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

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Running head: α-CELL-SPECIFIC MAFB CKO MICE

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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\textsuperscript{\Delta Gcg}). Not only α-cell number but also glucagon production were significantly decreased in Mafb\textsuperscript{\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 perform 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 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 [31, 43] 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 [43]. Recently, our laboratory demonstrated that MAFB is essential
for glucagon production and secretion in mouse pancreatic α-cells after birth by using
diabetes cell-specific Mafb-deficient models, Mafβflo::Ngn3-Cre (MafbEndo) and
Mafβflo::CAGG-CreER (MafbTAM) mouse models [26]. However, using a
pancreatic-specific Mafb conditional knockout (Mafβflo::Pdx-1-Cre, Mafbpan) 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 \textit{Gcg-Cre} mouse line is a powerful tool for future \(\alpha\)-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), and 117 newborns survived. Gcg knock-in was screened by PCR with AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific) 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’-TTGCCGGAAGCTAGAGTAA-3’ and R 5’-TTTGCCTTCCTGTTTTTGCT-3’; 3’ screening of Gcg-Cre, F 5’-TTGCCCGGAAGCTAGAGTAA-3’ and R 5’-TTTGCCCTTCCTGTTTTTGCT-3’; and 5’ screening of Gcg-Cre, F 5’-ATCAAGGAATTGCTCTGACCCGCTTTTAG-3’; and 5’ screening of Gcg-Cre, F 5’-CTGAAGGGACCTTTACCAGTGATGAG-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).

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
generated by crossing Gcg-Cre mice with R26GRR mice [21], while Mafb\textsuperscript{\textopencurlyquote;::Gcg-Cre
(Mafb\textsuperscript{\textopencurlyquote;}\textopencurlyquote;Gcg) mice were generated by crossing Gcg-Cre mice with Mafb\textsuperscript{\textopencurlyquote;}\textopencurlyquote; mice [34, 40].
R26GRR or Mafb\textsuperscript{\textopencurlyquote;} 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\textsuperscript{AGcg} 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). 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), rabbit anti-glucagon (1:500; 2760; Cell Signaling), guinea pig anti-glucagon
(1:1000; M182; Takara) 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 (1:1000; Life Technologies) were used to visualize the antigens. DNA was stained with Hoechst 33342 (Molecular Probes) in an aqueous fluorescent mounting medium (Fluoromount; Cosmo Bio). Slides were examined using a Biorevo BZ-9000 fluorescence microscope (Keyence), 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 Immunostaining 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 (Sigma, A6968) 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) 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<sup>em1(cre)Utr</sup>). 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 (Fig. 2B and 2C). 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 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 MafbΔEndo 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 MafbΔf mice to obtain MafbΔf::Gcg-Cre (MafbΔGcg) 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 MafbΔGcg mice (6 months) significantly decreased compared with control mice (Fig. 4A and 4B) (The percentage changes of glucagon-positive cells per islet in MafbΔGcg mice relative to control mice were 26.5% ± 5.3 % (MafbΔGcg) versus 100% ± 6.6% (control)), while the population of insulin-positive cells was comparable between the two groups (Fig. 4A and 4C) (The percentage changes of insulin-positive cells per islet in MafbΔGcg mice relative to control mice were 106.3% ± 4.5 % (MafbΔGcg) versus 100% ± 3.9% (control)). On the other hand, both islet architecture and total islet cell numbers remain unaffected in MafbΔGcg 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 MafbΔGcg mice was significantly decreased (Fig. 4D and 4E) (The percentage changes...
of ARX-positive cells per islet in \textit{Mafb}^{\Delta Gcg} mice relative to control mice were 33.5\% ± 4.0\% \textit{(Mafb}^{\Delta Gcg}) versus 100\% ± 11.1\% \textit{(control)}. In addition, the population of ARX-positive cells expressing glucagon in \textit{Mafb}^{\Delta Gcg} mice also decreased significantly (Fig. 4D and 4F) \textit{(The percentage changes of ARX-positive cells expressing glucagon per islet in \textit{Mafb}^{\Delta Gcg} mice relative to control mice were 51.2\% ± 7.3\% \textit{(Mafb}^{\Delta Gcg}) versus 100.0\% ± 1.1\% \textit{(control)}), suggesting that \textit{Mafb} deletion in \(\alpha\)-cells not only inhibits glucagon production but also suppresses the \(\alpha\)-cell lineage marker. The total cell number of islets was unaltered by \textit{Mafb} depletion \textit{(data not shown)}, implying that MAFB is essential for the terminal differentiation of pancreatic cells but not for the proliferation of pancreatic endocrine cells.

