



MafB Is Critical for Glucagon Production and Secretion in Mouse Pancreatic α Cells *In Vivo*

Megumi C. Katoh,^{a,b,c} Yunshin Jung,^{a,b,c} Chioma M. Ugboma,^{b,c,d} Miki Shimbo,^{b,c,e} Akihiro Kuno,^{a,b,c} Walaa A. Basha,^{b,c} Takashi Kudo,^{b,c} Hisashi Oishi,^{b,c*} Satoru Takahashi^{b,c,f,g,h}

^aPh.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, Ibaraki, Japan

^bDepartment of Anatomy and Embryology, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

^cLaboratory Animal Resource Center, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

^dMaster's Program in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

^eDoctoral Program in Biomedical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

^fLife Science Center, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Ibaraki, Japan

^gTransborder Medical Research Center, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

^hInternational Institute for Integrative Sleep Medicine (WPI-IIS), University of Tsukuba, Ibaraki, Japan

ABSTRACT The MafB transcription factor is expressed in pancreatic α and β cells during development but becomes exclusive to α cells in adult rodents. *Mafb*-null (*Mafb*^{-/-}) mice were reported to have reduced α - and β -cell numbers throughout embryonic development. To further analyze the postnatal function of MafB in the pancreas, we generated endocrine cell-specific (*Mafb* ^{Δ Endo}) and tamoxifen-dependent (*Mafb* ^{Δ TAM}) *Mafb* knockout mice. *Mafb* ^{Δ Endo} mice exhibited reduced populations of insulin-positive (insulin⁺) and glucagon⁺ cells at postnatal day 0, but the insulin⁺ cell population recovered by 8 weeks of age. In contrast, the Arx⁺ glucagon⁺ cell fraction and glucagon expression remained decreased even in adulthood. *Mafb* ^{Δ TAM} mice, with *Mafb* deleted after pancreas maturation, also demonstrated diminished glucagon⁺ cells and glucagon content without affecting β cells. A decreased Arx⁺ glucagon⁺ cell population in *Mafb* ^{Δ Endo} mice was compensated for by an increased Arx⁺ pancreatic polypeptide⁺ cell population. Furthermore, gene expression analyses from both *Mafb* ^{Δ Endo} and *Mafb* ^{Δ TAM} islets revealed that MafB is a key regulator of glucagon expression in α cells. Finally, both mutants failed to respond to arginine, likely due to impaired arginine transporter gene expression and glucagon production ability. Taken together, our findings reveal that MafB is critical for the functional maintenance of mouse α cells *in vivo*, including glucagon production and secretion, as well as in development.

KEYWORDS MafB, pancreatic islet, α cell, glucagon, PP cell, F cell

The pancreas is a secretory organ containing two major glands, an exocrine gland for digestive enzymes and an endocrine gland for pancreatic hormones. The endocrine pancreas is composed of small clusters of cells called the islets of Langerhans, which include insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells, pancreatic polypeptide (PP)-producing F cells (also known as PP cells), and ghrelin-producing ϵ cells. In particular, insulin and glucagon regulate glucose homeostasis: blood glucose levels decrease in response to insulin and increase in response to glucagon. Failure of this hormonal balance due to β -cell dysfunction causes hyperglycemia, a key feature of diabetes, which often accompanies α -cell impairment (1–3). Therefore, understanding pancreatic α - and β -cell biology produces deeper insights

Received 21 September 2017 Returned for modification 1 November 2017 Accepted 19 January 2018

Accepted manuscript posted online 29 January 2018

Citation Katoh MC, Jung Y, Ugboma CM, Shimbo M, Kuno A, Basha WA, Kudo T, Oishi H, Takahashi S. 2018. MafB is critical for glucagon production and secretion in mouse pancreatic α cells *in vivo*. *Mol Cell Biol* 38:e00504-17. <https://doi.org/10.1128/MCB.00504-17>.

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Satoru Takahashi, satoruta@md.tsukuba.ac.jp.

* Present address: Hisashi Oishi, Graduate School of Medical Sciences, Department of Comparative and Experimental Medicine, Nagoya City University, Aichi, Japan.

into hormonal regulation. Pancreatic islet development and specification, including α - and β -cell differentiation, are governed by various important transcription factors (4, 5). For example, Pdx1, a pancreatic progenitor marker, drives all pancreatic lineages. Ngn3, an endocrine progenitor marker, initiates pancreatic endocrine cell fates. Pax4 and Arx promote β - and α -cell specification and differentiation, respectively (4, 5). Finally, MafA is required for β -cell maturation and functional maintenance (6–8), and MafB plays a decisive role in α cells, although MafB is also involved in both α - and β -cell development during pancreas morphogenesis (9–11).

MafB, a member of the large Maf subfamily of basic leucine zipper (b-Zip) transcription factors, is first detected in the mouse embryonic pancreas from embryonic day 10.5 (7, 9) and becomes exclusively confined to α cells within 2 weeks of birth (8). In α cells, MafB activates glucagon gene expression through the G1 element of the glucagon promoter region (9); hence, glucagon is generally accepted as an α -cell marker (4, 5). Previous work demonstrated that *Mafb* knockout (*Mafb*^{-/-}) mouse embryos exhibit reduced α - and β -cell numbers with delayed onset of insulin expression in β cells, indicating the role of MafB in α - and β -cell differentiation (11). However, because the *Mafb*^{-/-} mutation in these mice was neonatal lethal due to defective respiratory rhythm (12), the postnatal function of MafB in pancreatic islets has thus far remained unknown. A recent study of pancreas-wide *Mafb*-deficient (*Mafb* ^{Δ panc}) mice revealed that insulin-positive (insulin⁺ [Ins⁺]) and glucagon⁺ (Glu⁺) cell numbers from postnatal day 1 (P1) neonates were also decreased (10). Interestingly, both cell types were restored by 2 weeks of age in these mutants, rendering α cells dysfunctional in response to low glucose levels and arginine stimulation *in vitro* (10). These results suggest that MafB is required only for maintaining α -cell function and not for glucagon production *per se*, implying that glucagon expression is compensated for by other factors. Given that MafB is a glucagon gene activator (9) and is heavily enriched in mature α cells (13), these results were intriguing. Additionally, frequent reports of diabetic patients displaying abnormal glucagon regulation highlight the importance of understanding α -cell physiology (1–3). Therefore, to confirm the postnatal role of MafB in pancreatic islet cells, we generated two types of conditional-knockout mice: endocrine cell-specific *Mafb* knockout (*Mafb* ^{Δ Endo}) and tamoxifen (TAM)-dependent *Mafb* knockout (*Mafb* ^{Δ TAM}) mice.

Here, we address an alternative *in vivo* role of MafB in postnatal pancreatic α cells. Both *Mafb* ^{Δ Endo} and *Mafb* ^{Δ TAM} mice failed to express glucagon in α cells, leading to low basal plasma glucagon levels. Moreover, *Mafb* deficiency disrupted glucagon secretory responses to α -cell stimuli in both mutants. Therefore, our findings demonstrate that MafB is critical for glucagon production during α -cell development and for α -cell functional maintenance in adult mice.

RESULTS

Embryonic deletion of *Mafb* in endocrine cells results in postnatal decreases in both Ins⁺ and Glu⁺ cell populations. To address the physiological function of MafB in postnatal pancreatic islets, we generated endocrine cell-specific *Mafb*-deficient mutant *Mafb*^{*ff*::*Neurogenin3*} (*Ngn3*)-Cre (*Mafb* ^{Δ Endo}) mice. We first performed immunohistochemical studies on pancreatic sections to explore the effects of *Mafb* loss on the postnatal development of pancreatic endocrine cells by examining insulin and glucagon protein expression. At P0, the fractions of Ins⁺ and Glu⁺ cells in *Mafb* ^{Δ Endo} islets were significantly reduced compared with *Mafb*^{*ff*} control mice (Fig. 1A to C) (control versus *Mafb* ^{Δ Endo}, Ins⁺, 100% \pm 6.9% versus 25.9% \pm 4.3%; Glu⁺, 100% \pm 10.0% versus 10.7% \pm 2.3%). Interestingly, the reduced population of Ins⁺ cells in *Mafb* ^{Δ Endo} pancreata recovered to nearly control levels as the mice aged (Fig. 1A and B) (control versus *Mafb* ^{Δ Endo}, 3 weeks, 100% \pm 2.6% versus 65.4% \pm 3.1%; 8 weeks, 100% \pm 2.5% versus 89.9% \pm 4.0%; 20 weeks, 100% \pm 1.8% versus 92.0% \pm 2.2%). However, the fraction of Glu⁺ cells in *Mafb* ^{Δ Endo} islets remained significantly reduced throughout postnatal development to 20 weeks of age compared with control groups (Fig. 1A and C) (control versus *Mafb* ^{Δ Endo}, 3 weeks, 100% \pm 8.3% versus 30.5% \pm 4.4%; 8 weeks, 100% \pm 9.7% versus 41.2% \pm 7.1%; 20 weeks, 100% \pm 6.2% versus 38.5% \pm 4.4%), while islet

