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Induced Quiescence of Lgr5+ Stem Cells in Intestinal **Organoids Enables Differentiation of Hormone-Producing Enteroendocrine Cells**

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Graphical Abstract



Highlights

- EGFR inhibition halts DNA replication and proliferation of Lgr5+ ISCs through MEK
- Lgr5+ ISCs reactivated from guiescence retain multilineage differentiation potential
- Combined EGFR/Wnt/Notch inhibition produces enteroendocrine cells with high purity
- RNA sequencing shows regional identity and heterogeneity in hormone-producing EECs

h.clevers@hubrecht.eu In Brief

Basak et al. identify signals to generate rare enteroendocrine cells (EECs) at high purity through manipulation of intestinal stem cell quiescence. Single-cell sequencing reveals a high level of heterogeneity in hormonal production, which is influenced by the regional identity of the intestinal organoid cultures.

Data Resources GSE80636





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Induced Quiescence of Lgr5+ Stem Cells in Intestinal Organoids Enables Differentiation of Hormone-Producing Enteroendocrine Cells

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SUMMARY

Lgr5+ adult intestinal stem cells are highly proliferative throughout life. Single Lgr5+ stem cells can be cultured into three-dimensional organoids containing all intestinal epithelial cell types at near-normal ratios. Conditions to generate the main cell types (enterocyte, goblet cells, Paneth cells, and M cells) are well established, but signals to induce the spectrum of hormone-producing enteroendocrine cells (EECs) have remained elusive. Here, we induce Lgr5+ stem cell quiescence in vitro by blocking epidermal growth factor receptor (EGFR) or mitogen-associated protein kinase (MAPK) signaling pathways in organoids and show that their quiescent state is readily reverted. Quiescent Lgr5+ stem cells acquire a distinct molecular signature biased toward EEC differentiation. Indeed, combined inhibition of Wnt, Notch, and MAPK pathways efficiently generates a diversity of EEC hormone-expressing subtypes in vitro. Our observations uncouple Wnt-dependent stem cell maintenance from EGF-dependent proliferation and provide an approach for the study of the elusive EECs in a defined environment.

INTRODUCTION

Lgr5+ stem cells self-renew constantly throughout life at the base of intestinal crypts (Clevers, 2013). Active Notch signaling in rapidly dividing daughters specifies an enterocyte fate. Alternatively, some daughters upregulate Notch ligands (i.e., DII1 and DII4) immediately after leaving the crypt base niche, concomitant with an exit from the cell cycle (van Es et al., 2012). The latter cells represent secretory progenitors that give rise to Paneth, goblet, and enteroendocrine cells (EECs).

Murine Lgr5+ intestinal stem cells divide on average every 21.5 hr (Schepers et al., 2011). A reserve stem cell population

has been shown to reside above the Paneth cells at the "+4" position (Montgomery et al., 2011; Muñoz et al., 2012; Potten et al., 1978; Powell et al., 2012; Sangiorgi and Capecchi, 2008; Schepers et al., 2011; Takeda et al., 2011; Yan et al., 2012). These cells are generally non-proliferative and can replace lost Lgr5+ stem cells. An elegant lineage-tracing strategy identified these label-retaining cells as non-cycling secretory progenitors (Buczacki et al., 2013). Indeed, these secretory progenitors and the +4 cells share several molecular markers, including Hopx, Bmi1, Lrig, and Tert expression (Montgomery et al., 2011; Muñoz et al., 2012; Powell et al., 2012; Schepers et al., 2011; Takeda et al., 2011; Yan et al., 2012). Moreover, dissection of Lgr5+ crypt populations with distinct cell-cycle features suggests that Lgr5^{low} cells with slow cell-cycle kinetics are secretory precursors (Basak et al., 2014). The presence and identity of "professional" quiescent intestinal stem cells has remained elusive.

Traditionally known as defensive units against microbial infections, Paneth cells also act as part of the niche for the juxtaposed Lgr5+ stem cells by secreting Wnt3 and epidermal growth factor (EGF) and by presenting the Notch ligands Dll1 and Dll4 (Pellegrinet et al., 2011; Sato et al., 2011). Mesenchyme surrounding the crypts also contributes to the niche by secreting Wnt2b as well as several BMP inhibitors (Aoki et al., 2016; Farin et al., 2012).

The murine intestinal organoid culture system (Sato et al., 2009) generates all principle cell types of the intestinal epithelium, including Lgr5+ stem cells. The system is based on substitution of in vivo niche components (i.e., the Wnt agonist R-spondin-1, EGF, and the BMP inhibitor Noggin). Matrigel mimics the extracellular matrix and provides the structural basis for self-organization. R-spondin-1 is a critical component that, through interaction with its Lgr4 and 5 receptors, amplifies the Wnt3 signal emanating from Paneth cells (de Lau et al., 2011).

Organoids can be programmed to produce relatively pure populations of most epithelial cell types. High-Wnt and high-Notch conditions favoring expansion of Lgr5+ stem cells can be mimicked by the addition of the GSK3 inhibitor CHIR99021 combined with the histone deacetylase (HDAC) inhibitor valproic acid (Yin et al., 2014). Enterocytes appear under conditions of

⁶Lead Contact

Wnt inhibition and Notch activation (Yin et al., 2014). The addition of Rank ligand promotes the fate of M cells, which cover Peyer's patches and transport luminal antigens via transcytosis (de Lau et al., 2012). Notch inhibition generally induces secretory fates. In the absence of Wnt, secretory goblet cells are formed (van Es et al., 2005), while in the presence of Wnt, Paneth cells appear (van Es et al., 2012; Yin et al., 2014).

EECs are rare, hormone-secreting cells that are also generated from Lgr5+ stem cells (Barker et al., 2007). Hormones expressed by EECs regulate a wide variety of physiological responses, including gastric emptying, release of pancreatic enzymes, blood glucose levels, and appetite and mood changes. Most commonly, subtypes are distinguished based on their secreted hormones and include somatostatin+ (Sst) D-cells, gastric inhibitory poplypeptide+ (Gip) K-cells, secretin+ (Sct) S-cells, cholecystokinin (Cck) I-cells, glucagon-like protein 1+ (GLP-1) L-cells, neurotensin+ (Nts) N-cells, and serotonin-producing enterochromaffin cells (Gunawardene et al., 2011). However, a single EEC may express multiple hormones at varying levels, underscoring a high level of heterogeneity (Egerod et al., 2012). In a recent single-cell-sequencing approach, we demonstrated that organoids faithfully generate the various EEC types and identified three additional subtypes of EECs: Tac1+/Cck+, Ucn3+, and Alb+/ Afp+ (Grün et al., 2015). G-protein-coupled taste receptors have been identified as regulators of hormone secretion in these cells (Janssen and Depoortere, 2013). Indeed, EECs can have direct luminal contact and sense the intestinal content with microvilli. Other EECs, the so-called closed-type cells, are not exposed to the lumen (Janssen and Depoortere, 2013). Their basal process (of varying length) may form synaptic contacts with enteric neurons to connect to the nervous system. While EECs clearly play crucial roles in controlling various aspects of intestinal function and organismal metabolism, their scarcity has posed a hurdle to their in-depth study. Here, we explore methods to program organoids toward EEC fates in vitro.

