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Uncovering the role of MAFB in glucagon production and secretion in pancreatic α-cells using a new α-cell-specific *Mafb* conditional knockout mouse model

Yu-Hsin CHANG[1,2], Megumi C. KATOH[1], Ahmed M. ABDELLATIF[3], Guli XIAFUKAITI[1,4], Abdelaziz ELZEFTAWY[5], Masami OJIMA[5], Seiya MIZUNO[5], Akihiro KUNO[1] and Satoru TAKAHASHI[1,5–8]

[1]*Department of Anatomy and Embryology, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[2]*Ph.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[3]*Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Mansoura University, 60 Elgomhoria st, Mansoura 35516, Egypt*
[4]*School of Comprehensive Human Sciences, Doctoral Program in Biomedical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[5]*Laboratory Animal Resource Center (LARC), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[6]*Life Science Center, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[7]*International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[8]*Transborder Medical Research Center, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*

Abstract: Cre/loxP is a site-specific recombination system extensively used to enable the conditional deletion or activation of target genes in a spatial- and/or temporal-specific manner. A number of pancreatic-specific Cre driver mouse lines have been broadly established for studying the development, function and pathology of pancreatic cells. However, only a few models are currently available for glucagon-producing α-cells. Disagreement exists over the role of the MAFB transcription factor in glucagon expression during postnatal life, which might be due to the lack of α-cell-specific Cre driver mice. In the present study, we established a novel *Gcg-Cre* knock-in mouse line with the *Cre* transgene expressed under the control of the preproglucagon (*Gcg)* promoter without disrupting the endogenous *Gcg* gene expression. Then, we applied this newly developed *Gcg-Cre* mouse line to generate a new α-cell-specific *Mafb* conditional knockout mouse model (*Mafb*$^{\Delta Gcg}$). Not only α-cell number but also glucagon production were significantly decreased in *Mafb*$^{\Delta Gcg}$ mice compared to control littermates, suggesting an indispensable role of MAFB in both α-cell development and function. Taken together, our newly developed *Gcg-Cre* mouse line, which was successfully utilized to uncover the role of MAFB in α-cells, is a useful tool for genetic manipulation in pancreatic α-cells, providing a new platform for future studies in this field.
Key words: α-cells, Cre/loxP, glucagon, MAFB, pancreatic islet

Introduction

The pancreas is a secretory organ composed of exocrine and endocrine glands that performs both digestive and hormonal functions. The endocrine pancreas is formed by three-dimensional clusters of cells called the islets of Langerhans (or the pancreatic islets). The pancreatic α-cells represent the second most abundant cell

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Corresponding authors: S. Takahashi. e-mail: satoruta@md.tsukuba.ac.jp
A. Kuno. e-mail: akuno@md.tsukuba.ac.jp

type in the pancreatic islets and secrete glucagon hormone to counteract the hypoglycemic action of postprandial insulin, thus maintaining normal blood glucose levels. It is well known that defects in insulin secretion and release by pancreatic β-cells are the major causes of diabetes mellitus. However, increasing evidence suggests that dysfunction of α-cells and dysregulated glucagon secretion contribute to dysglycemia in both type 1 and type 2 diabetes via exacerbating the chronic hyperglycemia caused by insulin deficiency [13, 42, 43]. In addition, recent studies showed the plasticity and interconversion of islet α-cells to β-cells, providing new insights for cell replacement and differentiation strategies for the treatment of diabetes [7, 8, 39]. In these regards, deciphering the physiology and pathology of α-cells holds great potential for the development of new therapies for diabetes.

The development, differentiation and maturation of pancreatic islets are governed by the sequential activation of a hierarchy of transcription factors [5, 44]. For example, PDX1 is the master transcription factor responsible for the specification of all pancreatic cell lineages [19]. NGN3 is necessary for the development of all endocrine cell lineages [17]. The antagonistic relationship between ARX and PAX4 is required for the subsequent differentiation of endocrine precursors [9, 11]. *MafA* and *MafB* are expressed at a delayed stage of development relative to other islet-enriched transcription factors [20]. *MafA* expression is required for maturation and the functional maintenance of β-cells [45]. A switch from MAFB to MAFA during islet development is critical for β-cell maturation in mice [3, 31]. MAFB is also critical for development and terminal differentiation in both α-cells and β-cells [2, 4, 12]. The importance of these transcription factors during the development of the endocrine pancreas has been identified through a number of transgenic and knockout mouse models.

MAFB is a basic leucine zipper (b-Zip) transcription factor belonging to the large MAF subfamily. *MafB* is expressed in both α-cells and β-cells in the developing pancreas from embryonic day 10.5 [4, 31] and is specifically restricted in α-cells in adult islets [5]. MAFB binds to the G1 element of the *Gcg* promoter together with other transcription factors, activating transcription of the *Gcg* gene and conferring α-cell specificity [4]. Recently, our laboratory demonstrated that MAFB is essential for glucagon production and secretion in mouse pancreatic α-cells after birth by using endocrine cell-specific *Mafb*-deficient models, *Mafb*$^{f/f}$*::Ngn3-Cre* (*Mafb*$^{\Delta Endo}$) and *Mafb*$^{f/f}$*::CAGG-CreER* (*Mafb*$^{\Delta TAM}$) mouse models [26]. However, using a pancreatic-specific *Mafb* conditional knockout (*Mafb*$^{f/f}$*::Pdx1-Cre, Mafb*$^{\Delta panc}$) mouse model, Conrad *et al.* reported a restoration of glucagon-positive α-cell count and islet glucagon content by 2 weeks and 8 weeks of age, respectively [12]. The discrepancy may result from different Cre drivers or mouse genetic backgrounds.

The Cre/loxP system is a site-specific recombination system that allows the conditional elimination or activation of a certain target gene in a specific tissue/cell and/or at the desired developmental time. Mouse models with pancreatic-specific Cre drivers have been broadly established for studying the development, function and pathology of pancreatic cells [30]. Of note, most of the endocrine cell-type-specific Cre driver mouse lines use progenitor cell-specific Cre drivers, such as *Pdx1-Cre* and *Ngn3-Cre*. In mouse lines utilizing endocrine genes to drive Cre expression, the majority of the reported lines use the insulin gene to manipulate the expression in β-cells. Only a few models are available for α-cells. To direct gene expression in α-cells, transgenic mouse lines carrying the Cre driver under the control of the *Gcg* promoter were established because the *Gcg* gene and glucagon specifically mark α-cells in the pancreas [2, 5]. However, due to the recombination efficiency, most of the reported *Gcg* transgenic models are not sufficient to reveal the phenotypes of target gene abrogation. Recently, two groups have developed new *Gcg-CreER*^{T2} knock-in mouse lines expressing a tamoxifen-inducible Cre recombinase from the endogenous *Gcg* gene locus [1, 36]. Both mouse lines exhibit a high specificity of *Cre* expression and recombination efficiency in pancreatic α-cells. However, the impacts of embryonic deletion of target genes on α-cells are difficult to be fully elucidated in these models due to the nature of the inducible system. In this regard, a new improved *Gcg-Cre* mouse line still awaits establishment.

