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# Use of Cancer Stem Cells to Investigate the Pathogenesis of Colitis-associated Cancer

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**Abstract:** Colitis-associated cancer (CAC) can develop in patients with inflammatory bowel disease with long-term uncontrolled inflammation. The mutational history and tumor microenvironment observed in CAC patients is distinct from that observed in sporadic colon cancer and suggests a different etiology. Recently, much attention has been focused on understanding the cellular origin of cancer and the cancer stem cells, which is key to growth and progression. Cancer stem cells are often chemo-resistant making them attractive targets for improving patient outcomes. New techniques have rapidly been evolving allowing for a better understanding of the normal intestinal stem cell function and behavior in the niche. Use of these new technologies will be crucial to understanding cancer stem cells in both sporadic and CAC. In this review, we will explore emerging methods related to the study of normal and cancer stem cells in the intestine, and examine potential avenues of investigation and application to understanding the pathogenesis of CAC.

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**Key Words:** colitis-associated cancer, stem cells, inflammation, enteroid, colonoid, APC, Lgr5, β-catenin

Colon cancer is the third most prevalent cancer in both men and women worldwide. The incidence of disease in men in the United States is 1 in 56,400 and 1 in 41,900 for women.[1] Genetic conditions, such as familial adenomatous polyposis and hereditary nonpolyposis colon cancer, account for ~5 to 10% of colon cancer cases, but age[1] and environmental factors including diet, alcohol intake,[2] obesity, and migration from a low-incidence country to a high-incidence country[3,4] all confer increased risk. A significant risk factor in the development of colon cancer is the presence of intestinal inflammatory conditions, including ulcerative colitis (UC) and Crohn's disease. Analysis of the risk of developing cancer in patients with UC has been evaluated by several studies and by meta-analyses[5–7] over the years. Estimates of incidence have been associated with duration of disease and extent of inflammation and were found to be upward of 30% in patients with disease for over 35 years.[6] However, recent studies using population-based patient cohorts have not revealed an increased incidence of cancer in patients with UC.[8–10] Nevertheless, age of onset between 20 and 39 years continued to demonstrate an increased relative risk of developing cancer after 20 years.[8] Moreover, mounting evidence demonstrates that antiinflammatory medications are protective against the development of cancer in UC.[11,12] This evidence provides support for an important role for chronic immune activation in the development of colon tumors.

Mutations in sporadic colon cancer occur in a generally predictable manner as outlined by Vogelstein.[13,14] Initiating mutations in the adenomatous polyposis coli (APC) gene are followed by mutations in Kirsten rat sarcoma (K-RAS) and p53 which lead to the development of adenoma and then cancer. However, in colitis-associated cancer (CAC), the mutational progression through tumorigenesis is less well defined. Mutations in APC are found less frequently in both low-grade and high-grade dysplasia in patients with CAC than in patients with sporadic colon cancer.[15,16] In CAC, the driving force behind tumor development is considered to be the inflammation itself.[17,18] APC functions in a protein complex that controls the ubiquitination and cellular localization of the transcription cofactor β-catenin. When β-catenin is bound by the inactivating complex, it is ubiquitinated and degraded. When the complex is disrupted, either by a mutated APC protein, or through phosphorylation of another member of

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the protein complex glycogen synthase kinase 3 beta, β-catenin is released and can translocate to the nucleus where it combines with transcription factor/lymphoid enhancer factor (TCF/LEF) transcription factors to mediate target gene transcription. β-catenin responsive genes include proteins involved in proliferation and prevention of apoptosis.[19–21] Therefore, any mutations, or the chronic activation of pathways that stabilize β-catenin allow a permissive environment for uncontrolled proliferation, a hallmark of cancer growth.