RESULTS

Inhibition of EGFR Signaling Abolishes Proliferation of Lgr5+ Stem Cells and Induces Their Quiescence

To understand how mouse Lgr5+ stem cells are kept in cycle, we manipulated key signaling pathways active in the crypt niche. The Lgr5^{GFPDTR} allele (Tian et al., 2011) is never silenced in Lgr5+ cells (see below) and is well suited for flow-cytometrybased quantification of Lgr5+ cell numbers. Combining flow cytometric analysis of Lgr5^{GFPDTR/+} organoids with antibody staining against KI67, a marker of cycling cells in all cellcycle phases, confirmed that the overwhelming majority (94.1% ± 2.1%) of the Lgr5+ cells cycle in ENR (EGF, Noggin and R-spondin-1) medium (Figures S1A and S1C). Wnt signaling is reported to induce cell-cycle progression through cyclin D₂ and c-Myc expression (Myant and Sansom, 2011). We inhibited Wnt signaling using two independent methods: (1) withdrawal of R-spondin1 from the culture medium and (2) IWP-2 treatment which inhibits Wnt3 secretion by Paneth cells (Figure S1A). R-spondin-1 withdrawal caused rapid loss of Lar5^{GFPDTR} expression (Figure S1A). IWP2 treatment (iWnt) poses a slower Wnt inhibition that depends on dilution of ligands through proliferation (Farin et al., 2016). Lgr5^{GFPDTR} expression was gradually downregulated while stem cells differentiated into KI67+ Lgr5- cells upon iWnt treatment (Figures S1A and S1B). Yet, the remaining Lgr5^{GFPDTR}+ cells maintained KI67 expression (63.5% ± 2.8% vs. 94.4% ± 2.1% in control; Figure S1C). Withdrawal of the BMP inhibitor Noggin or addition of the Notch inhibitor DAPT (iNotch) both induced a rapid decrease in Lgr5^{GFPDTR}+ cell numbers (Figure S1A) but did not affect proliferation of the remaining Lgr5^{GFPDTR}+ cells (82.3% \pm 1.4% in Noggin withdrawal and 45.1% \pm 10% in iNotch) (Figure S1C). Next, we inhibited EGF receptor (EGFR) signaling using gefitinib accompanied by withdrawal of EGF from the culture medium (iEGFR). While Lgr5^{GFPDTR} expression persisted (Figures S1A and S1D), the Lgr5^{GFPDTR}+ cells eventually lost KI67 expression (13.1% ± 1.0% remaining KI67+ cells) indicative of cell-cycle exit (Figures S1C and S1D). After 4 days of iEGFR treatment. Lgr5^{GFPDTR}+ cells comprised 44.4% ± 0.8% (vs. 13.6% \pm 6.5% in control) of the organoids when analyzed by fluorescence-activated cell sorting (FACS) (Figures S1A and S1D).

We then focused on the early events associated with EGFR inhibition (Figure 1A). Despite extensive apoptosis of the differentiated compartments of the organoid, buds resembling crypt structures survived iEGFR treatment for at least a week (Figures 1B and S2B). Fluorescent microscopy analysis using both Lgr5^{GFPiresCreER/+} (Figure S2A) and Lgr5^{GFPDTR/+} (Figure S2B) organoids confirmed that these buds contained Lgr5+ cells. Of note, the Lqr5^{GFPiresCreER} allele is well suited for lineage tracing and is the strongest GFP-expressing Lgr5 allele, yet it is stochastically silenced in some cells (Barker et al., 2007). We noticed that GFP levels increased upon iEGFR treatment (Figures S2A and S2B). The Rosa^{TCF-CFP} Wnt signal reporter allele (Serup et al., 2012) revealed that increased Lgr5 reporter expression coincided with high Wnt activity (Figure S2B). Confocal microscopy revealed that the cellular bridges connecting buds in normal organoid cultures (ENR) slowly converted into cellular debris in iEGFR cultures (Figure 1B). Typically, iEGFR cultures contained round, crypt-like bud structures with many Lgr5+ cells intermingled with Lgr5- cells (Figure 1C). We also noticed that organoids in iEGFR cultures were considerably smaller than controls (Figures 1C and 1D). Thus, iEGFR treatment results in smaller organoids mostly consisting of crypt-like buds with high Wnt signal strength and Lgr5 expression.

Next, we analyzed proliferation of organoids using immunofluorescence and confocal microscopy. The KI67 protein persisted for the first 24 hr but was lost from 48 hr onward (Figures 1E and 1F). Using a short pulse of ethynyldeoxyuridine (EdU) as a measure of S phase cells, we found that iEGFR lead to a rapid halt in DNA replication as early as 24 hr, which persisted for at least a week (Figures 1E and 1F). Consistent with exit from S phase and eventually from the cell cycle, labeling the DNA content of iEGFR-treated organoids using Hoechst DNA staining confirmed that all cells were in G₀/G₁ phase (Figure S2C). 4 days after iEGFR treatment, reconstitution of EGF signaling induced rapid cell-cycle entry within 24 hr (KI67+) and progression to the S phase within 48 hr (EdU+) (Figures 1G and S2D). Figure 1H further illustrates that Lgr5+ cells in iEGFR-treated organoids lacked the cell-cycle marker KI67 and the M phase marker pH3 and did not incorporate EdU, excluding that rare dividing cells persisted during iEGFR



treatment (Figure 1H). Altogether, our results reveal that iEGFR treatment abolishes proliferation of organoids and induces generation of quiescent Lgr5+ cells.

Stem Cell Potential Is Maintained in Reactivated Lgr5+ **Intestinal Stem Cells**

To test whether quiescent Lgr5+ cells maintain stem cell potential, we used Lgr5^{GFPiresCreER /+}Rosa^{LacZ/YFP} mice to lineagetrace Lgr5+ cells (Figure S2E). CreER induction using 4-OH tamoxifen (Tmx) led to rapid recombination of the Rosa^{LacZ} allele. Cre reporter that could be visualized by X-Gal staining (blue precipitate in Figure S2E). Quiescent Lgr5+ cells generated upon 4 days of iEGFR treatment. Tmx was introduced to the medium during the last day of the treatment and removed



ENR **iEGFR**

Figure 1. EGFR Inhibition Induces Cell-Cycle **Exit in Intestinal Organoids**

(A) Experimental setup for (B)-(H). Organoids were treated with either the EGFR inhibitor gefitinib in the absence of EGF (iEGFR) or DMSO in standard ENR medium (control) 1 week after plating in Cultrex® Basement Membrane Extract (BME). Samples were collected 1 day (d1), 2 days (d2), 4 days (d4), or 7 days (d7) after treatment.

(B) Bright-field images of intestinal organoids after 4 days of iEGFR treatment or culture in control (ENR) medium. Crypts and differentiated units are visible in ENR. while iEGFR-treated organoids mainly contain crypt-like structures that are placed closer to each other.

(C) GFP fluorescence of Lgr5^{GFPiresCreER/+} (green) organoids shows that Lgr5+ cells persist following iEGFR treatment.

(D) Quantification of circumference of organoids after 4 days of iEGFR treatment or in control cultures

(E) Analysis of the cell cycle in intestinal organoids. EdU was administered 1 hr prior to the sacrifice. Control (ENR) organoids continuously incorporate EdU (top panels) and express KI67 (bottom panels), while iEGFR-treated organoids exit the cell cycle over time

(F) Quantification of (E).

(G) Analysis of the cell cycle of iEGFR-treated organoids following reintroduction of EGF in the culture medium. KI67 expression and EdU incorporation were analyzed 1 day (d1), 3 days (d3), or 5 days (d5) after replating in ENR.

(H) Lgr5^{GFPiresCreER/+}+ cells exit the cell cycle upon 4 days of iEGFR treatment. Phospho-histone H3 (pH3) staining was used to visualize M phase. The graph at the bottom shows the quantification. DAPI was used to visualize the nuclei.

Scale bars, 50 um. Error bars represent SD. All fluorescent images are confocal sections. (B) and (C) are optical sections. (E), (G), and (H) are 3D reconstructions. See also Figures S1 and S2.

when Egf signaling was reactivated. Labeled and reactivated guiescent Lgr5+ cells gave rise to organoids entirely labeled with X-Gal, as visualized two passages after Tmx induction. As control, labeled

Dclk1^{GFPiresCreER}Rosa^{LacZ} cells (marking tuft cells) did not generate new organoids consistent with their differentiated nature (the rare blue cells are persisting Tuft cells). Since only stem cells can generate new organoids in intestinal organoid cultures (Sato et al., 2009), these findings indicated that quiescent Lgr5+ cells generated by EGFR inhibition retain their stemness.

To evaluate the cellular composition of iEGFR-treated organoids, we performed immunofluorescence analysis. Quantification of the number of marker-positive cells per organoid revealed that absolute numbers of LYZ+ Paneth cells and CHGA+ EECs were not significantly increased after 4 days in iEGFR (Figure 2A). Mucin-2 (MUC2) immunostaining revealed that a comparable amount of goblet cells were present following iEGFR treatment (Figure S2F). Tuft cells (intestinal M-cells) are rare



Figure 2. Differentiation Status of EGFR Inhibited and Reactivated Organoids Indicates Lineage Bias toward Enteroendocrine Cells

(A) Marker analysis of enteroendocrine cells (CHGA, green) and Paneth cells (LYZ, red) indicates that both cell types remain unchanged after 4 days of EGFR inhibition (iEGFR).

