



FULL LENGTH ARTICLE

Modeling colorectal tumorigenesis using the organoids derived from conditionally immortalized mouse intestinal crypt cells (ciMICs)

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Abstract Intestinal cancers are developed from intestinal epithelial stem cells (ISCs) in intestinal crypts through a multi-step process involved in genetic mutations of oncogenes and tumor suppressor genes. ISCs play a key role in maintaining the homeostasis of gut epithelium. In 2009, Sato et al established a three-dimensional culture system, which mimicked the niche microenvironment by employing the niche factors, and successfully grew crypt ISCs into organoids or Mini-guts *in vitro*. Since then, the intestinal organoid technology has been used to delineate cellular signaling in ISC biology. However, the cultured organoids consist of heterogeneous cell populations, and it was technically challenging to introduce genomic changes into three-dimensional organoids. Thus, there was a technical necessity to develop a two-dimensional ISC culture system for effective genomic manipulations. In this study, we established a conditionally immortalized mouse intestinal crypt (ciMIC) cell line by using a *piggyBac* transposon-based SV40 T antigen expression system. We showed that the ciMICs maintained long-term proliferative activity under two-dimensional niche factor-containing culture condition, retained the biological characteristics of intestinal epithelial stem cells, and could form intestinal organoids in three-dimensional culture. While *in vivo* cell implantation tests indicated that the ciMICs were non-tumorigenic, the ciMICs overexpressing oncogenic β -catenin and/or KRAS exhibited high proliferative activity and developed intestinal adenoma-like pathological features *in vivo*. Collectively, these findings strongly suggested that the engineered ciMICs should be used as a valuable tool cell line to dissect the genetic and/or epigenetic underpinnings of intestinal tumorigenesis.

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Introduction

Gastrointestinal cancers are developed from intestinal epithelial cells through a multi-step process involved in accumulative genetic mutations of oncogenes (such as the *CTNNB1* and *KRAS*) and tumor suppressor genes (such as the *APC* and *TP53*).^{1,2} Gastrointestinal cancers account for approximately 30% of the overall cancer incidences and mortalities worldwide. Histologically, the intestinal surface is covered by epithelium, which is organized into crypt-villus units and continuously renew throughout lifetime.^{1–3} Physiologically, the villus structural arrangement maximizes absorptive surface, whereas the crypts serve as the home of epithelial stem cells (also known as intestinal stem cells, ISCs). The ISCs proliferate vigorously, and their progeny cells migrate upward along basal membrane from the bottom of crypt to the top of villus, then become terminally differentiated cells and undergo apoptosis and shed into the lumen.^{3–5} This crypt-villus self-renew process occurs with a frequency of 7–10 days in mice, making ISCs as one of the fastest epithelial regenerative tissues under physiological condition in the body.^{3–5} Therefore, the reconstruction of

villus-crypt units would be an ideal model to dissect key signals in maintaining stemness and regulating proliferation and differentiation of ISCs, as well as to delineate the molecular and genetic bases of gastrointestinal cancers.^{4–7}

In their pioneering work, Sato et al established a three-dimensional culture system, which mimicked the niche microenvironment by employing the niche signaling molecules (EGF, Noggin, and R-spondin) and matrix gel, and successfully grew the isolated crypt ISCs into organoids (also known as Mini-guts) in dishes.⁸ Since then, the intestinal organoid technology has been used to delineate cellular signals that governed intestinal stem cell (ISC) self-renewal, proliferation and differentiation, and allowed long-term expansion of the ISCs in a form of Mini-guts.^{8–10} In order to efficiently deliver genes into cultured organoids, we and others previously accomplished transient and permanent transgene expression in organoids by using recombinant adenoviral and retroviral vectors, respectively.^{11,12} Nonetheless, the cultured organoids consist of heterogeneous cell populations, and yet it is technically challenging to effectively introduce genomic changes into three-dimensional cultured organoids using the current cutting-edge technologies such as CRISPR/Cas9 genome

editing system.^{13,14} Thus, there is a technical necessity to develop a two-dimensional ISC culture system for effective genomic manipulations.

We and others had demonstrated that SV40 large T antigen could effectively immortalize numerous primary mammalian cells.^{15–21} Furthermore, we demonstrated that the *piggyBac* transposon system could be used to effectively establish stable transgene expression in mammalian cells,²² and a *piggyBac* transposon-based SV40 large T antigen expression vector, pMPH86, was shown to immortalize mesenchymal stem cells with high efficiency and without any changes in the lineage-specific differentiation potential.²³ We hypothesized that a conversion of the crypt cells from three-dimensional culture of Mini-gut organoids into a stable two-dimensional monolayer culture through immortalization would enable us to genetically manipulate ISCs and model intestinal tumorigenesis with high efficiency.

Materials and methods

Cell culture, Mini-gut medium and chemicals

The Mini-gut medium contained advanced DMEM/F12 supplemented with N2 medium (100x stock), B27 medium (50x stock), penicillin/streptomycin (each at 100 unit/ml), 2 mM L-glutamine, and 10 mM HEPES (all from Invitrogen, Carlsbad, CA). RSPO2 conditioned medium was prepared from the HEK-293 cells stably expressing RSPO2 as described previously.¹¹ ENR medium was freshly prepared by mixing Mini-gut medium with EGF (final concentration 50 ng/ml, Sigma–Aldrich), Noggin (final concentration 100 ng/ml, PeproTech Co), RSPO2 conditioned medium (10% v/v), and ROCK inhibitor Y27632 (final concentration, 10 μ M, SelleckChem) immediately prior to use. The ciMICs and organoids were cultured with ENR medium. Growth factor reduced Matrigel was purchased from BD Bioscience. Unless indicated otherwise, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Generation and amplification of recombinant adenoviruses expressing GFP, RFP or *piggyBac* transposase (PBase)

Recombinant adenoviruses were generated using the AdEasy technology as described.^{23–25} The coding region of the *piggyBac* transposase was PCR amplified and subcloned into an adenoviral shuttle vector and subsequently used to generate recombinant adenoviruses in HEK-293 and its derivative lines 293 pTP and RAPA.^{26,27} The resultant adenoviruses were designated as AdPBase, which also co-expressed the GFP marker gene. Analogous adenoviruses AdGFP and RFP, which express GFP and RFP, respectively, were used as mock controls as previously described.^{18,28,29}

Mouse intestine crypt isolation and intestinal organoid culture

All animal experiments in this study were carried out by abiding by the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and

approved by the Institutional Animal Care and Use Committee (IACUC). Crypt isolation and organoid culture were carried out as previously described.¹¹ Briefly, after the CD1 mice or C57BL/6 J *Apc*^{Min+/-} mice (2-week old, male) were euthanized, the dissected jejunum and ileum were opened longitudinally, washed with cold PBS (with P/S), and cut into small (0.5–1.0 cm) pieces. The tissue bits were rocked in PBS with 2 mM EDTA for 30 min at 4 °C, and then switched to PBS with 54.9 mM D-sorbitol and 43.4 mM sucrose, followed by vortexing for 2 min and filtering through 100 μ m sterile cell strainers. The crypts were collected by centrifugation at 150g \times for 4 min in 4 °C.

