+ colonic IECs was clearly confirmed in DSS-colitis mice (Figure 5C) (Shimizu et al., 2014). Therefore, the DLL1+ population of ATOH1+ IEC-derived cells may be one of the candidate subpopulations that can form ATOH1+ICRs in the colon, especially under inflammatory conditions. However, it remains possible that the increase of ATOH1+ICR formation in DSS-colitis mice depends on the concerted contribution of other unknown subpopulations. A similar de-differentiation has been observed in the airway secretory lineage IECs, suggesting that re-acquiring stem cell properties is inversely related to their maturity (Tata et al., 2013).

Our analysis of ATOH1+ IECs in DSS-colitis mice successfully identified cytokine- or bacterial-component-induced NF-κB activation as a key event in promoting ATOH1+ICR formation (Figures 5D–5J), which is consistent with a previous study involving genetic NF-κB activation in villus IECs (Schwitalla et al., 2013). Because NF-κB pathway activation in secretory lineage-committed organoids appeared to upregulate the expression of Dll1 (Figure 5J), it may be possible that NF-κB signaling promotes ATOH1+ICR formation via generating an increased number of DLL1+ secretory progenitor cells.

In the AOM-DSS model, ATOH1+ IECs acquired the properties of tumor stem cells and appeared to contribute to the mosaic development of tumors (Figure 6C). At present, it remains uncertain to what extent these cells precisely contribute to the overall development and progression of these tumors. The contribution of ATOH1+ tumor cells to the overall tumor formation may be underestimated due to the limited labeling efficiency of the present lineage-tracing system. However, the clear mosaic pattern harboring both ATOH1+ and ATOH1− tumor stem cells may illustrate one of the reasons why colitis-associated tumors are highly resistant to chemotherapeutic approaches. Recently, it has been shown that human colorectal cancer cells can interconvert between differentiated tumor cells and tumor stem cells, thereby exhibiting resistance to tumor stem cell-specific ablation (Shimokawa et al., 2017). Further studies will be needed to confirm the contribution of ATOH1+ tumor cells to the heterogeneous development and progression of CACs found in IBD patients. Nevertheless, our results newly indicate ATOH1+ tumor cells as an additional therapeutic target cell population for CACs.

**EXPERIMENTAL PROCEDURES**

**Mice**

C57BL/6j mice and BALB/cAcl-nu/nu mice were purchased from Japan Clea. 

Atoh1Cre-PGR (Rose et al., 2009), Lgr5-EGFP-ires-creERT2 (Barker et al., 2007), Rosa26-LSL-tdTomato mice (Madisen et al., 2010), or ApcMin mice were purchased from the Jackson Laboratory. Apcfl/fl mice were provided by RIKEN BRC (Colnot et al., 2004). The primers used for genotyping are listed in Table S1. Male and female mice at 8–10 weeks of age were used. All animal experiments were approved by the Animal Care and Use Committee of Tokyo Medical and Dental University (0160326A and 0170276A). For the induction of Cre-mediated recombination, mifepristone (RU486, 2 mg/body, Sigma-Aldrich) or tamoxifen (TMX, 2 mg/body, Sigma-Aldrich) was injected intraperitoneally into mice carrying the Atoh1Cre-PGR allele or the Lgr5-EGFP-ires-creERT2 allele, respectively.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Okamoto et al., 2009). The primary antibodies employed for these assays are listed in Table S2. Antibodies required for the staining of ATOH1, HES1 (Ito et al., 2000), chromogranin A (CHGA), DCLK1, Ki-67, tdTomato, and β-CATENIN.

**ACCESSION NUMBERS**

Microarray data have been deposited in the Gene Expression Omnibus under accession numbers GEO: GSE81315 and GSE81451.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.11.006.

**AUTHOR CONTRIBUTIONS**

F.I., H.S., R.K., T. Nakata, and G.I. performed the histology experiments. F.I., S.F., S.A., K.S., and A.K. performed the organoid culture experiments under the supervision of T. Murano and T. Mizutani. Mouse experiments were performed by F.I. and S.N. under the supervision of S.O. and K.T. T. Nakamura and M.W. supervised the entire project. F.I. and R.O. wrote the manuscript.

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