Numerous aspects of inflammation can contribute to both tumor initiation and progression. Reactive oxygen species are important defensive factors that control infiltrating microbes and are increased in intestinal tissue from patients with inflammatory bowel disease.[22] However, reactive oxygen species can damage host cellular DNA and cause mutations.[23] Inflammation driven increases in cell proliferation during oxidative stress has recently been shown to be important in preventing DNA repair which, in turn, may contribute to increases in mutational frequencies.[24] Other aspects of inflammation that activate nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (NF-κB) can activate the β-catenin pathway.[25] Pro-inflammatory interleukin 1-beta (IL-1β) secretion from macrophages,[26] tumor necrosis factor alpha (TNFα),[27] and Toll-like receptor 4[28] have all been shown to signal through NF-κB and activate β-catenin. NF-κB signaling phosphorylates and inactivates glycogen synthase kinase 3 beta freeing β-catenin from ubiquitin-mediated proteolysis and allowing its stabilization and nuclear accumulation. The cross talk between these pathways protects cells from inflammation-induced apoptosis and is important for healing after injury. However, epithelial APC is required to maintain the homeostatic differentiation of cells during their movement through the crypt-villus axis. Loss of APC in intestinal epithelial cells prevents their differentiation and migration to the tips of the villi and subsequent loss into the lumen[29] demonstrating the importance of this pathway in controlling the natural lifecycle of enterocytes in the intestine. Thus, inflammation may activate pathways which mimic loss of APC in sporadic colon cancer.

Cancers are not a homogeneous collection of cells imbued with the same potential for infinite self-renewal. Cancers are a heterogeneous collection of cells in varying states of differentiation and with limited self-renewal potential analogous to their normal counterparts. Evidence for this lies in the inability of most tumor cells to engraft and grow in a new recipient. However, specific definable subsets of cancer cells have an increased ability to survive and propagate new tumors, and these have been termed "cancer stem cells." Stem cells are long lived cells which reside in all tissues and are capable of giving rise to tissue-specific progenitors and also to self-renew. Most current chemotherapeutics target the bulk cancer cells but are less effective at killing the cancer stem cells leading to recurrence after chemotherapy. As such, understanding the biology and niche factors that support cancer stem cells may provide new strategies to specifically target the stem cells and provide enduring protection from recurrence. In this review, we will examine the normal cellular function of intestinal stem cells, and how these functions may be coopted during transformation to drive cancer growth. We will also examine some of the emerging techniques that will allow a more in-depth study of the interaction of stem cells with other aspects of the environment and explore potential new uses for these technologies in understanding the pathogenesis of CAC.

## COLON CANCER STEM CELLS AND NORMAL STEM CELLS

There are 2 modes of developing a cancer stem cell: (1) *direct mutation* of the native stem cell itself. In this model, the stem cell acquires mutations which results in altered self-renewal kinetics and uncontrolled growth and altered differentiation of progeny to form the bulk cancer cells, or (2) *acquisition of stem properties* in differentiated cells through mutations in transit amplifying precursor populations allowing them to dedifferentiate into stem cells. Identification of the initiating cancer stem cells can be performed using several methods. In vivo, assays include using human primary tumors fractionated into subsets based on surface protein expression and injected into an immunocompromised host mouse in a limiting dilution xenograft transplant assay. This assay determines the frequency of cells in the original population which had the potential to initiate a tumor. An in vitro assay to test for stem cell properties of identified tumor cell subsets is performed by growing cells in serum free media supplemented with only epidermal growth factor (EGF) and basic fibroblast growth factor. Isolated cancer stem cells should be able to form floating spheres, whereas nonstem cells do not have the capacity to form spheres and will not grow in these assays. Using these techniques, early studies identified CD133[30,31] and CD166[32,33] as potential markers for colon cancer stem cells based on their ability to generate tumors in immunocompromised hosts and form spheres in serum-free conditions. Other markers including CD49f,[34] ALDH,[35] and CD44[36] have all been proposed as potential markers for colon cancer stem cells. However, the utility of these markers has been called into question in more recent studies.[37]

