A recellularized human colon model identifies cancer driver genes

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Refined cancer models are needed to bridge the gaps between cell line, animal and clinical research. Here we describe the engineering of an organotypic colon cancer model by recellularization of a native human matrix that contains cell-populated mucosa and an intact muscularis mucosa layer. This ex vivo system recapitulates the pathophysiological progression from APC-mutant neoplasia to submucosal invasive tumor. We used it to perform a Sleeping Beauty transposon mutagenesis screen to identify genes that cooperate with mutant APC in driving invasive neoplasia. We identified 38 candidate invasion-driver genes, 17 of which, including TCF7L2, TWIST2, MSH2, DCC, EPHB1 and EPHB2 have been previously implicated in colorectal cancer progression. Six invasion-driver genes that have not, to our knowledge, been previously described were validated in vitro using cell proliferation, migration and invasion assays and ex vivo using recellularized human colon. These results demonstrate the utility of our organoid model for studying cancer biology.

Clinical translation of basic cancer research is hindered by several obstacles. One arises from the intrinsic limitations of experimental systems, which may not demonstrate concordance with human studies; for example, conventional two-dimensional (2D) cell culture models do not maintain the interactions of tumor cells with the extracellular matrix (ECM) and the tissue microenvironment, which are essential for tumor pathogenesis. Similarly, animal models are expensive and time-consuming and may not have the resolution and sensitivity required to track the dynamics of cancer progression. In addition, because of species variation, animal models and humans may have different requirements for oncogenic transformation.

Another problem stems from the fact that tumors evolve heterogeneously; the large number of passenger mutations they accumulate can confound the identification of driver genes. Although high-throughput genetic approaches have facilitated the identification of genome-wide alterations in cancers, testing the role of each gene in cancer pathogenesis in the native environment is a crucial but difficult task, given the large number of low-frequency mutations in most cancer genomes.

Intestinal organoid systems can be used for modeling both normal and disease tissues. However, these systems usually lack native tissue structures and ECM and thus do not mimic vital aspects of intestinal physiology. Recent technical advances have made it possible to isolate natural cellular matrix with preserved ECM and normal 3D tissue architecture, providing a potentially useful approach for producing more physiologically relevant models of cancer.

Ridky et al. have successfully established ex vivo systems recapitulating the normal–neoplasia–invasion sequence with modified human epidermis, oropharynx, esophagus and cervix epithelial cells in a human 3D tissue environment. Here we describe an ex vivo model of the human colon that mimics physiological conditions by first decelluarizing normal human colon tissue under conditions that retain the tissue's complete geometry with a well-preserved ECM, relatively integral vascular network and intact muscularis mucosa, then reseeding with primary colonic epithelial cells, endothelial cells and myofibroblasts. We used the model to study colorectal cancer (CRC) progression by recellularizing the colon matrix with epithelial cells carrying mutations in genes known to be important in CRC progression, such as APC and KRAS. Similarly to CRC, this led to the development of neoplasia in situ with little submucosal invasion, whereas reseeding with cells carrying mutant APC and an activating mutation in KRAS (KRASG12D) led to the in the context of reduced transforming growth factor-β (TGF-β) signaling, and led to the development of invasive submucosal tumors. We used the ex vivo model to perform a Sleeping Beauty transposon mutagenesis screen to identify genes that cooperate with mutant APC in driving invasive neoplasia.

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RESULTS
Preparation of decellularized human colon matrix
Cellular components from fresh normal human colon tissue (~5 cm³) were removed by sodium dodecyl sulfate (SDS) treatment followed by washing with Triton X-100, resulting in the complete decellularization of the colon tissue (Fig. 1a–d). The DNA content of these acellular colon scaffolds was more than 95% lower than that found in normal colon (Supplementary Table 1 and Supplementary Methods), whereas no differences were observed in the quantities of the four main ECM proteins, glycosaminoglycan, collagen type I, laminin and fibronectin (Supplementary Table 1 and Fig. 1h).

The decellularized scaffolds successfully preserved the tissue architecture, main vasculature and crypt niches of the original organ (Fig. 1e–g). The removal of most cellular components was further confirmed by the observation that F-actin and cellular nuclei were undetectable in decellularized scaffolds by immunohistochemistry (Fig. 1h).

Recellularization of the acellular human colon matrix
A key feature that makes the ex vivo colon model useful for identifying CRC driver genes is that it has a genetically defined epithelium that is free of malignant origins and secondary genetic alterations. To recellularize the acellular colon matrix we first established primary cultures of the three main cellular components of the colon—epithelial cells, endothelial cells and myofibroblasts. Epithelial cells and myofibroblasts cells were obtained from routine colonoscopy patient samples, whereas endothelial cells were purchased from commercial sources. Epithelial cells were grown under conditions normally used for organoids to help retrain their stem cell component and differentiation potential. These cells were then modified with human telomerase reverse transcriptase (hTERT) to prevent premature senescence and ensure their long-term growth potential (Supplementary Fig. 1a). DNA sequencing of these primary colonic epithelial cells (hCECs) confirmed that they did not carry mutations in hotspot regions of APC, KRAS or TP53 (data not shown). When grown in 3D culture, these hCECs formed organoids with microcyst-like structures (Supplementary Fig. 1a) and were capable of self-renewal and multilineage differentiation (Supplementary Fig. 1b).