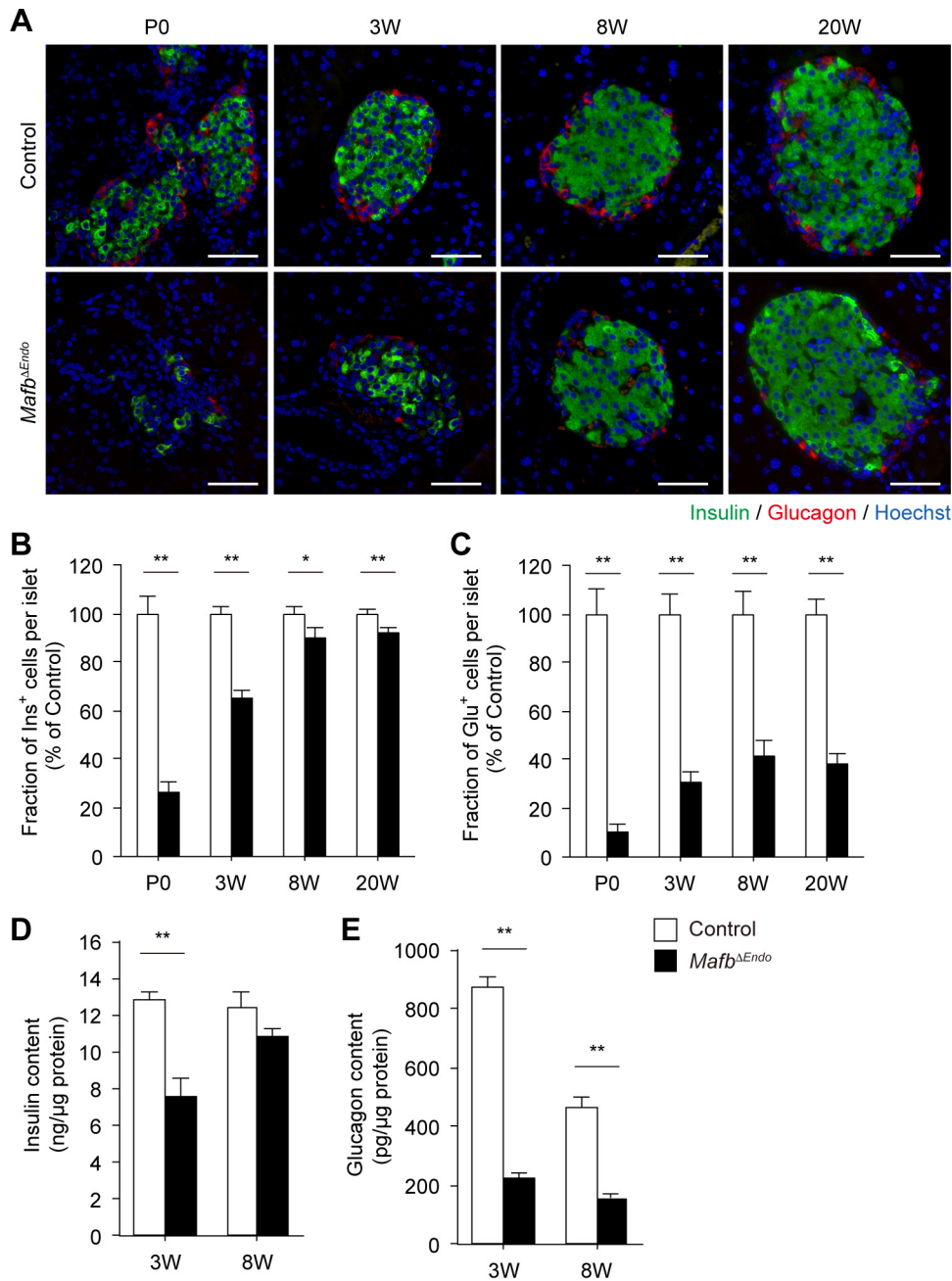


FIG 1 Embryonic deletion of *Mafb* in endocrine cells decreases the population of both Ins⁺ and Glu⁺ cells postnatally. (A) Immunostaining of insulin (green) and glucagon (red) in *Mafb*^{ΔEndo} and control (*Mafb*^{fl/fl}) pancreata from P0 and 3-, 8-, and 20-week-old (W) animals. Nuclei were stained with Hoechst 33342. Scale bars, 50 μm. (B and C) Fractions of Ins⁺ (B) and Glu⁺ (C) cells within islets in *Mafb*^{ΔEndo} and control pancreata (*n* ≥ 3). All values were normalized to age-matched controls. *, *P* < 0.05; **, *P* < 0.01. (D and E) Pancreatic insulin (D) and glucagon (E) contents in *Mafb*^{ΔEndo} and control pancreata from 3- and 8-week-old animals (*n* ≥ 4). The hormone content was normalized to the protein concentration. Means and SEM are shown. **, *P* < 0.01.

architecture and total islet cell numbers were unaffected (see Fig. S1A in the supplemental material). To confirm the reduction in insulin and glucagon production, we measured total insulin and glucagon contents in whole pancreata from 3- and 8-week-old animals (Fig. 1D and E). As expected, the insulin content in *Mafb*^{ΔEndo} pancreata was significantly reduced compared with control pancreata at 3 weeks of age but improved to approximately control levels at 8 weeks of age (Fig. 1D) (control versus *Mafb*^{ΔEndo}, 3 weeks, 12.8 ± 0.5 versus 7.6 ± 1.0 ng/μg protein; 8 weeks, 12.4 ± 0.8 versus 10.8 ± 0.5

ng/ μ g protein), consistent with the restoration of the Ins⁺ cell population (Fig. 1B). In contrast, the glucagon content in *Mafb* Δ *Endo* pancreata was severely compromised at both 3 and 8 weeks of age, with no sign of recovery to control levels (Fig. 1E) (control versus *Mafb* Δ *Endo*, 3 weeks, 869.7 \pm 37.8 versus 220.8 \pm 25.0 pg/ μ g protein; 8 weeks, 467.1 \pm 30.5 versus 157.9 \pm 16.3 pg/ μ g protein). Of note, this α -cell abnormality of *Mafb* Δ *Endo* mice did not affect the animals' growth, as the pancreas weight and body weight were both unaltered (see Fig. S1B and C in the supplemental material). These results suggest that the loss of *Mafb* during embryogenesis affects pancreatic endocrine cell development at early postnatal periods, leading to decreased populations of both Ins⁺ and Glu⁺ cells. However, only the α -cell defect persists into adulthood.

Endocrine cell-specific *Mafb* deficiency at the embryonic stage delays insulin production in β cells but suppresses α -cell development after birth. To more precisely investigate the role of MafB in postnatal islet cell development, we performed immunofluorescence staining to examine the expression of β - and α -cell fate markers that characterize cell identity. Pancreas sections from 3- and 8-week-old mice were costained for either insulin and Nkx6.1 in β cells (14) or glucagon and Arx in α cells (15) (Fig. 2A and D). The total Nkx6.1⁺ cell population remained unchanged, suggesting that *Mafb* ablation does not affect β -cell lineage differentiation (Fig. 2A and B) (control versus *Mafb* Δ *Endo*, 3 weeks, 100% \pm 8.1% versus 95.3% \pm 9.2%; 8 weeks, 100% \pm 6.8% versus 106.6% \pm 12.0%). At 3 weeks of age, only 60% of Nkx6.1⁺ cells expressed insulin in *Mafb* Δ *Endo* pancreata, whereas almost all Nkx6.1⁺ cells from control pancreata were also positive for insulin (Fig. 2A and C) (control versus *Mafb* Δ *Endo*, 98.4% \pm 0.6% versus 59.2% \pm 3.0%). However, this defect of decreased insulin was minimized by 8 weeks of age, at which point Ins⁺ β cells (Nkx6.1⁺ Ins⁺) accounted for greater than 90% of the total Nkx6.1⁺ cell population (Fig. 2A and C) (control versus *Mafb* Δ *Endo*, 99.0% \pm 0.2% versus 91.7% \pm 1.4%). These results suggest that embryonic *Mafb* deficiency in pancreatic islets causes delayed insulin production in β cells without affecting cell fate differentiation. Measurement of fasting blood glucose levels and glucose metabolism by intraperitoneal glucose tolerance test further supported our findings of delayed β -cell development. *Mafb* Δ *Endo* mice showed higher fasting blood glucose levels at P0, which were corrected to the control level by 8 weeks of age; delayed glucose tolerance in 4-week-old *Mafb* Δ *Endo* mice recovered to the control level at 8 weeks (see Fig. S2A to C in the supplemental material).

Conversely, the total Arx⁺ cell population in *Mafb* Δ *Endo* mice was reduced at both ages (Fig. 2D and E) (control versus *Mafb* Δ *Endo*, 3 weeks, 100% \pm 8.5% versus 74.1% \pm 6.6%; 8 weeks, 100% \pm 9.0% versus 59.7% \pm 3.8%). Similarly, 3-week-old *Mafb* Δ *Endo* mice displayed a considerably smaller population of Glu⁺ α cells (Arx⁺ Glu⁺), with fewer than 50% of Arx⁺ cells expressing glucagon (Fig. 2D and F) (control versus *Mafb* Δ *Endo*, 98.7% \pm 0.3% versus 47.5% \pm 3.6%). In contrast to β cells, this small population of Glu⁺ α cells was sustained even at 8 weeks of age without any sign of improvement (Fig. 2D and F) (control versus *Mafb* Δ *Endo*, 98.0% \pm 0.6% versus 51.4% \pm 6.0%). Thus, these findings demonstrate that *Mafb* ablation suppresses not only glucagon production but also the α -cell lineage, indicating that MafB plays a significant role in α -cell development. Interestingly, the remaining Glu⁺ α cells in the *Mafb* Δ *Endo* pancreas did not express MafB (Fig. 2G), suggesting an alternative glucagon production pathway independent of MafB, which could account for the minimal glucagon content level (Fig. 1E).

Loss of *Mafb* in adult mice impairs glucagon expression in mature α cells without affecting α -cell identity. Because MafB is a known regulator of glucagon transcription (9), we hypothesized that the suppression of glucagon expression is due to the deletion of *Mafb* rather than to the reduced Arx⁺ cell population in *Mafb* Δ *Endo* mice. To verify the effects of *Mafb* ablation on glucagon production, we generated conditional *Mafb* knockout (*Mafb* Δ *TAM*) mice after pancreatic islet cell maturation by tamoxifen (TAM) injection at 5 weeks of age. Immunohistochemistry revealed no significant changes in the organization of Ins⁺ and Glu⁺ cells in *Mafb* Δ *TAM* islets compared with *Mafb*^{fl/fl} control islets (Fig. 3A). Consistent with the undetectable MafB

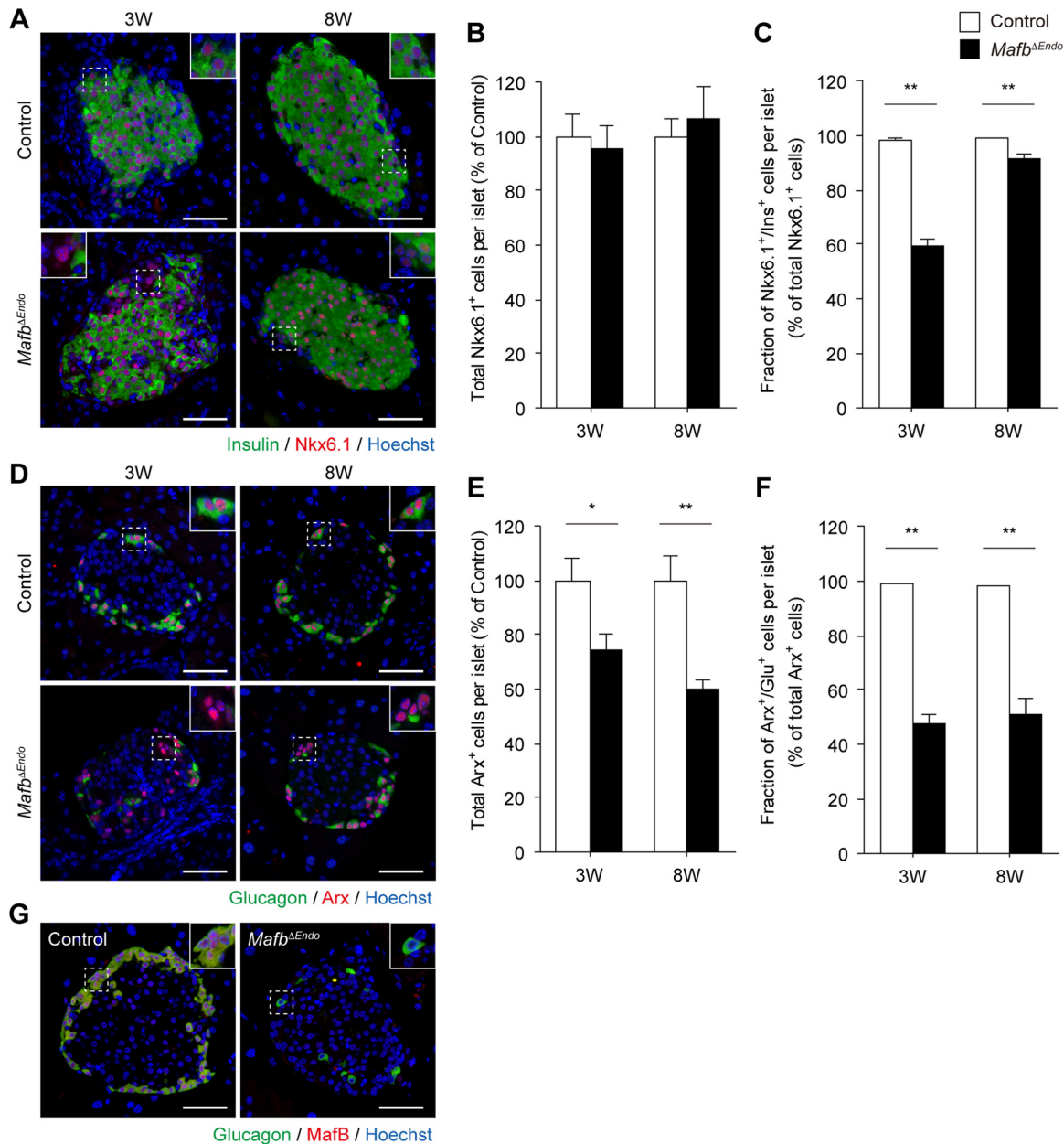


FIG 2 Endocrine cell-specific *MafB* deficiency at the embryonic stage delays insulin production in β cells but suppresses α -cell development after birth. (A) Insulin (green) and Nkx6.1 (red) immunoreactivity in *Mafb*^{ΔEndo} and control (*Mafb*^{f/f}) pancreata from 3- and 8-week-old animals. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. The insets are enlargements of the boxed areas. (B) Total Nkx6.1⁺ cells per islet normalized to age-matched controls ($n \geq 4$). (C) Fraction of Ins⁺ β cells among the total Nkx6.1⁺ cell population in *Mafb*^{ΔEndo} and control pancreata ($n \geq 4$). **, $P < 0.01$. (D) Immunofluorescence of glucagon (green) and Arx (red) in *Mafb*^{ΔEndo} and control pancreata from 3- and 8-week-old animals. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. (E) Total Arx⁺ cells per islet normalized to age-matched controls ($n \geq 4$). *, $P < 0.05$; **, $P < 0.01$. (F) Fraction of Glu⁺ α cells among the total Arx⁺ cell population in *Mafb*^{ΔEndo} and control pancreata ($n \geq 4$). **, $P < 0.01$. (G) Costaining of glucagon (green) and MafB (red) in 8-week-old *Mafb*^{ΔEndo} and control mice. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. Means and SEM are shown.