(B) Tuft cell numbers, quantified using DCLK1^{GFP} expression or with their characteristic apical actin bundles (visualized by Phalloidin and acetylated Tubulin staining), are increased after iEGFR treatment. Graph shows quantification.

(C) Experimental paradigm used to assess proliferation and differentiation potential of reactivated quiescent stem cells. Organoids were treated with EGFR inhibitor and subsequently replated in ENR medium (EGF reactivation) or medium without EGF (iEGFR release) to recover in two consecutive rounds.

(D) Proliferation (KI67 expression) is restored after recovery from the second iEGFR treatment, indicating cell-cycle inhibition is reversible. The number of enteroendocrine cells (CHGA+), but not Paneth cells (LYZ), was increased after consecutive iEGFR treatment. This was more pronounced after iEGFR release.

(E and F) Quantification of (D). (E) The number of KI67+ cells normalized to the circumference of the quantified sections. (F) Quantification of the absolute number of CHGA+ and LYZ+ cells per organoid.

(G) qPCR analysis of lineage markers in reactivated organoids that were cultured for 1 week in ENR following 4 days of iEGFR treatment.

Scale bars, 50 um. Error bars represent SD. All fluorescent pictures are 3D reconstructions confocal images, except for top panels in (D) that show optical confocal sections. See also Figure S2.

Next, we asked whether stem cells could survive repeated cycles of cell-cycle exit and entry (Figure 2C). Upon EGFR reactivation followed by washout and the addition of EGF, proliferation was

mechanosensory cells involved in response to parasitic invasion (Howitt et al., 2016). Apical actin bundles that are revealed by acetylated tubulin and F-actin (Phalloidin) staining distinguishes Tuft cells (Höfer and Drenckhahn, 1996). The number of Tuft cells per organoid increased upon iEGFR treatment (Figure 2B). We corroborated these results using the Dclk1^{GFPiresCreER} allele (Nakanishi et al., 2013), revealing that iEGFR treatment increased the absolute number of Dclk1+ Tuft cells 3.2-fold (11.3 ± 6.6 in ENR and 35.8 ± 8.8 in iEGFR; Figure 2B). GFPmarked cells almost invariably contained acetylated tubulin bundles confirming the specificity of the Dclk1 allele (Figure 2B). The absence of EEC, Paneth cell, and Tuft cell markers in ${\rm Lgr5}^{\rm GFPiresCreER} {\rm +}$ cells argued against upregulation of Lgr5 in differentiated cells (Figure S2G). Thus, continuous EGFR inhibition drives Lgr5+ cells into guiescence and leads to a loss of proliferating cells. However, this treatment provokes no change in the absolute number of differentiated cells, with the exception of inducing an increase in Tuft cell numbers.

restored to control levels (Figures 2C–2E). Some proliferation was even restored in the absence of exogenous EGF, likely due to endogenous EGF secreted by Paneth cells (Figures 2C–2E). These findings indicated that iEGFR-induced quiescence is reversible and that quiescent stem cells maintain their self-renewal potential.

While the absolute number of LYZ+ Paneth cells was not changed upon EGFR reactivation compared to the controls, CHGA+ EEC numbers were somewhat increased (Figures 2D and 2F). Similarly, absolute numbers of CHGA+ cells were higher in the absence of exogenous EGF, even though organoid size was considerably smaller compared to control organoids (Figures 2D, 2E, and S2H).

To corroborate these findings, we analyzed marker gene expression for key cell types in reactivated organoids using qPCR (Figure 2G; Table S5). After 1 week of reactivation, expression of proliferation markers Ki67 and Ccnb2 were restored to control levels. Moreover, lineage markers for Paneth cells (Lyz),



Figure 3. EGFR-Signaling-Induced Cell-Cycle Exit Is Mediated by the MAPK Signaling Pathway

(A) PathScan analysis of EGFR-inhibited organoids. AKT and ERK pathways are effectively inhibited after 1 hr of EGFR inhibition, which is maintained over 24 hr.

(B) Single inhibition of MEK (iMek) or ERK (iMek) as well as simultaneous inhibition of EGFR and ErbB-2 using afatinib yields similar results to gefitinib-induced EGFR inhibition. EdU is added to the culture medium 1 hr before the sacrifice. Middle panels show endogenous GFP expression from the Lgr5^{GFPiresCreER} allele. DAPI is used to visualize the nuclei in the bottom panels.

Scale bars, 50 um. Error bars represent SD. See also Figure S3.



Goblet cells (Gob5), and enterocytes (Alpi) were restored to nearnormal ratios. Expression of the EEC marker Chga was elevated upon reactivation (Figure 2G). Thus, all lineages could be generated from reactivated Lgr5+ cells, suggesting that EEC generation is enhanced by reduced EGF/EGFR signaling.

MAPK Signaling Downstream of EGFR Controls Intestinal Stem Cell Proliferation

Mitogen-associated protein kinase (MAPK) signaling is a major downstream target of EGFR signaling pathway and regulates cell-cycle progression. MAPK kinase (MEK) phosphorylates MAPK (ERK) to induce its nuclear localization and activation. Phosphatidylinositol 3-kinase (PI3K)/AKT pathway is also downstream of EGFR and is, for instance, implicated in neuroendocrine tumors (Banck et al., 2013). To quantify changes in ERK phosphorylation and AKT pathway activation, we used PathScan array analysis (Figures 3A and S3A). Phosphorylation of both ERK/2 and AKT at Thr306 and Ser473 was reduced as early as 1 hr after iEGFR treatment of organoids and remained low 24 hr after treatment (Figure 3A). S6 ribosomal protein phosphorylation, a target of AKT signaling, dropped after 3 hr, while mTOR and PTEN phosphorylation was reduced only after 24 hr (Figure 3A).

To evaluate the temporal change of ERK phosphorylation upon iEGFR treatment, we performed immunohistochemistry. iEGFR reduced ERK phosphorylation as early as 1 hr after treatment, consistent with the PathScan results (Figure S3B). However, we observed a gradual and partial recovery in phospho-ERK (pERK) levels within 48 hr, despite continuing quiescence (Figure S3B). Thus, we asked whether MEK/ ERK signaling is essential for cell-cycle progression of intestinal stem cells using small inhibitors for either MEK (PD0325901; Meki) or ERK (SCH772984;

Erki). Both inhibitors induced quiescence of Lgr5+ cells, implying that the ERK pathway downstream of EGFR is required for proliferation of Lgr5+ cells (Figure 3B). The use of afatinib, which inhibits both EGFR and ErbB2, yielded similar results (Figure 3B). These results indicated that inhibition of MAPK signaling could induce a reversible quiescent state in intestinal organoid stem cells, similar to iEGFR treatment. These data implied that decreased MAPK/ERK signaling suffices for cell-cycle exit of Lgr5+ cells.

RNA Sequencing Reveals the Molecular Signature of Quiescent Lgr5+ Stem Cells

To better understand the molecular characteristics of quiescent Lgr5+ cells, we performed bulk RNA sequencing on FACS-isolated control (DMSO) and quiescent (iEGFR treatment, day 4) Lgr5+ stem cells. We included both Lgr5^{GFPiresCreER/+} (n = 2) and Lgr5^{GFPDTR/+} (n = 2) organoids in our study to observe



