REVIEW

Growing Self-Organizing Mini-Guts from a Single Intestinal Stem Cell: Mechanism and Applications

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Recent examples have highlighted how stem cells have the capability to initiate morphogenesis in vitro; that is, to generate complex structures in culture that closely parallel their in vivo counterparts. Lgr5, the receptor for the Wnt-agonistic R-spondins, marks stem cells in multiple adult organs of mice and humans. In R-spondin–based three-dimensional cultures, these Lgr5 stem cells can grow into ever-expanding epithelial organoids that retain their original organ identity. Single Lgr5 stem cells derived from the intestine can be cultured to build epithelial structures that retain hallmarks of the in vivo epithelium. Here, we review the mechanisms that support this notable example of self-organization and discuss applications of this technology for stem cell research, disease modeling (e.g., for colorectal cancer and cystic fibrosis), and regenerative medicine.

The epithelium of the small intestine has a higher self-renewal rate than any other mammalian tissue, with a turnover time of less than 5 days. Intestinal stem cells reside near the bottom of the intestinal crypt. Their rapidly dividing, transit-amplifying (TA) daughter cells occupy the remainder of the crypts and flow onto the flanks of the villi, where they differentiate, absorb nutrients, and eventually die at the villus tips (Fig. 1A). The differentiated cell types include absorptive enterocytes, multiple secretory cells (Paneth cells, goblet cells, enteroendocrine cells, and tuft cells), and the M cells of Peyer's patches (*1*).

Stem Cells of the Intestinal Crypt

Cheng and Leblond (*2*) were the first to describe the slender crypt base columnar (CBC) cells at the crypt bottom, intercalated between the postmitotic Paneth cells (Fig. 1B). The Wnt target gene *Lgr5* is an excellent marker for CBC cells, in turn allowing the assessment of their position in the self-renewal hierarchy by genetic lineage tracing (*3*). Marked Lgr5$^+$ cells persist for the lifetime of a mouse, whereas their progeny include all differentiated cell lineages of the epithelium. Thus, Lgr5$^+$ cells represent cycling, long-lived, multipotent stem cells. A second, quiescent stem cell type is proposed to reside at position four (directly above the Paneth cells): These "+4 cells" (*1*) were originally identified as DNA label-retaining cells (*4*). Several markers have been described for these cells, such as Bmi-1, Hopx, mTert, and Lrig1 (*5*–*8*). Because the stem cell niche (microenvironment) and culture condition for +4 cells have

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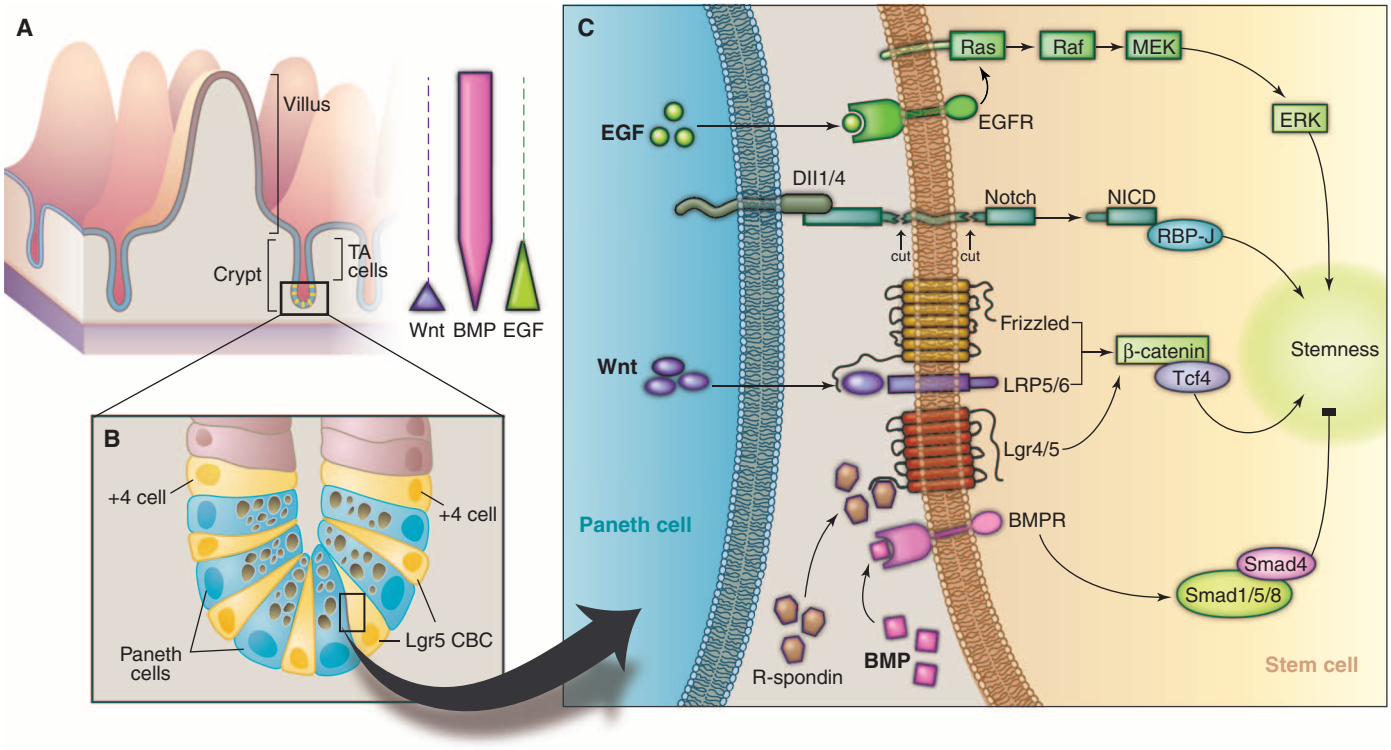

