nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-020-0298-4

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Cloning of ground-state intestinal stem cells from endoscopic biopsy samples

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'Adult' or 'somatic' stem cells harbor an intrinsic ability to regenerate tissues. Heterogeneity of such stem cells along the gastrointestinal tract yields the known segmental specificity of this organ and may contribute to the pathology of certain enteric conditions. Here we detail technology for the generation of 'libraries' of clonogenic cells from 1-mm-diamter endoscopic biopsy samples from the human gastrointestinal tract. Each of the 150–300 independent clones in a typical stem cell library can be clonally expanded to billions of cells in a few weeks while maintaining genomic stability and the ability to undergo multipotent differentiation to the specific epithelia from which the sample originated. The key to this methodology is the intrinsic immortality of normal intestinal stem cells (ISCs) and culture systems that maintain them as highly immature, ground-state ISCs marked by a single-cell clonogenicity of 70% and a corresponding 250-fold proliferative advantage over spheroid technologies. Clonal approaches such as this enhance the resolution of molecular genetics, make genome editing easier, and may be useful in regenerative medicine, unravelling heterogeneity in disease, and facilitating drug discovery.

Introduction

Adult stem cell–based drug discovery and regenerative medicine may lead to treatments and cures for numerous diseases and conditions that have defied conventional therapeutic approaches. Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), currently dominate stem cell research and clinical trials in regenerative medicine[1]. The drawbacks of these remarkable cells include teratoma formation, complicated and inefficient processes for directed commitment to desired lineages, and lack of regenerative capacity in derived lineages[2–7]. By contrast, 'adult' or 'somatic' stem cells harbor an intrinsic ability to regenerate tissues. Green and colleagues pioneered a system dependent on 3T3-J2 feeder cells for capturing p63+ epidermal stem cells[6] in their most immature and clonogenic form and showed that these cells can be differentiated, at will, to yield a stratified squamous epithelium. Subsequently, the Green protocol was successfully adapted for the cloning and expansion of p63+ stem cells from other stratified epithelia, including those of the thymus, cornea, and lung[7–9], and has become the basis of successful autologous stem cell transplants in thousands of patients with severe burns, blindness resulting from industrial accidents, and genetic blistering conditions[6,8,10]. However, the Green protocol does not support the cloning and expansion of the stem cells of the gastrointestinal tract.

Leveraging the remarkable success of Green's system of cloning stem cells of stratified epithelia, we developed a parallel system that enables the cloning of highly undifferentiated stem cells from 1-mm-diameter endoscopic biopsy samples from the human gastrointestinal tract[11–13] (Fig. 1). We demonstrated that these ground-state intestinal stem cells (gISCs) can be propagated to enormous

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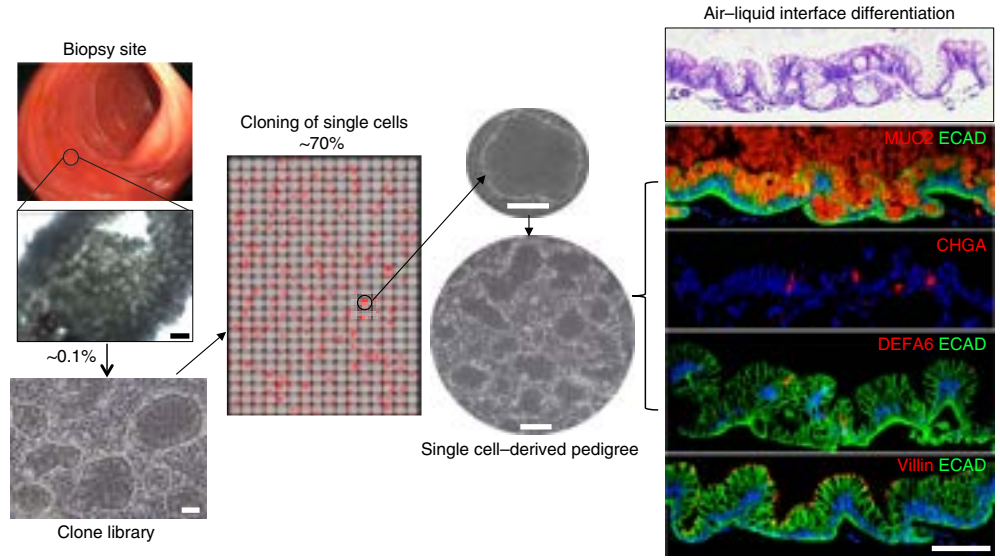


Fig. 1 | Schematic of cloning of gISCs from endoscopic biopsy samples. Left, endoscopic biopsy sample (1-mm diameter) from gastrointestinal mucosa is processed to yield a 'library' of clonogenic cells representing <0.1% of all epithelial cells. Scale bars, 100 μm. Center, libraries are further single-cell-sorted into 384-well plates and, right, clones are sampled for expansion as immature cells or differentiated to 3D epithelial-containing goblet cells ($MUC2^+$; red), endocrine cells ($CHGA^+$; red), Paneth cells ($DEFA6^+$; red), or enterocytes ($Villin^+$; red). See Table 1 for details of antibodies. Scale bar, 100 μm. ECAD, E-cadherin antibody. Adapted with permission from ref. [13], Elsevier.

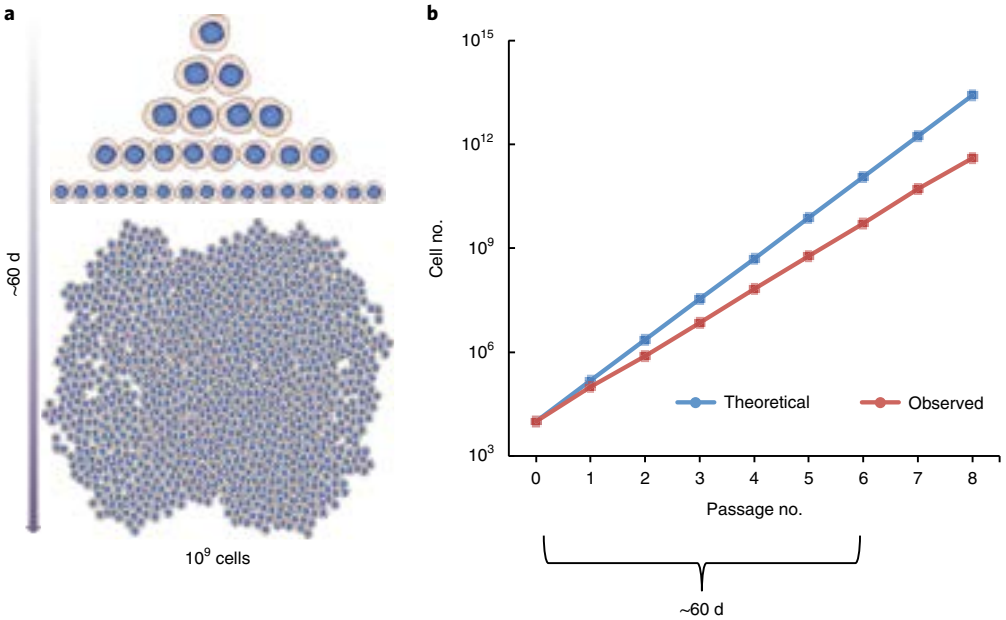


Fig. 2 | High clonogenicity facilitates the expansion of single cell–derived clones. a, High proliferation and symmetric division enable the expansion of a single gISC to 1 billion cells in 6 weeks. **b**, Theoretical expansion of a clone undergoing uniform proliferation over time compared with the representative expansion of a gISC clone (representative data; 6 clones were analyzed in total), reflecting only minor deviations. **a** adapted with permission from ref. [13], Elsevier.

numbers (Fig. 2) while maintaining genomic integrity (Fig. 3), epigenetically enforced fate commitment, and high clonogenicity. Our findings support the potential of gISCs in disease modeling and regenerative medicine. Herein, we present a detailed experimental protocol for the generation of gISCs from endoscopic biopsy samples.

