nature medicine

Sustained *in vitro* intestinal epithelial culture within a Wnt-dependent stem cell niche

Akifumi Ootani[1], Xingnan Li[1], Eugenio Sangiorgi[2], Quoc T Ho[1], Hiroo Ueno[3], Shuji Toda[4], Hajime Sugihara[4], Kazuma Fujimoto[5], Irving L Weissman[3], Mario R Capecchi[2] & Calvin J Kuo[1]

The *in vitro* analysis of intestinal epithelium has been hampered by a lack of suitable culture systems. Here we describe robust long-term methodology for small and large intestinal culture, incorporating an air-liquid interface and underlying stromal elements. These cultures showed prolonged intestinal epithelial expansion as sphere-like organoids with proliferation and multilineage differentiation. The Wnt growth factor family positively regulates proliferation of the intestinal epithelium *in vivo*. Accordingly, culture growth was inhibited by the Wnt antagonist Dickkopf-1 (Dkk1) and markedly stimulated by a fusion protein between the Wnt agonist R-spondin-1 and immunoglobulin Fc (RSpo1-Fc). Furthermore, treatment with the γ-secretase inhibitor dibenzazepine and neurogenin-3 overexpression induced goblet cell and enteroendocrine cell differentiation, respectively, consistent with endogenous Notch signaling and lineage plasticity. Epithelial cells derived from both leucine-rich repeat-containing G protein–coupled receptor-5–positive (*Lgr5*$^+$) and B lymphoma moloney murine leukemia virus insertion region homolog-1–positive (*Bmi1*$^+$) lineages, representing putative intestinal stem cell (ISC) populations, were present *in vitro* and were expanded by treatment with RSpo1-Fc; this increased number of *Lgr5*$^+$ cells upon RSpo1-Fc treatment was subsequently confirmed *in vivo*. Our results indicate successful long-term intestinal culture within a microenvironment accurately recapitulating the Wnt- and Notch-dependent ISC niche.

The surface of the intestine is lined by a simple columnar epithelium that undergoes complete regeneration every 5–7 d[1,2]. Underlying this profound regeneration are ISC populations[3], including pan-intestine *Lgr5*$^+$ ISCs at the crypt base[4] and small intestine *Bmi1*$^+$ ISCs at approximately four cell positions directly above the Paneth cells[5]. These ISCs divide to produce transit amplifying cells, which migrate toward the lumen and differentiate into absorptive enterocyte, goblet, Paneth and enteroendocrine lineages[2,6], followed by either extrusion into the luminal surface or Paneth cell phagocytosis[7].

Stem cells are generally influenced by a microenvironmental niche, typically comprised of epithelial and mesenchymal cells and extracellular substrates, which instructs either self-renewal or selective adoption of a particular cell lineage[8,9]. The ISC niche is notable for myofibroblasts adjacent to the crypt base, which are believed to elaborate paracrine signals regulating the neighboring ISCs[1,9,10]. Extracellular Wnt signals are absolutely required within the ISC niche, as deduced from the rapid ablation of proliferation and secondary loss of differentiation observed with the secreted Wnt inhibitor Dickkopf-1 (Dkk1)[11,12]. Notch signals are similarly essential, with stimulation amplifying the progenitor pool and inhibition resulting in large-scale conversion to post-mitotic goblet cells[13,14].

Many attempts have been made to produce culture systems that mimic normal intestinal epithelial growth and differentiation[15–17]. The most substantial obstacle has been the rapid initiation of apoptosis within a few hours after intestinal cells are removed from the basement membrane and underlying stroma[18,19]. Although explants of embryonic, neonatal or adult gut will develop successfully when transplanted into syngeneic or immunocompromised host animals[20], the tissue is largely inaccessible for experimental manipulation and time-series observation. Several studies have demonstrated intestinal epithelial growth and differentiation in organ culture, but the three-dimensional (3D) architecture of the tissue was not preserved, and preservation of epithelial viability lasting more than 10 d is difficult[21]. Recently developed 3D embryonic organoid cultures allow cellular differentiation and intestinal morphogenesis of the tissue[22]. However, these *in vitro* methods have been restricted to embryonic tissue and maintain cellular viability for less than 14 d. Thus, to date, the study of intestinal stem and progenitor cells has been largely dependent on *in vivo* approaches, and primary culture methodology is not commonly employed in studies of the intestine.

Here we describe a robust long-term methodology for primary mouse intestinal culture allowing sustained intestinal proliferation and multilineage differentiation over a range of 30 to >350 d, using neonatal tissue as starting material. Defining characteristics include the use of an air-liquid interface coupled with a 3D culture matrix, as well as recapitulation of both the cellular myofibroblast architecture and the rigorous Wnt and Notch dependence of the ISC niche. We further exploit this methodology to show the presence of putative ISC populations within these cultures and their *in vitro* modulation by the Wnt agonist RSpo1-Fc. These studies describe a method to enable study of both ISCs and the ISC niche, as well as general investigations of intestinal biology.

[1]Department of Medicine, Division of Hematology, Stanford University School of Medicine, Stanford, California, USA. [2]Howard Hughes Medical Institute and Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, Utah, USA. [3]Institute of Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California, USA. Departments of [4]Pathology and [5]Internal Medicine, Saga Medical School, Saga, Japan. Address correspondence to A.O. (aootani@stanford.edu) or C.J.K. (cjkuo@stanford.edu).

