Glucagon dysfunction in the liver induces hyperplasia of PP cells and the production of

glucagon and pancreatic polypeptide double-positive cells

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Short Title: Regulation of PP-cell proliferation in response to glucagon dysfunction

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Abstract

Understanding the mechanisms that regulate cellular identity and proliferation is crucial for

elucidating cellular functions. Under normal conditions, pancreatic endocrine cells express only

a single hormone, and their numbers are tightly regulated. Contrary to this general principle, our

study revealed a significant increase in glucagon (GCG) and pancreatic polypeptide (PP) double-

positive cells ($GCG^+ PP^+$ double-positive cells), along with hyperplasia of both PP and α cells in

proglucagon-deficient mice. Similarly, systemic glucagon receptor-deficient mice exhibited PP-

cell hyperplasia and an increase in $GCG^+ PP^+$ double-positive cells, with enhanced PP-cell self-

replication observed at 4 weeks and the appearance of $GCG^+ PP^+$ double-positive cells at 10 weeks.

Liver-specific glucagon receptor-deficient mice induced similar effects, which were linked to

hyperaminoacidemia. Elevated glutamine levels were found to promote $GCG^+ PP^+$ double-

positive cell formation via mTOR signaling, suggesting a molecular mechanism driving

pancreatic endocrine cell plasticity. Collectively, these findings indicate that increased plasma

amino acid levels caused by impaired glucagon action in the liver promotes the proliferation of α

cells and PP cells, and disturbs their cellular identity maintenance.

Introduction

Each organ in the body precisely regulates both the number and types of its cells, to ensure proper function. Similarly, the pancreatic islets, the endocrine component of the pancreas, maintain a delicate balance among four types of cells: α cells, β cells, δ cells, and pancreatic polypeptide (PP) cells. These cells respectively secrete glucagon (GCG), insulin (INS), somatostatin (SST), and PP, working synergistically to regulate nutrients homeostasis, including glucose [1, 2]. Disruptions in the regulation of cell proliferation or identity within the islets are closely linked to the onset of various diseases. For example, β-cell dysfunction or loss is a characteristic feature of type 1 and type 2 diabetes, resulting in insufficient insulin action [3, 4]. Conversely, the excessive proliferation of endocrine cells can result in endocrine neoplasms, which may impair normal pancreatic function and cause severe clinical complications [5, 6]. Understanding the molecular mechanisms underlying cell fate determination and proliferation is therefore essential for maintaining the normal regulation of pancreatic islet function.

One of the endocrine cells, α-cells, their own proliferation by secreting glucagon, which acts on the liver to modulate amino acid catabolism [7]. Consequently, mice lacking the proglucagon gene which encodes glucagon [8, 9] or glucagon receptor [10-13] exhibit a common

phenotype: enhanced α-cell proliferation and hyperplasia. Incidentally the proglucagon gene

encodes multiple proteins, including glucagon, glucagon-like peptide-1 (GLP-1) and others.

Although these proteins are derived from the same gene, they have distinct receptors. As a result,

proglucagon gene-deficient mice lack all the encoded proteins, leading not only to glucagon

dysfunction but also to dysfunction of GLP-1 and others. Hyperplastic α cells caused by glucagon

dysfunction have been reported to develop into glucagonomas over time, and to metastasize to

the liver [14-16]. The mechanism underlying α-cell hyperplasia in the context of glucagon action

deficiency is well characterized. Previous studies have shown that impaired glucagon signaling

in the liver induces α-cell hyperplasia [13, 17-23]. This phenomenon is triggered by increased

blood amino acid levels, particularly glutamine, owing to reduced amino acid uptake and

catabolism in the liver. Increased glutamine levels upregulate the glutamine transporter Slc38a5

in α cells, promoting enhanced glutamine uptake and subsequent activation of the mTOR pathway,

which is a key driver of α-cell hyperplasia [18-21]. Notably, treatment of hyperplastic α cells with

the mTOR inhibitor rapamycin inhibits amino acid-dependent α-cell replication, and facilitates

their transdifferentiation into β-like cells [18]. This suggests that glucagon deficiency may

enhance the plasticity of islet cells, enabling them to differentiate into other cell types. In addition

to α cells, an increase in cells expressing the *Ppy* gene, which encodes PP, has been reported in

glucagon activity-deficient mice [8, 23]. However, it remains unclear whether the number of PP

protein-expressing cells actually increases, and how this is associated with the increase in α cells.

We previously reported our analysis of *Ppy*-lineage cells by lineage tracing experiments

using *Ppy-Cre* mice [24]. We showed that approximately half of the α cells are derived from the

Ppy lineage, suggesting that about half of the α cells express the *Ppy* gene. Furthermore, we

utilized *Ppy*-DTA mice, in which the diphtheria toxin A fragment is specifically expressed in *Ppy*-

expressing cells, leading to their ablation. Interestingly, in the islets of these mice, not only were

PP cells completely depleted, but approximately 80% of the α cells were also lost. These findings

indicate that, under normal conditions, a substantial proportion of α cells express the *Ppy* gene,

even if they do not produce the PP protein. This suggests that α cells share certain properties with

PP cells. It is possible that the absence of PP protein expression in many α cells is regulated by a

mechanism that suppresses PP protein translation, thereby contributing to the maintenance of α-

cell identity.