Fig. 1. Histological location and biological interaction of intestinal stem cells and their niche. (**A**) Scheme of intestinal epithelial structure and stem cells. Spatial gradients of Wnt, BMP, and EGF signals are formed along the crypt axis. (**B**) Cartoon of the stem cell niche. Lgr5$^+$ intestinal CBC cells intimately adhere to Paneth cells and receive signals for stem cell maintenance. (**C**) Three signals (EGF, Notch, and Wnt) are essential for intestinal epithelial stemness, whereas BMP negatively regulates stemness. For full Wnt activation in the intestinal epithelium, R-spondin–Lgr4/5 signal is required. Currently, the source of R-spondin is unknown.

not yet been defined, we will not discuss them further. Very recently, genetic marking of DNA label-retaining cells has identified a rare, nondividing secretory precursor that coexpresses Lgr5 and all +4 markers. Located near crypt bottoms, this precursor undergoes terminal differentiation over periods of weeks. Upon tissue damage, it reverts into a cycling, Lgr5$^+$ stem cell CBC cell (*9*). Hereafter, we refer to the dividing Lgr5$^+$ stem cells as Lgr5-CBC cells to distinguish them from these much rarer, label-retaining, nondividing Lgr5$^+$ cells.

Every murine crypt contains ~15 Lgr5-CBC cells (*10*). An Lgr5-CBC cell divides every 24 hours and, through its TA daughters, generates 16 to 32 differentiated epithelial cells per day. Cell division generally occurs in a symmetrical fashion, after which individual daughter cells stochastically adopt stem cell or TA cell fates, depending on available niche space (*10*, *11*).

The Intestinal Stem Cell Niche

Intestinal homeostasis is tightly controlled by four well-characterized signaling pathways (Fig. 1, A and C). Wnt constitutes the key pathway to maintain stem cell fate and drive proliferation of stem- and TA cells (*12*). Paradoxically, Wnt also drives terminal differentiation of Paneth cells, that are always in direct contact with the stem cells (*13*). Wnt factors, when engaging their Frizzled-Lrp5/6 co-receptors, will induce stabilization of β-catenin. The latter molecule can then bind and activate the transcription factor Tcf4, thus activating a genetic program that supports stemness (Fig. 1C). Notch is also essential to maintain the undifferentiated state. When Notch signaling is blocked in proliferative stem and TA cells, these cells differentiate into secretory lineage cells (*14*). Dll1$^+$ Dll4$^+$ Paneth cells trigger signaling by Notch1/2 on neighboring stem cells, thus keeping them from secretory differentiation (*15*). Each day, stem cell daughters lose contact with the Dll1/4-expressing Paneth cells. Some of these cells down-regulate Notch and up-regulate Dll1, setting their own secretory fate (*16*). Such a secretory precursor presents Dll1 to six to eight neighboring Notch$^+$ TA cells, which will experience active Notch signals and stay fated toward the enterocyte lineage. In sum, Notch lateral inhibition controls the enterocyte-secretory switch. Figure 1C depicts how engagement of Notch by its ligand Dll1/4 leads to release of the Notch intracellular domain, which subsequently interacts with the nuclear effector RBP-J to suppress the gene program for secretory differentiation. Epidermal growth factor (EGF) signals exert strong mitogenic effects on stem and TA cells upon engagement of their EGF receptors (EGFRs). Indeed, the Ras/Raf/Mek/Erk signaling axis is active in crypt epithelium. Inhibition of Mek ablates intestinal stem cells (*17*). Finally, bone morphogenetic protein (BMP) signals are active in the villus compartment. When BMP signaling in the villus is inhibited by transgenic Noggin, cryptlike structures appear along the flanks of the villi (*18*), implying that BMP inhibition creates a crypt-permissive environment. Figure 1C shows how engagement of BMP receptors by BMP leads to complexes between Smad1/5/8 and Smad 4 to repress stemness genes in the nucleus.

