FCoR-Foxo1 Axis Regulates α-Cell Mass through Repression of Arx Expression

Noriko Kodani, Jun Nakae, Masaki Kobayashi, Osamu Kikuchi, Tadahiro Kitamura, Hiroshi Itoh

HIGHLIGHTS
FCoR increases DNA methylation of Arx promoter and decreases Arx expression
Loss-of-Fcor increases conversion from β- to α-cells
FCoR acetylates and inhibits Foxo1 in pancreatic β-cells
Inactivation of Foxo1 facilitates recruitment of Dnmt3a to Arx promoter

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FCoR-Foxo1 Axis Regulates α-Cell Mass through Repression of Arx Expression

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SUMMARY
Pancreatic endocrine cell development into differentiated α- and β-cells is highly regulated and involves multiple transcription factors. However, the mechanisms behind the determination of α- and β-cell masses remains unclear. We previously identified Foxo1 CoRepressor (FCoR), which inhibits Foxo1 by acetylation. Here we demonstrate that Fcor-knockout mice (FcorKO) exhibit significantly increased α-cell mass, expression of the master α-cell regulatory transcription factor Aristaless-related homeobox (Arx), which can be normalized by β-cell-specific FCoR overexpression (FcorKO-βFCoR), and exhibit β-to-α-cell conversion. Compared with FcorKO, β-cell-specific Foxo1 knockdown in the FcorKO (DKO) led to decreased Arx expression and α-cell mass. Foxo1 binding to Arx promoter led to DNA methyltransferase 3a (Dnmt3a) dissociation, Arx promoter hypomethylation, and increased Arx expression. In contrast, FCoR suppressed Arx through Foxo1 inhibition and Dnmt3a recruitment to Arx promoter and increased Arx promoter methylation. Our findings suggest that the FCoR-Foxo1 axis regulates pancreatic α-cell mass by suppressing Arx expression.

INTRODUCTION
Type 2 diabetes results from insulin resistance in peripheral insulin responsive tissues, along with relatively deficient insulin secretion from pancreatic β-cells because of β-cell dysfunction and/or decreased mass. Increased glucagon secretion from α-cells is also found in type 2 diabetes (Dunning and Gerich, 2007; Menge et al., 2011). This hyperglucagonemia may result from decreased insulin-induced inhibition of α-cells or from a relative increase in α-cell mass (Dor and Glaser, 2013). Although apoptosis and proliferation regulate β-cell mass, little is known about the regulation of α-cell mass. “Intra-islet plasticity” has been suggested to be involved in the mechanism for regulating β-cell and other endocrine cell masses (Ziv et al., 2013). Glucagon-expressing α-cells or β-cells can be converted to β-like cells by near-total genetic ablation of β-cells, forced Pax4 expression, or loss of Arx (Thorel et al., 2010) (Collombat et al., 2009) (Courtney et al., 2013). On the other hand, deletion of Dmnt1, Pdx1, or Foxo1 converts β-cells to α-cells or other endocrine cells (Dhawan et al., 2011) (Gao et al., 2014) (Talchai et al., 2012b). However, the precise molecular mechanism of conversions among endocrine cell types remains unknown.

Foxo1 may be a key molecule that determines endocrine cell fate at conversion. The loss of Foxo1 in neurogenin (Ngn)3-positive enteroendocrine progenitors or human fetal pancreatic explants gives rise to insulin-positive cells (Talchai et al., 2012a) (Bouchi et al., 2014). Furthermore, Foxo1 ablation in pancreatic and endocrine progenitors leads to expanded β-cell mass, whereas Foxo1 ablation in terminally differentiated β-cells does not have this effect (Talchai and Accili, 2015). These findings indicate that Foxo1 inhibits β-cell differentiation in endocrine progenitor cells. Foxo1 is phosphorylated and inhibited by insulin/IGF-1 through PI3-kinase/Akt and is translocated into the nucleus and activated by oxidative stress (Accili and Arden, 2004). Within the nucleus, Foxo1 is deacylated and activated by Sirt1 or Hdacs (Accili and Arden, 2004) (Wang et al., 2011) (Mihaylova et al., 2011) (Banks et al., 2011). We recently identified a Foxo1 corepressor (Foxo1 CoRepressor; FCoR) in adipocytes, which acetylates and inhibits Foxo1 activity (Nakae et al., 2012).

Here, we demonstrate that FcorKO exhibits significantly increased α-cell mass and expression of the master α-cell regulatory transcription factor Arx, which can be normalized by β-cell-specific FCoR overexpression, and that FCoR suppresses Arx expression through increased methylation of the Arx promoter region by inhibition on Foxo1 activity. In contrast, β-cell-specific Foxo1 knockout in the FcorKO led to decreased Arx expression and α-cell mass through loss of Foxo1-induced Arx expression. These findings indicate that the FCoR-Foxo1 axis regulates pancreatic α-cell mass via Foxo1 inhibition and suggest new avenues for the development of diabetes therapies.

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RESULTS

FCoR Is Expressed in Pancreatic Islets

FCoR is a Foxo1-binding protein that was originally identified in mouse adipose tissue. Null FcorKO exhibits glucose intolerance and insulin resistance (Figures S1A and S1B) (Nakae et al., 2012). Although insulin-resistant mice generally show increased insulin secretion as a result of compensated β-cell hypertrophy, we found that FcorKO exhibited decreased glucose-stimulated insulin secretion compared with control (Figure 1A). Additionally, compared with control, FcorKO showed significantly increased glucagon secretion at 15 min after glucose stimulation and in response to L-arginine (Figures 1B and 1C).

We next isolated islets and examined their insulin and glucagon secretion in response to glucose or L-arginine. Compared with control islets, islets from FcorKO exhibited significantly lower insulin secretion upon stimulation with glucose, whereas insulin secretion in response to L-arginine was similar in islets from FcorKO and control (Figure 1D), suggesting decreased glucose sensing and/or normal insulin secretion after depolarization. However, real-time PCR revealed that expression levels of Slc2a2 and Gck in isolated islets of FcorKO were similar to those of control (Figure S1C) and the insulin content of FcorKO islets was similar to that of control islets (Figure S1D). Therefore, as far as we investigated, we could not reveal the mechanism by which islets from FcorKO have the insensitivity to glucose. Additionally, glucagon secretion after stimulation with glucose was significantly greater in FcorKO islets compared with control islets (Figure 1E). These data suggested that the dysfunction of insulin and glucagon secretion in FcorKO was pancreatic endocrine cell-intrinsic.

In pancreatic tissue from C57Bl6J mice, we performed immunofluorescence analyses for FCoR and glucagon or insulin, which revealed FCoR expression in both α- and β-cells, although FCoR expression in postnatal α-cells was limited (Figure 1F). FCoR moves from cytosol to nucleus through phosphorylation by protein kinase A (PKA) and binds to and acetylates Foxo1 both in cytosol and nucleus (Nakae et al., 2012). On the other hand, the acetylation status of Foxo1 has been known to change intracellular and/or intranuclear localization. Furthermore, deacetylation of Foxo1 by Sirtuins overrides the phosphorylation-dependent nuclear exclusion of Foxo1 caused by growth factors and causes nuclear translocation of Foxo1, leading to activation of Foxo1 (Frescas et al., 2005). Acetylation of Foxo1 by FCoR in cytosol may also inhibit Foxo1 owing to cytosolic retention. Therefore, FCoRs may be active both in cytosol and nucleus. Additionally, to investigate whether FCoR expression in islets was modulated at insulin-resistant state, we examined FCoR protein expression in islets of mice fed a high-fat diet (HFD) or of Leprdb/db genetic background (Figure 1G). These data suggested that FCoR may play some physiological roles in islets.

Furthermore, immunofluorescence analyses in mouse embryos demonstrated co-localization of FCoR in both insulin- and glucagon-positive cells starting at embryonic day 14.5 (Figures 1I–1K). Although FCoR expression was increased in insulin-positive cells after embryonic day 16.5 and was in almost all insulin-positive cells after embryonic day 18.5, FCoR expression was decreased in glucagon-positive cells after embryonic day 14.5 (Figures 1I–1K). At embryonic day 14.5, FCoR was expressed in most of the glucagon-positive cells, which later decreased. In contrast, after embryonic day 18.5, all insulin-positive cells expressed FCoR (Figure 1K). FCoR was not co-localized within neurogenin 3 (Ngn3)-positive cells during these embryonic stages (data not shown). Foxo1 was expressed mainly in glucagon-positive cells until embryonic day 17.5. Furthermore, after that, Foxo1 was expressed in insulin-positive cells rather than in glucagon-positive cells (Figures S1E–S1H). These data suggest that FCoR expression occurs just after the development of pancreatic endocrine progenitors (Puri and Hebrok, 2010) and may be related to the development of pancreatic endocrine cell differentiation and that FCoR and Foxo1 may be co-localized in glucagon-positive cells before embryonic day 15.5.

Fcor Ablation Increases Arx Expression and α-Cell Mass

To investigate the effects of Fcor deletion on pancreatic islets, we examined α-cell and β-cell masses. Immunofluorescence analyses revealed significantly increased α-cell mass in FcorKO compared with control, with no significant difference in β-cell mass (Figures 2A–2C). The sizes did not differ between control and FcorKO islets (data not shown). The architecture of islets from FcorKO was normal, which indicated peripheral localization of α-cells (Figure 2A). In contrast, transgenic mice overexpressing FCoR specifically in β-cells in a FcorKO genetic background (FcorKO-βFcor, developed using RIP-Cre transgenic mice;
Figure 1. FCoR Is Expressed in Pancreatic Islets

(A) Insulin secretion during an intraperitoneal glucose tolerance test (IPGTT) in control (open circle, n = 7) and FcorKO (closed circle, n = 10). Data represent means ± SEM. *p < 0.05 by two-way ANOVA with Fisher’s test.

