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L-cell differentiation is induced by bile acids through GPBAR1 and paracrine GLP-1 and serotonin signaling

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Abstract

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, GLP-1 levels and improved glucose tolerance

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Data and Resource Availability

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

Author Contributions

M.L.L., T.W.S. and NP. conceived the study and designed experiments, N.P., M.L.L., G.S., K.L.E. and C.C. carried out the research, analyzed and interpreted the experiments, F.B., E.J.P.K and K.S. designed studies, F.B., B.M. and F.K. provided key samples and expertise, F.M.G., F.R., D.J.D. and K.S. provided essential mouse lines for the study and edited the manuscript. N.P., M.L.L., F.B. and T.W.S. wrote the manuscript with input from all other authors.

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in vivo. Further mechanistic examination revealed that the effect of L-3740 on L-cells required intact GLP-1 receptor and serotonin 5-HT4 receptor 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.

Keywords

bile acids; intestinal organoids; GLP-1; serotonin; L-cells; enterochromaffin cells

Introduction

Synthetic glucagon-like peptide 1 (GLP-1) analogues 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, represent 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 towards 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 regulation, such as cholecystokinine (CCK), neurotensin (NTS) and peptide YY (PYY). Somatostatin (SST) secreting cells and serotonin (5-HT) secreting cells, which constitute the majority of enteroendocrine cell population, develop as mostly separate branches.

We have previously shown that differentiation of L-cells can be pharmacologically increased *in vitro* and *in viv*o by a Notch inhibitor (1) and Ras homologue 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 chronic application of these drugs as 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

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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 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 co-expressed 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

Non-fasted male GLU-Venus mice with transgenic expression of yellow fluorescent protein (YFP) in L-cells (20), GPBAR1 knockout mice (12), their wild type littermates and GLP1R knockout mice (21) bred on C57BL/6J background were used for organoids studies at the age of 6 - 8-month 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 3-8 month-old mice, or significant differences between males and females. To our experience, organoids from 3 or 8 months old males and females show similar characteristics. In all experiments, littermates from the same cage were used as controls. C57BL/6 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 4 patients with no anti-cancer 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 Dulbecco's modified Eagle's medium/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 analysed after 48 h. By the time of the readout, the majority of organoids had 3-5 crypts. Duodenal organoids were generated and analyzed the same way. Human organoid lines were generated and cultured as described (23). The experiments were performed after the first passage.

LCA (Sigma Aldrich), L3740 (Merck-TGR5-A, ref. 17), tropisetron, RS-39604, BIMU 8 (all from Tocris), GPR40 agonist AM-1638 (24) and GPR119 agonist AR53 (AR231453, ref. 16, 24) were first dissolved in DMSO as 1mM stock, and diluted with the culture medium to 10, 20 or 50 μM for LCA and 1 μM for other compounds. DMSO (0.01%) was added to control wells. After 48 hours, the organoids were collected for qPCR 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 3-6 times in duplicates using organoids from 6 mice. The L-cell percentage in organoids was assessed in wholemount preparation from a 3D stack of 10-μm-thick digital sections, each containing 3-8 organoids, acquired on a Zeiss confocal microscope (LSM 780) at 20X magnification using Zen Software. Label-positive cells from each image were manually counted and expressed as percentage of cells calculated from nuclear labeling with DAPI, analyzed with Image J software. Number of measurements is presented in Figure legends.

GLP-1 secretion measurements

Organoids cultured in 96 well plates for 48 hours were washed 3 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 5 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 hours at 37°C, the medium was collected and assayed for GLP-1 content using GLP-1 V-plex MSD kit (Mesoscale). For glucose stimulation, the organoids were washed and pre-incubated with DMEM supplemented with 2% FBS containing no glucose and glutamine for 1 hour. Then DMEM containing 18 mM glucose and 2 mM L-glutamine or DMEM with no stimulants (control) was added. Experiment was performed 3-4 times. The values were normalized to the DNA content in the wells measured with DNA quantification kit (Sigma).

qPCR

Total RNA was extracted from organoids using mini RNA extraction kit (Qiagen). cDNA was synthesized using Superscript III kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed on a real-time PCR System (Bio-Rad) using SYBR green assays. We used Beta 2 microglobulin (*B2M*) as endogenous control gene (10). Mouse and human primer sequences were described earlier (3, 10) and for *Gpbar1*, *5Htr3a*, *5Htr3b* and *5Htr4* are presented in Table 1. Each gene expression was tested in 2-4 organoid lines from different mice generating 2-4 templates from each line from different passages (biological replicates, n) in independent experiments. The individual measurements were averaged from duplicated qPCR wells (technical replicates).