Endocrine cells generally follow the "one cell, one hormone" principle, but some cells are

known to express multiple hormones. In particular, during the early stages of human fetal

development (8–15 weeks of gestation), hormone double-positive cells, which co-express

multiple hormones, are frequently observed [25]. However, as fetal development progresses to

later stages, the number of these cells decreases, suggesting that they represent an intermediate

stage in endocrine cell differentiation. It is also suggested that immature cells may temporarily

express multiple hormones during differentiation. Furthermore, in the mature pituitary glands of

both fish and mammals, multi-hormonal cells, which produce and secrete multiple hormones

simultaneously, have been identified. In fish, these cells are thought to function to enable the

coordinated regulation of different hormones, which is necessary for adapting to changes in the

aquatic environment [26]. Additionally, in the pancreatic islets of mature mice, it has been

reported that some *PPY*-expressing cells co-express *INS*, *GCG*, or *SST* [27]. These cells exhibit a

mixed phenotypic profile, combining characteristics of β cells, α cells, and δ cells, and their gene

expression profiles overlap with those of these endocrine cell types. Notably, under diabetic stress

conditions, PP cells have been suggested to possess the plasticity to acquire insulin-producing

capacity. However, the physiological significance of these multi-hormonal cells remains largely

unknown.

In this study, we demonstrate that the hyperplasia of PP cells and GCG and PP double-

positive cells (GCG$^+$ PP$^+$ double-positive cells) occurs in proglucagon-deficient mice. We aimed

to identify the proteins encoded by the proglucagon gene that are responsible for inducing PP-cell

hyperplasia and the increase in GCG$^+$ PP$^+$ double-positive cells, as well as to elucidate the

underlying mechanisms.

Materials and methods

Animal care

The protocol for the animal experiments conducted in this study was formally approved by

the Animal Care and Ethics Committee of Gunma University (approval number: D21-040). Mice

were euthanized humanely by cervical dislocation following anesthesia with isoflurane (Fujifilm,

Osaka, Japan), which was administered via a rodent anesthesia inhalation system (NARCOBIT-

E, Natsume Seisakusho Co., Ltd., Tokyo, Japan). All efforts were made to minimize suffering.

Mice were housed in a specific-pathogen-free barrier facility, maintained under a 12-hour

light/dark cycle, and provided with unrestricted access to standard rodent chow (Oriental Yeast,

Tokyo, Japan) and water.

Mice

B6D2F1 mice were purchased from CLEA Japan (Kawasaki, Japan), ICR mice were

obtained from Charles River Japan (Yokohama, Japan), and C57BL/6JmsSlc mice were obtained

from Japan SLC, Inc. (Shizuoka, Japan).

Generation of *glucagon receptor* gene (*Gcgr*)-floxed mice

Gcgr-floxed mice were generated by the sequential electroporation method that we reported

previously [28]. Sequences of target crRNAs flanking exons 4 to 13 of the *Gcgr* gene, and

corresponding donor single-stranded oligodeoxynucleotides including loxP are shown in Table 1.

Fertilized eggs were isolated from superovulated B6D2F1 female mice, 21 h post administration

of human chorionic gonadotropin (hCG). The first electroporation to insert the 5′ loxP into intron

3 was conducted at the 1-cell stage (24–26 h post hCG); and the second electroporation to insert

the 3′ loxP to intron 13 was conducted at the 2-cell stage (42–44 h post hCG). The sequentially

electroporated embryos were then transferred to the oviducts of pseudopregnant ICR females. The

floxed alleles of the obtained mice were confirmed by sequencing analysis. For PCR-based

confirmation, the primers used for each Flox insertion site were as follows: for the left Flox

insertion site, the forward primer was 5′-CTTGAAGGAGAGGTGCCTTG-3′, and the reverse

primer was 5′-GGCCAGCAGGAGTACTTGTC-3′, and a 268-bp band is detected. For the right

Flox insertion site, the forward primer was 5′-TCTTTCTCCAGGGTCTGCTG-3′, and the reverse

primer was 5′-CAAGTTTCACCTTGGGTTCA-3′, and a 250-bp band is detected.

Generation of systemic *Gcgr*-knockout mice and liver-specific *Gcgr*-deficient mice

The Cytomegalovirus (CMV) early enhancer and chicken β-actin (CAG) promoter -Cre

transgenic mice (*CAG-Cre*) [29, 30] were crossed with *Gcgr*-floxed mice. To confirm the

deletion of exon 4 to exon 13 in the *Gcgr* gene by Cre recombinase, PCR was conducted using

the following primers: 5′-CTTGAAGGAGAGGTGCCTTG-3′, and 5′-

CAAGTTTCACCTTGGGTTCA-3′. If the deletion is present, a 155-bp PCR product was

detected. Liver-specific glucagon receptor-knockout mice were generated by breeding *Gcgr*-

floxed mice and with albumin-*Cre* mice [31].

Immunohistochemistry

After anesthesia with isoflurane (Fujifilm, Osaka, Japan), mice were perfused with normal

saline through the heart to remove blood cells from the blood vessels. Subsequently, they were

perfusion-fixed with 4% paraformaldehyde (PFA), and their pancreata were harvested. The

harvested pancreata were fixed in 4% PFA at 4 °C overnight. Subsequently, the pancreata were

transferred to 70% ethanol. The pancreas samples were embedded in paraffin, and the paraffin

pancreas blocks were sectioned at a thickness of 5 μm, and subjected to immunostaining.

Immunofluorescence staining was conducted following previously described methods [32]. The

primary antibodies used were guinea pig anti-insulin (Ready-to-use, IR002, Dako-Agilent, Santa

Clara, CA, USA), rabbit anti-glucagon (1:1,000, ab92517, Abcam, Cambridge, UK), rabbit anti-

Ki67 (1:200, ab16667, Abcam), rabbit anti-somatostatin (1:1,000, T-4103, Peninsula Laboratories,

San Mateo, CA, USA), rabbit anti-pS6 (1:200, 4858, Cell Signaling Technology, Danvers, MA,

USA), mouse anti-PP (1:1,000, 23-2D3, IBL, Gunma, Japan) [32], and chicken anti-GFP (1:1,000,

ab13970, Abcam). Tissue sections were incubated with the primary antibody, followed by a

secondary antibody (Alexa Fluor 488, ab150077, Abcam; A11001 and A11039, Thermo Fisher

Scientific, Waltham, MA, USA; Alexa Fluor 594, A11012 and A11005, Abcam; Alexa Fluor 647,

