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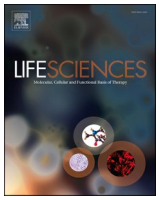

GPR41 and GPR43 modulate rodent pancreatic α-cell function and growth

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ABSTRACT

Objective: While SCFA receptors GPR41 and GPR43 regulate β-cell insulin secretion, their role in α-cells remains unknown despite hyperglucagonemia in type 2 diabetes (T2D). Thus, the current study aims to investigate the ability of synthetic GPR41 and GPR43 agonists to modulate α-cell physiology and responsiveness to nutrient challenge.

Methods: Using αTC1.9 cells and primary rat islets we investigated the role of SCFA receptors in glucagon expression and secretion under physiological and insulin resistant conditions associated with high-fat feeding (HFD) and lactation (L). The specific agonists AR420626 (AR) and (*S*)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl) butanamide (PA) were employed to study the mechanisms involved.

Results: Histological and flow cytometry analysis of islets demonstrated that GPR41 and GPR43 localized in α-cells. Treatment of αTC1.9 cells with the GPR41-agonist AR or GPR43-agonist PA increased *Gcg* expression and glucagon secretion at low glucose, while AR also potentiated glucagon release at high glucose. This effect was recapitulated in isolated islets demonstrating pertussis toxin sensitivity for both agonist effects. HFD-fed animals showed glucose intolerance, early fasting hyperglucagonemia and islet resistance to glucose inhibition of glucagon secretion together with enhanced expression of islet *Gpr41/43*. Stimulation of HFD islets with the synthetic agonists further increased *Gcg* expression. Pancreatic *Gpr41/43* levels were also transiently induced during lactation although only GPR41 activation of lactating rat islets up-regulated *Gcg* expression via $G\alpha_i$ and α-cell replication.

Conclusions: These findings position GPR41 as a promising therapeutic target for modulating hyperglucagonemia and improving glycemic control in T2D, supporting its translational relevance in diabetes intervention strategies.

1. Introduction

Glucagon, secreted by pancreatic α-cells, regulates blood glucose by stimulating hepatic glucose production during hypoglycemia. In diabetes, persistent glucagon secretion leads to hyperglucagonemia, driving excessive glucose output and worsening hyperglycemia [1,2]. This dysfunction appears early in type 2 diabetes (T2D) [3], highlighting α-cells as key targets for therapeutic intervention. Nevertheless, there is still a paucity of knowledge regarding the regulatory mechanisms that control glucagon secretion under normal physiological conditions and the factors influencing its release, which is essential to advance in the search for new therapies. Beyond the direct effect of glucose and other nutrients on α-cells, it is known that intra-islet communication from β- and δ-cells may also suppress glucagon release in response to secreted

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factors [4,5]. However, it has been suggested that the basal and post-prandial hypersecretion of glucagon in T2D may be related to disrupted action of gastrointestinal factors [6–8]. It is important to note that the communication between the gut and the pancreas, referred to as the entero-insular axis [9], has extensively been described [10]. Among the gut hormones released in response to nutrient ingestion, those capable of potentiating the glucose-induced insulin response are called incretins and mainly include glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). The insulinotropic and glucagonostatic effects of GLP-1 in combination with the glucagonotropic action of GIP, help to ensure consistent blood glucose levels throughout the day [8]. However, in addition to incretins, several gut bacteria metabolites, including short-chain fatty acids (SCFAs), can also modulate glucose homeostasis and energy metabolism. Dietary fibre that escapes digestion/absorption in the small intestine is fermented in the colon by the microbiota to produce SCFAs, the most abundant of which are acetate, propionate and butyrate [11]. SCFAs in general, and butyrate in particular, are the main source of energy for colonocytes, promote epithelial cell differentiation and growth, and modulate the maturation of the immune system, thus playing a central role in gut physiology and metabolism. Moreover, SCFAs can locally initiate cell-specific signalling cascades by activating the G protein-coupled free fatty acid receptors GPR41 (FFAR3) and GPR43 (FFAR2). GPR41 is coupled cytosolically to inhibitory signalling through $G\alpha_i$, whereas GPR43 is coupled to both $G\alpha_i$ and stimulatory signalling through $G\alpha_q$ [12,13]. In this way, SCFAs have been shown to directly stimulate the release of incretins in colonic enteroendocrine primary cells [14]. Specifically, it has been reported that the binding of SCFAs to GPR43 increases GLP-1 secretion from intestinal L-cells whereas global deletion of GPR43 in mice impairs glucose tolerance and reduces GLP-1 secretion [14–16]. Consistently, the fermentation of non-digestible carbohydrates in the gut also promotes the differentiation of L-cells in the small intestine and proximal colon, thereby increasing their density [17,18]. By increasing GLP-1 production and release, SCFAs indirectly modulate pancreatic β-cell function and insulin secretion. However, SCFAs can be rapidly absorbed, enter the portal and systemic circulation and signal in tissues other than L-cells, thus contributing to improved glucose homeostasis. Both GPR41 and GPR43 are known to be expressed in pancreatic islets [19–21]. Consistent with this, SCFAs may act directly on islet β-cells to regulate insulin secretion and survival. For example, long-term colonic administration of propionate in humans significantly improves β-cell function, as supported by increased circulating insulin not secondary to increased GLP-1 levels. Moreover, this effect seems to be via protein kinase C-dependent pathway [22]. However, studies in rodent and human islets focusing on the effects of acetate have reported that this SCFA stimulates [21,23], impedes [24] or has no effect on glucose-induced insulin secretion [19]. The reason for this divergence may be at least partly explained by the dual stimulatory ($G\alpha_q$) and inhibitory ($G\alpha_i$) activity downstream of GPR43, the major binding affinity receptor for acetate [12] but also should be note that GPR41 and GPR43 share endogenous ligands and can show differences in ligand efficacy between species [12]. On the other hand, GPR41 signalling only via inhibitory α_i negatively modulates insulin release in MIN6 or INS-1E β-cells or mouse islets [21]. Moreover, receptor expression on non-beta-cells within the islets [20] may also affect insulin secretion through paracrine effects [4,25]. In agreement, GPR43-agonists were recently reported to stimulate somatostatin secretion in mice [26], while the direct effects of both receptors on α-cells have yet to be explored. For all these reasons, the assignment of selective physiological roles to these receptors is limited and their real therapeutic potential against hyperglycaemia remains unsolved. In the present study, we aim to overcome these barriers by investigating the importance of GPR41 and GPR43 signalling in glucagon-secreting α-cells under both physiological and disease-related conditions, using specific synthetic agonists.

2. Material and methods

2.1. Animals and diets

Animal experiments were conducted according to Spanish legislation (Royal Decree 1201/2005 and 53/3013) on the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the Complutense University from Madrid (PROEX 215.8/21). Wistar rats, obtained from Janvier Labs (France), were housed in standard laboratory conditions (12 h light-dark cycle) with free access to water and a standard chow diet (SD; containing on caloric basis 11.1 % fat, 68.5 % carbohydrates and 20.3 % protein; ROD14, SodispanBiotech). The fatty acid profile of the SD diet is (% of total fat): 16 % saturated fatty acids, 20 % monounsaturated fatty acids and 49,4 % polyunsaturated fatty acids, 14,6 % unspecified. After 10 days of habituation, female rats were mated overnight with male rats. At birth, litter was standardized to eight pups per nursing dam to minimize effects of litter size on postnatal growth. The experiments were carried out in neonates on lactating days (L) 4, 14 and 18, and in 16–18 weeks old adult rats. The offspring used at adult age were randomly separated into two groups after weaning, one continued with the SD, and the other was fed with a high-fat diet for 14 weeks (HFD; 60.3 % fat, 21.4 % carbohydrates and 18.3 % protein; MD.2204, Envigo). The fatty acid profile of HFD (% of total fat): 36 % saturated fatty acids, 41 % monounsaturated fatty acids and 23 % polyunsaturated fatty acids. Rats were cohoused by diet and litters were mixed to avoid litter-specific effects of diet. Based on previous findings [27] that female rats develop more pronounced hyperglucagonemia and distinct metabolic responses to a HFD compared to males, this study focused exclusively on females to better model sex-specific impairments in glucagon regulation. All animals were weighed before sacrifice by decapitation. Blood was then collected, and serum was separated and stored frozen at −20 °C until analysis. The pancreas was quickly removed, weighed, and stored at −80 °C or fixed as indicated in Section 2.12.

2.2. Pharmacological treatments

The specific GPR41 agonist N-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamide (AR420626, AR) and the GPR43 agonist (*S*)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl) butanamide (PA) were both purchased from Sigma-Aldrich (catalog #SML1339 and #371725, respectively). For inhibition studies, pertussis toxin (PTX) and 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122), both purchased from Sigma-Aldrich, were added to the culture medium 1 h before the corresponding agonist at the doses indicated. cAMP production was induced by treatment with forskolin (purchased from Sigma-Aldrich) at the specified dose.

2.3. Cell culture

Alpha-TC1 cells (clone αTC1.9, ATCC CRL-2350) were grown in DMEM (Gibco, Invitrogen) supplemented with 4 mM L-glutamine, 19 mM $NaHCO_3$, 10 % FBS, 15 mM HEPES, 1 % penicillin/streptomycin, 0.1 mM non-essential amino acids, and a final glucose concentration of 3 g/L. Cells were kept at 37 °C in 95 % humidified air and 5 % CO_2.

2.4. Islet isolation

Adult pancreatic islets were isolated by collagenase digestion (collagenase P from Clostridium histoliticum; Sigma-Aldrich) as previously described [28]. Isolated islets, pending the experiments, were maintained overnight for recovery in medium RPMI 1640 containing 11 mM glucose (Gibco, Invitrogen) and supplemented with 1 % penicillin/streptomycin and 10 % FBS at 37 °C and 5 % CO_2.

Islets from neonates were obtained according to the method of

Hellerström et al. (Hellerström et al., 1979) with certain modifications as previously described [29]. The islets were maintained in medium RPMI 1640 (Gibco, Invitrogen) supplemented with 1 % penicillin/streptomycin and 10 % FBS for 2–3 days to ensure exocrine tissue removal.