The Lgr5-CBC cells are in intimate contact with Paneth cells. Paneth cells secrete bactericidal products as well as EGF and Wnt3. Moreover, they express the Notch ligands Dll1 and Dll4 on their surface (Fig. 1C). Paneth cell depletion in vivo results in concomitant loss of Lgr5-CBC cells in several animal models (*19*, *20*), with the exception of a conditional Math1 mutant, which eliminates Paneth cells yet retains its stem cells (*21*). It was then pointed out that *Math1* deletion in Lgr5-CBC cells relieves the in vivo dependence on Notch ligands, which are normally provided by Paneth cells and that are essential for Lgr5-CBC cell maintenance (*22*). Durand *et al*. noted

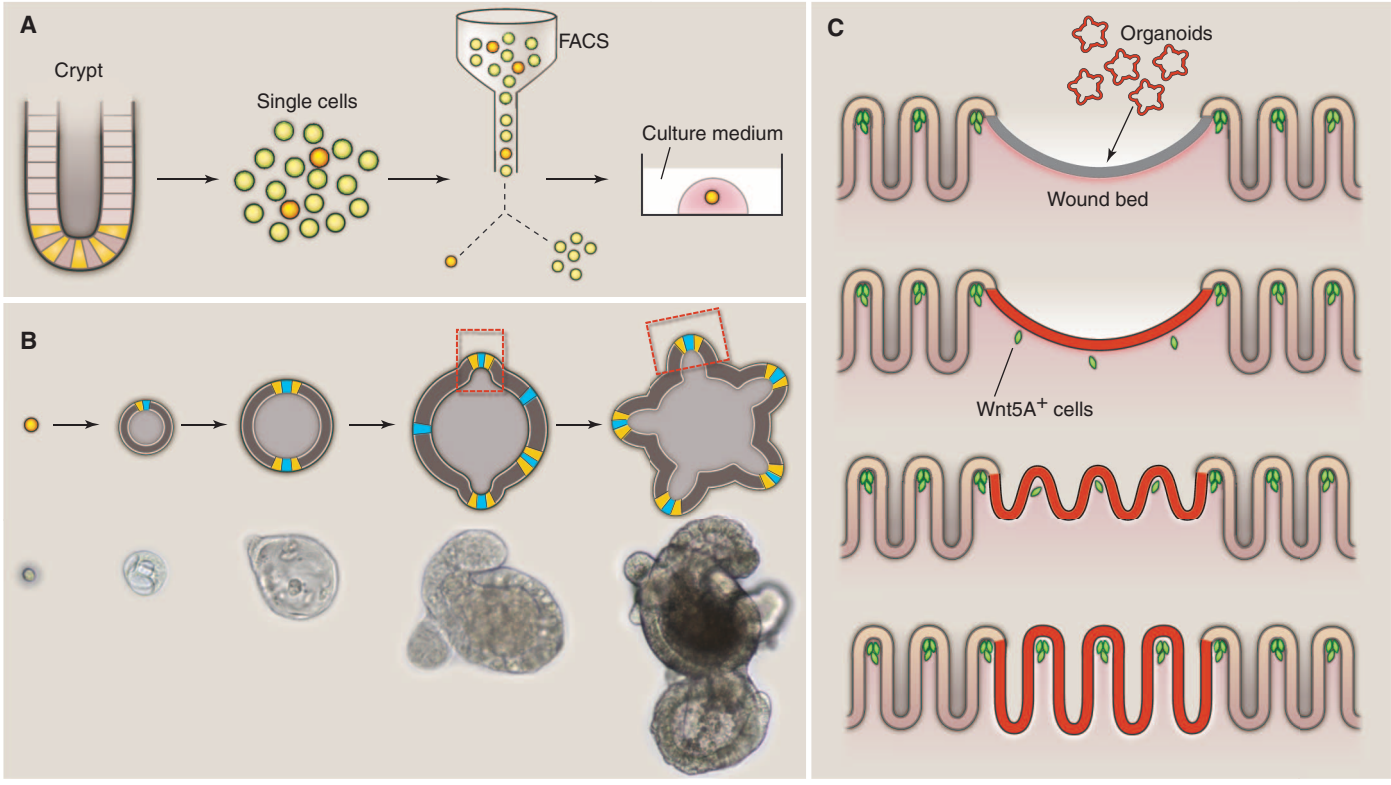

Fig. 2. Mini-gut culture system. (**A**) Lgr5$^+$ CBC cells genetically labeled by EGFP are sorted and embedded in Matrigel. The culture medium consists of EGF, Noggin, and R-spondin. FACS, fluorescence-activated cell sorting. (**B**) Time course of organoid growth. A single stem cell forms a symmetric cyst structure. The symmetry is broken by bud formation. The budding structure resembles a crypt. Lgr5$^+$ CBC cells are depicted in yellow, and Paneth cells are shown in blue. (**C**) Scheme showing the engraftment of intestinal oganoids. Organoids adhere to a de-epithelialized wound bed. Organoids form a flat epithelial layer, followed by crypt reconstruction. Wnt-5A$^+$ mesenchymal cells support crypt structure formation in the damaged area. Donor organoids are depicted in red.

that *Math1*-mutant mini-guts (see below) fail to grow in vitro, which implies a crucial in vitro dependence on another signal provided by Paneth cells, probably Wnt3. Indeed, $Wnt3^{-/-}$ crypts grow normally in vivo but fail to grow in vitro (*13*). Together, Paneth cells fulfill a stem cell niche function. In vivo, Wnts are also provided by surrounding mesenchyme (*13*), whereas R-spondins and BMP inhibitors are distinctly provided from nonepithelial sources.

Epithelial Mini-Gut Culture