A31573 and A31571, Abcam, all used at 1:1,000) together with the nuclear dye 4′,6-diamidino-

2-phenylindole (DAPI, Dojindo Molecular Technologies, Kumamoto, Japan). The sections were

then visualized using an FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Quantification of α- and PP-cell mass

The quantification of α-cell mass and PP-cell mass was performed as previously described

[24, 33]. Nonpancreatic tissues were removed from the pancreas, and the pancreas was weighed

before fixation in 4% paraformaldehyde for 6-hour. To obtain longitudinal sections from the tail

to the head of the pancreas, the pancreas was embedded in paraffin, and paraffin blocks were

prepared. From the entire pancreas, 6 sections per mouse were collected at 200-μm intervals, and

immunostained with an anti-glucagon or anti-PP antibody. The immunostained samples were

analyzed using a BX-9000 microscope and the BZII analysis application software (Keyence

Corporation, Osaka, Japan). The relative cross-sectional areas of α cells or PP cells were

determined by dividing the cross-sectional area occupied by α cells or PP cells by the total cross-

sectional area of the tissue. The total α-cell mass or PP-cell mass in the pancreas was calculated

by multiplying the relative cross-sectional area of each cell type by the weight of the pancreas.

Pancreata from four animals in each group were analyzed to determine the α-cell or PP-cell mass.

Islet culture

Following previously reported methods, islets were isolated from 10 week-old mice [34].

The islets were collected using a micropipette under a dissection microscope, and cultured

overnight in Roswell Park Memorial Instistute (RPMI) -1640 medium (11875093, Thermo Fisher

Scientific) supplemented with 1% penicillin/streptomycin. Subsequently, 100 islets were placed

on a 24-well plate coated with Matrigel (Corning Incorporated, Corning, NY, USA), and cultured

for 4 days. Islets in the control group were cultured in RPMI-1640 medium without L-glutamine

(32404, Thermo Fisher Scientific) supplemented with 500 μM L-glutamine. Islets in the

glutamine group were cultured in the same medium supplemented with 3,250 μM L-glutamine.

After the culture period, the islets were fixed with 4% PFA, followed by treatment with 15% and

30% sucrose solutions. Finally, a frozen block was prepared using Optimal Cutting Temperature

(OCT) compound, and the block was cryosectioned [32].

Mouse plasma collection

Prior to blood collection, the mice were weighed, and blood glucose levels were measured

using the Glutest Neo Alpha blood glucose meter (Sanwa Kagaku Kenkyusho Co. Ltd., Aichi,

Japan). Blood was collected by cutting the tails of the mice, and using a microhematocrit

heparinized capillary tube (22-362566, Thermo Fisher Scientific). The collected blood was then

transferred to a 1.5-mL tube. Subsequently, the collected blood was centrifuged at 6,200 rpm for

more than 5 minutes, and the supernatant was transferred to a new 1.5-mL tube, and frozen using

liquid nitrogen.

Metabolomics analysis

Widely targeted metabolomics analysis was performed as previously described [35, 36].

Briefly, frozen samples in 1.5-mL plastic tubes were homogenized in 300 µL of cold methanol

using φ3-mm zirconia beads and a freeze crusher (TAITEC Corp., Saitama, Japan) at 41.6 Hz for

2 min. The homogenates were mixed with 200 µL of methanol, 200 µL of H_2O, and 200 µL of

$CHCl_3$, and vortexed for 20 min at room temperature. The samples were then centrifuged at

15,000 rpm (20,000 g) for 15 min at 4 ℃. The supernatant was mixed with 350 µL of H_2O,

vortexed for 10 min at room temperature, and centrifuged at 15,000 rpm for 15 min at 4 ℃. The

aqueous phase was collected and dried down using a vacuum concentrator. The samples were

redissolved in 2 mM ammonium bicarbonate (pH 8.0) containing 5% methanol, and analyzed by

LC-MS/MS.

Chromatographic separations in an Acquity UPLC H-Class System (Waters, Milord, MA,

USA) were carried out under reverse-phase conditions using an ACQUITY UPLC HSS T3

column (100 mm × 2.1 mm, 1.8-µm particles, Waters) and under HILIC conditions using an

ACQUITY UPLC BEH Amide column (100 mm × 2.1 mm, 1.7-µm particles, Waters). Ionized

compounds were detected using a Xevo TQD triple quadrupole mass spectrometer coupled with

an electrospray ionization source (Waters). The peak area of a target metabolite was analyzed

using MassLynx 4.1 software (Waters).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 9.1.1 software

(GraphPad Software, San Diego, CA, USA). Results are presented as the mean ± standard error

of the mean (SEM). Differences between two groups were assessed using a two-tailed unpaired

Student *t*-test, and comparisons among three or more groups were performed using one-way

ANOVA followed by the Tukey test. A *p*-value of less than 0.05 was regarded as indicating a

statistically significant difference between groups.

Results

Induced hyperplasia of PP cells and formation of multi-hormonal cells in proglucagon gene-

deficient mice

Proglucagon gene-deficient mice ($Gcg^{gfp/gfp}$) lack the production of all hormones encoded

by the proglucagon gene, including glucagon and GLP-1, owing to the knock-in of the *gfp* gene

into the proglucagon gene locus [8]. $Gcg^{gfp/gfp}$ mice are known to demonstrate α-cell hyperplasia

similar to other mice with glucagon signaling deficiencies [37]. In $Gcg^{gfp/gfp}$ mice, GFP is

expressed in cells that would normally produce glucagon. Furthermore, it has been reported that

the expression level of *Ppy* mRNA is significantly elevated in the pancreatic islets of these mice