Received 8 December 2008; accepted 2 March 2009; published online 27 April 2009; doi:10.1038/nm.1951

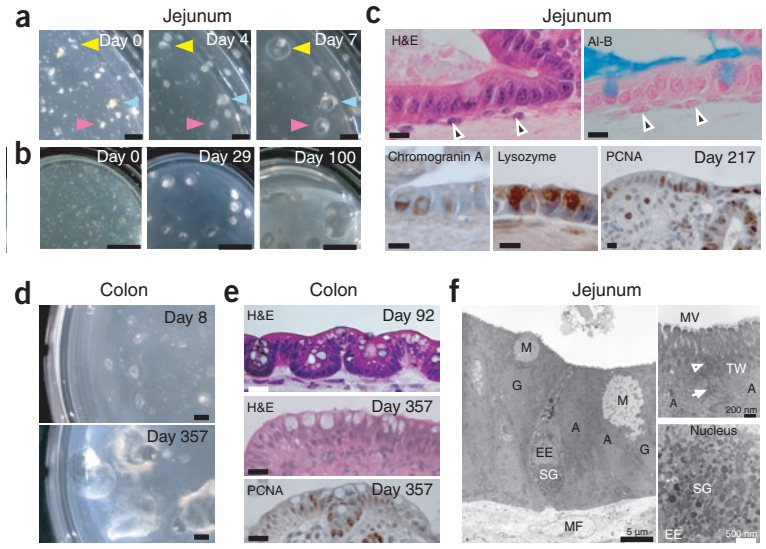

Figure 1 Long-term intestinal culture. (**a**) Time-course analysis of short-term air-liquid interface culture of neonatal small intestine. Stereomicroscopy shows the progressive growth of intestinal cultures, forming cyst-like structures in the collagen gel. Arrowheads indicate growth of individual spheres. Scale bars, 1 mm. (**b**) Long-term stereomicroscopy of air-liquid interface cultures of neonatal small intestine in collagen gel. The same field is visualized. Scale bars, 5 mm. (**c**) Histological analysis of small intestinal cultures (day 10). The wall of the intestinal spheres consists of a polarized epithelial monolayer and outer lining myofibroblasts (arrowheads). Tall-columnar absorptive enterocytes are visible on H&E staining. Alcian blue (Al-B) staining shows goblet cells with secreted mucus in the lumen. Chromogranin A immunohistochemistry reveals the presence of enteroendocrine cells. Lysozyme immunohistochemistry shows Paneth cells. PCNA staining reveals active proliferation of jejunal cultures at day 217. Scale bars, 10 µm. (**d**) Stereomicroscopy of long-term colon culture. Scale bars, 1 mm. The same field of view is depicted in both photographs. (**e**) Histology of long-term colonic cultures. Differentiated colonic epithelial cells are visible on H&E staining (top; day 92, middle; day 357). PCNA staining (bottom) shows continued active proliferation of cultured colonic mucosa at day 357. (**f**) Electron micrographs of jejunal culture (day 14). Goblet cells (G) contain mucus granules (M) in their apical cytoplasm. The epithelium is lined by myofibroblasts (MF). The apical surface of absorptive cells (A) shows microvilli (MV) ending on the terminal web (TW). Note the well organized tight junction (arrowhead) and desmosome (arrow) at the basolateral domain anchoring adjacent epithelial cells. Enteroendocrine cells (EE) contain secretory granules (SG) in their basal cytoplasm.

RESULTS

Establishment of a long-term intestinal culture system

3D culture of either small or large intestine from neonatal mice within a collagen gel with an air-liquid interface (**Supplementary Fig. 1a** online) yielded expanding cystic structures (termed intestinal spheres) on gross inspection within 7 d, following initial outer spindle cell growth (**Fig. 1a** and **Supplementary Fig. 1b**). Virtually all of these cultures showed growth for 30 d, with some growing to >350 d *in vitro* (the latest time point examined) (**Fig. 1b–e** and **Supplementary Figs. 2a** and **3a** online). The wall of the intestinal spheres consisted of a polarized epithelial monolayer with an apical, inner luminal surface and a basal outer surface in close proximity to myofibroblasts and the collagen matrix (**Fig. 1c,e**). The intestinal epithelial cells not only showed highly proliferative activity at extended time points (**Fig. 1c,e**) but also expressed numerous markers for multilineage differentiation to the absorptive enterocyte (lactase, maltase, sucrase and Na^+-K^+ ATPase), goblet (mucin-2), enteroendocrine (chromogranin A, serotonin and glucagon-like peptide-2) and Paneth cell (lysozyme, cryptdin and matrix metalloproteinase-7) lineages (**Fig. 1c** and **Supplementary Fig. 2a**). Underlying myofibroblasts expressed α-smooth muscle actin (**Supplementary Fig. 2b**). Ultrastructural examination revealed the fully differentiated microstructures of cultured intestinal epithelial cells, including microvilli, mucus granules and endocrine granules, as well as intracellular connections of junctional complexes (**Fig. 1f**). We have also been able to use small and large intestine from juvenile or adult mice up to 26 weeks of age (the oldest age evaluated) as starting material (**Fig. 2**). Although we have less experience with cultures of adult intestine, our preliminary studies indicate that their viability is much less extensive than with neonatal cultures.

Regardless of the age of the mouse cells used for the intestinal culture, both proliferative zones and differentiated zones were present (**Supplementary Fig. 3b,c**). Whereas proliferative zones were commonly observed within areas of monolayer (**Supplementary Fig. 3b**) within 2 weeks, crypt-like structures were also often produced within both small and large intestinal spheres (**Figs. 1e** and **2** and **Supplementary Fig. 3a,c**). Furthermore, villus-like protrusions were occasionally present in the jejunal spheres (**Fig. 2b**). The crypt-like structures showed marked proliferative activity; in contrast, the villus-like structures or differentiated zones were devoid of proliferating cell nuclear antigen (PCNA)-positive cells (**Fig. 2c,d** and **Supplementary Fig. 3b,c**). Accumulation of apoptotic sloughed cells positive for single-stranded DNA in the sphere lumen (**Supplementary Fig. 2c**) and BrdU pulse labeling (**Supplementary Fig. 2d**) revealed the rapid turnover and proliferation of intestinal epithelial cells in culture. Some of the intestinal spheres showed autonomous contraction within the outer surrounding muscle layer during culture days 5–14 (**Supplementary Video 1** online). The initial growth of myofibroblasts and the air-liquid interface microenvironment were essential for intestinal epithelial growth in this system (**Supplementary Fig. 1**). Although we did observe proliferative zones and viable tissue at >200 d of culture, they became more and more sporadic over time, as opposed to a more generalized presence in cultures <30 d old. Notably, viable intestinal tissue at such extended time points seemed to be obligately associated with a robust underlying stroma (**Fig. 1c,e**).