It has generally been thought impossible to establish long-term cultures from primary adult tissues without inducing genetic transformation. Indeed, the Hayflick limit indicates that somatic cells have a limited proliferative potential (*23*). Yet, we have recently established an in vitro culture system to grow three-dimensional (3D) intestinal epithelial organoids ("epithelial mini-guts") from a single Lgr5-CBC cell for periods greater than 1.5 years (*24*). We employed R-spondin-1, a Wnt signal enhancer, that was later discovered as the physiological ligand of Lgr5: The R-spondin–Lgr5 interaction augments Wnt signal strength initiated by the interaction of Wnt with Frizzled and Lrp (*25*, *26*) (Fig. 1C). R-spondins are crucial in vivo. Gut-specific depletion of Lgr4 and -5 leads to the demise of crypts (*26*, *27*), whereas exogenous R-spondin-1 induces hyperplasticity of crypts (*28*). The isolated crypts require Matrigel (BD Biosciences, San Jose, CA), a 3D laminin- and collagen-rich matrix that mimics the basal lamina. A cocktail of R-spondin, EGF, and Noggin represents the minimal, essential stem cell maintenance factor cocktail. For colon crypt culture, Wnt ligand is an additional factor required to maintain Lgr5-CBC cells, because the epithelium makes little, if any, Wnt. Of note, this approach also allows derivation of intestinal organoids from induced pluripotent stem cells (*29*). In an alternative approach, intestinal fragments containing epithelial and mesenchymal components from neonatal mice were grown for several months in a collagen gel with air-liquid interface in the presence of serum. The expanding cystic structures consisted of a simple epithelium of all cell types surrounded by myofibroblasts and were responsive to R-spondin-1 and Notch inhibition. They did not develop defined crypt- and villus-like domains (*30*).

Single crypts can be readily isolated from mouse or human intestine by EDTA-based Ca^{2+}/Mg^{2+} chelation. Such crypts grow into 3D organoids under the culture conditions described. Culturing of single stem cells is inefficient at 1 to 2% plating efficiency, whereas up to half of the stem cell–Paneth cells doublets form organoids in vitro (*19*). This doublet assay has allowed demonstration that Paneth cells monitor the metabolic state to fine-tune stem cell activity (*31*).