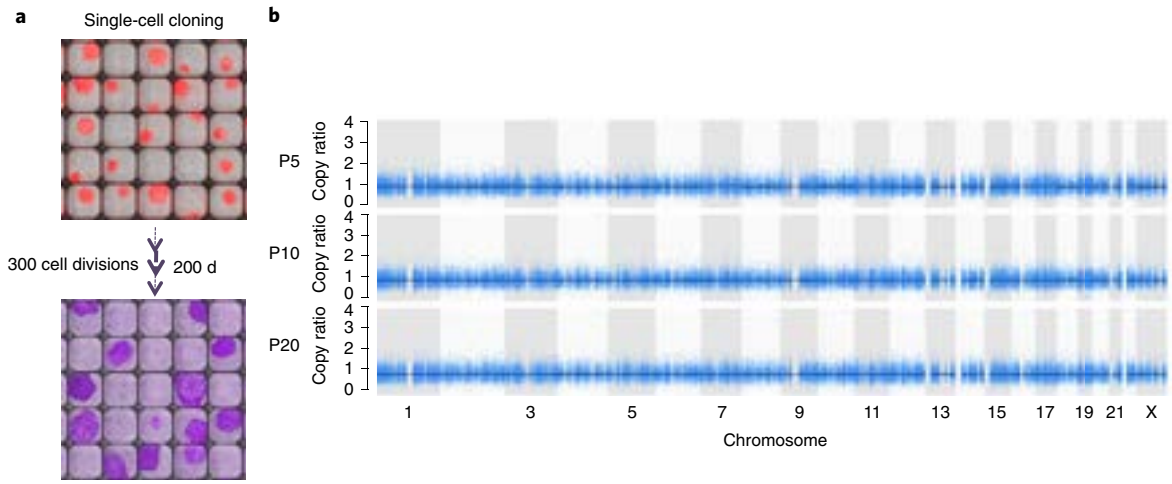


Fig. 3 | Conservation of high clonogenicity of gISCs over proliferative time. a, Clonogenicity assay based on FACS sorting into 384-well plates of passage 5 cells (P5; top) and passage 20 cells (P20; bottom), yielding similar results. **b**, Copy ratio of gISC clone, determined by whole-exome sequencing, showing absence of CNV events over continuous passaging. Chr, chromosome.

Development and applications of the protocol

The system developed by Green and colleagues for propagating epidermal stem cells[6,14–17] used a custom medium containing growth factors such as EGF and, importantly, an irradiated feeder cell layer of Swiss 3T3-J2 murine embryonic fibroblasts. About 1 in every 1,000 cells of a single-cell suspension of an epidermal biopsy sample forms 2D, circular colonies termed 'holoclones', which are composed of immature cells having a clonogenicity approaching 95% in subsequent passages. Expanded holoclones form a stratified epithelium upon transplant to abraded skin of immunodeficient mice or upon exposure to an air–liquid interface (ALI) in vitro, and have been the basis of autologous transplants for regenerative medicine treatments of damaged or mutant epithelia.

As adult stem cells residing in stratified epithelium and columnar epithelium require distinct signaling pathways for the maintenance of stemness, the medium devised by Green was not able to support adult stem cells in columnar epithelium. To overcome this obstacle, we developed and continue to improve media containing novel combinations of growth factors and regulators of an array of signaling pathways such as the TGF-β, Wnt/β-catenin, EGF, IGF, and Notch pathways[12,13] that support ISC cloning and maintenance in their most undifferentiated, ground state (referred to herein as "gISCs"). This new feeder-dependent system maintains gISCs as highly proliferative and clonogenic cells in culture so that they can quickly reach numbers necessary for downstream applications in a timely manner. Owing to their high clonogenicity and attending proliferation, a single gISC will yield >1 billion stem cells within 60–80 d in this system[18] (Fig. 2). Importantly, the apparent unlimited proliferative potential or 'immortality' of gISCs is evident in the absence of mutations or copy-number variation (CNV) events in pRB, CDKN2A, p53, or any of a host of tumor suppressor or proto-oncogenes[11] (Fig. 3). Furthermore, gISCs in this system retain their multipotent, region-specific differentiation capacity despite continuous cultivation for >1 year[11]. This cell-autonomous, region-specific commitment of stem cells along the intestinal tract suggests a developmentally established spectrum of stem cells that preserves the histological and functional properties that define these segments. Because there is not a unitary 'intestinal stem cell' or even one for each of the histologically recognized segments, we believe that this technology for cloning regionally committed gISCs offers real hope to permanently address both short-bowel syndrome (SBS) and non-SBS conditions using autologous regenerative medicine. Judging from lessons learned from the pioneering transplants of corneal epithelial stem cells for caustic eye burns[8], the high clonogenicity of gISCs will favor their use in regenerative therapies.

Comparing gISC cloning technology with organoids

For almost half a century, researchers have grown mammalian cells in collagen and laminin-rich, 3D hydrogels as 'organoids'[19–23], and this technology has facilitated fundamental advances in stem cell and developmental biology research. That being said, multiple studies have shown that the vast

majority of cells in an organoid are not stem cells, and those that are account for <1% of all organoid cells[13,23,24]. It is unclear at present how this low stem cell content will affect the utility of organoids for regenerative medicine, but the key indicator of success in corneal regeneration therapy was the ratio of stem cells in the transplant itself[8]. Apart from this potential concern, gISCs provide multiple practical advantages over the widely used organoids that largely stem from their high clonogenicity and the clonal platform this enables. For instance, the maintenance of gISCs with a >70% clonogenicity underlies the 250-fold proliferative advantage of gISCs over organoids that speeds the production of high-quality stem cells for downstream molecular analyses, transplant studies, genomic manipulation and drug screening. Moreover, gISCs naturally accommodate clonal approaches and the precision of these downstream studies, whereas clonal analyses with organoids are complex, labor intensive and largely confined to proof-of-concept experiments. The advantages of gISCs can be summarized by listing six routine processes with gISCs that are not practical with organoids: (i) generation of libraries of 100–300 independent ISC clones from a single 1-mm-diameter endoscopic biopsy sample of gastrointestinal mucosa; (ii) expansion of single gISCs to 1 billion highly clonogenic cells in 60 d; (iii) clonal determination of genomic profiles ahead of regenerative medicine applications; (iv) demonstration and validation of stable epigenetic commitment to region-specific differentiation of gISCs; (v) generation of accurate mucosal facsimiles with appropriate and accessible apical–basal polarity for drug and biologics testing; and (vi) rapid parsing of genome-edited populations for clonal analysis of variants.

Limitations of the protocol

We have successfully derived and cultured gISCs of human gastrointestinal epithelia from a large number of donors and have observed similar efficiency of cloning and long-term culture independent of donor age[11–13] (Supplementary Table 1). The condition of the 3T3-J2 feeder layer can play a defining role in the success of human ISC derivation, and this condition is ultimately dependent on adherence to rigid parameters of 3T3-J2 growth and expansion as defined below (Fig. 4). Not every investigator in the laboratory can or will work within these parameters. Another important limitation of this method is the tendency of gISCs to spontaneously differentiate if colonies are allowed to merge to confluence. Thus, to maintain the stemness of gISCs, the seeding density and confluency of the cultures need to be strictly monitored. In addition, gISCs tend to differentiate if they are seeded as clusters instead of single cells during passaging. Thus, thorough trypsinization and single-cell filtration or flow-sorting before seeding will be essential to maintaining the potential of these cells. Finally, it is critical to ensure the quality of gISCs before seeding them on Transwell membranes for ALI differentiation, transplanting them as xenografts, or subjecting them to genome-editing protocols.

Experimental design considerations

Herein, we present a step-by-step description of the whole process, including acquisition and processing of biopsy samples, gISC propagation and pedigree establishment, and purification of stem cells.

Acquisition and processing of endoscopic biopsy samples

It is critical to recognize that collection of biopsy samples must be compliant with institutional review board (IRB) regulations and guidelines, as well as requiring patient consent. The biopsy samples used for the experiments described here were obtained under informed consent as de-identified material under approved IRB protocols at the Connecticut Children's Medical Center (IRB approval no. HSC-MS-16-0330), the University of North Carolina (IRB approval no. 15-2813), the Baylor College of Medicine (IRB approval no. H-38746) or the National University of Singapore (IRB approval no. NHG DSRB ref.: 2014/00836). Under IRB-approved protocols, biopsy samples were collected with one pass of the biopsy forceps through a colonoscope. Captura disposable biopsy forceps (2.4 mm; Cook Medical), disposable jumbo biopsy forceps (2.8 mm; Boston Scientific) or equivalents were used, depending on the endoscopist's preferences. The biopsy samples weighed 8 mg on average. The biopsy samples should be immediately deposited in sterile, 15-ml conical tubes containing ice-cold wash buffer and transferred to the investigator's laboratory after de-identification and coding. Long-distance shipment under optimal conditions is feasible, and we routinely obtain samples in Houston from other cities in the United States or Asia; these are shipped on wet ice and take up to 7 d to reach us. A decline of clonogenicity might be expected with time, but we have not directly assessed this