[8, 23]. Based on this, we performed fluorescence immunostaining using our developed PP

antibody [32] to investigate PP protein dynamics. Fluorescence immunostaining was performed

on pancreatic islet sections from 40 week-old $Gcg^{gfp/gfp}$ mice. In these islets, in addition to α-cell

hyperplasia, PP-cell hyperplasia was observed (Fig 1A). Furthermore, many GFP[+] PP[+] double-

positive cells were detected. Since the expression level of *SST* mRNA was also elevated [8],

although not as prominently as *Ppy* mRNA, we conducted triple staining using antibodies for PP,

GFP, and SST. In the pancreatic islets of $Gcg^{gfp/gfp}$ mice, PP[+] GFP[+] SST[+] triple-positive cells were

identified (Fig 1B). In contrast, no PP^+ GCG^+ SST^+ triple-positive cells were observed in the

pancreatic islets of $Gcg^{+/+}$ mice when triple staining was performed using antibodies for PP, GCG,

and SST. These findings suggest that the absence of proteins encoded by the proglucagon gene

induces PP-cell hyperplasia and the formation of multi-hormonal cells. As the proglucagon gene

encodes multiple hormones, including glucagon, it was unclear as to exactly which hormone

deficiency caused the induction of PP-cell hyperplasia and the production of multi-hormonal cells.

Increased PP cells in glucagon receptor-deficient mice

To test whether glucagon, one of the proteins encoded by the proglucagon gene, is involved

in the above effects, *CAG-Cre* mice were crossed with *Gcgr*-floxed mice to produce *Gcgr* KO

($Gcgr^{\Delta/\Delta}$) mice (Fig 2A). We next investigated whether PP-cell hyperplasia and the production of

GCG^+ PP^+ double-positive cells occur in $Gcgr^{\Delta/\Delta}$ mice at 10 weeks of age. Compared to $Gcgr^{+/+}$

control mice, $Gcgr^{\Delta/\Delta}$ mice demonstrated substantial PP-cell hyperplasia, and the presence of

GCG^+ PP^+ double-positive cells (Fig 2B). Quantitative analysis demonstrated a significant

increase in both PP-cell mass and the ratio of GCG^+ PP^+ double-positive cells relative to GCG^+-

positive cells in $Gcgr^{\Delta/\Delta}$ mice compared with $Gcgr^{+/+}$ mice (Figs 2C and D). These findings suggest

that glucagon signaling deficiency induces PP-cell hyperplasia and the emergence of GCG$^+$ PP$^+$

double-positive cells.

265   Induction of PP-cell self-replication and an increase in GCG$^+$ PP$^+$ double-positive cells

266   owing to glucagon dysfunction

In 10 week-old $Gcgr^{\Delta/\Delta}$ mice, an increase in PP-cell mass and a rise in GCG$^+$ PP$^+$ double-

positive cells were observed. To assess how these changes progress at earlier stages, we examined

$Gcgr^{\Delta/\Delta}$ mice at 4 weeks of age. The PP cell mass in 4 week-old $Gcgr^{\Delta/\Delta}$ mice was significantly

higher than that in $Gcgr^{+/+}$ mice (Fig 3A and B). However, unlike the comparison at 10 weeks of

age (Fig 2B, right panel), the number of GCG$^+$ PP$^+$ double-positive cells in 4 week-old $Gcgr^{\Delta/\Delta}$

mice was not markedly increased compared to the control $Gcgr^{+/+}$ mice (Fig 3A, lower right panel).

Therefore, we quantitatively compared the number of GCG$^+$ PP$^+$ double-positive cells in $Gcgr^{\Delta/\Delta}$

mice between 4 and 10 weeks of age. The data showed that at 4 weeks of age, the number of

GCG$^+$ PP$^+$ double-positive cells was comparable to that in the control group. In contrast, at 10

weeks of age, the number of GCG$^+$ PP$^+$ double-positive cells was significantly higher in $Gcgr^{\Delta/\Delta}$

mice than in the control group (Fig 3C and D). Additionally, the number of GCG$^+$ PP$^+$ double-

positive cells in 4 week-old $Gcgr^{\Delta/\Delta}$ mice was significantly lower than that in 10 week-old $Gcgr^{\Delta/\Delta}$

mice. However, since an increase in PP cell mass was observed at 4 weeks of age, the increase in

PP cell mass was considered to be owning to factors other than an increase in double-positive

cells. To investigate this possibility, we analyzed PP-cell self-proliferation. Costaining analysis

was performed using antibodies against the proliferation marker Ki67, PP, and GCG to investigate

whether PP-cell self-proliferation was induced. The results showed that the number of $Ki67^+ PP^+$

GCG^- cells was significantly higher in $Gcgr^{\Delta/\Delta}$ mice than in $Gcgr^{+/+}$ mice (Figs 3E and F). These

findings suggest that PP-cell self-proliferation is enhanced in $Gcgr^{\Delta/\Delta}$ mice. Furthermore,

considering the temporal changes in PP-cell mass and the increase in $GCG^+ PP^+$ double-positive

cells, it is likely that at 4 weeks of age, the increase in PP-cell mass is primarily owning to the

enhanced self-replication of PP cells caused by glucagon dysfunction, and that the increase in

$GCG^+ PP^+$ double-positive cells subsequently becomes apparent at 10 weeks of age.

Induction of PP-cell hyperplasia and an increase in $GCG^+ PP^+$ double-positive cells is caused

by glucagon dysfunction in the liver

Next, the tissue responsible for the hyperplasia of PP cells and the increase in GCG^+ PP^+

double-positive cells resulting from glucagon dysfunction was investigated. Albumin-

Cre;Gcgr$^{f/f}$ (Alb-*Cre;Gcgr$^{f/f}$*) mice, lacking glucagon signaling specifically in the liver, which is

the primary site of glucagon action, were generated. As previously reported [13], 10 week-old

Alb-*Cre;Gcgr$^{f/f}$* mice demonstrated increased α-cell mass (S1 Fig). In addition, an increase in

PP-cell mass and the presence of GCG^+ PP^+ double-positive cells were observed (Figs 4A–C).