Recellularization of the colon matrix was done in several discrete steps (Fig. 2a). First, the large bowel mucosa with intact crypt niches and muscularis mucosa was mechanically separated from the submucosa by forceps (Supplementary Fig. 2a,b and Supplementary Video 1). This made it possible to reintroduce the hCECs, endothelial cells and myofibroblasts into their normal physiological locations within the acellular colon matrix (Fig. 2b). For populating the mucosa, a microinjection pipette was filled with endothelial cells (Supplementary Figs. 2c and 3b and Supplementary Video 2), and the cells were injected into the mucosa through the side of the tissue (0.1 million/cm²) (Supplementary Fig. 2d and Supplementary Video 3). After the endothelial cells started to proliferate, hCECs (0.2 million/cm²) were placed on top of the crypt surface and seeded into the crypt niches through gravity precipitation. Finally, colonic myofibroblasts (Supplementary Fig. 3a) (0.1 million/cm²) were seeded and grown on the muscularis mucosa, which was exposed through separation from the submucosa. In addition to the muscularis mucosa, the stroma surrounding the colonic crypts in the normal colon also contain myofibroblasts. Owing to technical limitations, however, we were unable to repopulate the mucosa pericryptal with myofibroblasts and focused instead on repopulating the muscularis mucosa layer with colonic myofibroblasts. The ex vivo colon was then generated by assembling together the mucosa and submucosa layers (Fig. 2b and Supplementary Fig. 2e,f). The process of decellularization and recellularization preserved native colon ECM and its mechanical and chemical properties (including intestinal fibers composed of laminin and collagen in the correct orientation), composition and microstructure (Supplementary Fig. 4a). Notably, the recellularized colon matrix retained the ultrastructural elements of the muscularis mucosa—the native barrier for the invasion of the malignant CRC submucosa—which was not substantially different in thickness from that found in native tissues (Fig. 1g and Supplementary Fig. 4b,c). The recellularized ex vivo colon not only was viable but also contained cells expressing LGR5, a stem cell marker in small intestine and colon, in addition to other differentiated cell types normally found in the colon (Supplementary Fig. 5). Physiologically similar to native ones, our ex vivo colon crypts contained dynamic proliferation capacity (Supplementary Figs. 5 and 6b), actively reproduced WNT–β-catenin signaling, which is essential for intestinal homeostasis (Supplementary Fig. 6a), and expressed the major mucin protein MUC2 (Supplementary Fig. 6c), indicative of secretory activity of the recellularized crypts. We also analyzed the growth capacity of the three cell lines in recellularized mucosa by Ki67 staining and observed that endothelial cells were the first cell type undergoing growth dormancy or senescence after the reconstituted mucosa were cultured for 8–10 weeks (data not shown).
Establishment of a CRC progression model

CRC develops through the stepwise selection of genetic changes. The initiating or gate-keeping mutation is biallelic loss of APC, which occurs in >80% of cases (Fig. 2a). This is followed by mutations in other genes, such as activating mutations in KRAS at the early- to intermediate-adenoma stages, and loss-of-function mutations in SMAD4 and TP53 at the intermediate-adenoma to adenocarcinoma stages (Fig. 2b). To determine whether it was possible to transform hCECs into tumor cells, we infected these cells with a lentivirus that expresses a small hairpin RNA that disrupts APC (APC-shRNA) and incubated in medium containing 500 mmol/L TGF-β (APC-KRAS−) or after treatment with TGF-β (APC-KRAS−TGF-β+). All of the recellularized tissues were analyzed after 7 weeks in culture. n = 10 independent matrices; P values determined by one-way ANOVA; error bars indicate mean ± s.e.m.

On the basis of these findings, we asked whether we could model CRC progression using the ex vivo human colon cancer model (Fig. 2a). Similarly to normal human colon (Fig. 2c), the acellular colon recellularized with unmodified hCECs formed a single-cell layer in crypt niches, and the cells were tightly attached to the basal membrane and stromal ECM (Fig. 2d). By contrast, when the acellular colon was recellularized with hCECs expressing APC shRNA (APC-shRNA hCECs), dysplasia was visible in the mucosa epithelium, a phenotype typically seen in early-stage adenomas, with which cells underwent fast proliferation to form multicellular layers and distorted crypt structures with multiluminal fusions (Fig. 2e,f). These dysplastic lesions formed in only 3 weeks and were restricted to the mucosa layer (P < 0.001, one-way ANOVA). The dysplasia grew larger without invasive events when acellular colon was recellularized with APC-shRNA hCECs in addition to activated KRASG12D (Fig. 2g,h). However, when the TGF-β signaling pathway was inhibited in acellular colon recellularized with APC-shRNA hCECs expressing activated KRASG12D, large adenomas developed, broke through the muscularis mucosa and invaded the submucosa, a key feature of malignant CRC (CRC) (P = 0.0024, one-way ANOVA). This malignant transformation occurred within 4 weeks after onset of the dysplastic lesions that formed with APC-shRNA hCECs.

Collectively, these results show that this type of organotypic colon cancer model, developed with human natural matrix and genetically defined primary colon cells, is capable of recapitulating key features of CRC initiation, progression and malignant transition from noninvasive neoplasia to invasive submucosal tumor. The different stages of oncogenic transformation can be generated relatively easily and within weeks in this model, correlating well with histological features

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Recellularization

Figure 3 Invasive adenomas induced by SB mutagenesis. (a) Steps used in the creation of invasive adenomas by SB. (b–e) Hematoxylin and eosin (H&E) staining (b, d) and immunostaining (c, e) of cytokeratin and fibronectin in CRC models recellularized with wild-type hCECs mutagenized SB (top) or APC shRNA-expressing hCECs mutagenized with SB (bottom). DAPI nuclear staining is shown in blue. Scale bars, 50 μm. (f) Quantification of invasive neoplasia in the two CRC models. All recellularized tissues were analyzed after 7 weeks in culture. n = 6 samples per group; P values determined by two-sided Student’s t-test. Error bars indicate mean ± s.e.m. (g–i) Immunostaining for cytokeratin and fibronectin showing progression from in situ neoplasia to submucosal invasion in SB-mutagenized hCECs. DAPI nuclear staining is shown in blue. Scale bars, 50 μm.