Approximately 500 crypts were mixed with 100 μ l Matrigel (per well), plated in 24-well plates, and incubated for 30 min in a 37 °C 5% CO₂ incubator to allow the Matrigel to polymerize. 500 μ l of organoid culture medium were then added to each well. The medium was changed every 3 days. For passaging, organoids were collected from Matrigel, mechanically dissociated into single-crypt domains by passing through a 1 cc syringe/needle (27G, BD Biosciences), mixed with freshly thawed Matrigel, and subsequently seeded into 24-well plates. The resultant 3D organoids were passaged every 5–7 days at a 1:4 split ratio and were continuously maintained in culture.

Establishment of the conditionally immortalized mouse intestinal crypt (ciMIC) cells using *piggyBac* transposon-mediated expression of SV40 T antigen

The immortalization of mouse intestinal crypt cells (MICs) was accomplished by using the *piggyBac* transposon-based SV40 T antigen expression vector pMPH86 as previously described.^{22,23} Briefly, the crypts were freshly collected as prescribed above, then resuspended in 500 μ l PBS, added with 300 μ l of 0.25% Trypsin EDTA and incubated in a 37 °C 5% CO₂ incubator for 10 min. The digested crypt organoids were transferred to 15 ml conical tubes and added with 5 ml of 10% FBS DMEM to inactivate Trypsin. The primary MICs were dissociated by pipetting up and down for multiple times, then seeded onto Matrigel-coated 6-well plate in ENR medium for 2–4 h. Once the MIC cells were attached, the medium was changed to 1 ml serum-free DMEM mixed with 100 μ l of Lipofectamine/pMPH86 mix (50 μ l/10 μ g) and placed in the 37 °C 5% CO₂ incubator. At 2–4 h after transfection, the DMEM and transfection mix were replaced with ENR medium, and approximately 10¹² pfu of AdPBase adenovirus were added to the transfected MIC cells as previously described.^{22,23} At 48 h after AdPBase infection, the transfected/infected MIC cells were replated and subjected to hygromycin selection (final concentration, 0.3 mg/ml). After multiple rounds of passaging and replating in ENR medium, a pool of conditionally immortalized mouse intestinal crypt cells, or designated as ciMICs, was established and kept in LN2 tanks for further uses.

Establishment of the stable ciMIC lines expressing firefly luciferase/GFP/LacZ (FGL), RFP, mutant β -catenin (mutBC) and/or KRAS (mutKRAS)

The N-terminal domain deletion mutant of human *CTNNB1*/ β -catenin (mutBC), human *KRAS*^{G12D} (mutKRAS), *red*

fluorescent protein (RFP), firefly luciferase (FLuc)-green fluorescent protein (GFP)-LacZ were PCR amplified and subcloned into our homemade piggyBac transposon-based expression vector pMPB4 as previously described,²² yielding the expression vectors pMPB4-mutBC (expressing mutant β -catenin), pMPB4-mutKRAS, pMPB4-mutBC/KRAS (expressing both mutant β -catenin and mutant KRAS), pMPB4-RFP (expressing RFP), and pMPB4-FGL (expressing firefly luciferase, GFP and LacZ). All PCR amplified fragments were verified by DNA sequencing.

For the establishment of stable lines, the ciMICs were seeded in T-25 cell culture flasks at sub-confluence, and transfected with mixes of purified pMPB4-based vectors (5 μ g) and Lipofectamine (5 μ g) for 4 h as previously described.^{30,31} The transfection was terminated by adding 8 ml of 10% FBS DMEM medium, and the cells were infected with AdPBse and incubated at 37 °C 5% CO₂ incubator. At 48 h after transfection/infection, the cells were selected with blasticidin (final concentration, 5 μ g/ml). The surviving and stable cells were scaled up and designated as the following stable lines, ciMIC-FGL, ciMIC-RFP, ciMIC-mutBC, ciMIC-mutKRAS, and ciMIC-mutBC/KRAS. These cell lines were stored in LN₂ tanks for further use.

Total RNA isolation and quantitative real-time-PCR (qPCR) analysis

Total RNA was isolated from the treated ciMICs by using the TRIzol Reagent (Invitrogen) and subjected to reverse transcription (RT) reactions with hexamer and MMuLV reverse transcriptase (New England Biolabs) as described.^{32,33} The RT cDNA products were further diluted and used as qPCR templates. The qPCR was carried out as previously described.³⁴ PCR primers were designed by using the Primer 3 program to amplify the genes of interest (product sizes: 100-200bp): *Gapdh* Forward: 5'-GGC TGC CCA GAA CAT CAT-3', Reverse: 5'-CGG ACA CAT TGG GGG TAG-3'; *Lgr5* Forward: 5'-CAC CAG CTT ACC CCA TGA CT-3', Reverse: 5'-CTC CTG CTC TAA GGC ACC AC-3'; *Bmi-1* Forward: 5'-TGT CCA GGT TCA CAA AAC CA-3', Reverse: 5'-TGC AAC TTC TCC TCG GTC TT-3'. All qPCR reactions were carried out in triplicate. All samples were normalized by the expression level of *Gapdh*.

Immunofluorescence staining of cultured ciMICs

Immunofluorescence staining was performed as described.^{15,18,35} Briefly, sub-confluent ciMICs were fixed with ice-cold methanol, made permeable with 1% NP-40, and blocked with 10% BSA, followed by incubating with primary antibodies against β -catenin (Santa Cruz Biotechnology, Dallas, TX), PCNA (Santa Cruz Biotechnology), Villin (Santa Cruz Biotechnology), and ZO-1 (Abcam, Cambridge, UK) for 60 min. After being washed, cells were incubated with RPE-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. Cell nuclei were visualized by DAPI staining. Stains without primary antibodies or with control IgG were used as negative controls. Staining results were examined under a fluorescence microscope.

Whole mount immunostaining of ciMIC-derived organoids

Whole mount immunostaining of organoids was carried out as previously reported.^{8,36} Briefly, ciMICs-derived organoids were transferred to a pre-chilled 24-well cell culture plate on ice, and the matrigel could melt and then completely removed. The organoids were then collected in 2.0 ml Eppendorf tubes and resuspended with small volumes of cold PBS. Individual organoids were generated by gently and repeatedly pipetting up-and-down of the organoid mix and fixed with 2% PFA/PBS at 4 °C overnight. The fixed individual organoids were washed with PBS and made permeable with 0.2% Triton X-100/PBS at room temperature for 30 min on a rocking platform. The organoids were then washed with PBS and blocked with 2% donkey serum/0.1% Tween-20/PBS for 30 min at room temperature. Subsequently, the organoids were recovered and incubated with primary antibodies against E-cadherin (Cell Signaling Technology, Danvers, MA), β -catenin, PCNA, Villin, Mucin 2 (Santa Cruz Biotechnology), and Lysozyme (Santa Cruz Biotechnology) at room temperature for 60 min. After being washed, cells were incubated with RPE-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. Cell nuclei were counterstained with DAPI. Stains without primary antibodies or with control IgG were used as negative controls. Staining results were recorded by using a fluorescence microscope.