(B) Glucagon secretion during the IPGTT (control, n = 11; FcorKO, n = 15) (B), and L-arginine tolerance test (ATT) (control, n = 5; FcorKO, n = 9) (C). Data represent means ± SEM. *p < 0.05 and **p < 0.005 by two-way ANOVA with Fisher’s test.

(F) Immunofluorescence of pancreatic islets for FCoR and glucagon or FCoR and insulin in islets from the 24-week-old C57Bl6J mouse. Scale bar, 20 μm.

(G) Immunofluorescence of pancreatic islets for FCoR and insulin (G) or FCoR and glucagon (H) immunostained with Arx antibodies. Scale bar, 20 μm.

(I) Representative images of pancreatic islets for FCoR and insulin (I) or FCoR and glucagon (J) from embryos at embryonic days 14.5 (E14.5), 16.5 (E16.5), and 18.5 (E18.5), and postnatal day 1 (P1). Scale bar, 20 μm.

(K) The percentage of embryonic cells stained with FCoR among glucagon- or insulin-positive cells at embryonic day 14.5 (percentage of FCoR-stained cells/glucagon-positive cells versus FCoR-stained cells/insulin-positive cells 100 ± 4.76: 63.6 ± 12.0, n = 3; 42 glucagon-positive cells, 193 insulin-positive cells counted, consistent for all comparisons), embryonic day 16.5 (34.8 ± 24.3: 84.8 ± 15.2, n = 3; 40 glucagon-positive cells, 36 insulin-positive cells), embryonic day 18.5 (34 ± 11.5: 100 ± 0, n = 3, 71 glucagon-positive cells, 86 insulin-positive cells counted), and P1 (31.3 ± 3.86: 100 ± 0, n = 3, 136 glucagon-positive cells, 78 insulin-positive cells counted). Data represent means ± SEM. *p < 0.05 by one-way ANOVA.

Figures S2A–S2D showed an α-cell mass similar to that of controls (Figures 2A–2C). Furthermore, to investigate the effects of FCoR on α-cell mass, we generated transgenic mice overexpressing α-cell-specific FCoR (αFcor) (Figures S2B, S3A, and S3B). Immunofluorescence demonstrated significantly decreased α-cell mass in αFcor compared with controls, but β-cell mass in αFcor was similar to that of control (Figure S3C). These findings indicated that FCoR plays an important role in regulating α-cell mass.

To investigate the mechanism behind the increased α-cell mass in FcorKO, we isolated islets from control, FcorKO, and FcorKO-βFcor and measured the expression levels of α-cell-specific genes. Real-time PCR revealed significantly increased expression of α-cell-specific genes (Gcg, Arx, and Mafb) in FcorKO compared with control (Figure 2D). In contrast, Gcg, Arx, and Mafb expressions in islets isolated from FcorKO-βFcor were similar to control levels (Figure 2D). Furthermore, Arx expression was significantly decreased in αFcor (Figure S3D). Moreover, immunofluorescence analyses of pancreas from 20- to 24-week-old adult mice revealed that the nuclear Arx protein was less detectable in α-cells from control mice, whereas nuclear Arx expression was present in most α-cells in FcorKO (Figures 2E and 2F). These data suggested that FCoR suppressed Arx expression in islets and that Fcor knockout led to increased Arx expression in islets.

To investigate whether the increased α-cell mass observed in FcorKO resulted from increased Arx expression during the embryonic stage, we performed immunofluorescence analysis with anti-Arx antibody. During pancreatic development, the master regulator of pancreatic islet differentiation and regeneration, Ngn3, was expressed in endocrine precursor cells starting at embryonic day 9.5 (Rukstalis and Habener, 2009). Subsequently, the Ngn3-positive cells that were also Arx positive differentiated into α-cells (Collombat et al., 2006). Immunofluorescence analysis revealed significantly more Arx-positive cells in FcorKO compared with controls on embryonic day 15.5 (Figures 2G and 2H). At embryonic day 15.5, there were no significant differences in numbers of insulin-positive cells between control and FcorKO (Figure 2I). Therefore, we counted the numbers of Arx-positive cells corrected by the numbers of insulin-positive cells counted. From embryonic day 16.5 to birth, endocrine cell lineages organize into clusters to form the islets (Habener et al., 2005; Rukstalis and Habener, 2009). Immunofluorescence of the fetal pancreas at embryonic day 17.5 revealed significantly more glucagon-positive cells in FcorKO compared with control (Figures 2J–2L). These results suggested that FCoR regulated Arx expression from the embryonic stage and controlled α-cell mass.

During development of pancreatic endocrine cells, a complex network of transcription factors, including Arx and Pax4, drives endocrine precursor cells toward the different endocrine cell fate. These two transcription factors mutually inhibit the other’s transcription and display antagonistic activities for proper endocrine cell lineage allocation (Collombat et al., 2005). Indeed, expression levels of the β-cell-specific gene Pax4 in islets isolated from FcorKO were significantly decreased compared with control and FcorKO-βFcor (Figure 2M). However, mature β-cell-specific marker genes (Pdx1, Mafa, Neurod, and Unc3) expression levels in islets from FcorKO were significantly increased compared with control and FcorKO-βFcor cells (Figure 2M).
Figure 2. FCor Regulates Arx Expression and \( \alpha \)-Cell Mass

(A) Immunofluorescence of insulin and glucagon in islets from control, FcorKO, and FcorKO-\( \beta \)FCor at age 24 weeks. Scale bar, 50 \( \mu \)m.

(B and C) Quantification of \( \alpha \)-cell (B) and \( \beta \)-cell masses (C) in control (\( n = 5 \) mice, 230 islets measured), FcorKO (\( n = 5 \) mice, 220 islets measured), and FcorKO-\( \beta \)FCor (\( n = 3 \) mice, 80 islets measured). Four sections \( \approx 200 \mu \)m apart were covered systematically by accumulating images from non-overlapping fields for whole pancreas sections. Data represent means \( \pm \) SEM. *p < 0.05 by one-way ANOVA.

(D) Expression levels of \( \alpha \)-cell marker genes in islets isolated from control (white bar) (\( n = 4 \)), FcorKO (black bar) (\( n = 4 \)), and FcorKO-\( \beta \)FCor (gray bar) (\( n = 4 \)) at the age of 20–24 weeks. Data represent means \( \pm \) SEM. *p < 0.05 by one-way ANOVA.

(E and F) Representative images of Arx and glucagon immunostaining of islets from control and FcorKO at age 20 weeks (E), and the percentages of Arx-positive cells among glucagon-positive cells (F). We have calculated Arx-positive and glucagon-positive cells in 3–5 sections from each 5 mice. Data represent means \( \pm \) SEM.

(F) Expression levels of \( \beta \)-cell-specific marker genes, Pax4, Mafa, and Ins1, were significantly increased in FCor-infected cells compared with LacZ-infected cells, although expression levels of the \( \alpha \)-cell-specific marker genes Mafb and Gcg were unaffected (Figure S4C). In contrast, Pax4 and Mafa expression levels were significantly decreased in FCor-knockdown cells compared with those in SCR-shRNA-infected cells. Expression levels of \( \alpha \)-cell-specific marker genes Mafb and Gcg tended to be increased in FCor-knockdown MIN6 cells but not statistically significantly (Figure S4E). These data indicated that FCor suppresses Arx and increases Pax4 expression.

FCor Regulates Arx Expression in Cell Lines

We next investigated the direct effects of FCor on Arx expression by infecting the \( \alpha \)-cell-line \( \alpha \)TC1 cells with adenoviruses encoding LacZ or FLAG-tagged FCor and examining the cellular expression of Arx.

Real-time PCR analysis revealed that Fcor expression level in \( \alpha \)TC1 cells was significantly lower than in the \( \beta \)-cell line MIN6 (Figure S4A). FCor overexpression in \( \alpha \)TC1 cells significantly and dose-dependently decreased Arx expression (Figure S4B). In contrast, expression levels of the \( \beta \)-cell-specific marker genes, Pax4, Mafa, and Ins1, were significantly increased in FCor-infected cells compared with LacZ-infected cells, although expression levels of the \( \alpha \)-cell-specific marker genes Mafb and Gcg were unaffected (Figure S4C). Furthermore, we examined the effects of FCor knockdown on Arx expression in MIN6 cells. Arx expression was significantly increased in MIN6 cells infected with adenovirus encoding short hairpin RNA (shRNA) targeting FCor (which showed 85\% knockdown of endogenous FCor), compared with that in MIN6 cells infected with adenovirus encoding SCR-shRNA (Figure S4D). In contrast, Pax4 and Mafa expression levels were significantly decreased in FCor-knockdown cells compared with those in SCR shRNA-infected cells. Expression levels of \( \alpha \)-cell-specific marker genes Mafb and Gcg tended to be increased in FCor-knockdown MIN6 cells but not statistically significantly (Figure S4E). These data indicated that FCor suppresses Arx and increases Pax4 expression.