Identification of genes driving invasive neoplasia

In previous studies, we used SB mutagenesis to model several types of cancer in mice, thereby identifying many novel cancer genes and cancer signaling pathways. We here extended the use of this transposon plasmid combined with 100 ng transposase plasmid produced higher copy numbers of transposon in the genome of hCECs (Supplementary Fig. 9a). Under these conditions, each transfected hCEC contains an average of 4 ± 3 (s.d.) transposon copies in its genome after 6 weeks of antibiotic selection (Supplementary Fig. 9b).

To determine whether we could use SB mutagenesis to identify genes driving invasive neoplasia in the ex vivo colon model, we used the modified SB transposition system to mutagenize APC-shRNA hCECs, which we then used to recellularize the acellular colon matrix (Fig. 3a). Within 6–7 weeks after recellularization, multiple tumors undergoing submucosal invasion could be seen in the recellularized colons, in contrast to those recellularized with unmutagenized APC-shRNA hCECs, which showed little submucosal invasion (Fig. 3b–e). The average number of tumors undergoing submucosal invasion formed in 10-cm² matrix was 5.5 ± 1.9 (s.d.) for mutagenized cells, significantly higher than the average number of 0.83 ± 0.75 for unmutagenized cells (Fig. 3f) (P = 0.002, Student’s t-test). SB-based mutagenesis thus appears to affect genes that drive the invasion of APC-mutant cells. Colon tumors in multiple disease stages were also observed in this ex vivo system, and single-cell resolution could be obtained from time-lapse tracking (Fig. 3g–i).

To identify genes mutated by SB that promote invasive neoplasia, we excised 21 invasive neoplasias from 15 recellularized colon matrices by laser-capture microdissection using a procedure that minimizes contamination from adjacent noninvasive tumor tissues (Supplementary Methods). Transposon junction fragments were then amplified by ligation-mediated PCR and the amplification products sequenced by Illumina sequencing. The distribution of read depths at the insertion sites indicated that the majority of insertions represented subclonal insertions. We therefore considered only the top 10% of insertions with the highest read depth to be clonal insertions. We then mapped these

Table 1 Candidate invasion-driver genes previously implicated as contributing to CRC progression

| Gene   | Unique insertions | Invasive neoplasia with insertions | Function                      |
|--------|-------------------|-----------------------------------|-------------------------------|
| AKT1P  | 1                 | 1                                 | AKT signaling                 |
| DCC    | 1                 | 1                                 | Cell adhesion and apoptosis signaling |
| EPHB1  | 1                 | 1                                 | Cell migration and adhesion   |
| EPHB2  | 1                 | 1                                 | Cell migration and adhesion   |
| FSTL5  | 1                 | 1                                 | Calcium ion binding           |
| JAK1   | 1                 | 1                                 | STAT signaling                |
| LATS2  | 2                 | 2                                 | P53 signaling                 |
| MAML3  | 1                 | 1                                 | NOTCH signaling               |
| MSH2   | 1                 | 1                                 | DNA mismatch repair           |
| NRCAM  | 1                 | 1                                 | Cell adhesion                 |
| PTPRD  | 2                 | 3                                 | Cell growth and differentiation |
| ROCK1  | 1                 | 1                                 | Cell motility                 |
| STAT3  | 1                 | 1                                 | STAT signaling                |
| TCF7L2 | 2                 | 1                                 | WNT signaling                 |
| TTN    | 1                 | 1                                 | Cell motility                 |
| TWIST2 | 1                 | 1                                 | Epithelial to mesenchymal transition |
| WNT7B  | 1                 | 1                                 | WNT signaling                 |

*Number of unique SB insertions in each gene; †number of independent invasive neoplasias with SB insertions in the same gene.
clonal insertion sites to the human genome and picked the sites that were located within 1,000 bp of a known transcript in RefGene. We subsequently confirmed the sequences of these transposon insertion sites by Sanger DNA sequencing. Collectively, these studies identified 38 candidate invasion-driver genes from 21 invasive neoplasias (Supplementary Table 2). Notably, 17 of the 38 genes have already been implicated as contributing to CRC progression, including TCF7L2, which functions in WNT1 signaling,40 MSH2, a DNA mismatch-repair gene,5,28 TWIST2, which is important for the epithelial–mesenchymal transition;2 JAK1 and STAT3, which function in JAK–STAT signaling33; and DCC, which is commonly deleted in CRC.34 (Table 1).