In vitro–generated organoids occur as cysts with a central lumen flanked by a simple, highly polarized villus epithelium. Multiple cryptlike structures project outward (Fig. 2B). The basal side of the cells is oriented toward the outside, touching the matrigel, whereas enterocyte brush borders form the luminal surface. Secretion by Paneth and goblet cells occurs toward the lumen. The organoids can be passaged weekly at a 1:5 ratio for at least 1.5 years, with a phenotype and karyotype that remain unchanged. Mechanically disrupted organoids rapidly reseal. Self-renewal kinetics and cell-type composition closely resemble the in vivo situation. Notably, a timer of unknown molecular nature remains active in the absence of the in vivo wear-and-tear: 2 to 3 days after terminal differentiation, the cells exfoliate into the lumen.

How normal are the epithelial mini-guts? To test this, large numbers of organoids were grown in vitro from a single Lgr5 stem cell (Fig. 2B) that was marked by a red fluorescent protein in the adult colon (*32*). These in vitro–expanded colon organoids were reintroduced per anum into the colons of multiple mice with chemical-induced mucosal lesions (Fig. 2C). The engrafted epithelial mini-guts regenerated epithelial patches that were indiscernible from surrounding recipient epithelium. The patches persisted for at least 6 months without changing their histologic appearance.

How Does an Lgr5 Stem Cell Establish Epithelial Mini-Gut Architecture?

When epithelial mini-guts derived from a Wnt reporter mouse (*Axin2-LacZ*) are grown under standard conditions, strong Wnt signals only occur adjacent to Paneth cells (*19*) (Fig. 3A). This fits with the notion that Paneth cells are the only source of Wnt in culture (*13*) and that R-spondin-1 that is ubiquitously present in the medium enhances these focal Wnt signals. Thus, sharp Wnt gradients surround Paneth cells. In a medium containing Wnt3A in addition to R-spondin-1, the Wnt gradient is lost and epithelial mini-guts become symmetric, round cysts, consisting of a homogeneous population of stem and progenitor cells (*19*). Epithelial mini-guts grown from adenomatous polyposis coli (APC)–mutant adenoma cells display the same symmetric shape (*19*), which is not surprising, because APC loss leads to constitutive Wnt pathway activation. These observations imply that the typical crypt-villus architecture is suppressed under conditions of homogeneous (rather than focal) Wnt signaling.

