Converting Adult Pancreatic Islet α Cells into β Cells by Targeting Both Dnmt1 and Arx

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Cell Metabolism

Converting Adult Pancreatic Islet α Cells into β Cells by Targeting Both Dnmt1 and Arx

Graphical Abstract

Highlights

- Adult mouse islet α cells convert rapidly into β cells after Dnmt1 and Arx loss
- RNA-seq reveals a strikingly similar gene expression in converted and native β cells
- Converted α cells acquire hallmark functional features of native β cells
- Glucagon+ cells lose DNMT1 and ARX and express β cell markers in human T1D islets

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In Brief

Chakravarthy et al. dissect the mechanisms maintaining α cell identity and reveal that simultaneous inactivation of the DNA methyltransferase Dnmt1 and the transcription factor Arx in adult mice drives the conversion of α- to β-like cells. In human T1D islets, glucagon+ cells lose DNMT1 and ARX expression and express β cell markers.

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Converting Adult Pancreatic Islet α Cells into β Cells by Targeting Both Dnmt1 and Arx

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SUMMARY

Insulin-producing pancreatic β cells in mice can slowly regenerate from glucagon-producing α cells in settings like β cell loss, but the basis of this conversion is unknown. Moreover, it remains unclear if this intra-islet cell conversion is relevant to diseases like type 1 diabetes (T1D). We show that the α cell regulators Aristalless-related homeobox (Arx) and DNA methyltransferase 1 (Dnmt1) maintain α cell identity in mice. Within 3 months of Dnmt1 and Arx loss, lineage tracing and single-cell RNA sequencing revealed extensive α cell conversion into progeny resembling native β cells. Physiological studies demonstrated that converted α cells acquire hallmark β cell electrophysiology and show glucose-stimulated insulin secretion. In T1D patients, subsets of glucagon-expressing cells show loss of Dnmt1 and ARX and produce insulin and other β cell factors, suggesting that Dnmt1 and Arx maintain α cell identity in humans. Our work reveals pathways regulated by Arx and Dnmt1 that are sufficient for achieving targeted generation of β cells from adult pancreatic α cells.

INTRODUCTION

Restoration of lost or diseased cells is a focus for intensive efforts in developmental and regenerative biology. Pancreatic islets are a paradigm for investigating organ regeneration, reflecting growth in our understanding of the development and maturation by the principal islet cell types (which include insulin [INS]+ β cells, glucagon [GCG]+ α cells, and somatostatin [SST]+ δ cells). Understanding the mechanisms maintaining islet cell fate and function is important for addressing the urgent challenge of restoring islet β cell and α cell function, which is compromised in diseases like type 1 diabetes (T1D). Prior studies have demonstrated that mouse α cells or δ cells can convert into insulin-producing cells following extreme experimental (>99%) β cell ablation; in the case of α cells, about 1% convert toward an insulin-producing fate, without detectable proliferation over a period of 6–7 months (Thorel et al., 2010; Chera et al., 2014). However, the genetic or epigenetic basis of this conversion, including the extent or heterogeneity of reprogramming by individual adult α cells, has not been elucidated. Thus, it remains unknown whether α cell gene targeting in adult mice could enhance conversion into β cells.

Maintenance of fate and function by adult cells likely reflects both genetic and epigenetic mechanisms (Morris and Daley, 2013). Prior studies demonstrate that the transcription factors MAFA, NKX6.1, and PDX1; the proinsulin-processing enzyme PCSK1/3; and, in mice, the glucose transporter encoded by Slc2a2 are essential regulators of β cell fate and mature function (Arda et al., 2013). By contrast, mouse and human islet α cells require Aristalless-related homeobox (Arx) to specify α cell fate and maintain production of hallmark factors like glucagon (Collombat et al., 2003, 2007; Kordowich et al., 2011; Papizan et al., 2011; Itoh et al., 2010; Mastracci et al., 2011). Ectopic expression of Pdx1, Nkx6.1, or Pax4 in α cells may be sufficient to induce β cell features in fetal or neonatal α cells (Yang et al., 2011; Collombat et al., 2009; Schaffer et al., 2013).