2.5. Flow cytometry

Adult islets were dissociated into single cells by mechanical and enzymatic dispersion using TrypLE™ Express Enzyme 1× (Gibco). Dissociated cells were fixed and permeabilized for 20 min at 4 °C with Fixation/Permeabilization solution (BD Biosciences). Fixed cells were then incubated with blocking solution [BD Perm/Wash™ buffer containing 1 % normal goat serum (*v/v*) and 2 % BSA (*w/v*)] for 15 min at 4 °C. After that, cells were incubated with mouse monoclonal anti-somatostatin antibody (GeneTex; 1:200) plus rabbit polyclonal anti-GPR41 antibody (Abcam; 1:100) or anti-GPR43 antibody (Abcam; 1: 500) in BD Perm/Wash™ buffer for 1 h at 4 °C and subsequently with secondary antibody conjugated with fluorescent dyes for 30 min at 4 °C (Alexa Fluor™ anti-rabbit 488 [1:500] or APC anti-mouse Invitrogen [1:2000]). Then, islet cells were incubated for 20 min at 4 °C with the conjugated antibodies PE rabbit monoclonal anti-insulin antibody (Abcam; 1:1000) and BV421 mouse monoclonal anti-glucagon antibody (BD Biosciences; 1:250) in BD Perm/Wash™ buffer. Cells were resuspended in PBS containing 1 % FBS and filtered. Unstained and single antibody controls were performed for every staining protocol and fluorescent minus one (FMO) controls were also used. Flow cytometry studies were performed in an LSR Fortessa Cell Analyzer (BD) and analyzed using FlowJo V10 Software (FlowJo) in LACISEP laboratory (CIEMAT, Madrid).

2.6. Cell viability measurement by crystal violet

Alpha-TC1.9 cells were seeded at a density of 10^5 cells/well in a 24-well plate, and the day after were treated with a range of concentrations (0.1–10 μM) of each agonist. Then, cells were incubated with crystal violet [0.2 % (*w/v*) in ethanol] for 20 min. Finally, cells were washed with water, allowed to dry and sodium dodecyl sulfate [1 % (w/v) SDS, PanReac AppliChem] added. Absorbance was measured in this supernatant at 570 nm.

2.7. Analysis of gene expression by RT-qPCR

Total RNA was extracted from the different cells or tissues using TRIzol Reagent (Gibco, Invitrogen) and reverse transcribed with a high-capacity cDNA reverse transcription kit (Life Technologies). Real-time qPCR for *Gcg, Sst, Gpr41* and *Gpr43* was performed with TaqMan probes (Applied Biosystems) following manufacturer's protocol. In other cases, forward and reverse primers were used to determine the relative abundance of *Pcsk1* (fw: TGGTGATTACACAGACCAGCG; rv: CTCCAAGGCCAGAGCAAAGA), *Pcsk2* (fw: AAGAAGACGCAGCCTACACC; rv: CCATCGGCTTGCCCAGTGTT), and *Ins* genes (fw: TCTTCTACACACCCAAGTCCCG; rv: AGTGCCAAGGTCTGAAGATCCC). Reactions were performed in duplicate and the target gene values were normalized to the expression of the endogenous reference Gapdh (TaqMan probe, Applied Biosystems). The comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) was used to calculate relative expression: [ΔCt = Ct (target gene) — Ct (Gapdh); ΔΔCt = ΔCt for any sample — ΔCt for the control] [30].

2.8. Ca^{2+} measurements

For intracellular Ca^{2+} measurements, alpha-TC1.9 cells were seeded on glass coverslips treated with poly-L-lysine (Sigma). Prior to fluorescence recordings, cells were loaded with 2 μM Fura-2/AM (Invitrogen) at room temperature for 1 h in a humidified atmosphere under a non-stimulatory concentration of 5.6 mM glucose. Recordings were performed in a constant-volume chamber with a Krebs-Ringer solution (141 mM NaCl, 5.5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4), with or without 2 mM $CaCl_2$, perfusion of 2 mL/min at 35–37 °C. Coverslips were perfused with a stimulatory concentration of 0.5 mM glucose for 10 min before perfusing 1 μM AR or 1 μM PA in combination with 0.5 mM glucose for 10 min. Cytoplasmic Ca^{2+} oscillations were recorded using an inverted fluorescence microscope (Zeiss Axiovert 200, Jana, Germany) equipped with a polychromator (TILL Photonics) to ensure a wavelength emission of 340 and 380 nm. Fluorescence intensity data were acquired using a Hamamatsu EMC9100 digital camera every 2.5 s and plotted with Aquacosmos software version 2.6 (Hamamatsu Photonics, Massy, France). Changes in cytosolic Ca^{2+} were represented as the ratio of the fluorescence emission intensities at 340 and 380 nm (F_{340}/F_{380}, ΔF; fluorescence arbitrary units). Intracellular Ca^{2+} variations were analyzed as peak amplitude and frequency of oscillations for 5 min after each stimulus.

2.9. Western blot

Pancreatic islets and αTC1.9 cells were lysed in lysis buffer containing 12.5 mM Tris pH 7.5, 1.25 mM EGTA, 1.25 mM EDTA, 0.25 % Triton x-100, 2 mM o-vanadate sodium, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptin, 2 mM benzamidine and 10 μg/mL of aprotinin. Protein extracts were quantified using the Bradford dye method (BioRad), and 25 μg was loaded in a 12 % SDS polyacrylamide gel for electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were incubated overnight at 4 °C with the corresponding primary antibodies: rabbit anti-GPR41 (ab236654, Abcam; 1: 10000 dilution) or mouse anti-GPR43 (sc-293202, Santa Cruz Biotechnology; 1: 500 dilution) and then with secondary anti-rabbit antibody (A6154, Sigma-Aldrich; 1: 1000 dilution) or anti-mouse (A4416, Sigma-Aldrich; 1: 4500 dilution) for 1 h at room temperature. Blots were visualized using a ChemiDoc™ Imaging System (Bio-Rad Inc.) and densitometric analysis was performed using Image J software (NIH, Bethesda, MA USA). Normalization of Western blot was ensured by reprobing the membranes with mouse anti-β-actin (A1978, Sigma-Aldrich; 1: 30000 dilution).

2.10. Glucagon secretion experiments

Freshly isolated islets were left to recover for 2 h at 37 °C and 5 % CO2 in Krebs-Ringer Bicarbonate buffer (KRB; NaCl 460 mM, NaHCO3 96 mM, KCl 20 mM, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$) supplemented with 0.5 % BSA and 5.6 mM glucose. Afterwards, batches of size-matched islets were exposed to 0.5 mM or 16.7 mM glucose for 1 h plus the corresponding agonists. The supernatant was collected and stored at −80 °C until glucagon analysis. Values were normalized per islet number [28]. For αTC1.9 cells, these were preincubated for 2 h with 500 μL of KRB secretion media with 5 mM glucose and then, incubated for another 1 h with the appropriate stimuli: 0.5 mM or 16.7 mM glucose plus agonists. Next, supernatant was also collected and used to measure glucagon secretion by ELISA. Aprotinin (20 mg/L; Sigma-Aldrich) was included in all media. Glucagon content was extracted from islets or cells in acid-ethanol solution (ethanol/water/HCl 12 N 74:25:1), centrifuged at 2500 rpm at 4 °C for 15 min, and supernatants were collected for later analysis. In all cases, glucagon concentration was measured by specific glucagon ELISA (10–1281-01, Mercodia) according to manufacturer's protocol. Total protein concentration was analyzed using the Bradford dye method (BioRad).

2.11. Glucose tolerance test

Following 16 h of fasting, adult female rats received an i.p. injection of D (+)-Glucose (2 g/kg body weight). Blood samples were obtained by tail vein puncture before or at 15, 30, 60 and 120 min after injection and glucose concentrations were measured with an Accu-Check® Aviva

blood glucose strips (Roche, Sigma-Aldrich).

2.12. Immunofluorescence staining

The whole pancreases were harvested and fixed in 4 % PFA for up to 24 h before embedding in paraffin. The immunofluorescence staining for islet hormones and receptors GPR41 and GPR43 was performed on 5 μm sections after being deparaffinized and rehydrated. The antigens were retrieved by heating the slides with sodium citrate buffer (10 mM $Na_3C_6H_5O_7$, Tween-20 0.05 %, pH 6) at 97 °C for 30 min. The slides were then cooled for 20 min at room temperature. Afterward, the slides were rinsed 3 times with PBS. Non-specific background was blocked by incubation with 2 % BSA and 1 % normal goat serum in PBS and 0.1 % Triton X-100 (PBS-T) for 1 h at room temperature. Sections were then incubated overnight at 4 °C with the following antibodies: guinea-pig anti-insulin (1:20 Abcam), mouse anti-glucagon (1:300, Sigma-Aldrich) and rabbit anti-GPR41 or rabbit anti-GPR43 (1:250 and 1:400, respectively; both from Abcam); all diluted in the aforementioned blocking buffer. After the incubation step, the slides were rinsed 3 times in PBS-T for 5 min each and probed with a 1:100 dilution of Alexa Fluor 594 conjugated goat anti-guinea pig, donkey anti-mouse 647 and donkey anti-rabbit 488 conjugated and a 1:1000 dilution of 10 mg/mL of DAPI solution (all from ThermoFisher Scientific, Inc.) in blocking buffer for 1 h at room temperature in dark. The sections were rinsed twice for 5 min each using PBS-T and covered with ProLong Gold Antifade Mountant (Invitrogen, ThermoFisher Scientific, Inc.). As negative control slides were incubated without primary antibody but with secondary AlexaFluor antibody. Images used for quantification were taken with a Leica SP-2 AOBS confocal microscope at 20× magnification from at least two non-adjacent sections of pancreas per rat, and four images were then used for each section to quantify. Images were taken from two independent experiments, and each experiment had 2–3 rats per group. The intensity of area stained was measured using Fiji (ImageJ software) [31]. For in toto islet immunostaining, 20 islets pretreated for 48 h with 1 μM of GPR41 agonist (AR) or GPR43 agonist (PA) were handpicked, placed in μ-Slide 8-well plates (80826; Ibidi) and processed for glucagon (same conditions as previously mentioned) and Ki67 (mAb IgG rabbit from Abcam, 1:100) immunostaining. Immunofluorescence was examined using the Leica SP-2 AOBS confocal microscope at 20× magnification. The percentage of replicating alpha cells was obtained by dividing the number of positive cells for Ki67 staining by the total number of glucagon-positive cells in each islet.

2.13. Analytical determinations

Serum insulin and glucagon levels were determined using a rat insulin ELISA kit (10–1250-01; Mercodia) and a rat glucagon ELISA kit (10–1281-01; Mercodia), respectively, according to the manufacturer's protocol. Analysis of serum SCFAs was performed by LC-QqQ-MS with stable-isotope dilution after chemical derivatization with dansylhydrazine based on a modified method [32]. Analysis was carried out at CEMBIO, Centre for Metabolomics and Bioanalysis, with the LC Instrument 1260 Infinity series (Agilent Technologies), coupled to a Triple Quadrupole analyzer (G6470A, Agilent) with an electrospray ionization source (ESI) in positive mode. Data was collected in dynamic multiple reaction monitoring.

2.14. Statistics

Statistical analysis was performed with GraphPad Prism 8 software (GraphPad Software, USA). Shapiro-Wilk and Kolmogorov-Smirnov test determined which samples followed a parametric distribution. For the comparison of two groups, significance was assessed using a 2-tailed Student's *t*-test, while one-way ANOVA followed by Bonferroni post hoc test was used to compare mean differences between three or more groups. Data were expressed as means ± SEM and the level of significance was $p < 0.05$.