A great step in understanding and identifying differences between cancer stem cells versus bulk cancer cells occurred through the identification of normal intestinal stem cell populations. Seminal work undertaken in the Clevers laboratory identified Lgr5 as a marker of the crypt base actively cycling stem cell which produces the transit amplifying pool under normal physiological conditions.[38] Lgr5 was identified as a potential stem cell–associated protein from a panel of wingless-related integration (Wnt) pathway genes that were selectively downregulated by an inducible, dominant negative Tcf1 or Tcf4 in colorectal cancer cell lines and because its expression localized to the crypt base, the putative site of stem cells.[39] To determine whether Lgr5 was a marker of stem cells, lineage tracing experiments were performed in transgenic mouse models. Mice carrying a $CreER^{T2}$ responsive element under the control of the Lgr5 gene were crossed to a Rosa26R mouse strain containing loxP sites flanking a stop codon in the lacZ gene. Activation of the Cre recombinase

in Lgr5 positive cells removes the stop codon from the lacZ gene allowing transcription of the β-galactosidase gene. Staining for the enzymatic activity of β-galactosidase allows visualization of the Lgr5 cells and all their progeny. Using this strategy, Lgr5 positive cells were demonstrated to cycle and generate all the cells in a crypt-villus axis including all the specialized subsets of enterocytes, such as Paneth, goblet, and enteroendocrine cells.[38] This was the first study of its kind tracing the lineage of an identified stem cell in the intestine. There are caveats to this initial discovery that will be explored later in this section.

Lgr5 marks the actively cycling crypt base columnar cells. However, another population of stem cells exists in the intestine, and these are the quiescent stem cells generally located at the +4 position relative to the crypt base. Quiescent stem cells divide infrequently compared with Lgr5-positive cells but are crucial for initiating repair during wound healing and after radiation injury—they are stem cells on reserve. Markers of these cells include Bmi1 (expressed mainly in the small intestine)[40] and Lrig1 (expressed in both small and large intestine).[41] There is a certain amount of overlap in the markers expressed by quiescent and actively cycling cells. Sorted Lgr5+ cells express some Lrig1 and Lrig1+ cells express some Lgr5 gene transcripts, although dual protein expression is rarely visualized.[41] Lineage tracing experiments performed in the above-mentioned studies[38] were all performed in mouse models that used tamoxifen to induce Cre recombinase activity. A recent study has called into question the direct impact of tamoxifen on the crypt base cells suggesting that these cells are susceptible to death after exposure to tamoxifen, and that lineage tracing experiments are biased in these models because they trace recombination events in cells that are not killed by the tamoxifen treatment.[42] Because of these findings, the identification and functional analysis of different populations of stem cells in the small intestine and colon has been complicated. However, the knowledge gained from these studies on the different markers, cycling kinetics, and repopulation potential of intestinal stem cells has greatly advanced the understanding of normal stem cell function and provides the basis to begin to explore malignant transformation in cancer.

Given that APC mutations are a key step in intestinal neoplasia, the cancer stem cell potential of Lgr5+ or Lrig1+ cells has been investigated through deletion of 1 or both copies of APC in those cell subsets. When both APC alleles were deleted simultaneously specifically in Lgr5+ cells, animals developed microadenomas within 8 days progressing rapidly to large tumors in the small intestine. By contrast, when APC was deleted in both alleles of noncrypt cells, the animals developed only microadenomas that did not often progress to large tumors even at late time points.[43] In a separate study, when APC was deleted in a single allele of the Lrig1+ cells, visible adenomas were present in the colon within 50 days and 100% of mice had adenomas at 100 days.[41] The longer kinetics of adenoma formation in the Lrig1 study was due to the induced loss of only a single copy of the APC gene. A longer period was required to lose function in the second copy of the APC gene. Loss of the second allele occurs stochastically in this model. In contrast to the study where loss of both alleles from Lgr5+ cells resulted in rapid adenoma formation, using a model of single APC allele loss, no tumors were observed when APC was lost from Lgr5+ cells in either the small intestine or the colon.[41] The reason for the discrepancy between the 2 studies is not clear but may be an illustration of stem cell hierarchy in action. Lrig1 cells have been described as quiescent reserve stem cells that can repopulate crypts after significant damage to the actively cycling Lgr5+ stem cell population.[41] In this model, wild-type quiescent Lrig1+ cells may be able to replace the mutated Lgr5+ cells and return the crypts to homeostasis, although this possibility was not tested in the study.

Mutations in the stem cell populations are an understandable and straightforward path to tumor formation. However, dedifferentiation and acquisition of stem cell function in previously differentiated villus cells can also lead to tumor formation. A recent study examined the ability of villus cells that were engineered with activated β-catenin to form adenomas in mouse models. This study identified that elevated NF-κB signaling in a permissive environment leads to rapid tumor formation.[44] This may be a particularly interesting model for understanding CAC as it involves inflammatory signaling to drive tumor progression.