Here, we describe a novel *Gcg-Cre* knock-in mouse line with constitutive *Cre* transgene expression under the control of the *Gcg* promoter without disrupting the endogenous *Gcg* gene expression and the application of this new *Gcg-Cre* model to generate a new α-cell-specific *Mafb* conditional knockout mouse model (*Mafb*$^{\Delta Gcg}$), aiming to ascertain the impact of MAFB on glucagon production as well as α-cell development and function. The *Gcg-Cre* driver is specifically expressed in α-cells in pancreatic islets with high recombination efficiency. Moreover, *Mafb*$^{\Delta Gcg}$ showed a defect in glucagon expression and reduced α-cell number. Both basal levels and the amount of secreted glucagon upon stimulation with arginine were found to be decreased. The consistency of these results with our previous observations verified the critical role of MAFB in glucagon production and secretion in the α-cell. In addition, our new *Gcg-Cre* mouse line is a powerful tool for future α-cell studies.

Materials and Methods

Generation of *Gcg-Cre* knock-in mice with the CRISPR/Cas9 targeting method

The *2A-Cre* sequence was integrated just before the stop codon of the *Gcg* gene via the CRISPR-Cas9 technique. The guide RNA sequence 5'- CCTCGTAGGAAATAGGTATTTCA-3' was selected and inserted into the entry site of *pX330-U6-Chimeric_BB-CBh-hSpCas9* (a kind gift from Dr. Feng Zhang, Addgene plasmid #42230). This plasmid was designated *pX330-Gcg*. The donor plasmid *pGcg/2A-Cre* contained the nuclear localization signal (*NLS*)-*Cre* and *2A* sequence of porcine teschovirus-1 (P2A). The 1.3-kb 5'-arm and the 1.4-kb 3'-arm were cloned into this vector. The strategy for generating bicistronic *Gcg-Cre* knock-in mice is shown in Fig. 1A.

Microinjection

A mixture of *pX330-Gcg* and *pGcg/2A-Cre* was microinjected into the male pronuclei of C57BL/6J fertilized oocytes. Living one-cell embryos were transferred to the oviduct of pseudopregnant ICR recipients (Charles River Laboratories, Kanagawa, Japan), and 117 newborns survived. *Gcg* knock-in was screened by PCR with AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) using the specific primers for *NLS-Cre* detection, F 5'- AAAATTTGCCTGCATTACCG-3' and R 5'-ATTCTCCCACCGTCAGTACG-3'; *Cas9* detection, F 5'-AGTTCATCAAGCCCATCCTG-3' and R 5'- GAAGTTTCTGTTGGCGAAGC-3'; donor transgene detection, F 5'-TTGCCGGGAAGCTAGAGTAA-3' and R 5'- TTTGCCTTCCTGTTTTTGCT-3'; 3' screening of *Gcg-Cre*, F 5'-TGGGAGAATGTTAATCCATATTGGCAGA-3' and R 5'-ATCAAGGAATTGTCTTGACCCGCTTTAG-3'; and 5' screening of *Gcg-Cre*, F 5'-CTGAAGGGACCTTTACCAGTGATGTGAG-3' and R 5'-ACAGAAGCATTTTCCAGGTATGCTCAGA-3'. The joint sequence between the 5' arm and the *NLS-Cre* sequence was confirmed by direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and with primer 5'- TTGCATCGACCGGTAATGCA-3' and analyzed on a 3500 Genetic Analyzer sequencing machine (Applied Biosystems, Waltham, MA, USA).

Animals

Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Resource Center at the University of Tsukuba. GRR/*Gcg-Cre* mice were

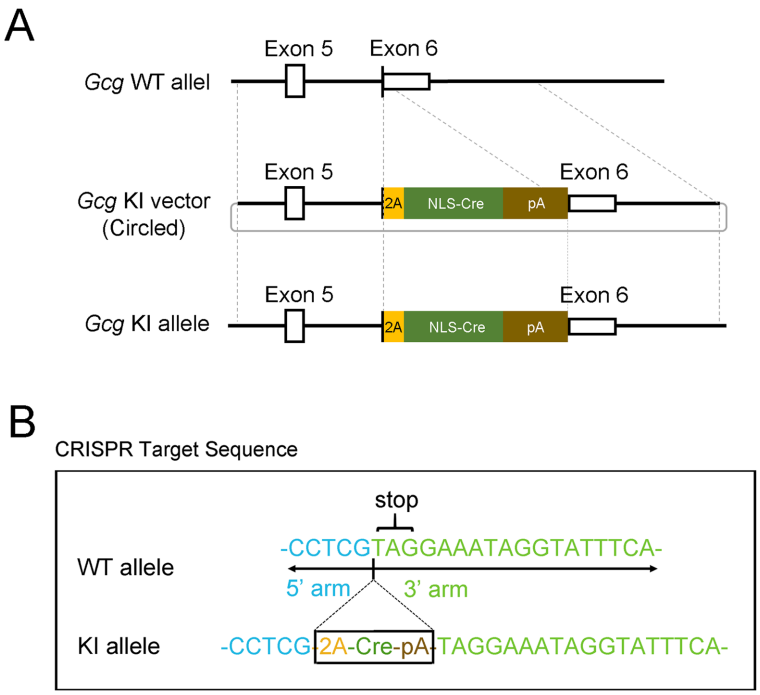

Fig. 1. Design of gene targeting for *Gcg-Cre* knock-in (KI) mice. (A) Schematic illustration of the WT allele, KI vector and KI allele of the *Gcg* gene. The *2A-NLS-Cre* was inserted just before the stop codon of the *Gcg* locus. (B) The sequence shows the 23-nt CRISPR target sequence (5'-CCTCGTAGGAAATAGGTATTTCA-3') containing a stop codon and protospacer adjacent motif (PAM). The 5'-homology arm ends at the final coding sequence of the *Gcg* gene, while the 3'-homology arm starts from the stop codon (TAG) of the *Gcg* gene.

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generated by crossing *Gcg-Cre* mice with R26GRR mice [21], while *Mafb*$^{f/f}$*::Gcg-Cre* (*Mafb*$^{\Delta Gcg}$) mice were generated by crossing *Gcg-Cre* mice with *Mafb*$^{f/f}$ mice [34, 40]. R26GRR or *Mafb*$^{f/f}$ mice were used as corresponding control mice in the experiments. Heterozygous GRR/*Gcg-Cre* mice were used in the subsequent studies. All animal handling and experiments were conducted under the approval and the supervision of the Institutional Animal Care and Use Committee of the University of Tsukuba.

Immunohistochemistry

Pancreatic, small intestinal and brain tissues of GRR/*Gcg-Cre* mice and pancreatic tissues of *Mafb*$^{\Delta Gcg}$ mice were fixed in 4% paraformaldehyde at 4°C overnight, processed, and embedded in OCT or paraffin, respectively. Five-micron sections were sliced and prepared for all histological analyses. For nuclear protein staining, antigen retrieval was performed by autoclaving using a target retrieval solution (Dako, Glostrup, Denmark). All sections were permeabilized with 0.1% Triton X-100 for 30 min, blocked in appropriate sera at room temperature for 1 h, and then incubated at 4°C overnight with appropriate primary antibodies, including guinea pig anti-insulin (1:500; ab7842, Abcam, Cambridge, UK), rabbit anti-glucagon (1:500; 2760; Cell Signaling Technology, Danvers, MA, USA), guinea pig anti-glucagon (1:1000; M182; Takara, Shiga, Japan) and rabbit anti-ARX (1:300; a kind gift from Drs. Kunio Kitamura and Kenichirou Morohashi, Kyushu University, Japan). Secondary antibodies conjugated to Alexa Fluor 488, 594 or 647 (1:1,000; Life Technologies, Carlsbad, CA, USA) were used to visualize the antigens. DNA was stained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) in an aqueous fluorescent mounting medium (Fluoromount; Cosmo Bio, Tokyo, Japan). Slides were examined using a Biorevo BZ-9000 fluorescence microscope (Keyence, Osaka, Japan), and images were acquired using BZ-II Analyzer software (Keyence).