potential differences in Lgr5 reporter expression. We also included sorted Tuft cells (using the Dclk1^{GFPiresCreER/+} allele) for comparison. Whole control organoids cultures were sequenced as a reference population. Hierarchical clustering and principal-component analysis (PCA) revealed that guiescent Lgr5+ cells were more similar to active Lgr5+ stem cells than to whole organoids or Tuft cells (Figures 4A and S4A). Differential gene expression analysis between active and guiescent Lgr5+ cells revealed 533 differentially regulated genes, 290 of which were enriched in guiescent Lgr5+ cells (false discovery rate [FDR] <0.01 Figures 4B and S4B; Table S1). Transcriptional targets of the Erk pathway (Etv4 [7.7×, p-adj < 0.001] and Etv5 [7.7×, p-adj < 0.001]) were downregulated in quiescent Lgr5+ stem cells, confirming efficient Erk inhibition (Figures 4C and S4B). Similarly, several cell-cycle-associated genes, such as Ccnb1 (2.1×, p-adj < 0.005) and Ccnb2 (1.9×, p-adj < 0.05), were decreased, consistent with cell-cycle arrest (Figure S4B). Gene Ontology (GO) analysis of the genes downregulated upon iEGFR treatment confirmed a clear loss of cell-cycle-associated genes (Figure S4C). In line with our reporter expression, we observed a significant increase in some of the well-known Wnt target genes, including Rnf43 (2.3×, p-adj < 0.005) and Lgr5 (2×, p-adj < 0.05) (Figure S4B). We also noticed a strong increase of members of the AP-1 family of transcription factors (Junb, Fos, and Fosb) in guiescent Lgr5+ cells (Figures 4C and S4B). Early markers for Paneth cells (Lyz1), enterocytes (Alpi1), and goblet cells (Muc2) remained unchanged (Figure 4C). Chga, expressed by EECs and their precursors, was 7.3-fold higher in quiescent compared to active Lgr5+ stem cells (Figure 4C). Similarly, while Dclk1 (6×, p-adj < 0.05) and some other Tuft cell markers increased upon iEGFR treatment, their levels were significantly lower in quiescent Lgr5+ cells than in Tuft cells (Figure 4C). These results confirmed our confocal analysis and highlighted key molecular changes in Lgr5+ stem cells upon quiescence entry.

The increase in per cell-Chga expression as well as the high CHGA+ cell numbers generated in the absence of EGF (Figures 3D and 3F) were reminiscent of the label-retaining secretory precursors (LRCs) described by Winton and colleagues (Buczacki et al., 2013). Indeed, gene set enrichment analysis (GSEA) revealed that the LRC signature is more similar to quiescent than to active Lgr5+ stem cells (Figure 4D; see STAR Methods). 12 out of 37 of the LRC genes were in the core enrichment group and included the EEC-related genes Chga, Chgb, Cldn4, Gip, and Ghrl2 (Table S2). Next, we analyzed the distribution of the "hallmarks" gene sets provided on the GSEA dataset (Figures 4D and S4D; Table S3). Analysis revealed an enrichment of "E2F targets" and "MYC targets V1 and V2" in active stem cells (Figure S4D). X2K transcription factor target analysis confirmed that 72% of the genes downregulated after iEGFR were targets of either MYC (62%) or E2F1 (38%) (Figure 4E). In addition, mTORC1-associated genes were downregulated upon EGFR inhibition (Figure 4D). The analysis also revealed a metabolic shift upon quiescence entry; genes associated with glycolysis, oxidative phosphorylation, and cholesterol metabolism were downregulated in guiescent stem cells (Figures 4D and S4D). On the other hand, quiescent stem cells were enriched in genes associated with tumor necrosis factor α (TNF- α) signaling via nuclear factor kB (NF-kB), interferon gamma response genes, and JAK-STAT3 signaling (Figure 4D; Table S3). In brief, GSEA analysis suggested that loss of proliferation might be driven by decreased of MYC/E2F1 activity. Quiescent stem cells downregulate several metabolic pathways and upregulate a signature related to TNF- α and JAK-STAT3 signaling (Figures 4D and S4D).

Combined Inhibition of the Wnt, Notch, and EGFR/MAPK Pathways Induces EEC Fate

We next aimed to establish a protocol for EEC differentiation. Inhibition of Notch signaling by DAPT treatment (iNotch) lead to a large increase in the number of LYZ+ Paneth cells (Figure 5A). Inhibition of Wnt secretion using IWP-2 (iWnt) in combination with iNotch abolished Paneth cell differentiation and induced EECs and goblet cells (Figure 5A). iEGFR treatment spared both Paneth cells and EECs (Figure 5A). Combined inhibition of WNT/Notch/EGFR pathways (iWnt/iNotch/iEGFR) resulted in a massive increase in EECs while inhibiting Paneth cell differentiation (Figure 5A). Similarly, inhibiting Mek together with Wnt and Notch signaling pathways (iWnt/iNotch/iMek) increased CHGA+ EEC numbers (Figure 5C). qPCR analysis confirmed that goblet cell differentiation induced by iWnt/iNotch treatment is countered by both iEGFR and iMek treatments (Figure S5A).

We used cleaved caspase-3 staining to evaluate cell death in these organoids. Only rare apoptotic cells were visible in the "crypt domain" of both standard and iWnt/iNotch/iMek-treated (24 hr) organoids. Similar to the controls, apoptosis was restricted to the "villus domain" upon iWnt/iNotch/iMek treatment (Figure S5D). These results implied that EECs are generated by altered cell-fate choice rather than massive apoptosis of remaining cell types.

We further analyzed the expression of EEC-related genes in differentiated organoids (Figure S5A). Expression of the pan-EEC marker Chga was 25-fold higher in iWnt/iNotch/iEGFRtreated organoids and over 100-fold higher in iWnt/iNotch/ iMek-treated organoids (Figure S5A). Concordantly, expression of Sst (55×), Gip (14×), Sct (5×), cholecystokinin (15×), and glucagon (Gcg/Proglucagon, 4×) mRNA were upregulated

(C) Boxplots displaying normalized expression values of marker genes.

Figure 4. RNA Sequencing Identifies Key Molecular Differences between Quiescent and Active Lgr5+ Stem Cells

⁽A) Hierarchical clustering of the whole transcript of sorted Lgr5+ cells using the Lgr5^{GFPDTR/+} (Lgr5^{DTR}), Lgr5^{GFPiresCreER/+} (Lgr5^{GFP}), and Tuft cells using the Dclk1^{GFPiresCreER/+} (Dclk1) organoids cultured in control medium (ENR) or upon EGFR inhibition (iEGFR) based on Pearson's correlation. Control organoids were added as a reference. Colors indicate Pearson correlation.

⁽B) Volcano plot comparing active and quiescent Lgr5 signatures. x axis shows adjusted p value (q value, in -log10), and y axis shows fold change (in log2). Each dots represents a gene; differentially expressed genes (false discovery rate < 0.01) are in red.

⁽D) Gene set enrichment analysis (GSEA). Fold change in gene expression in quiescent and active Lgr5+ stem cells is compared. Green line shows enrichment profile. Black bars show where genes from a given gene set are located (hit). NES, normalized enrichment score; FDR, false discovery rate.

⁽E) Expression2kinase (X2K) analysis showing key transcription factors targeting the active Lgr5+ stem cell signature. Error bars indicate SD. See also Figure S4 and Tables S1–S3.









upon iWnt/iNotch/iEGFR treatment, following a similar trend to iWnt/iNotch/iMek treatment (Figure S5A). Nts was the sole hormone analyzed that was expressed at control levels. Thus, our protocol generated high numbers of most subtypes of EECs (Egerod et al., 2012).

To visualize hormone production at the protein level, we used immunofluorescence (Figure 5B). We focused on the iWnt/iNotch/iMek condition, which yielded the highest CHGA+ cell numbers among the conditions tested (Figures 5C and 5D). The different EEC subtypes are rare in normal intestinal organoid cultures (Figure 5C). iWnt/iNotch/iMek treatment resulted in a robust increase in the number of CHGA, NTS, SEROTONIN, GIP, SCT, SST, and CCK+ cells (Figures 5C and 5D; Table S5). This implied that EECs induced in our culture system are functionally mature.

Regional Identity of Intestinal Organoids Determines EEC Heterogeneity

The intestinal tract displays regional differences in EEC subtype representation. We asked whether the regional origin of organoid cultures affects the EEC subtypes generated. Of note, a previous study demonstrated that gut organoids retain at least some aspects of their regional identity upon long-term culture (Middendorp et al., 2014). We established organoids from four different regions (duodenum to ileum) of the intestinal tract and analyzed EEC-related gene expression upon iWnt/iNotch/iMek using qPCR. iWnt/iNotch/iMek treatment induced Chga expression in all cultures when compared to standard culture conditions (Figure 5E). Nts- and Gcg-expressing cells predominantly reside in the distal small intenstine (SI) region, whereas Gip-expressing cells follow the opposite trend (Drucker and Nauck, 2006; Kitabgi and Freychet, 1978; Parker et al., 2009). Consistently, Nts and Gcg expression was much more strongly upregulated in the distal than the proximal organoids (Figure 5E). Conversely, while all regions upregulated Gip upon iWnt/iNotch/iMek treatment, levels were higher in organoids of a proximal origin. Organoids from all regions efficiently expressed Sst and Sct upon differentiation (Figure 5E). We conclude that while our induction protocol is applicable to organoids from all intestinal regions, the regional source of organoids affects the outcome in terms of specific EEC subtypes.