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In-feed GPBAR1 agonist treatment

Two groups of Glu-Venus mice were fed regular feed ground into powder during 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 during 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 hours (25), the mice received 60mg/kg of body weight of the compound per day (0.64 mg of L3740 per 1 g of mouse feed). The mice were fasted for 5 hours before the oral glucose tolerance test. Glucose (2g/kg of body weight) was given as oral gavage and blood samples were taken before and 15, 30, 60 and 120 minutes after the bolus. For plasma GLP-1 measurements, about 50 μL of blood was collected from retroorbital sinus from a separate cohort of L3740-treated and control mice before (0 min) and 10 minutes 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 caecum were collected and fixed in 4% PFA. The percentage of YFP-positive cells was calculated in 6-8 transverse cryosections per mouse as described above. Blood glucose was measured with a Contour-Next Bayer Glucometer. Area under the curve with baseline subtraction was calculated for glucose dynamics using GraphPad Prism 8.

In situ hybridization

Adult C57BL/6 male mice were euthanized by cervical dislocation, fragments of intestine were excised, rinsed and fixated by flushing with Bouin's fixative (50% ethanol and 5% acetic acid in dH2O). Hereafter, the intestines were cut open and rolled onto a toothpick to create so called "Swiss rolls". "Rolls" were fixated 24 hours 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 manufactures 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 2 groups was done using nonpaired 2-tailed Student's t-test, assuming normality of data. qPCR data were analyzed using Wilcoxon matched-pairs signed rank test for data from different organoid lines. P values less than 0.05 were considered significant (* < 0.05; ** <0.01; *** < 0.001). Data involving more than 2 groups were assessed by ANOVA with Bonferroni post-hoc test (GraphPad Prism 8).

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). Addition of 10 μM LCA to the culture medium for 48 h increased the L-cell number 2-fold (Figure 1A-B). Increasing the LCA concentration did not increase L-cell number further (Figure S1A-B), however, 20 and 50 μM LCA impaired crypt formation in organoids indicating toxicity, which was not observed with application of 10 μM LCA (Figure S1A). To avoid potential non-specific effects we used a potent, synthetic, non-detergent GPBAR1 agonist L3740 (17, Figure S1C). Incubation with 1 μM L3740 increased the number of L-cells by 2.5 fold within 48 hours (Figure 1C-D), while maintaining normal morphology of the organoids (data not shown). Immunostaining for GLP-1 confirmed that YFP positive cells produced GLP-1 (Figure S1D). L-cells were mostly localized to the crypt domains in both control and L3740-treated groups. We have 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 to the control (Figure S1E).

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 (Figure 1E), which is in agreement with the observed increase in L-cell number.

qRT-PCR 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* (Figure 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 (Figure S1F). 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 un-changed (Figure 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 non-secretory absorptive cells, enterocytes, was the same in control and L3740 group. 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 (Figure 1G). The expression of *Lgr5*, a stem cell marker, was unchanged (Figure 1G).

To directly test whether GPBAR1 is required for bile-acid induced L-cell differentiation we generated organoids from GPBAR1 knockout mice and their wild type littermates. Exposure to L3740 did not affect the number of L-cells (Figure 1H) nor the expression of *Gcg*, *Nd1* and *Ngn3* (Figure 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 (Figure 1J). Thus, both LCA and L3740 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 in-feed to GLU-Venus mice for 3 days. L3740-treated mice showed a modest increase in glucose tolerance after an oral glucose challenge (Figure 2A-B) and elevated basal GLP-1 plasma levels (0 min) and 10 minutes after the gavage (Figure 2C). Body weight in the control and treated group before and after the treatment was the same (Figure S1G). There was no difference in feed intake between the groups (2.6 ± 0.1 vs 2.7± 0.1 g/mouse/day, control and treated group, respectively, NS). L-cell number in the distal ileum was increased (Figure 2D-E) and accompanied by elevated expression of *Gcg* and *Ngn3* compared with controls (Figure 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 μM L3740 for 48 hours. Human organoid culture requires addition of Wnt3A and MAPK 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-cell were usually found in crypt domains, while in human organoids we did not observe a clear pattern for L-cell location (Figure 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 (Figure 3A). Vehicle-stimulated organoids only contained L-cells in 48 % of organoids, and the majority of those had only one or two L-cells regardless of the organoid size (Figure 3B). After L3740 treatment, L-cells were observed in 71 % of organoids (Figure 3C). Considering the irregular scattering of L-cells, we compared the frequency distribution of L-cells in organoid population. A shift towards more cells per organoid was detected in L3740-treated group (Figure 3C). The expression of GCG was increased in 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 (Figure 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 intestinal organoids from GLP-1 receptor deficient mice (GLP1RKO, ref. 21). The increase in L-cell number determined by immunostaining was completely eliminated in the absence of functional GLP-1 receptor signaling (Figure 4A). This indicates that the effect of GPBAR1 activation requires the endogenous GLP-1 receptor and that L-cell number is regulated by a positive feedforward mechanism.

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In an attempt to maximize the proposed effect of GLP-1 receptor activation, we used 100 μM liraglutide, a synthetic GLP-1 analogue, in the organoid culture medium and assessed the L-cell number. Liraglutide increased the number of L-cells (Figure S2A), but it is difficult to interpret this effect because the peptide also had a pronounced stimulatory effect on organoid growth (Figure S2B).