Further investigation of 40 week-old Alb-*Cre;Gcgr$^{f/f}$* mice demonstrated a more pronounced

increase in PP-cell mass (S2 Fig), as well as the presence of both GCG^+ PP^+ double-positive

cells (Fig 4D). Previous studies have reported that liver-specific glucagon dysfunction mice

exhibit α-cell hyperplasia. However, regarding β cells, while pancreatic enlargement

leads to an increase in total β-cell mass, β-cell mass per gram of pancreas and insulin

secretion levels remain unchanged compared to control mice [13]. In this study, PP and

INS double staining was performed using Alb-*Cre;Gcgr$^{f/f}$* mice, revealing the presence

of PP^+ INS^+ double-positive cells, which were not observed in control mice (Fig 4E).

These results suggest that hepatic glucagon dysfunction drives PP-cell hyperplasia and

the emergence of GCG^+ PP^+ and INS^+ PP^+ double-positive cells.

Induction of increased PP cell numbers and GCG⁺ PP⁺ double-positive cells owing to

hyperaminoacidemia

Since impaired glucagon action in the liver has been reported to elevate blood amino acid

levels [18, 20, 21], we examined whether amino acid levels were altered in the $Gcgr^{\Delta/\Delta}$ and Alb-

$Cre;Gcgr^{f/f}$ mice used in this study by measuring plasma amino acid concentrations. As expected,

plasma amino acid levels, including glutamine, were significantly increased in 10 week-old

$Gcgr^{\Delta/\Delta}$ and Alb-$Cre;Gcgr^{f/f}$ mice compared with control mice (S3 and S4 Figs). Next, we

investigated whether the addition of large amounts of glutamine would increase the number of PP

cells in the pancreatic islets and promote the formation of GCG⁺ PP⁺ double-positive cells. (Fig

5A). When pancreatic islets were cultured with a high concentration of glutamine, the proportions

of GCG⁺ positive and PP⁺ positive cells in the islets were significantly increased (Figs 5B and C).

Moreover, the ratio of GCG⁺ PP⁺ double-positive cells among GCG⁺ positive cells also increased

(Fig 5D). To determine whether the increase in GCG⁺ PP⁺ double-positive cells was mediated by

the mTOR pathway, we treated islets with the mTOR kinase inhibitor rapamycin. The glutamine-

induced increase in GCG⁺ PP⁺ double-positive cells was significantly suppressed by rapamycin

treatment (Figs 5E and F). These results suggest that the increased glutamine levels increase the

number of GCG^+ positive cells, PP^+ positive cells, and GCG^+ PP^+ double-positive cells in the

pancreatic islets via activation of the mTOR pathway. Finally, we investigated whether mTOR

was activated in GCG^+ PP^+ double-positive cells using tissues from $Gcgr^{\Delta/\Delta}$ mice. Consistent with

the results from isolated islets, GCG^+ PP^+ $pS6^+$ triple-positive cells were observed in these mice,

supporting the role of mTOR activation in the formation of GCG^+ PP^+ double-positive cells (S5

Fig).

Discussion

In this study, we investigated liver-specific glucagon receptor-deficient mice, to gain

additional insights into the effect of glucagon deficiency, beyond the previously reported

hyperplasia of α cells [10-13]. We found that impaired glucagon signaling in the liver also leads

to the hyperplasia of PP cells, and the emergence of GCG$^+$ PP$^+$ double-positive cells (Fig 4).

Additionally, we observed a significant increase in plasma amino acid levels in these mice

compared with the control groups (S3 Fig). Furthermore, when islets isolated from wild-type mice

were cultured with high concentrations of glutamine, one of the increased amino acids, we found

that the increase in PP cells and GCG$^+$ PP$^+$ double-positive cells is facilitated by activation of the

mTOR signaling pathway (Fig 5).

Previous studies have reported that expression of the *Ppy* gene is upregulated in

proglucagon-deficient and glucagon receptor-deficient mice [8, 23], however, whether PP protein

expression is indeed increased in these mice remained unclear. In this study, as shown in Fig. 2B,

we observed a significant increase in PP protein expression owing to glucagon signaling

insufficiency, accompanied by a marked increase in PP-cell mass (Fig 2C). Smith *et al.* reported

similar findings in their single-cell RNA sequencing study of islets from systemic glucagon

receptor-deficient mice, in which they found a significant increase in the PP-cell cluster in

addition to the α-cell cluster [23]. These results support the findings of our present study.

Regarding the mechanism underlying the increase in PP cells, as illustrated in Figs 3E and 3F

systemic glucagon receptor-deficient mice demonstrated a higher proportion of Ki67-positive

cells among PP$^+$ cells that were not stained with glucagon antibodies, compared with control wild-

type mice. The number of PP cells was also higher in the glucagon receptor-deficient mice than

in controls. These findings suggest that glucagon signaling insufficiency promotes an increase in

PP-cell mass through self-proliferation. The mechanism underlying PP-cell self-proliferation

remains unclear and represents a future research question. However, as shown in S6 Fig, in 4

week-old $Gcgr^{\Delta/\Delta}$ mice, pS6 signals were not detected in PP single-positive cells. This suggests

that a different pathway, distinct from the mechanism by which GCG$^+$ PP$^+$ double-positive cells

activate the mTOR signaling pathway to promote proliferation, may be involved. Previous studies

have reported that other pancreatic endocrine cells, such as α and δ cells, also demonstrate

enhanced proliferation under glucagon signaling deficiency, although the mechanisms differ. α

cells increase in number through self-proliferation [37], whereas δ cells expand through both self-

proliferation and duct-derived neogenesis [38]. In this study, we proposed that the increase in PP-

cell numbers is driven by both self-proliferation and an increase in multi-hormone-producing cells.