Fcor Ablation Causes \( \beta \)-to-\( \alpha \)-Cell Conversion

To examine whether the increased \( \alpha \)-cell mass in FcorKO resulted from increased conversion from \( \beta \)-cells to \( \alpha \)-cells, we performed a double immunostaining of EGFP and glucagon using control or FcorKO with the transgene RIP-Cre: EGFP (Figure S5). The confocal microscopy analyses demonstrated that EGFP was stained exclusively in the \( \beta \)-cell area in control mice. One EGFP-positive cell (0.17\%) was detected among 574 glucagon-positive cells of 38 islets from three control mice. In contrast, FcorKO exhibited cells co-stained with EGFP and glucagon, indicating conversion from \( \beta \)-cells to \( \alpha \)-cells. A total of 90 EGFP-positive cells (2.47\%) were detected among 3,644 glucagon-positive cells of 195 islets from five FcorKO mice (Figures 3A and 3B). Moreover, a 3D reconstruction of 2 stack images of pancreas from a 5-\( \mu \)m section also identified EGFP- and glucagon-double-positive cells (Figure 3C). Furthermore, double immunostaining of insulin and glucagon revealed a significantly increased number of double-positive cells in islet of FcorKO compared with control (Figures 3D and 3E). The presence of these cells indicates conversion from \( \beta \)-cells to \( \alpha \)-cells and bimodular and immature cells.

To explore the alternative mechanism that \( \alpha \)-cells may undergo expansion, we performed Ki67-staining in glucagon-positive cells from control and FcorKO mice at the ages of e15.5, e17.5, and adulthood. However, we detected no recognizable differences in glucagon-positive cells between control and FcorKO (Figure S6). These data indicate that \( \alpha \)-cell expansion in FcorKO does not result from increased...
proliferation of \( b \)-cells. Furthermore, to investigate whether \( b \)-cells proliferate more to compensate the loss of \( b \)-cells due to \( b \)-to-\( a \)-cell conversion, we performed double staining of insulin and Ki67 of islets using the same samples. However, there were also no significant differences in insulin-positive cells (data not shown). These indicate that there is no increased proliferation of \( b \)-cell in FcorKO mice. Moreover, no

Figure 3. Fcor Ablation Causes \( b \)-to-\( a \)-Cell Conversion
(A) \( b \)-Cell lineage tracing of control (CAG-CAT-EGFP: RIP-Cre) and FcorKO (FcorKO:CAG-CAT-EGFP: RIP-Cre) at age 24 weeks. The sections were examined with a confocal microscope (Olympus Fluoview FV3000). White arrows indicate EGFP- and glucagon-double-positive cells. Scale bar, 20 \( \mu \)m.
(B) The percentage of EGFP-positive cells among glucagon-positive cells in islets from control and FcorKO. In control mice, one EGFP-positive cell (0.17%) was detected among 574 glucagon-positive cells of 38 islets from three control mice. In contrast, 90 EGFP-positive cells (2.47%) were detected among 3,644 glucagon-positive cells of 195 islets from five FcorKO.
(C) Z sectioning and 3D reconstruction of a 5-\( \mu \)m section of pancreas from FcorKO:CAG-CAT-EGFP: RIP-Cre shows a glucagon- and EGFP-double-positive cell.
(D and E) Representative image of immunofluorescence for insulin and glucagon in pancreas at the age of 24 weeks (D), and the percentage of numbers of insulin-positive cells among glucagon-positive cells (E); control, 2 sections from each mouse (\( n = 3 \)), 0 insulin-positive cells among 46 islets counted; FcorKO, 2 sections from each mouse (\( n = 3 \)), 12 insulin-positive cells among 77 islets counted. Data represent means ± SEM. *\( p < 0.05 \) by one-way ANOVA. Scale bar, 50 \( \mu \)m.
Ngn3-positive cells were present in islets isolated from adult FcorKO mice, excluding the possibility that the converted cells were not attributable to dedifferentiated cells (data not shown). These results suggested that the conversion from β- to α-cells contributed to increased α-cell mass in FcorKO.

**FCor Is a Regulator of Foxo1 Activity in Islets**

FCor enhances the acetylation of Foxo1 and Foxo3, thus inhibiting their transcriptional activity (Nakae et al., 2012). Indeed, compared with control, islets isolated from FcorKO showed significantly greater deacetylation of transduced ADA-Foxo1, which was a constitutively nuclear mutant Foxo1 (Nakae et al., 2001), and stronger binding to Sirt1 (Figure 4A). Furthermore, immunofluorescence analyses revealed nuclear localization of endogenous Foxo1 in islets from FcorKO, in both α-cells and non-α-cells (Figure 4B), suggesting that Foxo1 may have been active. In addition, western blotting showed that endogenous Foxo1 protein expression level was significantly decreased in islets isolated from FcorKO compared with controls (Figure 4C), consistent with previous findings demonstrating that deacetylated Foxo1 is quickly ubiquitinated and degraded (Kitamura et al., 2005). These data indicate that FCor is one of the regulators of Foxo1 acetylation and intracellular localization in islets.

If Foxo1 activation in islets mediates the phenotypes of FcorKO, including Arx expression and α-cell mass, then additional knockout of Foxo1 specifically in β-cells could rescue the FcorKO phenotype. To examine this hypothesis, we generated double-knockout mice, β-cell-specific Foxo1KO (βFoxo1KO) in the background of FcorKO (DKO) (Figure 4D). Real-time PCR of islets isolated from each genotype revealed significantly decreased Foxo1 expression levels in βFoxo1KO and DKO mice compared with control and FcorKO. Foxo3 and Foxo4 expression levels were similar in islets isolated from each genotype, except for increased Foxo3 expression in FcorKO (Figure S7A).

Real-time PCR demonstrated that Arx expression level in islets isolated from DKO was significantly decreased compared with FcorKO and similar to controls. Furthermore, Arx expression level in islets isolated from βFoxo1KO was significantly lower than from any other genotyped islets (Figure 4E). Furthermore, immunofluorescence analyses also revealed that Arx staining in α-cells of FcorKO was more prominent than in control and DKO mice. In contrast, Arx staining in α-cells from βFoxo1KO was difficult to be detected (Figures S7B and S7C). These data indicate that increased expression of Arx in islets from FcorKO results from Foxo1 activation in islets and that Foxo1 may have an important role in the regulation of Arx expression. Furthermore, immunofluorescence analyses revealed that the α-cell mass in DKO was significantly decreased compared with that in FcorKO, although it was still significantly increased compared with controls (Figure 4F). The groups did not significantly differ in β-cell mass or number of islets (Figure 4G). These data indicate that increased Arx expression and α-cell mass with the FcorKO genotypes results from Foxo1 activation.

**Arx Is a Target Gene of Foxo1**

As described above, loss of Foxo1 in β-cells significantly decreased Arx expression compared with control, FcorKO, and DKO, although it did not affect the architecture of islets (Figures 4E–4G). These data led us to speculate that Foxo1 itself may affect endogenous Arx expression. To investigate the influence of Foxo1 on endogenous Arx expression, we infected MIN6 cells with adenovirus encoding constitutively nuclear HA-ADA Foxo1 (Nakae et al., 2001). Real-time PCR demonstrated that Arx expression was significantly increased compared with LacZ-infected control cells (Figure 5A). These data indicated that Foxo1 induced Arx expression. The murine Arx gene contains a conserved forkhead responsive element (FRE) in its promoter region (Figures S8A and S8B). ChIP assay results confirmed that constitutively nuclear Foxo1 mutant (HA-ADA) bound to the Arx promoter region, including FRE, in MIN6 cells (Figure 5B).

To assess whether Foxo1 directly induced Arx expression, we performed the Arx promoter assay in MIN6 cells using Arx promoter regions of various lengths. We found that FLAG-tagged constitutively nuclear mutant Foxo1 (FLAG-3AFoxo1) (Nakae et al., 2006) significantly increased the luciferase activity in the FRE-containing Arx promoter (Figure 5C) but did not do so in the truncated Arx promoter lacking FRE or in the Arx promoter with mutated FRE (Figure 5C). Moreover, site-directed mutagenesis of FRE in the Arx promoter completely abolished Foxo1-induced luciferase activity (Figure 5C).

We next conducted an electrophoretic mobility shift assay (EMSA) to further examine the ability of this putative FRE to bind Foxo1. A cMyc-tagged Foxo1 caused significant retardation of the FRE DNA (Figure 5D,
Figure 4. FCoR-Foxo1 Axis Regulates Arx Expression and α-Cell Mass

(A) Transduced Foxo1 desacetylation in islets isolated from FcorKO. Islets isolated from control and FcorKO were transduced with adenoviruses encoding HA-tagged ADA-Foxo1 and harvested at 36 h after transduction. Cell lysates were immunoprecipitated with anti-HA and blotted with the indicated antibodies. The left panel indicates representative western blotting, and the right panel indicates the quantitative analysis of acetylated HA-tagged ADA Foxo1 in islets. The intensity of each band was measured using NIH ImageJ, and the intensity of band of acetylated HA-tagged ADA Foxo1 was corrected by tubulin and calculated as the percentage of control. Data represent means ± SEM from three independent experiments. *p < 0.05 by one-way ANOVA.

(B) Representative immunofluorescence of endogenous FOX1 and glucagon-positive cells in islets from 16-week-old control and FcorKO. Scale bar, 20 μm. Insets represent endogenous nuclear Foxo1 and glucagon-negative (left panel) or glucagon-positive cells (right panel). Arrows indicate endogenous nuclear Foxo1-positive cells. Scale bar, 20 μm.