GLP-1 secretagogues induce L-cell differentiation through a paracrine cross-talk with serotonin signaling

In the intestinal epithelium, the GLP-1 receptor 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-HTR3 and 5-HTR4 (26), and 5-HTR4 blocker RS-39604. Both drugs abolished L3740-induced L-cell differentiation (Figure 4B). *In situ* hybridization demonstrated that the *Htr4* is expressed in a majority of epithelial cells of the small intestinal crypts (Figure 4C) and *Htr3,* as expected (27, 28), is expressed mainly in submucosal ganglia (Figure 4C). 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 (Figure 4D). To determine which differentiating cells in the crypt express *Htr4*, we performed in situ hybridization co-staining 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 (Figure 4E). 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 (Figure 4F). Importantly, BIMU 8 had no acute effect on GLP-1 release (Figure S2C). When tested in GLP1RKO mouse organoids and GPBAR1KO mouse organoids, BIMU 8 still increased the number of L-cells (Figure 4G and 4H), 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 (FFA1 or GPR40), AM-1638, and a lipid amide receptor GPR119 agonist, AR53. These two secretagogues were tested alone, or in combination with the 5-HTR4 antagonist. Both agents alone robustly increased the number of L-cells in organoids to a similar degree as L3740 (Figure 4I), while inhibiting 5HTR4 abolished the effect of both the FFAR1 agonist and the GPR119 agonist (Figure 4I). Thus, these findings indicate that serotonin signaling acts downstream of GLP-1 receptor 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 (Figure S3). 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, short chain fatty acids (10).

GLP-1 receptor has been implicated in cell proliferation and differentiation (31). As data from GLP1R-deficient mice indicate, GLP-1 receptor signaling specifically regulates L-cell differentiation. GLP-1 receptor 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, 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-1 receptor, indicating that serotonin acts downstream of GLP-1.

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 increase 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 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 a directing the differentiation in the intestinal epithelium into specific cell types is possible and GPBAR1 (and possibly other GPCRs) could be a target for modulation of L-cell number as a therapeutic strategy against type 2 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Arx	aristaless related homeobox
Dll1	delta-like protein 1
EEC	enteroendocrine cells
Foxa1 and 2	forkhead box A1 and A2
GLP-1	glucagon-like peptide 1
GPBAR1	G protein-coupled bile acid receptor 1
Hes1	hairy and enhancer of split-1
Htr (5HTR)	5-hydroxytryptamine receptors
iFABP	intestinal fatty-acid binding protein
ITF	intestinal trefoil factor

LCA	lithocholic acid
Lgr5	leucine-rich repeat-containing G protein-coupled receptor
Lyz1	lysozyme 1
MAPK	mitogen-activated protein kinase
Math1	mouse atonal homolog 1
NeuroD1	Neurogenic differentiation 1
Ngn3	neurogenin 3
Wnt	Wingless-type MMTV integration site family
YFP	yellow fluorescent protein

References

1. Petersen N, Reimann F, van Es JH, van den Berg BM, Kroone C, Pais R, Jansen E, Clevers H, Gribble FM, de Koning EJ. Targeting development of incretin-producing cells increases insulin secretion. J Clin Invest. 2015; 125(1):379–385. [PubMed: 25500886]
2. Basak O, Beumer J, Wiebrands K, Seno H, van Oudenaarden A, Clevers H. Induced Quiescence of Lgr5+ Stem Cells in Intestinal Organoids Enables Differentiation of Hormone-Producing Enteroendocrine Cells. Cell Stem Cell. 2017; 20(2):177–190. [PubMed: 27939219]
3. Petersen N, Frimurer TM, Terndrup Pedersen M, Egerod KL, Wewer Albrechtsen NJ, Holst JJ, Grapin-Botton A, Jensen KB, Schwartz TW. Inhibiting RHOA Signaling in Mice Increases Glucose Tolerance and Numbers of Enteroendocrine and Other Secretory Cells in the Intestine. Gastroenterology. 2018; 155(4):1164–1176.e2. [PubMed: 29935151]
4. Gehart H, Clevers H. Tales from the crypt: new insights into intestinal stem cells. Nat Rev Gastroenterol Hepatol. 2018; 16(1):19–34.
5. Jenny M, Uhl C, Roche C, Duluc I, Guillermin V, Guillemot F, Jensen J, Kedinger M, Gradwohl G. Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. EMBO J. 2002; 21:6338–6347. [PubMed: 12456641]
6. Schonhoff SE, Giel-Moloney M, Leiter AB. Minireview: development and differentiation of gut endocrine cells. Endocrinology. 2004; 145:2639–2644. [PubMed: 15044355]
7. Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev. 1997; 11(18):2323–2334. [PubMed: 9308961]
8. Beucher A, Gjernes E, Collin C, Courtney M, Meunier A, Collombat P, Gradwohl G. The homeodomain-containing transcription factors Arx and Pax4 control enteroendocrine subtype specification in mice. PLoS One. 2012; 7(5):e36449.doi: 10.1371/journal.pone.0036449 [PubMed: 22570716]
9. Ye DZ, Kaestner KH. Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice. Gastroenterology. 2009; 137:2052–2062. [PubMed: 19737569]
10. Petersen N, Reimann F, Bartfeld S, Farin HF, Ringnalda FC, Vries RG, van den Brink S, Clevers H, Gribble FM, de Koning EJ. Generation of L cells in mouse and human small intestine organoids. Diabetes. 2013; 63(2):410–420. [PubMed: 24130334]
11. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, Gribble FM. Short chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein coupled receptor FFAR2. Diabetes. 2012; 61:364–371. [PubMed: 22190648]
12. Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, Macchiarulo A, Yamamoto H, Mataki C, Pruzanski M, Pellicciari R, et al. TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab. 2009; 10(3):167–177. [PubMed: 19723493]

13. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, et al. A G protein-coupled receptor responsive to bile acids. J Biol Chem. 2003; 278:9435–9440. [PubMed: 12524422]
14. Harach T, Pols TW, Nomura M, Maida A, Watanabe M, Auwerx J, Schoonjans K. TGR5 potentiates GLP-1 secretion in response to anionic exchange resins. Sci Rep. 2012; 2:430.doi: 10.1038/srep00430 [PubMed: 22666533]
15. Glicksman C, Pournaras DJ, Wright M, Roberts R, Mahon D, Welbourn R, Sherwood R, Alaghband-Zadeh J, le Roux CW. Postprandial plasma bile acid responses in normal weight and obese subjects. Ann Clin Biochem. 2010; 47(Pt 5):482–4. [PubMed: 20595403]
16. Hauge M, Ekberg JP, Engelstoft MS, Timshel P, Madsen AN, Schwartz TW. Gq and Gs signaling acting in synergy to control GLP-1 secretion. Mol Cell Endocrinol. 2017; 449:64–73. [PubMed: 27908836]
17. Grunddal KV, Ratner CF, Svendsen B, Sommer F, Engelstoft MS, Madsen AN, Pedersen J, Nøhr MK, Egerod KL, Nawrocki AR, Kowalski T, et al. Neurotensin Is Coexpressed, Coreleased, and Acts Together With GLP-1 and PYY in Enteroendocrine Control of Metabolism. Endocrinology. 2016; 157(1):176–194. [PubMed: 26469136]
18. Donkers JM, Roscam Abbing RLP, van de Graaf SFJ. Developments in bile salt based therapies: A critical overview. Biochem Pharmacol. 2019; 161:1–13. [PubMed: 30582898]
19. Lund ML, Egerod KL, Engelstoft MS, Dmytriyeva O, Theodorsson E, Patel BA, Schwartz TW. Enterochromaffin 5-HT cells - A major target for GLP-1 and gut microbial metabolites. Mol Metab. 2018; 11:70–83. [PubMed: 29576437]
20. Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, Gribble FM. Glucose sensing in L cells: a primary cell study. Cell Metab. 2008; 8(6):532–539. [PubMed: 19041768]
21. Scrocchi LA, Brown TJ, MacLusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide receptor gene. Nat Med. 1996; 2:1254–1258. [PubMed: 8898756]
22. Sato T, Clevers H. Primary mouse small intestinal epithelial cell cultures. Methods Mol Biol. 2013; 945:319–328. [PubMed: 23097115]
23. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD, Clevers H. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology. 2011; 141(5):1762–1772. [PubMed: 21889923]
24. Hauge M, Vestmar MA, Husted AS, Ekberg JP, Wright MJ, Di Salvo J, Weinglass AB, Engelstoft MS, Madsen AN, Lückmann M, Miller MW, et al. GPR40 (FFAR1)—combined Gs and Gq signaling in vitro is associated with robust incretin secretagogue action ex vivo and in vivo. Mol Metab. 2015; 4:3–14. [PubMed: 25685685]
25. Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG. Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behav Genet. 2002; 32(6):435–443. [PubMed: 12467341]
26. VanDussen KL, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kolterud Å, Grosse AS, et al. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development. 2012; 139(3):488–97. [PubMed: 22190634]
27. Arranz B, Rosel P, San L, Sarró S, Navarro MA, Marcusson J. Characterization of the 5-HT4 binding site in human brain. J Neural Transm (Vienna). 1998; 105(6–7):575–86. [PubMed: 9826103]
28. Glatzle J, Sternini C, Robin C, Zittel TT, Wong H, Reeve JR Jr, Raybould HE. Expression of 5-HT3 receptors in the rat gastrointestinal tract. Gastroenterology. 2002; 123(1):217–226. [PubMed: 12105850]
29. Egerod KL, Engelstoft MS, Grunddal KV, Nøhr MK, Secher A, Sakata I, Pedersen J, Windeløv JA, Füchtbauer EM, Olsen J, Sundler F, et al. A major lineage of enteroendocrine cells coexpress CCK, secretin, GIP, GLP-1, PYY, and neurotensin but not somatostatin. Endocrinology. 2012; 153(12):5782–5795. [PubMed: 23064014]

30. Morimoto K, Watanabe M, Sugizaki T, Irie J, Itoh H. Intestinal Bile Acid Composition Modulates Prohormone Convertase 1/3 (PC1/3) Expression and Consequent GLP-1 Production in Male Mice. Endocrinology. 2016; 157(3):1071–1081. [PubMed: 26789236]