Crystal violet staining

Crystal violet staining assay was performed as previously described.³⁷ Briefly, sub-confluent ciMICs were fixed and stained with crystal violet (0.1% crystal violet in buffered formalin) for 20–30 min at room temperature. The stained cells were subjected to rinsing with tap water gently but thoroughly. The staining results were recorded under a bright field microscope.

Alkaline phosphatase (ALP) staining

The ciMICs or organoids were seeded in 24-well cell culture plates for the indicated time and fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min. The fixed cells or organoids were washed with PBS and stained with a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt. Histochemical staining was recorded using a bright field microscope.

Alcian blue staining

The ciMICs or organoids were fixed with 0.1% glutaraldehyde in PBS for 20 min at room temperature, rinsed with PBS three times, and stained with 1% Alcian blue (pH 2.5) for 2 h or overnight at room temperature. The stained cells or organoids were rinsed sequentially with 0.1 M HCl, PBS, and ddH₂O. The staining results were recorded under a bright field microscope.

Periodic acid Schiff (PAS) staining

The PAS staining was carried out as described.¹⁶ Briefly, sub-confluent ciMICs or organoids were fixed with 4% paraformaldehyde and stained with 0.5% periodic acid solution, followed by incubating with the Schiff's reagents. The stained cells or organoids were washed with tap water and then counterstained with hematoxylin solution, followed by being thoroughly rinsed with tap water. Positive stain (purple red) was recorded under a bright field microscope.

Subcutaneous implantation of the genetically modified ciMICs and xenogen bioluminescence imaging

The use and care of the animals were approved by the Institutional Animal Care and Use Committee (IACUC). The subcutaneous implantation assay was carried out as previously reported.^{18,21,30} Briefly, the exponentially growing ciMIC-FGL, ciMIC-mutKRAS, and ciMIC-mutBC cells were collected, resuspended in PBS or Matrigel, and subcutaneously injected into the flanks of athymic nude mice (3×10^6 cells/injection; $n = 5$ per group; 6-week old female, Harlan Laboratories, Indianapolis, IN). The mice injected with ciMIC-FGL cells were subjected to whole body bioluminescence imaging with the Xenogen IVIS 200 imaging system at the indicated time points for up to 55 days post implantation. The pseudo-images were obtained by superimposing the emitted light over the grayscale images of the animal. Average signals in photons/sec/cm²/steradian were calculated by using the Xenogen's Living Image software as previously described.^{17,38}

The mice injected with ciMIC-mutKRAS and ciMIC-mutBC cells, along with ciMIC-FGL cells as a control, were sacrificed at 4 weeks after implantation. Masses at the injection sites were retrieved for histologic analysis (see below).

Hematoxylin and eosin (H & E) histological evaluation

Briefly, the retrieved tissues were fixed in 10% buffered formalin and subjected to the paraffin embedding process. The embedded samples were sectioned and subjected to H & E staining procedure as described.³⁹

Statistical analysis

All measurements were performed in triplicate or repeat three times. Quantitative datum were presented as mean \pm standard deviation. Statistical differences between samples were determined by one-way analysis of variance (ANOVA). A statistical significance was defined with the p -value < 0.05 when a comparison was made.

Results and discussion

A conditionally immortalized mouse intestinal crypt (ciMIC) cell line can be effectively established

by using a *piggyBac* transposon-based SV40 T antigen expression system

In order to establish a user-friendly two-dimensional culture system for intestinal crypt progenitor cells, we sought to isolate mouse intestinal crypt cells and immortalize the crypt cells using our previously developed *piggyBac* transposon-mediated expression of SV40 T antigen system as outlined in Fig. 1A.^{22,23} Experimentally, we isolated mouse intestinal crypts (MICs) with high efficiency as described.¹¹ The isolated crypts were subjected to trypsin–EDTA digestion. While the primary MICs were able to survive and briefly expanded on Matrigel-coated cell culture plates (Fig. 1B and Fig. S1A), these cells underwent a rapid terminal differentiation by secreting mucus and eventually dying off upon passaging (Fig. S1B–D).

To immortalize primary MICs, we transfected the cells with pMPH86 vector and infected with AdPBBase with high efficiency (Fig. S2A). Stably transposed and immortalized cells were obtained upon hygromycin selection and could be passaged for generations (Fig. 1B and Fig. S2B). Furthermore, we tested the two-dimensional growth properties of the immortalized MICs under different growth media. We found that the immortalized MICs did not grow well in two-dimensional culture condition and exhibited significant spontaneous terminal differentiation phenotypes such as mucous production in both 1% FBS DMEM and 10% FBS DMEM (Fig. 2A). Similarly, while the immortalized MICs proliferated to certain extents in the Minigut medium, they grew most effectively in the ENR medium as shown in two-dimensional culture condition (Fig. 2A). These results indicate that the SV40 T antigen-mediated immortalization of the primary MICs is conditional, and that the long-term maintenance of these cells still requires the presence of intestinal stem cell niche factors in the ENR medium. Therefore, these cells were designated as conditionally immortalized mouse intestinal crypt (ciMIC) cells.

The ciMICs retain biological characteristics of intestinal epithelial stem cells

We also conducted biochemical staining assays and found that there were rare and sporadic cells stained positive for alkaline phosphatase (an apical brush border enzyme presented in villus enterocytes), Alcian blue (for mucin-producing cells), and periodic acid–Schiff (PAS) (for glycoproteins, glycolipids and mucins presented in goblet cells), whereas no strong eosin-positive cells were observed, suggesting the absence of cells of mesenchymal origin (Fig. 2B).