Cell counting

Cell counting was performed as previously described [26]. Briefly, tdsRed-positive, glucagon-positive; tdsRed-positive, glucagon-negative; insulin-positive; glucagon-positive; ARX-positive; and glucagon-positive, ARX-positive cells in each islet were counted manually in the microscopy images taken as described in the immunohistochemical staining section. To calculate the percentage of positive cells within islets, the number of positive-stained cells per islet was counted and divided by the total number of Hoechst-stained nuclei from the same islet. The proportions of ARX-positive, glucagon-positive cells were determined by dividing the number of double-stained cells per islet by the total number of ARX-stained cells. Approximately 19 to 22 representative islets from 3 mice per group were selected and counted. The counting was conducted using ImageJ software.

Arginine-stimulated glucagon secretion

Mafb$^{\Delta Gcg}$ (n=3) and *Mafb*$^{f/f}$ control (n=4) mice were fasted for 16 h. Then, 1 mg/ml of L-arginine monohydrochloride (A6969, Sigma, St. Louis, MO, USA) prepared in saline was injected intraperitoneally. Blood samples were collected from the venous vein in EDTA-containing Eppendorf tubes at 0, 2, 10 and 25 min post injection. Plasma glucagon levels were determined using an enzyme-linked immunosorbent assay kit (10 μl #10-1281-01, Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

Statistical analysis

All data are presented as the means and standard errors of the means (SEM). *P*-values were analyzed using a two-tailed Welch's *t*-test. *P*-values less than 0.05 were considered statistically significant.

Results

Generation of *Gcg-Cre* mice

To introduce the *Gcg*-knock-in into the *Gcg* gene, the ideal CRISPR cleavage site followed by a protospacer adjacent motif (PAM) sequence was selected just before the stop codon of the *Gcg* gene. The integration of the *Gcg*-knock-in vector into the *Gcg* allele target site was obtained by coinjection of the *pX330-Gcg* vector encoding Cas9 and the guide RNA (gRNA) sequence under the control of the U6 promoter and *pGcg/2A-Cre* encoding the Cre donor sequence into the pronuclei of the fertilized oocytes of C57BL/6J mice. Next, the genotypes of 117 surviving pups out of a total of 123 pups were confirmed by PCR designed to amplify the integrated knock-in fragment of *NLS-Cre*, *Cas9*, the donor vector, the 5'-homology arm, and the 3'-homology arm sequentially. Finally, the joint sequence between the 5'arm and *NLS-Cre* was examined by direct sequencing, resulting in the generation of seven independent founder mice. Among these founder lines, line 78 was selected for use in the following experiments in this study and was named the *Gcg-Cre* mouse (the official name is C57BL/6-*Gcg*$^{em1\ (cre)\ Utr}$). The strategy of bicistronic *Gcg-Cre* expression is summarized in Fig. 1.

Recombination in *Gcg-Cre* in pancreatic α-cells

To characterize the expression pattern and recombination efficiency of the transgene, *Gcg-Cre* mice were bred with R26GRR double reporter mice. R26GRR mice are double *Cre*-reporter mice for validating Cre/loxP site-specific recombination, expressing green fluorescence protein (GFP) before and red fluorescence protein (tdsRed) after Cre-mediated recombination [21]. Crossing *Gcg-Cre* mice with R26GRR mice resulted in a new GRR/*Gcg-Cre* mouse line. The emission of tdsRed in adult pancreatic sections after *Gcg-Cre* recombination was detected directly by epifluorescence. The pancreatic islets of R26GRR control mice showed GFP signal homogenously but no tdsRed fluorescence (Fig. 2A). On the other hand, pancreatic islets from GRR/*Gcg-Cre* mice exhibited a strong tdsRed signal (Fig. 2A), suggesting the high recombination efficiency of the *Gcg-Cre* driver in the pancreas.

In the pancreas, the expression of the *Gcg* gene is confined to α-cells. To determine whether *Gcg-Cre* recombination specifically occurs in α-cells, immunohistochemistry analysis using glucagon antibody was performed. As expected, tdsRed fluorescence signals coincide with glucagon-positive cells and more than 97% of tdsRed-positive cells are glucagon-positive cells, implying that the *Gcg-Cre* transgene expression is specific in the α-cells (Figs. 2B and C). On the other hand, the observed glucagon expression in GRR/*Gcg-Cre* mice suggests the intact *Gcg* gene, indicating that the *Gcg* gene expression was not disrupted by the insertion of the Cre driver in this *Gcg-Cre* mouse line.

Gcg-Cre recombination in the small intestine and brain

The *Gcg* gene encodes the proglucagon precursor peptide, which is processed posttranslationally to yield multiple products in different tissues. In addition to glucagon in the α-cells of pancreatic islets, the *Gcg* gene is also expressed in the L cells of the intestine [33] and the neurons of the brain [28]. To elucidate whether *Gcg-Cre* recombination also occurs in intestinal cells and neurons, sections from the duodenum, jejunum and ileum of the small intestine and sections from the brains of GRR/*Gcg-Cre* mice were examined. As expected, tdsRed-positive cells were sporadically observed in all three parts of the intestine (Fig. 3A) and in the nucleus of