As the PI3K/Akt/mTOR pathway is reduced upon iEGFR and may also affect EEC differentiation, we inhibited mTOR signaling using Azd8055 (iTOR) (Figure S5B). Inhibition of iTOR on a iWnt/ iNotch background did not further increase CHGA+ cell numbers. On the contrary, iTOR treatment abrogated the increase in CHGA+ cell numbers when combined with iWnt/iNotch/iEGFR treatment (Figure S5B). qPCR analysis revealed decreases in Chga, Sst, Gip, Sct, Cck, and Gcg upon iTOR treatment (Figure S5C). Thus, while its levels are reduced upon iEGFR treatment, iTOR signaling is required for efficient generation of EECs by our induction protocol.

To better characterize the quiescent stem cell (iMek and iEGFR) and EEC (iWnt/iNotch, iWnt/iNotch/iEGFR, and iWnt/iNotch/ iMek) induction protocols, we performed RNA sequencing on bulk cultures at 6 hr and 96 hr (Figure 6A). PCA and hierarchical clustering revealed three distinct groups (Figures 6A and S6A). First, all organoids treated for 6h clustered together with untreated organoids isolated at 6 hr and 96 hr. iMek- and iEGFRtreated organoids clustered closely together in PCA space, consistent with the notion that both induce quiescent Lgr5+ stem cells. iWnt/iNotch, iWnt/iNotch/iEGFR, and iWnt/iNotch/ iMek cultures were distinct at 96 hr (Figures 6A and S6A). Separate samples from the same treatment group clustered closely together, confirming the reproducibility of the treatments (Figures 6A and S6A). Expression of the Erk target gene Etv4 is lost at 6 hr in both iMek (7.2-fold; FDR < 0.001) and iEGFR (4.6-fold; FDR < 0.005), confirming efficient inhibition.

Next, we used our dataset to directly compare the effects of iEGFR and iMek treatments. We measured the number of differentially expressed genes (FDR < 0.01) to visualize the differences between samples. At both 6 hr and 96 hr, iEGFR (1,440 and 1,307 differentially expressed genes at 6 hr and 96 hr, respectively) and iMek (1,147 and 1,631 differentially expressed genes at 6 hr and 96 hr, respectively) treatments induced massive changes of the transcriptomes of the organoids (Figure S6B, red dots indicate differentially expressed gene). Transcriptomes of iMek- and iEGFR-treated cultures were almost identical at both time points (5 and 88 differentially expressed genes at 6 hr and 96 hr, respectively). iWnt/iNotch/iEGFR (3,847 differentially expressed genes) and iWnt/iNotch/iMek (3,166 differentially expressed genes) treatments were drastically different from controls at 96 hr. While organoids subjected to both treatments clustered together at 96 hr (Figure S6A), 267 genes were differentially expressed between EEC cultures differentiated with iWnt/iNotch/iEGFR versus iWnt/iNotch/iMek treatments. Most noticeable genes were goblet cell-related factors, such as Clca1 (2.4-fold, p < 0.001) and Zg16 (2.5-fold, p < 0.001; Figure 6B). In conclusion, while iEGFR and iMek treatments can be used interchangeably in the context of quiescent stem cell induction, iMek is more efficient in countering goblet cell differentiation.

Next, we scrutinized EEC differentiation. At 96 hr, Chga and Chgb expression were highly elevated in iWnt/iNotch/iEGFR

Figure 5. Derivation of a High-Purity EEC Culture

Scale bars, 50 um. Error bars indicate SD. See also Figure S5 and Table S5.

 ⁽A) Marker analysis of enteroendocrine cells (CHGA, green) and Paneth cells (LYZ, red). Organoids were treated for 4 days with the Notch inhibitor DAPT (iNotch), the inhibitor of Wnt secretion IWP-2 (iWnt), gefitinib (iEGFR), or a combination of these treatments. DMSO was used as a control. Images show optical sections.
 (B) Model shows critical signaling pathways manipulated in organoids for directed differentiation of intestinal stem cells.

⁽C) Inhibition of Mek signaling (iMek) together with Wnt and Notch signaling pathways (iWnt/iNotch/iMek) similarly increases enteroendocrine cell numbers (CHGA+). Neurotensin (NTS), serotonin, gastric inhibitory polypeptide (GIP), secretin (SCT), somatostatin (SST), and cholecystokinin (CCK)-positive cell numbers dramatically increase. Representative 3D reconstruction confocal images are shown.

⁽D) Quantification of the number of enteroendocrine cell markers per organoid upon iWnt/iNotch/iMek treatment.

⁽E) Regional identity of organoids is maintained in terms of enteroendocrine cell subtypes. Organoids were isolated from proximal-to-distal (#1–#4) small intestine. Distal organoids have higher levels of Nts and Gcg levels, while Gip is enriched proximally.



B Expression of key EEC and Goblet cell genes after 96h of treatment



Figure 6. Characterization of Organoids following Different Induction Regimens using RNA Sequencing

(A) Principal-component analysis (PCA) of the transcriptomes of samples treated with iMek, iEGFR, iWnt/iNotch, iWnt/iNotch/iEGFR, iWnt/iNotch/iMek, and DMSO-treated controls. Samples were analyzed after 6 hr (6h, square) or 96 hr (96h, circle) of treatment.

(B) Heatmap showing the expression of key genes related to enteroendocrine cell (EECs) and goblet cells at 96 hr. Color code shows the *Z* score for each gene along the whole dataset.

See also Figure S6.

and iWnt/iNotch/iMek treatments (Figure 6B). Similarly, most EEC genes, including Gip, Sst, Sct, Tac1, Tph1, and Reg4, were increased in both conditions. We noticed that expression of Cck, Gcg, Ghrl, and Reg3a was upregulated in iWnt/iNotch and not further enhanced by the addition of iEGFR and iMek (Figure 6B). Nts expression was not enriched following our EEC differentiation protocols, most likely because duodenum organoids were used. Even so, NTS was clearly expressed by rare cells (Figures 5D and 6B). In brief, both iWnt/iNotch/iEGFR and iWnt/iNotch/iMek conditions efficiently induce generation of multiple EEC subtypes, even though the ratio of the subtypes generated is different.

Single-Cell Sequencing Reveals Heterogeneous EECs in Reactivated Cultures

We previously used single-cell sequencing to reveal EEC subtypes in vivo (Grün et al., 2015). To elucidate the cellular composition of induced organoids and the extent of heterogeneity in hormone expression, we performed single-cell RNA sequencing (Figure 7). We sorted live single cells (without additional markers) from iWnt/iNotch/iEGFR- and iWnt/iNotch/iMek-treated organoids. Among the 289 cells that passed our filtering, we identified a cluster of 94 cells as enterocytes enriched in Aldob (4.9×, p-adj < 0.001), Apoa1 (12.6×, p-adj < 0.001), and Alpi (5.6×, p-adj < 0.001) (Figures S7A and S7B). These were interpreted as surviving post-mitotic enterocytes and were excluded from further analysis. Cells derived from both iWnt/iNotch/iEGFRand iWnt/iNotch/iMek-treated organoids were distributed similarly in t-distributed stochastic neighbor embedding (t-SNE) space and were analyzed together (Figure S7C).