Recapitulation of a Wnt- and Notch-dependent ISC niche

As Wnt signaling promotes maintenance of epithelial stem cells and early progenitor compartments[23], we hypothesized that the long-term growth of the intestinal cultures would be modulated by alteration of Wnt signals *in vitro*. We previously showed that Dkk1-dependent antagonism of extracellular Wnt signaling produces rapid cessation of intestinal epithelial proliferation and crypt loss in the adult mouse[12], consistent with findings in nonconditional villin-Dkk1–expressing transgenic mice[11]. Accordingly, addition of recombinant Dkk1 into the culture medium at the time of plating resulted in dose-dependent growth inhibition (**Fig. 3a,b**). Similarly, when we preestablished small or large intestine spheres without Dkk1 for 28 d followed by subsequent Dkk1 treatment for 5 d, we observed rapid degeneration of the epithelial layer (**Fig. 3c**). Conversely, to achieve gain-of-function Wnt activation, we used recombinant RSpo1-Fc, as studies have shown that it strongly augments intestinal proliferation *in vivo*[24]. Treatment with an RSpo1-Fc fusion protein (**Supplementary Fig. 4** online) produced a significant increase in the number and size of intestinal spheres, with a marked increase in the number of $PCNA^+$ proliferating cells with undifferentiated features, whether in neonatal cultures (**Fig. 3d–h**) or those derived from

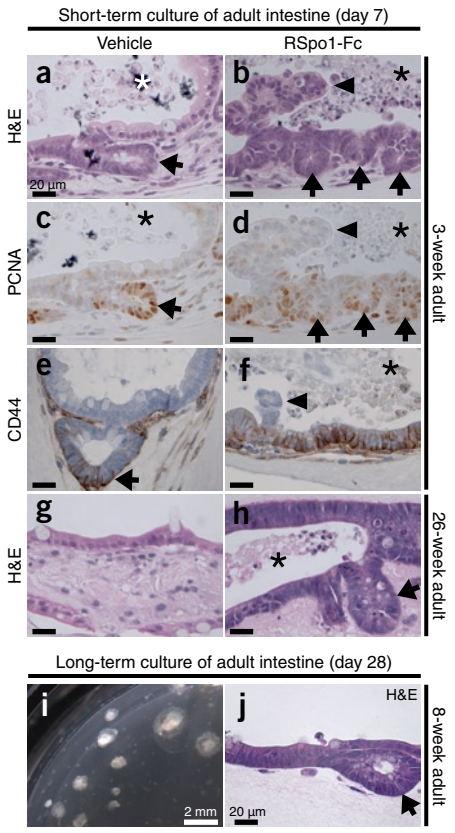

Figure 2 Intestinal cultures from juvenile and adult mice. (**a**–**h**) Histology of jejunal culture at day 7 from 3-week-old (**a**–**f**) or 26-week-old mice (**g**,**h**). Staining for H&E (**a**,**b**,**g**,**h**), PCNA (**c**,**d**) or CD44 (**e**,**f**) is depicted. (**i**,**j**) RSpo1-Fc treatment permitted longer-term jejunal culture (day 28) from 8-week-old adult intestine. Stereomicroscopy (**i**) and H&E staining (**j**) is depicted. Arrows indicate highly proliferative, $PCNA^+$ crypt-like structures, which invaginated from the sphere wall into the surrounding collagen matrix. Arrowheads indicate quiescent $PCNA^-$ villus-like protrusions. Numerous sludged or dead cells are present in the sphere lumen, indicated by the asterisk.

adult 3- to 26-week-old mice (**Fig. 2b,d,h** and **Supplementary Fig. 5a** online). Furthermore, RSpo1-Fc treatment extended the ability to culture adult intestine to 28 d (**Fig. 2i,j**), which seemed to be otherwise restricted to approximately 7–10 d without RSpo1-Fc treatment (data not shown). Expression of the β-catenin and T cell factor target genes *Cd44* and *Myc* in cultured intestinal epithelial cells was strongly induced by RSpo1-Fc, confirming functional stimulation of Wnt signaling (**Fig. 2e,f** and **Supplementary Fig. 5b**). Thus, Wnt gain- and loss-of-function studies indicated both accurate recapitulation of the Wnt-dependent ISC niche in culture, as well as Wnt responsiveness of the cultured intestinal epithelium.

Notch genes encode large, single-transmembrane receptors regulating a broad spectrum of cell fate decisions; in the intestine, Notch governs secretory lineage fate and maintains the proliferative progenitor state[13,14]. Notch inhibition with either γ-secretase inhibitors[25] or by conditional targeting of the Notch pathway transcription factor recombination signal-binding protein for immunoglobulin κ J region[13] induce marked goblet cell hyperplasia *in vivo*. Accordingly, treatment of preestablished small intestine cultures with the γ-secretase inhibitor dibenzazepine[25] for 5 d produced complete conversion of the epithelial layer into terminally differentiated goblet cells, as determined by morphology, periodic acid–Schiff (PAS) staining and the absence of mitotic PCNA staining (**Fig. 4a** and **Supplementary Fig. 6** online). We observed identical results with dibenzazepine treatment of large intestinal cultures (**Supplementary Fig. 6**). These results suggested accurate *ex vivo* recapitulation of the Notch-dependent ISC niche with preservation of endogenous Notch signaling within the intestinal sphere culture.

We further examined the plasticity of the intestinal sphere cultures with respect to the enteroendocrine lineage. The helix-loop-helix transcription factor neurogenin-3 (Ngn3) regulates enteroendocrine fate, with overexpression increasing intestinal enteroendocrine cell number[26–28]. Adenoviral Ngn3 overexpression in jejunal cultures was sufficient to induce an approximately threefold increase in the number of chromogranin A–positive enteroendocrine cells versus a control adenovirus expressing an antibody IgG2α Fc fragment[12] (**Fig. 4b**). Both Ngn3-mediated enteroendocrine cell differentiation and dibenzazepine-mediated goblet cell differentiation argue for the substantial plasticity of the cultured epithelium and the potential for its modulation by viral or small molecule approaches.

