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L-Cell Differentiation Is Induced by Bile Acids Through GPBAR1 and Paracrine GLP-1 and Serotonin Signaling

Mari Lilith Lund,[1] Giovanni Sorrentino,[2] Kristoffer Lihme Egerod,[1] Chantal Kroone,[3] Brynjulf Mortensen,[4] Filip Krag Knop,[1,4,5,6] Frank Reimann,[7] Fiona M. Gribble,[7] Daniel J. Drucker,[8] Eelco J.P. de Koning,[9,10] Kristina Schoonjans,[2] Fredrik Bäckhed,[1,11] Thue W. Schwartz,[1,12] and Natalia Petersen[1]

Glucagon-like peptide 1 (GLP-1) mimetics are effective drugs for treatment of type 2 diabetes, and there is consequently extensive interest in increasing endogenous GLP-1 secretion and L-cell abundance. Here we identify G-protein–coupled bile acid receptor 1 (GPBAR1) as a selective regulator of intestinal L-cell differentiation. Lithocholic acid and the synthetic GPBAR1 agonist, L3740, selectively increased L-cell density in mouse and human intestinal organoids and elevated GLP-1 secretory capacity. L3740 induced expression of *Gcg* and transcription factors *Ngn3* and *NeuroD1*. L3740 also increased the L-cell number and GLP-1 levels and improved glucose tolerance in vivo. Further mechanistic examination revealed that the effect of L3740 on L cells required intact GLP-1 receptor and serotonin 5-hydroxytryptamine receptor 4 (5-HT4) signaling. Importantly, serotonin signaling through 5-HT4 mimicked the effects of L3740, acting downstream of GLP-1. Thus, GPBAR1 agonists and other powerful GLP-1 secretagogues facilitate L-cell differentiation through a paracrine GLP-1–dependent and serotonin-mediated mechanism.

Synthetic glucagon-like peptide 1 (GLP-1) analogs and dipeptidyl peptidase 4 (DPP-4) inhibitors today constitute effective components of type 2 diabetes treatment. Both strategies potentiate the action of the GLP-1 system, leading to beneficial effects on insulin and glucagon secretion, food intake, and nutrient metabolism. Consequently, increasing the number of intestinal endocrine L cells, the main source of GLP-1, represents an attractive alternative strategy for augmenting GLP-1 bioavailability (1–3).

Differentiation of L cells is a continuous process in the intestine. Constantly renewing intestinal epithelium has the capacity to expand the population of L cells if the appropriate signals are present (4). In the crypts of the intestine, stem cells expressing Lgr5 give rise to proliferating transit-amplifying cells. Each one of these cells can become a secretory cell progenitor expressing a specific lineage marker Math1 or an absorptive cell progenitor labeled by Hes1 expression—a process controlled by Notch signaling. Next, some of these secretory cell progenitors start expressing Ngn3, which defines endocrine progenitors (5). At the next stage, new transcription factors direct the maturation toward a certain hormonal cell type (6), where NeuroD1, Arx, Foxa 1, and Foxa 2 are associated with differentiation into L cells (7–9). In addition to GLP-1, L cells secrete other hormones important for metabolic

[1]Novo Nordisk Foundation Center for Basic Metabolic Research Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[2]Laboratory of Metabolic Signaling, Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

[3]Department of Thrombosis and Hemostasis, Leiden University Medical Centre, Leiden, the Netherlands

[4]Center for Clinical Metabolic Research, Gentofte Hospital, University of Copenhagen, Hellerup, Denmark

[5]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[6]Steno Diabetes Center Copenhagen, Gentofte, Denmark

[7]Institute of Metabolic Science and Medical Research Council Metabolic Diseases Unit, University of Cambridge, Addenbrooke's Hospital, Cambridge, U.K.

[8]Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada

[9]Department of Medicine, Leiden University Medical Centre, Leiden, the Netherlands

[10]Hubrecht Institute/Koninklijke Akademie van Wetenschappen (KNAW) and University Medical Center Utrecht, Utrecht, the Netherlands

[11]Department of Molecular and Clinical Medicine at Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[12]Laboratory for Molecular Pharmacology, Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Corresponding author: Natalia Petersen, napt@novonordisk.com

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regulation, such as cholecystokinin (CCK), neurotensin, and peptide YY (PYY). Somatostatin (SST)-secreting cells and serotonin (5-hydroxytryptamine [HT])-secreting cells, which constitute most of the enteroendocrine cell population, develop as mostly separate branches.

We previously showed that differentiation of L cells can be pharmacologically increased in vitro and in vivo by a Notch inhibitor (1) and Ras homolog family member A-associated coiled-coil–containing protein kinases (ROCK) inhibitor (3) through a general increase in the proportion of secretory cells. The subsequent increase in L-cell number resulted in amplified GLP-1 secretion, which augmented insulin responses and restored glucose tolerance in a mouse model of type 2 diabetes (1,3). However, the therapeutic potential of Notch and ROCK inhibitors used to manipulate L-cell differentiation is limited due to adverse effects during long-term application of these drugs because their effects are not selective to the L-cell lineage. On the other hand, short-chain fatty acids (SCFAs), endogenous products of intestinal microbiota metabolism, selectively enhance differentiation and consequently the number of L cells (9,10). SCFAs are also effective GLP-1 secretagogues (11), and thus, we hypothesized that locally amplified GLP-1 secretion drives L-cell differentiation. Among other natural GLP-1 secretagogues, secondary bile acids, synthesized by intestinal microflora from primary bile acids, are powerful stimulants of GLP-1 release through activation of the G–protein–coupled bile acid receptor 1 (GPBAR1) (12–14). Lithocholic acid (LCA) has the highest affinity to GPBAR1 among secondary bile acids (13). In humans, plasma concentrations of bile acids are at the low micromolar range (15). Intracellularly, GPBAR1 activates the Gs signaling pathway resulting in increased cAMP, which is particularly effective in stimulating release of GLP-1 (16) and other coexpressed peptide hormones from the L cell, including PYY and neurotensin (17). Therefore, synthetic GPBAR1 agonists are promising drug candidates acting locally in the intestine to enhance GLP-1 secretion (18). In this study, the effect of GPBAR1 activation on L-cell number, GLP-1 secretion, and gene expression of L-cell lineage markers was tested in the small intestine organoid platform. The observed positive effect of GPBAR1 agonists on L-cell differentiation was dependent on GLP-1 signaling and, surprisingly, mimicked by downstream serotonin signaling conceivably through paracrine stimulation from neighboring enterochromaffin cells (19).