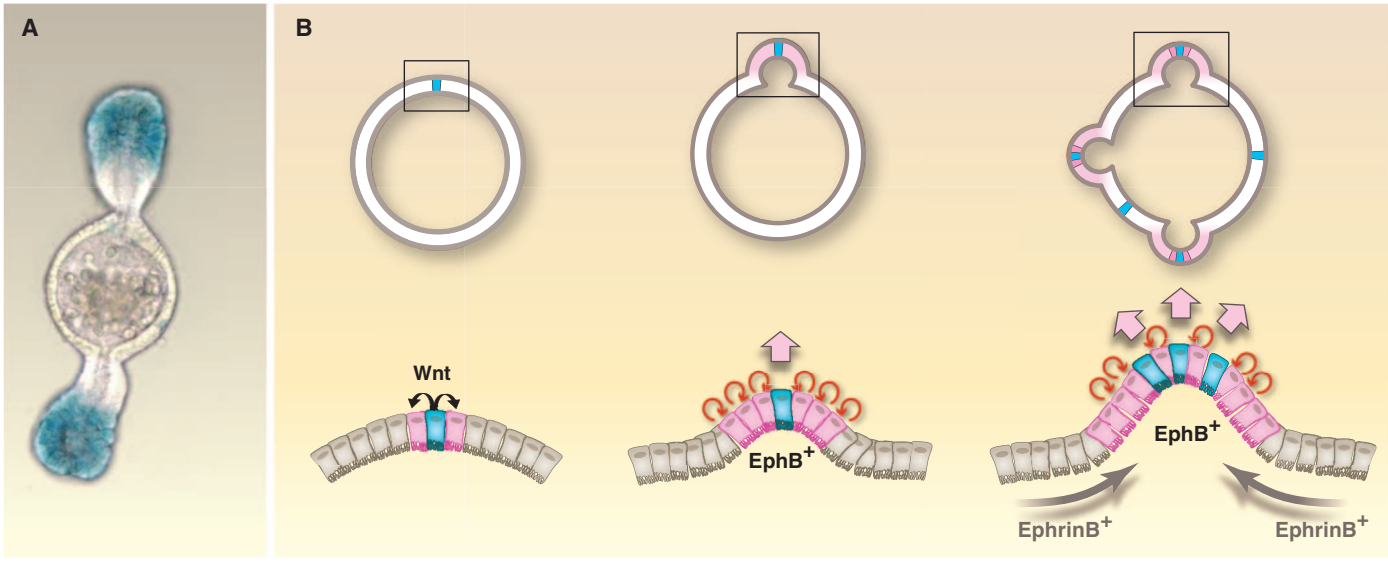

Fig. 3. Mechanism of the self-organizing architecture of a mini-gut. (**A**) An organoid derived from Axin2-LacZ knock-in mice. Axin2-LacZ (blue) expression recapitulates Wnt activation. (**B**) Wnt activation induces local proliferation and EphB expression. Local cell expansion and Eph-Ephrin repulsive force generate bud formation. Wnt-producing Paneth cells are depicted in blue; Wnt-activated cells are shown in pink.

When a single cultured stem cell is followed over time, a small symmetric cyst forms. The stochastic appearance of a Paneth cell constitutes the "symmetry-breaking" event (Fig. 3B): A bud forms around the cell; within 2 to 3 days, this bud develops into a cryptlike structure with stem and Paneth cells. Every nondifferentiated cell that touches a Paneth cell is (or becomes) an Lgr5-CBC cell (*16*, *19*), presumably driven by potent Wnt and Notch signals from the Paneth cell.

The proliferative stem cell–Paneth niche pushes itself outward from the central cyst (movie S1). This process is most likely driven by repulsive EphB-EphrinB interactions, as described in vivo (*33*). EphB2 and -B3 are Wnt target genes, expressed by Paneth and stem cells. When TA cells move toward the villus in vivo, they travel down the Wnt gradient. As a consequence, they differentiate and gradually replace EphB expression by expression of the counterstructures, EphrinBs. Translated to the situation in the mini-gut, cells that sit at the Wnt source (Paneth cells and their direct neighbors, the Lgr5-CBC cells) are $EphB^+$. Because all other cells in the central cyst do not experience Wnt signals and express the counterstructure EphrinB, the $EphB^+$ stem cells and Paneth cells are expelled from the cyst and create a bud (Fig. 3B). In these cryptlike buds, the Wnt3-producing (and, therefore, $EphB3^+$) Paneth cells are sorted toward the bottom. Thus, a Wnt-gradient is automatically created along the crypt axis. Proliferating TA cells are mechanically pushed toward the lumen by younger TA cells, thus experiencing rapidly decreasing Wnt levels. This drives their terminal differentiation into one of the villus-epithelial cell types, with Notch lateral inhibition acting as the enterocyte-secretory fate switch.

How Stem Cell Numbers Are Controlled

Touching a Paneth cell appears necessary and sufficient to acquire and maintain the Lgr5-CBC cell phenotype. Thus, a key parameter that controls crypt homeostasis is the number of Paneth cells. Strong Wnt signals, in the absence of Notch signals, drive formation of new Paneth cells (*13*, *34*). Because Paneth cell–derived Wnt can, in turn, generate more stem and Paneth cells, a Wnt-driven positive-feedback loop could potentially induce ever-expanding crypts. Two E3 ligases (Rnf43 and Znrf3) are encoded by stem cell–specific Wnt target genes (*35*). These two E3 ligases down-regulate Wnt receptors, thus serving as feedback inhibitors of the Wnt pathway (*35*, *36*). When this negative-feedback loop is broken by deletion of the two E3 ligase genes, a rapidly expanding Paneth–stem cell zone is observed (*35*). Thus, Rnf43 and Znrf3 serve in the homeostatic control of crypt size by negatively regulating Wnt signal strength. The strength of the EGF signal also affects stem cell numbers. Deletion of Lrig1, a negative regulator of EGFR family members, leads to increased stem cell numbers in vivo and in culture (*17*).

Epithelial Mini-Guts as Experimental Tools

The ability to grow epithelial mini-guts from single cells can be used as a surrogate marker of stemness, allowing the study of (the interrelationship of) intestinal stem cell types (*6*, *37*–*39*, *40*). Although cycling Lgr5-CBC cells grow under standard conditions (*24*), the addition of Wnt3A can coerce proliferative progenitors, such as the $Dll1^+$ secretory precursor (*16*), or even the nondividing Paneth precursors (*9*, *41*), to revert to a stem cell phenotype and generate epithelial mini-guts.