3. Results

3.1. GPR41 and GPR43 receptors are both expressed in pancreatic α-cells

In order to identify in which islet-cell type SCFA receptors, GPR41 and GPR43, were expressed, we performed histological and flow cytometry analysis on adult rat pancreatic islets. Immunohistochemistry examination of pancreatic sections showed that GPR41 appears localized mainly in glucagon-positive α-cells although a lighter staining signal was also detected in β-cells (Fig. 1A). However, GPR43 immunofluorescence signal overlapped neither with glucagon nor with insulin-positive cells but with other minority islet cell suggesting expression in somatostatin-producing δ-cells (Fig. 1B). For this reason, we then performed flow cytometry analysis to more accurately identify and quantify the staining intensity in each specific islet cell type. To this end, isolated rat islets were dispersed and incubated with antibodies against glucagon, insulin and somatostatin as well as GPR41 or GPR43 receptors. The gated strategy is represented in Fig. 1C-D. Side scatter (SSC) and forward scatter (FSC) indicated the relative differences in complexity and size of the cells, respectively. This property allowed us to easily visualize the different populations being β-cells more granulated and larger than α- or δ-cells whereas the last two others were distinguished between them by the higher size of δ-cells (Supplemental Fig. 1). Immunostaining for GPR41 of rat islet cell preparations revealed the presence of this receptor in the three types of cells but with significantly higher levels in α- and δ-cells compared to β-cells (Fig. 1C). GPR43 staining was also detected in all islet cells, although δ-cells showed more than 7-fold higher intensity than α- or β-cells, in line with immunofluorescence results (Fig. 1D).

3.2. GPR41 and GPR43 agonists regulate the expression of identity genes of α-cells and glucagon secretion

In line with results depicted in Fig. 1, RT-qPCR verified that *Gpr41* and *Gpr43* genes were abundantly expressed in isolated rat islets and the rat β-cell line INS-1, but also in the mouse cell line αTC1.9 (Fig. 2A). Likewise, Western blot analysis detected both receptors at the protein level in αTC1.9 cells (Fig. 2B).

To determine the role of GPR41 and GPR43 activation in α-cell function, we treated αTC1.9 cells with the selective GPR41-specific agonist, AR or GPR43-specific agonist, PA. Both agonists demonstrated non-toxicity in the 0.1–1 μM concentration range, as monitored by the crystal violet assay, but a partial although significant reduction in cell density of 27.6 % and 37.4 % for GPR41 and GPR43 agonists respectively when incubated at the higher dose of 10 μM (Fig. 2C). Hence, the two lowest doses were chosen for the following experiments. We then analyzed whether the agonists were able to directly regulate the expression of key α-cell genes (Fig. 2D). Treatment with either agonist for 24 h induced a 3-fold increase in the expression of proglucagon (*Gcg*) in αTC1.9 cells. Consistent with this, activation of GPR41 and GPR43 almost abolished the expression of pro-hormone convertase 1 (*Pcsk1*), which is involved in the cleavage of the proglucagon gene to glucagon-like peptide 1 (GLP-1). On the contrary, the GPR41 agonist (AR) significantly increased *Pcsk2* gene expression, the convertase responsible for the cleavage of proglucagon to glucagon. Finally, only the GPR43 agonist (PA) significantly reduced the expression of *Gpr41* at long term, whereas both specific ligands were able to down-regulate the expression of *Gpr43*.

Then, we examined whether activation of these receptors affected glucagon secretion from αTC1.9 cells (Fig. 3A). In agreement with the literature [33], αTC1.9 cells were able to respond to increasing glucose concentration from 0.5 mM to 16.7 mM, reducing glucagon release by almost half of that measured at low glucose concentration (statistical non-reflected in the graph, unpaired *t*-test $p = 0.0028$). When α-cells

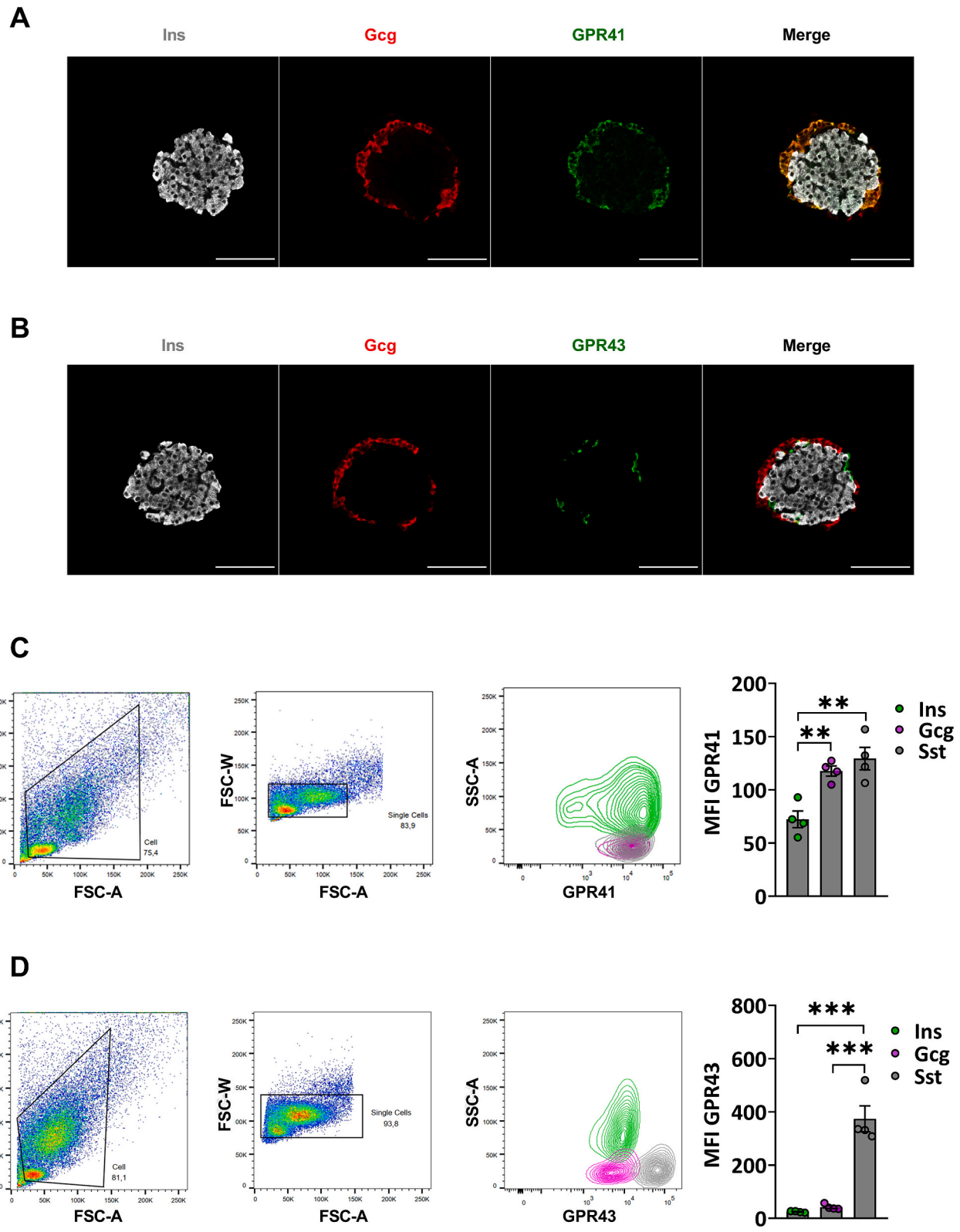

Fig. 1. Localization of GPR41 and GPR43 receptors in pancreatic tissue sections by histological and flow cytometry analysis. Confocal microscopy images of islets triple immunostained with insulin (in white), glucagon (in red) and G-protein–coupled receptor, GPR41 (A) or GPR43 (B) (in green). Scale bar (20×) = 100 μm. Representative diagram of the gating strategy to identify the expression of GPR41 (C) and GPR43 (D) in the pancreatic α-, β- and δ-cell populations from rat islets. First, cells were gated based on size and granularity by FSC-A/SSC-A gating to eliminate debris and clumped cells. Then singlets (single cell signals) were identified by FSC-A/FSC-W gating, followed by GPR41^{+} and GPR43^{+} on pancreatic islet cells. Finally, the fluorescence intensity of G-protein-coupled receptors in the three major subsets of endocrine cells was quantitatively analyzed, normalized to the relative cellular autofluorescence intensity. Data are means ± SEM for each experimental group ($n = 4$). $^{**}p < 0.01$; $^{***}p < 0.001$ between glucagon or somatostatin vs. insulin. Gcg: glucagon; Ins: insulin; Sst: somatostatin.

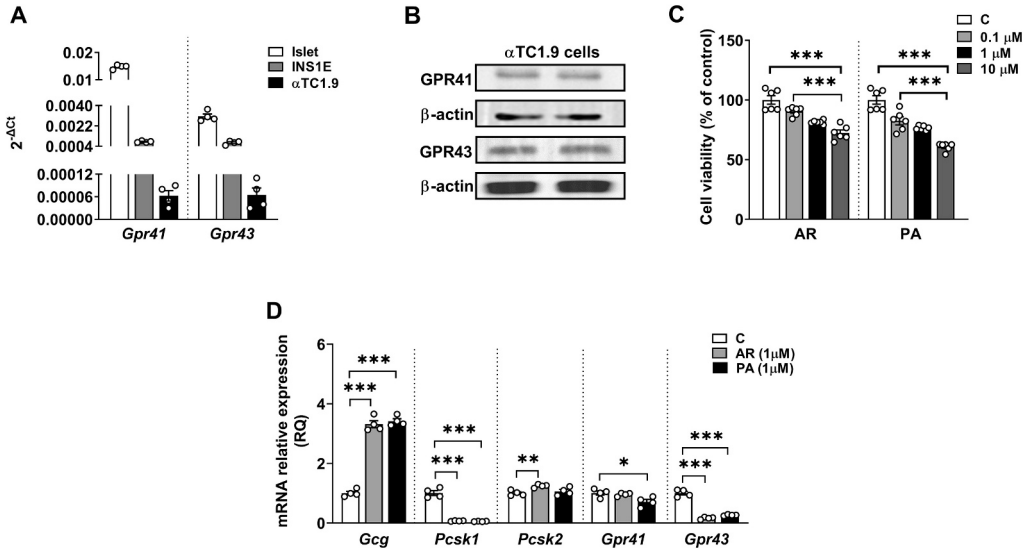

Fig. 2. Role of GPR41 and GPR43 agonists on the gene expression profile of αTC1.9 cells. (A) RT-qPCR analysis of *Gpr41* and *Gpr43* gene expression in rat islets, INS-1 cells and αTC1.9 cells ($n = 4$). (B) Western blot of GPR41 and GPR43 in αTC1.9 cells. (C) Cell viability of αTC1.9 cells incubated 24 h with different concentrations of GPR41 and GPR43 agonists monitored by crystal violet assay and represented as the percentage of control cells non-incubated with agonist ($n = 6$). ***$p < 0.001$ for each dose of agonist vs C group or other dose of same stimulus. (D) RT-qPCR analysis of the *Gcg*, *Pcsk1*, *Pcsk2*, *Gpr41* and *Gpr43* genes after incubation of cells for 24 h with 1 μM of GPR41 or GPR43 agonist. (n = 4). Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). *$p < 0.05$; **p < 0.01; ***p < 0.001 compared with C group. All data are means ± SEM for each experimental group. C: non-stimulated control; AR: GPR41 agonist; PA: GPR43 agonist.

were treated with the GPR41 agonist (AR), we observed no effect on glucagon secretion at either low or high glucose levels with 0.1 μM concentration, but a relevant increase when α-cells were incubated with 1 μM of agonist for 1 h. GPR43 agonist (PA) stimulated glucagon secretion only at low glucose. The glucagon content of α-cells was not affected by acute stimulation with the agonists at any dose (Fig. 3B).