expression in adult β cells (8, 13), we did not observe any differences in the Ins⁺ cell population (Fig. 3A and B) (control versus *Mafb*^{ΔTAM}, 3 weeks post-TAM, 100% \pm 2.6% versus 96.2% \pm 2.5%; 11 weeks post-TAM, 100% \pm 1.8% versus 95.1% \pm 2.9%), and therefore, no differences in fasting blood glucose and plasma insulin levels (data not shown). In contrast, the percentage of Glu⁺ cells in *Mafb*^{ΔTAM} islets was drastically reduced at 3 weeks post-TAM injection and was further reduced by 11 weeks post-injection (Fig. 3A and C) (control versus *Mafb*^{ΔTAM}, 3 weeks post-TAM, 100% \pm 6.6%

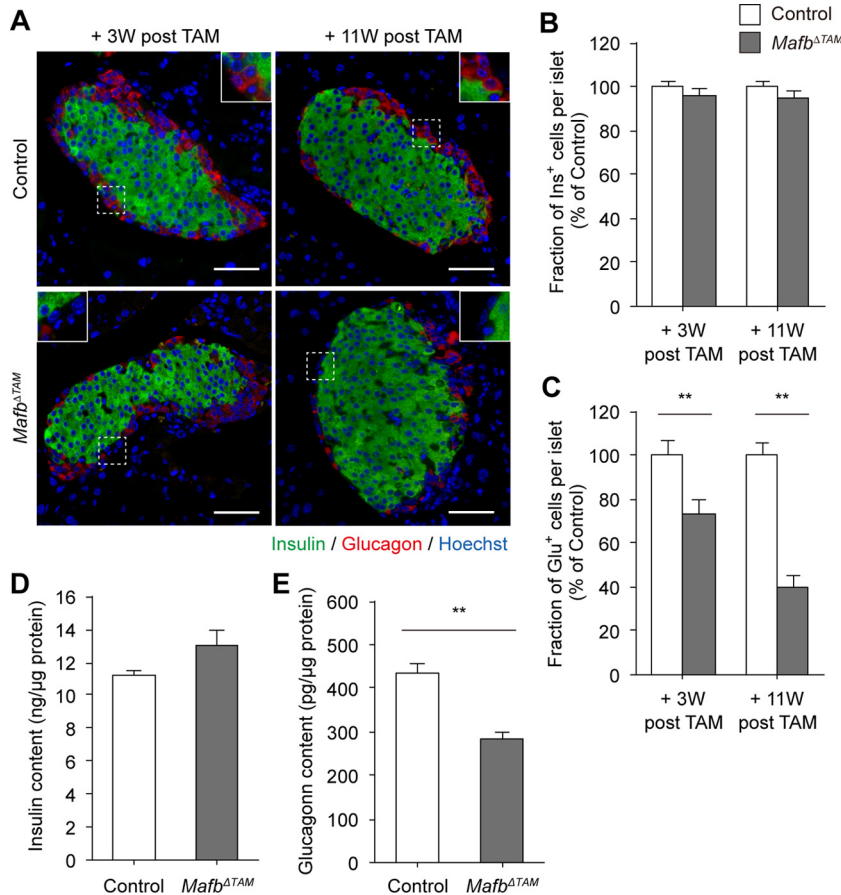


FIG 3 Loss of *Mafb* in adult mice impairs glucagon expression in mature α cells. (A) Immunostaining of insulin (green) and glucagon (red) in *Mafb*^{ΔTAM} and control (*Mafb*^{+/+}) pancreata from mice 3 and 11 weeks post-TAM injection. TAM was injected at 5 weeks of age. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. The insets are enlargements of the boxed areas. (B and C) Fraction of Ins⁺ (B) and Glu⁺ (C) cells among islets in *Mafb*^{ΔTAM} and control pancreata ($n \geq 4$). All the values were normalized to age-matched controls. **, $P < 0.01$. (D and E) Pancreatic insulin (D) and glucagon (E) contents in *Mafb*^{ΔTAM} and control pancreata from mice 3 weeks post-TAM injection ($n = 6$). Hormone contents were normalized to the total protein concentration. **, $P < 0.01$. Means and SEM are shown.

versus $73.3\% \pm 6.0\%$; 11 weeks post-TAM, $100\% \pm 6.0\%$ versus $40.0\% \pm 4.7\%$). Additionally, as anticipated, insulin content levels were not altered by *Mafb* deletion in TAM-injected *Mafb*^{ΔTAM} pancreata 3 weeks postinjection (Fig. 3D) (control versus *Mafb*^{ΔTAM}, 11.2 ± 0.4 versus 13.0 ± 0.9 ng/ μ g protein), whereas glucagon levels were approximately 65% of control levels (Fig. 3E) (control versus *Mafb*^{ΔTAM}, 437.3 ± 18.6 versus 283.8 ± 17.6 pg/ μ g protein). Taken together, these results indicate that *Mafb* deficiency in adult mice alters hormone expression in α cells but not in β cells, demonstrating that sustained MafB function is required for normal glucagon production by α cells from postnatal development to maturity.

We further investigated whether *Mafb* ablation in adult mice influences islet cell identity. At 3 and 11 weeks post-TAM injection, the total Nkx6.1⁺ cell population in *Mafb*^{ΔTAM} pancreata did not differ from that in the control (Fig. 4A and B) (control versus *Mafb*^{ΔTAM}, 3 weeks post-TAM, $100\% \pm 10.9\%$ versus $104.5\% \pm 7.9\%$; 11 weeks post-TAM, $100\% \pm 8.4\%$ versus $96.4\% \pm 5.7\%$). In addition, almost all Nkx6.1⁺ cells expressed insulin, albeit at slightly lower levels in *Mafb*^{ΔTAM} pancreata than in the controls (Fig. 4A and C) (control versus *Mafb*^{ΔTAM}, 3 weeks post-TAM, $98.8\% \pm 0.4\%$ versus $97.2\% \pm 0.5\%$; 11 weeks post-TAM, $98.4\% \pm 0.4\%$ versus $96.4\% \pm 0.5\%$). These results suggest that MafB has no significant effects on mature β cells. Conversely, although the total Arx⁺ cell population was similar to that of the control (Fig. 4D and E) (control

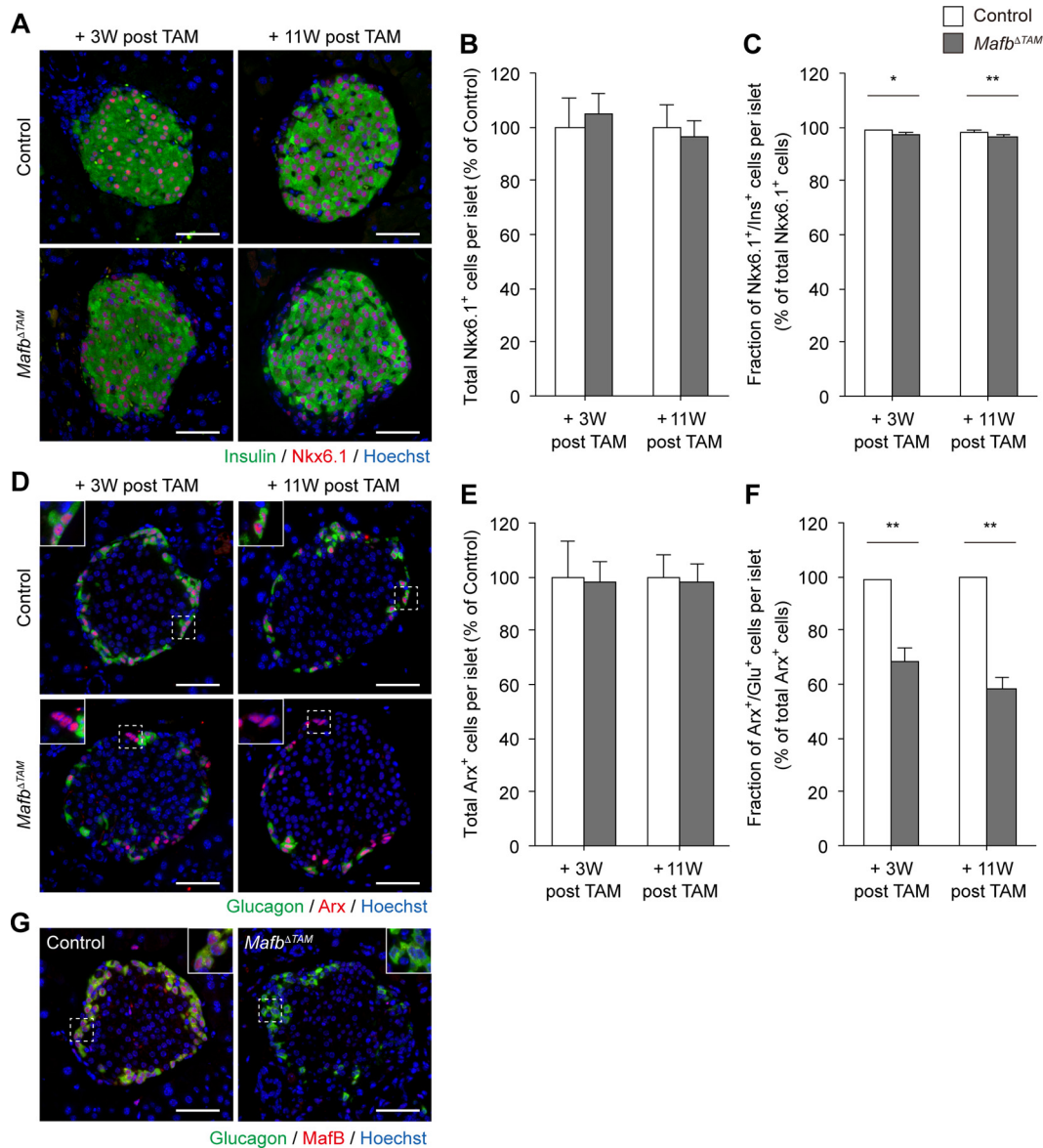


FIG 4 Adult onset ablation of *Mafb* does not affect α -cell identity. (A) Insulin (green) and Nkx6.1 (red) immunoreactivity in *Mafb*^{ΔTAM} and control (*Mafb*^{f/f}) pancreata from mice 3 and 11 weeks post-TAM injection. TAM was injected at 5 weeks of age. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. (B) Total Nkx6.1⁺ cells per islet normalized to age-matched controls ($n \geq 4$). (C) Fraction of Ins⁺ β cells among the total Nkx6.1⁺ cell population in *Mafb*^{ΔTAM} and control pancreata ($n \geq 4$). *, $P < 0.05$; **, $P < 0.01$. (D) Immunofluorescence of glucagon (green) and Arx (red) in *Mafb*^{ΔTAM} and control pancreata from mice 3 and 11 weeks post-TAM injection. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. The insets are enlargements of the boxed areas. (E) Total Arx⁺ cells per islet normalized to age-matched controls ($n \geq 4$). (F) Fraction of Glu⁺ α cells among the total Arx⁺ cell population in *Mafb*^{ΔTAM} and control pancreata ($n \geq 4$). **, $P < 0.01$. (G) Costaining of glucagon (green) and MafB (red) in *Mafb*^{ΔTAM} and control mice 3 weeks post-TAM injection. Nuclei were stained with Hoechst 33342. Scale bars, 50 μ m. Means and SEM are shown.

versus *Mafb*^{ΔTAM}, 3 weeks post-TAM, 100% \pm 13.1% versus 98.1% \pm 7.4%; 11 weeks post-TAM, 100% \pm 8.1% versus 98.4% \pm 6.6%), a marked decrease in the Glu⁺ α -cell population was noted (Fig. 4D and F) (control versus *Mafb*^{ΔTAM}, 3 weeks post-TAM, 98.6% \pm 0.4% versus 68.8% \pm 4.4%; 11 weeks post-TAM, 99.4% \pm 0.3% versus 58.3% \pm 4.1%). This reduced proportion of Glu⁺ α cells was not caused by α -cell apoptosis, as confirmed by negative signals in the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (see Fig. S3A in the supplemental material). Furthermore, the total islet cell number in *Mafb*^{ΔTAM} pancreata remained unchanged (see Fig. S3B in the supplemental material), as was observed in *Mafb*^{ΔEndo} mice.