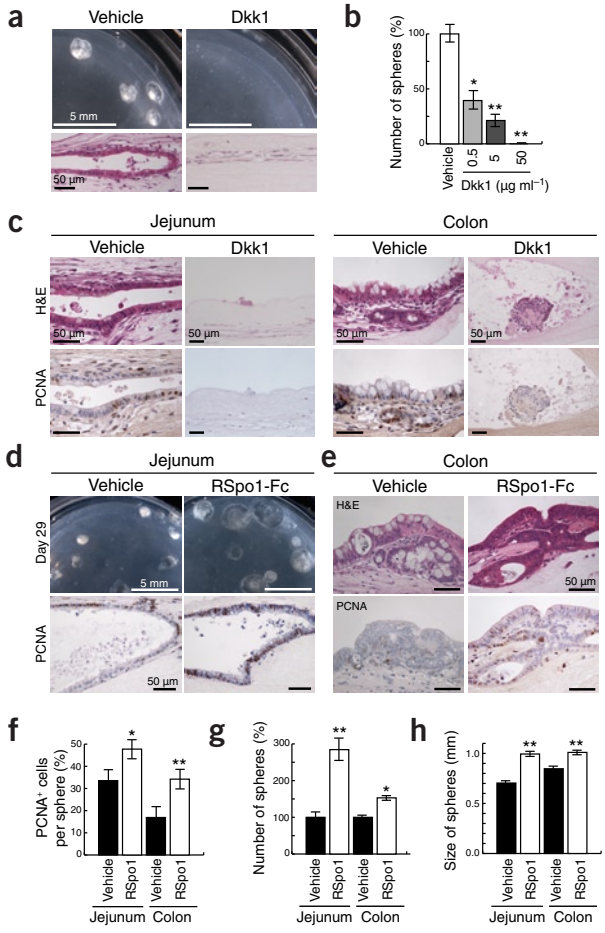

Figure 3 Wnt signaling regulates proliferation of cultured intestinal epithelium. (**a**–**c**) Dkk1 inhibits intestinal epithelial growth. (**a**) Stereomicroscopy and histology of Dkk1-treated jejunal cultures. Cultures were maintained with Dkk1 (50 μg ml^{-1}) for 7 d followed by incubation without Dkk1 for an additional 28 d. (**b**) Dose-dependent inhibition of jejunal sphere growth by Dkk1. Cultures were treated with various concentrations of Dkk1 on days 0–7. The number of spheres was counted. Error bars indicate s.e.m.; $n \geq 7$. $^*P < 0.05$ versus vehicle control (VC); $^{**}P < 0.01$ versus VC. (**c**) H&E and PCNA staining after Dkk1 treatment of pre-established intestinal cultures. Cultures were preestablished for 28 d followed by Dkk1 treatment for 5 d . (**d**–**h**) RSpo1-Fc treatment of intestinal epithelial cultures. (**d**) Stereomicroscopic visualization and PCNA staining of the jejunal cultures. RSpo1-Fc was included once weekly until harvest at day 29. (**e**) H&E and PCNA staining of colon cultures that were preestablished for 28 d followed by RSpo1-Fc treatment for 5 d. (**f**–**h**) Proliferative activity (**f**), number (**g**) and size (**h**) of jejunal and colon spheres. RSpo1-Fc was included once weekly for 5 weeks (**f**) or from days 0–7 (**g**,**h**). Error bars indicate s.e.m.; $n \geq 7$. $^*P < 0.05$ versus VC; $^{**}P < 0.01$ versus VC.

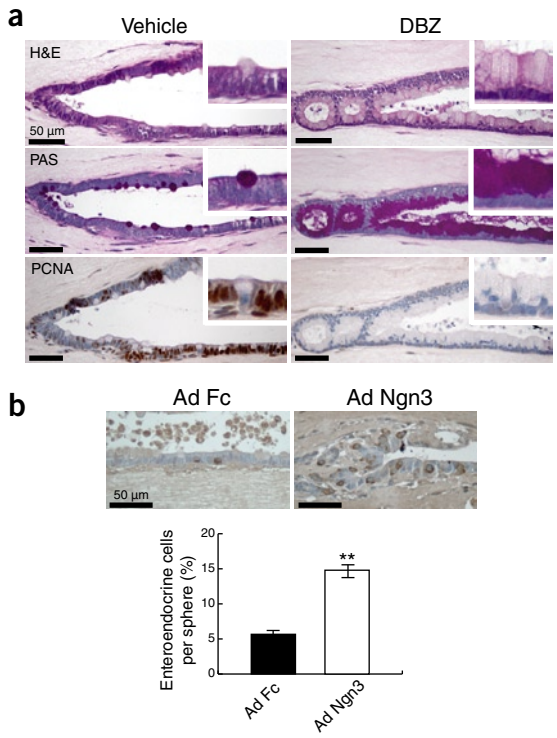

Figure 4 Notch and Neurogenin-3 regulate intestinal cell fate *in vitro*. (**a**) Treatment with Notch inhibitor dibenzazepine (DBZ) in neonatal jejunal cultures. H&E and PAS staining show morphological differentiation and mucus secretion of the epithelial cells. PCNA staining reveals active proliferation of the cultured cells. (**b**) Adenoviral expression of Neurogenin3 (Ad Ngn3) or a control immunoglobulin Fc fragment (Ad Fc) in jejunal cultures. Immunohistochemistry of chromogranin A demonstrates enteroendocrine cell differentiation *in vitro* (top). Percentages of enteroendocrine cells per sphere were analyzed (bottom). Error bars represent s.e.m.; $n \geq 8$ per condition. $^{**}P < 0.01$ versus Ad Fc.

Presence of putative ISCs and effects of RSpo1-Fc

Mosaic analyses of developing intestine have revealed that intestinal epithelium is initially generated as a well mixed population of multiple progenitors at birth that becomes exclusively monoclonal within each crypt by postnatal day 14, suggesting specification of ISCs by the niche during this period[29,30]. Accordingly, intestinal cultures from tetrachimeric mice with mosaic expression of distinct fluorescent proteins[30] showed progressively demarcated clonal fluorescence domains consistent with ISC specification (**Supplementary Fig. 7** online).