RESEARCH DESIGN AND METHODS
Animals
Nonfasted male GLU-Venus mice with transgenic expression of yellow fluorescent protein (YFP) in L cells (20), GPBAR1 knockout (KO) mice (12), their wild-type littermates, and GLP 1 receptor (GLP-1R) KO mice (21) bred on C57BL/6J background were used for organoids studies at the age of 6–8 months, and GLU-Venus female mice were used for in vivo testing at the age of 3–4 months. Mice of different age were used to reduce costs for mouse colony

maintenance. We did not expect a difference in the mechanisms of intestinal cell differentiation in 3- to 8-month-old mice or significant differences between males and females. In our experience, organoids from 3- or 8-month-old males and females show similar characteristics. In all experiments, littermates from the same cage were used as controls. C57BL/6 mice were purchased from Charles River. All animal experiments were approved by the Danish Animal Inspectorate (2018-15-0201-01424).

Human Intestinal Samples
Human jejunum fragments were obtained from Roux-en-Y gastric bypass operations from four patients with no anticancer treatment (Sydvestjysk Hospital, Esbjerg, Denmark). All patients participated with informed consent, with the research protocol approved by the National Research Ethics Committee (H-18015120).

Mouse and Human Organoid Culture and Experiment Design
Mice were sacrificed by cervical dislocation. Small-intestine crypts were isolated from the ileum by EDTA incubation (22) and seeded in Matrigel in advanced DMEM/F12 containing 100 units/mL penicillin/streptomycin, 10 mmol/L HEPES, 2 mmol/L GlutaMAX, supplements N2 and B27, 50 ng/mL murine epidermal growth factor (all from Life Technologies), 1 mmol/L N-acetylcysteine (Sigma-Aldrich), 100 ng/mL murine Noggin, and 500 ng/mL murine R-spondin-1 (PeproTech). For maintenance, organoids were split every 4–6 days and cultured in 48-well plates. Test compounds were added into the culture medium, and the organoids were analyzed after 48 h. By the time of the readout, most of the organoids had three to five crypts. Duodenal organoids were generated and analyzed the same way. Human organoid lines were generated and cultured as previously described (23). The experiments were performed after the first passage.

LCA (Sigma-Aldrich), L3740 (Merck-TGR5-A) (17), tropisetron, RS-39604, BIMU 8 (all from Tocris), GPR40 agonist AM-1638 (24), and GPR119 agonist AR53 (AR231453) (16,24) were first dissolved in DMSO as 1 mmol/L stock, and diluted with the culture medium to 10, 20, or 50 μmol/L for LCA and 1 μmol/L for other compounds. DMSO (0.01%) was added to control wells. After 48 h, the organoids were collected for quantitative (q)PCR analysis or fixed in 4% paraformaldehyde for GLP-1 immunostaining (for human, GPBAR1 KO, wild-type, and GLP-1R KO mouse organoids) (10) and microscopy analysis. The experiment was performed three to six times in duplicates using organoids from six mice. The L-cell percentage in organoids was assessed in whole mount preparation from a three-dimensional stack of 10-μm-thick digital sections, each containing three to eight organoids, acquired on a Zeiss confocal microscope (LSM 780) at ×20 magnification using Zen Software. Label-positive cells from each image were manually counted and expressed as the percentage of cells calculated from nuclear labeling with DAPI, analyzed with

ImageJ software. The number of measurements is presented in Figure legends.

GLP-1 Secretion Measurements

Organoids cultured in 96-well plates for 48 h were washed three times in Advanced DMEM/F12 with HEPES and L-glutamine. Medium with stimulants (L3740, LCA, or BIMU 8) and control medium (without stimuli) was added to the wells with five replicates for each condition (unless otherwise indicated) and slowly shaken on an orbital shaker after addition of the medium and before the collection. After 2 h at 37°C, the medium was collected and assayed for GLP-1 content using the GLP-1 V-Plex Meso Scale Discovery kit (Meso Scale Diagnostics). For glucose stimulation, the organoids were washed and preincubated with DMEM supplemented with 2% FBS containing no glucose and glutamine for 1 h. Then DMEM containing 18 mmol/L glucose and 2 mmol/L L-glutamine or DMEM with no stimulants (control) was added. The experiment was performed three to four times. The values were normalized to the DNA content in the wells measured with a DNA quantification kit (Sigma-Aldrich).

qPCR

Total RNA was extracted from organoids using the mini RNA extraction kit (Qiagen). cDNA was synthesized using the SuperScript III kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Real-time qPCR was performed on a real-time PCR System (Bio-Rad) using SYBR green assays. We used beta-2-microglobulin (*B2M*) as the endogenous control gene (10). Mouse and human primer sequences were described previously (3,10) and for *Gpbar1*, *5Htr3a*, *5Htr3b*, and *5Htr4* are presented in Supplementary Table 1. Each gene expression was tested in two to four organoid lines from different mice generating two to four templates from each line from different passages (biological replicates, *n*) in independent experiments. The individual measurements were averaged from duplicated qPCR wells (technical replicates).