(C) Endogenous Foxo1 protein expression level was decreased in islets isolated from FcorKO compared with control. The top panel indicates representative western blotting, and the bottom panel indicates the quantitative analysis of endogenous Foxo1 in islets. The intensity of each band was measured using NIH ImageJ, and the intensity of band of endogenous Foxo1 was corrected by tubulin and calculated as the percentage of control. Data represent means ± SEM from three independent experiments. *p < 0.05 by one-way ANOVA.

(D) Representative double immunofluorescence of islets from 24-week-old controls and from FcorKO, DKO, and βFoxo1KO using anti-glucagon and anti-FCoR, anti-Foxo1, or anti-insulin antibodies. Scale bar, 50 μm.

(E) Arx expression level in islets isolated from 20- to 24-week-old controls (n = 4) and from β Foxo1KO (n = 4), FcorKO (n = 4), and DKO (n = 4). Data represent means ± SEM from three independent experiments. *p < 0.05 and **p < 0.005 by one-way ANOVA.

(F and G) Quantification of α-cell (F) and β-cell masses (G) in 20- to 24-week-old control (n = 3 mice; 70 islets measured), βFoxo1KO (n = 3; 61 islets measured), FcorKO (n = 4; 102 islets measured), and DKO (n = 4; 98 islets measured). Data represent means ± SEM. *p < 0.05 and **p < 0.005 by one-way ANOVA.

FCoR Induces DNA Methylation of the Arx Promoter Region

Epigenetic regulation in the Arx promoter region controls Arx expression. The Arx promoter region is methylated, and its expression is suppressed in β-cells, whereas in α-cells, this promoter is hypomethylated and Arx is expressed. One well-studied methylation locus is the CpG-rich UR2 region located upstream of the transcription start site, from −2,103 to around −1,992 nt (Papizan et al., 2011). Bisulfite sequencing analysis of the Arx upstream promoter region revealed that a vast majority of CpG dinucleotides in the UR2 region were methylated in MIN6 cells, but hypomethylated in αTC1 cells (Figure S9A), consistent with previous findings (Dhawan et al., 2011). Intrinsic FCoR expression level was low in αTC1 cells and high in MIN6 cells (Figure S4A). Therefore, to investigate the effects of FCoR on Arx methylation, we overexpressed FCoR in αTC1 cells (through infection with adenovirus encoding FLAG-FCoR) and we knocked down Fcor in MIN6 cells (through infection with adenovirus encoding FCoR-targeting shRNA). Bisulfite sequencing of the UR2 region revealed that methylation in the UR2 locus was significantly increased in FCoR-overexpressing αTC1 cells (Figure 6A) and was significantly decreased in FCoR-knockdown MIN6 cells (Figure 6B). Methylation of the CpG-rich UR2 region enables binding of methyl CpG binding protein 2 (MeCP2) to this area (Dhawan et al., 2011). We next conducted a chromatin immunoprecipitation (ChIP) assay in αTC1 cells infected with adenovirus encoding FLAG-FCoR or LacZ. The results revealed that MeCP2 bound to the UR2 regulatory region of the Arx promoter, whereas we observed no binding in αTC1 cells infected with adenovirus encoding LacZ (Figure S9B).

DNA methylation requires conducive histone modifications at target genes (Martin and Zhang, 2007). For this reason, we explored histone modification associated with Arx in αTC1 cells overexpressing FCoR. A ChIP assay using anti-H3K4 trimethylation (H3K4me3) antibody, which is implicated in activation of transcription (Kouzarides, 2007), in αTC1 cells overexpressing FCoR showed significantly low levels of H3K4me3 at the UR2 region of Arx promoter compared with control αTC1 cells. This result indicates that FCoR overexpression induces a repressed chromatin state of the UR2 region of Arx promoter (Figure S9C). Furthermore, a ChIP assay using anti-H3K9 trimethylation (H3K9me3) showed significantly low levels of this histone modification at the Arx locus in Fcor-knockdown MIN6 cells compared with SCR-MIN6 cells (Figure S9D). Because H3K9me3 is implicated in the silencing of euchromatic genes as well as in forming silent heterochromatin, which is transcriptionally inert (Berger, 2007), a low level of H3K9me3 in the Arx UR2 region of Fcor-knockdown MIN6 cells indicates an open chromatin state and active transcription of Arx.
Figure 5. **Arx Is a Target Gene of Foxo1**

(A) Foxo1 induces Arx expression in MIN6 cells. MIN6 cells were transduced with adenoviruses encoding LacZ (white bar) or HA-ADA-Foxo1 (gray bar) and harvested at 48 h after transduction. Data represent means ± SEM from three independent experiments. *p < 0.05 and **p < 0.005 by one-way ANOVA.

(B) ChIP assay of MIN6 cells transduced with adenoviruses encoding LacZ (white bar) or HA-ADA-Foxo1 (gray bar) and harvested at 48 h after transduction. Samples were subjected to immunoprecipitation with anti-HA, followed by PCR amplification of the UR2 region. Data represent means ± SEM from three independent experiments. *p < 0.05 by one-way ANOVA.

(C) Effect of Foxo1 on Arx promoter activity. Data were obtained from 10 experiments and are shown as mean ± SEM of the fold-change from mock vector-transfected activity. Data represent means ± SEM from three independent experiments. **p < 0.005 by one-way ANOVA.

(D) EMSA of Foxo1 binding to DNA. The DNA probe was a DNA fragment of 31 base pairs covering the consensus Foxo1-binding site (−1552/−1547 nt) of the mouse Arx promoter (lanes 1–6). A mutant DNA with an altered Foxo1-binding motif was used as a control (lanes 7–9). The position of the gel-retarded complex is indicated as A and that of the super-shifted complex is indicated as B.

(E) Oligonucleotide probes corresponding to the Foxo1-binding site of the Arx promoter were incubated with nuclear extracts in the absence or presence of increasing amounts of unlabeled wild-type (lanes 1–3) or mutant oligonucleotide (lanes 4–6). The position of the gel-retarded complex is indicated as A.
Figure 6. FCoR Regulates DNA Methylation of the Arx Promoter
(A) Bisulfite-sequencing analysis of the UR2 region of the Arx promoter in zTC1 cells transduced with adenoviruses encoding LacZ (n = 45) or FLAG-FCoR (n = 50). Quantification of the bisulfite-sequencing data is shown as %DNA methylation.
Figure 6. Continued
(B) Bisulfite-sequencing analysis of the UR2 region of the Arx promoter in MIN6 cells transduced with adenoviruses encoding shRNA of scramble sequence (SCR) (n = 45) or FcoR knockdown sequence (n = 50). Quantification of the bisulfite-sequencing data is shown as %DNA methylation. Data represent means ± SEM. *p < 0.05 by one-way ANOVA. (C and D) Bisulfite-sequencing analysis of the UR2 region of the Arx promoter in islets from control (n = 12, 263 colonies sequenced), FcorKO (n = 12, 274 colonies sequenced) and FcorKO-βFcor (n = 10, 192 colonies sequenced). (C), and quantification of the bisulfite-sequencing data is shown as %DNA methylation (D). Data represent means ± SEM. *p < 0.05 by one-way ANOVA.

These data suggest that FCoR can lead to increased DNA methylation and silent chromatin structure of the UR2 region of Arx promoter.

To confirm the effects of FCoR on methylation of the UR2 region of the Arx promoter in vivo, we performed bisulfite sequencing analysis using islets isolated from control, FcorKO, and FcorKO-βFcor. Although bisulfite sequencing data using whole islets may be less informative, the size of islets and β-cell mass of FcorKO were similar to that of control. Therefore, we thought that whole islets bisulfite sequencing data could indicate endogenous status of DNA methylation of Arx promoter in β-cells. Compared with islets from control and FcorKO-βFcor, islets from FcorKO showed significantly fewer methylated sites (Figures 6C and 6D). Higher DNA methylation in islets isolated from FcorKO-βFcor may be the result of excessive expression level of FCoR compared with endogenous FCoR expression level (Figure S2B). These data indicated that FCoR induced methylation of the UR2 region of the Arx promoter.

Foxo1 Inhibits Methylation of the Arx Promoter
As described above, DNA methylation of Arx promoter largely affects Arx gene expression level. Therefore, to investigate whether Foxo1 influences DNA methylation of the Arx promoter or not, we performed bisulfite sequencing to examine the methylation status of Arx promoters from islets isolated from control, FcorKO, DKO, and βFoxo1KO. The results revealed significantly increased methylation of the UR2 region of Arx in islets from βFoxo1KO compared with control, FcorKO, and DKO. Methylation of the Arx promoter in islets from DKO was similar to that of controls and was significantly increased compared with FcorKO (Figures 7A and 7B). These data indicated that Foxo1 decreased Arx promoter methylation and FCoR inhibits Foxo1-induced Arx hypomethylation.

Finally, to examine the molecular mechanism through which Foxo1 decreased methylation of the UR2 region of the Arx promoter, we performed a ChIP assay using MIN6 cells transduced with adenoviruses encoding LacZ or HA-ADAFoxo1. Our results showed significantly decreased Dnmt3a binding to the UR2 region in MIN6 cells transduced with adenovirus encoding HA-ADAFoxo1 compared with LacZ (Figure 7C). In contrast, the ChIP assay revealed that FCoR overexpression in αTC1 cells led to increased recruitment of Dnmt3a (but not Dnmt1) to the UR2 region of the Arx promoter (Figure 7D). These data indicated that Foxo1 bound to and released Dnmt3a from the UR2 region of the Arx promoter and that FCoR increased Dnmt3a recruitment to the UR2 region of the Arx promoter, leading to its increased DNA methylation.