The prolonged expansion and proliferation within the intestinal sphere cultures (>350 d) strongly suggested the presence of functional ISCs. We sought to demonstrate the presence of *Lgr5*$^+$ and *Bmi1*$^+$ epithelial cell populations, which are putative ISCs[4,5], in the cultures as well as to exploit this system to explore their previously unexamined regulation by extracellular Wnt signals[4,5]. We thus exposed jejunal cultures from *Bmi1*$^{Cre\text{-}ER/+}$;*Rosa26*$^{LacZ/+}$ mice[5] to 7 d of tamoxifen. In this model, tamoxifen temporally induces expression of *Cre* recombinase in *Bmi1*-expressing cells while simultaneously inducing expression of β-galactosidase from the ubiquitously active *Rosa26* locus. Consequently, cells expressing *Bmi1* and their progeny are permanently labeled upon tamoxifen treatment. Under these conditions, RSpo1-Fc treatment, as opposed to vehicle controls, robustly induced detectable LacZ$^+$ epithelial clusters corresponding to *Bmi1*$^+$ cells and their progeny, consistent with a previously undocumented responsiveness of the *Bmi1*$^+$ lineage to extracellular Wnt signals (**Fig. 5a,b**).

As the *Lgr5* gene, encoding an orphan G protein–coupled receptor, has been validated as a robust panintestinal ISC marker[4], we examined *Lgr5*-expressing epithelial cell populations in both small and large intestinal cultures using fluorescent *in situ* hybridization (**Fig. 5c**). This revealed *Lgr5*$^+$ cells in both jejunum and colon cultures that were more numerous with RSpo1-Fc treatment (**Fig. 5d**), paralleling increased mitotic index and growth of the cultured intestinal spheres (**Fig. 3d–h**). Confirming the predictive nature of the culture system, we further observed increased numbers of *Lgr5*$^+$ cells in small and large intestinal crypts *in vivo* after systemic adenoviral RSpo1-Fc expression (**Fig. 5e** and **Supplementary Fig. 8a** online), paralleling marked crypt hyperplasia and increased mitotic index (**Fig. 5f** and **Supplementary Fig. 8b,c**).

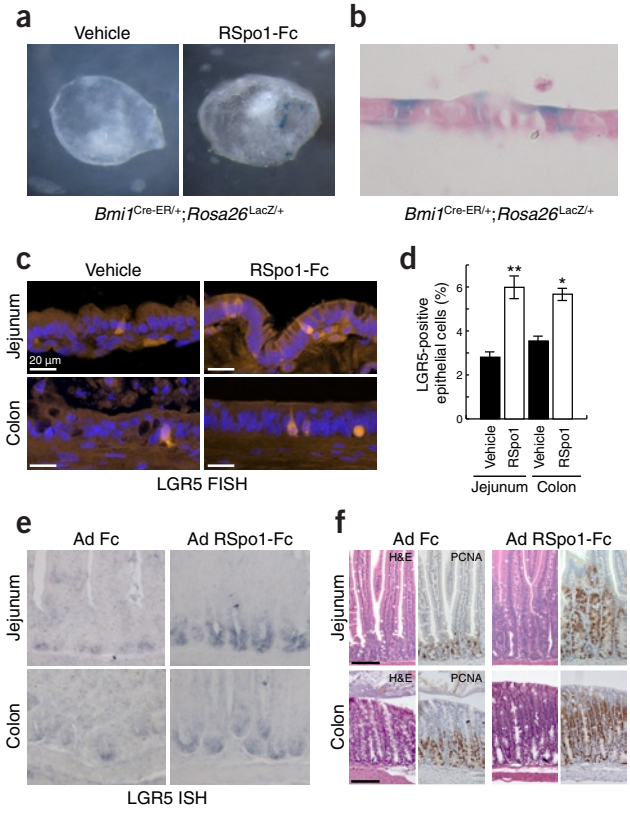

Figure 5 Putative intestinal stem cell populations with or without R-spondin1 treatment in culture. (**a**,**b**) Jejunal cultures contain *Bmi1* lineage–derived cells. (**a**) Whole-mount LacZ staining of intestinal spheres. *Bmi1*$^+$ cells and their progeny are identified as blue LacZ$^+$ patches in RSpo1-Fc treatment versus vehicle control. (**b**) Frozen section of LacZ-stained intestinal spheres showing LacZ$^+$ *Bmi1* lineage–derived cells and their progeny in the epithelial layer of RSpo1-Fc-treated cultures. (**c**) Fluorescent *in situ* hybridization (FISH) for *Lgr5* (orange) with simultaneous DAPI nuclear stain (blue) at culture day 35. (**d**) Percentage of *Lgr5*$^+$ cells as a fraction of epithelial cells after RSpo1-Fc treatment compared to vehicle control at day 35. RSpo1-Fc was added once weekly for 5 weeks. Error bars indicate s.e.m.; $n \geq 4$. $^*P < 0.05$ versus vehicle control. (**e**) *In situ* hybridization (ISH) for *Lgr5 in vivo* from mice 7 d after single intravenous administration of adenoviruses encoding RSpo1-Fc (Ad RSpo1-Fc) or antibody Fc control (Ad Fc). (**f**) Histology of jejunum and colon from Ad RSpo1-Fc–treated– or Ad Fc–treated mice. H&E and PCNA staining of intestine 7 d after single intravenous injection of Ad RSpo1-Fc or Ad Fc is depicted (**e**,**f**). Scale bars, 100 μm.

DISCUSSION

This study describes a robust long-term methodology for either small or large intestinal culture, allowing sustained intestinal proliferation and multilineage differentiation for a range of 30 d to >350 d. The lack of long-term methodology for primary intestinal culture has been a substantial obstacle to exploration of intestinal stem and progenitor cell biology and more general questions of physiology, despite attempts to recapitulate normal intestinal epithelial growth and differentiation *in vitro*[15–17]. Monolayer cultures have been complicated by rapid apoptosis[18,19], whereas organoid cultures have historically been unable to preserve viability lasting more than ~10 d[21]. More recently developed 3D organoid cultures have been restricted to embryonic tissue and again have shown restricted viability, typically less than 14 d[22]. Consequently, implantation of dissociated intestinal material into syngeneic hosts or immunocompromised mice, either directly or with an intervening culture period, has been the only available tool with which to demonstrate the presence and potential of stem cells and progenitors in cultured intestine[17].