In order to gain a deeper understanding of the molecular mechanism involved in the secretion of glucagon induced by stimulation of the GPR41 and GPR43 receptors, we then studied the effect of a 1 μM concentration of each compound, the GPR41 agonist (AR) and the GPR43 agonist (PA), on intracellular Ca^{2+} oscillations in the pancreatic α-cell line αTC1.9 using the radiometric Ca^{2+} probe FURA-2/AM. For this, cells were perfused for 10 min with 0.5 mM glucose, followed by 10 min with 0.5 mM glucose plus agonist. The effects of the agonists were analyzed in the last 5 min for each cell, using the initial oscillations in response to 0.5 mM glucose as a control situation. GPR41 agonist (AR) stimulated intracellular Ca^{2+} signalling in the α-cell in response to low glucose, significantly increasing both the average peak amplitude and the frequency of intracellular Ca^{2+} oscillations (Fig. 3C). In contrast, GPR43 agonist (PA) only induced an increase in the average peak amplitude but had no effect on the frequency of Ca^{2+} oscillations in response to low glucose (Fig. 3D). To determine whether the Ca^{2+} oscillations induced by each compound were dependent on the availability of extracellular Ca^{2+}, we next performed experiments in the absence of extracellular Ca^{2+}. Analysis of the intracellular Ca^{2+} signal in the absence of extracellular Ca^{2+} gave no response with either agonist (Fig. 3E-F), indicating that the mechanism underlying the effect of GPR41 and GPR43 receptor activation on glucagon secretion requires Ca^{2+} entry into the cell.

3.3. GPR41 and GPR43 agonists affect the expression of common islet-related genes, α-cell glucagon secretion and proliferation

To test the relevance of these findings in whole islets, we treated isolated rat islets with the specific GPR41 or GPR43 agonists. Consistent with our findings in αTC1.9 cells, 1 μM of GPR43 agonist for 24 h was enough to induce the expression of proglucagon in pancreatic islets whereas GPR41 agonist showed no effect at the same dose (Fig. 4A). Neither of the agonists were able to increase insulin or somatostatin expression under these same conditions of incubation. Supporting the potential of these receptors in modulating islet α-cell function, both agonists stimulated glucagon release not only at low glucose concentration but also at high glucose (Fig. 4B). This effect was not due to an increase in islet glucagon content (Fig. 4C) but to activation of the secretory machinery.

It has been well established that GPR41 is coupled to G-protein subtype α_i, while GPR43 connects with both subtypes α_i and α_q in many cells. To establish the downstream coupling of GPR41 and GPR43 in alpha cells, we analyzed whether treatment with the specific $G\alpha_i$ inhibitor pertussis toxin (PTX) had any effect on agonist-induced glucagon secretion (Fig. 4D). Pre-treatment of islets with PTX for 1 h prior to GPR41 or GPR43 addition significantly decreased glucagon release in both cases. In contrast, inhibition of PLC, the target protein of $G\alpha_q$, with the inhibitor U73122 had no effect on GPR41 induction of glucagon secretion, as expected, but interestingly, it promoted GPR43-mediated glucagon release from islets. These results suggest that $G\alpha_q$ signalling acts as a brake of glucagon secretory machinery and point out the relevant role of $G\alpha_i$ for effective glucagon secretion signalling. Consistent with $G\alpha_i$ coupling of glucagon release, forskolin (FSK)-stimulated cAMP production further increased GPR41 agonist effects but not those of GPR43 agonist (Fig. 4D).

Finally, to determine whether activation of GPR41 or GPR43 signalling pathway may have any effect on α-cell mass maintenance, isolated pancreatic islets were incubated for 48 h in the presence or absence of 1 μM agonists. We then quantified by immunofluorescence the number of glucagon$^+$ α-cells per islet and the number of Ki67$^+$ α-cells to calculate the percentage of α-cells that had undergone proliferation (Fig. 4E-F). Treatment of islets with GPR41 agonist significantly induced α-cell proliferation, almost doubling the rate of the unstimulated control islets. However, incubation of islets with GPR43 agonist had no effect on α-cell mass. We cannot rule out that the lack of effect of PA compound might be related to desensitization of GPR43 by reduction of its expression after long-term exposure, as we have previously shown in Fig. 2.

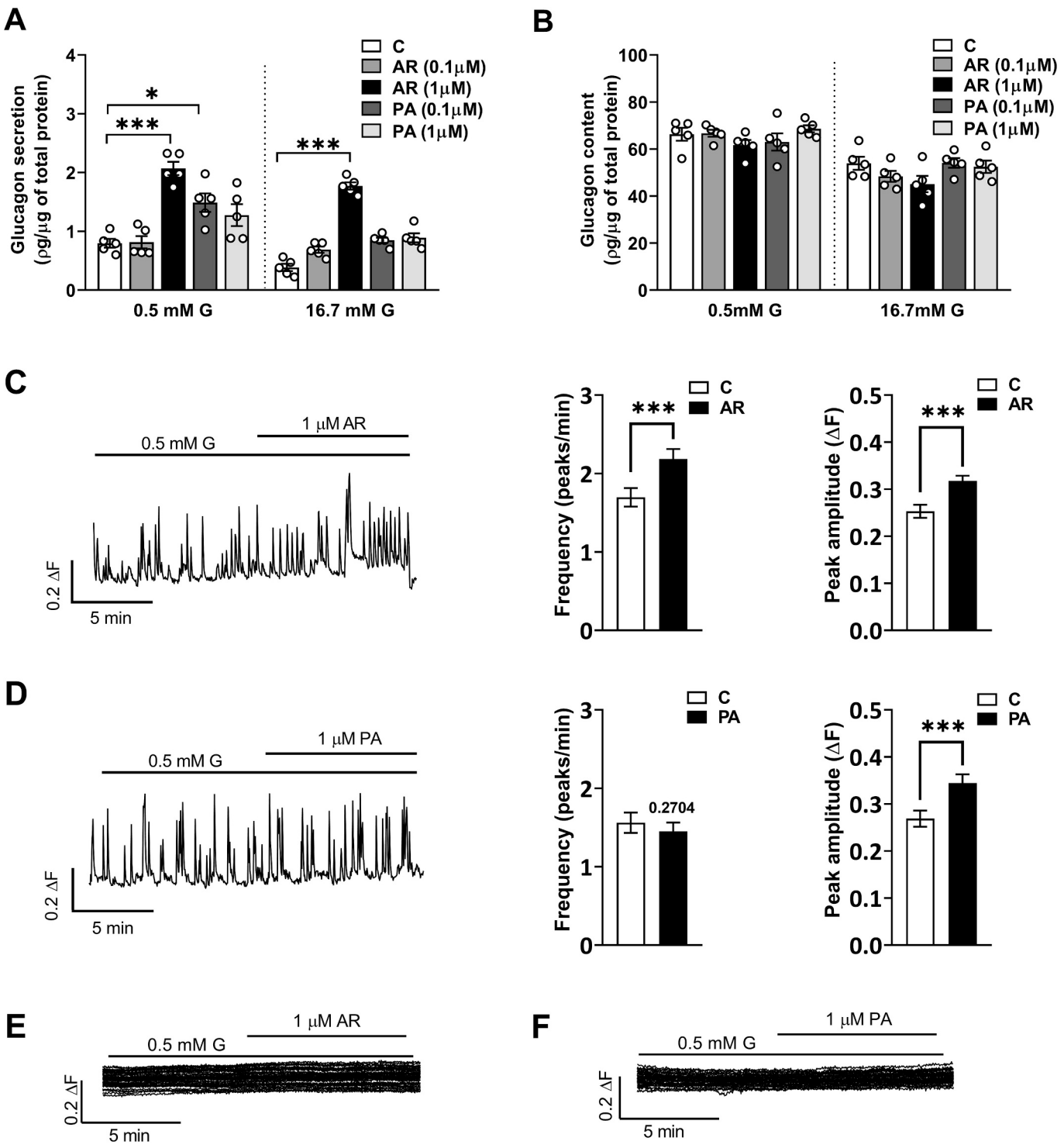

Fig. 3. Effect of in vitro treatment of αTC1.9 cells with GPR41 and GPR43 agonist on glucagon secretion and Ca^{2+} signalling. (A) Glucagon release from αTC1.9 cells normalized by total protein, exposed to 0.5 mM glucose and 16.7 mM glucose. (B) Glucagon content normalized by total protein from αTC1.9 cells exposed to 0.5 mM glucose and 16.7 mM glucose ($n = 5$). *$p < 0.05$; ***$p < 0.001$ compared with C group at the same glucose concentration but without agonists. (C) Representative recording of intracellular Ca^{2+} oscillations of αTC1.9 cells in the presence of 0.5 mM glucose and 0.5 mM glucose with 1 μM of GPR41 agonist. The graphs on the right show the oscillatory frequency and peak amplitude of Ca^{2+} oscillations elicited by 0.5 mM glucose with or without 1 μM of GPR41 agonist ($n = 184$ individual cells analyzed from 14 different recordings, obtained across 5 independent experiments). (D) Representative recording of intracellular Ca^{2+} oscillations of αTC1.9 cells in the presence of 0.5 mM glucose and 0.5 mM glucose with 1 μM of GPR43 agonist. The graphs on the right show the oscillatory frequency and peak amplitude of Ca^{2+} oscillations elicited by 0.5 mM glucose with or without 1 μM of GPR43 agonist ($n = 153$ individual cells analyzed from 14 different recordings, obtained across 5 independent experiments). ***$p < 0.001$ compared with C group at the same glucose concentration but without agonists. (E) Recordings of intracellular Ca^{2+} response of αTC1.9 cells in the presence of 0.5 mM glucose and 0.5 mM glucose with 1 μM of GPR41 agonist, without extracellular Ca^{2+}. Traces correspond to 139 individual cell recordings from 3 different experiments. (F) Recordings of intracellular Ca^{2+} response of αTC1.9 cells in the presence of 0.5 mM glucose and 0.5 mM glucose with 1 μM of GPR43 agonist, without extracellular Ca^{2+}. Traces correspond to 116 individual cell recordings from 3 different experiments. All data are mean ± SEM for each experimental group. C: non-stimulated control; AR: GPR41 agonist; PA: GPR43 agonist.