We further analyzed the expression of intestinal epithelial and proliferation markers

Using immunofluorescence staining assay, we easily detected the expression of the Wnt signaling mediator β -catenin,⁴⁰ the cell proliferation marker PCNA, the intestinal epithelium marker villin, and the epithelium tight junction protein ZO-1⁴¹ (Fig. 2C). Furthermore, we conducted qPCR analysis and found that the intestinal stem cell niche markers *Lgr5*⁴² and *Bmi1*⁴³ were readily detected in the PO

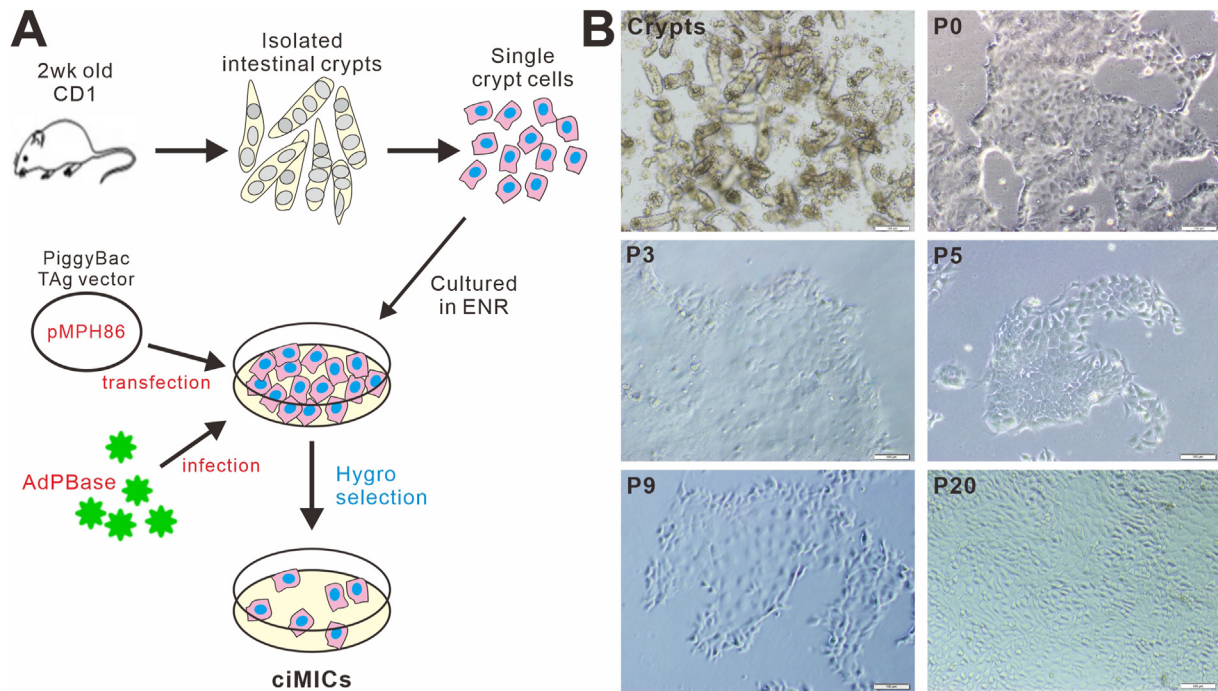


Figure 1 Establishment of the conditionally immortalized mouse intestinal crypt (ciMIC) cell line for two-dimensional culture. **(A)** Schematic representation of the essential steps for generating the ciMIC cell line using pMPH86, a piggyBac transposon-based vector that expresses SV40 T antigen and hygromycin resistance marker (see methods for details). **(B)** Representative morphological features of the isolated mouse intestinal crypts and the different passages of the ciMICs in two-dimensional culture (ENR medium). Scale bar = 100 μm .

(Primary MIC cells) and P9 passages of the ciMICs (Fig. 2D). Taken together, these results demonstrate that the ciMICs maintain long-term proliferative activity in two-dimensional culture and retain biological characteristics of intestinal epithelial stem cells.

The ciMICs retain the capability of forming intestinal organoids in three-dimensional culture

We next tested whether the ciMICs would have the potential to form “mini-gut” or crypt organoids that mimicked intestinal epithelial structure in three-dimensional culture. By following the schematic outlined in Fig. 3A, we mixed the exponentially growing ciMICs with Matrigel at a pre-determined cell density in multi-well plates and found that more than 70% of the ciMICs formed organoids with lumen 8 days after being cultured in three-dimensional Matrigel condition (Fig. 3B and C). We also compared the morphological features of organoids derived from wild-type primary MICs, ciMICs and the MICs from *Apc*^{Min+/-} mice, and found that, while the organoids from wild-type crypt budded extensively, the ciMIC-derived organoids resembled more closely to those derived from *Apc*^{Min+/-} mice (Fig. 3D), as previously reported.^{44,45} Furthermore, we tracked the organoid forming process from a single ciMIC cell to a fully-developed lumen organoid in 8 days (Fig. 3E).

We further characterized the ciMIC-derived organoids and found that these organoids were stained strongly

positive for ALP, PAS and Alcian blue (Fig. 4A and Fig. S3). The immunofluorescent staining further revealed the presence of fully-polarized enterocytes represented by villin⁺ mature brush borders, Paneth cells represented by lysozyme⁺ cells, and Goblet cells represented by Mucin 2⁺ cells, in addition to the positive staining of β -catenin (Wnt signaling pathway), E-cadherin (cell adhesion) and PCNA (cell proliferation) (Fig. 4B). Taken together, these results demonstrated that the ciMICs could form intestinal organoids with high efficiency in three-dimensional culture, while the ciMICs might consist of a pool of crypt progenitor cells with diverse differentiation potential.

Modeling colorectal tumorigenesis can be accomplished by introducing activated oncogenes into the ciMICs

One of the most attractive applications for the ciMICs is to carry out efficient genetic manipulations, either gene overexpression, silencing gene expression, or gene deletion/genome editing through CRISPR/Cas9 techniques, in ciMICs in two-dimensional culture. In order to accomplish effective and stable gene expression, we chose the piggyBac transposon system (Fig. 5A), which could also accommodate the expression of multiple genes and/or genes with large coding sequences as previously described.^{22,23} It has been well established that oncogenic activation of Wnt/ β -catenin signaling and KRAS play essential roles in the early