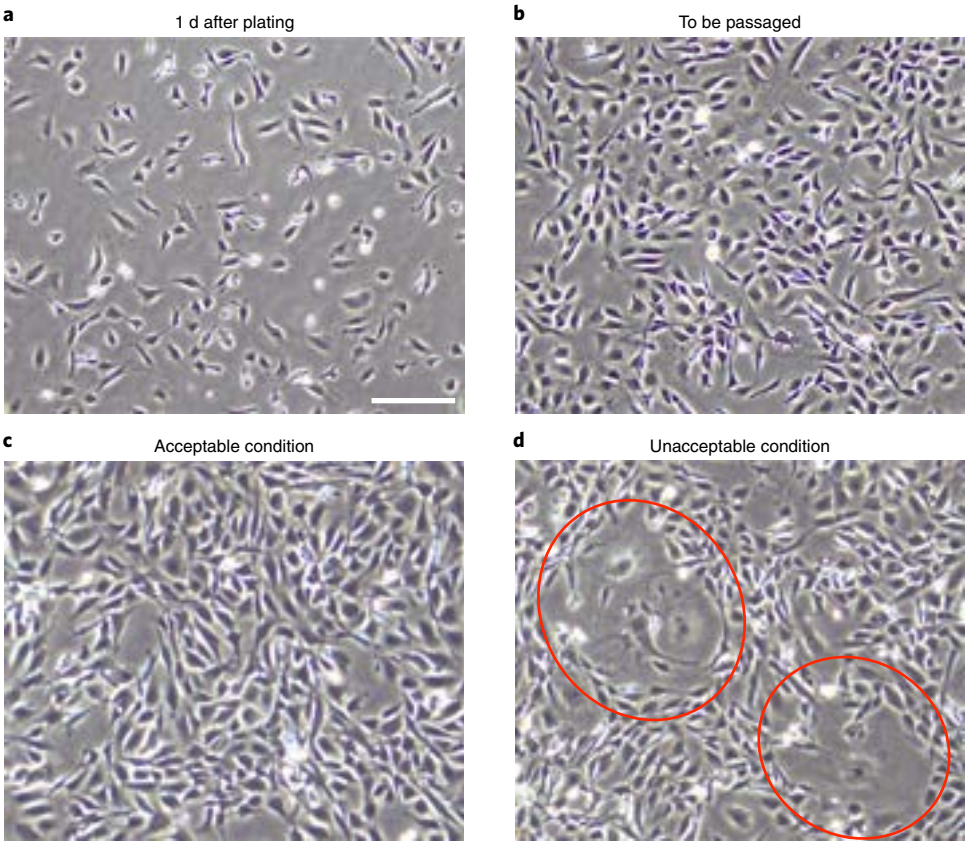


Fig. 4 | Representative images of 3T3-J2 cells. a, One day after seeding, the 3T3-J2 cells attach, spread and start proliferating. **b**, The 3T3-J2 cells should be passaged when the cells reach 80–90% confluence, with space apparent between cells. **c**, For irradiation, 3T3-J2 cells can proliferate to 95% confluence before harvest. The space between cells should still be visible in some regions. The cells should appear uniform and show contact inhibition. **d**, Giant 3T3-J2 cells with extensive cytoplasmic domains indicate an unhealthy condition (red circles); in this case, the culture should be discarded. Scale bar, 100 μm.

issue. Before receiving biopsy samples, 3T3-J2 feeder cells should be prepared according to the protocol below. Preferably, the biopsy samples would be processed immediately upon arriving at the laboratory. We have processed hundreds of biopsy samples from human small intestine and colon and demonstrated a success rate of at least 95% independent of age and sex[13] (Supplementary Table 1). Processing of biopsy samples from ascending, transverse and descending colon is uniformly successful, whereas that of samples from small intestine is somewhat more variable but still >90%.

Expanding gISCs and setting up single cell-derived clones

The gISC colonies appear 7–10 d after seeding, and generally 100–300 independent colonies form from a typical 1-mm-diameter biopsy sample that harbors 300.000–900,000 epithelial cells at all stages of differentiation (Fig. 1). The cells should be passaged within 10 d, before the 3T3 J2 feeder cell lawn ages. Immature gISC colonies are compact and composed of highly proliferative cells with high nucleus/cytoplasm ratio. These gISCs are highly clonogenic (>70%) and consequently proliferate to >1 billion cells in 6–8 weeks. Inappropriate handling, such as use of aged or non-optimal 3T3 J2 feeders, will lead to a differentiation of gISCs marked by an increased cytoplasm/nucleus ratio and expression of differentiation markers, as well as a loss of clonogenicity and proliferation potential. As long as the protocols that we provide here are followed, the establishment of gISC clones is highly efficient. For cloning of gISCs from libraries of gISC colonies, we favor FACS-aided sorting from single-cell suspensions into 384-well plates previously seeded with 3T3-J2 feeder cells (Fig. 1; Supplementary Video 1). In 7–14 d, single cell–derived colonies can be observed and can be sampled and expanded to discrete lines for all downstream applications. We suggest freezing each passage of an gISC clone in liquid nitrogen until needed.

Table 1 | Antibodies used for immunofluorescence

Target	Cat. no.	Species	Dilution	Vendor	RRID no.
Primary antibodies					
Villin	ab52102	Rabbit	1:300	Abcam	AB_883445
E-Cadherin	AF648	Goat	1:400	R&D Systems	AB_355504
Mucin2	sc-515032	Mouse	1:400	Santa Cruz Biotechnology	AB_2815005
Chromogranin A	ab15160	Rabbit	1:350	Abcam	AB_301704
Defensin alpha 6	HPA019462	Rabbit	1:350	Sigma-Aldrich	AB_1847595
Secondary antibodies					
Alexa Fluor 488 donkey anti-goat IgG	A11055	Donkey	1:500	Invitrogen	AB_2534102
Alexa Fluor 594 donkey anti-mouse IgG	A21203	Donkey	1:500	Invitrogen	AB_141633
Alexa Fluor 594 donkey anti-rabbit IgG	A21207	Donkey	1:500	Invitrogen	AB_141637

Functional characterization of gISCs

We find that gISCs harbor intrinsic immortality, indicated by unlimited self-renewal ability and conditional, multipotent differentiation. To examine clonogenicity of gISC clones, single-cell suspensions can be FACS-sorted to single cells in 384-well plates, which typically yield a 70% rate of colony formation in wells for stem cells from the gastrointestinal tract (Fig. 1). Owing to the remarkably consistent clonogenicity across numerous passages, gISCs can achieve a proliferative expansion that is close to the theoretical limit (Fig. 2, Supplementary Video 2). Single clones selected from these 384-well plates can be expanded as clonal populations and validated by in vitro differentiation in ALI cultures that recapitulate intestinal epithelia comprising $Muc2^+$ goblet cells, $CHGA^+$ endocrine cells, $DEFA6^+$ Paneth cells and $Villin^+$ enterocytes (Fig. 1, Table 1). We note that these ALI cultures yield 3D epithelia whose histology is remarkably similar to the in situ histology of the corresponding regions of the gastrointestinal tract. From histology alone, the ALI cultures of gISCs appear to yield facsimiles of the gastrointestinal mucosa superior to those reported for organoids (Fig. 1). Alternatively, gISC clones can be readily adapted to organoid cultures by seeding into Matrigel and growth in established organoid media (Fig. 5). Using validated ISC clones, we also note that seeding 2,000 single cells in parallel into either ground-state or organoid cultures yields 1,500 colonies in the 2D, gISC cultures, and ~1,000 spheroids in organoid cultures. Subsequent passaging showed that although the gISCs maintain their 70% clonogenicity, cells from organoids showed a marked decline of clonogenicity, suggesting gISC culture comprises a uniform population of stem cell cells, whereas organoid culture comprises a majority of differentiated cells or transit-amplifying cells[23].

We previously demonstrated that gISCs display remarkable genomic stability during long-term culture[11]. For instance, when we examined copy-number and single-nucleotide variation in gISC clones across extended periods of continuous proliferation to 1 billion cells (6–8 passages of 10 d each), we found no chromosomal aneuploidy, no CNV events >10 kb, and very few single-nucleotide variations, none of which impacted known proto-oncogenes or tumor suppressor genes[11]. After 20 passages, this situation changed in some clones, and this should be borne in mind. Ample numbers of cells for downstream applications can be generated before this point. We collected DNA from single cell–derived gISC clones at P5 (50 d in culture), P10 (100 d in culture) and P20, and subjected the DNA for whole-exome sequencing. On the basis of the somatic allelic CNVs called by the best-practices pipeline of GATK v.4.0.4 (https://gatk.broadinstitute.org/hc/en-us), we did not observe any chromosomal aneuploidies during the passaging of selected clones (Fig. 3).

Separation of gISC from 3T3-J2 feeder cells

Some downstream applications require the elimination of feeder cells from gISCs, which can be achieved by several routes. gISCs can be labeled with GFP, using retroviral or lentiviral transduction, and then readily separated by FACS sorting from feeder cells. As shown in Fig. 6, live-cell sorting based on green fluorescence can efficiently separate GFP-labeled gISC from mouse feeder cells.