31. Koehler JA, Baggio LL, Yusta B, Longuet C, Rowland KJ, Cao X, Holland D, Brubaker PL, Drucker DJ. GLP-1R agonists promote normal and neoplastic intestinal growth through mechanisms requiring Fgf7. Cell Metab. 2015; 21(3):379–391. [PubMed: 25738454]

32. De Vadder F, Grasset E, Mannerås Holm L, Karsenty G, Macpherson AJ, Olofsson LE, Bäckhed F. Gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks. Proc Natl Acad Sci U S A. 2018; 115(25):6458–6463. [PubMed: 29866843]

33. Mawe GM, Hoffman JM. Serotonin signaling in the gut--functions, dysfunctions and therapeutic targets. Nat Rev Gastroenterol Hepatol. 2013; 10(8):473–86. [PubMed: 23797870]

34. Muñoz J, Stange DE, Schepers AG, van de Wetering M, Koo BK, Itzkovitz S, Volckmann R, Kung KS, Koster J, Radulescu S, Myant K, et al. The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. EMBO J. 2012; 31(14):3079–3091. [PubMed: 22692129]

35. Sonne DP, Hansen M, Knop FK. Bile acid sequestrants in type 2 diabetes: potential effects on GLP1 secretion. Eur J Endocrinol. 2014; 171(2):R47–65. [PubMed: 24760535]

36. Gioiello A, Rosatelli E, Nuti R, Macchiarulo A, Pellicciari R. Patented TGR5 modulators: a review (2006 - present). Expert Opin Ther Pat. 2012; 22(12):1399–414. [PubMed: 23039746]

37. Delzenne NM, Neyrinck AM, Bäckhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol. 2011; 7(11):639–646. [PubMed: 21826100]

38. Makki K, Deehan EC, Walter J, Bäckhed F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. Cell Host Microbe. 2018; 23(6):705–715. [PubMed: 29902436]

39. Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. Nature. 2016; 535(7610):56–64. [PubMed: 27383980]

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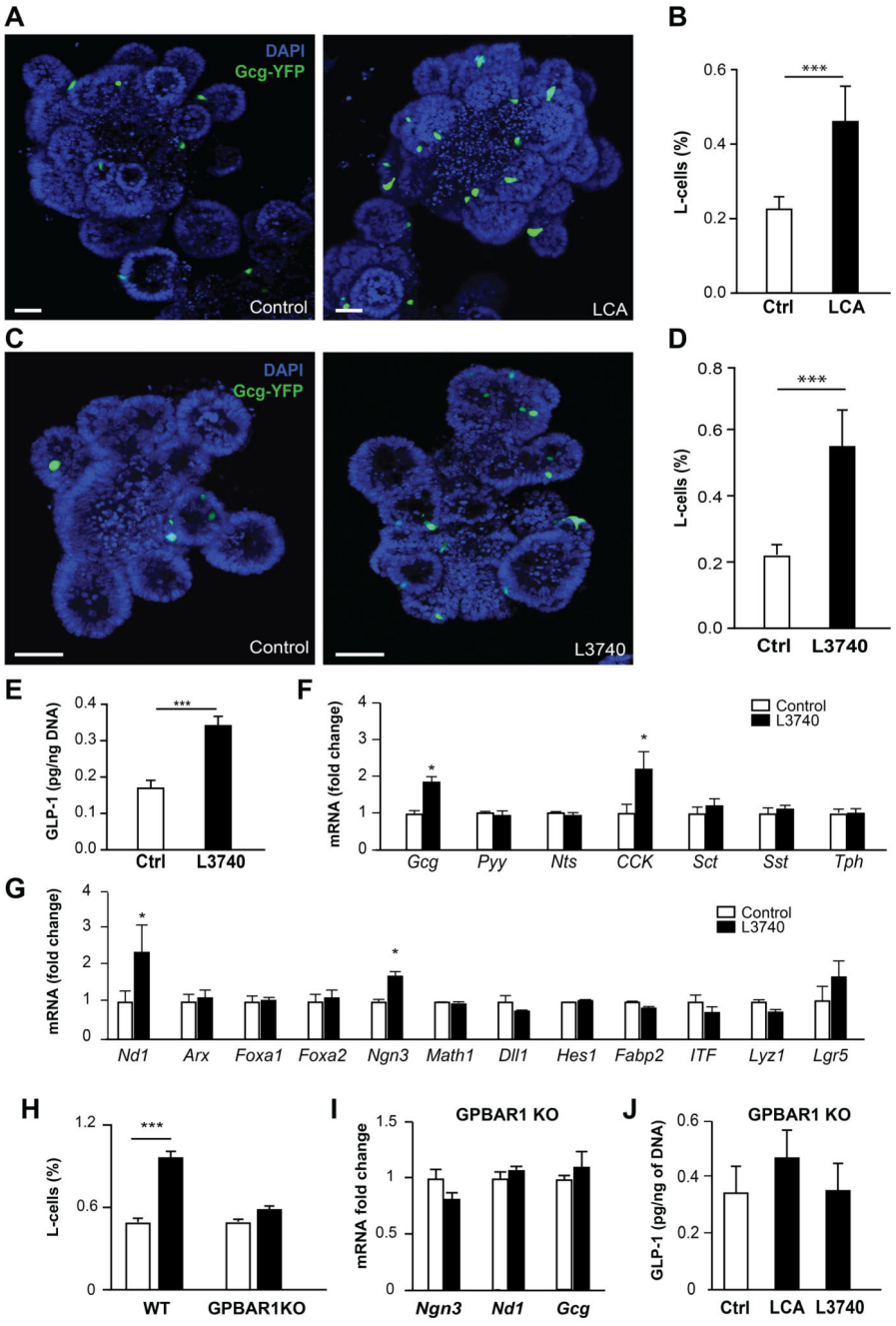