Most of these candidate invasion-driver genes were mutagenized in a single invasive neoplasia (Supplementary Table 2). However, six genes (CSTF3, GRM8, KDM2B, LAT2, PAX7 and TCF7L2) were mutagenized in two invasive neoplasias, and one (PTPRD) was mutagenized in three invasive neoplasias. In the case of CSTF3, GRM8, KDM2B, PAX7 and TCF7L2, SB was inserted at the same nucleotide site in each invasive neoplasia, indicating that these invasive neoplasias were generated from the same clone of SB-mutagenized cells. For LAT2 and PTPRD, two invasive neoplasias for each gene were identified in which SB was located at different nucleotide sites (Supplementary Table 2), suggesting that these neoplasias were derived from different clones of SB-mutagenized cells. LAT2 encodes a putative serine–threonine kinase that physically interacts with MDM2 to inhibit p53 ubiquitination and promote p53 activation.35,36 Expression of PTPRD, which encodes protein–tyrosine phosphatase receptor-delta, is downregulated in highly invasive cancers and has been shown to suppress colon cancer cell migration in coordination with CD44.37 Moreover, 55% of the identified candidate invasion-driver genes (21/38) are mutated in ≥5% human CRC patients and/or show a twofold change in mRNA expression level in CRC patients (Supplementary Table 2; RNA-seq data available at http://firebrowse.org). Collectively, these results suggest that insertional mutations in these genes were selected because of their influence on invasive cell growth.

Although the majority of genes identified by SB in solid tumors are tumor suppressor genes, some, such as TWIST2, JAK1, ROCK1 and STAT3 are more likely to be oncogenes on the basis of published studies. However, owing to the limited amounts of invasive tissue obtained from laser-capture microdissection and the potential RNA degradation the procedure may cause, the samples were sufficient for isolating only enough DNA to PCR amplify and sequence transposon insertion sites. It was therefore not possible to determine whether SB insertion in these genes resulted in activation or inactivation of their expression. Because these genes have already been implicated in CRC, we focused our validation studies on the 21 genes identified that have not previously been implicated in CRC.

In initial experiments, we used small interfering RNA (siRNA) to knockdown expression of these 21 genes in APC-shRNA hCECs or SW480 colon cancer cells and measured the effect of the knockdown on cell proliferation, migration and invasion. We used siRNA-mediated knockdown because the majority of genes identified by SB in solid tumors are tumor suppressor genes, and we chose the SW480 cell line because it was derived from an early-stage adenocarcinoma that harbors a mutation in APC. As a positive control, we generated an siRNA against LAT2, a known tumor suppressor identified in the screen, in addition to five randomly chosen negative control genes (Table 2). As expected, LAT2 scored positive in vitro cell proliferation and invasion assays, whereas none of the negative control genes scored positive in any assay (Table 2). Among the 21 candidate invasion-driver genes, 9 scored positive in one or more assays in hCECs and/or SW480 cells (Table 2).

We further validated the 9 genes that scored positive using the ex vivo colon model, along with LAT2 as a positive control and CSTF3, which did not score positive in any of the in vitro assays, as a negative control (Table 2). We generated shRNAs to stably downregulate these 11 genes in APC-shRNA hCECs, which we then used to recellularize acellular colon matrices. Among these genes, six (ASXL2, CAMTA1, DDX20, FXR1, MITF and PAX7) significantly promoted submucosal invasion in the recellularized colon model, whereas CSTF3 scored negative (Supplementary Fig. 10a,b). Notably, 85% of invasive neoplasia samples (18/21) harbored at least one mutated candidate invasion-driver gene that was previously implicated in CRC progression and/or functionally validated in this study (Supplementary Table 3). In addition, two or more driver genes that were previously implicated in CRC progression and/or functionally validated in this study were identified in 8 of the 21 samples, suggesting potential collaboration among these genes in the invasion events we observed. Collectively, these results suggest that SB mutagenesis in the ex vivo colon model can identify genes important for neoplastic cell invasion.

**DISCUSSION**

We describe here a tissue-engineering method that enables unbiased forward genetic screens in human tissues under physiologically relevant conditions. This method is powered by the 21 candidate invasion-driver genes that were previously implicated in CRC progression and/or functionally validated in this study. Among these genes, six (ASXL2, CAMTA1, DDX20, FXR1, MITF and PAX7) significantly promoted submucosal invasion in the recellularized colon model, whereas CSTF3 scored negative (Supplementary Fig. 10a,b). Notably, 85% of invasive neoplasia samples (18/21) harbored at least one mutated candidate invasion-driver gene that was previously implicated in CRC progression and/or functionally validated in this study (Supplementary Table 3). In addition, two or more driver genes that were previously implicated in CRC progression and/or functionally validated in this study were identified in 8 of the 21 samples, suggesting potential collaboration among these genes in the invasion events we observed. Collectively, these results suggest that SB mutagenesis in the ex vivo colon model can identify genes important for neoplastic cell invasion.
conditions. The decellularized human native colon matrix provides major tissue-relevant elements, including complex tissue structure, cell–matrix interactions and physiological colocation of multiple types of differentiated cells. This makes the model more desirable than conventional assays of cell migration or invasion through synthetic Matrigel or collagen layers, which does not mimic real tissues. We used systems that are simpler than the bioreactor-and-medium formula used to generate native functional organs for orthotopic transplantation but fulfill minimal requirements for developing tissue-level tumor models, enabling low-cost and large-scale cancer studies. Currently, only a few animal models have been developed to study malignant events in late-stage CRC. The introduction of pathologically paired genetic elements into the ex vivo model enabled recapitulation of a number of features associated with CRC progression. Malignant transformation also occurred rapidly, within 6–7 weeks after recellularization, further enhancing the value of this ex vivo model as a complement to current cancer models.