Using RaceID2 (Grün et al., 2016), we identified 12 distinct clusters of cells (Figures 7A and 7B). k-medoids clustering of the Pearson correlation of cellular transcriptomes revealed a clear separation between clusters as well as possible heterogeneity within clusters (e.g., 7 and 8, Figure 7A). Differential gene expression analysis revealed signature genes for each cluster. which we used to classify cell types (Table S4). The most prominent clusters ("3" [53 cells] and "4" [35 cells]) expressed the pan-EEC markers Chga and Chgb (Figures 7C and S7D). Chga and Reg4 expression formed a gradient, both being higher in cluster 4. Hormonal production in these Chgb high clusters was best defined by Tac1 and Tph1 expression, both markers of enterochromaffin cells (Figures 7B, 7C, and S7D). Tac1 encodes for the hormone substance P, while Tph1 encodes for the rate-limiting enzyme in serotonin synthesis (Egerod et al., 2012; Grün et al., 2015). Substance P and serotonin may act as neurotransmitters exciting the connected enteric neurons (Latorre et al., 2016). The other clusters displayed relatively low levels of Chga and Chgb transcripts but included cells expressing peptide hormones (Figures 7C and S7D). Cluster 2 (21 cells) was marked by Gip expression (74×) that is expressed by K-cells. Fabp5 was also highly enriched in this cluster (12.6×), consistent with its role in Gip secretion (Shibue et al., 2015). Members of cluster 5 (nine cells) expressed very high levels of Sst (182×), identifying them as D-cells (Figure 7C). Ghrelin (Ghrl) expression was present in more than one cluster but was highest in cluster 6 (19×, three cells). We also noticed that Islet1 (IsI-1; 9.7×) was co-expressed with Ghrl in these cells. Islet1 plays an important role in cell fate specification, and its loss leads



t-SNE map of RaceID2 clusters

Figure 7. Single-Cell Transcriptome Profiling Reveals Heterogeneity among Induced EECs

(A) Heatmap displaying k-medoids clustering of Pearson's correlation of the whole transcriptome of individual live organoid cells from iWnt/iNotch/iEGFR and iWnt/iNotch/iMek experiments after filtering. Numbers indicates clusters. The colors code for Pearson's correlation.

(B) t-SNE map depicting individual cells and cluster numbers assigned by RaceID2.

(C) Heatmap displaying the log2 transformed color-coded transcript counts of respective genes related to the enteroendocrine lineage.

(D) t-SNE map displaying the heterogeneous expression of Ecn3 and Tac1 transcripts by Chga high cells.

See also Figure S7 and Table S4.

to impaired glucose homeostasis (Terry et al., 2014). Cells in cluster 7 (18 cells) all highly expressed Cck (55.7×).

One of the early inducers of EEC differentiation is neurogenin-3 (Neurog3), which is followed by Neurod1. Neurog3 (5.2×) expression was highest in cluster 9 (six cells) and in some cells of cluster 3 that were most similar to cluster 9. Virtually all EEC clusters contained Neurod1-expressing cells (Figure 7B). Given the temporal expression of these transcription factors, we propose that cluster 9 represents EEC progenitors, which through Neurod1 generate a panel of EECs. Cluster 1 (18 cells) was enriched in goblet cell- and Paneth cell-related genes, such as Agr2 (33×), Muc2 (26×), Ttf3 (23×), and Defa24 (28×). Despite the filtering, some enterocyte-like cells expressing Aldob and Mt1/2 remained (cluster 8, seven cells). Dclk1 and Trpm5 expression identified cluster 10 (15 cells) as Tuft cells (Figures 7B and S7C). In total, 145/289 cells (50% of all cells) analyzed were EECs or their progenitors, confirming the efficiency of our induction protocol.

Since multiple hormones can be co-expressed in the same cell, we addressed the heterogeneity of hormone expression at the single-cell level (Figure 7C). Focusing on EEC-related gene expression, we identified occasional expression of multiple different hormones in a single cell (Figure 7B). This was in line with our previous report on EECs from freshly isolated intestinal epithelium (Grün et al., 2015). A prominent example is cluster 7, where Cck+ cells also expressed Gcg (28.2×), Ghrl (5.3×), or Pyy (11.4×). Consistently, I-cells have been reported to co-express Cck with other hormones at varying levels (Egerod et al., 2012). Transcriptomes of Sst+ cells were more homogeneous, co-expressing low levels of Gip and Cck, while one cell co-expressed Ghrl only. We previously reported partial overlap between Cck+ and Tac1+ cells (Grün et al., 2015). Consistently, some of the Tac1+ cells in clusters 3 and 4 expressed low levels of Cck (Figures 6B and 6C). Similar to their in vivo counterparts, EECs induced with our protocol contained Chga+ Tac1+ Ucn3+, Chga+ Tac1+ Ucn3-, and Chga+ Tac1- Ucn3- cells (Figure 7D). Thus, EECs generated in our cultures recapitulate EEC heterogeneity seen in the intestinal epithelium in vivo. Taken together, our single-cell analysis indicated that the protocol induces EEC fates in ${\sim}50\%$ of organoid cells based on marker gene expression.

DISCUSSION

Here, we identify EGF signaling as an indispensible driver of Lgr5+ stem cell proliferation in organoids. Under conditions where Wnt signaling is untouched but EGF signaling is blocked, actively dividing Lgr5+ stem cells convert into quiescent Lgr5+ cells that retain expression of various Wnt target genes. This cellular state can be maintained for up to a week. Yet, the simple restoration of EGF signaling converts the quiescent cells back into their normal active stem cell state. In organoids as well as in crypts, Lgr5+ cells are always the direct neighbors of the Wnt3-secreting Paneth cells (Sato et al., 2011). In this setting, Wnt3 does not diffuse over distances, but is loaded directly onto the Lgr5+ stem cells (Farin et al., 2016). The quiescent Lgr5+ stem cells remain juxtaposed to the Paneth cells in iEGFR treated organoids and are thus exposed to high local Wnt signals. Indeed, three independent Wnt target gene alleles as well as gene expression analyses confirmed robust Wnt signaling upon EGFR inhibition. In sum, our results show that maintenance of stem cell fate requires Wnt, but not EGF, whereas stem cell proliferation depends on the combination of Wnt and EGF. Whether guiescent stem cells are more competent to remain in the niche when in competition with dividing stem cells remains an open question.

Previous studies have identified quiescent cells located close to the zone of differentiation at the +4 position with stem cell potential (Clevers, 2013). We have reported the existence of Dll1+ secretory precursors at this position (van Es et al., 2012). Using a histone label retention assay, Doug Winton's group identified a chromatin-label-retaining population with secretory differentiation potential. These LRCs share a signature with crypt base columnar cells (CBCs), including the expression of Lgr5, but express significant levels of some of the secretory lineage genes, such as Chga (Buczacki et al., 2013). Taken together, these secretory precursors represent transient states yet can de-differentiate into stem cells when the need arises and can thus be considered facultative stem cells (Buczacki et al., 2013; van Es et al., 2012). A similar situation exists for the abundant enterocyte precursors in the crypt (Tetteh et al., 2016).

We noticed a slight bias of quiescent Lgr5+ cells (induced in culture) toward expression of EEC markers, such as Chga, which made them reminiscent of the in vivo Lgr5+-label-retaining cells identified by Doug Winton. EGFR signaling has been shown to be essential for the production of goblet cells (Heuberger et al., 2014). Our current data show that simultaneous inhibition of enterocyte, Paneth, and goblet cell fate by inhibiting Notch, Wnt, and EGFR signaling, respectively, is the key to the generation of EECs.

This culture system may yield answers toward some of the major outstanding questions about the biology of the enigmatic EECs. It is unclear what signals drive the fate specification of the different subtypes of EECs. It is not known if the physiological processes that are controlled by specific EEC subtypes in turn feed back into the formation of the pertinent subtypes of EECs. Little is known about the triggers that lead to secretion of hormones beyond the identification of a handful of receptors and their ligands (Janssen and Depoortere, 2013). EEC-derived hormones have been implied in conditions of major importance such as depression, glucose insensitivity/diabetes, and obesity (Latorre et al., 2016). A detailed mechanistic understanding of the biology of EECs can be derived using this culture system and may yield insights with broad therapeutic impact.

A detailed description of the materials and methods used in the study is given in the STAR Methods.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.11.001.

AUTHOR CONTRIBUTIONS

O.B., J.B., and H.C. designed the experiments and wrote the manuscript; J.B. performed the cell culture experiments; O.B. and K.W. performed RNA-sequencing experiments under the supervision of A.v.O.; O.B., J.B., and K.W analyzed the data; J.B. was supervised by O.B. and H.C; and H.S. contributed the Dclk1 knockin allele.