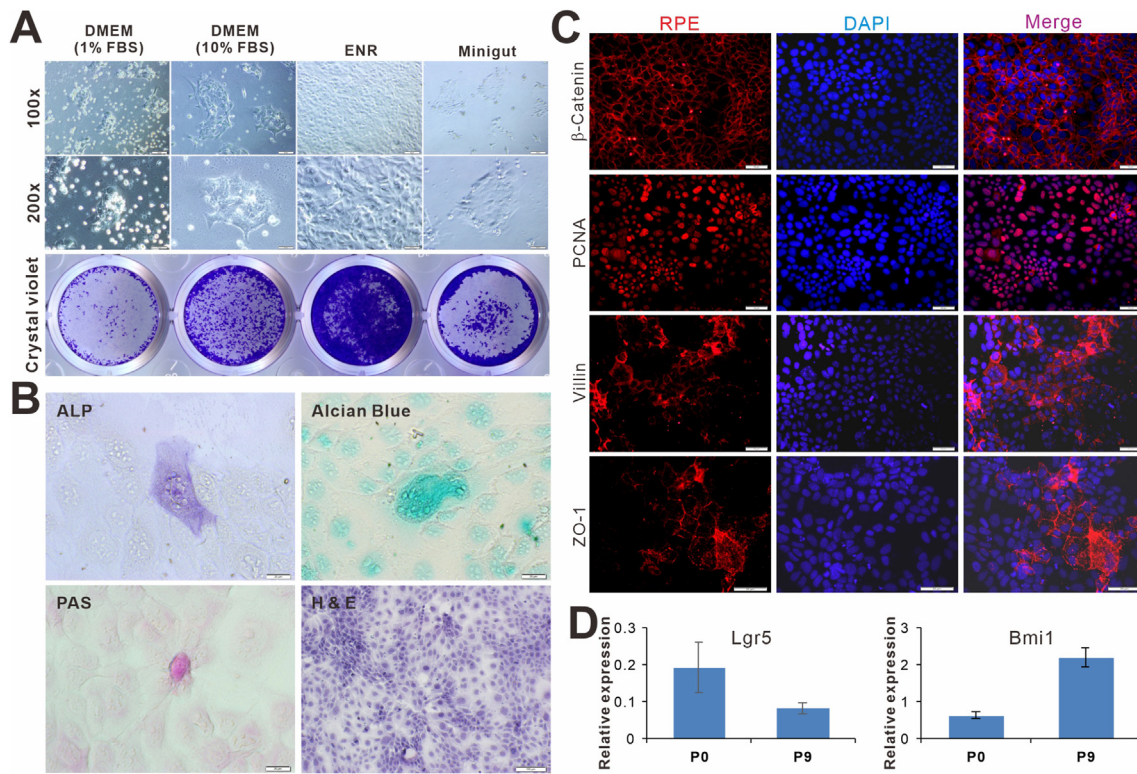


Figure 2 Biochemical and biological characterization of the ciMICs in two-dimensional culture. **(A)** The proliferation characteristics of ciMICs in different growth media. Subconfluent ciMICs were grown in 1% FBS DMEM, 10% FBS DMEM, ENR medium and Minigut medium for 3 days and subjected to crystal violet staining. Representative results are shown. Scale bar: 100 μm (100x), 50 μm (200x). **(B)** Biochemical staining of intestinal crypt cells in two-dimensional culture of ciMICs. No eosin staining was observed in H & E staining. Scale bar: H & E = 100 μm , the others = 20 μm . **(C)** Immunofluorescence staining of intestinal epithelial and proliferation markers. Subconfluent ciMICs were subjected to immunostaining with primary antibodies against β -catenin, PCNA, villin and ZO-1, and visualized with RPE-labeled secondary antibodies. Cell nuclei were counterstained with DAPI. Scale bar = 50 μm . **(D)** Quantitative analysis of Lgr5 and Bmi1 expression in the passages 0 and 9 of ciMICs ($N = 3$).

stage of intestinal tumorigenesis.¹ We previously constructed a constitutively activated mutant β -catenin with the deletion of its N-terminal destruction domain (i.e., mutBC),^{46,47} as well as the common point mutation of KRAS^{G12D} in *piggyBac* vectors. Using these vectors, we generated several stable ciMIC lines, including ciMIC-FGL (expressing firefly luciferase, GFP and LacZ), ciMIC-RFP (expressing RFP), ciMIC-mutBC (expressing active mutant β -catenin), ciMIC-mutKRAS (expressing mutant KRAS), and ciMIC-mutBC/KRAS (expressing both mutant β -catenin and mutant KRAS).

We found that the transduced marker transgenes GFP and RFP were highly expressed in ciMIC-FGL and ciMIC-RFP cells, respectively (Fig. 5B and Fig. S4A), and yet retained their ability to form organoids in three-dimensional culture condition (Fig. 5C and Fig. S4B). As expected, overexpression of the activated oncogenes β -catenin and/or KRAS in ciMIC-mutBC, ciMIC-mutKRAS and ciMIC-mutBC/KRAS drove a multi-layer growth phenotype in two-dimensional over-confluent culture, while the control ciMICs stopped growth once reaching confluence (Fig. 5D). Under the three-dimensional culture condition, all four lines were shown to form organoid structures with similar

efficiency (Fig. 5E). Cell masses, different from lumen organoid, were observed in ciMIC-mutBC, ciMIC-mutKRAS and ciMIC-mutBC/KRAS groups.

Since ciMICs were immortalized by SV40 T antigen, we further investigated whether the ciMICs were tumorigenic *in vivo* using the firefly luciferase (FLuc) labeled ciMICs, ciMIC-FGL cells. Considering the possibility that the nutrient microenvironment might impact the survival and/or growth of the ciMIC derived cells *in vivo*, we firstly mixed the ciMIC-FGL and ciMIC-mutBC cells with PBS separately, then injected the mix subcutaneously into the flanks of athymic nude mice. The bioluminescence signals lasted up to 2 weeks at the ciMIC-FGL/PBS injection sites (Fig. 6A), suggesting the ciMICs might not possess any significant short-term tumorigenic potential in immuno-compromised mice. In order to assess the long-term tumorigenicity of the ciMICs, we continued to monitor the injection sites for up to 7 weeks and no bioluminescence signals were detected (Fig. 6A). Meanwhile, no masses formed for both iMIC-FGL and iMIC-BC injection sites. Collectively, these results strongly indicate that ciMICs may not be tumorigenic even though they can be maintained in long-term two-dimensional culture.