However, a minimum level of glucagon expression was also observed even in the absence of MafB in *Mafb*^{ΔTAM} pancreata (Fig. 4G), consistent with that in *Mafb*^{ΔEndo} mice, which suggests the presence of a MafB-independent pathway for glucagon production. Overall, our results indicate that MafB is critical for maintaining α -cell function in adult mice, confirming the principal role of MafB in glucagon expression.

MafB expression during embryonic development favors α -cell lineage commitment by suppressing F-cell differentiation. Because, MafB affects the development of both α and β cells, whose precursors are shared with F and δ cells, respectively, we determined to examine MafB's involvement in the other islet cell types. We first performed immunostaining of somatostatin (Som)-producing δ cells and pancreatic polypeptide (PP)-producing F cells to verify any changes in the cell population. In both 3- and 8-week-old *Mafb*^{ΔEndo} pancreata, Som⁺ cell populations remained unaffected (see Fig. S4A and B in the supplemental material). However, the PP⁺ cell population was increased approximately 3.5-fold in comparison to the control at both ages (Fig. 5A and B) (control versus *Mafb*^{ΔEndo}, 3 weeks, 100% \pm 16.6% versus 387.7% \pm 45.9%; 8 weeks, 100% \pm 26.4% versus 352.1% \pm 39.2%). Because F cells share a common precursor with α cells, to investigate whether the increased PP⁺ cell population comes from cell fate change induced by *Mafb* deletion in α cells, we conducted immunohistochemistry by costaining with glucagon/PP or Arx/PP antibodies. Surprisingly, the majority of PP expression did not merge with glucagon signal (Fig. 5C) but consistently merged with Arx expression regardless of genotypes (Fig. 5D), suggesting that PP⁺ cells are mainly generated from Arx⁺ Glu⁻ cells. Although PP⁺ Glu⁺ double-positive cells were rarely observed, as previously reported (16), this subpopulation remained unaltered in both groups, excluded from the source of the Arx⁺ PP⁺ cell population. More strikingly, the significant 3.5-fold increase in the PP⁺ cell population was equivalent to the decrease in the Glu⁺ cell fraction (Fig. 1C), suggesting that the diminished glucagon-producing cell population could be compensated for by an increased PP-producing cell population. These differences (approximately 3.5-fold change) were consistently observed in both 3- and 8-week-old *Mafb*^{ΔEndo} mice, whereas *Mafb*^{ΔTAM} mice 11 weeks post-TAM injection had no change in any cell population, including δ cells (data not shown) and F cells (Fig. 5E and F) (control versus *Mafb*^{ΔEndo}, 100% \pm 12.5% versus 105.0% \pm 9.9%). Given that the total islet cell numbers did not change (see Fig. S1A in the supplemental material), increased numbers of PP⁺ cells could have differentiated from Arx⁺ Glu⁻ precursor cells. In sum, these results indicate that the population change is probably not attributable to cell fate conversion but rather to alterations of cell fate specification during the developmental stage, suggesting the importance of MafB in α -cell lineage specification due to suppressing F-cell fate differentiation (Fig. 5G).

MafB is a key regulator of glucagon gene expression. To explore MafB-regulated genes involved in α - and β -cell differentiation, as well as hormone production and secretion, we performed real-time quantitative PCR (qPCR) on pancreatic islet cDNA. Islets were isolated from 10-week-old *Mafb*^{ΔEndo} mice and from TAM-injected *Mafb*^{ΔTAM} mice at 3 weeks postinjection. The mRNA expression analysis revealed the successful deletion of *Mafb* in both mutant models (Fig. 6A and B) and the consequent decrease in glucagon gene (*Gcg*) expression (Fig. 6C and D). *Mafb*^{ΔEndo} islets exhibited a marked decrease in *Arx* transcription that was equivalent to the reduced number of Arx⁺ cells observed via immunohistochemistry (Fig. 2E), while a moderate decrease in the expression of *Brn4*, another gene that is highly enriched in α cells (α -cell-enriched gene) (13), was also noted (Fig. 6C). In addition, the expression of β -cell-enriched genes (i.e., *Ins2*, *Pdx1*, and *Nkx6.1*) (13) was significantly lower in *Mafb*^{ΔEndo} islets (Fig. 6C), which might explain the partial recovery of insulin production indicated by the slight reduction in the Ins⁺ cell population and insulin content (Fig. 1B and D). Thus, these results support our conclusion that *Mafb* deficiency during embryogenesis suppresses α -cell development and glucagon expression but delays β -cell terminal differentiation. Moreover, the increase in the PP⁺ cell population in *Mafb*^{ΔEndo} mice was further supported

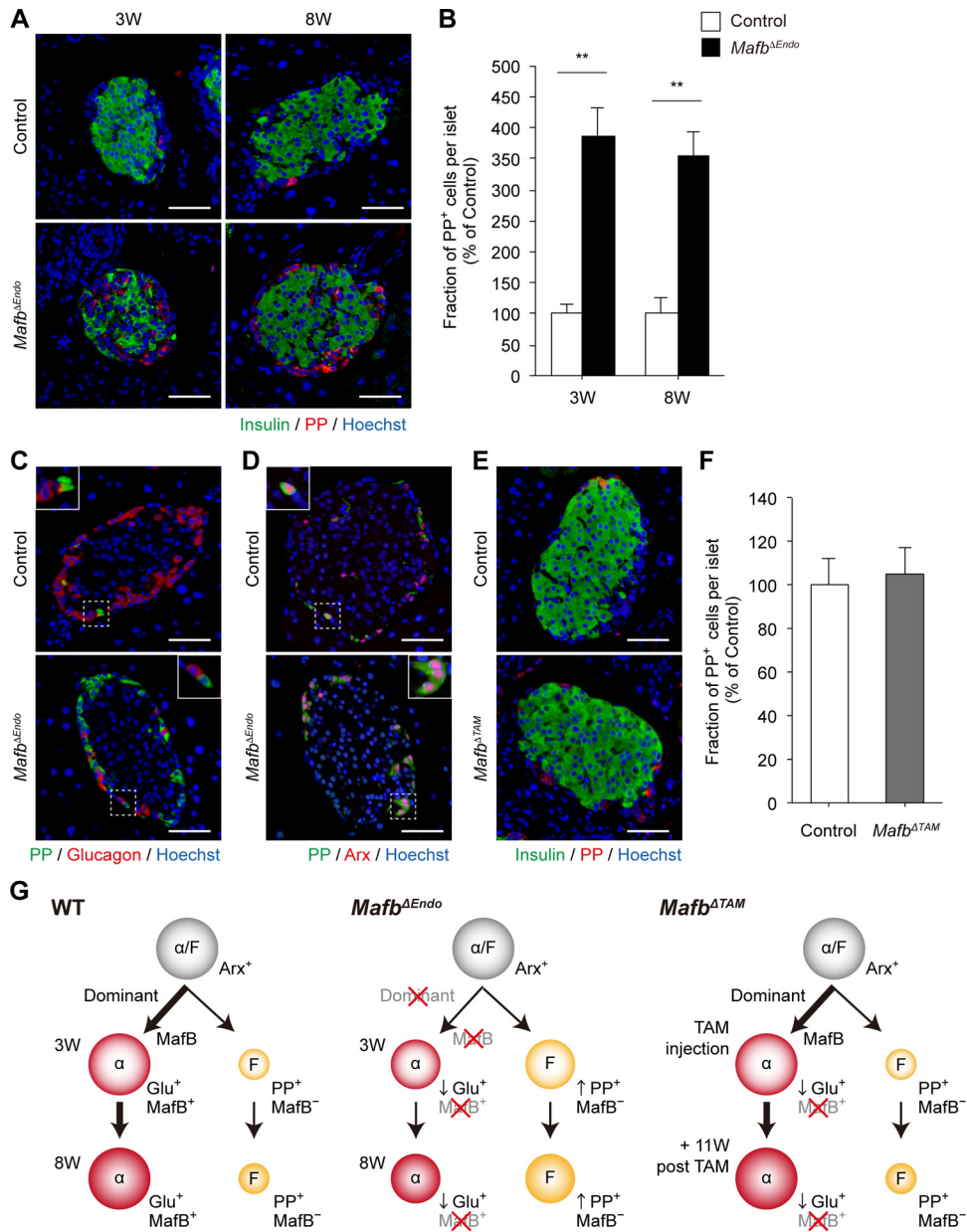


FIG 5 The PP⁺ cell population is increased in *Mafb*^{ΔEndo} pancreata. (A) Immunostaining of insulin (green) and PP (red) in *Mafb*^{ΔEndo} and control (*Mafb*^{f/f}) pancreata from 3- and 8-week-old animals. Nuclei were stained with Hoechst 33342. Scale bars, 50 μm. (B) Fraction of PP⁺ cells within islets in *Mafb*^{ΔEndo} and control pancreata (n ≥ 4). **, P < 0.01. (C and D) Investigation of cell fate conversion by costaining PP (green) with glucagon (C) (red) or Arx (D) antibodies in *Mafb*^{ΔEndo} and control pancreata from 8-week-old animals. Nuclei were stained with Hoechst 33342. Scale bars, 50 μm. The insets are enlargements of the boxed areas. (E) Insulin (green) and PP (red) immunoreactivity in *Mafb*^{ΔTAM} and control pancreata from mice 11 weeks post-TAM injection. TAM was injected at 5 weeks of age. Nuclei were stained with Hoechst 33342. Scale bars, 50 μm. (F) Fraction of PP⁺ cells within islets in *Mafb*^{ΔTAM} and control pancreata (n ≥ 4). (G) Model of α-cell and F-cell lineage specification from α/F precursor cells in wild-type (WT), *Mafb*^{ΔEndo}, and *Mafb*^{ΔTAM} mutants. Means and SEM are shown.

by an upregulated *Ppy* mRNA level in the islets (Fig. 6C). Interestingly, *Mafb*^{ΔEndo} islets also showed decreased expression of the ghrelin gene (*Ghrl*) (Fig. 6C). Given that some ghrelin⁺ cells are reported to express MafB in wild-type embryos and the neonatal mouse pancreas (10, 17), *Mafb* depletion at the embryonic stage may have affected ghrelin⁺ cell subpopulation development. Conversely, *Mafb*^{ΔTAM} islets did not display any differences in the expression of α- and β-cell-enriched genes (i.e., *Arx*, *Brn4*, *Ins2*, *Pdx1*, and *Nkx6.1*) or any other islet hormone genes (i.e., *Sst*, *Ppy*, and *Ghrl*) (Fig. 6D) (13),