To overcome the difficulties described above, we have maintained minced intestinal fragments in a 3D matrix scaffold of type I collagen gel under an air-liquid interface. With growing evidence that the 3D matrix environment has a crucial role in facilitating the behavior of stem cells and tissue morphogenesis, organotypic 3D cultures have great advantages over both conventional two-dimensional cell approaches and animal models[31–33]. The major features of 3D collagen gel models are derived from their ability to mimic normal tissue organization to induce appropriate polarity of epithelial cells, to induce behavior of fibroblasts including extracellular matrix remodeling and to induce signaling between cell-cell and cell-matrix interactions[34,35]. Additionally, air-liquid interface methods allow long-term culture of various epithelial cell types via improved oxygenation *in vitro*[36–38]. In the current method, substitution of conventional immersed conditions for an air-liquid interface markedly decreases viability, although sporadic short-term sphere formation and growth can be observed. Our results suggest that our 3D culture system enables mouse intestinal fragments to recapitulate intestinal epithelial growth and differentiation within the microenvironment of an *in vitro* ISC niche.

Adult or somatic stem cells generally have limited function without their niche[8]. Our method allows primary intestinal epithelium to be cultured in close apposition to myofibroblasts, which have been proposed to be the candidate niche supporting ISCs and influencing intestinal epithelial growth[1,9,10]. Indeed, the growth of individual intestinal spheres was highly correlated with the preceding growth of myofibroblasts, which lined the basal surface of the cultured intestinal epithelium. Thus, appropriate stromal cell growth seems to permit long-term culture and multilineage differentiation of intestinal epithelial cells without implantation into syngenic or immunocompromised host animals. Our results by no means exclude the possibility of culturing intestinal epithelium without a cellular niche, which could certainly be achieved with appropriate extracellular signals.

Cultures <30 d old were predominantly comprised of viable, robustly proliferating tissue, which was present but increasingly sporadic at extremely long culture durations of >200 d. Although we obtained the most prolonged culture with neonatal intestine, we observed limited culture for 7 to 10 d without RSpo1-Fc treatment and up to 4 weeks with RSpo1-Fc treatment using adult mouse intestine as starting material. These aforementioned limitations of long-term and adult culture may be secondary either to progressive deficits in either the stromal myofibroblasts or other niche components *in vitro* or to cell-intrinsic deficits in ISCs, transit amplifying cells or both.

Accurate recapitulation of the *in vivo* Wnt and Notch dependence of the ISC niche represents a prominent feature of the intestinal sphere cultures. Indeed, Dkk1 and dibenzazepine treatment phenocopied the intestinal effects of *in vivo* Wnt and Notch inhibition on proliferation and goblet cell differentiation, respectively[11–13,25]. At the same time, the endogenous Wnt and Notch signaling within the intestinal cultures was sufficient to support vigorous expansion, and exogenously added RSpo1-Fc conferred a further induction of intestinal epithelial growth, even allowing limited expansion of adult intestinal cultures. In this regard, RSpo1-Fc induced expansion of transit amplifying cell populations, which express the Wnt target genes *Cd44* and *Myc* in concert with $PCNA^+$ proliferative activity. This preservation of the ISC niche probably underlies the successful support of long-term proliferation and differentiation observed in the current studies. These studies further illustrate the utility of extracellular Wnt agonists, particularly R-spondin-1, as a potent growth factor that markedly enhances the efficacy of intestinal culture.

The observed prolonged growth and differentiation (>350 d) suggest the presence of ISCs or extremely long-lived transit amplifying cells in the sphere cultures. Our detection of both $Lgr5^+$ cells as well as cells derived from the $Bmi1^+$ lineage within the intestinal cultures is consistent with the former possibility. The potential regulation of ISCs by extracellular Wnt signals has been a plausible but previously untested hypothesis. The observed increase in both $Lgr5^+$ lineage– and $Bmi1^+$ lineage–derived cells by RSpo1-Fc treatment *in vitro* is consistent with the direct regulation of ISCs by Wnt signals, although we can not exclude the alternative possibilities that transit amplifying cells expressing these markers may have arisen in culture, or that RSpo1-Fc–induced $Lgr5^+$ cells merely reflect increased Wnt-dependent gene expression rather than increased ISC number. We observed similar increases in crypt $Lgr5^+$ cells after *in vivo* adenovirus RSpo1-Fc treatment, demonstrating the predictive nature of the *in vitro* culture system, although in both cases our *in situ* hybridization analyses only allow conclusions regarding *Lgr5* mRNA and not Lgr5 protein.

The availability of a robust intestinal culture system supporting the expression of populations expressing ISC markers, accurately recapitulating the ISC niche in both small and large intestine and applicable to both neonatal and adult tissues should greatly facilitate the study of intestinal stem cells and niche-ISC interactions. This model could also potentially be used to study intestinal epithelial interactions with other heterologous cell types, including neurons, smooth muscle, endothelial cells and immune cells. Furthermore, the enablement of primary intestinal culture should have widespread application to general studies of intestinal biology, including investigations of physiology, host-pathogen interactions, neoplasia, tissue engineering and regenerative medicine.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nm/

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We are grateful to members of the Kuo laboratory, C. Chartier, R. Nusse, L. Chia, M. Lee and M. Pech for helpful discussions. We are indebted to M. Amieva for video recording, J. Yuan, P. Chu, H. Ideguchi and S. Nakahara for technical assistance, M. Kay (Stanford University) for adenovirus expressing Ngn3, P. Soriano (Mount Sinai School of Medicine) for Rosa26-1 vector, J. Chen (Stanford University) for Wnt reporter cells, R. Nusse (Stanford University) for Wnt3a L cells, and M. Selsted (University of California–Irvine) and E. Sibley (Stanford University) for antibodies specific for cryptidin and intestinal hydrolases, respectively. A.O. was a California Institute for Regenerative Medicine Scholar. X.L. was supported by a Dean's Fellowship from Stanford University, and H.U. was supported by the Floren Family Fund. This work was supported by US National Institutes of Health grant R01 DK069989-01, California Institute for Regenerative Medicine RS1-00243-1 and the Broad Medical Research Foundation (C.J.K.), US National

Institutes of Health grant R01 CA86065-06 and the Smith Family Fund (I.L.W.), the Ichiro Kanehara Foundation, Kato Memorial Trust for Nambyo Research and Nagao Memorial Trust (A.O.), and Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology for Scientific Research No. 20592023 (S.T.). We acknowledge the generous support of the Stanford University Digestive Disease Center (US National Institutes of Health grant P30 DK56339; H.U. and C.J.K.).