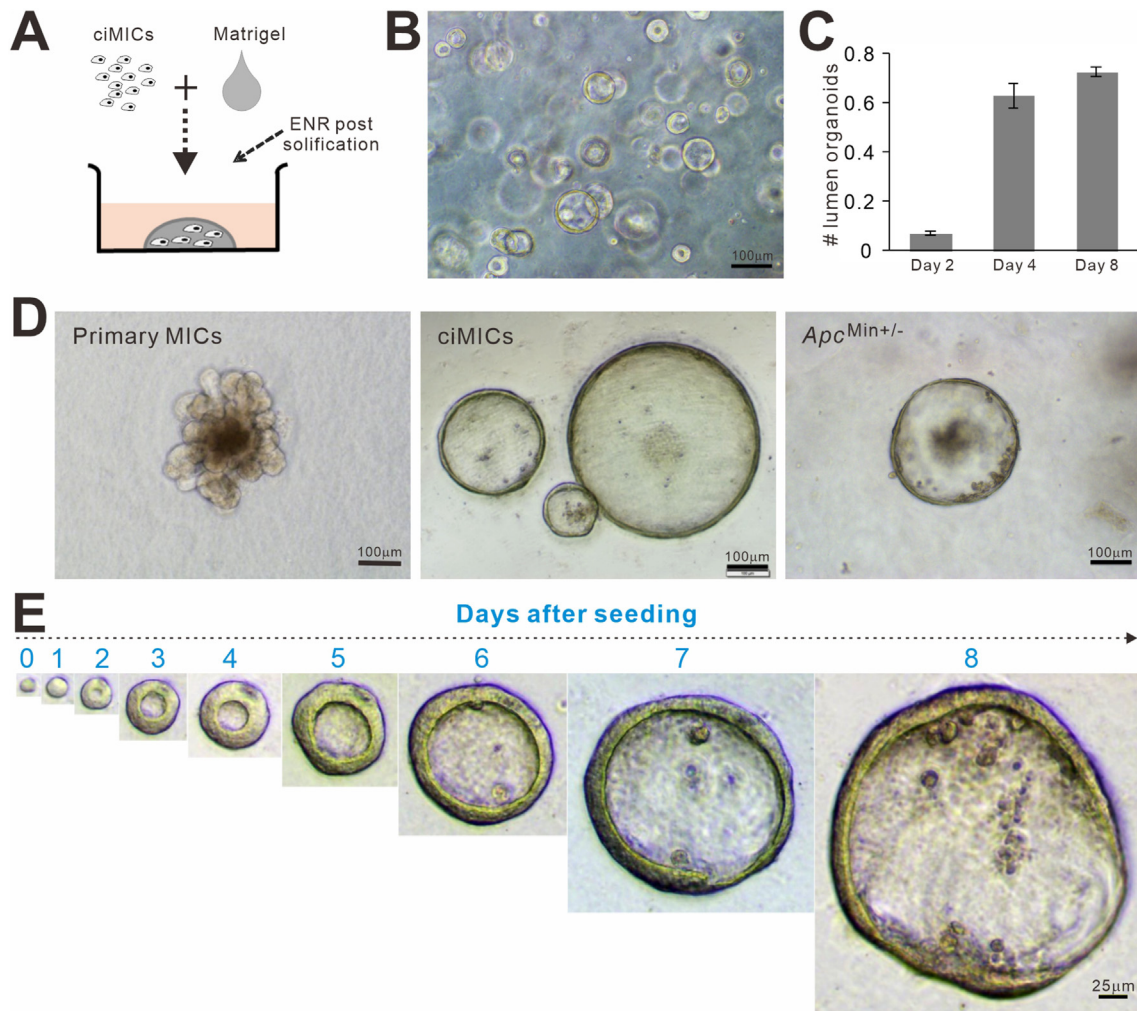


Figure 3 Development of three-dimensional crypt organoids from the ciMICs. **(A)** Schematic representation of sphere-cyst-like organoid formation in three-dimensional culture using ciMICs. **(B)** Morphological features of the organoids derived from ciMICs at 3 days after seeded in Matrigel. **(C)** Average rate of lumen-forming organoids at the indicated time points for ciMICs seeded in Matrigel. **(D)** Morphological features of the organoids derived from primary MICs, ciMICs and the MICs derived from *Apc* $min \pm$ mice. Representative images are shown. **(E)** Tracing the growth of organoid derived from a single ciMIC cell in three-dimensional culture condition. Representative images are shown. Scale bar: B, D = 100 μ m; E = 25 μ m.

Lastly, we investigated whether the ciMICs could be used to model intestinal tumorigenesis upon the introduction of appropriate oncogenes. To ensure the ciMICs could survive better *in vivo*, we mixed ENR medium or Matrigel and subcutaneously injected the oncogene-expressing ciMICs (ciMIC-mutBC and ciMIC-mutKRAS) and the control ciMIC-FGL cells into the flanks of nude mice for four weeks. As shown in Fig. 6B, apparent masses were readily retrieved from the ciMIC-mutBC injection sites, while only varied small masses were retrieved from the ciMIC-FGL mixed with Matrigel injection sites. No masses were detected at the ciMIC-FGL/ENR medium injection sites. Furthermore, histologic evaluation revealed that, while intestinal epithelium and adenoid structures were established in the ciMIC-FGL group, the masses retrieved from the ciMIC-mutBC and ciMIC-mutKRAS injection groups exhibited highly proliferative, intestinal adenoma-like pathological and histological features (Fig. 6C). Thus, these *in vivo* findings strongly

suggest that, while non-tumorigenic themselves, the ciMICs may be a valuable tool to dissect the genetic underpinnings of intestinal tumorigenesis.

Organoids may serve as valuable models for the development and treatment of human diseases

In this study, we developed the ciMIC cell line for long-term two-dimensional culture of intestinal crypt stem cells, which could be used for basic cancer research, microbiota research, drug screening, and translational research for personalized medicine. The ciMICs were derived from intestinal crypt cells and possessed the characteristics of physiologically normal immature and stem/progenitor cells, including epithelial cell phenotype, niche factor dependence in two-dimensional culture, robust organoid-forming capability, and multi-lineage differentiation potential.