DISCUSSION
Our present results confirmed that FCoR plays important roles in the regulation of pancreatic α-cell mass. FCoR expression starts around embryonic day 14.5 in both insulin- and glucagon-positive cells. However, FCoR expression declines in glucagon-positive cells, so that by embryonic day 18.5, it is restricted to insulin-positive cells. This differential pattern promotes increased Arx expression in glucagon-positive cells and decreased Arx expression in insulin-positive cells, resulting in determination of terminally mature endocrine cells.

Of note, FCoR and Foxo1 are expressed in both insulin- and glucagon-positive cells during differentiation. We could not co-stain cells with FCoR and Foxo1 because antibodies for both FCoR and Foxo1 that we used are anti-rabbit antibodies. However, as the developmental phase proceeds, Foxo1 expression becomes restricted in insulin-positive cells, as does FCoR. Interestingly, Foxo1 was strongly co-stained with glucagon-positive cells at embryonic day 15.5, the early phase of endocrine progenitor cells. In contrast, FCoR expression in glucagon-positive cells after embryonic day 14.5 was declined. These data are consistent with the previous report of Foxo1 expression in embryonic glucagon-positive cells (Kitamura et al., 2009) and that Foxo1 may be activated due to loss of FCoR in α-cell-committed cells and facilitate
α-cell differentiation at early phase of endocrine progenitor cells. FCoR loss results in decreased acetylation and increased activation of Foxo1, namely, increased nuclear Foxo1 because FCoR is a co-repressor and main acetyltransferase for Foxo1 in islets. From the present study, the most important significance of nuclear Foxo1 from Fcor loss in pancreatic endocrine cells was the induction of Arx expression. Especially, in fetal pancreatic endocrine cells, until embryonic day 14.5, FCoR was expressed in both α- and β-cells. Therefore, FCoR can inactivate Foxo1, and Arx expression is suppressed in pancreatic endocrine precursors. However, after embryonic day 14.5, FCoR expression in α-cells gradually declined, leading to Foxo1 activation and Arx induction, resulting in fate determination similar to α-cells.

Indeed, loss of Fcor causes Foxo1 activation and increased Arx expression in both differentiating pancreatic α-cells and insulin-positive β-cells, increasing both the α-cell generation and the conversion of β-to-
α-cells, ultimately raising the proportion of α-cell mass. However, the architecture of islets from the FcorKO genotype is almost normal, indicating the peripheral localization of α-cells. Several cases of β- to α-cell conversion have demonstrated that α-cells are localized in the core of the islets (Lu et al., 2018). Furthermore, the ratio of EGFP-positive cells among glucagon-positive cells was relatively low (2.47%). This low ratio of β-to-α-cell conversion might result from the period of activation of rat insulin promoter in RIP-Cre mice. It has been reported that rat insulin promoter was active in terminally differentiated β-cells (Talchai and Accili, 2015). In contrast, Fcor deletion is constitutive and occurs before defining a particular cell fate of pancreatic endocrine cells. Therefore, we could detect β-to-α-cell conversion only in adult β-cells. These findings indicate that conversion from β-cells to α-cells in the FcorKO genotype might occur in the earlier stages during differentiation of pancreatic endocrine cells and that the architecture of islets therefore should be normal. However, it is also possible that the increase in α-cell mass is due to unrestrained growth of those cells in the earlier stages during differentiation of pancreatic endocrine cells because increased Foxo1 activity due to loss-of-Fcor in embryonic α-cells may lead to α-cell hyperplasia (Kitamura et al., 2009), although we could not detect increased proliferation of α-cells in FcorKO at embryonic day 15.5.

The use of RIP-Cre to trigger FCoR overexpression specifically in β-cells of FcorKO (FcorKO-βFcor) normalized the proportion of α-cell mass attributable to the inhibition of Arx expression in cells that would otherwise have been converted from β- to α-cells. In contrast, the use of RIP-Cre to specifically delete Foxo1 in terminally differentiated β-cells does not alter the proportion of α-cells (Talchai and Accili, 2015) because decreased Arx expression due to loss-of-Foxo1 from terminally differentiated β-cells does not influence the β-cell fate. On the other hand, in αFcor, FCoR overexpression in glucagon-positive cells significantly decreased the α-cell mass compared with controls. This effect may become manifest just before the establishment of differentiated pancreatic endocrine cells, because endogenous glucagon-positive cells are reportedly detectable at embryonic day 9.5, before detection of the first insulin-positive cells at embryonic day 10.5–11 (Herrera et al., 2002). These findings suggest that FCoR overexpression using Glucagon-Cre leads to a decrease in the α-cell lineage itself and thus to a significantly decreased α-cell mass compared with controls. Of interest, DKO mice exhibited significantly greater α-cell mass compared with controls, although Arx expression level was normalized. Because FcorKO represents null knockout mice, in these animals, deletion of Fcor has occurred not only in the β-cell lineage but also in the α-cell lineage, resulting in increased α-cell lineage during fetal development of pancreatic endocrine cells. Therefore, even if deletion of Foxo1 specifically in Fcor-deleted β-cells prevents β-to-α-cell conversion, it does not affect α-cell lineage increases. These data indicate that FCoR regulates Arx expression through Foxo1. However, we cannot exclude the possibility that other factors mediate the function of FCoR in islets. Further investigation should be needed.

During the development of pancreatic endocrine cells, next to Ngn3 induction, a complex network of transcription factors, including Arx and Pax4, differentially promotes the specific endocrine fates. These two transcription factors, Arx and Pax4, have mutually antagonistic effects. The amount of Pax4 expression is up-regulated in Arx mutant mice, whereas the Arx expression is increased in Pax4-deficient pancreas (Collombat et al., 2003). This is consistent with the present study. Pax4 expression in FcorKO islets was significantly decreased compared with control islets. Furthermore, overexpression of FCoR in αTC1 cells significantly increased Pax4 expression but decreased Arx expression. In contrast, knockdown of Fcor in MIN6 cells significantly increased Arx expression but decreased Pax4 expression. However, interestingly, FcorKO islets exhibit significantly increased expression of mature β-cell markers, including Pdx1, Mafa, Neurod, and Ucn3, even if Pax4 expression is decreased. These findings in FcorKO islets are not consistent with decreased Mafa expression in Fcor-knockdowned-MIN6 cells. We speculate that decreased Mafa expression in Fcor-knockdowned-MIN6 cells is an acute effect of Fcor knockdown but that Fcor deletion in FcorKO is constitutive and has chronic effects on gene expression in islets, leading to compensatory induction of mature β-cell markers. Indeed, Mafa expression is regulated by several transcription factors, including Foxa2, Nkx2.2, and Pdx1, and glucose levels (Vanderford, 2011). Therefore, chronic effects resulting from Fcor deletion may affect expression levels of mature β-cell markers because null FcorKO exhibits glucose intolerance due to insulin resistance in adipose tissues (Nakae et al., 2012). Furthermore, these findings might indicate that the effect on glucose-stimulated insulin secretion in FcorKO may come from an α-cell-centric phenotype and these changes in β-cell-maturity-markers may be compensatory, resulting in the generation of dysfunctional α-cells, or the generation of dysfunctional β-cells, leading to decreased
insulin secretion. Indeed, bihormonal or immature cells were increased significantly in islets of FcorKO compared with control. However, further investigation is needed to elucidate the precise molecular mechanism by which loss-of-Fcor induces the generation of dysfunctional β-cells. Furthermore, as already reported, activation of Foxo1 in pancreatic β-cells leads to protection of β-cells against oxidative stress through formation of a complex with the promyelocytic leukemia protein Pml and Sirt1 to activate expression of Mafa and Neurod (Kitamura et al., 2005) (Kobayashi et al., 2012). This finding is consistent with our present data, which indicate increased expression levels of Mafa and Neurod in islets isolated from FcorKO.

The molecular mechanism by which DNA methylation regulates Arx expression has been reported. In one proposed model, a repressor complex that includes Nkx2.2, Grg3, and Dnmt3a preferentially recruits HDAC1 to the Arx promoter to silence Arx expression specifically in the pancreatic β-cells, preventing β-to-α-cell conversion (Dhawan et al., 2011; Papizan et al., 2011). Of interest, the FRE in the Arx promoter is close to the consensus Nkx2.2-binding site (Figure S8A). The binding of Foxo1 to the FRE may dissociate the repressor complex from the Arx promoter, leading to Arx promoter hypomethylation and induction of Arx expression. In contrast, inhibition of Foxo1 by FCoR may make the repressor complex assemble on the Arx promoter, leading to hypermethylation as shown in the present study. Furthermore, our present findings confirmed that the binding of Foxo1 to the Arx promoter led to Dnmt3a dissociation from the Arx promoter. However, we performed bisulfite sequencing analysis using whole islets from each genotype. Therefore, these data may be affected by differences in the α-/β-cell ratios. But, because β-cell mass of each genotype is similar and the absolute value of α-cell mass is relatively low, the differences of α-/β-cell ratios may slightly affect the results of bisulfite sequencing data.