Specifically studying stem cell populations in tumors that arise in patients with CAC or those in mouse tumors from inflammatory cancer models including AOM-DSS and IL-$10^{-/-}$ mice may reveal novel insights into the differences in the development of CAC cancers compared with sporadic cancers. Use of the Lgr5-EGFP mouse model has been an asset for tracking stem cells in these studies. An interesting use of new confocal laser endomicroscopy has traced the appearance and progression polyps after AOM-DSS treatment in Lgr5-EGFP mice.[45] Using this new technology, the authors were able to follow the mislocalization of Lgr5-EGFP to luminal areas after AOM-DSS treatment through repeated imaging. They found that 75% of polyps that grew in this model arose from areas with luminal EGFP expression before observable polyp formation, which could indicate that an early event in tumor formation in the AOM-DSS model involves the retention of Lgr5 expression in the villus, or the movement of Lgr5+ cells out of their crypt niche. Another study has examined the gene expression profiling of AOM-DSS–induced tumors separated into Lgr5-EGFP high and low populations.[46] This analysis confirmed that EGFP+ cells were a minority in colon tumors, as they are in normal tissue. Consistent with their function in normal tissue, tumor-derived EGFP high cells had an overexpression of Wnt pathway genes. An interesting area of investigation in these samples may be to look for gene signatures associated with the ongoing inflammation and general stress responses in the stem cells in these models. Because inflammation is critical to the progression of these tumors, we speculate that the stem cell response to the inflammation may be critical to driving tumor progression, and may provide novel pathways that can be targeted to improve susceptibility of the cancer stem cells to chemotherapy regimens.

Genetic models of knockouts in specified cell types have been very useful in illustrating some of the prerequisites of tumor

formation, and the importance of the type of cell where the deletions or mutations take place. However, the use of mouse models is extremely expensive and time consuming, and as evidenced with the findings on the effect of tamoxifen on the crypt cells, the manipulations themselves may be skewing the findings. As such, the development of in vitro culture techniques to grow primary intestinal cells which differentiate into all the cell types and faithfully recapitulate location-specific function may increase the ease of study of the transforming events that occur during tumorigenesis. In particular, they allow reductionist and mechanistic approaches to address how cancer stem cells function.

## EMERGING IN VITRO TECHNIQUES TO STUDY TUMORIGENESIS

Growth of primary epithelial cells has long been a technically difficult culture method. For this reason, most studies investigating epithelial responses have been conducted using colon adenoma or carcinoma-derived cell lines. However, study of the initiating events in tumorigenesis cannot be performed in previously transformed cell lines. Recent advances in evaluating the crypt stem cell niche helped to elucidate the conditions required to allow intestinal stem cells to grow in vitro.[39,47,48] Using a cocktail of recombinant proteins including a WNT ligand binding partner (R-spondin1), BMP inhibitor (Noggin) and epithelial growth factor (EGF), crypt structures as well as single isolated Lgr5+ cells can be grown in a semisolid laminin and collagen gel (Matrigel). These cultures (enteroid in the small intestine and colonoid from the colon) form crypt and villus regions and differentiate into all the epithelial subsets according to their in vivo stoichiometry.[47] Cultures grown from stem cells isolated from various regions of the intestine (duodenum, jejunum, and ileum) retain their region-specific differences in protein expression including Fe transporters exclusively found in the duodenum, high levels of sucrase isomerase in the jejunum, bile acid transporters in the ileum, and high carbonic anhydrase expression in the colon.[49,50] The ease (but not the cost) of growing these cultures has greatly advanced the field of epithelial stem cell biology. Cultures can be grown out of normal or cancerous tissue from murine, human,[51] and porcine sources.[52]

The utility of these cultures for drug screening and personalized medicine has been demonstrated by several studies. Cultures can be grown from patient samples and recapitulate their in vivo physiological characteristics in culture. For example, normal rectal-derived colonoids induce rapid swelling in response to forskolin. When colonoids derived from patients with cystic fibrosis with defined mutations in the cystic fibrosis transmembrane conductance regulator anion channel protein were examined, they demonstrated limited ability to swell in response to forskolin; thus, the function of genetically perturbed human tissue can be studied ex vivo.[53]