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Li, L. & Xie, T. Stem cell niche: structure and function. *Annu. Rev. Cell Dev. Biol.* **21**, 605–631 (2005).
2. Barker, N., van de Wetering, M. & Clevers, H. The intestinal stem cell. *Genes Dev.* **22**, 1856–1864 (2008).
3. Potten, C.S., Booth, C. & Pritchard, D.M. The intestinal epithelial stem cell: the mucosal governor. *Int. J. Exp. Pathol.* **78**, 219–243 (1997).
4. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003–1007 (2007).
5. Sangiorgi, E. & Capecchi, M.R. Bmi1 is expressed *in vivo* in intestinal stem cells. *Nat. Genet.* **40**, 915–920 (2008).
6. Scoville, D.H., Sato, T., He, X.C. & Li, L. Current view: intestinal stem cells and signaling. *Gastroenterology* **134**, 849–864 (2008).
7. Cheng, H. & Leblond, C.P. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am. J. Anat.* **141**, 537–561 (1974).
8. Scadden, D.T. The stem-cell niche as an entity of action. *Nature* **441**, 1075–1079 (2006).
9. Crosnier, C., Stamataki, D. & Lewis, J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat. Rev. Genet.* **7**, 349–359 (2006).
10. Williams, E.D., Lowes, A.P., Williams, D. & Williams, G.T. A stem cell niche theory of intestinal crypt maintenance based on a study of somatic mutation in colonic mucosa. *Am. J. Pathol.* **141**, 773–776 (1992).
11. Pinto, D., Gregorieff, A., Begthel, H. & Clevers, H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev.* **17**, 1709–1713 (2003).
12. Kuhnert, F. *et al.* Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc. Natl. Acad. Sci. USA* **101**, 266–271 (2004).
13. van Es, J.H. *et al.* Notch/γ-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959–963 (2005).
14. Fre, S. *et al.* Notch signals control the fate of immature progenitor cells in the intestine. *Nature* **435**, 964–968 (2005).
15. Pageot, L.P. *et al.* Human cell models to study small intestinal functions: recapitulation of the crypt-villus axis. *Microsc. Res. Tech.* **49**, 394–406 (2000).
16. Kaeffer, B. Mammalian intestinal epithelial cells in primary culture: a mini-review. *In Vitro Cell. Dev. Biol. Anim.* **38**, 123–134 (2002).
17. Bjerknes, M. & Cheng, H. Intestinal epithelial stem cells and progenitors. *Methods Enzymol.* **419**, 337–383 (2006).
18. Frisch, S.M. & Francis, H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124**, 619–626 (1994).
19. Sträter, J. *et al.* Rapid onset of apoptosis *in vitro* follows disruption of β_1-integrin/matrix interactions in human colonic crypt cells. *Gastroenterology* **110**, 1776–1784 (1996).
20. Booth, C., O'Shea, J.A. & Potten, C.S. Maintenance of functional stem cells in isolated and cultured adult intestinal epithelium. *Exp. Cell Res.* **249**, 359–366 (1999).
21. Perreault, N. & Beaulieu, J.F. Primary cultures of fully differentiated and pure human intestinal epithelial cells. *Exp. Cell Res.* **245**, 34–42 (1998).
22. Abud, H.E., Watson, N. & Heath, J.K. Growth of intestinal epithelium in organ culture is dependent on EGF signalling. *Exp. Cell Res.* **303**, 252–262 (2005).
23. Blanpain, C., Horsley, V. & Fuchs, E. Epithelial stem cells: turning over new leaves. *Cell* **128**, 445–458 (2007).
24. Kim, K.A. *et al.* Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* **309**, 1256–1259 (2005).
25. Milano, J. *et al.* Modulation of notch processing by γ-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol. Sci.* **82**, 341–358 (2004).
26. Schonhoff, S.E., Giel-Moloney, M. & Leiter, A.B. Neurogenin 3–expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev. Biol.* **270**, 443–454 (2004).
27. López-Díaz, L. *et al.* Intestinal neurogenin 3 directs differentiation of a bipotential secretory progenitor to endocrine cell rather than goblet cell fate. *Dev. Biol.* **309**, 298–305 (2007).
28. Wang, J. *et al.* Mutant neurogenin-3 in congenital malabsorptive diarrhea. *N. Engl. J. Med.* **355**, 270–280 (2006).
29. Schmidt, G.H., Winton, D.J. & Ponder, B.A. Development of the pattern of cell renewal in the crypt-villus unit of chimaeric mouse small intestine. *Development* **103**, 785–790 (1988).
30. Ueno, H. & Weissman, I.L. Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev. Cell* **11**, 519–533 (2006).
31. Griffith, L.G. & Swartz, M.A. Capturing complex 3D tissue physiology *in vitro*. *Nat. Rev. Mol. Cell Biol.* **7**, 211–224 (2006).
32. Yamada, K.M. & Cukierman, E. Modeling tissue morphogenesis and cancer in 3D. *Cell* **130**, 601–610 (2007).
33. Bryant, D.M. & Mostov, K.E. From cells to organs: building polarized tissue. *Nat. Rev. Mol. Cell Biol.* **9**, 887–901 (2008).
34. Nelson, C.M. & Bissell, M.J. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* **22**, 287–309 (2006).
35. Pampaloni, F., Reynaud, E.G. & Stelzer, E.H. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.* **8**, 839–845 (2007).
36. Bingmann, D. & Kolde, G. PO2-profiles in hippocampal slices of the guinea pig. *Exp. Brain Res.* **48**, 89–96 (1982).
37. Pruniéras, M., Regnier, M. & Woodley, D. Methods for cultivation of keratinocytes with an air-liquid interface. *J. Invest. Dermatol.* **81**, 28s–33s (1983).
38. Toda, S. *et al.* A new organotypic culture of thyroid tissue maintains three-dimensional follicles with C cells for a long term. *Biochem. Biophys. Res. Commun.* **294**, 906–911 (2002).