When comparing the systemic glucagon receptor-deficient mice at 4 weeks of age (Fig 3B) with

those at 10 weeks of age (Fig 2C), we observed a significant increase in PP-cell mass at 10 weeks

of age. Corresponding to this substantial change, the number of $GCG^+ PP^+$ double-positive cells

in 4 week-old glucagon receptor-deficient mice was comparable to that in wild-type mice (Fig

3D). However, in 10 week-old glucagon receptor-deficient mice, the number of $GCG^+ PP^+$

double-positive cells robustly increased compared with that in both wild-type mice and 4 week-

old glucagon receptor-deficient mice (Fig 3D). We propose that this increase in $GCG^+ PP^+$ double-

positive cells accounts for the substantial increase in PP-cell mass observed in 10 week-old

glucagon receptor-deficient mice.

Owing to glucagon dysfunction in the liver, the hyperplasia of PP cells and the emergence

of multi-hormone-producing cells were observed (Fig 4), associated with an increase in blood

amino acid levels. Similar to α-cell hyperplasia, the increased amino acids levels may have

triggered these phenomena [18, 20, 21]. Supporting this hypothesis, culturing isolated islets in a

high-glutamine medium resulted in an increased number of PP cells (Fig 5C) and an increase in

$GCG^+ PP^+$ double-positive cells (Fig 5D). Furthermore, we compared PP-cell mass in 10 week-

old systemic glucagon receptor-deficient mice (Fig 2D) and liver-specific glucagon receptor-

deficient mice (Fig 4C). Despite being the same age, there was a significant difference in the PP-

cell mass between the two groups. A potential explanation for this difference is the variation in

plasma amino acid levels between the two groups. As shown in S3 Fig, plasma amino acid levels

in systemic glucagon receptor-deficient mice were higher than those in liver-specific glucagon

receptor-deficient mice. Particularly in terms of glutamine levels, systemic glucagon receptor-

deficient mice demonstrated significantly higher values (S4 Fig). We believe that this difference

in amino acid levels accounts for the variation in PP-cell mass of these mice at the same age.

Our results demonstrate that increased blood amino acid levels resulting from impaired

glucagon action in the liver activates the mTOR pathway in α cells, leading to the emergence of

GCG^+ PP^+ double-positive cells. Previous studies have shown that forced expression of

Aristaless-related homeobox (ARX) protein, a key transcription factor that determines the fate of

α cells [39-41], in the embryonic pancreas or developing islet cells leads to a marked increase in

α cells, accompanied by the loss of β and δ cell. Interestingly, under these conditions, the number

of PP cells also increases substantially [39]. In humans with ARX mutations, β, δ, and ghrelin-

secreting ε cells are present normally, whereas α and PP cells are deficient [42]. During

development, α and PP cells differentiate from a common α/PP precursor [40, 43, 44]. This

suggests that their differentiation may also depend on transcription factors other than ARX.

However, the mechanisms that distinguish these two cell types and maintain their respective

identities remain unclear. Since some transcription factors, such as ARX, are commonly active in

both cell types, it is believed that under normal conditions, PP protein expression is suppressed in

α cells to preserve their identity. Collectively, our findings suggest that suppression of the mTOR

pathway in α cells inhibits the expression of factors, including PP proteins, that drive PP-cell

characteristics. Future studies should focus on elucidating how the mTOR pathway regulates these

factors in α cells. Furthermore, we demonstrated that glucagon dysfunction leads to the emergence

of multi-hormone-producing cells, including PP^+ INS^+ double-positive cells (Fig 4E) and PP^+

GFP^+ SST^+ triple-positive cells (Fig 1B). However, the precise mechanistic link between glucagon

dysfunction and the appearance of these cells remains unclear.　　Interestingly, a subset of pituitary

cells is known to produce multiple hormones and function under specific physiological conditions

such as growth, stress response, and reproduction. Gene expression analysis further suggests that

these multihormone cells originate from undifferentiated or plastic cells [26].　　Given these

findings, one hypothesis is that the increase in blood amino acid levels due to impaired glucagon

action in the liver activates the mTOR pathway in α cells, leading to enhanced cell proliferation

and increased cellular plasticity. Consequently, α cells may shift to a more undifferentiated state,

resulting in an increased emergence of multi-hormone-producing cells, including GCG^+ PP^+

double-positive cells. Future studies are needed to elucidate how these multi-hormone-producing

cells influence the progression and regulation of pathological conditions caused by glucagon

dysfunction.

In conclusion, impaired glucagon signaling activates the mTOR pathway via

hyperaminoacidemia, particularly elevated glutamine levels, inducing not only α-cell hyperplasia

but also PP-cell hyperplasia and the formation of multi-hormone-producing cells. Inhibition of

mTOR suppresses these changes. This study reveals a novel mechanism by which glucagon

dysfunction alters pancreatic endocrine cell composition, highlighting the critical role of mTOR

in endocrine cell plasticity.

Conflicts of interest

428   The authors declare that they have no conflicts of interest associated with this study.

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 Figure legends

 Fig. 1. Deficiency of proteins encoded by the proglucagon gene induces PP-cell hyperplasia

 and the formation of multi-hormone-producing cells

 (A) Representative immunofluorescence staining of GCG or GFP (green), PP (red), and nuclei

 (DAPI; blue) in pancreas sections from 40 week old $GCG^{+/+}$ mice and $Gcg^{gfp/gfp}$ mice. A magnified

 view of the boxed region is shown on the very right. (B) Representative immunofluorescence

 staining of PP (blue), GCG or GFP (green), and SST (red) in pancreas sections from 40 week-old

 $Gcg^{+/+}$ mice and $Gcg^{gfp/gfp}$ mice (B). Scale bars represent 30 μm or 50 μm.