Transposon mutagenesis provides an unbiased high-throughput genetic tool for cancer gene discovery. Although SB mutagenesis has been used to model different types of cancers in mice, to our knowledge no previous study has shown that SB is capable of inducing cancer under close-to-native human conditions. Identification of genes driving invasive neoplasia can provide new drug targets and diagnostic markers. However, identifying and studying genes driving malignancy still remains a challenge. The ex vivo colon model provides a tractable system that recapitulates the dynamics of malignant transformation.

In a forward genetic screen, we used this ex vivo model to identify 38 candidate genes driving CRC submucosal invasion through cooperation with mutant APC, demonstrating the effectiveness of the ex vivo model as a functional platform for identifying genes driving tumor cell invasion. One of the genes identified, ASXL2, encodes additional sex combs-like 2 (ASXL2). ASXL family members are scaffolding proteins that function as epigenetic regulators by recruiting polycomb-group repressor complexes (PRC) and trithorax-group (trxG) activator complexes to DNA. They also have roles in histone modification by assembling transcription factors to specific genetic domains. ASXL2 and ASXL1, along with BRCA1 and YY1, are binding partners of BAP1, a nuclear deubiquitinating enzyme and tumor suppressor that functions mainly during metastasis. One of the genes identified, ASXL2, has been correlated with poor prognosis in prostate cancer, pancreatic cancer and breast cancer. Additionally, ASXL1 is involved in the malignant progression of CRC with microsatellite instability.

Another gene identified, CAMTA1, is a putative tumor suppressor gene in neuronal cancers. Its expression decreases glioblastoma cell growth by stimulating expression of the anti-proliferative peptide NPPA. CAMTA1 also regulates neuroblastoma cell mobility by increasing the expression of β3 tubulin, microtubule-associated protein 2 and neurofilament light chain. Notably, low expression of CAMTA1 is observed frequently in CRC and reported to be an independent indicator for poor patient survival. Consistent with previous findings, both our in vitro and ex vivo functional assays showed significantly increased cell proliferation and invasion in hCCECs and SW480 cells with decreased CAMTA1 expression, further indicating a potential tumor-suppressor role for CAMTA1 in CRC malignant transformation.

We observed that 8 of 21 neoplasia samples had mutations in two or more candidate invasion-driver genes previously implicated in CRC progression and/or functionally validated in the present study. These combined mutations may explain how the invasion events occurred. Investigation of the potential cooperative relationships of gene mutations found in the same samples will enable understanding of CRC progression and might eventually help develop novel therapies.

Because of the heterogeneity generated during tumor evolution and the genetic diversity that occurs among individuals, the 38 candidate genes identified here may be specific, to some extent, to the patients from whom the primary colonic epithelial cells were derived. Theoretically, this model system should enable the discovery of a broader range of novel cancer genes by deriving epithelial cells from multiple patients or by generating initial pools of matricigenized cells covering different mutation profiles through varying rounds of SB mutagenesis.

Although mutations in tumors are now routinely identified on a genome-wide scale, elucidating the roles of these genetic alterations in tumorigenesis is still challenging. The ex vivo colon cancer model complements existing cell line systems and animal models for multi-dimensionally testing the mechanistic roles of recurrent human cancer mutations. Our model and tractable biomimetic systems, such as organoid systems using human and mouse intestinal tissues, will aid understanding of the mechanisms of CRC progression at different stages and development of novel therapeutics. Our model also offers opportunities to create specialized physiological microenvironments for mimicking clinical diseases—for example, by incorporating engineering of vascular networks, the immune system and organ-specific microbes. In addition, our approach might be extended to the generation of other types of ex vivo organs, such as lung, liver, skin and kidney. It could also be applied in other areas of oncology, from developing biomarkers for diagnosis and prognosis to screening drugs, chemicals, pathogens and toxins for personalized medicine.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequence Read Archive: sequence data have been deposited under accession code SRX746204.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.J.C., Z.W., R.S., N.G.C., N.A.J. and M.L.S. conceived the concept, designed the experiments and wrote the manuscript. H.J.C., Z.W., A.B., D.J.S., P.B., L.W., Y. M., S.A.C., S.C., E.H. and L.C.-G. performed the experiments and data analyses. J.S., Z.W., S.M.L., X.S., N.G.C. and N.A.J. contributed to bioinformatics analyses.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Arrowsmith, J. Trial watch: Phase II failures: 2008–2010. Nat. Rev. Drug Discov. 10, 328–329 (2011).
2. Gout, S. & Huot, J. Role of cancer microenvironment in metastasis: focus on colon cancer. Cancer Microenviron. 1, 69–83 (2008).