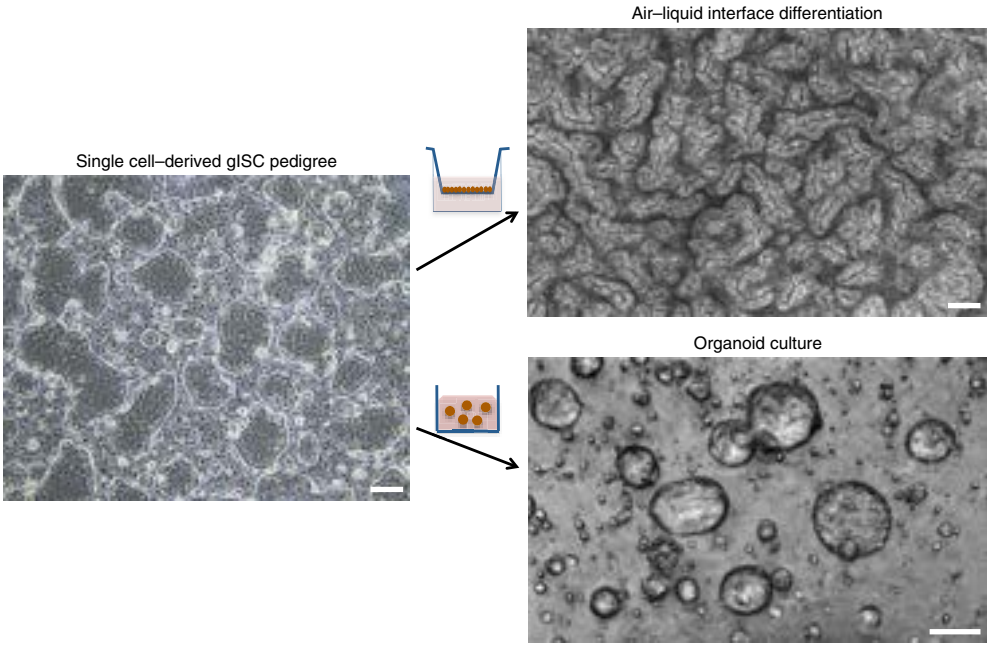


Fig. 5 | Adaptation of gISC clone to ALI differentiation or organoid culture format. Left, 2D colonies of a single gISC clone 10 d after seeding. Right, 3D intestinal mucosal resulting from 10-d ALI differentiation of an gISC clone (top) and 3D organoids 10 d after gISC seeding into Matrigel in organoid culture medium. Scale bars, 200 μm.

Alternatively, murine 3T3-J2 feeder cells can be depleted from co-cultures with gISCs by using Feeder Removal MicroBeads (Miltenyi Biotec), LS columns, and a MidiMACS separator, with human-specific PCR primers indicating a 99% purity of gISCs (Table 2). In unpurified samples, we can detect 25–40% of mouse DNA. By contrast, after purification, we observed <1% of mouse DNA (Supplementary Methods). This level of purity ensures the integrity of DNA and RNA from these gISCs in the analyses of clonal heterogeneity, genome stability and genome editing.

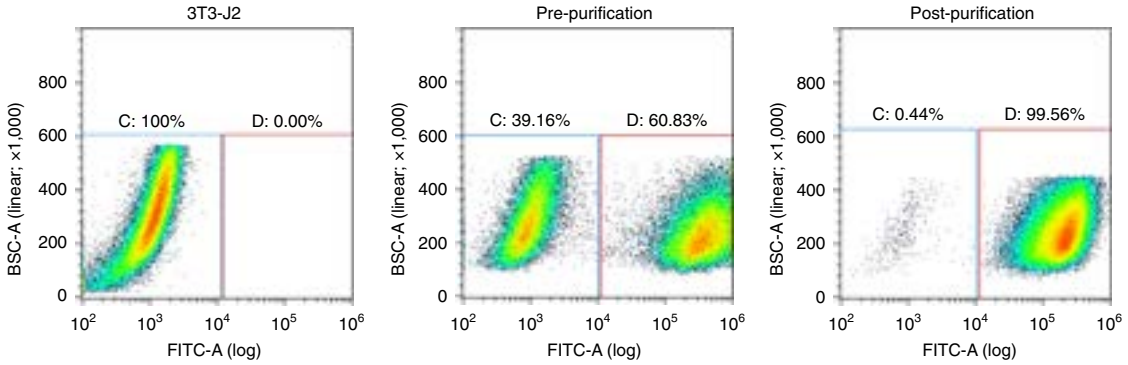
Materials

Biological materials

- Endoscopic biopsy samples from human gastrointestinal mucosa **! CAUTION** Ethical approval is required for acquisition and use of human-derived samples; collection must conform to relevant institutional and national regulations, and patient consent is required. Our biopsy samples were collected under IRB approval from Connecticut Children's Medical Center (IRB approval no. HSC-MS-16-0330), the University of North Carolina (IRB approval no. 15-2813), the Baylor College of Medicine (IRB approval no. H-38746) and the National University of Singapore (National Healthcare Group (NHG) Domain Specific Review Board (DSRB) IRB approval no. 2014/00836).
- Resected human intestine tissue collected as surgical waste **! CAUTION** Ethical approval is required for acquisition and use of human-derived samples; collection must conform to relevant institutional and national regulations, and patient consent is required. Our biopsy samples were collected under IRB approval from the MD Anderson Cancer Center (IRB approval no. LAB06-0893).
- 3T3-J2 fibroblasts. The 3T3-J2 fibroblast cell line[25] was generously provided by H. Green. 3T3-J2 cells can also be purchased commercially (https://web.expasy.org/cellosaurus/CVCL_W667; https://www.biocompare.com/11023-Cells-Strains/8563571-3T3-J2-Cell-Line/) **▲ CRITICAL** The cell lines used in your research should be regularly checked to ensure that they are authentic using short tendem repeat (STR) profiling and that they are not infected with mycoplasma.

Reagents

- DMEM without L-glutamine, without sodium pyruvate (DMEM$^-$; 4.5 g/liter glucose; Invitrogen, cat. no. 11960-044)
- F12 (Gibco, Life Technologies, cat. no. 11765-054)
- HEPES (Gibco, Life Technologies, cat. no. 15630-080)
- Gentamycin (Gibco, Life Technologies, cat. no. 15710-064)



	3T3-J2		Pre-purification		Post-purification	
Name	Events	Parent%	Events	Parent%	Events	Parent%
C	49,800	100	27,452	39.15785	365	0.436619
D	0	0	42,643	60.82646	83,226	99.5562

Fig. 6 | FACS profiling of GFP-labeled gISCs for monitoring of purification. Top, from left to right, FACS profile of 3T3-J2 cells, FACS profile of co-culture of 3T3-J2 cells and gISCs, and FACS profile of co-cultured cells following removal of feeder cells. Bottom, quantification of 3T3-J2 in FACS gate C in 3T3-J2 cultures (left), in co-cultures with gISCs (in FACS gate D), and following removal of 3T3-J2 cells. See Supplementary Figs. 1–4 for gating strategy and numerical data. BSC-A, back scatter area.

Table 2 | Primers used for assessment of DNA purity

Primer	Species	Forward sequence	Reverse sequence
MHC1	Human	ATC ATG ACT CTC ACC CTC CA	TCA ACC CTT TTG CTT ACC TG
MICA1	Mouse	AGG GTC TGT GAG ATC CAT GA	CTG CAT GCA TAG CGT GAT AG

- Fungizone (Gibco, Life Technologies, cat. no. 15290018)
- Bovine calf serum (BCS) (HyClone, cat. no. SH130072.03)
- Fetal bovine serum (FBS) (HyClone, cat. no. SH30396.03)
- Penicillin–streptomycin (Invitrogen, cat. no. 15140-122) **! CAUTION** Penicillin–streptomycin is a health hazard if swallowed. It should be disposed of by appropriate routes. Consult safety and hazard sheets provided by the seller.
- L-glutamine (Invitrogen, cat. no. 25030-081)
- 0.05% Trypsin–EDTA (Invitrogen, cat. no. 25300-062)
- TrypLE Express enzyme (1×; Gibco, Invitrogen, cat. no. 12605-010)
- DPBS, no calcium, no magnesium ($DPBS^{-}$; 1 ×; Invitrogen, cat. no. 14190-144)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. 41640-500ml)
- Trypan blue solution (0.4% (wt/vol) in $DPBS^{-}$, pH 7.5 ± 0.5; Corning, cat. no. 25-900-CI) **! CAUTION** Trypan blue solution is acutely toxic if swallowed and is a health hazard. Consult safety and hazard sheets provided by the seller.
- Collagenase, type IV (1g; Life Technologies 17104-019)
- Expansion medium (StemECHO202; Tract Pharmaceuticals, cat. no. 044102)
- Bullet enhancer (StemECHObullet002; Tract Pharmaceuticals, cat. no. 044002)
- Differentiation medium (StemECHO204; Tract Pharmaceuticals, cat. no. 044104)
- Neutralization medium (StemECHO200; Tract Pharmaceuticals, cat. no. 044100)
- Growth-factor-reduced Matrigel (Corning, cat. no. 354230)