In-Feed GPBAR1 Agonist Treatment

Two groups of Glu-Venus mice were fed regular feed ground into powder for 4 days before the experiment. The body weight was monitored for potential weight losses due to the feed change. Mice with stable body weight were randomized so that one group received L3740-containing feed for 3 days, and the other continued with the same powder feed (control). Assuming that mice consumed at least 2.8 g of feed during 24 h (25), the mice received ~60 mg/kg body wt of the compound per day (0.64 mg L3740 per 1 g mouse feed). The mice were fasted for 5 h before an oral glucose tolerance test. Glucose (2 g/kg body wt) was given as an oral gavage, and blood samples were taken before and 15, 30, 60, and 120 min after the bolus. For plasma GLP-1 measurements, blood (~50 μL) was collected from the retro-orbital sinus from a separate cohort of L3740-treated and control mice before (0 min) and 10 min after

the glucose bolus. The total GLP-1 Meso Scale Discovery assay (cat. no. K150JVC-4) was used for determination of plasma GLP-1 concentrations. Mice were euthanized, and fragments of ileum 2 cm from cecum were collected and fixed in 4% paraformaldehyde. The percentage of YFP-positive cells was calculated in six to eight transverse cryosections per mouse as described above. Blood glucose was measured with a Contour-Next Glucometer (Bayer). Area under the curve with baseline subtraction was calculated for glucose dynamics using GraphPad Prism 8 software.

In Situ Hybridization

Adult C57BL/6 male mice were euthanized by cervical dislocation, and fragments of intestine were excised, rinsed, and fixated by flushing with Bouin fixative (50% ethanol and 5% acetic acid in deuterium H_2O). The intestines were cut open and rolled onto a toothpick to create so-called Swiss rolls. "Rolls" were fixated 24 h in 4% paraformaldehyde and subsequently embedded in paraffin. In situ hybridization was performed on 5-μm sections using RNAscope Multiplex Fluorescent Assay (ACDBio) with the probes for Htr4 (Mm-Htr4, #408241), Htr3a (Mm-Htr3a, #411141), Lgr5 (Mm-LGR5-C3, #312171-C3), Math1 (Mm-Atoh1-C3, #408791-C3), CD133 (Mm-Prom1-C3, #412221-C3), and Ngn3 (Mm-Neurog3-C2, #422401-C2) following manufacturer's instruction. Htr4, CD133, and Ngn3 were applied as a triple labeling. Images of staining were acquired on LSM 780 using Zen Software.

Statistics

Quantitative results are presented as mean values with SEM. Comparison of two groups was done using the nonpaired two-tailed Student *t* test, assuming normality of data. qPCR data were analyzed using the Wilcoxon matched-pairs signed rank test for data from different organoid lines. *P* values of <0.05 were considered significant. Data involving more than two groups were assessed by ANOVA with Bonferroni post hoc test using GraphPad Prism 8 software.

Data and Resource Availability

The data sets generated during the current study are available from the corresponding author on reasonable request.

RESULTS

Activation of GPBAR1 Increases L-Cell Number in Intestinal Organoids

To investigate whether GPBAR1 activation could increase L-cell number, we applied LCA to organoids generated from GLU-Venus mouse ileum crypts, where L cells are labeled by transgenic YFP expression (20). The addition of 10 μmol/L LCA to the culture medium for 48 h increased the L-cell number twofold (Fig. 1A and B). Increasing the LCA concentration did not increase L-cell number further (Supplementary Fig. 1A and B); however, 20 and 50 μmol/L LCA impaired crypt formation in

organoids, indicating toxicity, which was not observed with application of 10 μmol/L LCA (Supplementary Fig. 1A). To avoid potential nonspecific effects, we used L3740, a potent, synthetic, nondetergent GPBAR1 agonist (17) (Supplementary Fig. 1C). Incubation with 1 μmol/L L3740 increased the number of L cells by 2.5-fold within 48 h (Fig. 1C and D), while maintaining normal morphology of the organoids (data not shown). Immunostaining for GLP-1 confirmed that YFP-positive cells produced GLP-1 (Supplementary Fig. 1D). L cells were mostly localized to the crypt domains in the control and L3740-treated groups. We also tested the effect of L3740 on L-cell numbers in duodenal organoids and found a 1.7-fold increase in the L-cell percentage in the treated group compared with the control group (Supplementary Fig. 1E).

Next, we tested GLP-1 secretion from GPBAR1-treated organoids in response to a combination of natural stimulants of GLP-1 release—glucose and L-glutamine—and observed a 1.8-fold increase in the secretory capacity of the cultures compared with the control (Fig. 1E), which is in agreement with the observed increase in L-cell number.