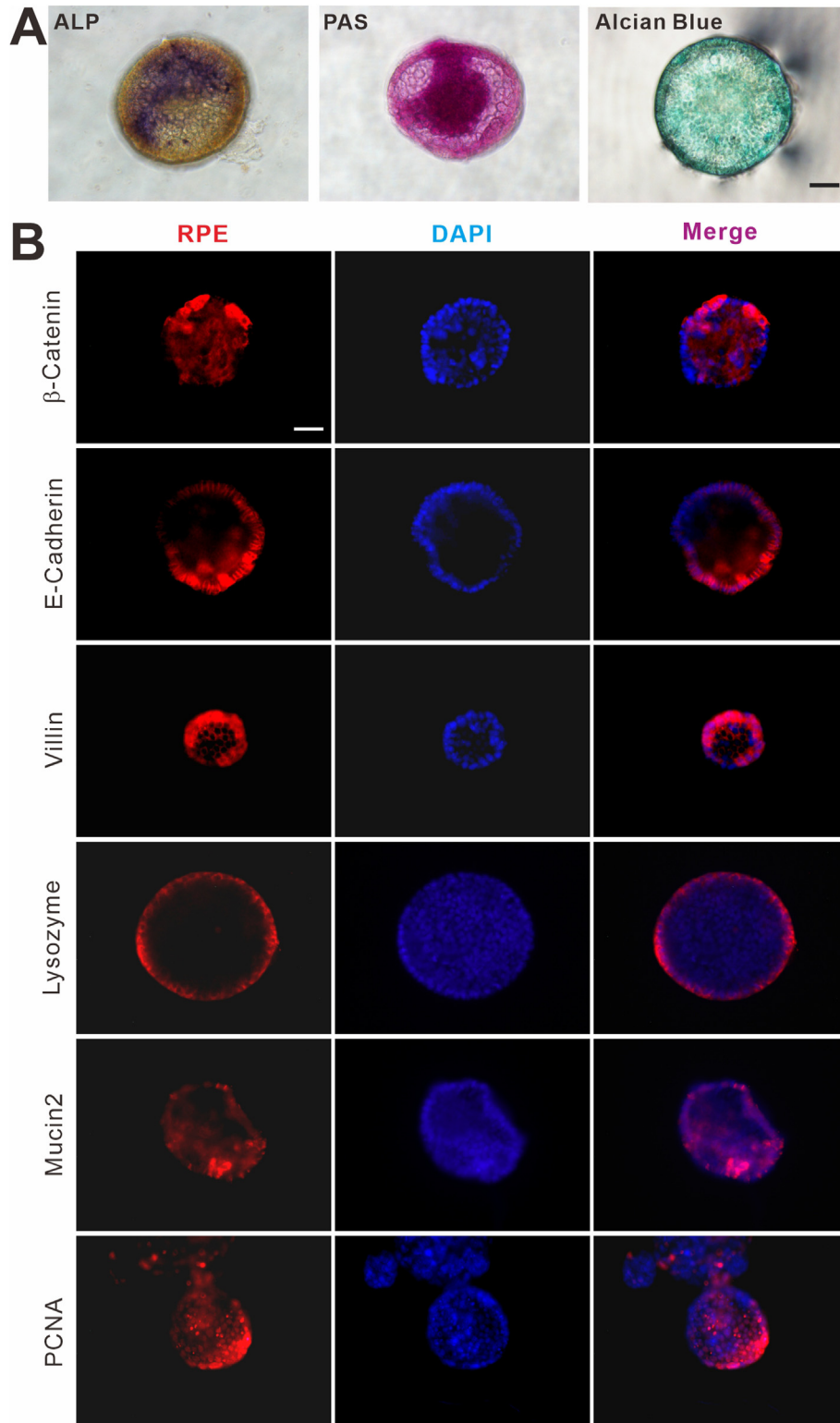


Figure 4 Characterization of the intestinal organoids derived from the ciMICs in three-dimensional culture. **(A)** ALP, PAS and Alcian blue stains of ciMIC-derived organoids in a whole-mount manner. **(B)** Immunofluorescence staining of intestinal epithelial markers and important proteins (β -Catenin, E-Cadherin, Villin, Lysozyme, Mucin 2, PCNA) in ciMIC-derived organoids in a whole-mount manner. Representative results are shown. Scale bar = 50 μ m.

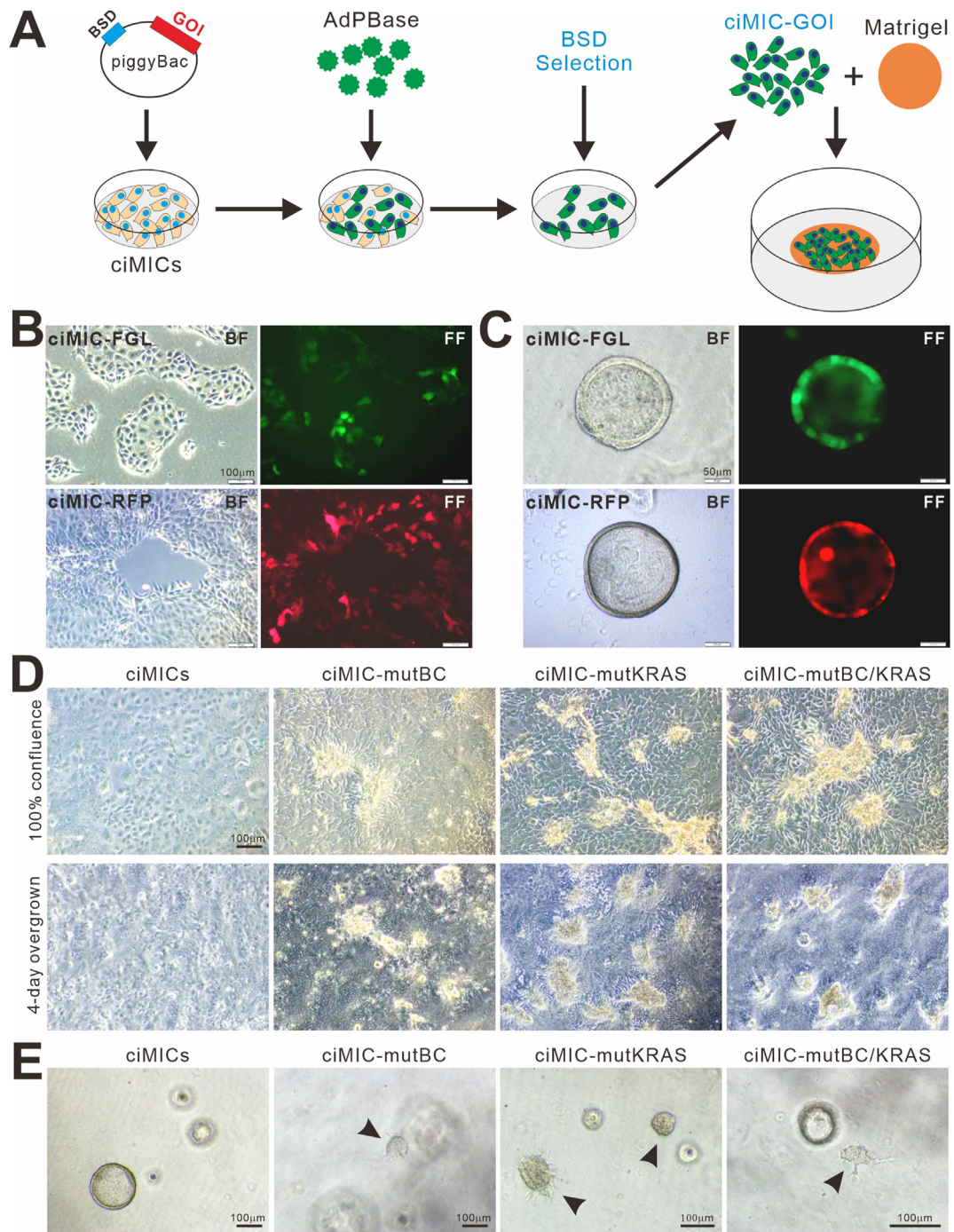


Figure 5 The piggyBac transposon-mediated stable introduction of transgenes and mutant oncogenes into ciMICs. **(A)** Schematic depiction of the piggyBac transposon-mediated stable expression of transgenes. GOI, gene of interested; BSD, blasticidin resistance marker. **(B & C)** Characterization of ciMIC-FGL and ciMIC-RFP lines in two-dimensional culture **(B)** and three-dimensional organoid culture under bright field **(BF)** and fluorescence field **(FF)** conditions. **(D & E)** Characterization of ciMICs stably expressing activating mutant oncogenes in two-dimensional **(D)** and three-dimensional **(E)** organoid culture conditions. Representative images are shown. Scale bar: **B, D, E** = 100 μm, **C** = 50 μm.