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STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit anti-Lysozyme (EC3.2./.17)	DAKO	A0099, RRID: AB_2341230
goat anti-Chromogranin A (C-20)	Santa Cruz	Sc-1488, RRID: AB_2276319
mouse anti-Ki67	BD PharMingen	550609, RRID: AB_393778
rabbit anti-phospho-Histone 3(pH3, Ser10)	Millipore	06-570, RRID: AB_310177
mouse anti-Cytokeratin 20 (KS20.8)	DAKO	M7019, RRID: AB_2133718
goat anti-Cholestocystokin	Santa Cruz	Cat# sc-21617, RRID: AB_2072464
rabbit anti-Neurotensin	Santa Cruz	Cat# sc-20806, RRID: AB_2155562
goat anti-Secretin	Santa Cruz	Cat# sc-26630, RRID: AB_656130
goat anti-Somatostatin	Santa Cruz	Cat# sc-7819, RRID: 2302603
goat anti-Serotonin	Abcam	ab66047, RRID: AB_1142794
rabbit anti-Gastric inhibitory polypeptide	Abcam	ab22624-50, RRID: AB_2109683
mouse anti-acetylated Tubulin	Santa Cruz	Sc-23950, RRID: AB_628409
Mouse anti-mucin2 (clone CCP58)	Monosan	MONX10515
Alexa Fluor 488 goat anti-mouse IgG (H+L)	Thermo Fisher scientific	A11029, RRID: AB_2534088
Alexa Fluor 488 donkey anti-rabbit IgG (H+L)	Thermo Fisher scientific	A21206, RRID: AB_2535792
Alexa Fluor 488 donkey anti-goat IgG (H+L)	Thermo Fisher scientific	A11055, RRID: AB_2534102
Alexa Fluor 568 goat anti-rabbit IgG (H+L)	Thermo Fisher scientific	A11036, RRID: AB_10563566
Alexa Fluor 647 donkey anti-rabbit IgG (H+L)	Thermo Fisher scientific	A31573, RRID: AB_2536183
AlexaFluor 647 donkey anti-mouse IgG (H+L)	Thermo Fisher scientific	A31571, RRID: AB_162542
Alexa Fluor 647 goat anti-rat IgG (H+L)	Thermo Fisher scientific	A21247, RRID: AB_10563568
Envision+ System –HRP polymer anti-mouse	DAKO	K4001
eFluor-660 conjugated rat anti-KI67 (Clone:SolA15)	eBiosciences	50-5698-80, RRID: AB_2574234
Chemicals, Peptides, and Recombinant Proteins		
DAPI solution (1mg/ml)	Thermo Fisher scientific	62248
AlexaFluor 647 Phalloidin	Thermo Fisher scientific	A22287
Vectashield	Vector Labs	H-1000
Gefitinib	Santa Cruz Biotechnology	sc-202166
Azd8055	Selleckchem	S1555
SCH772984	Selleckchem	S7101
IWP-2	Stemgent	130-105-335
DAPT	Sigma Aldrich	D5942
PD0325901	Sigma Aldrich	PZ0162
Critical Commercial Assays		
Click-iT Assay Kit	Thermo Fisher scientific	C10340
Deposited Data		
Single cell (Figure 7) and bulk sequencing data of sorted cells (Figure 4) or organoids (Figure 6)	This paper	GEO: GSE80636
Experimental Models: Organisms/Strains		
Lgr5-GFPiresCreER	Barker et al., 2007	Hans Clevers, clevers@hubrecht.eu
Lgr5-GFPDTR	Tian et al., 2011	Frederic J. de Sauvage, Department of Molecular Biology, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA
Dclk1-GFPiresCreER(Nakanishi et al., 2013)	Nakanishi et al., 2013	Hiroshi Seno, seno@kuhp.kyoto-u.ac.jp
B6;129S4-Gt(ROSA)26Sor ^{tm1Sor} /J	Soriano, 1999	https://www.jax.org/strain/003309
Gt(ROSA)26Sor ^{tm10.1(Tcf/Lef-CFP)Mgn}	Serup et al., 2012	Kenneth S. Zaret, zaret@upenn.edu

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
RaceID2	Grün et al., 2016	https://www.github.com/dgrun/StemID
DESeq	Anders and Huber, 2010	https://www.bioconductor.org/packages/release/ bioc/html/DESeq.html
Deseq2	Love et al., 2014	https://www.bioconductor.org/packages/release/ bioc/html/DESeq2.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for reagents should be directed to Prof. Hans Clevers at clevers@hubrecht.eu.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Strains Used to Initiate Organoid Cultures

Primary organoid cultures used in this study were derived from Lgr5^{GFPiresCreER/+} (Barker et al., 2007), Lgr5^{GFPDTR/+} (Tian et al., 2011), Dclk1^{GFPiresCreER/+} (Nakanishi et al., 2013) and Rosa^{TCF-CFP/+} (Gt(ROSA)26Sor^{tm10.1}(Tcf/Lef-CFP)Mgn) mice (Serup et al., 2012). For lineage tracing experiments, organoids were derived from the Lgr5^{GFPiresCreER/+};Rosa^{LacZ/YFP} and Dclk1^{GFPiresCreER/+};Rosa^{LacZ/+} mice. All mice were bred on a C57BL/6 background. All animal procedures and experiments were performed in accordance with national animal welfare laws under a project license obtained from the Dutch Government, and were reviewed by the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW). All rodents are housed in a barrier facility in conventional cages and are changed without using a change stations. All personnel entering the barrier must wear protective clothing (including head caps, specials clogs). All animals are received directly from approved vendors (Charles River) or generated in house. Animals arriving from other sources must pass the GDL –quarantine for screening or by embryo-transfer. After screening these SPF mice are housed in micro isolator cages and are transferred to the Hubrecht laboratory.

METHOD DETAILS

Organoid Culture

The basic culture medium (advanced Dulbecco's modified Eagle's medium/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax, B27 [Life Technologies, Carlsbad, CA] and 1 mM N-acetylcysteine [Sigma]) was supplemented with 50 ng/ml murine recombinant epidermal growth factor (EGF; Peprotech, Hamburg, Germany), R-spondin1 (conditioned medium, 5% final volume), and Noggin (conditioned medium, 5% final volume), called ''ENR'' medium. Conditioned media were produced using HEK293T cells stably transfected with HA-mouse Rspo1-Fc (gift from Calvin Kuo, Stanford University) or after transient transfection with mouse Noggin-Fc expression vector. Advanced Dulbecco's modified Eagle's medium/F12 supplemented with penicillin/streptomycin, and Glutamax was conditioned for 1 week.

Organoids were derived from the duodenum of the Lgr5^{GFPiresCreER/+} (Barker et al., 2007), Lgr5^{GFPDTR/+} (Tian et al., 2011), Dclk1 ^{GFPiresCreER/+} (Nakanishi et al., 2013) and Rosa^{TCF-CFP/+} (Gt(ROSA)26Sor^{tm10.1}(Tcf/Lef-CFP)Mgn) mice (Serup et al., 2012). For experiment displayed in Figure 5E, organoids were derived from 4 different regions spanning the proximal-distal axis of the intestine. For lineage tracing experiments, organoids were derived from the Lgr5^{GFPiresCreER/+};Rosa^{LacZ/YFP} and Dclk1^{GFPiresCreER/+};Rosa^{LacZ/+} mice.

Organoids were plated in BME (Trevigen) and treated with the EGFR inhibitor Gefitinib (5 μ M; Santa Cruz Biotechnology), EGFR and ErbB-2 inhibitor Afatinib (10 μ M, Selleckchem), MEK inhibitor PD0325901 (5 μ M; Sigma Aldrich) or ERK inhibitor SCH772984 (10 μ M, Selleckchem) while EGF was withdrawn from the medium. Wnt secretion was inhibited with IWP-2 (1,5 μ M; Stemgent) and Notch with DAPT (10uM, Sigma Aldrich). All treatments were performed on organoids 5-7 days after passaging. For EGFR reactivation experiments, organoids were replated in fresh BME and ENR medium to make sure EGFR inhibitor is washed away. For the repeated EGF withdrawal experiment in Figures 2C–2E, EGF was omitted in the medium during reactivation. For mTOR inhibition, Azd8055 (Selleckchem) was added to the medium at 0.1mM concentration. For induction of Cre-ER activity, organoids were treated overnight with 4-OH tamoxifen (1uM). All control organoids were treated with similar concentrations of the compound dissolvent, dimethyl sulfoxide (DMSO). During treatments, cells were imaged using an EVOS microscope (Electron Microscopy Sciences).