Figure 1. Activation of GPBAR1 by the bile acid LCA and a synthetic agonist increases L-cell number in organoids.

(A) Representative images of control and organoids treated with 20 μM LCA. L-cells are labeled by Gcg-YFP expression in this and following images unless otherwise indicated. Nuclei labeled by DAPI. Bar 50 μm. (B) L-cell numbers in control organoids (n = 70) and organoids treated with LCA (n = 78). In this and following figures data are mean ± SEM (unless otherwise indicated). * $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$. (C) Representative images of control and L3740-treated organoids. Bar 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 mM glucose and 2 mM L-glutamine. Data from 3 experiments performed with 5 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*, *Foxa1/2* – L-cell transcription factors (n = 4-10 experiments in each series). (H) L-cell numbers, identified by immunostaining, in control and GPBAR1KO and wild type mouse organoids after 48-h-treatment with L3740 (n = 21-25). (I) Expression of L-cell differentiation markers in control and L3740-treated GPBAR1 knockout organoids. n = 3 for each series. (G) GLP-1 release in response to LCA and L3740 in GPBAR1 knockout organoids. n = 5 for each series.

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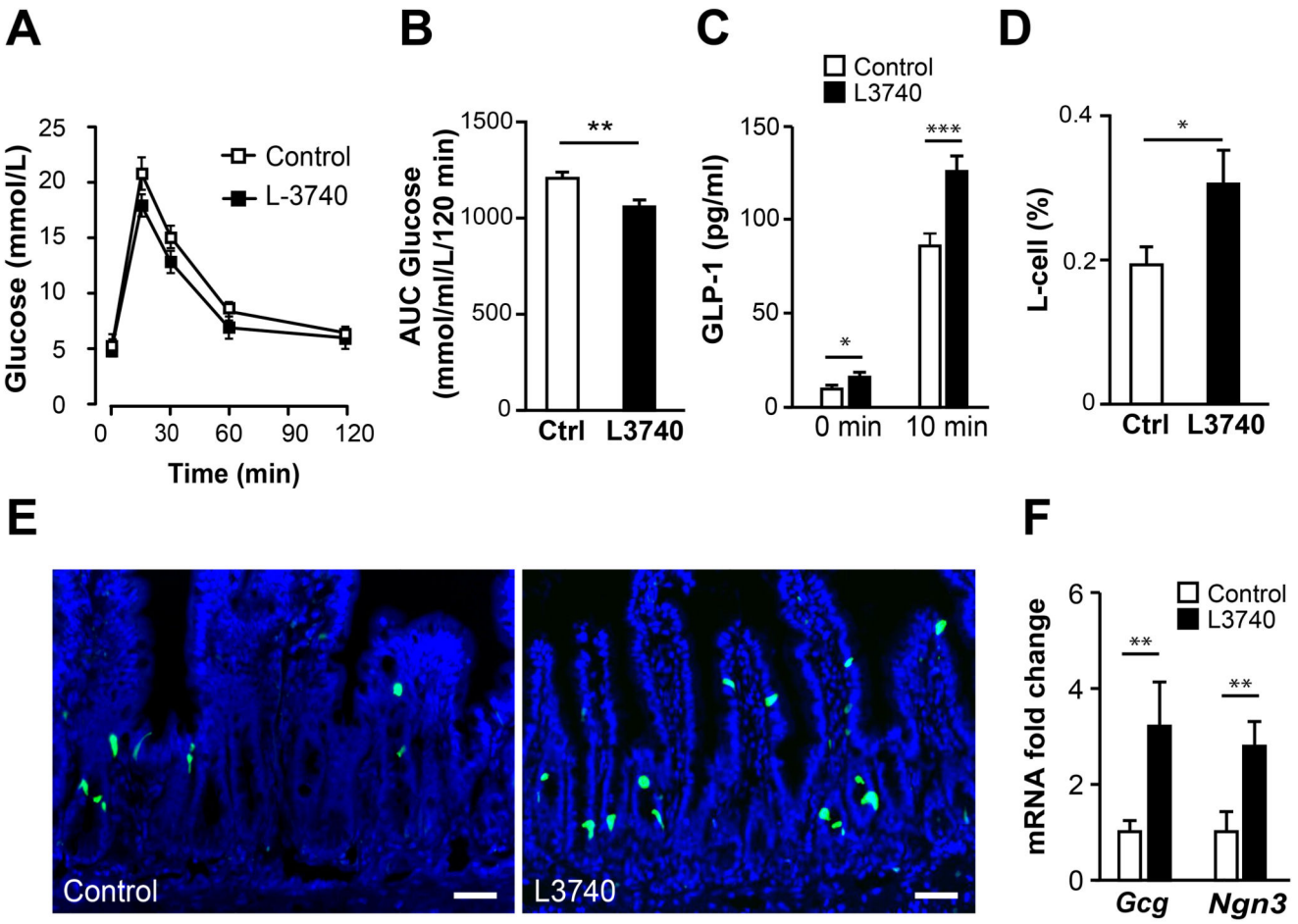