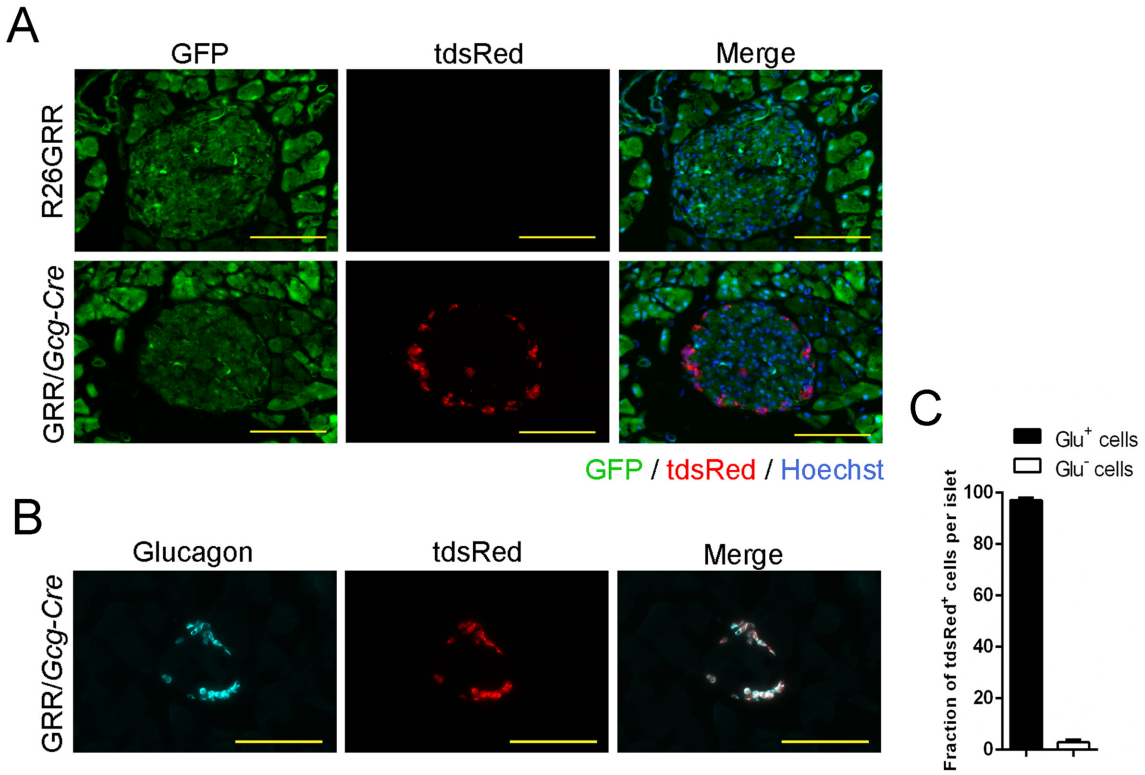

Fig. 2. Efficiency and specificity of *Gcg-Cre* recombination in the pancreatic α-cells of GRR/*Gcg-Cre* mice. (A) R26GRR mice show ubiquitous EGFP expression and no tdsRed expression without Cre recombination. EGFP is excised, and tdsRed is expressed in the adult pancreas of GRR/*Gcg-Cre* mice. (B) Colocalization of tdsRed signals with glucagon signals in the pancreatic islets of GRR/*Gcg-Cre* mice. Nuclei were counterstained with Hoechst 33342. Scale bars, 100 μm. (C) Fraction of glucagon-positive (Glu^+) and glucagon-negative (Glu^-) cells among tdsRed-positive ($tdsRed^+$) cells in the pancreatic islets GRR/*Gcg-Cre* mice (n=3).

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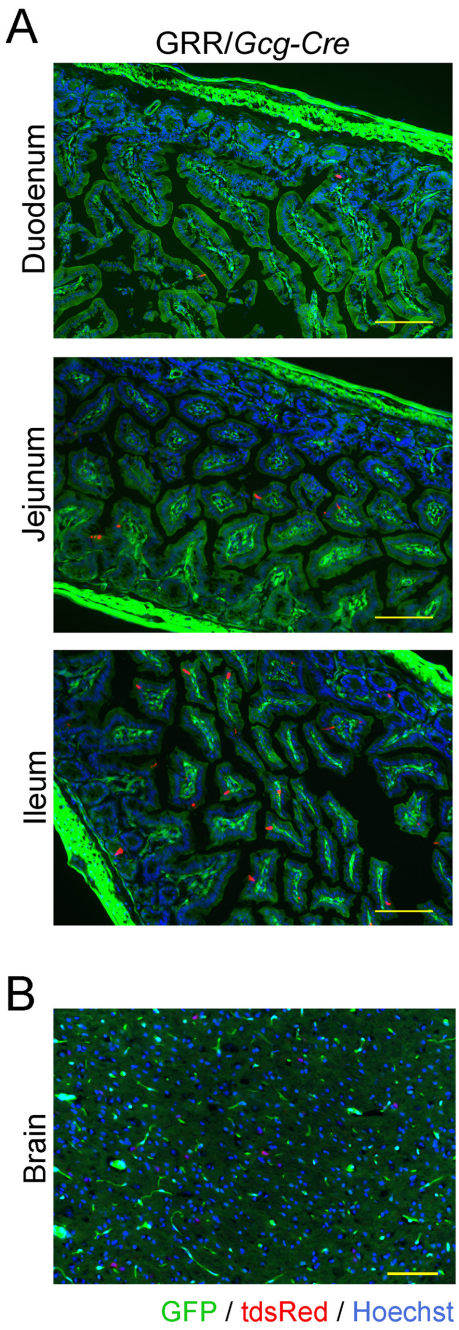

Fig. 3. *Gcg-Cre* recombination in the intestine and brain of GRR/*Gcg-Cre* mice. Sections from the (A) duodenum, jejunum and ileum of the intestine and (B) the NST of brainstem of GRR/*Gcg-Cre* mice exhibit tdsRed signal. Nuclei were stained with Hoechst 33342. Scale bars, 100 μm.

solitary tract (NST) of brainstem (Fig. 3B) but not in the control sections (data not shown), suggesting the successful recombination of *Gcg-Cre* in these tissues.

Loss of *Mafb* in glucagon-positive α-cells results in decreases in the glucagon-positive cell population and the suppression of α-cell development

A previous study by our laboratory indicated that embryonic deletion of *Mafb* in pancreatic endocrine cells leads to persistent postnatal decreases in the glucagon-positive cell population in $Mafb^{\Delta Endo}$ mice throughout postnatal development to adulthood [26]. To manipulate *Mafb* gene expression specifically in α-cells and to elucidate the role of MAFB in α-cell development, *Gcg-Cre* mice were crossed with $Mafb^{f/f}$ mice to obtain $Mafb^{f/f}$*::Gcg-Cre* ($Mafb^{\Delta Gcg}$) mice, in which *Mafb* is specifically deleted in α-cells. Consistent with the previous observation, the fraction of glucagon-positive cells in pancreatic islets in the adult $Mafb^{\Delta Gcg}$ mice (6 months) significantly decreased compared with control mice (Figs. 4A and B) (The percentage changes of glucagon-positive cells per islet in $Mafb^{\Delta Gcg}$ mice relative to control mice were 26.5 ± 5.3% ($Mafb^{\Delta Gcg}$) versus 100 ± 6.6% (control)), while the population of insulin-positive cells was comparable between the two groups (Figs. 4A and C) (The percentage changes of insulin-positive cells per islet in $Mafb^{\Delta Gcg}$ mice relative to control mice were 106.3 ± 4.5% ($Mafb^{\Delta Gcg}$) versus 100 ± 3.9% (control)). On the other hand, both islet architecture and total islet cell numbers remain unaffected in $Mafb^{\Delta Gcg}$ mice. These results reveal that the embryonic deletion of *Mafb* specifically in α-cells results in persistent defects in glucagon-positive cells.

To elucidate the physiological role of MAFB during α-cell development more precisely and to characterize cell identity, immunohistochemical staining was performed to examine the expression of *Arx*. ARX is a transcription factor considered an α-cell fate marker for its crucial role in α-cell differentiation and the maintenance of glucagon production [9, 11]. The population of ARX-positive cells per islet in $Mafb^{\Delta Gcg}$ mice was significantly decreased (Figs. 4D and E) (The percentage changes of ARX-positive cells per islet in $Mafb^{\Delta Gcg}$ mice relative to control mice were 33.5 ± 4.0% ($Mafb^{\Delta Gcg}$) versus 100 ± 11.1% (control)). In addition, the population of ARX-positive cells expressing glucagon in $Mafb^{\Delta Gcg}$ mice also decreased significantly (Figs. 4D and F) (The percentage changes of ARX-positive cells expressing glucagon per islet in $Mafb^{\Delta Gcg}$ mice relative to control mice were 51.2 ± 7.3% ($Mafb^{\Delta Gcg}$) versus 100.0 ± 1.1% (control)), suggesting that *Mafb* deletion in α-cells not only inhibits glucagon production but also suppresses the α-cell lineage marker. The total cell number of islets was unaltered by *Mafb* depletion (data not shown), implying that MAFB is essential for the terminal differentiation of pancreatic cells but not for the proliferation of pancreatic endocrine cells.