Organoids that are grown from mouse intestine are amenable to all standard experimental manipulations used for cell lines, including long-term storage by freezing, transfection of DNA and of small interfering RNA, and infection with recombinant retro- and lentiviruses (*42*). They can be analyzed by immunohistochemistry and confocal immunofluorescence, gene expression microarray, and mass spectrometry. Floxed alleles can be deleted in culture (for instance, using CreERT2 in conjunction with tamoxifen) and the effect followed in real time. The identification of Lgr4 and -5 as receptors of R-spondins has rested on the generation of organoids mutant in these genes (*26*, *27*, *43*). Other genes studied in organoids include Toll-like receptor 4 (*44*), Troy (*45*), and YAP (*46*).

The organoid technology is well suited to the study of cell-biological phenomena that require a closed epithelial structure with a physiological, polarized topology. Mizutani *et al*. studied P-glycoprotein (an efflux transporter acting at the intestinal epithelium) in mini-guts. The transporter was observed at the apical (luminal) membrane in organoids and actively pumped rhodamine 123 toward the luminal space (*47*).

Organoids recapitulate the complete stem cell differentiation hierarchy and allow the in vitro

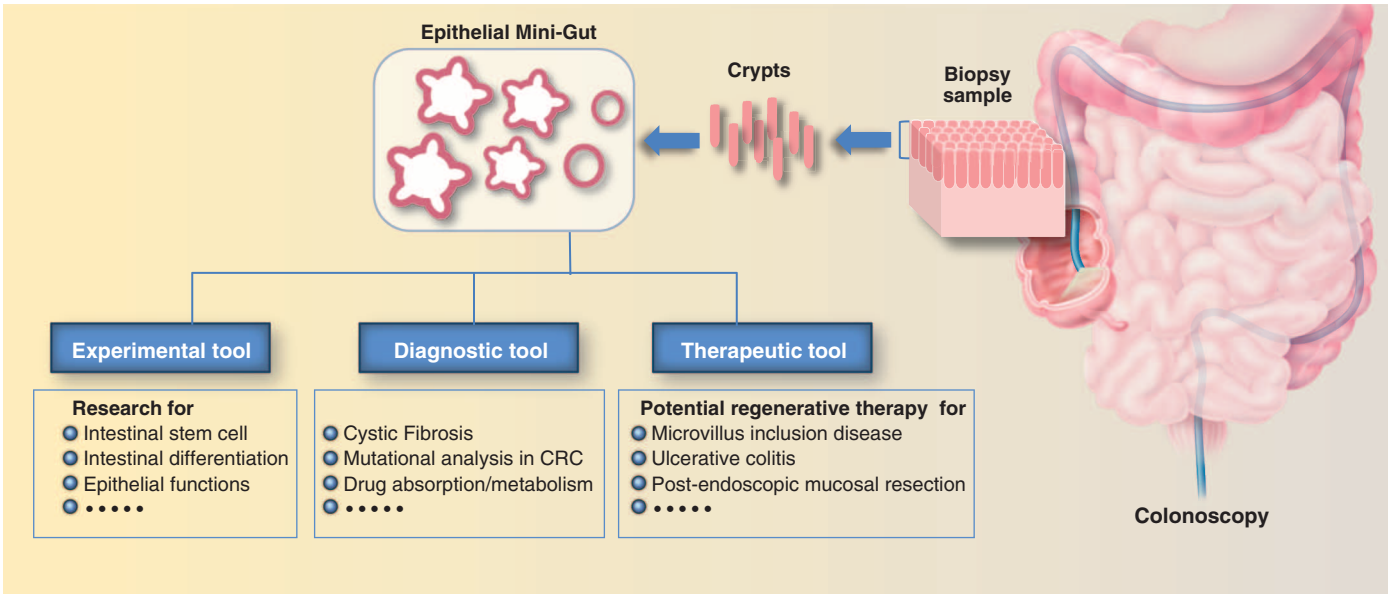

Fig. 4. Basic and clinical applications of an epithelial mini-gut. An epithelial mini-gut is efficiently established from a single (3 to 5 mm^2) endoscopic biopsy sample. EDTA chelation releases ~3000 crypts from a biopsy sample. An epithelial mini-gut grows logarithmically and expands 1000-fold within a month. Three applications of epithelial mini-guts are as follows: (i) As an experimental tool. Genetic manipulation, gene expression analysis, live imaging, and other standard biological analyses can be employed for normal and patient-derived epithelial mini-guts. (ii) As a diagnostic tool. Patient-derived epithelial mini-guts recapitulate in vivo intestinal epithelial functions and genetic signatures. Efficient expansion of pure epithelial cells provides a high-quality source for deep sequencing or functional assays. (iii) As a therapeutic tool. Epithelial mini-gut transplantation may become a feasible regenerative therapy.