Equipment

- Inverted microscope (Nikon, model no. Eclipse Ts2)
- CO_2 incubator (Eppendorf/New Brunswick Galaxy 48R incubator; Eppendorf, cat. no. CO48R230 or Panasonic, cat. no. KM-CC17RU2)
- Tissue culture biological safety cabinet (class II; LabGard ES; NuAire, cat. no. NU-437)

- pH meter (Five Easy FE20; Mettler Toledo, cat. no. 10385343)
- Fine forceps (student Dumont no. 5 forceps; Interfocus, cat. no. 91150-20)
- Fine scissors (straight/large loop; Interfocus, cat. no. 14040-10)
- Pasteur pipettes (Scientific Laboratory Supplies, cat. no. PIP4172)
- Sterile disposable scalpels (Dynarex, cat. no. 4122)
- Tissue culture–grade plates (Corning, cat. no. CLS3506)
- Nalgene rapid-flow sterile disposable filter units with PES membrane (1,000 ml, 0.2μm; Fisher Scientific, cat. no. 09-741-03)
- Steriflip filtration units (50 ml; Millipore, cat. no. C3238)
- Cell strainer (40 μm; Corning, cat. no. 07-201-430)
- Cell strainer (30 μm; Miltenyi Biotec, 130-041-407)
- QuadroMACS Starting Kit (LS) (Miltenyi Biotec, cat. no. 130-091-051)
- Transwell multiple-well plate with 0.4-μm-pore polyester membrane inserts (Fisher Scientific, cat. no. 07-200-154)
- Tissue culture–treated microplates (96 wells, flat, clear, polystyrene; Fisher Scientific, cat. no. 07-200-90)
- Cell sorter (Sony, cat. no. SH800S)
- X-ray irradiation machine (RadSource, model no. RS1800, cat. no. 1087)
- Mini shaking incubator (WISBiomed, cat. no. BS-ICH1)
- Centrifuge (Eppendorf, model no. 5810 R)
- Hemocytometer with cover glass (Daigger, cat. no. EF16034F)
- CoolCell freezing container (Corning, cat. no. 432008)
- Freezer (−80 °C; VWR, cat. no. 76307-942)
- Liquid nitrogen tank (VWR, cat. no. 10027-532)
- Tissue culture dishes (Corning, 6-well plates, cat. no. 08-772-1B; 12-well plates, cat. no. 08-772-29; 24-well plates, cat. no. 08-772-1; 48-well plates, cat. no. 07-200-86; 100-mm dish, cat. no. 08-772-22; 60-mm dish, cat. no. 430589; 150-mm plates, Corning 08-772-24; 24-well Transwell inserts, cat. no. 3470)
- Plates (96 well; Greiner Bio-One, cat. no. 65500)
- Filters (0.22 μm; Corning 09-741-08)
- Pipette tips
- Feather scalpels
- Nylon mesh strainer (100 μm)
- Cell sorter

Reagent setup

3T3-J2 culture medium

To 440 ml of DMEM$^-$, add 50 ml of BCS, 5 ml of penicillin–streptomycin and 5 ml of L-glutamine. Filter the medium with a 0.22-μm filter. The culture medium can be stored at 4 °C for up to 1 month.

! CAUTION Be sure to use BCS for the 3T3-J2 culture medium. Do not use FBS.

3T3-J2 freezing medium

To 700 ml of DMEM$^-$, add 200 ml of BCS and 100 ml of DMSO. Pass the medium through a 0.22-μm filter. Make aliquots and store at −80 °C for up to a year.

Wash buffer

To 950 ml of F12 medium, add 50 ml of FBS, 10 ml of penicillin–streptomycin, 10 ml of gentamycin and 1 ml of Fungizone. Filter the medium with 0.22 μm filter. The wash buffer can be stored at 4 °C for up to 1 month.

Digestion buffer

Dissolve 1 mg of collagenase in 10 ml of wash buffer in a 37 °C water bath for 30 min. Filter the medium with a 0.22-μm filter. Prepare fresh digestion buffer each time. Use approximately 2–3 ml of digestion buffer for each biopsy sample.

gISC growth medium

To prepare gISC growth medium (complete expansion medium; StemECHO202 + StemECHObullet002), add 0.25 ml of StemECHObullet002 to 250 ml of StemECHO202. Mix thoroughly and store at 4 °C for up to 1 month.

gISC freezing medium
To prepare 100 ml of ISC freezing medium, add 10 ml of DMSO and 10 ml of FBS to 80 ml of gISC growth medium. Filter the medium using a 0.22-μm filter. Make aliquots and store at −80 °C for up to 1 year.

Diluted Matrigel
Thaw one aliquot of Matrigel at 4 °C overnight packed in ice. Pipette 2 ml of Matrigel into 8 ml of cold (4 °C) 3T3-J2 culture medium. Swirl the tube to mix the contents completely, store at 4 °C and use within 1 week.

Equipment setup

Humidified incubator
Set to 37 °C, 7.5% CO_2.

Matrigel-coated plates
Chill tissue culture plates and pipette tips at 4 °C overnight. Add diluted Matrigel to the wells of a tissue culture plate in a biological safety cabinet. Incubate for a minimum of 30 min at 37 °C. Plates should be used immediately to plate feeder cells.

Procedure

! CAUTION All procedures should be undertaken in class II biological safety cabinets to ensure sterility.
! CAUTION All procedures used for handling human tissues should assume potential contamination of tissue with human pathogens (e.g., HBV, HCV, HIV, *Clostridium difficile*) and should be in compliance with IRB-approved protocols.

Reviving 3T3-J2 cells from frozen stock ● Timing 3 d

1 Clean the hood with 70% ethanol and turn on a UV light for 15 min before the cell culture experiment.
! CAUTION This should be repeated before and after each cell culture session.
2 Warm 3T3-J2 culture medium in a water bath (37 °C) for 30 min before thawing the vial.
3 Retrieve a cryovial of 3T3-J2 cells from frozen cell storage and thaw the vial (each vial should contain 10^6 cells) in water bath.
4 Gently transfer cells with a 1,000-μl pipette to a 50-ml Falcon tube and add 30 ml of 3T3-J2 culture medium drop by drop while swirling the tube slowly.
▲ CRITICAL STEP Thaw the cells quickly in the water bath and swab the vial with 70% ethanol before bringing it into the hood. Dilute freezing medium slowly, drop by drop. Do not pipette the cells up and down.
5 After resuspension, seed the cells into a 15-cm tissue culture plate.
▲ CRITICAL STEP Do not centrifuge to wash away the freezing medium.
6 Gently shake the plates with cells in the hood to evenly distribute the cells.
▲ CRITICAL STEP Flow the medium left and right five times and let it sit for 5 s or until the medium is stabile. Then flow toward the back and front five times in the hood.
7 Repeat Step 6 three times in the incubator.
▲ CRITICAL STEP Do not change the location once the plates are placed in the incubator. This step is very important to evenly distributing the cells in the plate.
8 Change the medium the day after thawing the frozen vial. After that, change medium every 2 or 3 d.
▲ CRITICAL STEP It is important to change the medium the day after thawing the vial because the freezing medium contains DMSO, which is harmful to cells.
9 Proceed to the next step to passage the cells when they become 80% confluent. Generally 80% confluency will be reached 3 d after seeding, however, monitoring is required.
▲ CRITICAL STEP Do not continue culturing if 3T3-J2 cells grown past confluence. Abandon them and thaw a new vial.
? TROUBLESHOOTING

Passaging 3T3-J2 cells for expansion ● Timing variable

10 Warm 3T3-J2 culture medium and 0.05% Trypsin–EDTA in a water bath (37 °C) for 30 min before use.

11 Remove the plates from the incubator, check the 3T3-J2 cells under a microscope and gently put the plates into a cleaned hood.

12 Remove the medium, add 30 ml of $DPBS^{-}$ to thoroughly wash away the serum, and add 5 ml of warmed 0.05% Trypsin–EDTA.
▲ CRITICAL STEP It is important to evenly spread the Trypsin–EDTA in the plates.

13 Incubate the cells with Trypsin–EDTA in incubator at 37 °C for 5 min.
▲ CRITICAL STEP Do not leave the cells in Trypsin–EDTA for >10 min.