In the present study, the findings of significantly decreased acetylation of transduced Foxo1 in FcorKO islets are notable. Deacetylated Foxo1 can be imported to the nucleus, where it can bind to the FREs of its target genes, and should be active transcriptionally (Matsuzaki et al., 2005) (Qiang et al., 2010). The main acetyltransferases of Foxo1 are reported to be CBP, p300, and PCAF (Brunet et al., 2004) (Motta et al., 2004) (Yoshimochi et al., 2010). However, little is known about the regulation of Foxo1 acetylation in islets, although the mechanism by which Foxo1 in islets is deacetylated is well described (Kitamura et al., 2005). The present data indicate that FCoR is one of the regulators of Foxo1 acetylation in islets and works upstream of Foxo1.

In conclusion, here we provide evidence that the FCoR-Foxo1 axis is an important determinant in regulating the identities of pancreatic α-cell. Elucidating the molecular mechanisms underlying the regulation of α- and β-cell masses will be an important step toward developing strategies to cure diabetes. Our present findings indicate that FCoR offers a promising target molecule for the development of therapies for type 2 diabetes.

Limitations of the Study
In the present study, we demonstrated that FCoR increased DNA methylation of Arx promoter using the whole islets. However, to clarify DNA methylation of Arx promoter in β-cells, we should perform bisulfite sequencing analysis using the sorted β-cells. Furthermore, ChIP analysis of Dnmt3a should be performed using isolated islets. These experiments will reveal the conversion of β- to α-cells more precisely.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.100798.

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Supplemental Information

FCoR-Foxo1 Axis Regulates α-Cell Mass through Repression of Arx Expression

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**Transparent Methods**

**Antibodies and Cell Cultures**

Anti-FCoR antiserum has been described previously (Nakae et al., 2012). We purchased anti-FLAG (M2) and anti-tubulin from Sigma; anti-cMyc (9E10) from Santa Cruz Biotechnology, Inc; anti-FOXO1 (L27) and anti-acetylated lysine polyclonal antibodies from Cell Signaling Technology; anti-insulin from Dako; and anti-glucagon (ab92517) from Abcam. Anti-Arx antibody was a gift from Dr. Ken-ichirou Morohashi and Dr. Kanako Miyabayashi (Kyushu University, Fukuoka, Japan) (Kitamura et al., 2002) (Miyabayashi et al., 2013), and anti-EGFP was a gift from Dr. Naoki Mochizuki (National Cerebral and Cardiovascular Center, Osaka, Japan). Anti-DNMT1 (60B1220.1) was purchased from Novus Biological; anti-DNMT3A (ab13888) from Abcam; anti-Sirt1 (07-131) from Merck Millipore; anti-HA (12CA5) from Santa Cruz Biotechnology, Inc.; anti-MeCP2 (#3456) from Cell Signaling; anti-Histone H3 (tri methyl K4) (ab213224) from Abcam; anti-Histone H3 (tri methyl K9) (ab176916) from Abcam; and anti-Ki67 (RM-9106-S1) from Thermo Fisher Scientific. HEK293, aTC1, and MIN6 cells were cultured as described previously (Nakae et al., 1999) (Efrat et al., 1988) (Ishihara et al., 1993).

**Available Mice**

RIP-Cre(Herrera, 2000), Glucagon-Cre (Herrera, 2000), FcorKO (Nakae et al., 2012), Foxo1\textsuperscript{floxed/floxed} (Paik et al., 2007), and CAG-CAT-EGFP (Kawamoto et al., 2000) mice have been previously described elsewhere. Foxo1\textsuperscript{floxed/floxed} mice were obtained from Dr. Ronald DePinho, University of Texas M.D. Anderson Cancer Center, courtesy of Dr. Domenico Accili, Columbia University.

**Animal Studies, Analytical Procedures, and Intraperitoneal Glucose and Insulin Tolerance Tests**

For the following experiments, we used only male mice, which are more susceptible to insulin resistance and diabetes. All experimental protocols using mice were approved by the animal ethics committee of the Keio University School of Medicine. Animals were fed a standard chow diet and water ad libitum, and were housed in sterile cages in a barrier animal facility with a 12-h light/12-h dark cycle. Wild-type littermates were used as controls. At weaning (4 weeks of age), the mice were started on a high-fat diet (HFD). We continued to provide the same diet, and blood glucose and insulin level measurements and glucose and insulin tolerance tests were performed as described previously (Nakae et al., 2008). All assays were performed in duplicate, and each value represents the mean of two independent determinations.
Generation of Tissue-specific FCoR Transgenic Mice

We ligated a BspE1-BamHI-BglIII adaptor (5'-CCGGAGGATCCCA-3') with pAcGFP1-C1 (Clontech) treated with BspE1 and BglII. We amplified a BglII- and EcoRI-treated PCR fragment using 5'-GGGGAGATCTTAGACTACAAGACGATGAC-3' and 5'-GGGGGAATTCTAGCATGCCTTATAGTCCC-3', with pFLAG-CMV2-FCoR (Nakae et al., 2012) as a template, and ligated this fragment with BglII- and EcoRI-treated pAcGFP1-C1 (pAcGFP1-C1-FLAG-FCoR). Next, EcoRI- and Smal-treated β-globin pA of the pBS II SK(+)CAG-CAT-EGFP vector (Kawamoto et al., 2000) was ligated with EcoRI- and Smal-treated pAcGFP1-C1-FLAG-FCoR (pAcGFP1-C1-FLAG-FCoR-β-globin pA). Finally, the BamHI-treated pAcGFP1-C1-FLAG-FCoR-β-globin pA fragment was ligated with the BamHI-treated pBS II SK(+)CAG-CAT-EGFP vector. The transgene was excised using SalI and S Aval, gel-purified, and injected into fertilized eggs from BDF1 x C57BL/6 mice. The resulting embryos were implanted into CD-1 foster mothers, and the offspring were screened for transgene transmission by PCR. Ten independent transgenic lines were obtained, among which seven founders transmitted the transgene through the germ line. To obtain F1 mice, we crossed each founder with C57BL/6 mice. We then crossed the F1 mice with Albumin-Cre transgenic mice (Okamoto et al., 2007). We sacrificed one mouse from each line in which the transgene was transmitted, and examined the transgene expression in the liver. We ultimately obtained two independent Fcor transgenic lines. The primers used for genotyping were: 5'- ATG GAC TAC AAA GAC GAT GAC –3' and 5'-CTGCACCGGTCTCATCCTTTC-3'.

RNA Isolation and Real-time PCR

We isolated total RNA from tissues and cells using the SV Total RNA Isolation System (Promega) following the manufacturer's protocol. Real-time PCR was performed as previously described (Nakae et al., 2008). The primers used in this study are described in Supplementary Table 1.

Insulin and Glucagon Secretion Studies

Pancreatic islets were isolated by collagenase digestion. The isolated pancreatic islets were preincubated for 15 min in KREBS buffer (119 mM NaCl; 2.5mM CaCl$_2$; 1.19mM KH$_2$PO$_4$; 1.19mM MgSO$_4$; 10mM HEPES, pH 7.4; and 2% bovine serum albumin) with 2.8 mM glucose, and then stimulated for 1 h with glucose or L-arginine at the indicated concentration. We measured the insulin and glucagon released in the incubation buffer using the rat insulin radioimmunoassay kit (RI-13K, Millipore) and glucagon
radioimmunoassay kit (GL-32K, Millipore), respectively. The amounts of insulin secretion were normalized according to the cellular DNA content. For measurement of islet insulin content, islets were solubilized in acid-ethanol solution (74% ethanol, 1.4% HCl) overnight at 4°C before insulin measurement. The amounts of insulin content were normalized according to the numbers of islets.

Bisulfite Sequencing Analysis

Bisulfite conversion of DNA isolated from αTC1 or MIN6 cells, or from isolated male mice pancreatic islets, was subjected to bisulfite conversion using the EZ DNA Methylation™ Kit (Zymo Research). Bisulfite-treated DNA samples were used for PCR using Arx UR2 primers (Dhawan et al., 2011). PCR products were gel purified using the QIAquick® Gel Extraction Kit (QUIAGEN). Purified products were cloned using the Mighty TA-cloning Kit (Takara), and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher) with the M13 Primer RV (CAGGAAACAGCTATGAC).

Chromatin Immunoprecipitation (ChIP) Assay

αTC1 or MIN6 cells were seeded onto 10 cm-culture dishes and transduced with adenovirus encoding constitutively nuclear HA-tagged ADA-Foxo1. At 36-48 h after transduction, the cells were fixed with 1% formaldehyde at 37°C for 1 h. The DNA solution for ChIP PCR was prepared according to the protocol for the Chip Assay Kit (Upstate). Immunoprecipitation was performed using anti-HA (12CA5; Santa Cruz Biotechnology, Inc.), anti-MeCP2 (#3456; Cell Signaling), anti-Histone H3 (tri methyl K4) (ab213224, Abcam), anti-Histone H3 (tri methyl K9) (ab176916, Abcam), anti-DNMT1 (60B1220.1, Novus Biological), anti-DNMT3A (ab13888, Abcam), or an equal amount of normal mouse IgG (Santa Cruz Biotechnology, Inc). Samples were subjected to PCR amplification of the UR2 region of the Arx promoter (Dhawan et al., 2011).