Mafb deletion in α-cells effectively abrogates glucagon secretion upon arginine stimulation

To elucidate the effects of the loss of *Mafb* on α-cells

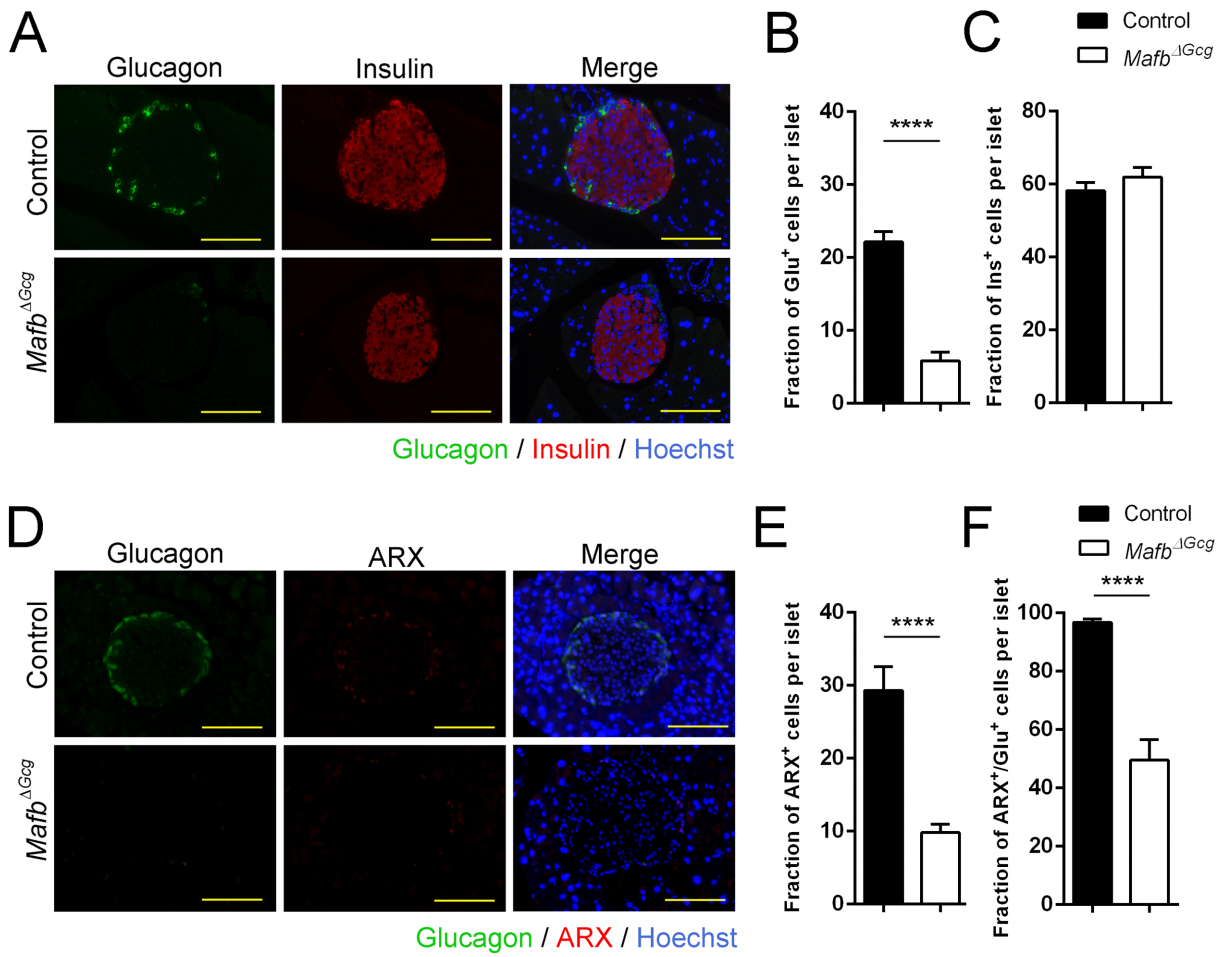

Fig. 4. Deletion of *Mafb* specifically in α-cells decreases the population of glucagon-positive cells and suppresses α-cell development. (A) Immunostaining of glucagon (green) and insulin (red) in $Mafb^{\Delta Gcg}$ (n=3) and control ($Mafb^{f/f}$; n=3) pancreatic sections from mice at 6 months of age. Nuclei were counterstained with Hoechst 33342. (B and C) Fraction of glucagon-positive (Glu^+) (B) and insulin-positive (Ins^+) (C) cells within islets in $Mafb^{\Delta Gcg}$ and control pancreatic sections. (D) Immunostaining of glucagon (green) and ARX (red) in pancreatic sections from $Mafb^{\Delta Gcg}$ (n=3) and control (n=3) mice at 6 months of age. Nuclei were counterstained with Hoechst 33342. (E and F) Fraction of ARX-positive (ARX^+) cells (E) and glucagon-positive α-cells among the total ARX-positive (ARX^+/Glu^+) cell population (F) within islets in $Mafb^{\Delta Gcg}$ and control mice. Scale bars, 100 μm. ****, $P<0.0001$.

from a functional aspect, amino-acid-stimulated glucagon secretion was examined. To this end, arginine was injected intraperitoneally in 8-week-old $Mafb^{\Delta Gcg}$ mice and control littermates after overnight fasting. After arginine stimulation, the plasma glucagon levels rapidly increased in control mice within 2 min (68.3 ± 12.8 pg/ml) and reached a peak at 10 min (74.8 ± 10.9 pg/ml) after arginine administration (Fig. 5). In contrast, the basal states of glucagon levels secreted by $Mafb^{\Delta Gcg}$ mice were significantly lower than those secreted by control mice at the pre-exposure time point (or time 0) (control versus $Mafb^{\Delta Gcg}$, 27.0 ± 0.9 versus 6.9 ± 1.6 pg/ml). In addition, the stimulated glucagon levels peaked at 2 min (32.5 ± 6.3 pg/ml) and cannot be retained to 10 min (25.4 ± 3.0 pg/ml), failing to reach the levels comparable to the control mice by the end of the experimental conditions. Notably, the plasma glucagon levels of an additional $Mafb^{\Delta Gcg}$ mouse were only slightly induced and were even lower than the detection limit of the ELISA at 0 and 25 min post arginine injection (data not shown), providing strong evidence that ablation of *Mafb* in α-cells disrupts glucagon secretion not only in the basal states but also upon stimulation with amino acids in mice. However, it is notable that the reduction in arginine-stimulated glucagon secretion in $Mafb^{\Delta Gcg}$ mice is not necessarily an indication of α-cells dysfunction since the amount of arginine-stimulated glucagon was not normalized with total pancreatic glucagon contents. The reduction of glucagon secretion in $Mafb^{\Delta Gcg}$ mice might simply reflect the decreased number of α-cells.