Real-time qPCR analysis of specific cell markers was used as an indirect measure of representation of different cell types. We found increased expression of Gcg and Cck transcripts in L3740-treated organoids but not of other L-cell hormones such as Pyy and Nts (Fig. 1F). We tested the expression of Tph1, a marker of serotonin-secreting enterochromaffin cells, and found no difference between the control and treated group. Because the Tph1 gene encodes a key enzyme for serotonin synthesis and not the final product, we also determined the enterochromaffin cell representation and found no changes after the treatment with L3740 (Supplementary Fig. 1F). Levels of mRNA transcripts encoding the transcription factors NeuroD1 and Ngn3 associated with L-cell endocrine specification was also elevated, whereas expression of Arx, Foxa1, and Foxa2 remained unchanged (Fig. 1G). Expression of secretory cell progenitor markers Math1 and Dll1 did not change, indicating that the increase in L-cell number was modulated after the Notch-mediated secretory cell specification (26). Accordingly, the expression of Hes-1, a marker of progenitor of nonsecretory absorptive cells, enterocytes, was the same in control and L3740 groups. No changes in the expression levels of I-fabp and Itf (enterocyte and goblet cell markers, respectively) were observed, and the expression of Lyz1, a Paneth cell marker, was slightly reduced (Fig. 1G). The expression of Lgr5, a stem cell marker, was unchanged (Fig. 1G).

To directly test whether GPBAR1 is required for bile acid-induced L-cell differentiation, we generated organoids from GPBAR1 KO mice and their wild-type littermates. Exposure to L3740 did not affect the number of L cells (Fig. 1H) or the expression of Gcg, Nd1, and Ngn3 (Fig. 1I) in the absence of functional GPBAR1 signaling. Consistent with these findings, L3740 and LCA did not induce GLP-1 secretion from organoids lacking GPBAR1 (Fig. 1J). Thus, LCA and L3740 both selectively induced L-cell differentiation through mechanisms requiring functional GPBAR1 signaling.

L3740 Increases L-Cell Numbers In Vivo

To investigate whether GPBAR1 stimulation can amplify L-cell numbers in vivo, L3740 was administered o GLU-Venus mice for 3 days in their feed. L3740-treated mice showed a modest increase in glucose tolerance after an oral glucose challenge (Fig. 2A and B) and elevated basal GLP-1 plasma levels (0 min) and 10 min after the gavage (Fig. 2C). Body weight in the control and treated group before and after the treatment was the same (Supplementary Fig. 1G). There was no difference in feed intake between the groups (2.6 ± 0.1 vs. 2.7 ± 0.1 g/mouse/day in control and treated groups, respectively; NS). L-cell number in the distal ileum was increased (Fig. 2D and E) and accompanied by elevated expression of Gcg and Ngn3 compared with controls (Fig. 2F). This indicates that the GPBAR1 agonist L3740 stimulates formation of new endocrine-committed intestinal cells in vivo in mice.

GPBAR1 Stimulation Increases L-Cell Abundance in Human Organoids

We next investigated the effect of GPBAR1 activation in human intestinal epithelium. Human ileal organoids were treated with 1 μmol/L L3740 for 48 h. Human organoid culture requires the addition of Wnt3A and mitogen-activated protein kinase inhibitors, which interfere with normal cell differentiation, and the percentage of mature cells in human organoids consequently is much lower than in an intact intestine or in mouse organoids (23). In mouse organoids, L cells were usually found in crypt domains, whereas in human organoids, we did not observe a clear pattern for L-cell location (Fig. 3B). GPBAR1 stimulation induced a 1.6-fold increase in the L-cell number compared with control cells (i.e., similar to what was observed in the murine organoids) (Fig. 3A). Only 48% of vehicle-stimulated organoids contained L cells, and most of those had only one or two L cells regardless of the organoid size (Fig. 3B). After L3740 treatment, L cells were observed in 71% of organoids (Fig. 3C). Considering the irregular scattering of L cells, we compared the frequency distribution of L cells in the organoid population. A shift toward more cells per organoid was detected in the L3740-treated group (Fig. 3C). The expression of GCG was increased in the L3740-treated group as well as expression of NEUROD1, but we did not detect changes in NGN3 expression, probably because the overall expression of NGN3 was surprisingly low in the human organoids (Fig. 3D). These data indicate that L3740 stimulates L-cell development also in human intestinal epithelium.