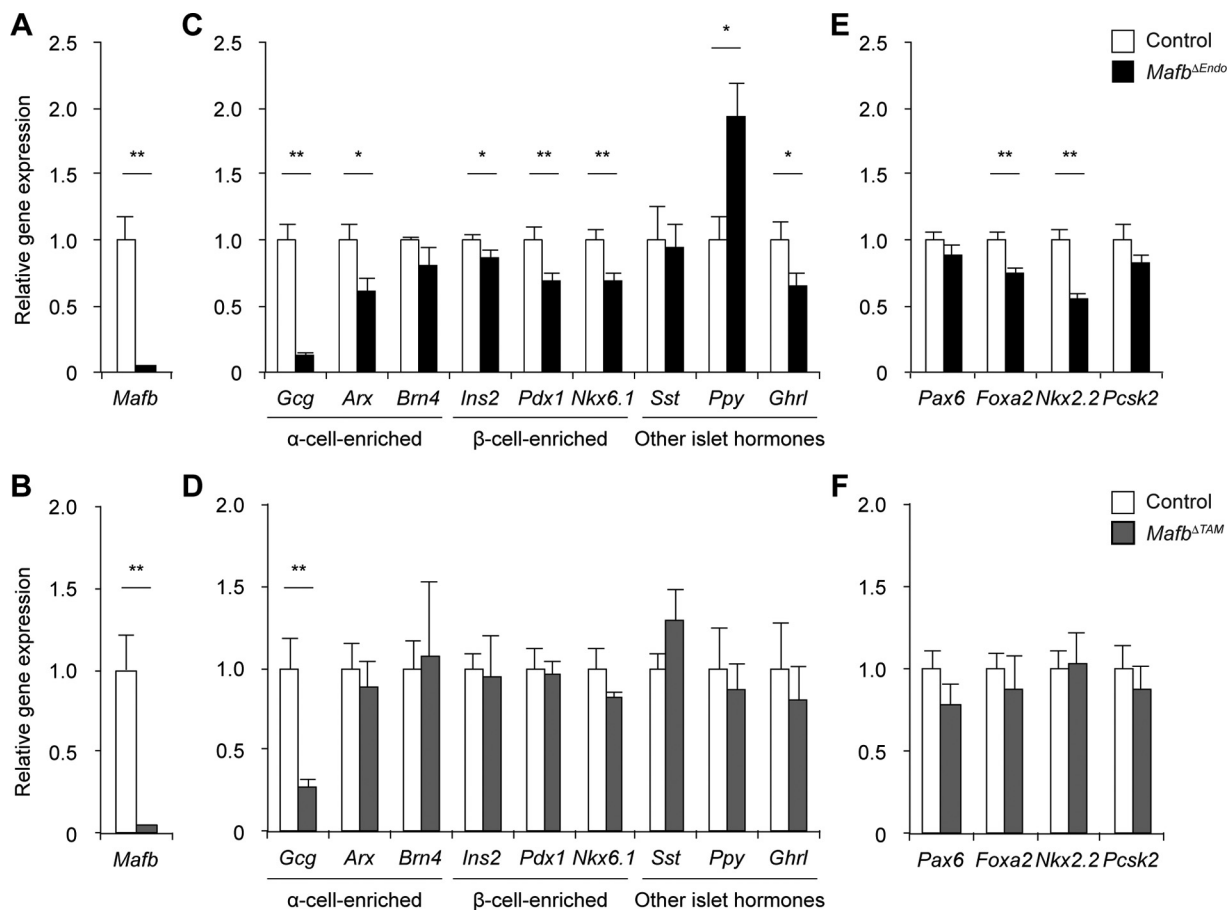


FIG 6 *MafB* is a key regulator of glucagon gene expression. Gene expression comparisons were performed by qPCR in pancreatic islets isolated from 10-week-old *Mafb*^{ΔEndo} (A, C, and E) and *Mafb*^{ΔTAM} (B, D, and F) mice (*n* ≥ 5) 3 weeks post-TAM injection. TAM was injected at 5 weeks of age. The *Mafb* gene (A and B), islet hormone and cell identity genes (C and D), and glucagon production/ α -cell differentiation regulatory genes (E and F) were analyzed. The primers are listed in Table 1. *, *P* < 0.05; **, *P* < 0.01. Means and SEM are shown.

consistent with the immunostaining results (Fig. 3B, 4B and E, and 5F). These data confirm our finding that *Mafb* ablation in adult mice impairs glucagon expression in α cells but does not affect other islet cells.

In light of previous work showing the complete recovery of glucagon expression upon *Mafb* deletion (10), we wanted to address a potential compensatory mechanism of glucagon production in the absence of *Mafb*. To this end, we further inspected gene expression levels of other transcription factors known to regulate glucagon and/or α -cell differentiation, including *Pax6* (18), *Foxa2* (19, 20), *Nkx2.2* (21), and the peptide-processing gene *Pcsk2* (22). Two of four glucagon/ α -cell regulatory genes (i.e., *Foxa2* and *Nkx2.2*) were significantly reduced in *Mafb*^{ΔEndo} islets, whereas the other two genes (i.e., *Pax6* and *Pcsk2*) exhibited a trend toward reduced expression compared with controls (Fig. 6E). This finding suggests that *Mafb* deletion in developing endocrine cells leads to the downregulation of other glucagon/ α -cell regulators, which in turn exacerbates glucagon reduction. Nevertheless, we cannot exclude the possibility of an effect of β cells, as these factors are also highly conserved in β cells (13) and because *Mafb*^{ΔEndo} islets showed a decreased trend of β -cell-enriched gene expression (Fig. 6C). However, in *Mafb*^{ΔTAM} islets, none of the glucagon/ α -cell regulator genes were affected (Fig. 6F), suggesting that *MafB* in adult mice is the major controller of α -cell functional maintenance independently of other glucagon/ α -cell regulators, such as *Pax6*, *Foxa2*, *Nkx2.2*, and *Pcsk2*. If these unaffected genes represented α -cell populations, then this could explain the residual glucagon production in *Mafb*-deficient (*Mafb*^{ΔEndo} and *Mafb*^{ΔTAM}) animals (Fig. 1E and 3E).

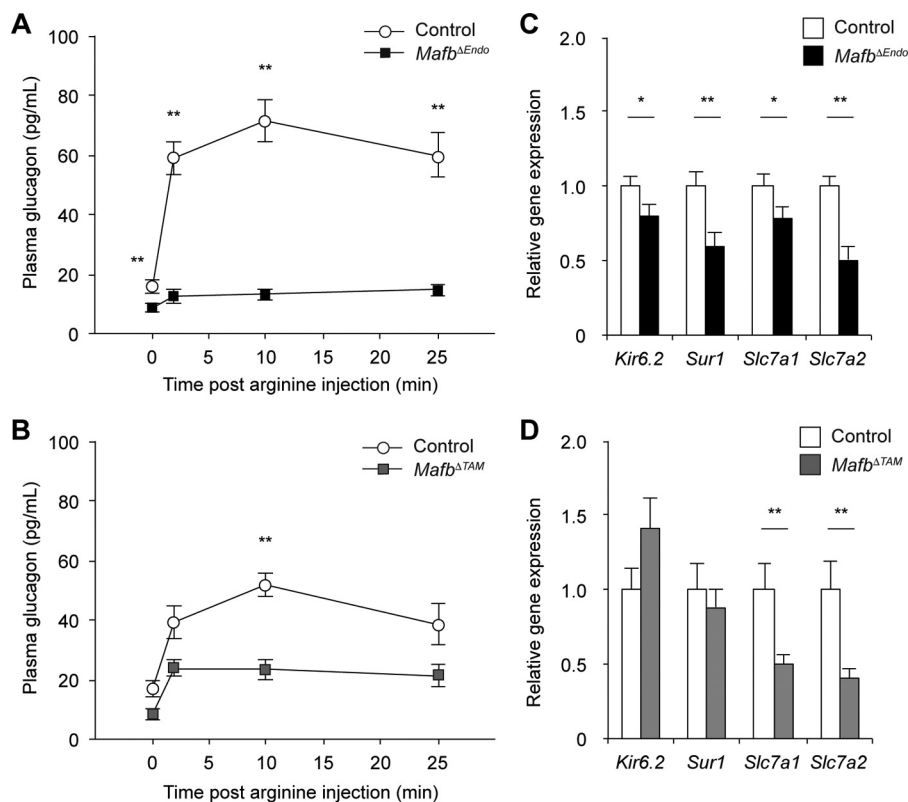


FIG 7 *Mafb* deletion impairs glucagon secretion upon α -cell stimulation. (A and B) Glucagon secretion was measured after intraperitoneal injection of L-arginine (1 mg/g) in 8-week-old *Mafb*^{ΔEndo} ($n \geq 8$) (A) and *Mafb*^{ΔTAM} (B) male mice ($n \geq 4$) 3 weeks post-TAM administration. TAM was injected at 5 weeks of age. **, $P < 0.01$. (C and D) qPCR analysis of glucagon secretion machinery gene expression was performed on pancreatic islets isolated from 10-week-old *Mafb*^{ΔEndo} (C) and *Mafb*^{ΔTAM} (D) mice ($n \geq 5$) 3 weeks post-TAM injection. *, $P < 0.05$; **, $P < 0.01$. Means and SEM are shown.

***Mafb* deletion disrupts glucagon secretion in response to α -cell stimuli.** To address the functional competence of *Mafb*-deficient α cells, we analyzed glucagon secretion in response to amino acid stimulation (23) in both groups of mutant mice. Eight-week-old *Mafb*^{ΔEndo} and *Mafb*^{ΔTAM} male mice at 3 weeks post-TAM treatment were injected with 1 mg/g L-arginine intraperitoneally after overnight fasting (Fig. 7A and B). In the basal state, plasma glucagon levels in both mutant groups were significantly lower than in control groups (Fig. 7A and B) (control versus *Mafb*^{ΔEndo}, 15.7 ± 2.1 versus 8.6 ± 1.3 pg/ml; control versus *Mafb*^{ΔTAM}, 17.0 ± 2.8 versus 8.3 ± 1.8 pg/ml). These results are presumed to reflect the constantly observed reduced glucagon production phenotype and suggest the importance of MafB in maintaining the basal glucagon production/secretion. After arginine stimulation, a rapid increase in plasma glucagon levels was observed within 2 min and peaked at 10 min after arginine administration in all the control groups (Fig. 7A and B). However, both groups of *Mafb*-deficient mice failed to secrete glucagon to the control level, although *Mafb*^{ΔTAM} mice exhibited slightly higher levels (Fig. 7A and B). Moreover, plasma glucagon peaked at 2 min after arginine injection in both mutants and remained unaltered (Fig. 7A and B), suggesting possible defects in the cellular machinery that regulates glucagon secretion. To test whether arginine uptake via cationic amino acid transporters and/or the glucagon-secreting machinery was affected, real-time qPCR was performed on isolated pancreatic islets from 10-week-old *Mafb*^{ΔEndo} and *Mafb*^{ΔTAM} mice 3 weeks post-TAM injection (Fig. 7C and D). Since β cells were persistently affected by *Mafb* ablation in *Mafb*^{ΔEndo} mice, both cationic amino acid transporter genes (i.e., *Slc7a1* and *Slc7a2*) and ATP-sensitive potassium (K^{ATP}) channel subunit genes (i.e., *Kir6.2* and *Sur1*) involved in glucagon secretion (23) were downregulated (Fig. 7C). However, *Mafb*^{ΔTAM}

islets exhibited significant reductions in *Slc7a1* and *Slc7a2* but not *Kir6.2* and *Sur1* (Fig. 7D). Therefore, impaired arginine-stimulated glucagon secretion likely reflects reduced cationic amino acid transporter gene expression in both mutants and can be partially attributed to reduced K^{ATP} channel subunits in *Mafb* ^{Δ Endo} mice. Overall, these data suggest that *Mafb* ablation leads to insufficient glucagon secretion under basal and stimulated conditions, supporting the role of MafB in α -cell development and function.