14 Add 10 ml of warmed 3T3-J2 culture medium to the plate to neutralize the Trypsin–EDTA following 5 min of incubation. Pipette up and down five times to obtain a single cell solution.

15 Collect the cells into a 50-ml Falcon tube and spin down at 300*g* for 5 min at 4 °C.

16 Remove the supernatant carefully and resuspend the cell pellet with a fresh 5 ml of 3T3-J2 growth medium.

17 Count the cells using hemocytometer by mixing 10 µl of cells with 10 µl trypan blue, and using a hemocytometer to count the cells.
▲ CRITICAL STEP If you are working on a number of tubes, all should be combined in one container and mixed gently by swirling before counting.

18 Calculate the number of cells using the following equations:

$$\text{Avg. cell counts of the four corner squares} \times 10^4\text{, corrected for dilution factor} = \text{cells/mL}$$
$$\text{Total Cells} = \text{cells/ml} \times \text{amount of total volume of medium in mL}$$

19 Determine the number of 15-cm plates to seed with 500,000 cells per plate and reserve medium as necessary.

20 Seed 500,000 cells per plate and shake the plates in the incubator.
▲ CRITICAL STEP Add 30 ml of warmed growth medium to the required number of plates and then add 500 µl of cell suspension into the plates. Shake the tube continuously to ensure the cells do not accumulate at the bottom. Shake the plate left and right five times and let it sit for 5 s or until the medium is stabile, and then shake back and forth five times in the hood. Repeat the process of shaking three times in the incubator.
? TROUBLESHOOTING

21 When the cells are 80% confluent, passage them for further expansion by repeating Steps 10–20. When you have sufficient cells, proceed to the next stage to irradiate and freeze the cells.
? TROUBLESHOOTING

Irradiating and freezing irradiated 3T3-J2 cells ● Timing 1 d

▲ CRITICAL Freeze as many early-passage 3T3-J2 cells as you will need for your experiments. We freeze ~10^6 cells per vial, but as many as 4×10^6 and as few as 5×10^5 can be frozen in one vial.

22 When the confluency of 3T3-J2 cells reaches 90–95%, change the medium.

23 The next day, repeat Steps 10–16 to passage cells and place the cells in a 50-ml Falcon tube on ice.
▲ CRITICAL STEP Move immediately to the next step of irradiation.

24 Irradiate the 3T3-J2 cells at 2,000 rad.
▲ CRITICAL STEP The duration required to administer 2,000 rad (20 Gy) must be calculated each time according to the facility records.

25 Following the irradiation, put the container back on the ice and immediately count the number of viable cells using a hemocytometer and trypan blue (as described in Steps 17 and 18).

26 Spin down cells at 300*g* for 5 min at 4 °C.

27 Resuspend cells in freezing medium very gently to freeze 6×10^6 cells/vial. Use 0.5 ml of freezing medium per vial.
▲ CRITICAL STEP 3T3-J2 cells are extremely fragile after irradiation and need to be handled with extreme care. Do not pipette the cells up and down to mix the cells. Swirl the container of cells very gently for mixing.

28 Put the cells in the CoolCell freezer box at −80 °C overnight for gradual freezing.

29 Transfer the cryovials with cells to a liquid nitrogen tank or deep freezer the next day for long-term storage.
■ PAUSE POINT 3T3-J2 cells can be stored indefinitely at −80 °C.
? TROUBLESHOOTING

Table 3 | The seeding density of 3T3-J2 cells and gISCs in different tissue culture dishes

Plates	6-well	12-well	24-well	48-well	96-well	100 × 20 mm	60 × 15 mm	24-Transwellwell plate
Surface area (cm^2)	9.6	3.8	2	1.1	0.32	58.95	19.5	0.3
No. of feeder cells	1.5M–1.8M	600k–720k	300k–400k	120k–150k	50k–60k	9M–11M	3M–3.6M	300k–400k
No. of ISCs	50k–100k	15k–30k	8k–15k	5k–7k	1k–3k	0.4M–0.6M	0.1M–0.6M	250k–300k

k, thousand; M, million.

Preparing feeders for ISCs ● Timing 2 d

30 Thaw growth-factor-reduced Matrigel on ice and dilute in cold 3T3-J2 growth medium at a concentration of 25%.
▲ CRITICAL STEP Be sure to use pre-cooled tips to add Matrigel to the cold growth medium in order to prevent the solidification of the Matrigel during this process.

31 Precool the tissue culture plates at −20 °C for 15 min.

32 Add diluted Matrigel to the cold plate, swirl the plate to evenly distribute the diluted Matrigel and pipette out the extra Matrigel.

33 Place the plates in a 37 °C incubator for 30 min to allow the Matrigel layer to solidify.

34 Seed irradiated 3T3-J2 cells in the presence of 3T3-J2 culture medium and shake the plates with cells in a 37 °C incubator to evenly distribute the cells (see Table 3 for recommended cell numbers). We recommend using these 3T3-J2-seeded plates 24 h after seeding and within 72 h of seeding.

35 Change the 3T3-J2 medium each day after seeding.

gISC derivation from a 1-mm-diameter biopsy sample ● Timing 11 d

36 Wash the human intestinal biopsy sample (transferred from the hospital in cold wash buffer on ice) with 5 ml of cold wash buffer in a 15-ml Falcon tube, centrifuge at 300*g* for 7 min at 4 °C, and then remove the medium carefully without disturbing the biopsy sample. Repeat the washing procedure three times.
▲ CRITICAL STEP Washing the biopsy sample thoroughly can help to avoid potential contamination. However, it is critical not to lose the biopsy sample during washing steps, so pay particular attention to the step of aspirating the medium.

37 Pipette the biopsy sample into a tissue culture Petri dish and mince the tissue between two Feather scalpels. Mince until the tissue resembles a paste.
▲ CRITICAL STEP This mincing step is imperative and will determine cloning efficiency.

38 Digest tissue in digestion buffer (1.5 ml of digestion buffer per biopsy sample) for ~30 min on a 37 °C rocker set to 100 r.p.m. Every 10 min, pipette the mixture up and down to break up any aggregated clumps.
▲ CRITICAL STEP At the end of digestion, you should not observe any undigested pieces. Any remaining, undigested tissue fragments can be removed by passing the minced tissue through a 100-μm Nylon mesh strainer.

39 At the end of digestion, add 10 ml of cold wash buffer to each tube containing digested tissue. Mix the contents by inverting the tubes ten times.

40 Centrifuge the digested tissue at 300*g* for 7 min at 4 °C, remove the wash buffer carefully and repeat the washing step five times.
▲ CRITICAL STEP The pellet of the biopsy sample is very small, so the wash buffer should be saved and rechecked prior to discarding.

41 After the last wash with wash buffer, spin down the digested biopsy sample at 300*g* for 7 min at 4 °C and wash one more time with 10 ml of cold StemECHO neutralization medium.
▲ CRITICAL STEP Do not skip this step. It is critical to removing all residual wash buffer.

42 Following centrifugation at 300*g* for 7 min at 4 °C, resuspend the pellet in 1 ml of gISC growth medium completely by pipetting up and down ten times.

43 Seed the suspension of each biopsy sample in one well of a 24-well tissue culture dish pre-coated with 20% growth-factor reduced Matrigel and irradiated 3T2 J2 feeder cells (from Step 35). Return the plates to incubator.
? TROUBLESHOOTING

44 Change the gISC growth medium every 2 d.

45 When the ISC colonies are visible (in about 1 week), wash the culture with DPBS$^-$ twice, add 500 µl of TrypLE, and incubate the cells with TrypLE in the incubator at 37 °C for 15 min.
? TROUBLESHOOTING

46 Pipette the cells gently up and down five times to dissociate them into single cells. If the cells still look clumpy under a microscope, put the plate back into the incubator for another 15 min.

47 Add 5 ml of warmed StemECHO neutralization medium *quickly* to the well-trypsinized cells and pipette up and down ten times.
▲ **CRITICAL STEP** Do not add neutralization medium dropwise because it will lead to the re-aggregation of stem cells.

48 Centrifuge the cells at 300*g* for 7 min at 4 °C, and completely resuspend the pellet in 1 ml of gISC growth medium by pipetting up and down ten times. Pass the cell solution through a 40-µm cell strainer and seed as single cells into one well of a 24 well plate pre-coated with growth factor-reduced Matrigel and irradiated 3T3-J2 feeder cells (from Step 35).
▲ **CRITICAL STEP** We always recommend freezing half of the stem cells at this step (as described in Step 50).

49 Change the medium every 2 d. Three days later, individual ISC clones should be observable. In about 10 d, the stem cell culture should be ready for the next step (passaging, freezing, or generation of single cell–derived pedigrees).