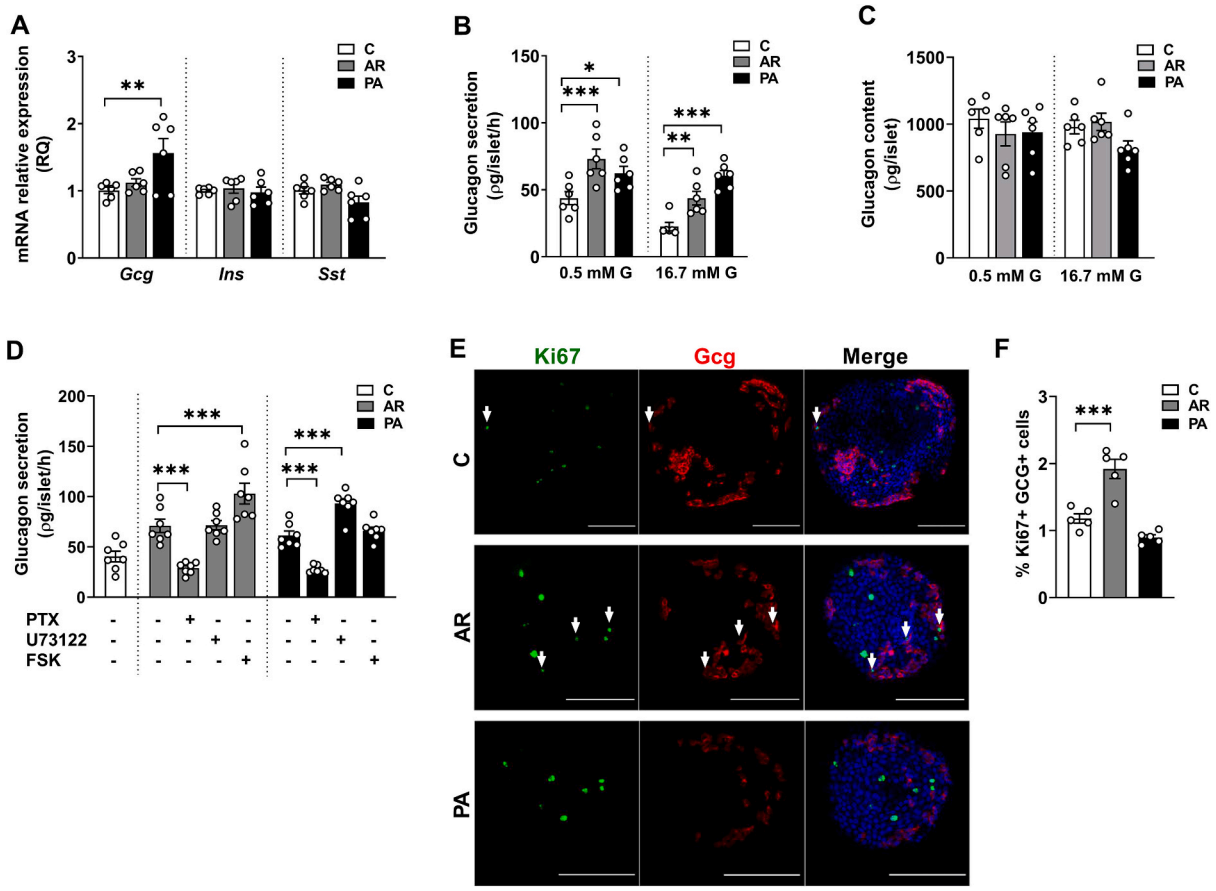

Fig. 4. GPR41 and GPR43 agonists effect on the expression of common islet-related genes, glucose-modulated glucagon secretion and α-cell proliferation in rat islets. (A) RT-qPCR analysis of the *Gcg*, *Ins* and *Sst* gene expression after incubation of islets for 24 h with 1 μM of GPR41 or GPR43 agonist (n = 6). Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). **p < 0.01 compared with non-stimulated C group. (B) Glucagon secretion from islet in response to GPR41 or GPR43 agonist (1 μM) normalized by islet number (fifteen islets per point) at 0.5 mM glucose and 16.7 mM glucose (n = 6). *p < 0.05; ***p < 0.001 compared with C group at the same glucose concentration. (C) Glucagon content from isolated islets normalized by islet number (15 islets per point) exposed at 0.5 mM glucose and 16.7 mM glucose (n = 6). Similar sized islets were selected. (D) Glucagon secretion in rat islets pre-treated for 1 h with PTX (100 ng/mL), U73122 (20 μM) or FSK (10 μM) and then stimulated for an additional 1 h with or without GPR41 or GPR43 agonist (1 μM) at 0.5 mM glucose. ***p < 0.001 compared with the same agonist-treated group but no pretreated with pharmacological inhibitors (PTX, U73122) or inductor (FSK) (*n* = 7). (E) Confocal microscopy images of Ki67 (in green) colocalized with glucagon positive cells (in red) from complete isolated islets incubated for 48 h with 1 μM of GPR41 or GPR43 agonist. Scale bar (20×) = 100 μm. (F) Percentage of $Ki67^{+}Gcg^{+}$ cells. Three sections per islet (n = 5). ***p < 0.001 compared with non-stimulated C group. All data are means ± SEM for each experimental group. C: non-stimulated control; AR: GPR41 agonist; PA: GPR43 agonist.

3.4. Role of GPR41 and GPR43 signalling in the response of islet α-cells to high-fat diet

In order to determine whether GPR41 and GPR43 might have any function in the α-cell response of islets to high-fat feeding, Wistar rats were fed a 60 % high-fat diet (HFD) after weaning and for 14 weeks (Fig. 5). Despite HFD did not cause obesity in Wistar rats (Fig. 5A), it promoted the development of overt glucose intolerance compared with standard diet (SD)-fed group (Fig. 5B). Circulating insulin levels were not elevated after 14 weeks on the diet (Fig. 5C), whereas HF-feeding induced the appearance of fasting hyperglucagonemia (Fig. 5D) and an elevated glucagon:glucose ratio (Fig. 5E), suggesting that the relationship between glucagon and glucose was impaired in HFD rats. To examine whether HFD phenotype was associated, at least in part, to defects in pancreatic islets we analyzed the gene expression of main islet hormones (Fig. 5F). Islets from long-term HF-fed rats showed enhanced expression of proglucagon and insulin genes while reduction of somatostatin levels. We then assessed glucose-stimulated glucagon secretion from isolated islets (Fig. 5G). Although HFD islets maintained certain capacity to reduce glucagon release under high glucose concentrations (16.7 mM), glucagon secretion under both low (0.5 mM) and high-glucose conditions was significantly greater than in islets from SD rats, which is consistent with the glucose intolerance exhibited by HF-fed rats during GTTs. To note, dysregulated glucagon secretion did not seem to be related to changes in islet glucagon content (Fig. 5H).

It is well known that HFD favors gut dysbiosis and alters microbial-derived metabolite production such as SCFAs. Thus, we next analyzed serum concentration of main SCFAs, acetate, propionate and butyrate, together with other minor bacterial end-products as valerate or isovalerate by LC-QqQ-MS (Fig. 5I). As expected, HFD significantly reduced serum acetate levels, but it did not alter circulating levels of the other SCFAs. In a previous study, McNelis et al. [21] described increased gene expression of GPR43 in islets from mice fed with HFD. Likewise, we observed herein a significant induction of both GPR41 and GPR43 gene expression in islets from 14-week HF-fed rats (Fig. 5J). Consistent with these results, immunofluorescence identification of $GPR41^{+}$ or $GPR43^{+}$ cells in total pancreas (Fig. 5K) and quantification of staining intensity (Fig. 5L) demonstrated higher levels of both receptors in islets obtained from HFD animals.

Treatment of HFD islets with GPR41 or GPR43 agonists for 24 h further increased proglucagon expression, although GPR41 ligand also caused a reduction in insulin gene expression (Fig. 6A). However, when we performed experiments of α-cell function, we observed no effect on glucagon release or glucagon content by stimulation of HFD islets with