DISCUSSION

In the present study, we demonstrate that MafB regulates glucagon production and secretion in postnatal α cells *in vivo* by using two different mouse models: endocrine cell-specific (*Mafb* ^{Δ Endo}) and TAM-dependent (*Mafb* ^{Δ TAM}) *Mafb* knockout mice. Both *Mafb* ^{Δ Endo} and *Mafb* ^{Δ TAM} mice exhibited decreased populations of Glu⁺ α cells (Fig. 1C and 3C) and, consequently, reduced glucagon production (Fig. 1E and 3E) and secretion (Fig. 7A and B). Although α -cell fate was not changed, embryonic *Mafb* deletion further reduced the Arx⁺ cell population (Fig. 2E) and increased the PP⁺ cell population to compensate for decreased α -cell differentiation (Fig. 5B). Moreover, both groups of mutant mice failed to respond to arginine (Fig. 7A and B), potentially due to the compromised expression of cationic amino acid transporter genes involved in arginine uptake (Fig. 7C and D). Therefore, our findings clearly demonstrate the contribution of MafB to α -cell development at the neonatal stage and to the maintenance of α -cell function during adulthood *in vivo*.

Consistent with previous reports (10, 11), *Mafb* ablation only delayed β -cell development, given that MafB is not expressed in postnatal β cells (8, 13). However, our observations in α cells consistently disagreed with the prior work of Conrad et al. (10). Pancreas-specific *Mafb* conditional-knockout (*Mafb*^{*fl/fl*}::*Pdx1*-Cre *Mafb* ^{Δ panc}) mice recover Glu⁺ α cells by 2 weeks of age and islet glucagon content by 8 weeks of age (10). This discrepancy may reflect the use of mice with different genetic backgrounds in the present study, whereas the difference in the Cre drivers used is unlikely to have contributed, because we also generated a *Mafb* conditional-knockout mouse with *Pdx1*-Cre (24), which exhibited the same phenotype as *Mafb* ^{Δ Endo} mice (data not shown). Indeed, the *Mafb*^{*fl/fl*} mice used in the present study (25, 26) and by Conrad et al. (10, 27) were generated in mouse embryonic stem (ES) cells from different genetic backgrounds, e.g., C57BL/6J and 129S4/SvJae, which may explain the phenotypic variations.

Nevertheless, the recovery of α -cell numbers and glucagon content in *Mafb* ^{Δ panc} mice (10) led us to explore the secondary effects on glucagon production in the absence of *Mafb*, which indeed was constantly observed in both groups of mutant mice (Fig. 2G and 4G). Various transcription factors have been reported to regulate glucagon expression in α cells, including MafB (9), c-Maf (28), Pax6 (18), Foxa1 and Foxa2 (19, 20), NeuroD1 (29), Isl1 (30), and Brn4 (31). However, MafB and Brn4 are the only two factors known to be α cell specific, whereas the genes encoding the other factors are expressed in both α and β cells (13). Interestingly, a previous study in *Brn4*-null mice observed no significant impact on glucagon gene expression, synthesis, and secretion, suggesting that Brn4 is dispensable for glucagon regulation (32). Conversely, the relationship between MafB and glucagon expression has been clearly addressed (33, 34). For example, either overexpression of *Arx* in pancreatic progenitor cells or deletion of *Pdx1* in β cells leads to an increase in the α -cell population, which is frequently associated with MafB and glucagon expression (33, 34). This finding raises the possibility that MafB dominates the regulation of α -cell activity, including glucagon production and secretion, although a minimal level of glucagon is maintained through a MafB-independent pathway. In the *Mafb* ^{Δ TAM} islet gene analysis, *Pax6*, *Foxa2*, *Nkx2.2*, and *Pcsk2* mRNA levels remained unchanged, with no indication of rescuing depleted glucagon gene expression (Fig. 6F). Thus, *Mafb* ablation is exclusively responsible for glucagon reduction, supporting our hypothesis that MafB is the principal transcriptional activator of the glucagon gene in α cells.

Interestingly, the population of PP⁺ cells was increased at the cost of decreasing the

population of α cells. At early pancreas morphogenesis, α /F precursor cells (Arx^+) preferentially differentiate toward the α -cell lineage, while a small number of cells are designated for a F-cell fate (15, 33). However, in *Mafb* ^{Δ Endo} mice, a significant population of Arx^+ Glu^- cells are differentiated into PP-producing cells at 3 weeks of age, and their fate is maintained throughout the developmental stage until 8 weeks of age (Fig. 5A to D). These results suggest that α /F precursor cells might have been favored to promote F-cell lineage differentiation in the absence of MafB, indicating a potential role of MafB in the early α -cell fate decision during islet development by inhibiting F-cell differentiation (Fig. 5G).

Although α -cell dysfunction is specifically addressed in the work of Conrad et al., the specific glucagon-secretory machinery affected by *Mafb* ablation was not identified (10). In both of our groups of mutant mice, cationic amino acid transporter genes (i.e., *Slc7a1* and *Slc7a2*) were significantly decreased (Fig. 7C and D), suggesting that impaired arginine-stimulated glucagon secretion is likely caused by reduced arginine transporter gene expression. Based on the text-mining application from SABiosciences and the UCSC Genome Browser, *Foxa2* is predicted to target these transporter genes. However, *Foxa2* expression was not reduced in our qPCR analysis results (Fig. 6F), indicating that the reduction in *Slc7a1* and *Slc7a2* expression is independent of *Foxa2* and suggesting an alternative mechanism for the regulation of cationic amino acid transporter gene expression.

Understanding α -cell functional activity will not only increase our knowledge of islet physiology but will also have translational implications, given that the regulation of glucagon secretion and action has been shown to ameliorate diabetes symptoms (35, 36). Extremely high plasma glucagon concentrations are observed in insulin-deficient states, such as type 1 diabetes, advanced type 2 diabetes, and diabetic ketoacidosis (2, 37). Therefore, inhibiting glucagon signaling may potentially reduce diabetic hyperglycemia, as demonstrated in animal studies. For example, mice with either a disrupted glucagon receptor (38, 39) or defective glucagon synthesis (40, 41) tend to display reduced fasting blood glucose levels and improved glucose tolerance compared with control mice, even without treatment. Furthermore, glucagon receptor knockout mice are resistant to diabetes upon streptozotocin (STZ)-induced β -cell destruction (42). In addition, recent work using a peptide-based glucagon receptor antagonist reported improved metabolic control of genetically or directly induced obesity-related diabetes in mice (43). Therefore, our discovery may potentiate glucagon regulation in diabetes through *Mafb* gene regulation.

Unexpectedly, *Mafb* ^{Δ Endo} β cells exhibited residual effects, which were supposedly caused by delayed β -cell terminal differentiation, with β -cell populations, insulin content, and β -cell transcript levels all minimally but consistently reduced (Fig. 1B and D and 6C). These results potentially highlight the supporting role of MafB in postnatal β -cell development. A recent study by van der Meulen et al. introduced the novel concept of a "virgin β -cell" subpopulation that contains functionally immature β cells characterized as Ins^+ $MafB^+$ Ucn^- (44). This minor virgin β -cell subpopulation can transdifferentiate into functional β cells and is found in both mouse and human islets (44). Therefore, the authors concluded that MafB-expressing virgin β cells may be the source for β -cell regeneration through transdifferentiation in adult islets (44). Moreover, another study by Cheng et al. demonstrated β -cell regeneration from a novel mesenchymal cell population after nearly total ablation of preexisting β cells by a high dose of STZ in adult rodents (45). Interestingly, these newly formed β cells expressed MafB and vimentin but not Pdx1 and Nkx6.1 and were thus immature. However, the cells gradually acquired functional maturity and became bona fide β cells (45). These findings suggest that β -cell regeneration is preceded by MafB expression in β cells, which is lost upon functional maturity. Thus, the role of MafB in facilitating β -cell development cannot be neglected.

In contrast to mice, MAFB expression in human β cells is retained postnatally (13, 46), thereby implicating human MAFB in β -cell maintenance and/or activity. Notably, severe reductions in MAFB levels are observed in human type 2 diabetic islets, sug-

gesting that β -cell failure is associated with low MAFB expression (47). In addition, *in vitro* work using a human β -cell line (EndoC- β H1) also demonstrated that MAFB is required for glucose-stimulated insulin secretion in human β cells, indicating that mature human β cells require MAFB for their functional activity (48). However, clinical reports of patients carrying MAFB mutations did not include their blood glucose levels or HbA1c status (49–51), creating the impression that these patients are normoglycemic. Further investigation will be required to clarify the role of MAFB in human β cells.

In conclusion, our findings indicate that MafB is critical for glucagon production in α cells to maintain development and function. Therefore, this study yields insights into islet physiology in glucagon regulation through MafB in α cells, thereby providing a platform for understanding glucagon control under pathophysiological conditions.

MATERIALS AND METHODS

Animals. Mice were maintained under specific-pathogen-free conditions in the Laboratory Animal Resource Center at the University of Tsukuba, Ibaraki, Japan. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba. Endocrine cell-specific and TAM-dependent MafB knockout mice were generated by crossing MafB^{fl/fl} mice (25, 26) with either Ngn3-Cre (52) or CAGG-CreER (stock number 004682; The Jackson Laboratory) (53) transgenic mice. MafB^{fl/fl} mice were generated in a C57BL/6J strain background, as described previously (25, 26). The Ngn3-Cre transgenic mouse strain was kindly provided by Shosei Yoshida (Division of Germ Cell Biology, National Institute for Basic Biology, Japan). In this study, MafB^{fl/fl}::Ngn3-Cre and MafB^{fl/fl}::CAGG-CreER mice are referred to as MafB Δ Endo and MafB Δ TAM mice, respectively. MafB^{fl/fl} littermates were used as controls. To activate the Cre recombination system in the CAGG-CreER strain, 5-week-old MafB Δ TAM mice and their control groups were injected intraperitoneally with 75 mg of TAM/kg for 5 consecutive days (54). TAM (Sigma) was first dissolved in ethanol and then mixed with corn oil as described previously (55).

Immunohistochemistry. Pancreatic tissues were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C, processed, and embedded in paraffin. Then, 2- μ m sections were sliced and prepared according to standard methods. For nuclear protein staining, sections were soaked in 0.3% Triton X-100–PBS solution, followed by heat-induced epitope retrieval using target retrieval solution (Dako) and a pressure cooker. All the sections were blocked in appropriate sera for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: guinea pig anti-insulin (1:500; Abcam), rabbit antiglucagon (1:2,000; 2760; Cell Signaling), guinea pig anti-glucagon (1:1,000; M182; TaKaRa), rabbit anti-Arx (1:250; a generous gift from Kunio Kitamura and Kenichirou Morohashi, Kyushu University, Japan) (56), mouse anti-Nkx6.1 (1:250; F55A10; Developmental Studies Hybridoma Bank), rabbit anti-MafB (1:100; IHC-00351; Bethyl), rabbit anti-pancreatic polypeptide (1:1,000; ab113694; Abcam), goat anti-pancreatic polypeptide (1:50; ab77192; Abcam), and rabbit antisomatostatin (1:50; 18-007; Zymed). Antigens were visualized using appropriate secondary antibodies conjugated to Alexa Fluor 488 or 594 (1:1,000; Life Technologies), and nuclei were labeled with Hoechst 33342 (Molecular Probes). Tissue specimens were mounted with Fluoromount (Diagnostic BioSystems). All images were acquired on a fluorescence microscope (Bioevo BZ-9000; Keyence).