Discussion

Mouse models have been broadly established for pan-

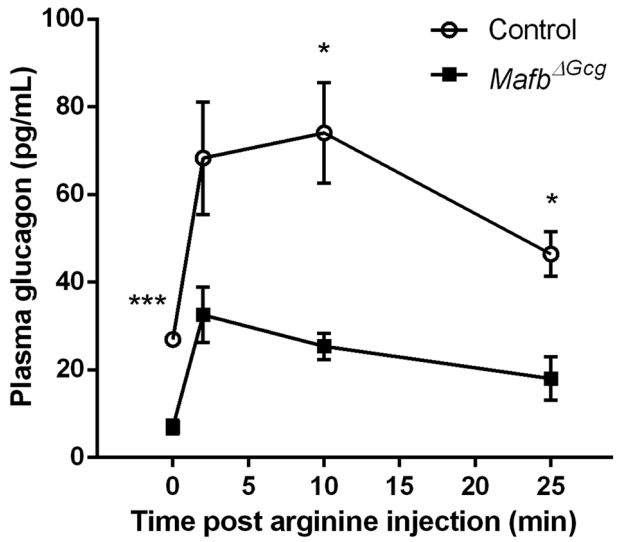

Fig. 5. Arginine-induced glucagon stimulation test of *Mafb*$^{\Delta Gcg}$ mice. Plasma glucagon levels were stimulated in overnight-fasted 8-week-old *Mafb*$^{\Delta Gcg}$ (n=3) and control (n=4) mice following an intraperitoneal injection of 1 mg/ml L-arginine. *, $P<0.05$, ***, $P<0.005$.

creatic studies due to the development of Cre/loxP site-specific recombination systems that allow the ablation or activation of specific genes in a spatial and/or temporal manner. For gene manipulation specifically in α-cells in pancreatic islets, the *Gcg* gene has been selected to generate Cre driver lines. Compared to the availability of *Ins1-Cre* driver lines utilized for β-cell study, the models for α-cells are limited even though many laboratories put efforts into establishing *Gcg-Cre* mouse lines. The first reported *Gcg-Cre* mouse line carries the *Cre* transgene driven by the 1.6-kb fragment of the rat glucagon gene promoter [24]. Although recombination was first reported to be efficient in glucagon-positive α-cells, later studies exhibited relatively lower recombination efficiency in this transgenic mouse line [37, 41], which may be caused by transgene silencing [30]. Another *Gcg-Cre* mouse line utilized an 8-kb region of the mouse *Gcg* promoter, and codon-optimized Cre (iCre) was generated [35], though off-target recombination in β-cells was observed in this model. There were also other *Gcg-Cre* mouse lines generated by the bacterial artificial chromosome (BAC) reported for gene manipulation in pancreatic α-cells, intestinal L cells or hindbrain neurons [15, 32].

Recently, two *Gcg-CreER*^{T2} mouse lines generated by knock-in strategies were developed by two research groups [1, 36]. Both *Gcg-CreER*^{T2} mice show a specific expression of the *CreER*^{T2} transgene in pancreatic α-cells and high tamoxifen-mediated recombination efficiency. Shiota *et al.* also described a new *Gcg*^{iCre} mouse line generated by the same strategy as their *Gcg*^{CreERT2} line [36]. Since the *Cre* transgene was substituted for the beginning 22 amino acids of exon 2 of proglucagon in both the *Gcg*^{CreERT2} and *Gcg*^{iCre} models, possible effects of this replacement on *Gcg* gene products may exist. Although the plasma glucagon levels and pancreatic glucagon contents in *Gcg*$^{CreERT2/w}$ mice were comparable to those of the *Gcg*$^{w/w}$ control mice, a 60% decrease in *Gcg* in the transcript levels in *Gcg*$^{CreERT2/w}$ mice was observed. On the other hand, the *Gcg-CreER*^{T2} mouse line developed by Ackermann *et al.* showed noticeable levels of Cre leakage before tamoxifen induction, which is probably due to a combination of the high expression of *CreER*^{T2} from the *Gcg* gene.

In the present study, we established a new bicistronic *Gcg-Cre* knock-in mouse line by using the CRISPR/Cas9 targeting method for gene manipulation in α-cells. In this model, Cre is knocked-in before the stop codon of the *Gcg* gene, not only protecting the integrity of the *Gcg* gene but also enabling the control of the *Cre* transgene by the endogenous transcriptional regulatory elements of the *Gcg* gene (Fig. 1). The observed glucagon expression in GRR/*Gcg-Cre* mice supports the intact *Gcg* gene, indicating that the *Gcg* gene expression was not disrupted by the insertion of the Cre driver in this *Gcg-Cre* mouse line (Fig. 2B). Previously, we reported an *Ins1-Cre* driver mouse line that was established using the same knock-in strategy [22]. The *Cre* transgene is integrated before the stop codon of exon 2 of the *Ins1* gene. The results of the glucose tolerance test showed no significant difference among *Ins1*$^{cre/cre}$, *Ins1*$^{Cre/+}$ and *Ins1*$^{+/+}$, indicating that the *2A-Cre* fusion does not affect gene and normal cell function. In addition, a porcine teschovirus-1 P2A peptide which was reported to possess high recombinase activity, enhancing the efficiency of cleavage and providing a more reliable expression of the appended gene was applied in bicistronic *Cre* expression in our new *Gcg-Cre* mice [27]. As expected, the efficient and specific recombination of the Cre driver in the pancreatic α-cells was demonstrated by using the GRR/*Gcg-Cre* mouse line, as more than 97% of tdsRed-expressing cells are glucagon-positive cells (Figs. 2B and C). In addition to the α-cells of the endocrine pancreas, Cre recombination in GRR/*Gcg-Cre* mice was also observed in the intestine and brain (Fig. 3), in which other *Gcg* gene products are expressed, suggesting that our *Gcg-Cre* mouse model is also a prospective tool for studies in intestinal L cells and neurons. Further experiments are required to examine whether *Gcg-Cre* recombination is confined to glucagon peptide-expressing cells in these tissues.

A number of transcription factors, including MAFB, c-MAF, PAX6, FOXA2, FOXA1, ARX, NEUROD1,

ISL1 and BRN4, have been reported to regulate α-cell development and glucagon production [18]. Among these factors, ARX, PAX6 and FOXA2 are considered the most important based on the observations that mutant mice lacking *Pax6* and *Foxa2* exhibit only a few α-cells, while *Arx* mutant mice have no α-cells [11, 29, 38]. In regard to the role of MAFB, a recent study using pancreatic cell-specific *Mafb*-deficienct mice ($Mafb^{\Delta panc}$) demonstrated that despite the decrease in glucagon-positive cell numbers from postnatal day 1 neonates, the glucagon-positive cells were restored by 2 weeks of age, implying that glucagon expression is compensated for by other factors [12]. In our previous [26] and current studies, endocrine cell-specific and α-cell-specific *Mafb* conditional knockout mouse models both showed significantly reduced glucagon expression (Fig. 4A) and secretion (Fig. 5) in adult stage, suggesting that MAFB is also one of the indispensable factors regulating glucagon gene expression in α-cells. Since the new α-cell-specific Cre mouse model generated in this study showed the same phenotype as our previous study, the discrepancy between our study and the study by Conrad *et al.* most likely reflects the different mouse genetic backgrounds.

A fundamental question to be addressed to understand the α-cell development is whether these factors act independently or form an interrelated gene regulating network [16]. A deficiency of *Pax6* or *Foxa2* does not decrease *Arx* gene expression [23, 29]. Similarly, the ablation of the *Foxa2* gene also does not alter the expression of *Pax6* and *Arx* [29]. On the other hand, ARX has been shown to alter α-cell differentiation but not glucagon gene expression [16]. We demonstrated that ARX-positive cells and *Arx* gene expression were reduced in the absence of *Mafb*. The expression of *Foxa2* is also affected [26]. Taken together, these findings suggest that MAFB is the principal factor transactivating other factors required in α-cell development and the glucagon pathway. In addition, among these transcription factors known to interact with the promoter of the rodent *Gcg* gene, MAFB and BRN4 are the only two factors specific to the α-cells [6]. BRN4 was demonstrated to be dispensable for glucagon regulation [25], while *MafB* and glucagon expression was shown to be positively correlated. The expression of *MafB* and glucagon increases upon the overexpression of *Arx* in pancreatic progenitor cells or the deletion of *Pdx1* in β-cells [10, 14], again implying the dominant potential of MAFB to regulate α-cell activity.