ONLINE METHODS

Mice. We used C57BL/6 mice (Jackson Laboratories) aged from postnatal day 0 up to 26 weeks throughout our experiments. We generated tetrachimeric mice as previously described[30]. We generated *Bmi1*$^{Cre\text{-}ER/+}$;*Rosa26*$^{LacZ/+}$ mice as previously described[5]. All animal experimental protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

Three-dimensional intestinal culture system. We opened the mouse small and large intestines (optimally from postnatal days 0–2) lengthwise and washed them in PBS to remove all luminal contents. We minced a 1-cm segment immediately and extensively on ice with iris scissors. We embedded the minced tissues in a 3D collagen gel using a double-dish culture system as previously described[38–40]. We poured a 1-ml collagen gel solution (Cellmatrix Type I-A, Nitta Gelatin) into a 30-mm dish (Millicell-CM, Millipore), the inner dish, with a hydrophilic polytetrafluoroethylene membrane bottom to form an acellular layer. Next, we placed a 1-ml collagen gel solution containing a total of 0.1 g minced tissues on the acellular layer in the dish. We placed this inner dish into a 60-mm outer dish containing 1.5 ml Ham's F12 medium supplemented with 20% FCS and 50 μg ml^{-1} gentamicin (Gibco). We carried out the culture assembly for 7–365 d at 37 °C in a humidified atmosphere of 5% CO_2 in air, and we changed the medium every 7 d. When appropriate, we included mouse RSpo1-Fc (500 ng ml^{-1}) or Dkk1 (50 μg ml^{-1}) in the outer dish medium with change of medium every 1–7 d as indicated in the **Figure 3** legend. See the **Supplementary Methods** online for detailed protocols.

Dibenzazepine treatment of cultures. We treated cultures with RSpo1-Fc at culture days 0 and 2. At day 7, we added dibenzazepine (10 μM, Calbiochem) daily with medium change until cell collection at day 12.

Adenoviral infection of cultures. We treated jejunal cultures with RSpo1-Fc at culture days 0 and 2. At day 14, we microinjected 1×10^7 particles of adenovirus encoding *Neurogenin-3* in 0.25 μl PBS into the lumen of preestablished spheres using glass-pulled pipettes; we collected the cells at day 26.

Protein purification. We purified the mouse RSpo1-Fc fusion protein containing a C-terminal mouse antibody IgG2α Fc fragment (**Supplementary Fig. 4**) and mouse Dkk1 bearing an amino-terminal hemagglutinin tag and carboxyl-terminal Flag and 6× His tags[12] from the conditioned medium of stably transfected 293T cells using protein A affinity and Ni-agarose chromatography, respectively.

Adenoviral expression of RSpo1-Fc *in vivo*. We inserted the complementary DNA encoding the mouse RSpo1-Fc fusion protein into the E1 locus of E1$^-$E3$^-$ adenovirus type 5 as previously described[12] to generate Ad RSpo1-Fc. We gave adult C57BL/6 mice a single intravenous injection of 3×10^8 plaque-forming units Ad RSpo1-Fc, resulting in hepatic transduction and systemic transgene expression from liver secretion. We collected tissue samples at 7 d after injection.

Histology and immunohistochemistry. We fixed samples with 4% paraformaldehyde overnight, paraffin-embedded them and sectioned them. We stained deparaffinized sections with H&E, PAS and alcian blue. We used antibodies to the following proteins: PCNA (1 in 100; Dako), CD44 (1 in 100; PharMingen), c-Myc (1 in 100; Santa Cruz), lysozyme (1 in 1,000; Dako), cryptdin (1 in 10; gift from M. Selsted), matrix metalloprotease-7 (1 in 100; Cell Signaling), Na^+-K^+ ATPase (1 in 100; Novus Biologicals), mucin-2 (1 in 100; Santa Cruz), chromogranin A (1 in 100; ICN), serotonin (1 in 40; Abcam), glucagon-like peptide-2 (1 in 500; Biomol), single-stranded DNA (1 in 100; Dako) and α-smooth muscle actin (1 in 200; Epitomics). Rabbit antibodies to rat lactase, maltase, and sucrase antisera (1 in 2,000) were kindly provided by E. Sibley. Antibody to cryptidin was a kind gift from M. Selsted. We immunostained deparaffinized sections by the avidin-biotin complex immunoperoxidase method[39]. For transmission electron microscopy, we fixed samples with 2.5% gluteraldehyde and 1% osmic acid, dehydrated them with alcohol, embedded them in epoxy resin as described previously[39].

Detection of *Bmi1*$^+$ cells. We cultured jejunal cultures from *Bmi1*$^{Cre\text{-}ER/+}$;*Rosa26*$^{LacZ/+}$ neonatal mice with or without RSpo1-Fc for 14 d. We added tamoxifen (0.5 μM, Sigma) to both cultures from days 8–14 and collected the cells at day 14. We fixed cultured cells for 5 min in PBS with 4% paraformaldehyde, 0.2% gluteraldehyde, 2 mM $MgCl_2$, 25 mM EGTA pH 7.5 and 0.02% Nonidet P40 (USB Corporation), rinsed them three times in 2 mM $MgCl_2$ in PBS and then stained them for 24 h at 4 °C with 1 mg ml^{-1} X-Gal (Research Organics) with 2 mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 0.01% Na-deoxycholate and 0.02% NP-40 in PBS. We rinsed the culture assemblies three times in PBS and then post-fixed them in 4% paraformaldehyde at 4 °C overnight. We cryopreserved the stained intestinal spheres in 30% sucrose, embedded them in optimal cutting temperature medium (Tissue-Tech) and froze them on dry ice. We counterstained frozen sections with nuclear fast red (Vector laboratories).

Statistical analysis. We analyzed data obtained from four to nine independent experiments by Student's *t* test. We expressed results as means ± s.e.m. and considered them significant when $P < 0.05$.

39. Ootani, A., Toda, S., Fujimoto, K. & Sugihara, H. Foveolar differentiation of mouse gastric mucosa *in vitro*. *Am. J. Pathol.* **162**, 1905–1912 (2003).
40. Amemori, S. *et al.* Adipocytes and preadipocytes promote the proliferation of colon cancer cells *in vitro*. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G923–G929 (2007).

doi:10.1038/nm.1958