Figure 2. In-feed L3740 treatment increases oral glucose tolerance, GLP-1 secretion and L-cell number in mice.

(A) Plasma glucose excursion during an oral glucose tolerance test after 5 hour fasting and. L3740, black squares; control (vehicle), white squares. n = 10 for each series. (B) Area under the curve from data in panel A (AUC). (C) Plasma GLP-1 concentrations in control and L3740-treated mice and 10 minutes after glucose. n = 8 for each series. (B) L-cells (Gcg-YFP) in the ileum of control and L3740 treated mice. Nuclei labeled by DAPI. Bar 50 μm. (C) L-cell percentage in mouse ileum sections. n = 4 for each series. (D) Expression of *Gcg* and *Ngn3* (n = 4 for each series).

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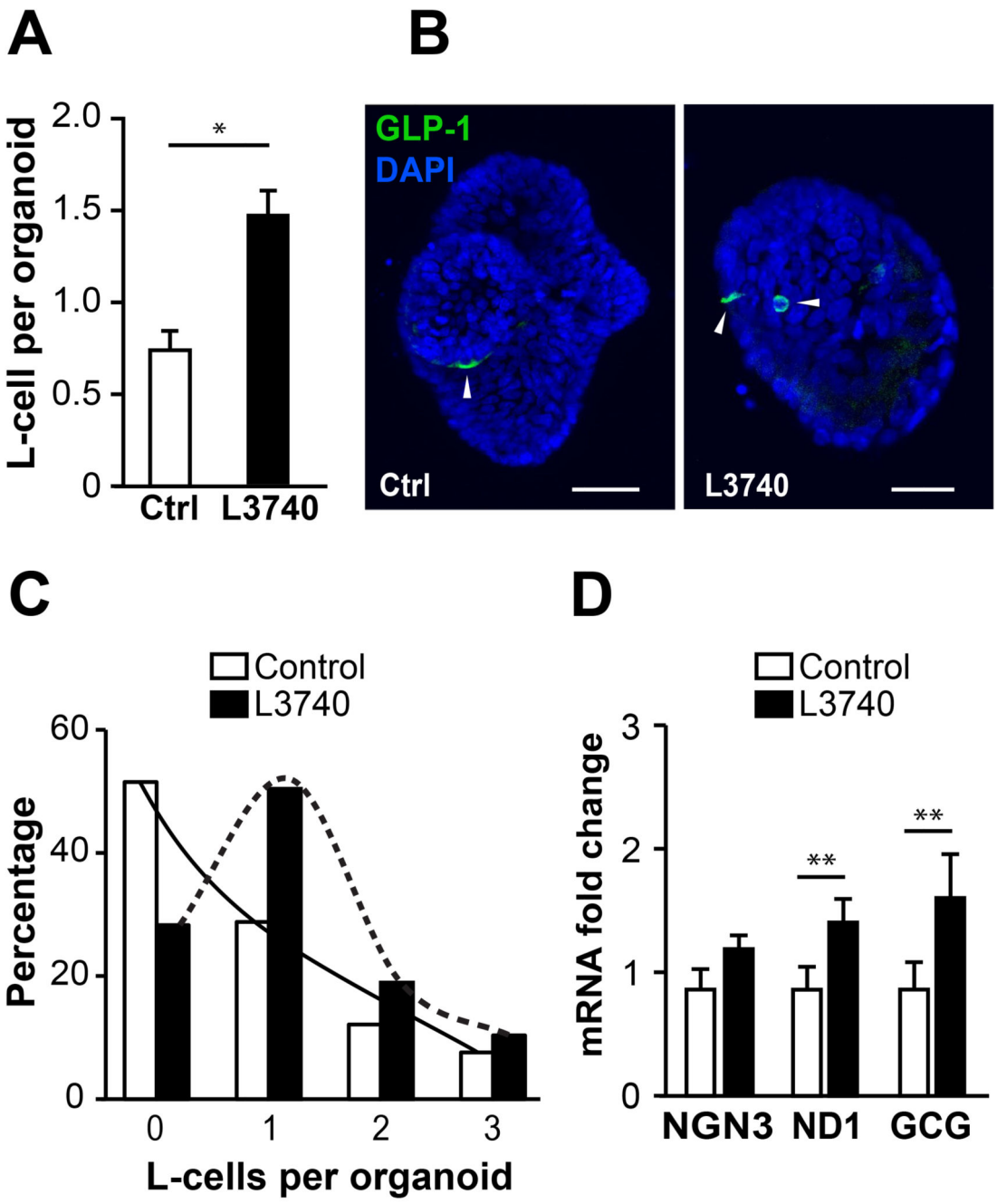

Figure 3. GPBAR1 stimulation increases L-cell abundance in human organoids.