Another example of the use of these new culture methods using patient samples was recently published.[54] Biopsy samples collected from patients with inflammatory bowel disease and healthy patients were grown and reliably passaged successively and frozen down as stock cultures. The cultures in this study were grown in conditioned media from transfected L cells secreting recombinant Wnt3a, R-spondin3, and Noggin (L-WRN–conditioned media). These cultures grew mostly as undifferentiated spheres, and the culture media could be manipulated by reducing the concentration of conditioned media and by adding a Notch inhibitor (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT)) to favor differentiation of cells into goblet cells or Paneth cells—although both types of cells were not shown to be produced concurrently using a single cocktail. The undifferentiated spheres could be dissociated, plated onto transwells, and grown as a monolayer. When the investigators added DAPT to the media favoring goblet cell differentiation, the cells secreted a mucus layer comparable with that found in-vivo.[54,55] Growing stem cells in a transwell will further facilitate studies that require polarization of the epithelium and access to the apical and basolateral sides separately. By contrast, when cells are grown as spheres, it makes it difficult to stimulate the apical (inner) surface of the enteroid/colonoid. This study also examined the ability of different strains of *Escherichia coli* to adhere to the apical side of the monolayers. They were able to demonstrate that known adherent and nonadherent strains, as determined by studies using cell lines, behaved similarly with the primary monolayers. However, using the enterohemorrhagic *E. coli* O157:H7 strain, the cell line demonstrated limited adherence, whereas primary epithelial monolayers from both terminal ileum and rectum demonstrated adherence to the bacteria, which is likely to be more similar to what happens in vivo with this known intestinal pathogen. Others studies are beginning to be published examining epithelial cell death,[56] and rotavirus infection[57] and the monolayer cultures are amenable to study most host–pathogen interactions in primary and patient-derived samples.

These studies illustrate the usefulness of these cultures in personalized medicine. Samples derived from patients can be used in specific assays to screen for responsiveness to drug regimens and for susceptibility to infection by different strains of bacteria. This technique may be especially advantageous in cancer care. Cultures derived from patient samples can be screened for responsiveness to various chemotherapy regimens and compared for effective killing of the bulk tumor cells, but also, the assays may be manipulated to monitor the killing of specific stem cell populations.

## INDUCED PLURIPOTENT STEM CELLS

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to dedifferentiate into embryonic stem cells or into an embryonic stem cell-like state, and they are able to generate cells from the 3 germ layers (endoderm, mesoderm, and ectoderm). This line of research is fairly recent, going back barely a decade. In 2006, the Yamanaka laboratory published the first study about reprogramming mouse somatic stem cells into iPSCs.[58] They discovered that introducing only 4 genes (Oct 4, Sox2, c-Myc, and Klf4) into mouse

fibroblasts by retroviral transduction could achieve cell pluripotency. This breakthrough in stem cell research merited Yamanaka the Nobel Prize in 2012. Shortly after the first report of mouse iPSCs in 2007, Yamanaka[59] and Thompson[60] independently published the first studies reprogramming human somatic cells into iPSCs. Cancer cells and stem cells share the characteristics of increased self-renewal capacity and proliferative signaling, so studying iPSCs may shed significant light on the molecular processes that create a permissive environment for uncontrolled growth.

Several methods have been published to generate iPSCs, and they differ mainly in the delivery route to reprogram the cells, the starting cellular source to be reprogrammed, or the chemical environment in which these cells are kept in culture.[61–64] Retroviral and lentiviral vectors are commonly used to reprogram cells because of their efficiency of delivering genes and ease of use. However, use of these particles has the potential to activate oncogenes during the integration of the foreign DNA into the host, which may theoretically lead to tumorigenesis.