Downstream assays

50 Follow option A to freeze cells, option B to establish a gISC pedigree, and option C to differentiate cells at an ALI.

(A) **Freezing of ISC clones** ● **Timing 4 h**

(i) Trypsinize ISC clones as described in Steps 45–47 and count the cells using a hemocytometer. We recommend freezing 100,000 cells per vial in 500 µl of freezing medium, which will be sufficient for recovery in 1 well of a 6-well plate.
▲ **CRITICAL STEP** It is critical to count the ISCs and record the cell number on the freezing vial. Seeding too few or too many cells after thawing will negatively affect the quality of stem cell culture.

(B) **Establishment of a gISC pedigree** ● **Timing 14 d**

(i) When the ISC culture reaches ~60% confluency (<100 cells in each colony), trypsinize the ISC clones by following Steps 45–47.

(ii) Gently pipette up and down to achieve a single-cell suspension and pass the cells through a 30-µm cell strainer.

(iii) Remove mouse feeder cells using a QuadroMACS Starting Kit (LS) according to the manufacturer's instructions. Briefly, cells are trypsinized to a single-cell suspension, incubated with antibody-coated microbeads for 15 min at 4 °C, and then run through an LS column to collect the stem cell–containing flow-through.

(iv) Use a cell sorter to sort single cells into individual wells of a 96-well plate previously coated with 30% growth factor-reduced Matrigel and seeded with the feeder cells.

(v) Monitor the cells daily. It generally takes ~5 d to observe colony formation in the 96-well plates.

(vi) In ~7–10 d, before the colonies become confluent and within the 2-week period of optimal 3T3-J2 lawn integrity, wash the culture with DPBS$^-$ twice, add 30 µl of TrypLE, and incubate the cells with TrypLE in the incubator at 37 °C for 15 min.
▲ **CRITICAL STEP** Do not add >30 µl of TrypLE.

(vii) Gently pipette the cells up and down ten times to dissociate them into single cells. If the cells still look microscopically clumpy, put the plate back into the incubator for another 15 min.

(viii) Add 100 µl of warmed gISC growth medium quickly to the well-trypsinized cells and pipette up and down ten times.
▲ **CRITICAL STEP** Pipette carefully to avoid cross-contamination.

(ix) Transfer the resuspended cells to 1 well of a 24-well plate that has been coated with Matrigel and seeded with feeder cells.
▲ **CRITICAL STEP** Make sure the colony is trypsinized to single cells before reseeding.

(x) Expand these single cell–derived ISCs to billions of cells within a few weeks.
▲ **CRITICAL STEP** Freeze early-passage ISCs as described in Step 50(A).
? TROUBLESHOOTING

(C) **Differentiating gISCs in ALI culture** ● Timing **10 d**

(i) Prepare the Transwell inserts in a 24-well plate by coating the inserts with 30 µl of 30% growth-factor-reduced Matrigel. Gently shake the plate to make sure that the Matrigel solution evenly covers the surface of the inserts. Remove excess Matrigel solution, and incubate the plate at 37 °C for 30 min.

(ii) Add 700 µl of 3T3-J2 growth medium to each well of the 24-well plate, and then place the Matrigel-coated inserts into the wells.

(iii) Prepare 200,000 irradiated 3T3-J2 cells in 200 µl of growth medium (from Step 29) and seed onto each insert.

(iv) Incubate the plate at 37 °C overnight in the incubator.

(v) The next day, aspirate the gISC growth medium from the ISC plate and rinse with $DPBS^{-}$ thoroughly.

(vi) Trypsinize with TrypLE Express enzyme (1×) for 10–20 min.

(vii) Gently pipette up and down to achieve a single-cell suspension. Add StemECHO neutralization medium to neutralize the trypsin and pass the cells through a 30-µm cell strainer.

(viii) Remove the mouse feeder cells using a QuadroMACS Starting Kit, as described in Step 50B(iii).

(ix) Determine the cell number as described in Step 18.

(x) To start an ALI culture, remove the medium from a Transwell plate and change to gISC growth medium.

(xi) Prepare 200,000–300,000 ISCs in 200 µl of gISC growth medium and seed each insert.

(xii) Incubate Transwell inserts in a 37 °C incubator.

(xiii) Change the gISC growth medium every day.

(xiv) In 3–4 d, the ISCs in the insert should reach confluency. Remove the gISC growth medium from the inserts by careful pipetting to create ALI culture. Change the medium in the Transwell plate to StemECHO differentiation medium and incubate in a 37 °C incubator.

(xv) Change the medium every day and continue the cultures for an additional 10–12 d to induce differentiation.

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 4.

Table 4 | Troubleshooting table

Step	Problem	Possible reason	Solution
9, 20, 21	3T3-J2 cells lose contact inhibition	Cells might spontaneously transform	Contact inhibition is a unique feature of 3T3-J2 cells and it is an essential characteristic indicating the suitability of these cells as feeders for adult stem cells. 3T3-J2 cells that have lost this feature should not be used any longer. Start from a new vial of early-passage 3T3-J2 cells
29	Significant rise in the saturation density of 3T3-J2 cells	Continuous passaging of 3T3-J2 cells from over-confluent flasks	It is normal to notice a slight rise in the saturation density of the flask as you increase passage number, but the maximum number of cells you should get from a just-confluent 15-cm plate^2 is ~8×10^6 to 10×10^6. If you are getting on the order of 15×10^6 to 20×10^6 cells, then your feeders are not being passaged properly. This can affect the quality of your ISC cultures Therefore, immediately switch to a new vial of 3T3-J2 cells These guidelines should be strictly adhered to
43	Poor feeder cell condition	Feeder cell death after irradiation	Shake the tube containing irradiated 3T3-J2 cells regularly and gently so that cells do not become concentrated at the bottom of the tube while adding cells to the freezing vials
		Inaccurate cell counting	Use one vial of freshly irradiated cells to check the cell condition and density each time after irradiation
		Insufficient Matrigel coating	Use fresh Matrigel and be sure to coat for >30 min
45, 50B	gISCs lose clonogenicity	Cells differentiate	You should not observe a decline of clonogenicity during long-term culture. Loss of clonogenicity indicates a loss of stemness of the culture. If this occurs, you then should start a new culture from early-passage ISCs
50C	Leakage in the ALI	gISCs are not in optimal condition before seeding or the structure was damaged during medium changes	Five days following removal of the medium from the insert, you should observe intestine-like structures. If leakage is observed, you need to restart the experiment

Timing

Steps 1–9, reviving 3T3-J2 cells from frozen stock: 3 d
Steps 10–21, passaging 3T3-J2 cells for expansion: variable
Steps 22–29, irradiating and freezing irradiated 3T3-J2 cells: 1 d
Steps 30–35, preparing feeders for ISCs: 2 d
Steps 36–49, deriving ISCs from a 1-mm-diameter biopsy sample: 11 d
Step 50A, freezing of ISC clones: 4 h
Step 50B, establishment of a single cell–derived pedigree: 14 d
Step 50C, inducing gISCs to differentiate in an ALI culture: 10 d

Anticipated results

Herein, we provide a protocol that details how to derive and expand cells from standard human endoscopic biopsy samples. This 2D culture system enables the generation of libraries of 100–300 independent gISC clones from clinically standard, 1-mm-diameter biopsy samples of human intestinal mucosa. We anticipate that this culture medium system will support the expansion of gISC clones in a highly undifferentiated and clonogenic state to practically unlimited numbers in vitro and specifically 1 billion cells in ~60 d (Fig. 2). Importantly, the gISC culture medium system described here confers a host of advantages over either induced pluripotent stem cells or the organoid approach, both of which yield relatively few stem cells among many differentiated cells. Moreover, these single cell–derived clones can be induced to differentiate into mucosa-like structures, including all appropriate cell lineages, such as enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Fig. 1). The high clonogenicity of gISCs confers a uniquely high rate of 'expandability' for experimental manipulation, regenerative medicine, and drug discovery. Taken together, this is a remarkably simple culture system for generating unlimited numbers of genetically stable and regionally committed gISCs from any patient for analysis via multiple technologies.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The corresponding authors will provide all data presented in the article and address technical questions upon reasonable request.

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Acknowledgements

This work was supported by grants from the Cancer Prevention Research Institute of Texas (CPRIT; RR15014 to W.X. and RR15088 to F.M.), the National Institutes of Health (1R01DK115445-01A1 to W.X.; U24CA228550 and 1R01CA241600-01 to F.M.), the US Dept. of Defense (W81XWH-17-1-0123 to W.X.; W81XWH-19-1-0127 to C.P.C.), the Singapore National Medical Research Council (to K.Y.H. and F.M.), a University of Texas Presidential Award (to W.X.) and an American Gastroenterology Association Research and Development Pilot Award in Technology (to W.X.). W.X. and F.M. are CPRIT Scholars for Cancer Research. We thank all the members of the Xian–McKeon laboratory for helpful discussions and support. We thank H. Green and J. Rosen for advice and support. We thank H. Green for the generous gift of the 3T3-J2 fibroblast cell line.