For the induction of enteroendocrine differentiation, cells were either cultured in standard culture conditions (ENR). 5 days after plating in BME, medium was removed and organoids were washed with PBS before re-embedding in BME. The cocktail for EEC differentiation included: IWP2 (1,5 μM; Stemgent), DAPT (10 μM, Sigma Aldrich) and MEK inhibitor PD0325901 (1 μM; Sigma Aldrich) or Gefitinib (5 μM; Santa Cruz Biotechnology).

Immunostainings

Whole organoids were collected by gently dissolving the BME in ice-cold PBS, and subsequently fixed overnight at 4°C in 4% paraformaldehyde (Sigma). Next, organoids were permeabilized and blocked in PBS containing 0,5% Triton X-100 (Sigma) and 2% normal donkey serum (Jackson ImunoResearch) for 30 min at room temperature. Organoids were incubated for 2 hr at room temperature in blocking buffer containing primary antibodies. Primary antibodies used were rabbit anti-Lysozyme (1:500; DAKO), goat anti-Chromogranin A (1:500; Santa Cruz), mouse anti-Ki67 (1:250; BD PharMingen), rabbit anti-phospho-Histone 3 (pH3 Ser10, 1:1000; Millipore), mouse anti-Cytokeratin 20 (1:1000; Dako), goat anti-Cholestocystokin (sc-21617,1:100; Santa Cruz), rabbit anti-Neurotensin (sc-20806,1:100; Santa Cruz), goat anti-Secretin (sc-26630,1:100; Santa Cruz), goat anti-Somatostatin (sc-7819, 1:100; Santa Cruz), goat anti-Serotonin (ab66047, 1:1000, Abcam), rabbit anti-Gastric inhibitory polypeptide (ab22624-50, 1:500, Abcam) and mouse anti-acetylated tubulin (1:100; Santa Cruz). Organoids were incubated with the corresponding secondary antibodies Alexa488, 568 and 647 conjugated anti-rabbit, anti-goat and anti-mouse (1:1000; Molecular Probes), in blocking buffer containing DAPI (1;1000, Invitrogen), or with Alexa 647 conjugated Phalloidin (Thermo Fisher scientific, 1:2000). EdU incorporation was visualized using the Click-iT Assay Kit (Thermo Fisher), after 1 hr pre-incubation with EdU (10uM). LacZ staining was performed as previously described (Barker et al., 2007). Alexa 647 conjugated Phalloidin (Thermo Fisher scientific, 1:2000) was added together with the secondary antibodies. Sections were embedded in Vectashield (Vector Labs) and imaged using a Sp5 and Sp8 confocal microscope (Leica). Image analysis was performed using ImageJ software.

FACS Sorting

For FACS analysis of Lgr5 and KI67 expression, Lgr5^{GFPDTR/+} organoids were first dissociated into single cells through mechanical disruption, after 15 min of Trypsin treatment at 37°C (TrypLE Express; Life Technologies). Single cells were fixed on ice using 4% paraformaldehyde for 30 min, and washed 3 times in PBS. Cells were permeabilized in PBS containing 0,5% Triton X-100 for 30 min, and were stained with an eFluor-660 conjugated rat anti-KI67 (1:1000; eBioscience) antibody for 30 min on ice. For cell cycle analysis, cells were stained in 1ug/ml Hoechst 33342 (ThermoFisher). Subsequently, stained cells were analyzed on a BD FACS Calibur (BD Biosciences).

For RNA-sequencing analysis in Figures 4 and 7, organoids were dissociated and immediately sorted using a BD FACS Aria (BD Biosciences). For bulk sequencing experiments in Figure 4, up to 5000 cells were sorted in Trizol in eppendorf tubes. For single cell sequencing experiment, cells were sorted as single cells into 384-well plates containing ERCC spike-ins (Agilent), RT primers (Hashimshony et al., 2012) and dNTP (Promega).

RNA Isolation

For RNA-sequencing of sorted cells in bulk, cells were sorted into Trizol (Life Technologies) and total RNA was isolated according to the manufacturer's instructions, with the following alterations. RNA was precipitated overnight at -20° C, with 2ug glycogen (Life Technologies). No additional RNA isolation step was used for cells sorted into 384-wells. For quantitative PCR analysis, RNA was isolated from organoids using the RNAeasy kit (QIAGEN) as instructed in the manufacturers protocol. For bulk sequencing experiment described in Figure 6, organoids were treated in triplicate for 6 or 96 hr in 48-well plates, collected and washed in PBS. RNA was isolated using Trizol as described above. 10 ng RNA was used as starting material for sequencing reactions.

Quantitative PCR

PCR analysis was performed using the SYBR-Green and Bio-Rad systems as described (Muñoz et al., 2012). PCR reactions were performed in triplicate with a standard curve for every primer. Changes in expression were calculated using CFX manager software (Bio-Rad). Primers were designed using the NCBI primer design tool.

Single-Cell and Bulk Sequencing

RNA samples were prepared using a modified version of the CEL-seq protocol as described previously (Grün et al., 2015; Hashimshony et al., 2012). RNA pellets were dissolved in primer mix and incubated for 2 min at 70°C. Cells sorted into 384-well were directly lysed at 65°C for 5 min. cDNA libraries were sequenced on an Illumina NextSeq500 using 75-bp paired-end sequencing. Data processing is described below.

PathScan Analysis

Organoids that were Gefitinib treated for 1h, 3h, 6h or 24h were collected in ice cold DMEM in medium, and lysed according to manufacture instructions (PathScan Akt Signaling Antibody Array Kit with chemoluminescent, Cell Signaling Technology). Lysates were processed according to protocol. Readout of chemoluminescent readout was performed on ImageQuant LAS 4000 (GE Healthcare Life Sciences). Signal intensities were quantified using ImageJ software. Quantification was performed by calculating intensity of each antigen signal relative to independent time point specific control antigens.

QUANTIFICATION AND STATISTICAL DETAILS

Analysis of RNA-Sequencing Data

Paired-end reads were quantified as described before (Grün et al., 2015) with the following exceptions. Reads that did not align or aligned to multiple locations were discarded. For analysis of the bulk sequencing, unique molecular identifiers (UMIs) were ignored; instead read counts for each transcript were determined by the number of reads that uniquely mapped to that transcript. This count was divided by the total number of reads that mapped to all transcripts and multiplied by one million to generate the reads-per-million (RPM) count. RPM was used in preference of RPKM because CEL-seq only allows 3' end sequencing. Differential gene expression was evaluated using the DESeq (Anders and Huber, 2010) and Deseq2 (Love et al., 2014) packages in R platform. Cut-offs in Figure 4 used were an adjusted p value < 0,1 and FDR < 0,1 and at least 2-fold difference to the compared population. To prevent samples with no reads disabling ratiometric analysis, all 0 reads were converted into 0,1 reads prior to ratio calculation and log2 conversion. Gene ontology analysis was performed using the Revigo (Supek et al., 2011) and Gorilla (Eden et al., 2009) software.

Single-Cell Data Analysis

Single-cell sequencing data was analyzed as described previously (Grün et al., 2015). In brief, 288 cells sorted from iNotch/iWnt/iEGFR treated organoids were sequenced in parallel. Cells with less than 1000 unique reads were discarded and samples were down-sampled. Genes with maximum expression less than 5 following down-sampling were discarded. Exclusion of Enterocytes was achieved by discarding samples with more than 8 transcripts of Apoa1.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed following producers' instructions (http://software.broadinstitute.org/gsea/). A ranked list comparing the fold changes between quiescent and active Lgr5+ stem cells was created and compared to the label retaining cell gene set (Basak et al., 2014; Buczacki et al., 2013) and the 'HALLMARKS' gene set available on the server (http://software. broadinstitute.org/gsea/). Expression2kinase (X2K) software was used to identify the transcription factors targeting the active Lgr5+ stem cell signature (Chen et al., 2012).

DATA AND SOFTWARE AVAILIBILITY

Data Resources

The data generated in this paper has been deposited in the Gene Expression Omnibus (GEO) under accession number GEO: GSE80636.

The list of differentially expressed genes between quiescent and active Lgr5+ stem cells are described in Table S1.

Results of the GSEA analysis using the label retaining cell gene set are described in Table S2. GSEA results for the 'HALLMARKS' gene sets are reported in Table S3.

Differentially expressed genes for each cluster described in the single cell analysis are reported in Table S4. The qPCR primers used in this study are in Table S5.