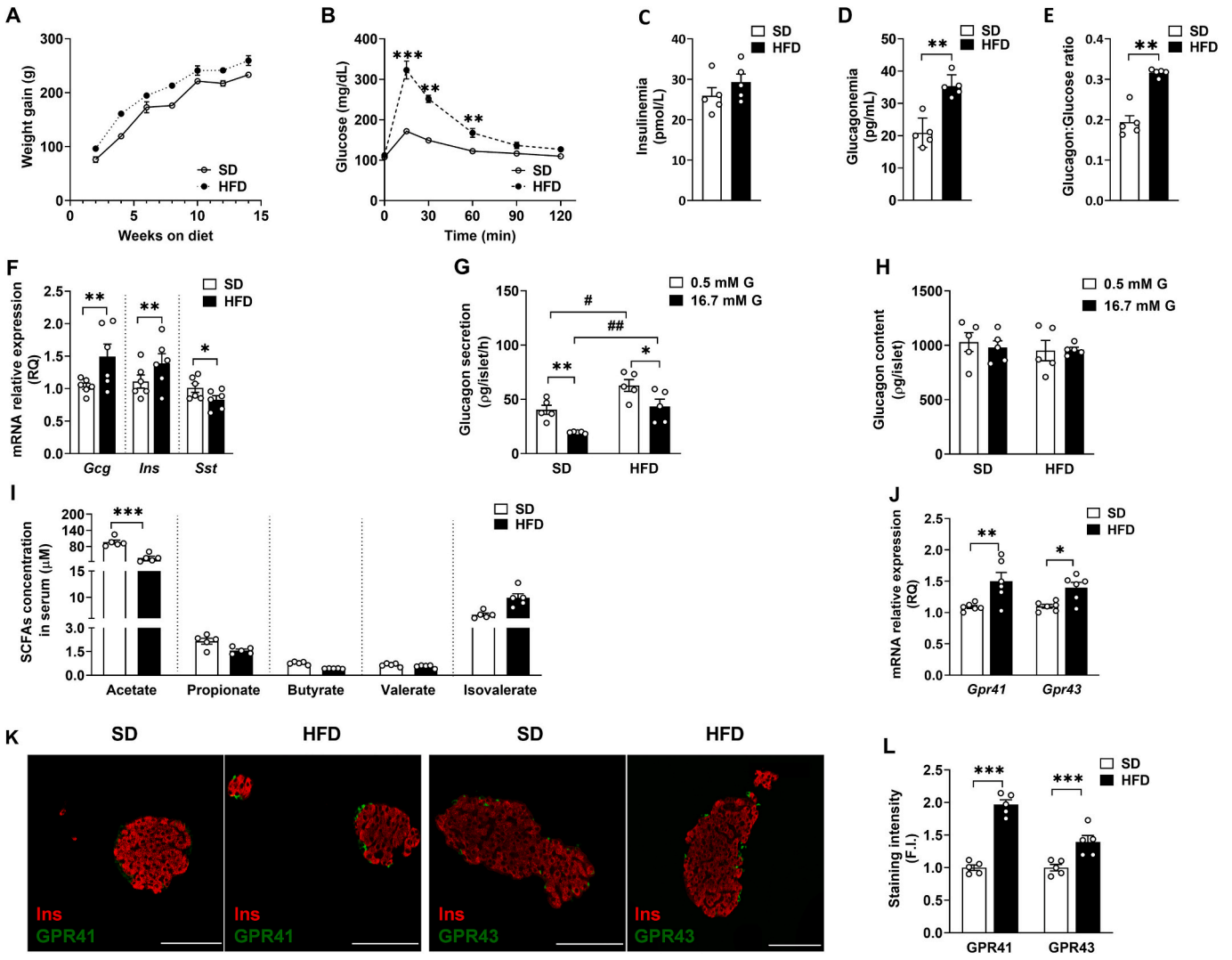

Fig. 5. Metabolic characterization and study of the hormonal profile and serum levels of SCFAs in rats fed a high-fat diet for 14 weeks. (A) Weight gain in rats for 14 weeks of dietary treatment. (B) Glucose tolerance tests (GTTs) were performed by intraperitoneal glucose administration (2 g/kg body weight) in overnight (16 h)-fasted rats after 14 weeks on diet. Serum insulin levels (C) and glucagon (D) after 14 weeks on HFD. (E) The glucagon:glucose ratio in SD and HFD rats. (A-E) All data are means ± SEM for each experimental group (n = 5). **p < 0.01; ***p < 0.001 compared with SD group. (F) RT-qPCR analysis of the *Gcg*, *Ins* and *Sst* gene expression in islets from SD and HFD rats (n = 6). Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). All data are means ± SEM for each experimental group. *p < 0.05; **p < 0.01 compared with SD group. (G) Glucagon secretion and (H) content of SD and HFD islets exposed to 0.5 mM or 16.7 mM glucose; in both the result was normalized by islet number (15 islets per point). All data are means ± SEM for each experimental group (n = 5). *p < 0.05; **p < 0.01 compared with C group at the same glucose concentration. #p < 0.05; ##p < 0.01 between groups with the same type of diet stimulated with different glucose concentrations. (I) Serum levels of SCFAs were measured by high-performance liquid chromatography in SD and HFD rats (n = 5). (J) RT-qPCR analysis of the *Gpr41* and *Gpr43* gene expression in islets from SD and HFD rats (n = 6). Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). (K) Confocal microscopy representative images of pancreatic sections from SD-fed and HFD-fed rats, immunostained against insulin (red) and GPR41 or GPR43 (in green). Scale bar (20×) = 100 μm. (L) Quantification with ImageJ of fluorescence intensity levels of GPR41 and GPR43 in the total area of pancreas analyzed from SD and HFD rats, expressed as fold induction (F.I.) relative to SD group (n = 5 pancreas). (I-L) All data are means ± SEM for each experimental group. *p < 0.05; **p < 0.01; ***p < 0.001 compared with SD group. SD: standard diet; HFD: high-fat diet; AR: GPR41 agonist (1 μM); PA: GPR43 agonist (1 μM).

either agonist (Fig. 6B-C). Taken together, these results point out that islets from HFD rats have an autonomous glucagon secretion defect and lack responsiveness to GPR41 and GPR43 stimulation.

3.5. Modulation of SCFAs receptors expression and function in pancreatic islets during lactation

We have previously described [28] that α-cell mass markedly increases soon after birth due to α-cell hyperplasia and increased α-cell to β-cell ratio into the islets of lactating rats. This event was accompanied by physiological hyperglucagonemia. In this regard, breastfeeding is also a critical window for gut microbiota colonization in infants, facilitating the establishment of a healthy microbial community capable of fermenting dietary fibre to produce short-chain fatty acids (SCFAs) [34,35]. Accordingly, the time of lactation in rodents represents an interesting period of natural endocrine pancreas maturation well suited to study the role of SCFA receptors for α-cell growth and function. For this reason, we conducted experiments along lactation on days 4 (L4), 14 (L14) and 18 (L18).

Based on previous data from metataxonomic analysis of lactating fecal samples [34], we first analyzed the changes in the relative abundance of SCFA-producing bacteria (Fig. 7A) and we found a progressive increase along the lactation period in bacteria able to produce acetate, mainly represented by *Akkermansia, Bacteroides, Gemella* and

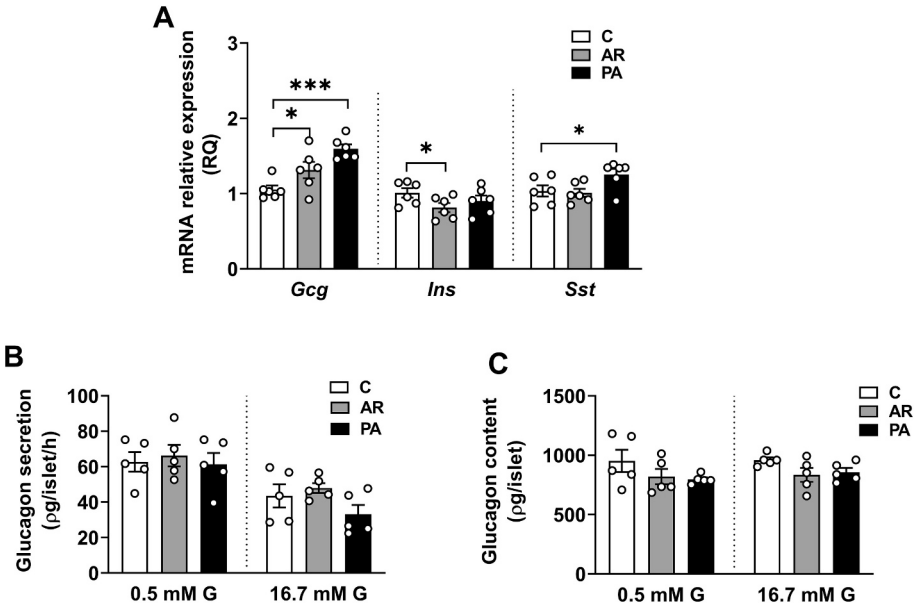

Fig. 6. Effects of GPR41 and GPR43 agonists on expression of common islet-related genes and glucagon secretion from islets of rats fed a high fat diet. (A) RT-qPCR analysis of the *Gcg*, *Ins* and *Sst* gene expression (n = 6). Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). *p < 0.05; ***p < 0.001 compared with non-stimulated C group. (B) Glucagon secretion from HFD islets in response to GPR41 or GPR43 agonist (1 μM) at 0.5 mM glucose and 16.7 mM glucose, normalized by islet number (15 islets per point) (n = 5). (C) Glucagon content of the same islets used to perform glucagon secretion analysis in (B) (n = 5). All data are means ± SEM for each experimental group. C: non-stimulated control; AR: GPR41 agonist; PA: GPR43 agonist.

Parabacteroides genera, and a tendency for increased propionate-producing bacteria in L14 and L18 animals. Consistent with this analysis, our results showed a significant increase in serum levels of acetate with age, whereas a decrease in circulating concentration of butyrate on days L14 and L18 compared to L4, together with a marked increase in levels of the branched-chain fatty acid isovalerate on days L14 and L18 (Fig. 7B). Meanwhile, pancreatic *Gpr41* and *Gpr43* mRNA levels were found sharply elevated during lactation compared to expression levels at adulthood (Fig. 7C), with a preferential localization detected in the endocrine pancreas (Fig. 7D) and following a distribution pattern similar to adult islets. This peak in the expression of *Gpr41* and *Gpr43* during lactation coincides with a similar timing to that of *Gcg* (pancreatic α-cell marker) (Fig. 7E) and the appearance of hyperglucagonemia compared to levels found in adults (99.88 ± 17.03 pg/mL; Fig. 7F).

We further sought to investigate the contribution of GPR41 and GPR43 signalling to α-cell growth using a primary culture of neonatal isolated islets (L4) (Fig. 8). Only incubation with 1 μM of the specific GPR41 agonist (AR) for 24 h significantly promoted the expression of *Gcg*, *Ins* and *Sst* (Fig. 8A). Similarly, stimulation of islets with GPR41 agonist (AR) resulted in a 3-fold increase in the proliferation rate of α-cells compared to the percentage found in unstimulated islets (Fig. 8B-C). However, consistent with what we observed in adult rat islets (Fig. 4E), incubation with 1 μM of the GPR43 agonist (PA) for 48 h had no effect on the proliferation of α-cells from L4 lactating rats (Fig. 8B-C). This lack of effect of GPR43 signalling may in part be due to the fact that long-term stimulation with both agonists induces resistance in the GPR43 receptor by decreasing its expression, as we observed in the αTC1.9 cell line (Fig. 2D).

Finally, pre-treatment of L4 islets with the α_i inhibitor PTX significantly reduced the effect of the GPR41 agonist on *Gcg* and *Ins* gene expression (Fig. 8D). Surprisingly, blocking GPR41 signalling via the α_i subunit further enhanced *Sst* expression.