 Fig 2. Glucagon signaling deficiency induces PP-cell hyperplasia and the appearance of

 GCG$^+$ PP$^+$ double-positive cells

 (A) Schematic diagram of the loxP target site. (B) Representative immunofluorescence staining

 of GCG (green), PP (red), and nuclei (DAPI; blue) in pancreas sections from 10 week-old $Gcgr^{+/+}$

 mice and $Gcgr^{\Delta/\Delta}$ mice. Magnified images of the boxed region are shown at the bottom. Scale bar

 represents 30 μm. PP-cell mass (C) and the proportion of GCG$^+$ PP$^+$ cells among the GCG$^+$ cells

 (D) in 10 week-old $Gcgr^{+/+}$ mice and $Gcgr^{\Delta/\Delta}$ mice (n = 4–7 mice). Data are shown as the mean

± SEM, and analyzed by the two-tailed unpaired Student *t*-test. ***p* < 0.01, ****p* < 0.001.

Fig 3. PP-cell hyperplasia involves both the self-proliferation of PP cells and an increase in

GCG$^+$ PP$^+$ double-positive cells

(A) Representative immunofluorescence staining of GCG (green), PP (red), and nuclei (DAPI;

blue) in pancreas sections from 4 week-old *Gcgr$^{+/+}$* mice and *Gcgr$^{\Delta/\Delta}$* mice. Scale bar represents

30 μm. (B) PP-cell mass in 4week-old *Gcgr$^{+/+}$* mice and *Gcgr$^{\Delta/\Delta}$* mice (n = 6 mice each). (C)

Representative immunofluorescence staining of GCG (green), PP (red), and nuclei (DAPI; blue)

in pancreas sections from 4 week-old and 10 week-old *Gcgr$^{+/+}$* mice and *Gcgr$^{\Delta/\Delta}$* mice. Scale bar

represents 30 μm. (D) Proportions of GCG$^+$ PP$^+$ cells among GCG$^+$ cells in 4 week-old and 10

week-old *Gcgr$^{+/+}$* mice and *Gcgr$^{\Delta/\Delta}$* mice (n = 4 mice each). (E) Representative

immunofluorescence staining of PP (blue), GCG (green), and Ki67 (red) in pancreas sections from

4 week-old *Gcgr$^{+/+}$* mice and *Gcgr$^{\Delta/\Delta}$* mice. Scale bar represents 30 μm. A magnified image of the

boxed region is shown on the very right. (F) Proportions of Ki67$^+$ PP$^+$ GCG$^-$ cells among PP$^+$

cells in 10 week-old *Gcgr$^{+/+}$* mice and *Gcgr$^{\Delta/\Delta}$* mice (n = 4 mice each).   Data are shown as the

mean ± SEM, and analyzed by the two-tailed unpaired Student *t*-test (B and F) or one-way

ANOVA followed by the Tukey test (D). $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Fig 4. Glucagon dysfunction in the liver induces PP-cell hyperplasia and the appearance of

GCG$^+$ PP$^+$ double-positive cells

(A) Representative immunofluorescence staining of GCG (green), PP (red), and nuclei (DAPI;

blue) in pancreas sections from 10 week-old $Gcgr^{f/f}$ mice and Alb-$Cre;Gcgr^{f/f}$ mice. Scale bar

represents 30 μm. The PP-cell mass (B) and the proportion of GCG$^+$ PP$^+$ cells among GCG$^+$ cells

(C) in 10 week-old $Gcgr^{f/f}$ mice and Alb-$Cre;Gcgr^{f/f}$ mice (n = 5 mice each) are shown. (D)

Representative immunofluorescence staining of GCG (green), PP (red), and nuclei (DAPI; blue)

in pancreas sections from 40 week-old $Gcgr^{f/f}$ mice and Alb-$Cre;Gcgr^{f/f}$ mice. (E) Representative

immunofluorescence staining of INS (green), PP (red), and nuclei (blue) in pancreas sections from

40 week-old $Gcgr^{f/f}$ mice and Alb-$Cre;Gcgr^{f/f}$ mice. Scale bar represents 30 μm. In (B) and (C),

data are shown as the mean ± SEM, and analyzed by the two-tailed unpaired Student t-test. $*p <$

0.05, $**p < 0.01$. In C, D, and E, magnified images of the boxed regions are shown in the bottom

row.

Fig 5. GCG$^+$ PP$^+$ double-positive cells are increased in isolated islets upon incubation in high

concentrations of glutamine, and this increase is mediated by the mTOR pathway

(A) Representative immunofluorescence staining of PP (red), GCG (white), and nuclei (blue) in

pancreas islets from 10 week-old wild-type mice, untreated (Ctrl, upper panels) or incubated with

a high concentration glutamine (Gln, lower panels). Scale bar represents 30 μm. Magnified

images of the boxed region are shown at the bottom. The proportion of GCG$^+$ cells among islet

cells (B) (n = 10 islets), the proportion of PP cells among islet cells (C) (n = 10 islets), and the

proportion of GCG$^+$ PP$^+$ cells among GCG$^+$ islet cells (D) (n = 10 islets) from 10 week-old wild-

type mice, either untreated (Ctrl) or incubated in a high concentration of glutamine (Gln). (E)

Representative immunofluorescence staining of PP (red) and GCG (white) in pancreatic islets

isolated from 10 week-old wild-type mice. Untreated islets (Ctrl, left panel), islets incubated with

a high concentration of glutamine (Gln, middle panel), and islets incubated with both a high

concentration of glutamine and rapamycin (Gln+Rap, right panel) are shown. Scale bar represents

30 μm. (F) Proportions of GCG$^+$ PP$^+$ cells among GCG$^+$ cells of the islets (n = 10 islets) from 10

week-old wild-type mice, which were untreated (Ctrl), incubated with a high concentration of

glutamine (Gln), or incubated with both a high concentration of glutamine and rapamycin

(Gln+Rap). Data are shown as the mean ± SEM, and analyzed by the two-tailed unpaired Student

t-test (B–D) or one-way ANOVA followed by the Tukey test (F). **$p < 0.01$, ***$p < 0.001$, ns,

not significant.