Generating iPSCs from malignant tissues from patients may provide a tool for patient-based in vitro models to study cancer progression and response to chemotherapy.[65] Miyoshi et al[66] showed that reprogramming gastrointestinal cancer cell lines conferred sensitivity to chemotherapeutic agents compared with the parental lines. Despite the technical difficulties, Kim et al[67] created a patient-derived iPSC line from pancreatic ductal adenocarcinoma and showed that injection of these iPSCs into immunodeficient mice resulted in early stage neoplastic transformation that progressed to a more invasive form of pancreatic neoplasia. This study is an example of the use of iPSCs to create a model for in vitro study of cancer initiation and progression.

Alternatively, iPSCs can be derived from healthy patients and differentiated into intestinal organoids.[68–70] Organoids derived from iPSCs have an associated surrounding mesenchyme of α-SMA positive myofibroblasts surrounding the main body of the organoids. In contrast, enteroids and colonoids derived directly from intestinal tissue are purely epithelial. The addition of myofibroblasts in the iPSC culture allows for the investigation of the impact of these cells in different disease settings.

Myofibroblasts are important mediators of fibrosis in inflamed tissues, which is a major complication in patients with Crohn's disease. Rodansky et al have used this interesting property of iPSC-derived organoids to investigate the interaction between myofibroblasts in the epithelial environment to fibrosis-inducing signaling through TGFβ. Increased gene expression of fibrotic proteins such as COL1A1 (collagen, type I, alpha 1) and FN1 (fibronectin 1) by the myofibroblasts was observed in response to TGFβ and was prevented by the antifibrotic agent spironolactone.[71] Future studies using this type of iPSCs-derived organoid culture may provide insight into aspects of the interaction between epithelial cells and myofibroblasts during inflammation and critically, during healing and transformation—all of which could potentially be modeled in vitro.

The potential usefulness of iPSCs to investigate the initiation, progression, and response to treatment of cancer is promising, especially at the individual patient level. However, because of the technical difficulty in the methodology to generate iPSCs, more studies are needed to advance in the field.

## UTILITY OF ENTEROIDS/COLONOIDS IN STUDYING NONEPITHELIAL ASPECTS OF CAC DEVELOPMENT

Recently, it has become possible to genetically manipulate enteroids and colonoids using lentivirus vectors[72] as well as using the new clustered regularly interspersed short palindromic repeat (CRISPR)–CRISPR-associated protein 9 (Cas9) genome editing (CRISPR/Cas9) technology.[73,74] In addition, lentiviral-GFP constructs can be efficiently transduced into enteroid cultures and transfection monitored by live cell immunofluorescence.[72] This technique allows for the generation of genetically manipulated cultures in as little as 2 weeks. Two recent studies have used the CRSIPR/Cas9 system to investigate the tumorigenic potential of common cancer-associated mutations. The authors in both studies sequentially induced common mutations in human-derived colonoids and determined their ability to grow independently of exogenously added growth factors. They hypothesized that the absence of growth factors was analogous to the loss of environmental regulation for growth which characterizes cancers and metastases. The authors further demonstrated the ability of the transformed colonoids to grow in vivo by transplanting them into an immunocompromised host. Colonoids with 4 to 5 mutations in tumor suppressors *APC*, *TP53*, *SMAD4* and tumor promoters *K-RAS*,[74] and *PIK3CA*[73] were generated and found to develop into invasive tumors resembling carcinomas in most recipients. However, colonoids generated with only 3 mutations (*APC, K-RAS, TP53*) produced small adenoma-like growths in only a minority of recipients—thus recapitulating the sequence of events that happen in the transition from dysplastic crypts, to polyps, to colon cancer.[74] Stem cell markers in these engineered tumors were identifiable by in situ hybridization, indicating that the transplanted transformed cells had maintained their stem cell phenotype. The utility of being able to manipulate epithelial stem cells to investigate the outcome of different genetic transformations on subsequent loss of environmental regulation will be advantageous in comparison with mouse models due in part to their ease of construction and also due to the decreased time and money required to generate the models.

CACs have a more diverse repertoire of mutations in their tumors, and also a different stepwise progression from adenomas to carcinoma compared with sporadic cancers. This modeling system could be used to investigate CAC-specific mutations and chronic activation of inflammatory pathways in enteroids and colonoids and may provide enormous insight into the pathogenesis of the inflammation-specific aspects of CAC tumorigenesis. Derivation of colonoids from patients with CAC and their response to growth in an inflammatory environment may also

provide insight into the way inflammatory pathways are coopted to actually support, rather than fight, tumor growth.