4. Discussion

A major role for glucagon in the pathophysiology of diabetes is well accepted. However, the mechanisms involved in the control of α-cell function remain fragmentary, making it difficult to understand how changes in α-cell behavior progress from pre-diabetes to overt diabetes. This study provides new insights into the role of SCFA-specific receptors, GPR41 and GPR43, in modulating glucagon production and release from α-cells. Most studies of SCFA receptors in the pancreas have focused mainly on β-cells [19,21,23,24], while the potential effect of these receptors on α-cell physiology has been completely neglected, thereby losing the overall view of islet function as a whole. We show that both receptors are highly expressed in α-cells, at levels similar to β-cells for GPR43 or even higher in the case of GPR41. Interestingly, GPR43 was found to be much more highly expressed in δ-cells than in α- or β-cells, suggesting an important function in the paracrine connection between these three endocrine cells. In line with this, Orgaard et al. [26] provided evidence that GPR43 agonists stimulate somatostatin secretion in mice. Moreover, the in vitro and ex vivo approaches performed in this study demonstrate for the first time that the activation of GPR41 and GPR43 stimulates glucagon secretion by a direct effect independent of glucose concentration. Glucose over ~4 mM suppresses glucagon secretion after its uptake and metabolism in α-cells [36], which leads to an increase in intracellular ATP and closure of K_{ATP} channels, membrane depolarization and reduction in action potential amplitude. This culminates in reduced activation of voltage-gated Ca^{2+} channels and consequently reduced exocytosis of glucagon-containing granules [36]. However, our results suggest that glucose plays only a permissive role in the effect described for the specific agonists of GPR41 and GPR43, since both agonists were able to potentiate glucagon release from α-cells and pancreatic islets not only at low glucose but also at inhibitory high glucose levels. In agreement with our data, the SCFA acetate has previously been described to enhance glucagon secretion in perfused rat pancreas [37] but the mechanisms involved were not explored. In the present study, GPR41- and GPR43-dependent glucagon secretion appears to be coupled, at least in part, to the PTX-sensitive Gαi in rodent islets. We observed that preincubation of islets with PTX significantly attenuated the stimulatory effects of AR or PA on glucose-mediated glucagon release. Since Gαi is typically coupled to adenylate cyclase, these results would imply that AR and PA are likely to reduce cAMP levels in rat islets. Paradoxically, the addition of forskolin to isolated

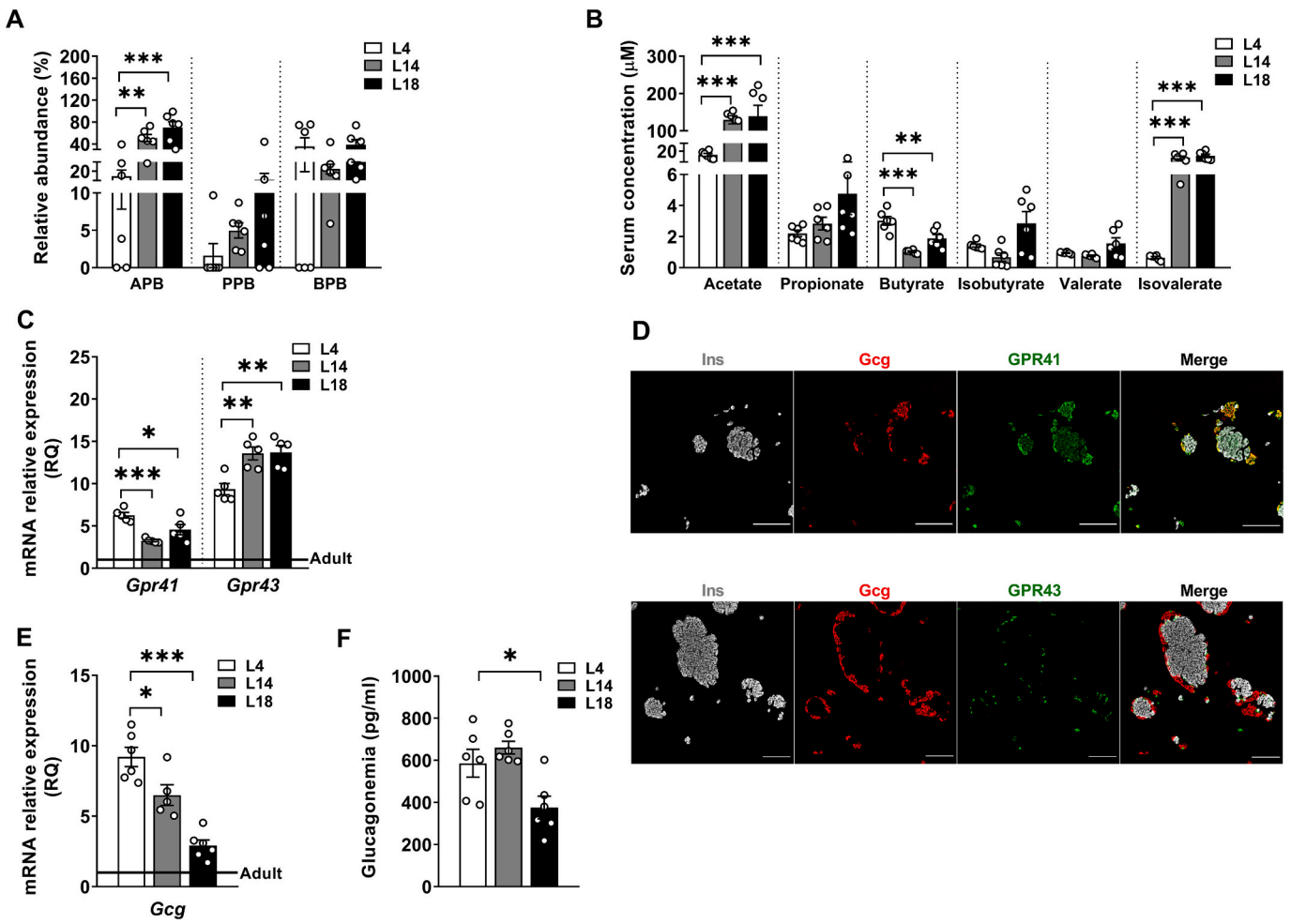

Fig. 7. The production of SCFAs and the expression of their pancreatic receptors are strongly induced during lactation. (A) Relative abundance of acetate-, butyrate- and propionate-producing bacteria. Data are represented as the sum of all SCFA-producing bacterial genera expressed as the percent of total reads (n = 6). (B) Serum levels of SCFAs (μM) were measured by high-performance liquid chromatography in lactating rats (n = 6). (C) RT-qPCR analysis of *Gpr41* and *Gpr43* gene expression in pancreatic tissues from lactating rats, normalized to adult expression levels (n = 5). (D) Confocal microscopy representative images of pancreatic sections from lactating rats (L4), immunostained against insulin (in white), glucagon (in red) and G-protein–coupled receptor, GPR41 or GPR43 (in green). Scale bar (20×) = 100 μm. (E) RT-qPCR analysis of *Gcg* gene expression in pancreatic tissues from lactating rats, normalized to adult expression levels (n = 5–6). (F) Serum glucagon levels in lactating rats (n = 6). (C, E) Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). All data are means ± SEM for each experimental group. *p < 0.05; ** p < 0.01; ***p < 0.001 compared to L4 or L14. Lactating day 4 (L4), 14 (L14) or 18 (L18). Acetate-producing bacteria (APB), butyrate-producing bacteria (BPB), propionate-producing bacteria (PPB).

islets further amplified agonist-induced glucagon secretion, indicating the existence of additional mechanisms. These results contrast with the canonical concept that cAMP-mediated activation of protein kinase A (PKA) or the low-affinity cAMP sensor Epac2 are essential for glucagon secretion [36,38] and that paracrine signals such as somatostatin, negatively influence hormone release by reduction of intracellular cAMP levels [39,40]. However, there are still many conflicting data on α-cell cAMP regulation. In the absence of high concentrations of cAMP, α-cell exocytosis and glucagon secretion is maintained due to Ca^{2+} influx through N-type voltage dependent Ca^{2+} channels whereas elevation of cAMP in the presence of forskolin or adrenaline causes a switch in Ca^{2+} channel dependence towards L-type Ca^{2+} channel [41,42]. Consistent with this, under our conditions, the GPR41 agonist compound AR stimulated Ca^{2+} signalling in the α-cells in response to low glucose, significantly increasing the frequency of intracellular Ca^{2+} oscillations. Interestingly, other authors have demonstrated that activation of cystic fibrosis transmembrane conductance regulator (CFTR) by cAMP indeed decreases glucagon secretion through its effects on the cell membrane potential [43] and K_{ATP} channels [44], whereas pharmacological inhibition of forskolin-activated CFTR increased glucagon secretion at all glucose concentrations [43]. All these data highlight the complexity of the mechanisms that regulate α-cell physiology. It should therefore be considered that the induction of hormone secretion by Gαi may involve a number of other mechanisms beyond cAMP regulation. In this respect, it is worth mentioning the work of Ang et al. [45], who used proximity ligation assays to show that the GPR41-GPR43 heteromer has a different signalling pathway from its corresponding homomers. The heteromer exhibited enhanced intracellular Ca^{2+} signalling and recruitment of beta2-arrestin, but it lost the capability to inhibit cAMP production, implying a unique signalling pattern. Thus, the coexistence of GPR41 and GPR43 receptors in pancreatic α-cells suggests that their activation may differentially affect glucagon secretion and cell mass, or even that the presence of both receptors is necessary for the full activity of each.

Besides regulation of hormone secretion, G protein-coupled receptors (GPCRs) are known to be associated with several mitogen-activated protein kinases (MAPKs), including extracellular-related kinase-1/2 (Erk1/2), c-Jun N-terminal kinase (JNK) and p38MAPK. In particular, Erk1/2 are important regulators of cell proliferation, and their activation by GPR41 and GPR43 has been shown to be PTX-sensitive in different cell lines, suggesting the involvement of Gαi