(A) L-cell numbers in control (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), nuclei labeled by DAPI. Bar 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 Chi-Square contingency test. (D) Expression of NGN3, NEUROD1 (ND1) and GCG (n = 4 for each series, generated from 2 human organoid lines).

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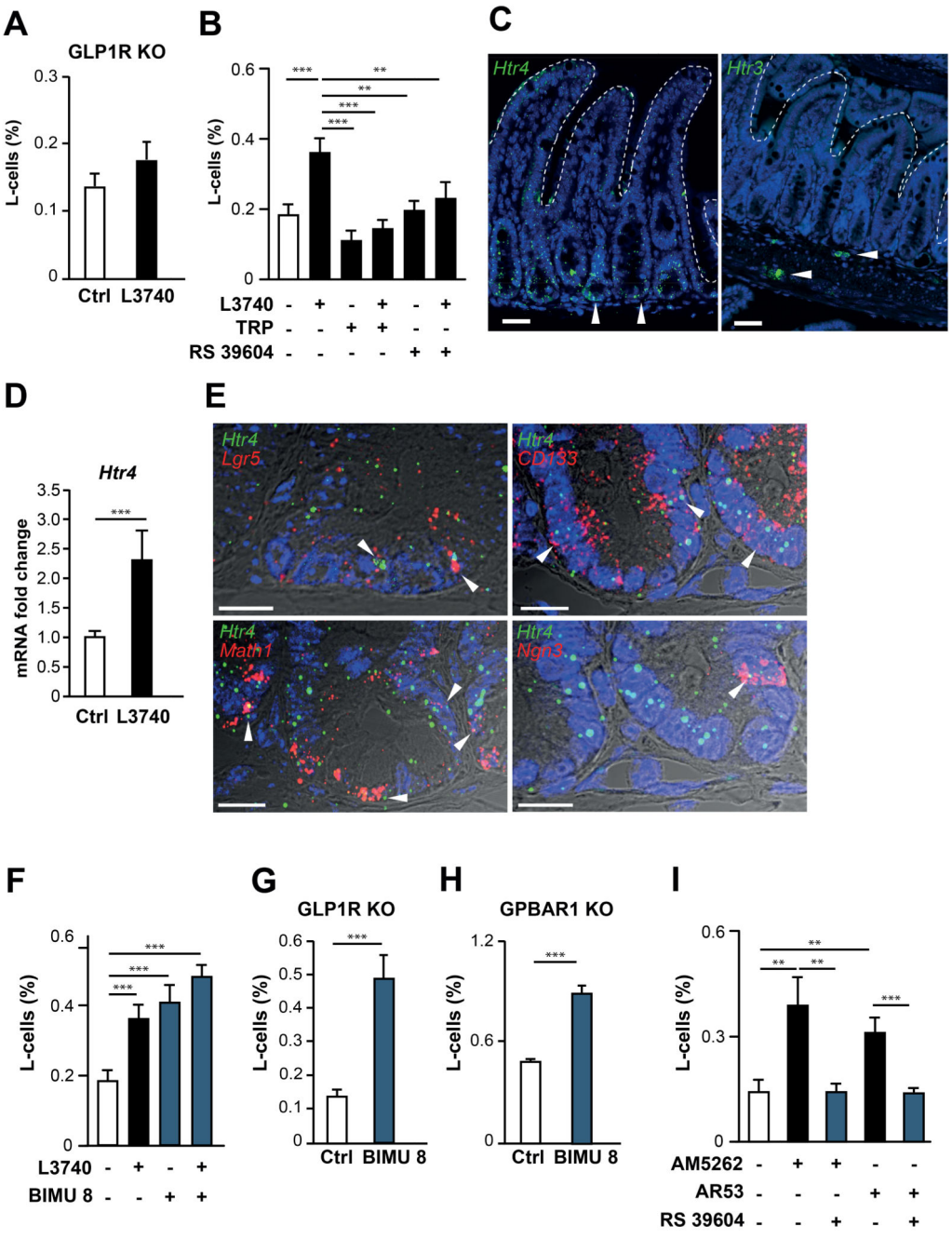

Figure 4. GPBAR1 induction of L-cell differentiation requires cross-talk with serotonin signaling.

(A) Effect of L3740 in GLP1RKO mouse organoids. n = 47 for control 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. Bar 50 μm. (D) L3740 increases *Htr4* expression in organoids. n = 7 for control and n = 6 for L3740-treated series. (E) Co-expression 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). Bar 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) BIMU8 increases L-cell numbers in GPBAR1KO 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.

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