In summary, we generated an improved CRISPR/Cas9-mediated bicistronic knock-in *Gcg-Cre* mouse strain without disrupting the endogenous *Gcg* gene. The Cre recombinase of this mouse line is specifically expressed in glucagon-positive cells in pancreatic islets. The application of the new *Gcg-Cre* mice verifies the dominant role of MAFB in glucagon production and secretion in α-cells by generating the $Mafb^{\Delta Gcg}$ mouse line. These results suggest that our newly developed *Gcg-Cre* mouse line is a useful tool for genetic manipulation in pancreatic α-cells, providing a new platform for future studies in this field.

Conflict of Interest

The authors declare no competing financial interests.

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References

1. Ackermann, A.M., Zhang, J., Heller, A., Briker, A. and Kaestner, K.H. 2017. High-fidelity *Glucagon-CreER* mouse line generated by CRISPR-Cas9 assisted gene targeting. *Mol. Metab.* 6: 236–244. [Medline] [CrossRef]
2. Artner, I., Blanchi, B., Raum, J.C., Guo, M., Kaneko, T., Cordes, S., Sieweke, M. and Stein, R. 2007. MafB is required for islet beta cell maturation. *Proc. Natl. Acad. Sci. USA* 104: 3853–3858. [Medline] [CrossRef]
3. Artner, I., Hang, Y., Mazur, M., Yamamoto, T., Guo, M., Lindner, J., Magnuson, M.A. and Stein, R. 2010. MafA and MafB regulate genes critical to beta-cells in a unique temporal manner. *Diabetes* 59: 2530–2539. [Medline] [CrossRef]
4. Artner, I., Le Lay, J., Hang, Y., Elghazi, L., Schisler, J.C., Henderson, E., Sosa-Pineda, B. and Stein, R. 2006. MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. *Diabetes* 55: 297–304. [Medline] [CrossRef]
5. Benitez, C.M., Goodyer, W.R. and Kim, S.K. 2012. Deconstructing pancreas developmental biology. *Cold Spring Harb. Perspect. Biol.* 4: a012401. [Medline] [CrossRef]
6. Benner, C., van der Meulen, T., Cacéres, E., Tigyi, K., Donaldson, C.J. and Huising, M.O. 2014. The transcriptional landscape of mouse beta cells compared to human beta cells reveals notable species differences in long non-coding RNA and protein-coding gene expression. *BMC Genomics* 15: 620. [Medline] [CrossRef]
7. Chakravarthy, H., Gu, X., Enge, M., Dai, X., Wang, Y., Damond, N., Downie, C., Liu, K., Wang, J., Xing, Y., Chera, S., Thorel, F., Quake, S., Oberholzer, J., MacDonald, P.E., Herrera, P.L. and Kim, S.K. 2017. Converting Adult Pancreatic Islet α Cells into β Cells by Targeting Both Dnmt1 and Arx. *Cell Metab.* 25: 622–634. [Medline] [CrossRef]
8. Chung, C.H., Hao, E., Piran, R., Keinan, E. and Levine, F. 2010. Pancreatic β-cell neogenesis by direct conversion from mature α-cells. *Stem Cells* 28: 1630–1638. [Medline] [CrossRef]
9. Collombat, P., Hecksher-Sørensen, J., Broccoli, V., Krull, J., Ponte, I., Mundiger, T., Smith, J., Gruss, P., Serup, P. and Mansouri, A. 2005. The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specifica-