Cell counting. Following immunofluorescence staining, the different cell types in each islet were manually counted in the islet microscopy images. For each cell type, 20 to 40 representative islets from 3 to 6 mice per group were counted. To calculate the fraction of hormone⁺ cells within islets, the number of hormone⁺ cells per islet was manually determined using ImageJ software and divided by the total number of Hoechst⁺ nuclei from the same islet (percent hormone⁺ cells/islet Hoechst⁺ nuclei) and then normalized to the control group. The fractions of Nkx6.1⁺ Ins⁺ and Arx⁺ Glu⁺ cells were determined by dividing the number of double-positive cells per islet by the total numbers of Nkx6.1⁺ (Nkx6.1⁺ Ins⁻ and Nkx6.1⁺ Ins⁺) and Arx⁺ (Arx⁺ Glu⁻ and Arx⁺ Glu⁺) cells, respectively. Total Nkx6.1⁺ and Arx⁺ cell counts were normalized to their corresponding controls.

Measurement of pancreatic insulin and glucagon contents. Whole pancreata were collected from 3- and 8-week-old MafB Δ Endo and MafB Δ TAM mice 3 weeks post-TAM injection and homogenized in ice-cold acetic-ethanol buffer (1.5% HCl in 75% ethanol) as previously described (57). Total insulin and glucagon contents in pancreatic tissue extracts were measured via enzyme-linked immunosorbent assay (ELISA) (Morinaga Mouse Insulin ELISA kit, M1102; Mercodia Glucagon 10 μ l ELISA kit, 10-1281-01) according to the manufacturer's instructions. Each pancreatic content was normalized by the total protein concentration per sample, which was determined with Bradford reagent (Thermo Fisher Scientific).

Isolation of pancreatic islets. Pancreatic islets were isolated from 10-week-old MafB Δ Endo and MafB Δ TAM mice 3 weeks post-TAM injection as described previously, with slight modifications (58). Briefly, after clamping the common bile duct at the intestine, the pancreas was inflated with 1 mg/ml collagenase type V (Wako) diluted in Krebs-Ringer bicarbonate (KRBH) buffer (129.4 mM NaCl, 5.2 mM KCl, 1.3 mM KH₂PO₄, 1.3 mM MgSO₄, 2.7 mM CaCl₂, 24.8 mM NaHCO₃, 10 mM HEPES, pH 7.4). The distended pancreas was transferred into a collecting tube with additional fresh collagenase solution and then incubated at 37°C for 20 min with gentle shaking. After the second wash with ice-cold KRBH buffer containing 0.5% bovine serum albumin (BSA), islets were hand picked under a stereomicroscope.

qPCR. Total RNA was extracted in Isogen (Nippon Gene) from pancreatic islets isolated from 10-week-old MafB Δ Endo and MafB Δ TAM mice 3 weeks post-TAM injection. cDNA was synthesized according

TABLE 1 Primer sequences for real-time qPCR

Gene	Primer sequence (5'→3')	
	Forward	Reverse
<i>Hprt</i>	TTGTTGTTGGATATGCCCTTGACTA	AGGCAGATGGCCACAGGACTA
<i>Mafb</i>	TGAATTTGCTGGCACTGCTG	AAGCACCATGCGGTTTCATACA
<i>Gcg</i>	AGGGACCTTTACCAGTGATGT	AATGGCGACTTCTTCTGGGAA
<i>Arx</i>	TCCGGATACCCCACTTAGCTT	GACGCCCTTTCTTTAAGTG
<i>Brn4</i>	CTCGCCGCACACTAACCAT	GCTCCAGCATAACCGCTCAC
<i>Ins2</i>	GCTTCTTCTACACACCCATGTC	AGCACTGATCTACAATGCCAC
<i>Pdx1</i>	TTCCCGAATGGAACCGAGC	GTAGGCAGTACGGGTCCTCT
<i>Nkx6.1</i>	CAGACCCACGTTCTCTGGAC	TGACCTGACTCTCCGTCATCC
<i>Sst</i>	GAGCCCAACCAGACAGAGAA	GAAGTTCTTGACCCAGCTT
<i>Ppy</i>	TACTGCTGCCTCTCCCTGTT	CCAGGAAGTCCACCTGTGTT
<i>Ghrl</i>	GAAGCCACCAGCTAAACTGC	GCCTGTCCGTGGTTACTTGT
<i>Pax6</i>	ATATGTCGACAGCTCCAGCATGCAGAAC	TGCCCAGAATTTTACTCACACAA
<i>Foxa2</i>	GAGCACCATTACGCCTTCAAC	AGGCCTTGAGGTCCATTTTGT
<i>Nkx2.2</i>	ATGTGCTGACCAACACAAA	TCACCGGACAATGACAAGGA
<i>Pcsk2</i>	AATGACCCCTACCATACCC	GAGGAGGCTTCGATGATGTC
<i>Kir6.2</i>	GTAGGGGACCTCCGAAAGAG	TGGAGTCGATGACGTGGTAG
<i>Sur1</i>	CTGGTCTCAGCAGCACAT	GGAActCTGGGACGAGACA
<i>Slc7a1</i>	ATCGTACTTCAAGCGTGGC	CCATGGCTGACTCCTTCACG
<i>Slc7a2</i>	ATGGCTTTACAGGGACGTTG	GCGTTAAAGCTGCAGAAACC

to the protocol of the QuantiTect reverse transcription kit (Qiagen). Real-time qPCRs were performed in duplicate on a Thermal Cycler Dice real-time system (TaKaRa) with SYBR green PCR master mix (TaKaRa). The expression of all target genes was normalized to *Hprt*. The primers used in this study are listed in Table 1.

Arginine-stimulated glucagon secretion. Eight-week-old *Mafb^{ΔEndo} Mafb^{ΔTAM}* male mice 3 weeks post-TAM injection and the corresponding controls were fasted for 16 h overnight. On the following morning, 1 mg/g of L-arginine (Sigma) was injected intraperitoneally, and venous blood was collected at 0, 2, 10, and 25 min postinjection in heparinized tubes (Drummond Scientific Company). Plasma glucagon levels were determined using the Mercodia Glucagon 10- μ l ELISA kit (10-1281-01).

Statistical analysis. All data are presented as means and standard errors of the mean (SEM). For statistical significance between *Mafb* mutants and controls, a minimum of three biological replicates were analyzed using Welch's *t* test, and a *P* value of <0.05 was considered significant. To compare longitudinal data from the arginine-stimulated glucagon secretion test, *P* values were calculated using Welch's *t* test, followed by Holm's correction for multiple comparisons.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/MCB.00504-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

We thank S. Yoshida and K. Kitamura, as well as K. Morohashi, for their generous gifts of *Ngn3*-Cre transgenic mice and rabbit anti-Arx antibody, respectively. We also thank M. Ojima for technical assistance; A. Tagami, R. Fujii, S. Fuseya, and R. Suzuki for helping with the cell count analysis; and T. Kunath and R. Koshida for proofreading the manuscript.

This work was supported by JSPS Kakenhi grants JP26221004 and 16H06276.

REFERENCES

- Unger RH, Aguilar-Parada E, Müller WA, Eisentraut AM. 1970. Studies of pancreatic α cell function in normal and diabetic subjects. *J Clin Invest* 49:837–848. <https://doi.org/10.1172/JCI106297>.
- Dunning BE, Gerich JE. 2007. The role of α -cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. *Endocr Rev* 28:253–283. <https://doi.org/10.1210/er.2006-0026>.
- Menge BA, Grüber L, Jørgensen SM, Deacon CF, Schmidt WE, Veldhuis JD, Holst JJ, Meier JJ. 2011. Loss of inverse relationship between pulsatile insulin and glucagon secretion in patients with type 2 diabetes. *Diabetes* 60:2160–2168. <https://doi.org/10.2337/db11-0251>.
- Benitez CM, Goodyer WR, Kim SK. 2012. Deconstructing pancreas developmental biology. *Cold Spring Harb Perspect Biol* 4:a012401. <https://doi.org/10.1101/cshperspect.a012401>.
- van der Meulen T, Huisin MO. 2015. Role of transcription factors in the transdifferentiation of pancreatic islet cells. *J Mol Endocrinol* 54:R103–R117. <https://doi.org/10.1530/JME-14-0290>.
- Zhang C, Moriguchi T, Kajihara M, Esaki R, Harada A, Shimohata H, Oishi H, Hamada M, Morito N, Hasegawa K, Kudo T, Engel JD, Yamamoto M, Takahashi S. 2005. MafA is a key regulator of glucose-stimulated insulin secretion. *Mol Cell Biol* 25:4969–4976. <https://doi.org/10.1128/MCB.25.12.4969-4976.2005>.