Construction of Arx Promoter-directed Luciferase Reporter Vectors

From mouse genomic DNA, we PCR amplified several DNA fragments containing the mouse Arx promoter using the following primers: forward primers, 1813Ccr2 5’-GGGGAGATCTTCCACCATTGAGGTACGGAAAAC-3’ and 1432Ccr2 5’-GGGGAGATCTAGAGAGGAAAACCTGGGCTTGGATT-3’ (underlining indicates the BglII recognition site); reverse primer, 5’-GGGGAAGCTTGCTCGTTCTTTCCTTGGGCTC -3’ (underlining indicates the HindIII recognition site). Their nucleotide sequences were verified by DNA sequencing. Then the Arx promoter fragments were treated with BglII- and HindIII-treated luciferase reporter pGL3-
Basic vector (Promega, Madison, WI).

**Site-directed Mutagenesis**

To alter the consensus Foxo1 response element (FRE) in the Arx promoter of the PGL3-Basic vectors, we performed site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Mutant 1 vector was constructed using the following primers: 5’-ATCCAAGAGTTATGCAGAGGGGAATGGCTGGGGGAAAAATC-3’ and 5’-GATTTTTCCCCAGCCATCCCCATCTGCATAACTCTTGGAT-3’ (mutant bases are indicated by bold font and underlining). The Mutant 2 vector was constructed using the following two primers: 5’-ATCCAAGAGTTATGCAGAGGGGAATGGCTGGGGGAAAAATC-3’ and 5’-GATTTTTCCCCAGCCATCCCCATCTGCATAACTCTTGGAT-3’ (mutant bases are indicated by bold font and underlining). The Mutant 3 vector was constructed using the following two primers: 5’-ATCCAAGAGTTATGCAGAGGGGAATGGCTGGGGGAAAAATC-3’ and 5’-GATTTTTCCCCAGCCATCCCCATCTGCATAACTCTTGGAT-3’ (mutant bases are indicated by bold font and underlining). Mutated nucleotides were confirmed by DNA sequencing.

**Luciferase Assay**

For the mouse Arx promoter luciferase assay, MIN6 cells were plated onto 12-well culture dishes. Cells were grown to 70%-80% confluence, and transfections were performed using 1.5µg of pGL3/Basic-Arx reporter vector, and/or 0.6µg of pFLAG-CMV-2 empty vector or constitutively nuclear FLAG-CMV-2-CNFoxo1 expression vectors(Nakae et al., 2006). The synthetic renilla luciferase reporter vector (phRL-SV40; Promega) (10ng) was used as an internal control of transfection efficiency. After transfection, cells were cultured in DMEM containing 15% foetal calf serum and 0.0005% β-mercaptoethanol. Following a 36-h incubation, the cells were harvested for the luciferase assay.

**Electrophoretic Mobility Shift Assay (EMSA)**

From HEK293T cells transfected with PCMV5/cMyc-WTfoxo1, we isolated nuclear extracts using the NE-PER extraction reagents (Pierce). The DNA probe was a 31-bp DNA fragment covering the consensus FRE (-1565/-1535 nt) of the mouse Arx promoter: (5’-AGAGTTATGCAGATTTTATGGCTGGGGGGA-3’). The mutant probe contained five bases substitutions within the consensus FRE (underlined letters): 5’-
AGAGTTATGCAGA GGGGGTGCTGGGGGGA -3'. The oligonucleotides were labelled with ³²P-ATP using the MEGALABEL™ DNA 5'-End Labeling Kit (Takara Bio, Inc., Shiga, Japan). EMSA was performed as previously described (Nakae et al., 2003). For the super shift assay, we incubated anti-cMyc or normal mouse IgG with nuclear extracts at 4°C for 20 min, added labeled probes, and performed another 20-min incubation at room temperature. Competitor wild-type and mutant probes were incubated in the reaction mixture for 20 min prior to the addition of the radiolabelled probe.

Construction of Adenoviral Vectors and Adenoviral Transduction

Previous reports describe the adenoviral vectors encoding cMyc-FCoR (Nakae et al., 2012) or HA-ADA-Foxo1 (Nakae et al., 2001), and the adenovirus vectors for FCoR knockdown (Nakae et al., 2012). To construct an adenoviral vector encoding FCoR, we amplified the FLAG-FCoR cDNA fragment using the pFLAG-CMV2-FCoR expression vector (Nakae et al., 2012) as a template and the following primers: 5’- GG GCTAGC (NheI) ATG GACTACAAAGACGATGACGAC-3’ (sense) and 5’-GG GCTAGC (NheI) CTAGCACATGCCTTATGTC-3’ (antisense). After treatment with NheI, the PCR fragment was subcloned into a NheI-treated pShuttle2 vector (Clontech). Following sequencing of the vectors to confirm that they had the intended sequences, and confirming protein expression in HEK293 by transient transfection, a fragment treated with I-CeuI- and PI-SceI was subcloned into Adeno-X viral DNA (pAdeno-X-FLAG-FCoR) (Clontech). The adenovirus vector was generated by transfecting HEK293 cells with the pAdeno-X-FLAG-FCoR plasmid. αTC1 or MIN6 cells were grown to 70%-80% confluence, and then transduced every day with adenovirus preparations encoding cMyc-FCoR, FLAG-FCoR, or FCoR-shRNA at different MOI for 7-8 h and removed from the culture dish. The cells were harvested after 48 h.

Western Blotting

We homogenized tissues and lysed cells in buffer containing 50mM Tris HCl (pH 8.0), 250mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (Roche Diagnostics). The lysis solution was centrifuged to remove insoluble material, and the proteins in 30 µg of lysate were separated using 8% or 14 % SDS-PAGE. Western blotting was performed using the indicated antibodies. Immunoprecipitation was performed as previously described (Cao et al., 2006).

Immunohistochemistry, Immunofluorescence and Histological Analysis

For histological analysis, dissected pancreases were fixed in 10 % paraformaldehyde
and embedded in paraffin blocks. Tissues were cut in 5µm section by a microtome and used for immunohistochemical staining. 4 pancreatic sections from 3-7 mice from each genotype were sampled 150-200 µm apart. For α- and β-cell, each section was covered systematically by accumulating images from non-overlapping fields with fluorescence microscope (BZ-9000, KEYENCE) using a 10 x objective for whole pancreas sections. All morphometric analyses were performed using ImageJ software. Briefly, individual channels were converted to 8 bits grayscale and measurement scale was converted from pixels to microns. An identical threshold was applied to all images from the same channel to exclude background signals and further converted to binary format before automated analysis of weight. Sections in the lineage tracing study were imaged on Olympus Fluvowview V3000 confocal microscope or KEYENCE BZ-X800 fluorescent microscope at 400X. Z-stack images were taken using KEYENCE BZ-X800 and was transformed into 3D images.

**Statistical Analysis**

We calculated descriptive statistics using one-way ANOVA with the Fisher’s test. All data are expressed as mean ± standard error. Significance was set at p<0.05.

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Figure S1

**A** IPGTT

**B** ITT

**C** Relative mRNA abundance (\(\text{Slc2a2} / \text{Gapdh}\))

**D** Insulin content (ng/Islets)

**E** Foxo1, Insulin, merge

**F** % of Foxo1-positive cells

**G** Foxo1, Glucagon, merge

**H** % of Foxo1-positive cells
Figure S1. Related to Figure 1. Glucose Metabolism and Foxo1 Expression in Fetal Pancreas.
(A) Intraperitoneal glucose tolerance test (IPGTT) of control (n=11) and FcorKO (n=12) at the age of 20 weeks. Data are means ± SEM. *P < 0.05 by two-way ANOVA with Fisher's test.
(B) Insulin tolerance test (ITT) of control (n=11) and FcorKO (n=14) at the age of 20 weeks. Data are represented as % of basal glucose level and means ± SEM. *P < 0.05 by two-way ANOVA with Fisher's test.
(C) Expression levels of Glut 2 (Slc2a2) and Glucokinase (Gck) in islets isolated from control (white bar) (n=4) and FcorKO (black bar) (n=4) at the age of 20-24 weeks. Data represent means ± SEM.
(D) Insulin content of islets isolated from control (n=4) and FcorKO (n=4) at the age of 20-24 weeks. Data are represented as insulin content (ng) per islet and means ± SEM.
(E)(F) Representative images of pancreatic islets for Foxo1 and insulin from embryos (n=3) at embryonic days 15.5 (E15.5), 17.5 (E17.5), and postnatal week 5 (E) and the percentages of Foxo1-positive cells among insulin-positive cells (F). Data represent means ± SEM. * P <0.05 by one-way ANOVA. Scale bar, 20 µm.
(G)(H) Representative images of pancreatic islets for Foxo1 and glucagon from embryos (n=3) at embryonic days 15.5 (E15.5), 17.5 (E17.5), and postnatal week 5 (G) and the percentages of Foxo1-positive cells among glucagon-positive cells (H). Data represent means ± SEM. * P <0.05 by one-way ANOVA. Scale bar, 20 µm.
A. β-cell targeting

βFcor → RIP → Cre → β-globin pA

CAG → CAT → FLAG-Fcor

B. 1: control
2: βFcor
3: αFcor

FCoR

FLAG

Tubulin

C. Control | βFcor

| FLAG | Glucagon | DAPI |

1: control
2: βFcor
3: αFcor

D. Fcor+/−

CAG → CAT → FLAG-Fcor

β-globin pA

FcorKO

FcorKO-βFcor

βFcor

Figure S2
Figure S2. Related to Figure 2. Generation of β-Cell-Specific Fcor Transgenic Mice
(A) Construction of β-cell-specific Fcor transgenic mice (βFcor).
(B) Western blotting of islets isolated from control, βFcor, and αFcor with anti-FCoR, anti-FLAG, and anti-tubulin antibodies.
(C) Representative images of immunofluorescence of islets from control and βFcor using anti-FLAG and anti-glucagon antibodies. Scale bar, 20 µm.
(D) Generation of FcorKO-βFcor.
A. Glucagon Cre-α-cell targeting

\[ \alpha \text{-cell targeting} \quad \text{Glucagon} \quad \text{Cre} \quad \beta \text{-globin pA} \]