The ciMICs should represent a pool of mouse intestinal crypt cells at immature status, which avoids selection bias caused by cell surface marker-based cell sorting process. Interestingly, although ciMICs are not tumorigenic, the sphere cysts of ciMIC-derived organoids are distinct from

the typical villus-crypt architecture formed by organoids derived from primary crypts. In fact, the ciMIC-derived organoids share a great deal of similarity with that derived from the *Apc*^{Min+/-} organoids as previously described.^{45,48} We also attempted but failed to promote budded

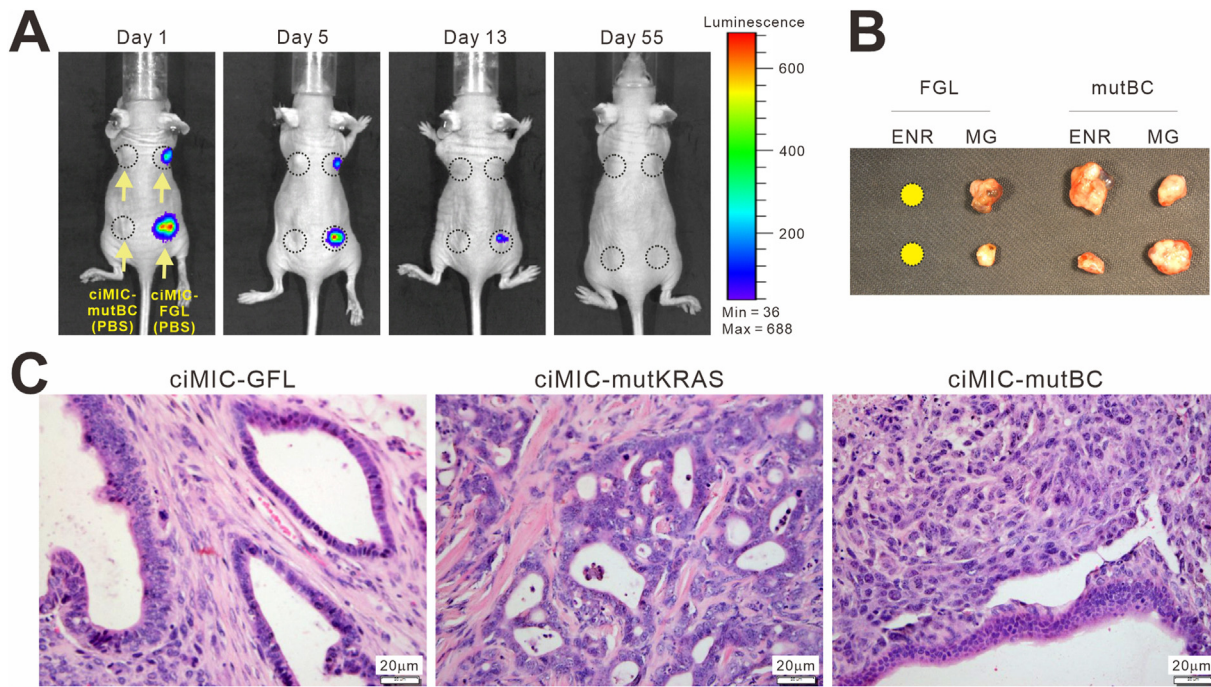


Figure 6 The ciMICs are non-tumorigenic but oncogene-expressing ciMICs develop tumor-like phenotype. **(A)** Non-tumorigenic activity of ciMICs. The ciMIC-FGL cells and ciMIC-BC were resuspended in PBS and injected subcutaneously into the flanks of athymic nude mice and imaged at the indicated time points. The injection sites are indicated with dotted circles. Representative results are shown ($n = 3$). **(B)** Subcutaneous mass formation by ciMIC-mutBC cells. The ciMIC-FGL and ciMIC-mutBC cells were resuspended in ENR medium (ENR) or Matrigel (MG) and injected subcutaneously into the flanks of athymic nude mice. The mice were sacrificed at 4 weeks after implantation, and detectable masses were retrieved from the injection sites for histologic analysis. Masses were also retrieved from the mice injected with ciMIC-mutKRAS cells (data not shown). **(C)** H & E staining of the masses retrieved from the mice injected with the ciMIC-FGL, ciMIC-mutKRAS and ciMIC-mutBC cells. Representative results are shown.

organoids from ciMIC by raising Noggin concentrations in the culture medium (data not shown). Currently, we do not have any satisfactory explanations for such phenotypic changes in the ciMIC-derived organoids. Interestingly, the organoids derived from mutKRAS are devoid of lumen structure, indicating that a loss of self-organization ability of organoids may be a symbol of malignant transformation.

Intestinal organoid culture technology represents a remarkable breakthrough for intestinal stem cell research,^{8–10,49} which has subsequently been extended to other human and mouse tissues, including stomach, liver, pancreas, brain, lung, prostate, retina, salivary gland, breast, and taste bud.^{4,9} Organoid technology offers a valuable tool to bridge the gap between two-dimensional cell culture and *in vivo* animal models.^{4,9,50} The pseudo-physiological conditions can recapitulate the three-dimensional microenvironment for organ/tissue development. Theoretically, organoids can be used to model human diseases, predict drug responses, and serve as a platform for drug development.⁵⁰ Patient-derived organoids (or PDOs) can be used to optimize treatment strategies for individual patients as a part of the personalized medicine. Furthermore, organoid technology may be applied to regeneration medicine and transplantation to overcome the shortage of healthy donor tissues and inherent immunological rejection by establishing isogenic organoids from patient biopsies.⁵⁰ Thus, organoid

technology holds great promise for translational and clinical applications.

In summary, in order to overcome the technical challenge of genetic manipulations in three-dimensional intestinal organoids, we established a conditionally immortalized mouse intestinal crypt (ciMIC) cell line by using a *piggyBac* transposon-based SV40 T antigen expression system. We showed that the ciMICs maintained long-term proliferative activity in two-dimensional niche factor-containing culture, retained the biological characteristics of intestinal epithelial stem cells, and were capable of forming intestinal organoids in three-dimensional culture. While the *in vivo* cell implantation studies indicated that the ciMICs were not tumorigenic, the ciMICs overexpressing oncogenic β -catenin and/or KRAS exhibited high proliferative activity and developed intestinal adenoma-like pathological features *in vivo*.

The establishment of ciMIC cell line make culture intestinal epithelia *in vitro* conveniently to a great extent. It firstly lead the intestinal epithelia research into the era of 2D culture, which provide a new dimension for intestine biomedical researchers. Remarkably, the routine cell biology experiments based on ciMIC could perform in 2D and 3D easily and economically. The manipulating of ciMIC's genome as what we did in this study make the potential applications of ciMICs fascinating. It include but not limit to bowel cancer initiation and development, intestinal

infectious disease, the gut microbiome, intestinal stem cell, inflammatory bowel disease, drug screen based on iMIC derived experimental model, etc. Taken together, these findings strongly suggest that the ciMICs should be a valuable tool cell line for physiological and pathological research of intestine.

Conflict of Interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2021.01.004>.

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