GPBAR1 Induction of L-Cell Differentiation Requires Intact GLP-1 Signaling

We hypothesized that activation of GPBAR1 promotes L-cell differentiation by chronically increasing secretion of GLP-1. To test this, the effect of L3740 was probed in
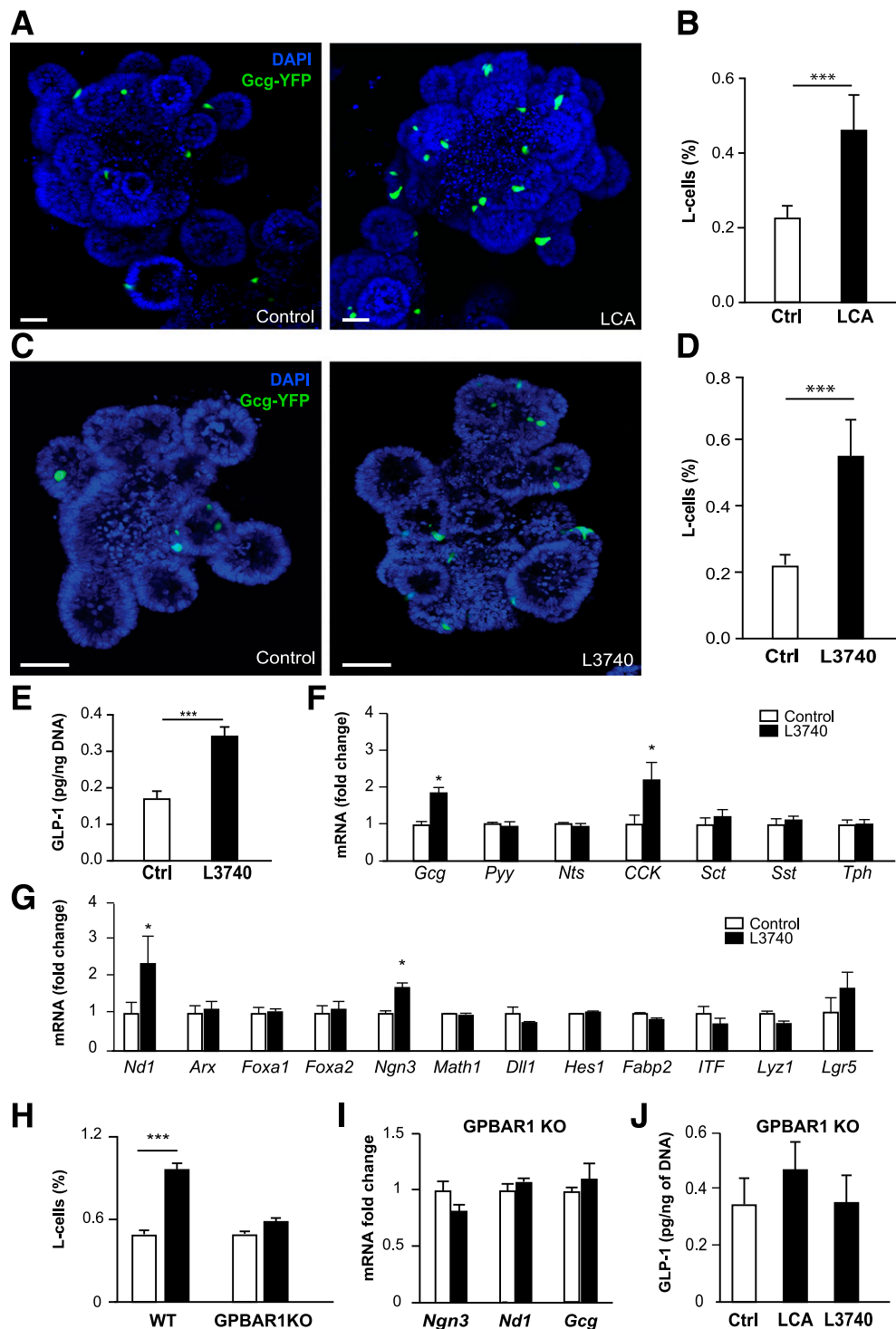
Figure 1—Activation of GPBAR1 by the bile acid LCA and a synthetic agonist increases L-cell number in organoids. *A*: Representative images of control and organoids treated with 10 μmol/L LCA. L cells are labeled by Gcg-YFP expression in this and following images unless otherwise indicated. Nuclei labeled by DAPI. Scale bars, 50 μm. *B*: L-cell numbers in control (Ctrl) organoids (*n* = 70) and organoids treated with LCA (*n* = 78). In this and following figures, the data are mean ± SEM (unless otherwise indicated). *C*: Representative images of control and L3740-treated organoids. Scale bars, 50 μm. *D*: L-cell numbers in control (*n* = 38) and L3740-treated organoids (*n* = 30). *E*: GLP-1 secretion from L3740-treated organoids stimulated by 18 mmol/L glucose and 2 mmol/L L-glutamine. Data from three experiments were performed with five replicas. *F*: Gene expression of intestinal hormones in L3740-treated organoids (*n* = 4–10 in each series). *G*: Gene expression of intestinal cell type markers and transcription factors directing L-cell development. *Lgr5* and *CD133*, proliferating cells; *ITF*, goblet cells; *Lyz1*, Paneth cells; *I-Fabp*, enterocytes; *Ngn3*, *NeuroD1*, *Arx*, and *Foxa1/2*, L-cell transcription factors (*n* = 4–10 experiments in each series). *H*: L-cell numbers, identified by immunostaining, in control and GPBAR1 KO and wild-type (WT) mouse organoids after 48-h treatment with L3740 (*n* = 21–25). *I*: Expression of L-cell differentiation markers in control and L3740-treated GPBAR1 KO organoids (*n* = 3 for each series). *J*: GLP-1 release in response to LCA and L3740 in GPBAR1 KO organoids (*n* = 5 for each series). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
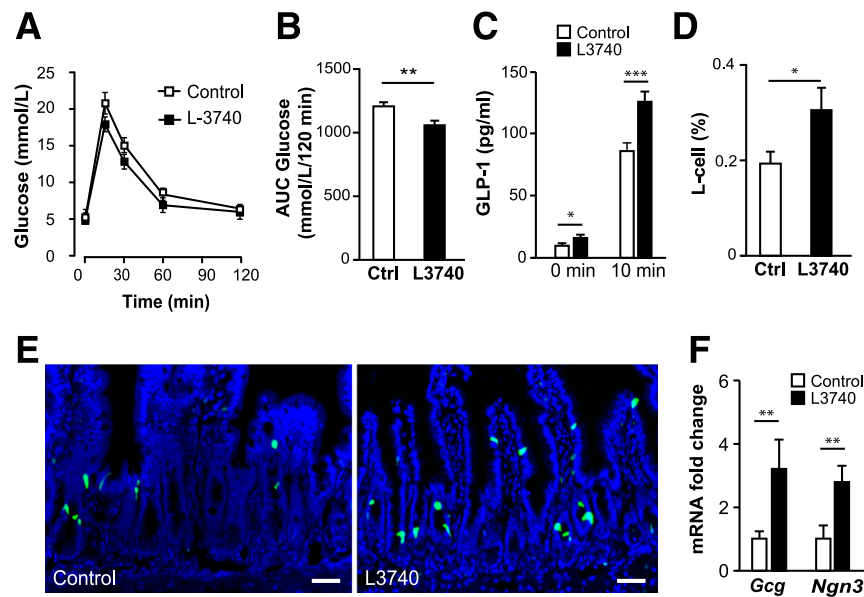
Figure 2—In-feed L3740 treatment increases oral glucose tolerance, GLP-1 secretion, and L-cell number in mice. *A*: Blood glucose excursion during an oral glucose tolerance test after L3740 treatment (*n* = 10 for each series). *B*: Area under the curve (AUC) from data in panel *A*. Ctrl, control. *C*: Plasma GLP-1 concentrations in control and L3740-treated mice before the test and 10 min after glucose gavage (*n* = 8 for each series). *D*: L-cell percentage in mouse ileum sections (*n* = 4 for each series). *E*: L cells (Gcg-YFP, green) in the ileum of control and L3740-treated mice. Nuclei labeled by DAPI (blue). Scale bars, 50 μm. *F*: Expression of *Gcg* and *Ngn3* in mouse ileal epithelium (*n* = 4 for each series). *P < 0.05, **P < 0.01, ***P < 0.001.