B. Control | αFcor

| FLAG | Glucagon | DAPI |

C. α-cell mass

| Control | αFcor |
|---------|-------|
| 0.035   | 0.04  |

β-cell mass

| Control | αFcor |
|---------|-------|
| 2.0     | 2.5   |

D. Relative mRNA abundance (Fold of basal state)

| Pdx1 | Ngn3 | Arx | Pax4 | Foxa1 |
|------|------|-----|------|-------|
| 1.5  | 1.2  | 1.0 | 2.0  | 3.0   |

Figure S3
Figure S3. Related to Figure 2. Generation of α-Cell-Specific Fcor Transgenic Mice
(A) Construction of α-cell-specific Fcor transgenic mice (αFcor).
(B) Representative images of immunofluorescence of islets from control and αFcor using anti-FLAG and anti-glucagon antibodies. Scale bar, 10µm.
(C) Quantification of α-cell and β-cell mass in control (n=7 mice; 174 islets measured) and αFcor (n=5 mice; 166 islets measured).
(D) Gene expression in islets isolated from control (white bar) and αFcor (gray bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.
Figure S4. Related to Figure 2. Endogenous Expression Levels of Islet-specific Genes in Cell Lines.

(A) Fcor Expression in αTC1 and MIN6 Cells. Fcor expression in αTC1 and MIN6 cells. Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(B) Fcor and Arx expression in αTC1 cells transduced with adenoviruses encoding LacZ (white bar) and FLAG-FCoR (gray bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(C) Expression levels of islet-specific genes in αTC1 cells transduced with adenoviruses encoding LacZ (white bar) and FLAG-FCoR (grey bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(D) The effect of FCoR knockdown on Arx expression in MIN6 cells. Fcor and Arx expression in MIN6 cells transduced with adenoviruses encoding scramble-shRNA (Scr) (white bar) and Fcor knockdown-shRNA (FcorKD) (black bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(E) Expression levels of islet-specific genes in MIN6 cells transduced with adenoviruses encoding scramble-shRNA (Scr) (white bar) and Fcor knockdown-shRNA (FcorKD) (black bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.
Figure S5
Figure S5. Related to Figure 3. β-Cell Tracing Analysis of FcorKO
Construction of β-cell tracing mice.
**Figure S6**

- **Glucagon**
- **DAPI**
- **Ki67**
- **DAPI**
- **merge**

**Control**

**FcorKO**
Figure S6. Related to Figure 3. Glucagon and Ki67 staining of fetal pancreas from control and FcorKO.
Representative images of fetal pancreas for glucagon and Ki67 from control and FcorKO at embryonic day 15.5. Scale bar, 20µm.
Figure S7
Figure S7. Related to Figure 5. Expression of Foxo Family Genes and Arx in Islets.

(A) Gene Expression Levels of Foxo Family Members in Islets. Gene expression levels of Foxo family members in islets isolated from 16-week-old controls (n=4), FcorKO (n=4), DKO (n=4), and βFoxo1KO (n=4). Data represent means ± SEM from three independent experiments. *P<0.05, and **P<0.005 by one-way ANOVA.

(B, C) Arx expression in islets from control, FcorKO, βFoxo1KO, DKO. Representative images of pancreatic islets for Arx and glucagon (B), or Arx and insulin (C) from control, FcorKO, βFoxo1KO, DKO at the age of 24 weeks. Scale bar, 50 µm.
Figure S8

A

Foxo responsive element (FRE) (-1552～-1547)

GAGTTATGCAGATGTTTA
TGGCTGGGGGGAAAAATCCCAGTTAGGTATCCTTGTATT

Mouse Arx

GAGTTATGCAGATGTTTA
TGGCTGGGGGGAAAAATCCCAGTTAGGTATCCTTGTATT

B

FRE(-1576～-1571)

Human ARX

GAGTTATGCAGATTGTTTA
TGGCTGAAAAAAATCCCAGTTATGCATCCTTGTATT

Mouse Arx

GAGTTATGCAGATTGTTTA
TGGCTGGGGGGAAAAATCCCAGTTATGCATCCTTGTATT

FRE(-1552～-1547)
Figure S8. Related to Figure 6. Analysis of Mouse Arx Promoter Region
(A) Sequencing data of the Arx promoter region around the Foxo responsive element and NKX2.2 binding element.
(B) Conservation between human and mouse sequences of the Arx promoter region near the Foxo responsive element (FRE).
Figure S9

A

\[ \alpha \text{TC} \]

\[ \text{MIN6} \]

B

|          | LacZ | FCoR |
|----------|------|------|
| MeCP2    |      |      |
| IgG      |      |      |
| Input    |      |      |

C

|          | H3K4me3 |
|----------|---------|
| LacZ     |         |
| FCoR     | ![Graph](image) |

D

|          | H3K9me3 |
|----------|---------|
| SCR      | ![Graph](image) |
| Fcor KD  | ![Graph](image) |
Figure S9. Related to Figure 4. DNA Methylation and Histone Modification of Arx Promoter Region.

(A) Methylation Status of the UR2 Region of the Arx Promoter in αTC1 and MIN6 Cells. Bisulfite sequencing analysis of the UR2 region of Arx promoter in αTC1 and MIN6 cells.

(B) ChIP assay showing the binding of MeCP2 to the UR2 region of Arx promoter in αTC1 cells transduced with adenoviruses encoding LacZ or FLAG-FCoR, and harvested 48 h after transduction.

(C)(D) ChIP assay comparing the levels of H3K4 trimethylation (H3K4me3) (C) and H3K9 trimethylation (H3K9me3) (D) at the UR2 region of the Arx promoter in αTC1 cells transduced with adenoviruses encoding LacZ or FCoR (C), and in MIN6 cells transduced with adenoviruses encoding scramble shRNA (SCR) or Fcor-knockdown shRNA (Fcor KD) (D).

Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.
Table S1. Related to Figure 2. Primers used for real-time PCR

| Gene symbol | Sense                  | Antisense                  |
|-------------|------------------------|----------------------------|
| Arx         | GGCCGGAGTGCAAGAGTAAAT  | TCGATGCAGTAGGAGGAGAGC      |
| Pax4        | AGGAGGACTTTTTGGAATG    | ACCTGTGCGGTAGTAGCGT        |
| Pdx1        | TTCCGGAATGGGACCGGAGC   | GCCTGAACCTTGTGAGGATTT      |
| Slc2a2      | TCGAAGCAGACAGTGCCAGGA  | TCGAAGCAGACAGTGCCAGGA      |
| Mafa        | AGGAGGAGTGTACCGACTG    | CTTCTCGCTTCACAGATGTA      |
| Neurod      | ATGACCAAAATCCTACAGCGGAGG | TCTGCCGCTTGTTGCTCGT    |
| Ucn3        | AAGGCTTCCCAAGTTCTTA    | GAGGCTGCTTTGATGCTATC      |
| Ins1        | CACTTCTTACTCCCATCGT    | ACCACAAGATGCTTGGACAC      |
| Ins2        | GCTTCTTCTACACACCAGTTC  | AGCAGTCTACATACATGCCAC      |
| Neurog3     | AGTGCTCAGTTCCAAATCCCA | CGGCTTCTCGCTTGGCTG        |
| Gcg         | GATCATTTCCAGCTTCCAG    | CTGGTAAAGGTCCCTTCAGC      |
| Irx1        | CTGCGGCTACCCGCAATACC   | ATACATGCTAGCAGGAAGGTT      |
| Irx2        | CCAAGCCCCGTAACCTCCTC   | GTCCGAGCTGAGCTATC         |
| Mafb        | TCGACCTTCTCAAGTTGGAGC  | GAGATGGGTCTCGGTTGCTAG      |
| Foxa1       | ATGAGAGCAGACTGAGAACA   | TCAATGAGTTCTACGAGACCA      |
| Fcor        | ATATGCTTGCTGTTGCGCCA   | CGGTGACGTCGCCCTAC         |
| Dnmt1       | AAGAATGGTGTTGCTACAGC   | CATCCAGTGTTGCTCCCTTG      |
| Dnmt3a      | GAGGGAATCGAGCCACACCCAC | CTGGAAGGTGAGCTTGGACA      |
| Tet1        | ACACAGTGTGCTAATGCA     | AGCATGAAAGCCAGGATCCAG      |
| Tet2        | AGAGAAGACAAATCGAGAGTCCG | CCTCCGTACTCCCAAATC      |
| Foxo1       | GTCCTGGGGGAAATGTAAGT   | AGCCTGACACCAGTGCTG         |
| Foxo3a      | CAAACAGGCTACTTTGTCGCC  | GTTGTGATGATCACCAGGAGGCT   |
| Foxo4       | TGACACTCGGCCAGATCTAGC  | AGGACAGGTGTGAGAAGGATG     |
| Gapdh       | CATCCTGGGGCTACACTGGAGA | TGAGGCCCCATGGGATCCATGA    |