ARTICLES
3. Bozic, I. et al. Accumulation of driver and passenger mutations during tumor progression. Proc. Natl. Acad. Sci. USA 107, 18545–18550 (2010).
4. Jones, S. et al. Comparative lesion sequencing provides insights into tumor evolution. Proc. Natl. Acad. Sci. USA 105, 4283–4288 (2008).
5. The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337 (2012).
6. Copeland, N.G. & Jenkins, N.A. Deciphering the genetic landscape of cancer—from genes to pathways. Trends Genet. 25, 455–462 (2009).
7. Li, X. et al. Oncogenic transformation of diverse gastrointestinal tissues in primary organoid culture. Nat. Med. 20, 769–777 (2014).
8. Matano, M. et al. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. Nat. Med. 21, 256–262 (2015).
9. Jung, P. et al. Isolation and in vitro expansion of human colon stem cells. Nat. Med. 17, 1225–1227 (2011).
10. Gilbert, T.W., Sellaro, T.L. & Badylak, S.F. Decellularization of tissues and organs. Biomaterials 27, 3675–3683 (2006).
11. Ott, H.C. et al. Regeneration and orthotopic transplantation of a bioartificial lung. Nat. Med. 16, 927–933 (2010).
12. Ott, H.C. et al. Perfusion-decellularized matrix: using nature’s platform to engineer a bioartificial heart. Nat. Med. 14, 213–221 (2008).
13. Ridky, T.W., Chow, J.M., Wong, D.J. & Khavari, P.A. Invasive three-dimensional organotypic neoplasia from multiple normal human epithelia. Nat. Med. 16, 1450–1456 (2010).
14. Tuveson, D.A. et al. Endogenous oncogenic Kras<G12D> stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5, 375–387 (2004).
15. Biswas, S. et al. Transforming growth factor-β receptor type II inactivation promotes the establishment and progression of colon cancer. Cancer Res. 64, 4687–4692 (2004).
16. Trobbridge, P. et al. TGF-β receptor inactivation and mutant Kras induce intestinal neoplasms in mice via a β-catenin-independent pathway. Gastroenterology 136, 1342–1355 (2009).
17. Lohi, J. et al. Laminins, tenascin and type VII collagen in colorectal mucosa. Histochem. J. 28, 431–440 (1996).
18. Vermeulen, L. et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc. Natl. Acad. Sci. USA 105, 13427–13432 (2008).
19. Liotta, L.A. Tumor invasion and metastases: role of the basement membrane. Warner-Lambert Parke-Davis Award lecture. Am. J. Pathol. 117, 339–348 (1984).
20. Lau, W., Barker, N. & Clevers, H. WNT signaling in the normal intestine and colorectal cancer. Front. Biosci. 12, 471–491 (2007).
21. Fearon, E.R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell 61, 759–767 (1990).
22. Waed, L.D. et al. The genomic landscapes of human breast and colorectal cancers. Science 318, 1108–1113 (2007).
23. Oshima, H. et al. Suppressing TGFβ signaling in regenerating epithelia in an inflammatory microenvironment is sufficient to cause invasive intestinal cancer. Cancer Res. 75, 766–776 (2015).
24. Copeland, N.G. & Jenkins, N.A. Harnessing transposons for cancer gene discovery. Nat. Rev. Cancer 10, 696–706 (2010).
25. Dupuy, A.J., Akagi, K., Largaespada, D.A., Copeland, N.G. & Jenkins, N.A. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. Nature 436, 221–226 (2005).
26. March, H.N. et al. Insertional mutagenesis identifies multiple networks of cooperating genes driving intestinal tumorigenesis. Nat. Genet. 43, 1202–1209 (2011).
27. Takada, H. et al. Transposon mutagenesis identifies genes and evolutionary forces driving gastrointestinal tract tumor progression. Nat. Genet. 47, 142–150 (2015).
28. Grabundzija, I. et al. Comparative analysis of transposable element vector systems in human cells. Mol. Ther. 18, 1200–1209 (2010).
29. Brett, B.T. et al. Novel molecular and computational methods improve the accuracy of insertion site analysis in Sleeping Beauty-induced tumors. PLoS One 6, e24668 (2011).
30. Angus-Hill, M.L., Elbert, K.M., Hidalgo, J. & Capecchi, M.R. T-cell factor 4 functions as a tumor suppressor whose disruption modulates colon cell proliferation and tumorigenesis. Proc. Natl. Acad. Sci. USA 108, 4914–4919 (2011).
31. Behrens, J. et al. Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. Science 280, 596–599 (1998).
32. Yu, H. et al. Twist2 is a valuable prognostic biomarker for colorectal cancer. World J. Gastroenterol. 19, 2404–2411 (2013).
33. Xiong, H. et al. Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. Neoplasia 10, 287–297 (2008).
34. Forcet, C. et al. The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. Proc. Natl. Acad. Sci. USA 98, 3416–3421 (2001).
35. Aylon, Y. et al. Lats2 null mice show increased spontaneous gut tumorigenesis and have a positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. Genes Dev. 20, 2687–2700 (2006).
36. Yabula, N. et al. Structure, expression, and chromosome mapping of LATS2, a mammalian homologue of the Drosophila tumor suppressor gene lat-warts. Genomics 63, 263–270 (2000).
37. Funato, K., Yamazumi, Y., Oda, T. & Akiyama, T. Tyrosine phosphatase PTPRD suppresses colon cancer cell migration in coordination with CD44. Exp. Ther. Med. 2, 457–463 (2011).
38. Chen, H.J. et al. Comprehensive models of human primary and metastatic colorectal tumors in immunodeficient and immunocompetent mice by chemokine targeting. Nat. Biotechnol. 33, 656–660 (2015).
39. Katoh, M. Functional and cancer genomics of ASXL family members. Blood 119, 4316–4321 (2001).
40. Brett, B.T. et al. Novel molecular and computational methods improve the accuracy of insertion site analysis in Sleeping Beauty-induced tumors. PLoS One 6, e24668 (2011).
41. Henrych, K.O. et al. CAMTA1, a 1p36 tumor suppressor candidate, inhibits growth and activates differentiation programs in neuroblastoma cells. Cancer Res. 71, 3142–3151 (2011).
42. Kim, M.Y. et al. Recurrent genomic alterations with impact on survival in colorectal cancer identified by genome-wide array comparative genomic hybridization. Gastroenterology 131, 1913–1924 (2006).
Decellularization of human colon tissues. All normal colon tissues in this study were from discarded surgical normal colon from CRC surgical resections. Pathological analysis was used to check the normal origin by tissue morphology. Briefly,9 fresh patient colon tissues were collected in Medium 199 supplemented with 200 U/ml penicillin and 200 mg/ml streptomycin immediately after patient operative resection. Fat and blood clots were removed from tissues and rinsed 10 times in sterile PBS. Samples were cut into 5 cm × 2 cm pieces and incubated in sterile 1% SDS (Fisher Scientific) in deionized water for 4–6 h at room temperature with gentle shaking. Sterile 1% Triton X-100 (Sigma-Aldrich) in deionized water was applied to rinse the tissues for 1 h and the acellular matrix was then washed in sterile PBS containing penicillin, streptomycin and amphotericin at 37 °C. PBS was changed once every 30 min for the first 5 h and twice each day for the remaining 5 d. The decellularized matrix can be freshly used for the following recellularization or stored at −80 °C for up to 6 months. Molecular characterizations were performed using immunostaining in F-actin (Invitrogen, fluorescent phallotoxins) for cytoskeleton, DAPI (Invitrogen) for cell nuclei, collagen I (Novus Biologicals, NB600-408), laminin (Sigma-Aldrich, HPA001895) and fibronectin (Sigma-Aldrich, F0916) for three main ECM proteins in native tissues and acellular matrices.