intestinal organoids from GLP-1R KO mice (21). The increase in L-cell number determined by immunostaining was completely eliminated in the absence of functional GLP-1R signaling (Fig. 4*A*). This indicates that the effect of GPBAR1 activation requires the endogenous GLP-1R and that L-cell number is regulated by a positive feed forward mechanism.

In an attempt to maximize the proposed effect of GLP-1R activation, we used 100 μmol/L liraglutide, a synthetic GLP-1 analog, in the organoid culture medium and assessed the L-cell number. Liraglutide increased the number of L cells (Supplementary Fig. 2*A*), but interpreting this effect is difficult because the peptide also had a pronounced stimulatory effect on organoid growth (Supplementary Fig. 2*B*).

GLP-1 Secretagogues Induce L-Cell Differentiation Through a Paracrine Cross Talk With Serotonin Signaling

In the intestinal epithelium, the GLP-1R is almost exclusively and highly expressed by the large population of enterochromaffin cells producing serotonin, or 5-hydroxytryptamine (5-HT) (19). Thus, we hypothesized that differentiation of L cells could be mediated through serotonin release. We stimulated L-cell differentiation in mouse organoids with L3740 in the presence of two different serotonin receptor inhibitors—tropisetron, a prototype rather broad-spectrum serotonin antagonist blocking 5-HT receptor 3 (5-HTR3) and 5-HT receptor 4 (5-HTR4) (26), and 5-HTR4 blocker RS-39604. Both drugs abolished L3740-induced L-cell differentiation (Fig. 4*B*). In situ hybridization demonstrated that the *Htr4* is expressed in most epithelial cells of the small

intestinal crypts (Fig. 4*C*), and *Htr3*, as expected (27,28), is expressed mainly in submucosal ganglia (Fig. 4*C*). In view of these facts and because 5-HTR4 inhibition was sufficient to inhibit the effect of L3740, we focused on the 5-HT4 receptor. Correspondingly, we found that L3740 treatment increased the expression of *Ht4r* in the organoids (Fig. 4*D*). To determine which differentiating cells in the crypt express *Htr4*, we performed in situ hybridization costaining for Htr4 and stem cell marker Lgr5, transit-amplifying cell marker CD133, secretory progenitor marker Math1, and endocrine progenitor marker Ngn3. We found that Htr4 was present in all these cell types (Fig. 4*E*). This is in accordance with the capacity of these cells to differentiate into L cells, which supports our discovery of a role for serotonin in regulating the number of L cells.

Next, we incubated organoids with the 5-HTR4 agonist BIMU 8 alone and in combination with L3740. BIMU 8 produced a similar increase in the number of L cells as L3740, and combination of the two did not result in an additive or synergistic effect (Fig. 4*F*). Importantly, BIMU 8 had no acute effect on GLP-1 release (Supplementary Fig. 2*C*). When tested in GLP-1R KO mouse organoids and GPBAR1 KO mouse organoids, BIMU 8 still increased the number of L cells (Fig. 4*G* and H), further supporting that the increase in L-cell numbers by L3740 was an indirect effect mediated by activation of serotonin receptors.

Based on the key role of GLP-1 signaling in the effect of GPBAR1 activation on L-cell differentiation, we tested other efficient L-cell secretagogues: a synthetic agonist of free fatty receptor 1 (FFAR1 or GPR40), AM-1638, and a lipid amide receptor GPR119 agonist, AR53. These two secretagogues were tested alone or in combination with
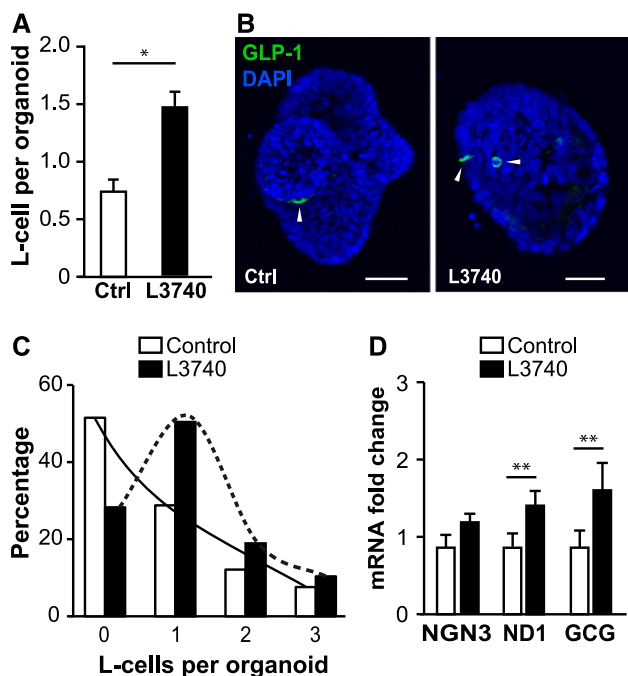
Figure 3—GPBAR1 stimulation increases L-cell abundance in human organoids. *A*: L-cell numbers in control (Ctrl; *n* = 66) and L3740-treated human small intestine organoids (*n* = 44). *B*: Representative images of control and L3740-treated human organoids. L cells are labeled by GLP-1 immunostaining (white arrows), and nuclei are labeled by DAPI. Scale bars, 50 μm. *C*: A shift in the organoid distribution by the cell number after treatment with L3740. Percentages of organoids with various L-cell numbers are presented from data in panel *A*. $P < 0.05$ by χ^2 contingency test. *D*: Expression of NGN3, NEUROD1 (ND1), and GCG (*n* = 4 for each series, generated from two human organoid lines). *$P < 0.05$, **$P < 0.01$.