Currently, most enteroid and colonoid cultures have been purely epithelial derived, and were generated in this manner specifically to examine the epithelial responses in the absence of any contaminating immune response. But the intestine is not made solely of epithelial cells, and other cell types are important in modulating the function of the epithelium and shape the microenvironment surrounding the epithelium. To model more complex interactions which occur within the intestinal environment, additional cell types will need to be included in the culture system. Hemocytes (macrophage-like cells in *Drosophila*) were recently described to have an important role in providing crucial pro-proliferative signals to stimulated intestinal stem cells after inflammation. Regulation of this interaction was important for wound healing but was also shown to play a role in dysplasia in aging flies.[75] The importance of interactions between monocytes and stem cells was also demonstrated in a mouse explant model. The authors found that monocytes and epithelial cells were directly in contact with each other and that MyD88 signaling in the monocytes promoted crypt cell proliferation and hypothesized that this was likely a mechanism of protection after injury.[76] A recent study has cultured enteroids with peripheral antigen-specific T cells and noted that both cells can be cultured together for greater than 4 weeks, and that the cocultured T cells go on to develop projections between the epithelial cells consistent with the physical location and function of normal in vivo intraepithelial lymphocytes. Enteroids derived from iFABP-tOVA mice, which produce OVA peptide under the intestinal fatty acid-binding protein promoter in mature enterocytes, and $CD8^+$ OVA transgenic T cells were cocultured, and the response of T cells to OVA peptide presented in MHC class I by the epithelial cells could be monitored. Proliferation was measured in the T cells after culture and was only detected when iFABP-tOVA, not WT enteroids, were cultured with transgenic T cells. Interesting differences were noted when naïve or previously activated T cells were added to the cultures. Antigen presentation by the enteroids seemed to decrease proliferation induced by previously activated T cells, as compared with naïve T cells and the authors postulated that presentation of antigen to activated T cells by enteroids may dampen T cell responses.

This study, and similar future studies harnessing the power of enteroid and colonoid cultures in combination with immune cells will allow unprecedented knowledge to be garnered about the interactions between immune cells and their impact on epithelial cells and vice versa. For the study of CAC, this is especially important given the critical role of inflammation in the process. We envisage that studies using genetic knockouts in either epithelial or immune compartments will more accurately distinguish the roles played by innate immune recognition (Toll-like receptors or NODs), chronic inflammatory cytokine stimulation (IL-6 or TNF$\alpha$), and the role of different subsets of immune cells on the growth and transformation potential of epithelial stem cells. A possible experimental paradigm may be to use the lentiviral or CRISPR/Cas9 system to induce early genetic transformations observed in inflammation-associated dysplasia such as *TP53*, and *TFGBRII* mutations which were observed in gastric tissue infected with *Helicobacter pylori*.[77] The enteroids and colonoids could then be cultured long term with immune cells or inflammatory cytokines and the impact on the stem cells proliferative capacity, ability to differentiate, and their potential for chromosome instability could be measured. Conversely, the addition of current therapeutic treatments on the cultures could be assessed for their ability to prevent further epithelial transformation.

## CONCLUDING REMARKS

Knowledge of the biology of the intestine has progressed significantly in the last decade. The field now has at its disposal a number of powerful techniques to investigate numerous aspects of the intestinal environment. The understanding gleaned using in vitro cultures of primary epithelial cells in enteroid or colonoid cultures can then be followed up with sophisticated cell type specific deletions or mutations in animal models. We anticipate that knowledge of the specific protective mechanisms used by normal stem cells can be used to understand cancer stem cells and will enable targeted therapies to efficiently eradicate these chemoresistant cells. Specifically in CACs, the use of cultures containing other elements of the intestine, such as immune cells in enteroid/colonoid cultures and myofibroblasts in iPSC organoids will revolutionize the understanding of the complex interactions occurring during cellular transformation and inflammation. Finally, the ability to easily and reproducibly grow primary patient-derived cells could have a major impact on future diagnostic and therapeutic decision making.

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