Culture of primary human colon cells. Normal colon biopsies (0.5–1 cm3) without visible adenomas by pathology were obtained from patients undergoing colonoscopy screening. The protocol of isolation and culture of human colon epithelial cells (hCECs) was slightly modified from previous studies.9,43 Briefly, colon specimens were immersed in cold X medium (HyClone) supplemented with 2% penicillin–streptomycin immediately after the operative procedure and rinsed with sterile PBS with antibiotics and antimycotic (Invitrogen) five times. The tissues were minced into small pieces (~1 mm3 in size), and crypts were gently extracted by digestion in X medium containing collagenase type XI (150 U/ml, Sigma-Aldrich), dispase neutral protease (200 ng/ml, R&D Systems) and Noggin (50 ng/ml, Peprotech) in collagen–I-coated flasks (BD Scientific) and incubated at 33 °C with 5% CO2. After 48 h culture, fibroblast inhibitory reagent (Human Colon FibroOut, CHI Scientific) was added to the culture medium for 2 d to reduce fibroblast growth. After cell colonization was observed, cells were transferred with retroviral hTERT (ATCC hTERT Immortalization Kit). The hCECs were then grown in 3D Matrigel culture and checked for their ability to form organoids. The hCECs were also characterized for their expression of the stem cell marker Lgr5 (OrigenGene, TAS03316) and differentiation markers using anti-Villin, Abcam, ab739; anti-MUC2, Sigma-Aldrich, HPA006179; anti-CHGA, Sigma-Aldrich, HPA017369; anti-A33, Sigma-Aldrich, HPA018858 and anti-β-catenin, Santa Cruz, sc1496 (for immunocytochemistry) and anti-pan-cytokeratin, Sigma-Aldrich, c2562; anti-Ki67, Cell Signaling, 9027; anti-β-catenin, Abcam, ab16051 (for immunohistochemistry)). Co-culture with human colon fibroblasts was required to develop tight-junction (anti-TJP1, Sigma-Aldrich, HPA001636) in hCECs. Using primers previously described41–43, DNA sequencing of IRR indicated no mutations in hotspot regions of APC, KRAS and TP53 genes.

Human colonic myofibroblasts were cultured as follows. Briefly, biopsies were placed in ice-cold DMEM supplemented with 3% FBS, 1-glutamine (2 mmol/l), sodium pyruvate (1 mmol/l) and 2% penicillin and streptomycin immediately after operative procedures and then rinsed with sterile PBS with antibiotics and antimycotic (Invitrogen) five times. Then biopsies were minced into small pieces (1 mm3) and dispersed by mechanical testing with pipetting. The tissue mixture was washed three times in medium and subject to centrifugation at 2,000 r.p.m. for 5 min. The tissue explants including the tissue fragments were then placed on culture dishes and covered with DMEM supplemented with 3% FBS, 1-glutamine (2 mmol/l), sodium pyruvate (1 mmol/l) and 1% penicillin and streptomycin and maintained at 37 °C in a 5% CO2 incubator. Once fibroblast-like cells appeared and began to colonize, the remaining tissue fragments were discarded. The fibroblast strains were characterized by immunostaining with human α-smooth muscle actin (Abcam, ab5694) and used for follow-up experiments within five passages.

Human colonic microvascular endothelial cells were purchased and maintained in culture according to the company instructions (ScienCell Research Laboratories, 2930). Primary cells were characterized for the endothelial marker CD31 (Sigma-Aldrich, CWH005175SM1) and used within ten passages.

Recellularization of acellular colon matrix. The mucosa layer of the decellularized colon matrix was physically separated from the submucosa using forceps, and the human endothelial cells were seeded by microinjection of 5,000 cells/side into four sides (the front, back, left and right sides) of a 0.25-cm2 (0.5 cm × 0.5 cm) mucosa layer using Eppendorf TransferMan NK micromanipulators under invert microscope (Nikon Diaphot). After 5 d culture with gentle shaking in endothelial cell medium, 5 × 104 hCECs were seeded on the 0.25-cm2 mucosa evenly with cells seated in crypt niches and maintained in culture for 10 d in a 1:1 mixture of epithelial cell medium with endothelial cell basal medium. After the myofibroblasts were seeded in the opposite surface of mucosa (the muscularis mucosa layer), the tissues were turned over with epithelial cell medium up and continued to culture for another 15–20 d. This orientation with crypt niches facing bottom also facilitated crypt repopulation with epithelial cells by neutral gravity. Last, the mucosa layers were assembled with the submucosa part, and the whole ‘sandwich tissues’ returned for continuous culture with mucosal crypts up in a 1:1 mixture of epithelial cell medium with endothelial cell basal medium until CRC in different stages developed.