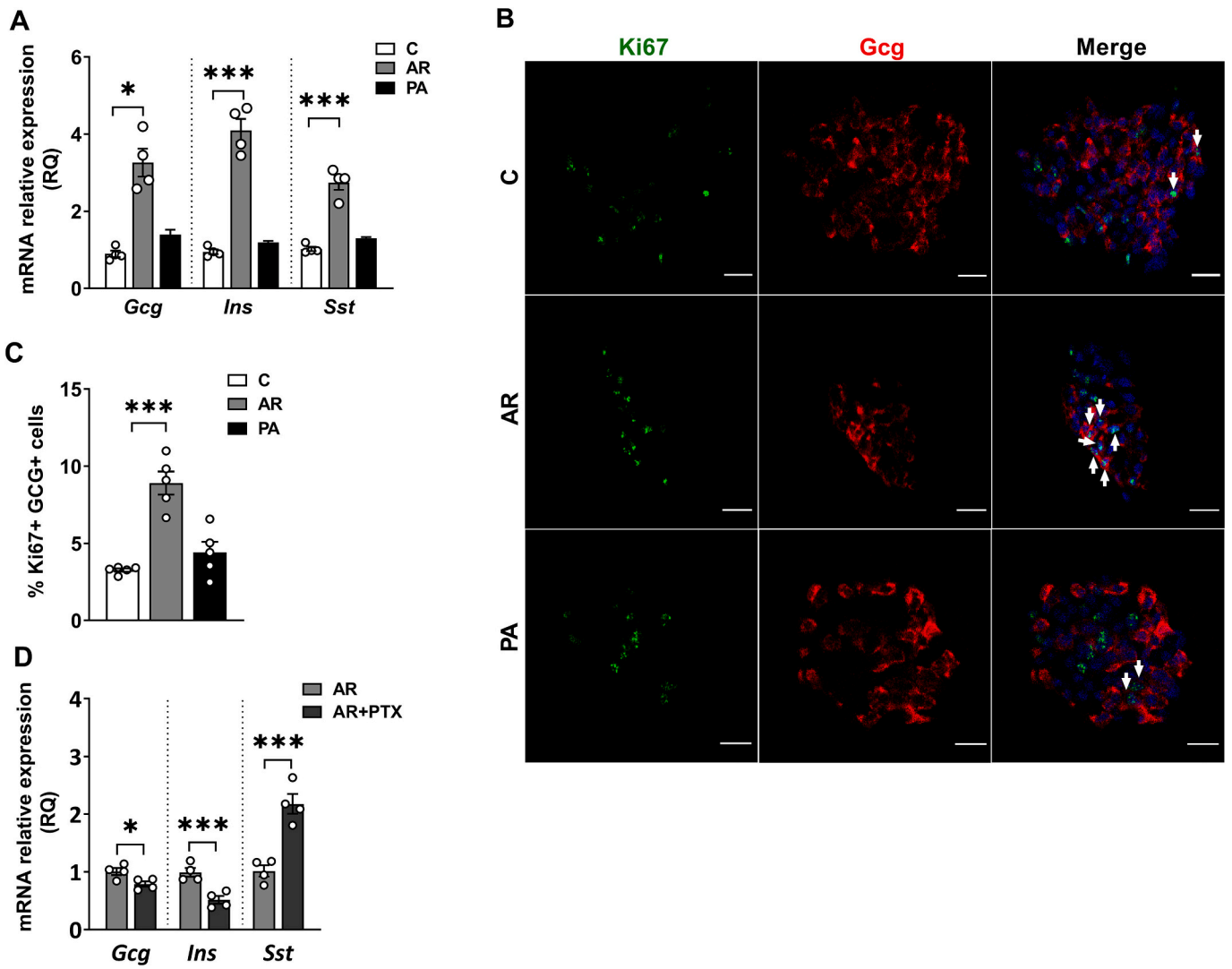

Fig. 8. Activation of GPR41 and GPR43 receptors affects hormone expression and α-cell proliferation in islets from L4 rats. (A) RT-qPCR analysis of the *Gcg*, *Ins* and *Sst* gene expression from lactating rat islets incubated during 24 h in the presence of 1 μM of each receptor agonist (n = 4). (B) Confocal microscopy images of Ki67 (in green) colocalized with glucagon positive cells (in red) after incubation of complete isolated L4 islets for 48 h with 1 μM of GPR41 or GPR43 agonist. Scale bar (20×) = 100 μm. (C) Percentage of $Ki67^{+}Gcg^{+}$ cells. Three sections per islet were analyzed (n = 5 islets/group). *p < 0.05; ***p < 0.001 compared with non-stimulated C group. (D) RT-qPCR analysis of the *Gcg*, *Ins* and *Sst* gene expression pre-treated for 1 h with PTX (100 ng/mL) and then stimulated for an additional 1 h with GPR41 and GPR43 agonists (1 μM) (n = 4). *p < 0.05; ***p < 0.001 compared with the same agonist-treated group but no pretreated with pharmacological inhibitor (PTX). All data are means ± SEM for each experimental group. (A, D) Data were normalized with the housekeeping gene *Gapdh* and relative expression was quantified using the comparative $2^{-\Delta\Delta CT}$ (RQ). C: non-stimulated control; AR: GPR41 agonist; PA: GPR43 agonist. PTX: pertussis toxin, Gαi inhibitor. Lactating day 4: L4.

[46]. Similarly, we found that activating GPR41 with AR significantly increased the number of proliferating α-cells in rat islets, whereas PA treatment failed to produce a similar response. This discrepancy was likely due to receptor-specific desensitization mechanisms, as long-term incubation with the specific GPR43 agonist downregulated its own receptor expression, thereby attenuating GPR43 signalling capacity and concealing any potential proliferative activity. The existence of additional mechanisms also linked to GPR43 desensitization cannot be discarded [46]. Consistent with our findings, McNellis et al. [21] reported that 48-h incubation of intact islets with the endogenous ligand of GPR43, acetate, or the specific agonist PA at the same dose as we used did not achieve statistical significance in thymidine incorporation, suggesting that this lack of effect was not exclusive to the allosteric agonist PA, but also to the orthosteric endogenous ligand. In line with this, sustained propionate stimulation of neutrophils caused GPR43 desensitization as well [47].

The relevance of both receptors for β-cell mass maintenance has been largely studied in knockout mouse models with controversial results. Deficiency of *Gpr41* and *Gpr43* has been reported to not alter [21,48] or decrease [49] β-cell mass under normal diet, whereas it has been observed reduced β-cell area and proliferation upon high-fat dietary challenge [21,49]. However, in all these studies, direct evidence for effects of GPR41 or GPR43 on α-cell growth and function is lacking. For this reason, we next systematically studied the regulation of pancreatic α-cells under different states of insulin resistance and the possible involvement of SCFA receptors signalling. First, we focused on a well-recognized rodent model of diet-induced prediabetes. HF-fed rats became glucose intolerant, hyperglucagonemic and showed impaired glucose-dependent inhibition of glucagon secretion after 14-weeks on diet. We previously outlined impaired metabolic regulation of glucagon in HFD rats, even when a more moderate dietary challenge was employed, and in a sex-specific manner, with significantly more severe hyperglucagonemia observed in females than in males [27]. This rationale underpinned the decision to limit the current study to females.

Despite the absence of overt hyperglycaemia or hyperinsulinemia at the time of the study, hyperglucagonemia was already observed in the fasted state, indicating that glucagon release disturbance represents a primary event in the pathophysiology of diabetes. These findings are consistent with previous observations of diet-induced obese mice, which have also demonstrated fasting hyperglucagonemia [39,50], although others have detected irregular glucagon levels during the postprandial period as well [3]. The presence of abnormal fasting or fed glucagon levels has also been documented in T2D patients [1,2,51].

On the other hand, our ex vivo results obtained with pancreatic islets from rats fed a HFD demonstrated a pronounced hypersecretion of glucagon, which was evident at both low and high glucose levels. Furthermore, HFD-islets exhibited upregulated insulin and glucagon expression, while somatostatin expression was diminished. An analysis of somatostatin secretion in response to glucose was not conducted in the present study. However, a similar prediabetic animal model was employed by Kellard et al. [39], who described hyperglucagonemia associated with impaired somatostatin secretion and α-cell paracrine resistance to somatostatin. Therefore, we cannot rule out the possibility of defects in intra-islet paracrine regulation in our animals.

Human trials and experimental murine models have shown that decreased SCFAs production have detrimental effects for health. In a cross-sectional study of 18 obese women, Layden et al. [52] found that serum concentrations of acetate, but not propionate or butyrate, were inversely associated with both fasting and postprandial insulin levels and visceral adipose tissue. Similarly, in rodents a negative relationship between the increased abdominal circumference, which represents the degree of visceral obesity, with decreased concentrations of SCFAs on a high-fat/high-sucrose diet has been reported as well [53]. Accordingly, we present here a reduction in circulating acetate levels in conjunction with glucose intolerance resulting from the administration of HFD. Interestingly, as previously described by other researchers in the context of physiological [54] and pathological [23] insulin resistance, our group observed an increased expression of *Gpr41* and *Gpr43* in islets from HF-fed rats. This modulation of GPR41 and GPR43 receptors may represent a compensatory mechanism in response to the observed decrease in circulating acetate levels under HF-feeding conditions. Furthermore, stimulation of HFD islets with GPR41 and GPR43 agonists led to an increase in glucagon expression but did not further enhance glucagon secretion. This suggests that the islets' responsiveness to GPR41 and GPR43 activation is constrained by a ceiling effect, where glucagon secretion is already maximally stimulated due to chronic metabolic stress, leaving little or no capacity for additional agonist-induced secretion. Thus, it is tempting to infer that glucagon hypersecretion in HFD female rats could be partially a consequence of increased SCFAs receptor expression, which may enhance glucagon synthesis and contribute to elevated baseline secretion levels under chronic metabolic stress.

Pancreatic *Gpr41* and *Gpr43* mRNA levels also increased transiently during the early postnatal period, in agreement with results reported by others in mice [55] and rabbits [56]. This temporal pattern of expression could be caused by the development of gut microbiota and the progressive SCFA-producing capacity of rats with age. Due to the initial low density of the gut microbiota, the concentration of SCFAs is quite low shortly after birth [34]. Subsequently, the gastrointestinal tract is colonized by a variety of bacteria, including *Lactobacilli*, *Streptococci* and *Enterobacteria*, which are known to produce SCFAs and other minor metabolites [34,57]. Thus, the richer diversity and higher abundance of gut microbiota directly related to milk ingestion [34] induces a large increase in the levels of SCFAs, which may then up-regulate the expression of their receptors not only locally in the colon but also in a variety of other tissues. However, in many different studies, including ours, the expression levels of *Gpr41* and *Gpr43* in the pancreas were down-regulated with growth [55,56], despite fully mature gut microbiota. This suggests that GPR41 and GPR43 receptors may have specific functions in the pancreas during early development, especially before weaning, consistent with the findings of Kimura et al. [55] who observed reduced *Gpr43* expression in the pancreas of offspring of germ-free mothers. In line with this, the parallelism found between the increase in the pancreatic expression of SCFAs receptors and that of circulating glucagon during lactation, together with the expansion of the α-cell mass during this period [28], invite to consider a role of these receptors for the normal growth and function of alpha cells. This idea was strengthened when stimulation of neonatal L4 islets with the specific agonist AR markedly enhanced *Gcg* gene expression and α-cell replication in a Gαi-dependent manner.

In summary, this study provides novel insights into the distinct roles of GPR41 and GPR43 in pancreatic α-cell function, an area that remains poorly understood. By using selective receptor agonists to isolate their individual contributions, we were able to dissect receptor-specific effects on glucagon secretion and α-cell biology. A key strength of our approach is that findings in the αTC1.9 cell line were validated in primary α-cells and whole islets, thereby enhancing the physiological relevance of our conclusions. Nonetheless, a key limitation is that selective agonists, unlike endogenous SCFAs, may not fully replicate the physiological context of receptor activation. Despite this, the overlapping ligand specificity and functional promiscuity of GPR41 and GPR43 make it difficult to differentiate their individual roles using endogenous ligands alone. Our findings therefore lay an essential foundation for future studies employing more physiologically relevant models, including human islets and induced pluripotent stem cell (iPSC)-derived α-like cells, to further define the contribution of SCFA receptors to metabolic regulation. This will help to translate mechanistic insights from rodent models into human islet physiology, thereby enhancing the translational relevance of our findings. Thus, while further investigation is needed to fully elucidate the roles of GPR41 and GPR43 in nutrient sensing, our results highlight the critical function of these receptors, particularly GPR41, in α-cell growth and activity, supporting its potential as a therapeutic target for modulating glucagon secretion and improving glycemic control in diabetes.

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CRediT authorship contribution statement

A. Sánchez-Roncero: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **T. Fernández-Marcelo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **A.A. Pérez-Serna:** Methodology, Formal analysis, Data curation. **P. Martínez-Oca:** Methodology, Formal analysis, Data curation. **O. Alberquilla-Fernández:** Methodology, Data curation. **R. Sánchez-Domínguez:** Methodology, Data curation. **J.C. Segovia:** Methodology, Data curation. **F. Escrivá:** Writing – review & editing, Supervision. **B.G. Gálvez:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **C. Álvarez:** Writing – review & editing, Supervision, Conceptualization. **L. Marroqui:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **E. Fernández-Millán:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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