7. Nishimura W, Kondo T, Salameh T, El Khattabi I, Dodge R, Bonner-Weir S, Sharma A. 2006. A switch from MafB to MafA expression accompanies differentiation to pancreatic β -cells. *Dev Biol* 293:526–539. <https://doi.org/10.1016/j.ydbio.2006.02.028>.
8. Artner I, Hang Y, Mazur M, Yamamoto T, Guo M, Lindner J, Magnuson MA, Stein R. 2010. MafA and MafB regulate genes critical to β -cells in a unique temporal manner. *Diabetes* 59:2530–2539. <https://doi.org/10.2337/db10-0190>.
9. Artner I, Le Lay J, Hang Y, Elghazi L, Schisler JC, Henderson E, Sosa-Pineda B, Stein R. 2006. MafB: an activator of the glucagon gene expressed in developing islet α - and β -cells. *Diabetes* 55:297–304. <https://doi.org/10.2337/diabetes.55.02.06.db05-0946>.
10. Conrad E, Dai C, Spaeth J, Guo M, Cyphert HA, Scoville D, Carroll J, Yu W-M, Goodrich LV, Harlan DM, Grove KL, Roberts CT, Powers AC, Gu G, 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. <https://doi.org/10.1152/ajpendo.00285.2015>.
11. Artner I, Bianchi B, Raum JC, Guo M, Kaneko T, Cordes S, Sieweke M, Stein R. 2007. MafB is required for islet β cell maturation. *Proc Natl Acad Sci U S A* 104:3853–3858. <https://doi.org/10.1073/pnas.0700013104>.
12. Bianchi B, Kelly LM, Viemari J-C, Lafon I, Burnet H, Bévengut M, Tillmanns S, Daniel L, Graf T, Hilaire G, Sieweke MH. 2003. MafB deficiency causes defective respiratory rhythmogenesis and fatal central apnea at birth. *Nat Neurosci* 6:1091–1100. <https://doi.org/10.1038/nn1129>.
13. Benner C, van der Meulen T, Cacères E, Tigyi K, Donaldson CJ, Huisling MO. 2014. The transcriptional landscape of mouse β cells compared to human β cells reveals notable species differences in long non-coding RNA and protein-coding gene expression. *BMC Genomics* 15:620. <https://doi.org/10.1186/1471-2164-15-620>.
14. Sander M, Sussel L, Conners J, Scheel D, Kalamaras J, Dela Cruz F, Schwitzgebel V, Hayes-Jordan A, German M. 2000. Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of β -cell formation in the pancreas. *Development* 127:5533–5540.
15. Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P. 2003. Opposing actions of Arx and Pax4 in endocrine pancreas development. *Genes Dev* 17:2591–2603. <https://doi.org/10.1101/gad.269003>.
16. Huang YH, Sun MJ, Jiang M, Fu BY. 2009. Immunohistochemical localization of glucagon and pancreatic polypeptide on rat endocrine pancreas: coexistence in rat islet cells. *Eur J Histochem* 53:81–85.
17. Nishimura W, Rowan S, Salameh T, Maas RL, Bonner-Weir S, Sell SM, Sharma A. 2008. Preferential reduction of β cells derived from Pax6-MafB pathway in MafB deficient mice. *Dev Biol* 314:443–456. <https://doi.org/10.1016/j.ydbio.2007.12.009>.
18. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P. 1997. Pax6 is required for differentiation of glucagon-producing α -cells in mouse pancreas. *Nature* 387:406–409. <https://doi.org/10.1038/387406a0>.
19. Kaestner KH, Katz J, Liu Y, Drucker DJ, Schütz G. 1999. Inactivation of the winged helix transcription factor HNF3 α affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes Dev* 13:495–504. <https://doi.org/10.1101/gad.13.4.495>.
20. Gauthier BR, Schwitzgebel VM, Zaiko M, Mamin A, Ritz-Laser B, Philippe J. 2002. Hepatic nuclear factor-3 (HNF-3 or Foxa2) regulates glucagon gene transcription by binding to the G1 and G2 promoter elements. *Mol Endocrinol* 16:170–183. <https://doi.org/10.1210/mend.16.1.0752>.
21. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS. 1998. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic β cells. *Development* 125:2213–2221.
22. Furuta M, Zhou A, Webb G, Carroll R, Ravazzola M, Orci L, Steiner DF. 2001. Severe defect in proglucagon processing in islet α -cells of pro-hormone convertase 2 null mice. *J Biol Chem* 276:27197–27202. <https://doi.org/10.1074/jbc.M103362200>.
23. Quesada I, Tuduri E, Ripoll C, Nadal A. 2008. Physiology of the pancreatic α -cell and glucagon secretion: role in glucose homeostasis and diabetes. *J Endocrinol* 199:5–19. <https://doi.org/10.1677/JOE-08-0290>.
24. Gu G, Dubauskaite J, Melton DA. 2002. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* 129:2447–2457.
25. Tran MTN, Hamada M, Nakamura M, Jeon H, Kamei R, Tsunakawa Y, Kulathunga K, Lin Y-Y, Fujisawa K, Kudo T, Takahashi S. 2016. MafB deficiency accelerates the development of obesity in mice. *FEBS Open Bio* 6:540–547. <https://doi.org/10.1002/2211-5463.12058>.
26. Shichita T, Ito M, Morita R, Komai K, Noguchi Y, Ooboshi H, Koshida R, Takahashi S, Kodama T, Yoshimura A. 2017. MAFB prevents excess inflammation after ischemic stroke by accelerating clearance of damage signals through MSR1. *Nat Med* 23:723–732. <https://doi.org/10.1038/nm.4312>.
27. Yu W-M, Appler JM, Kim Y-H, Nishitani AM, Holt JR, Goodrich LV. 2013. A Gata3-MafB transcriptional network directs post-synaptic differentiation in synapses specialized for hearing. *Elife* 2:e01341. <https://doi.org/10.7554/eLife.01341>.
28. Gosmain Y, Avril I, Mamin A, Philippe J. 2007. Pax-6 and c-Maf functionally interact with the α -cell-specific DNA element G1 in vivo to promote glucagon gene expression. *J Biol Chem* 282:35024–35034. <https://doi.org/10.1074/jbc.M702795200>.
29. Dumonteil E, Laser B, Constant I, Philippe J. 1998. Differential regulation of the glucagon and insulin I gene promoters by the basic helix-loop-helix transcription factors E47 and BETA2. *J Biol Chem* 273:19945–19954. <https://doi.org/10.1074/jbc.273.32.19945>.
30. Wang M, Drucker DJ. 1995. The LIM domain homeobox gene islet-1 is a positive regulator of islet cell-specific proglucagon gene transcription. *J Biol Chem* 270:12646–12652. <https://doi.org/10.1074/jbc.270.21.12646>.
31. Hussain MA, Lee J, Miller CP, Habener JF. 1997. POU domain transcription factor brain 4 confers pancreatic α -cell-specific expression of the proglucagon gene through interaction with a novel proximal promoter G1 element. *Mol Cell Biol* 17:7186–7194. <https://doi.org/10.1128/MCB.17.12.7186>.
32. Hussain MA, Miller CP, Habener JF. 2002. Brn-4 transcription factor expression targeted to the early developing mouse pancreas induces ectopic glucagon gene expression in insulin-producing β cells. *J Biol Chem* 277:16028–16032. <https://doi.org/10.1074/jbc.M107124200>.
33. Collombat P, Hecksher-Sørensen J, Krull J, Berger J, Riedel D, Herrera PL, Serup P, Mansouri A. 2007. Embryonic endocrine pancreas and mature β cells acquire α and PP cell phenotypes upon Arx misexpression. *J Clin Invest* 117:961–970. <https://doi.org/10.1172/JCI29115>.
34. Gao T, McKenna B, Li C, Reichert M, Nguyen J, Singh T, Yang C, Pannikar A, Doliba N, Zhang T, Stoffers DA, Edlund H, Matschinsky F, Stein R, Stanger BZ. 2014. Pdx1 maintains β cell identity and function by repressing an α cell program. *Cell Metab* 19:259–271. <https://doi.org/10.1016/j.cmet.2013.12.002>.
35. Unger RH, Cherrington AD, Wollheim C, Wang Z, Unger R, Friedman J. 2012. Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. *J Clin Invest* 122:4–12. <https://doi.org/10.1172/JCI60016>.
36. Sandoval DA, D'Alessio DA. 2015. Physiology of proglucagon peptides: role of glucagon and GLP-1 in health and disease. *Physiol Rev* 95:513–548. <https://doi.org/10.1152/physrev.00013.2014>.
37. Müller WA, Faloona GR, Unger RH, Faloona G, Unger R, Derot M. 1973. Hyperglucagonemia in diabetic ketoacidosis. *Am J Med* 54:52–57. [https://doi.org/10.1016/0002-9343\(73\)90083-1](https://doi.org/10.1016/0002-9343(73)90083-1).
38. Parker JC, Andrews KM, Allen MR, Stock JL, McNeish JD. 2002. Glycemic control in mice with targeted disruption of the glucagon receptor gene. *Biochem Biophys Res Commun* 290:839–843. <https://doi.org/10.1006/bbrc.2001.6265>.
39. Gelling RW, Du XQ, Dichmann DS, Romer J, Huang H, Cui L, Obici S, Tang B, Holst JJ, Fledelius C, Johansen PB, Rossetti L, Jelicks LA, Serup P, Nishimura E, Charron MJ. 2003. Lower blood glucose, hyperglucagonemia, and pancreatic α cell hyperplasia in glucagon receptor knockout mice. *Proc Natl Acad Sci U S A* 100:1438–1443. <https://doi.org/10.1073/pnas.0237106100>.
40. Hancock AS, Du A, Liu J, Miller M, May CL. 2010. Glucagon deficiency reduces hepatic glucose production and improves glucose tolerance in adult mice. *Mol Endocrinol* 24:1605–1614. <https://doi.org/10.1210/me.2010-0120>.
41. Hayashi Y. 2011. Metabolic impact of glucagon deficiency. *Diabetes Obes Metab* 13:151–157. <https://doi.org/10.1111/j.1463-1326.2011.01456.x>.
42. Lee Y, Wang M-Y, Du XQ, Charron MJ, Unger RH. 2011. Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes* 60:391–397. <https://doi.org/10.2337/db10-0426>.
43. O'Harte FPM, Franklin ZJ, Irwin N. 2014. Two novel glucagon receptor antagonists prove effective therapeutic agents in high-fat-fed and obese diabetic mice. *Diabetes Obes Metab* 16:1214–1222. <https://doi.org/10.1111/dom.12360>.
44. van der Meulen T, Mawla AM, DiGruccio MR, Adams MW, Nies V, Dölleman S, Liu S, Ackermann AM, Cáceres E, Hunter AE, Kaestner KH, Donaldson CJ, Huisling MO. 2017. Virgin β cells persist throughout life at

- a neogenic niche within pancreatic islets. *Cell Metab* 25:911–926.e6. <https://doi.org/10.1016/j.cmet.2017.03.017>.
45. Cheng Y, Kang H, Shen J, Hao H, Liu J, Guo Y, Mu Y, Han W. 2015. β -Cell regeneration from vimentin+/MafB+ cells after STZ-induced extreme β -cell ablation. *Sci Rep* 5:11703. <https://doi.org/10.1038/srep11703>.
 46. Dai C, Brissova M, Hang Y, Thompson C, Poffenberger G, Shostak A, Chen Z, Stein R, Powers AC. 2012. Islet-enriched gene expression and glucose-induced insulin secretion in human and mouse islets. *Diabetologia* 55:707–718. <https://doi.org/10.1007/s00125-011-2369-0>.
 47. Guo S, Dai C, Guo M, Taylor B, Harmon JS, Sander M, Robertson RP, Powers AC, Stein R. 2013. Inactivation of specific β cell transcription factors in type 2 diabetes. *J Clin Invest* 123:3305–3316. <https://doi.org/10.1172/JCI65390>.
 48. Scoville DW, Cyphert HA, Liao L, Xu J, Reynolds A, Guo S, Stein R. 2015. MLL3 and MLL4, ethyltransferases bind to the MAFA and MAFB transcription factors to regulate islet β -cell function. *Diabetes* 64:3772–3783. <https://doi.org/10.2337/db15-0281>.
 49. Dworschak GC, Draaken M, Hilger A, Born M, Reutter H, Ludwig M. 2013. An incompletely penetrant novel MAFB (p.Ser56Phe) variant in autosomal dominant multicentric carpotarsal osteolysis syndrome. *Int J Mol Med* 32:174–178. <https://doi.org/10.3892/ijmm.2013.1373>.
 50. Mehawej C, Courcet J-B, Baujat G, Mouy R, Gérard M, Landru I, Gosselin M, Koehrer P, Mousson C, Breton S, Quartier P, Le Merrer M, Faivre L, Cormier-Daire V. 2013. The identification of MAFB mutations in eight patients with multicentric carpo-tarsal osteolysis supports genetic homogeneity but clinical variability. *Am J Med Genet A* 161:3023–3029. <https://doi.org/10.1002/ajmg.a.36151>.
 51. Park JG, Tischfield MA, Nugent AA, Cheng L, Di Gioia SA, Chan W-M, Maconachie G, Bosley TM, Summers CG, Hunter DG, Robson CD, Gottlob I, Engle EC. 2016. Loss of MAFB function in humans and mice causes Duane syndrome, aberrant extraocular muscle innervation, and inner-ear defects. *Am J Hum Genet* 98:1220–1227. <https://doi.org/10.1016/j.ajhg.2016.03.023>.
 52. Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T, Nabeshima YI. 2004. Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Dev Biol* 269:447–458. <https://doi.org/10.1016/j.ydbio.2004.01.036>.
 53. Hayashi S, McMahon AP. 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 244:305–318. <https://doi.org/10.1006/dbio.2002.0597>.
 54. Nagao M, Cheong CW, Olsen BR. 2016. Col2-Cre and tamoxifen-inducible Col2-CreER target different cell populations in the knee joint. *Osteoarthritis Cartil* 24:188–191. <https://doi.org/10.1016/j.joca.2015.07.025>.
 55. Metzger D, Chambon P. 2001. Site- and time-specific gene targeting in the mouse. *Methods* 24:71–80. <https://doi.org/10.1006/meth.2001.1159>.
 56. Kitamura K, Yanazawa M, Sugiyama N, Miura H, Iizuka-Kogo A, Kusaka M, Omichi K, Suzuki R, Kato-Fukui Y, Kamiirisa K, Matsuo M, Kamijo S, Kasahara M, Yoshioka H, Ogata T, Fukuda T, Kondo I, Kato M, Dobyns WB, Yokoyama M, Morohashi K. 2002. Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. *Nat Genet* 32:359–369. <https://doi.org/10.1038/ng1009>.
 57. im Walde SS, Dohle C, Schott-Ohly P, Gleichmann H. 2002. Molecular target structures in alloxan-induced diabetes in mice. *Life Sci* 71:1681–1694. [https://doi.org/10.1016/S0024-3205\(02\)01918-5](https://doi.org/10.1016/S0024-3205(02)01918-5).
 58. Lacy PE, Kostianovsky M. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16:35–39. <https://doi.org/10.2337/diab.16.1.35>.