\textit{Mafb} deletion in \(\alpha\)-cells effectively abrogates glucagon secretion upon arginine stimulation

To elucidate the effects of the loss of \textit{Mafb} on \(\alpha\)-cells from a functional aspect, amino-acid-stimulated glucagon secretion was examined. To this end, arginine was injected intraperitoneally in 8-week-old \textit{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 \textit{(68.3 ± 12.8 pg/ml)} and reached a peak at 10 min \textit{(74.8 ± 10.9 pg/ml)} after arginine administration (Fig. 5). In contrast, the basal states of glucagon levels secreted by \textit{Mafb}^{\Delta Gcg} mice were significantly lower than those secreted by control mice at the pre-exposure time point \textit{(or time 0)} \textit{(control versus \textit{Mafb}^{\Delta Gcg}, 27.0 ± 0.9 versus 6.9 ± 1.6 pg/ml)}. In addition, the stimulated glucagon levels peaked at 2 min \textit{(32.5 ± 6.3 pg/ml)} and cannot be retained to 10 min \textit{(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 \textit{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∆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∆Gcg mice might simply reflect
the decreased number of α-cells.
Discussion

Mouse models have been broadly established for pancreatic 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 $\alpha$-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 $\beta$-cell study, the models for $\alpha$-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 $\alpha$-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 $\beta$-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 $\alpha$-cells, intestinal L cells or hindbrain neurons [15, 32].

Recently, two $Gcg$-$CreERT^2$ mouse lines generated by knock-in strategies were developed by two research groups [1, 36]. Both $Gcg$-$CreERT^2$ mice show a specific expression of the $CreERT^2$ transgene in pancreatic $\alpha$-cells and high tamoxifen-mediated recombination efficiency. Shiota et al. also described a new $Gcg^{Cre}$ mouse line generated by the same strategy as their $Gcg^{CreERT^2}$ line [36]. Since the $Cre$ transgene was substituted for the beginning 22 amino acids of exon 2 of proglucagon in both the $Gcg^{CreERT^2}$ and $Gcg^{Cre}$ models, possible effects of this
replacement on Gcg gene products may exist. Although the plasma glucagon levels
and pancreatic glucagon contents in Gcg<sup>CreERT2/w</sup> mice were comparable to those of
the Gcg<sup>w/w</sup> control mice, a 60% decrease in Gcg in the transcript levels of Gcg<sup>CreERT2</sup>
was observed. On the other hand, the Gcg-Cre<sup>ERT2</sup> 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 Cre<sup>ERT2</sup> 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 Ins<sup>1cre/cre</sup>, Ins<sup>1Cre/+</sup>
and Ins<sup>1+/+</sup>, 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 (Fig. 2B and 2C). 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-deficient mice (Mafb∆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<sup>ΔGcg</sup> 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.
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Conflict of interest

The authors declare no competing financial interests.
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**Figure legends**

**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.

**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) Fractions of glucagon-positive (Glu⁺) and glucagon-negative (Glu⁻) cells among tdsRed-positive (tdsRed⁺) cells in the pancreatic islets GRR/*Gcg-Cre* mice (n=3).

**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.

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

Fig. 5. Arginine-induced glucagon stimulation test of $Maft^\Delta Gcg$ mice. Plasma glucagon levels were stimulated in overnight-fasted 8-week-old $Maft^\Delta Gcg$ (n=3) and control (n=4) mice following an intraperitoneal injection of 1 mg/ml L-arginine. *, $P<0.05$, ***, $P<0.005$. ***, $P<0.0005$. ***, $P<0.0001$. ***, $P<0.0001$.
Fig. 1
A

|    | GFP | tdsRed | Merge |
|----|-----|--------|-------|
| R26GRR | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| GRR/Ggc-Cre | ![Image](image4) | ![Image](image5) | ![Image](image6) |

GFP / tdsRed / Hoechst

B

|    | Glucagon | tdsRed | Merge |
|----|----------|--------|-------|
| GRR/Ggc-Cre | ![Image](image7) | ![Image](image8) | ![Image](image9) |

C

**Fig. 2**
A

GRR/Gcg-Cre

Duodenum

Jejunum

Ileum

B

Brain

GFP / tdsRed / Hoechst

Fig. 3
Fig. 4
Fig. 5