the 5-HTR4 antagonist. Both agents alone robustly increased the number of L cells in organoids to a similar degree as L3740 (Fig. 4*I*), while inhibiting 5-HTR4 abolished the effect of both the FFAR1 agonist and the GPR119 agonist (Fig. 4*I*). Thus, these findings indicate that serotonin signaling acts downstream of GLP-1R signaling, enabling regulation of intestinal cell differentiation into L cells.

DISCUSSION

Based on studies on secondary bile acids produced by intestinal microbiota, we here propose a general mechanism through which GLP-1 secretagogues stimulate L-cell differentiation and thereby increase L-cell number in the intestinal epithelium. This mechanism involves paracrine GLP-1 stimulation of serotonin secretion and downstream activation of serotonin receptors conceivably on progenitor cells in the intestinal crypts (Supplementary Fig. 3). Activation of GPBAR1 increased L-cell differentiation in human and mouse intestinal organoids, supported by increased expression of transcription factors guiding L-cell differentiation. Interestingly, the GPBAR1 agonist L3740 only increased the expression of *Gcg* and *Cck* (which is known to be produced by L-cells to some degree), but not PYY

and Nts, which are expressed in certain populations of L cells, and these results may reflect the prolonged maturation time for these populations (17). As a large fraction of enteroendocrine cell subsets express *Gcg* (29), GPBAR1 activation can further stimulate *Gcg* expression and prohormone convertase 1/3 (30), thereby converting more cells into GLP-1–producing cells. Thus, GBPAR1 modulates L-cell differentiation selectively in contrast to earlier methods to increase the number of L cells (1–3). Using GPBAR1-deficient mouse organoids, we demonstrate that the effect indeed was specific for GPBAR1 when L3740 was applied. However, other efficient GLP-1 secretagogues acting on FFAR1 (GPR40) and GPR119 mimicked the effect of L3740 on the L-cell number. This is likely due to high enrichment of GPBAR1, FFAR1, and GPR119 (20) in L cells and the ability of L cells to secrete large amounts of GLP-1 upon its Gs activation. Previously, similar effects on L-cell abundance have been shown for FFAR2 and FFAR3, receptors for other microbial metabolites, SCFAs (10).

GLP-1R has been implicated in cell proliferation and differentiation (31). As data from GLP1R-deficient mice indicate, GLP-1R signaling specifically regulates L-cell differentiation. GLP-1R is mainly expressed within intestinal intraepithelial lymphocytes, enteric neurons, and enterochromaffin cells in the intestinal epithelium, and GLP-1 stimulates release of serotonin (19). The role of serotonin in growth and maturation of intestine has previously been described, although these effects were attributed to the interaction of enterochromaffin cells with enteric neurons (32,33). Here we show that 5-HTR4 is predominantly expressed in the crypt in stem cells, supporting earlier data (34), but also in secretory and endocrine progenitor cells. Importantly, the serotonin receptor antagonist abolished the increase in L-cell number induced by GLP-1 secretagogues. In agreement, activation of serotonin signaling increased L-cell number regardless of the presence of GPBAR1 and GLP-1R, indicating that serotonin acts downstream of GLP-1.

The GPBAR1 agonist L3740 stimulated formation of new endocrine-committed intestinal cells in vivo in mice. The increased glucose tolerance and GLP-1 levels in L3740-treated mice indicates that the increase in L-cell abundance was physiologically significant. It is possible that positive influence of bile acids on energy expenditure and improved glucose metabolism (35) are partially caused by increased L-cell numbers. Together with our data from human organoids, this suggests that GPBAR1 may be a potential therapeutic target for increased endogenous GLP-1 production in humans with type 2 diabetes. The current limitation of use of GPBAR1 agonists as antidiabetic agents is gall bladder dysfunction (18,36). However, altering the intestinal microbiota in favor of microorganisms producing bile acids (37–39) to activate GPBAR1 locally appears to be an intriguing possibility.