Ligation-mediated PCR amplification and preparation for Illumina sequencing. The transposon insertion sites were amplified by ligation-mediated PCR (LM-PCR) and prepared for Illumina sequencing as described29. Briefly, total genomic DNA was digested with NlaIII (IR) or Alul (IRL), and fragments were linked with double-stranded adaptor by ligation. Primers used to generate adapters were as follows:

| Primer Sequence | Position |
|-----------------|----------|
| IR | 5′-TAAATACGACTCACTATAGGG-3′ |
| IRR | 5′-ATCTAATGACGACGATGATCTACTATAGGG-3′ |
| IRL | 5′-GACTATCCGAGATGATCTACTATAGGG-3′ |

Secondary PCR was performed to amplify IR and IRL by adding barcodes, which made it possible to sequence multiple samples together, and then the DNA samples are sent for Illumina sequencing (Illumina HiSeq 2500). Secondary PCR primers for IR and IRL:

| Primer Sequence | Position |
|-----------------|----------|
| 5′-AGATCGAGACGACGATGATCTACTATAGGG-3′ | SB linker + |
| 5′-GACTATCCGAGATGATCTACTATAGGG-3′ | SB linker − |

Analysis of SB insertion sites in invasive neoplasia. For analysis of transposon insertion sites, LM-PCR was performed to specifically amplify the integrated transposon-human genomic junction fragments for Illumina sequencing (HiSeq 2500, single-end reads). Raw read sequence data were first demultiplexed according to the 6-hp barcodes, then LM-PCR primer was trimmed from the read sequences. The trimmed read sequences were then mapped to the reference human genome hg19 (UCSC Genome Browser, http://hgdownload.cse.ucsc.edu/goldenPath/hg19/chromosomes/) using the BWA program (version 0.7.5a). Low mapping quality reads (MAPQ < 30 MQ) were filtered out, and only those reads with their mate read with MAPQ > 30 MQ were kept for subsequent analyses. Among the high-mapping-quality reads, about one-quarter to one-half were mapped to TA dinucleotide sites (varying

ONLINE METHODS

Study approval. Use of patient tissues was approved by the Institutional Review Board (IRB) of Weill Cornell Medical College and the IRB of the Cleveland Clinic, and informed consent was obtained for each participant. All animal protocols were approved by the IACUC committees of Weill Cornell Medical College and Cornell University.
between different samples). Further analysis was focused on these consensus SB insertion sites. From the distribution of read depth at the insertion sites, it was clear that the majority of the insertion sites resulted from background insertion events or PCR artifacts. We considered the top 10% of the insertion sites with the deepest read counts as clonal insertion sites. We mapped these clonal insertion sites in each sample to the human genome annotation file, RefGene, and picked the sites that were located within 1,000 bp of known transcripts in RefGene to cover both the core promoter and proximal promoter elements. In addition to this, Sanger DNA sequencing of chain-terminating dideoxynucleotides (Genewiz) was used to confirm the transposon insertion sites.

Statistical analyses. Sample sizes for all figures and tables were estimated on the basis of previous studies. For mouse experiments, animals were randomly assigned to experimental groups, and no animals were excluded from the analyses. For each set of experiments, samples were prepared for all experimental arms at the same time. All statistical tests were two-sided. No adjustments were made for multiple comparisons. Investigators were blinded to experimental allocations among different experimental arms for all experiments. All parametric statistical analyses, data were determined to be normally distributed by the D’Agostino–Pearson test. For all parametric and nonparametric tests, variances were similar between groups being compared.

For comparison between experimental and control groups at specific time points or tissue sites (Figs. 1–3, Supplementary Figs. 4, 6 and 8–10 and Supplementary Table 1), two-sided Student’s t-tests, two-sided Mann–Whitney (MW) tests and Fisher’s exact tests were used. All cells were purchased from ATCC between 2013 and 2015, and cells derived from patients were used within 10 passages and confirmed negative for mycoplasma.

43. Roig, A.I. et al. Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro. Gastroenterology 138, 1012–1021 (2010).
44. Gomes, C.C. et al. Assessment of TP53 mutations in benign and malignant salivary gland neoplasms. PLoS One 7, e41261 (2012).
45. Groden, J. et al. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 66, 589–600 (1991).
46. Lière, A. et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 66, 3992–3995 (2006).
47. Ruiz-Ponte, C., Vega, A., Carracedo, A. & Barros, F. Mutation analysis of the adenomatous polyposis coli (APC) gene in northwest Spanish patients with familial adenomatous polyposis (FAP) and sporadic colorectal cancer. Hum. Mutat. 18, 355 (2001).
48. Maston, G.A., Evans, S.K. & Green, M.R. Transcriptional regulatory elements in the human genome. Annu. Rev. Genomics Hum. Genet. 7, 29–59 (2006).
49. Chen, H.J. et al. Chemokine 25-induced signaling suppresses colon cancer invasion and metastasis. J. Clin. Invest. 122, 3184–3196 (2012).
Author Correction: A recellularized human colon model identifies cancer driver genes

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