Author contributions

W.X., Y.Y., M.D. and F.M. wrote the manuscript with input from all other authors. J.A.A., K.Y.H., J.K.H., J.S.H., F.A.S. and C.P.C. provided biopsy material; M.D., Y.Y., Y.Z. and R.N. cloned gISCs; J.X., S.W. and R.M. performed informatics analyses; A-A.L., Y.Q. and K.G. performed xenografts and ALI cultures; W.R. and R.N. performed the FACS analyses; and all authors collaborated on the concepts of this work.

Competing interests

W.X., F.M., M.D. and M.V. have filed patents related to the technology used in the present work. W.X., M.V. and F.M. have financial interests in Tract Pharmaceuticals, Inc.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-020-0298-4.

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Received: 30 September 2019; Accepted: 16 January 2020;
Published online: 01 April 2020

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☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	No software was used.
Data analysis	Excel, ImageJ, ZEN 2 (blue edition), Python2.7 and Graphpad Prism 5.0 softwares were used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All the datasets we used are publicly available datasets generated by other groups with accession codes indicated in the paper. All the raw data associated with all the figures and supplementary figures are available upon request. There is no restrictions on data availability.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was reported in the 'Statistical analysis' section of the Methods and figure legends. The sample size for quantifying the number of neurons and single-mRNA molecules was based on previous publications for biological experiments as well as the property of our data, i.e. clear separation between excitatory neurons and inhibitory neurons. For single-nucleu RNA-seq datasets, there are 3227 single nuclei and 19,550 single nuclei in each dataset, which are enough based on previous single-nucleus RNA-seq analysis.
Data exclusions	In order to avoid biases in the analysis and reduce the amount of noise, the bottom 5% low quality samples (samples with less reads across the transcriptome) in DroNc-Seq dataset were discarded as they were considered to have been damaged during the experimental procedure.
Replication	In order to ensure our experimental findings can be reliably reproduced, we include appropriate number of animals, sections, and neurons as listed in the figure legends. Further, three independent experiments were repeated with similar results. Also, the staining has been repeated by three persons in the lab independently. In order to ensure our experimental findings can be reliably reproduced, we include appropriate number of animals, sections, and neurons as listed in the figure legends. Further, three independent experiments were repeated with similar results. Also, the staining has been repeated by three persons in the lab independently.
Randomization	The experimental groups were allocated by the animal genotype, human case information, and the treatment. The covariants of age, gender, culture days, and vehicle were controlled as the same between experimental groups. Further, the sections, coverslips and neurons within each group were selected randomly. 10 images per sm-FISH stained section was randomly taken from the superficial layers of the entorhinal cortex or BA9 region. 60 excitatory and 60 inhibitory neurons from 3 human non-AD and AD sections were randomly selected for comparing the mean intensity of BAG3. Also, 5 excitatory and 5 inhibitory neurons from each coverslip (n = 11 coverslips each group) were randomly taken for comparing the endogenous 12E8 tau+ punta in neurites. Additionally, 20 images per coverslip at 1,024 × 1,024 resolution were taken randomly from all the orientations of the coverslip for the tau seeding experiment. The number of TBR1+ EX and GAD1+ IN neurons with tau inclusions were quantified blindly.
Blinding	The experimenter was blind to the experimental groups when they performed the immunostaining and sm-FISH as well as the counting of neurons and the single-mRNA molecules, and the comparison of the mean intensity of BAG3.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	The Tau RD-P301S-YFP lentivirus and DS9 clone cell lines were provided by Dr. Marc Diamond. The BAG3 shRNA and the shRNA-resistant BAG3 were provided by Dr. Gail Johnson. All the brain tissue were provided by New York Brain Bank and Banner Sun Health Research Institute Brain and Body Donation Program.

Antibodies

Antibodies used

Human conformation-dependent tau (MC1, 1:750) and human/murine phospho-tau pSer396/ Ser404 (PHF1, 1:500) monoclonal antibodies were provided by Dr. Peter Davies. Mouse anti-phosphorylated tau Ser262 and/or Ser356 (12E8, 1:2000) antibody is a kind gift from Dr. Philip Dolan. Human/murine phospho-tau pSer202/Thr205 (AT8, Cat# MN1020, 1:500) and pThr212/Ser214 (AT100, Cat# MN1060, 1:500) monoclonal antibodies, rabbit anti-phospho-tau pSer422 (pS422, Cat# 44-764G, 1:250) and parvalbumin (PVALB, Cat# PA5-18389, 1:1000) polyclonal antibodies, Alexa Fluor dye-labeled cross-absorbed goat and donkey secondary antibodies (Cat# A-11029, A-11037, A-11007, A-11058, and A-21202, 1:1000) were purchased from Thermo Fisher Scientific. Rabbit anti-TBR1 (Cat# ab31940, 1:250) and SATB2 (Cat# ab92446, 1:250) polyclonal antibodies were purchased from Abcam. Rat anti-somatostatin (SST) (Cat# MAB354, 1:100) and mouse anti-NeuN (Cat# MAB377, 1:250) monoclonal antibody and goat anti-GAD1 (Cat# AF2086, 1:100) polyclonal antibody were purchased from Millipore and R&D Systems, respectively. Rabbit anti-calretinin (CALB2) (Cat# 7697, 1:1000), IBA-1 (Cat# 019-19741, 1:500), and GFAP (Cat# G9269, 1:2500) polyclonal antibodies were purchased from Swant, Wako and Sigma-Aldrich, respectively. Rabbit anti-TBR1 (Cat# 20932-1-AP, 1:250) and rabbit anti-BAG3 (Cat# 10599-1-AP, 1:100) polyclonal antibody were purchased from Proteintech Group.

Validation

All of them have been used in previous publications and validated by the manufacturers or research scientists.
MC1: validated in Neurobiol Aging. 2000 Sep-Oct;21(5):719-27; https://www.alzforum.org/antibodies/tau-mc1
PHF1: validated in https://www.alzforum.org/antibodies/tau-phos-ser396ser404-phf-1
12E8: validated in https://www.alzforum.org/antibodies/tau-12e8
AT8: validated in https://www.thermofisher.com/antibody/product/Phospho-Tau-Ser202-Thr205-Antibody-clone-AT8-Monoclonal/MN1020
AT100: validated in https://www.thermofisher.com/antibody/product/Phospho-Tau-Thr212-Ser214-Antibody-clone-AT100-Monoclonal/MN1060
pS422: validated in https://www.thermofisher.com/antibody/product/Phospho-Tau-Ser422-Antibody-Polyclonal/44-764G
PVALB: validated in https://www.thermofisher.com/antibody/product/Parvalbumin-Antibody-Polyclonal/PA5-18389
TBR1: validated in http://www.ptglab.com/Products/TBR1-Antibody-20932-1-AP.htm#validation
SATB2: validated in https://www.abcam.com/satb2-antibody-epncir130a-ab92446.html
SST: validated in http://www.emdmillipore.com/US/en/product/Anti-Somatostatin-Antibody-clone-YC7,MM_NF-MAB354#overview
NeuN: validated in http://www.emdmillipore.com/US/en/product/Anti-NeuN-Antibody-clone-A60,MM_NF-MAB377
GAD1: validated in https://www.rndsystems.com/products/human-mouse-rat-gad1-gad67-antibody_af2086
calretinin : validated in https://www.swant.com/?p=products&c=1.2
IBA-1: validated in http://www.wako-chem.co.jp/english/labchem/product/life/Antilba1/index.htm
GFAP: validated in https://www.sigmaaldrich.com/catalog/product/sigma/g9269?lang=en&region=US
BAG3: validated in https://www.ptglab.com/products/BAG3-Antibody-10599-1-AP.htm#validation

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

The species, strain, sex and age of the laboratory animals used in this study have been clearly written in the paper. The F1 mouse offspring (both males and females at 22 and 30+ months old, strain FVB/N:C57BL/6) were used as experimental animals. All animals were maintained on a 12 hr light/dark cycle with food and water provided ad libitum. All animal experiments were performed in accordance with national guidelines (National Institutes of Health) and approved by the Institutional Animal Care and Use Committee of Columbia University. Primary cortical neuron culture were prepared from and embryonic day 16-18 embryos from C57BL/6 mice.

Wild animals

This study does not include wild animals.

Field-collected samples

This study does not include the samples collected from the field.