Supporting information

S1 Fig. α-cell mass in 10 week-old *Gcgr*^{f/f} mice and Alb-*Cre*:*Gcgr*^{f/f} mice

Data are shown as the mean ± SEM, and analyzed by the two-tailed unpaired Student *t*-test. *$p <$

0.05; n = 4 each.

S2 Fig. PP-cell mass in in 40 week-old *Gcgr*^{f/f} mice and Alb-*Cre*:*Gcgr*^{f/f} mice

Data are shown as the mean ± SEM, and analyzed by the two-tailed unpaired Student *t*-test. *$p <$

0.05. n = 6 each.

S3 Fig. Plasma levels of individual amino acids in 10 week-old *Gcgr*^{f/f}, Alb-*Cre*:*Gcgr*^{f/f},

***Gcgr*^{+/+}, and *Gcgr*^{Δ/Δ} mice**

Data are shown as the mean ± SEM (n = 4–7 mice each).

S4 Fig. Plasma glutamine levels in 10 week-old *Gcgr*^{f/f}, Alb-*Cre*:*Gcgr*^{f/f}, *Gcgr*^{+/+}, and *Gcgr*^{Δ/Δ}

mice

Data are shown as the mean ± SEM, and analyzed by one-way ANOVA followed by the Tukey

test. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$; ns, not significant.

S5 Fig. Activation of the mTOR pathway in GCG⁺ PP⁺ cells from 40 week-old *Gcgr*^{+/+} mice

and *Gcgr*^{Δ/Δ} mice

Representative immunofluorescence staining of GCG (green), pS6 (red), and PP (white) from 40

week-old *Gcgr*^{+/+} mice and *Gcgr*^{Δ/Δ} mice. Scale bar represents 30 μm. Enlarged images of the

boxed region are shown on the very right.

S6 Fig. Comparison of the number of PP⁺ positive-cells based on the presence or absence of

glucagon and pS6 signals in 4 week-old *Gcgr*^{+/+} mice and *Gcgr*^{Δ/Δ} mice

(A) Representative immunofluorescence staining of GCG (green), pS6 (red), and PP (white) from

4 week-old *Gcgr*^{+/+} mice and *Gcgr*^{Δ/Δ} mice. Scale bar represents 30 μm. Enlarged images of the

boxed region are shown on the very right. (B) Changes in the number of PP⁺ positive cells based

on the presence or absence of glucagon and pS6 signals in 4 week-old $Gcgr^{+/+}$ mice and $Gcgr^{\Delta/\Delta}$

micen (n = 25 islets). Data are shown as the mean ± SEM, and analyzed by one-way ANOVA

followed by the Tukey test. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant.

Locus	Target	ssODN
Left loxP	GcgrL4 (5'- ATGGCAAGATCACTGGCCAG -3')	GcgrL4loxP (5'-AAATGTTGCTGGGCTGGAGAGGGAGGTAGTCATGGCATCTAGATGGCAAGATCACTGGCCAATATTATAACTTCGTATAATGTATGCTATACGAAGTTATAGGGGATGCCCCTTGTGGCCCATATCAGGGAGGCAGCCCCTTTAGATGCAGGCTCCGTGT -3')
Right loxP	GcgrR6 (5'- GAATTCCCTGAGGACCTAGG -3')	GcgrR6loxP (5'-GGCCATTTGTCACCAGGAAAGGCAAGGACCACCTCATACACTGAATTCCCTGAGGACCTAATAACTTCGTATAGCATACATTATACGAAGTTATCTCGAGGGGGGACCTGACATCCAACACATGCAACCTTAATAATGGGGATGGAGATATGGTCATACC -3')

Table 1. Sequences of target crRNAs flanking exons 4 to 13 of the *Gcgr* gene, and corresponding donor single-stranded oligodeoxynucleotides (ssODNs), including loxP

Fig. 1. Deficiency of proteins encoded by the proglucagon gene induces PP-cell hyperplasia and the formation of multi-hormone-producing cells

Fig 2. Glucagon signaling deficiency induces PP-cell hyperplasia and the appearance of GCG$^+$ PP$^+$ double-positive cells

Fig 3. PP-cell hyperplasia involves both the self-proliferation of PP cells and an increase in GCG⁺ PP⁺ double-positive cells

Fig 4. Glucagon dysfunction in the liver induces PP-cell hyperplasia and the appearance of GCG⁺ PP⁺ double-positive cells

Fig 5. GCG$^+$ PP$^+$ double-positive cells are increased in isolated islets upon incubation in high concentrations of glutamine, and this increase is mediated by the mTOR pathway

S1 Fig. α-cell mass in 10 week-old *Gcgr*^{f/f} mice and Alb-*Cre*:*Gcgr*^{f/f} mice

S2 Fig. PP-cell mass in in 40 week-old *Gcgr*^{f/f} mice and Alb-*Cre*:*Gcgr*^{f/f} mice

S3 Fig. Plasma levels of individual amino acids in 10 week-old *Gcgr*^{f/f}, Alb-*Cre*:*Gcgr*^{f/f}, *Gcgr*^{+/+}, and *Gcgr*^{Δ/Δ} mice

S4 Fig. Plasma glutamine levels in 10 week-old *Gcgr*^f/f, **Alb-**_Cre_:_Gcgr_^f/f, *Gcgr*^+/+, **and** *Gcgr*^Δ/Δ **mice**

S5 Fig. Activation of the mTOR pathway in GCG[+] PP^{+} cells from 40 week-old *Gcgr*^{+/+} mice and *Gcgr*[Δ/Δ] mice

S6 Fig. Comparison of the number of PP⁺ positive-cells based on the presence or absence of glucagon and pS6 signals in 4 week-old *Gcgr^{+/+}* mice and *Gcgr^{Δ/Δ}* mice