In conclusion, bile acids may act as regulators of L-cell number in the intestinal epithelium. Here we show a specific
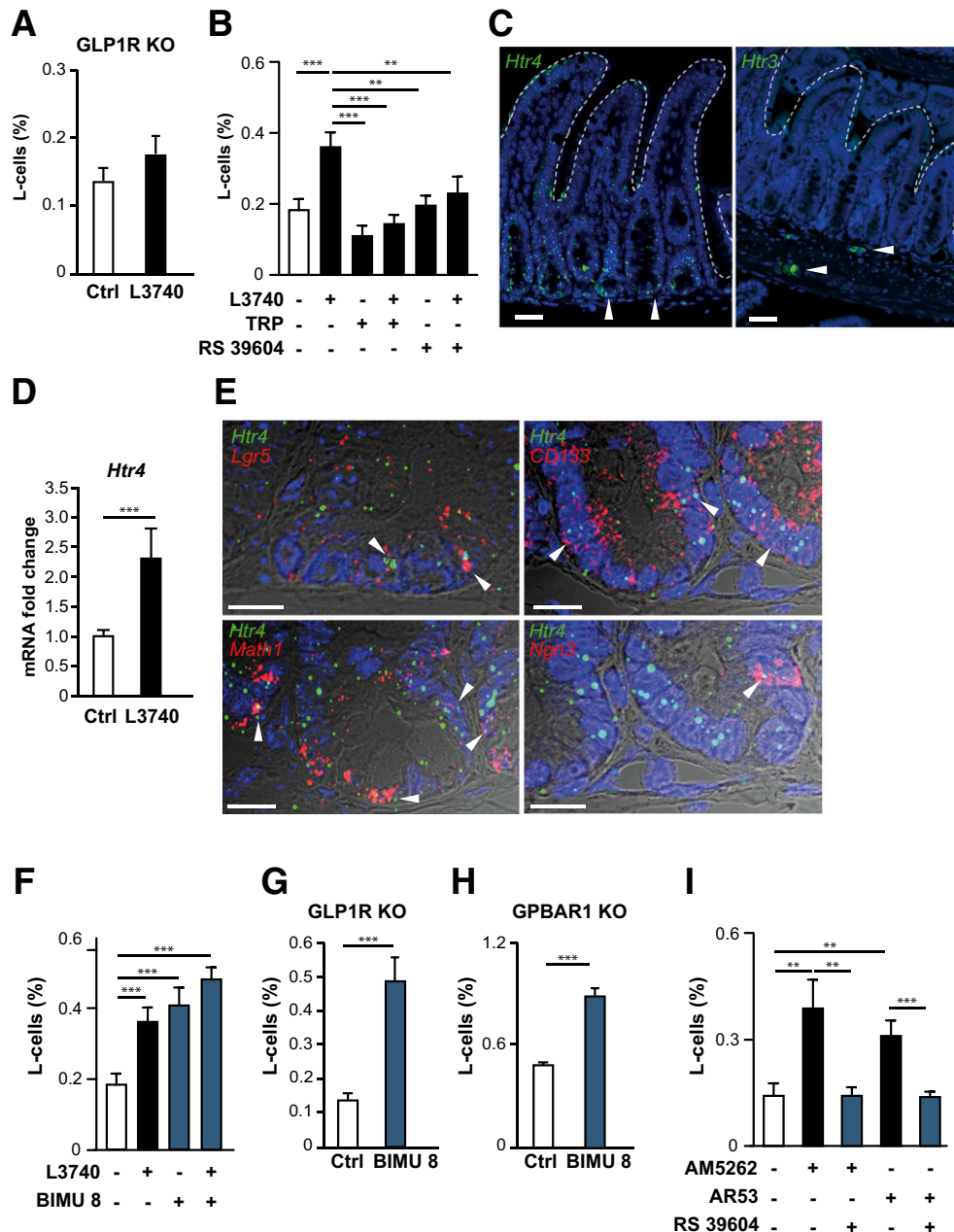
Figure 4—GPBAR1 induction of L-cell differentiation requires cross talk with serotonin signaling. *A*: Effect of L3740 in GLP1R KO mouse organoids ($n = 47$ for control [Ctrl] and $n = 58$ for L3740-treated series). *B*: Effect of 5-HTR3 and 5-HTR4 inhibitor tropisetron (TRP) and 5-HTR4 inhibitor RS-39604 in organoids alone and in combination with L3740. *C*: *Htr4* and *Htr3* in mouse ileum identified by in situ hybridization. Nucleus labeling by DAPI (blue). White arrows indicate positive *Htr3* staining outside of the epithelial layer. Scale bars, 50 μm. *D*: L3740 increases *Htr4* expression in organoids ($n = 7$ for control and $n = 6$ for L3740-treated series). *E*: Coexpression of *Htr4* and cell markers *Lgr5*, *CD133*, *Math1*, and *Ngn3* in mouse ileal crypts identified by in situ hybridization. White arrows indicate positive *Htr4* staining. Nuclei are labeled with DAPI (blue). Scale bars, 20 μm. *F*: Effect of 5-HT4R agonist BIMU 8 and L3740 on the L-cell number ($n = 17$–35 in panels *A* and *B*). *G*: BIMU 8 increases L-cell number in GLP1R KO mouse organoids, while L3740 has no effect on the L cells ($n = 47$ for control and $n = 20$ for L3740-treated series). *H*: BIMU 8 increases L-cell numbers in GPBAR1 KO mouse organoids ($n = 21$ for control and $n = 23$ for L-3740-treated series). *I*: FFAR1 agonist AM-1638 and GPR119 agonist AR53 have a similar effect on the number of L cells, while addition of RS-39604 counteracts this effect ($n = 33$–42). **$P < 0.01$, ***$P < 0.001$.

target, GPBAR1, which increases the differentiation into L cells but does not alter other cell ratios in intestinal epithelium. We propose a model whereby GLP-1 levels contribute to control of L-cell abundance through serotonin signaling, explaining the ability of the body to adjust the L-cell numbers to compensate for changes in nutrient availability. Finally, our study demonstrates that directing the differentiation in the intestinal epithelium into specific cell types is possible and that GPBAR1 (and possibly other G-protein–coupled receptors) could be a target for

modulation of L-cell number as a therapeutic strategy against type 2 diabetes.

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