tion at the expense of the alpha- and beta-cell lineages in the mouse endocrine pancreas. *Development* 132: 2969–2980. [Medline] [CrossRef]
10. Collombat, P., Hecksher-Sørensen, J., Krull, J., Berger, J., Riedel, D., Herrera, P.L., Serup, P. and Mansouri, A. 2007. Embryonic endocrine pancreas and mature beta cells acquire alpha and PP cell phenotypes upon Arx misexpression. *J. Clin. Invest.* 117: 961–970. [Medline] [CrossRef]
11. Collombat, P., Mansouri, A., Hecksher-Sorensen, J., Serup, P., Krull, J., Gradwohl, G. and Gruss, P. 2003. Opposing actions of Arx and Pax4 in endocrine pancreas development. *Genes Dev.* 17: 2591–2603. [Medline] [CrossRef]
12. Conrad, E., Dai, C., Spaeth, J., Guo, M., Cyphert, H.A., Scoville, D., Carroll, J., Yu, W.M., Goodrich, L.V., Harlan, D.M., Grove, K.L., Roberts, C.T. Jr., Powers, A.C., Gu, G. and Stein, R. 2016. The MAFB transcription factor impacts islet α-cell function in rodents and represents a unique signature of primate islet β-cells. *Am. J. Physiol. Endocrinol. Metab.* 310: E91–E102. [Medline] [CrossRef]
13. Dunning, B.E., and Gerich, J.E. 2007. The role of alpha-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. *Endocr. Rev.* 28: 253–283. [Medline] [CrossRef]
14. Gao, T., McKenna, B., Li, C., Reichert, M., Nguyen, J., Singh, T., Yang, C., Pannikar, A., Doliba, N., Zhang, T., Stoffers, D.A., Edlund, H., Matschinsky, F., Stein, R. and Stanger, B.Z. 2014. Pdx1 maintains β cell identity and function by repressing an α cell program. *Cell Metab.* 19: 259–271. [Medline] [CrossRef]
15. Gaykema, R.P., Newmyer, B.A., Ottolini, M., Raje, V., Warthen, D.M., Lambeth, P.S., Niccum, M., Yao, T., Huang, Y., Schulman, I.G., Harris, T.E., Patel, M.K., Williams, K.W. and Scott, M.M. 2017. Activation of murine pre-proglucagon-producing neurons reduces food intake and body weight. *J. Clin. Invest.* 127: 1031–1045. [Medline] [CrossRef]
16. Gosmain, Y., Cheyssac, C., Heddad Masson, M., Dibner, C. and Philippe, J. 2011. Glucagon gene expression in the endocrine pancreas: the role of the transcription factor Pax6 in α-cell differentiation, glucagon biosynthesis and secretion. *Diabetes Obes. Metab.* 13:(Suppl 1): 31–38. [Medline] [CrossRef]
17. Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F. 2000. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc. Natl. Acad. Sci. USA* 97: 1607–1611. [Medline] [CrossRef]
18. Gromada, J., Franklin, I. and Wollheim, C.B. 2007. Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocr. Rev.* 28: 84–116. [Medline] [CrossRef]
19. Gu, G., Dubauskaite, J. and Melton, D.A. 2002. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* 129: 2447–2457. [Medline] [CrossRef]
20. Hang, Y., and Stein, R. 2011. MafA and MafB activity in pancreatic β cells. *Trends Endocrinol. Metab.* 22: 364–373. [Medline] [CrossRef]
21. Hasegawa, Y., Daitoku, Y., Sekiguchi, K., Tanimoto, Y., Mizuno-Iijima, S., Mizuno, S., Kajiwara, N., Ema, M., Miwa, Y., Mekada, K., Yoshiki, A., Takahashi, S., Sugiyama, F. and Yagami, K. 2013. Novel ROSA26 Cre-reporter knock-in C57BL/6N mice exhibiting green emission before and red emission after Cre-mediated recombination. *Exp. Anim.* 62: 295–304. [Medline] [CrossRef]
22. Hasegawa, Y., Hoshino, Y., Ibrahim, A.E., Kato, K., Daitoku, Y., Tanimoto, Y., Ikeda, Y., Oishi, H., Takahashi, S., Yoshiki, A., Yagami, K., Iseki, H., Mizuno, S. and Sugiyama, F. 2016. Generation of CRISPR/Cas9-mediated bicistronic knock-in ins1-cre driver mice. *Exp. Anim.* 65: 319–327. [Medline] [CrossRef]
23. Heller, R.S., Stoffers, D.A., Liu, A., Schedl, A., Crenshaw, E.B. 3rd., Madsen, O.D. and Serup, P. 2004. The role of Brn4/Pou3f4 and Pax6 in forming the pancreatic glucagon cell identity. *Dev. Biol.* 268: 123–134. [Medline] [CrossRef]
24. Herrera, P.L. 2000. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* 127: 2317–2322. [Medline] [CrossRef]
25. Hussain, M.A., Miller, C.P. and Habener, J.F. 2002. Brn-4 transcription factor expression targeted to the early developing mouse pancreas induces ectopic glucagon gene expression in insulin-producing beta cells. *J. Biol. Chem.* 277: 16028–16032. [Medline] [CrossRef]
26. Katoh, M.C., Jung, Y., Ugboma, C.M., Shimbo, M., Kuno, A., Basha, W.A., Kudo, T., Oishi, H. and Takahashi, S. 2018. MafB Is Critical for Glucagon Production and Secretion in Mouse Pancreatic α Cells *In Vivo*. *Mol. Cell. Biol.* 38: e00504-17. [Medline] [CrossRef]
27. Kim, J.H., Lee, S.R., Li, L.H., Park, H.J., Park, J.H., Lee, K.Y., Kim, M.K., Shin, B.A. and Choi, S.Y. 2011. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* 6: e18556. [Medline] [CrossRef]
28. Larsen, P.J., Tang-Christensen, M., Holst, J.J. and Orskov, C. 1997. Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. *Neuroscience* 77: 257–270. [Medline] [CrossRef]
29. Lee, C.S., Sund, N.J., Behr, R., Herrera, P.L. and Kaestner, K.H. 2005. Foxa2 is required for the differentiation of pancreatic alpha-cells. *Dev. Biol.* 278: 484–495. [Medline] [CrossRef]
30. Magnuson, M.A., and Osipovich, A.B. 2013. Pancreas-specific Cre driver lines and considerations for their prudent use. *Cell Metab.* 18: 9–20. [Medline] [CrossRef]
31. Nishimura, W., Kondo, T., Salameh, T., El Khattabi, I., Dodge, R., Bonner-Weir, S. and Sharma, A. 2006. A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. *Dev. Biol.* 293: 526–539. [Medline] [CrossRef]
32. Parker, H.E., Adriaenssens, A., Rogers, G., Richards, P., Koepsell, H., Reimann, F. and Gribble, F.M. 2012. Predominant role of active versus facilitative glucose transport for glucagon-like peptide-1 secretion. *Diabetologia* 55: 2445–2455. [Medline] [CrossRef]
33. Reimann, F., Habib, A.M., Tolhurst, G., Parker, H.E., Rogers, G.J. and Gribble, F.M. 2008. Glucose sensing in L cells: a primary cell study. *Cell Metab.* 8: 532–539. [Medline] [CrossRef]
34. Shichita, T., Ito, M., Morita, R., Komai, K., Noguchi, Y., Ooboshi, H., Koshida, R., Takahashi, S., Kodama, T. and Yoshimura, A. 2017. MAFB prevents excess inflammation after ischemic stroke by accelerating clearance of damage signals through MSR1. *Nat. Med.* 23: 723–732. [Medline] [CrossRef]
35. Shiota, C., Prasadan, K., Guo, P., El-Gohary, Y., Wiersch, J., Xiao, X., Esni, F. and Gittes, G.K. 2013. α-Cells are dispensable in postnatal morphogenesis and maturation of mouse pancreatic islets. *Am. J. Physiol. Endocrinol. Metab.* 305: E1030–E1040. [Medline] [CrossRef]
36. Shiota, C., Prasadan, K., Guo, P., Fusco, J., Xiao, X. and Gittes, G.K. 2017. Gcg ^{CreERT2} knockin mice as a tool for genetic manipulation in pancreatic alpha cells. *Diabetologia* 60: 2399–2408. [Medline] [CrossRef]
37. Solomou, A., Meur, G., Bellomo, E., Hodson, D.J., Tomas, A., Li, S.M., Philippe, E., Herrera, P.L., Magnan, C. and Rutter, G.A. 2015. The Zinc Transporter Slc30a8/ZnT8 Is Required in a Subpopulation of Pancreatic α-Cells for Hypoglycemia-induced Glucagon Secretion. *J. Biol. Chem.* 290: 21432–21442. [Medline] [CrossRef]
38. St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P. 1997. Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* 387: 406–409. [Medline] [CrossRef]

39. Thorel, F., Népote, V., Avril, I., Kohno, K., Desgraz, R., Chera, S. and Herrera, P.L. 2010. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature* 464: 1149–1154. [Medline] [CrossRef]
40. Tran, M.T., Hamada, M., Nakamura, M., Jeon, H., Kamei, R., Tsunakawa, Y., Kulathunga, K., Lin, Y.Y., Fujisawa, K., Kudo, T. and Takahashi, S. 2016. MafB deficiency accelerates the development of obesity in mice. *FEBS Open Bio* 6: 540–547. [Medline] [CrossRef]
41. Tudurí, E., Denroche, H.C., Kara, J.A., Asadi, A., Fox, J.K. and Kieffer, T.J. 2014. Partial ablation of leptin signaling in mouse pancreatic α-cells does not alter either glucose or lipid homeostasis. *Am. J. Physiol. Endocrinol. Metab.* 306: E748–E755. [Medline] [CrossRef]
42. Unger, R.H., Aguilar-Parada, E., Müller, W.A. and Eisentraut, A.M. 1970. Studies of pancreatic alpha cell function in normal and diabetic subjects. *J. Clin. Invest.* 49: 837–848. [Medline] [CrossRef]
43. Unger, R.H., and Cherrington, A.D. 2012. Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. *J. Clin. Invest.* 122: 4–12. [Medline] [CrossRef]
44. van der Meulen, T., and Huising, M.O. 2015. Role of transcription factors in the transdifferentiation of pancreatic islet cells. *J. Mol. Endocrinol.* 54: R103–R117. [Medline] [CrossRef]
45. Zhang, C., Moriguchi, T., Kajihara, M., Esaki, R., Harada, A., Shimohata, H., Oishi, H., Hamada, M., Morito, N., Hasegawa, K., Kudo, T., Engel, J.D., Yamamoto, M. and Takahashi, S. 2005. MafA is a key regulator of glucose-stimulated insulin secretion. *Mol. Cell. Biol.* 25: 4969–4976. [Medline] [CrossRef]