study of cell fate determination. Inhibition of Notch signaling in vitro (*42*, *48*), for instance, phenocopies the effects of the same manipulation in vivo (*14*), resulting in the massive production of goblet cells. Local, retrovirally driven production of Wnt3 converts the recombinant Wnt3-expressing cells into Paneth cells (*13*). Peyer's patch M cells are normally absent in organoid culture. Simple addition of RANKL, essential for M cells in vivo (*49*), robustly induces M cell formation. The minute numbers of M cells in the gut have previously precluded their detailed study. This may now be overcome by this in vitro system.

The technology described here has been used to study how a regenerating colon reestablishes its repetitive crypt architecture. Wnt5a is focally expressed by intercrypt mesenchyme, driving the overlying epithelium to differentiate (Fig. 2C). Wnt5a-soaked beads block local growth when placed onto organoids (*50*). Malignant transformation can also be studied in organoids. Introduction of activating Wnt pathway mutations generates organoids that are spheroid and no longer require R-spondin-1 (*19*, *35*, *51*, *52*). Along these lines, deletion of the EGF inhibitor *Lrig1* allows the mutant organoids to grow without EGF (*17*).

Epithelial Mini-Guts as Disease Models

Protocols have been developed to grow human epithelial mini-guts from biopsies (*53*) or single human EphB2$^+$ stem cells (*54*). Human intestinal epithelial cells are more difficult to grow and require small-molecule inhibitors for activin-like kinase and p38 mitogen-activated protein kinase, in addition to the mouse colon epithelial mini-gut culture condition. The protocol has been applied for the study of cystic fibrosis (CF), a disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Raising adenosine 3′,5′-monophosphate levels by forskolin opens the channel and results in membrane depolarization of wild-type mice, but not of *Cftr*-knockout mice (*55*). Along these lines, forskolin induces a robust swelling of wild-type mini-guts, but not of mini-guts derived from CF patients (*56*). The function of the common, temperature-sensitive CFTR-F508del mutant is restored at 27°C and by addition of experimental CFTR corrector compounds. The simple and robust assay can be performed within weeks after biopsy. It may facilitate diagnosis, drug development, and personalized medicine approaches in CF.

The culture protocol also allows the establishment of organoids from primary human adenomas and colorectal cancers (*53*). Because the success rate of establishing the cultures from individual patient samples is near 100%, this allows the prospective generation of large "living biobanks," side-by-side with healthy tissue from the same individual. This presents the opportunity to bridge the current experimental gap between deep-sequencing efforts in human colon cancer (*57*) and patient outcome.

The notable ability of single intestinal Lgr5-CBC cells to grow into structures that faithfully recapitulate the self-renewing intestinal epithelium offers a broad range of experimental approaches. In its current guise, the technology is restricted to epithelial phenomena. Future applications may include the study of immune-mediated diseases (by reconstitution with components of the immune system) or infectious diseases (by coculture with pathogens). Because intestinal organoids can be expanded indefinitely from single stem cells, the technology may present a safe venue for gene therapy approaches: The offspring of individual stem cells, transfected in culture, can be analyzed at the clonal level. This allows the selection of individual mini-guts with safe integrations, which can then be expanded for subsequent transplantation. Because biopsies taken from live donors can serve as the tissue source, this approach could solve ethical and logistical issues associated with organ transplantation and may represent a safe complement to embryonic or induced pluripotent stem cell–based strategies.

In sum, intestinal stem cells possess the notable capacity to form epithelial structures in vitro that closely resemble the self-renewing crypt-villus architecture of the gut. These mini-guts can be expanded for periods of years and are amenable to essentially all experimental technologies that have been developed for cell lines. A variety of applications of mini-guts have been reported in studies of stem cell behavior, gene function, and disease modeling (Fig. 4). Thus, mini-guts present an opportunity to exploit in a variety of ways the distinct morphogenetic characteristics of adult stem cells.

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Acknowledgments: We thank I. Nathke and P. Appleton (University of Dundee) for providing a movie of mini-guts and A. Takano (Keio University) for figure preparation. T.S. and H.C. are inventors on several patent applications pertaining to the organoid technology (WO/2010/090513 and WO/2012/168930).

Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6137/1190/DC1
Movie S1

10.1126/science.1234852