Surprisingly, studies of Arx inactivation in adult mouse glucagon-producing pancreatic cells have not detected clear evidence of direct α to β cell conversion (Courtney et al., 2013; Wilcox et al., 2013). In a prior study of doxycycline (Dox)-induced Arx inactivation in mice (Courtney et al., 2013), lineage tracing reflected a schedule of constitutive Dox exposure and did not distinguish ductal cell from α cell progeny. This study concluded that Arx loss in adult mice induced a program of β cell neogenesis resembling embryonic islet development, in which ductal cells expressed the embryonic islet regulator Neurogenin3 and then Glucagon and Insulin. In other work, continuous Arx inactivation from embryonic stages led to the development of polyhormonal cells (Wilcox et al., 2013). Thus, it remains unclear whether targeted Arx inactivation specifically in adult mouse α cells could induce loss of α cell features and acquisition of β cell properties.
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A
Control or αAKO → Dox (3 wk) → No Dox (0, 4 or 12 wk) → Assessment

B

C

D

E

Gcg
YFP
MafB

Gcg
YFP

Nkx6.1

Ins
YFP

MafB

Ins
Dapi

Ins
YFP

Pdx1

F

G

H

I

J

K

L

M

n = 4 mice

n = 4 mice

n = 4 mice

n = 3 mice

% YFP cells

% YFP cells

% YFP + cells

% YFP + cells

% YFP + cells

% YFP + cells

100

75

50

25

0

4 week chase

12 week chase

Gcg *Ins Neg

Gcg *Ins +

Gcg

Gcg + Sst Neg

Gcg + Sst +

% Ki67 + cells

% total YFP cells

0.5

1.0

ns

ns

control

4 wk

12 wk

(legend on next page)
In humans with T1D, blunted glucagon output in the setting of severe hypoglycemia is a frequent complication and suggests that islet α cell fate and/or function may be attenuated by disease (Cryer et al., 2003; Pietropolo, 2013). However, the molecular basis of this α cell dysfunction remains unclear.

Regulation of islet epigenetics by DNA methylation appears to be an important regulatory mechanism during α and β cell differentiation and maturation (Papizan et al., 2011; Avrahami et al., 2015; Dhawan et al., 2011, 2015), and prior studies report an unexpected degree of similarity in gene expression and chromatin modifications of α cells and β cells in mice and humans (Arda et al., 2016; Bramswig et al., 2013; Benitez et al., 2014; Morâın et al., 2012). Adult α cells and other islet cells express enzymes like DNA methyltransferase 1 (DNMT1), suggesting a requirement for these factors in maintaining α cell fate (Avrahami et al., 2015; Dhawan et al., 2011; Benitez et al., 2014). Although DNMT1 activity is best understood in the context of maintaining epigenetic “memory” in proliferating cells, recent studies demonstrate DNMT1 function in non-dividing cells (Dhawan et al., 2011). However, direct testing of in vivo DNMT1 requirements in α cells has not been described.

Here, we report that simultaneous inactivation of Arx and Dnmt1 in mouse α cells promotes efficient conversion of α cells into progeny resembling β cells in multiple ways, including insulin production, global gene expression, hallmark electrophysiology, and insulin secretion in response to glucose stimulation. Studies of glucagon+ cells in islets from a subset of humans with T1D similarly reveal loss of ARX and DNMT1, with a gain of β cell features.

RESULTS

Altered Cell Fates after Arx Loss in Adult Mouse α Cells

To determine if Arx loss in vivo directly alters adult α cell fate, we developed systems for simultaneous in vivo Arx inactivation and lineage tracing in mouse α cells (Experimental Procedures; Figure S1A, available online). We used previously described mice (Thorel et al., 2010) harboring a doxycycline-inducible Gcg-driven reverse tet transactivator (Gcg-rtTA) to direct Cre recombinase expression from a Tet-O-Cre transgene in Gcg+ α cells. Cre then activates lineage-independent YFP transgene expression from the Rosa26 locus. Intercrossed generated α cell-inducible Arx knockout (ziAKO) mice (Figure S1A) harboring a Cre-recombinase-sensitive floxed Arx allele (Fulp et al., 2008) and the three alleles described above. Briefly, in ziAKO islets, Dox exposure should stimulate Cre recombinase expression specifically in Gcg+ α cells. Cre then inactivates the floxed Arx allele and activates YFP transgene expression from the Rosa26 locus.

Over 90% of Gcg+ cells were labeled with yellow fluorescent protein (YFP) in 2-month-old control Gcg-rtTA, Tet-O-Cre, Rosa26-YFP animals exposed to Dox for 3 weeks or ziAKO animals exposed to Dox for 3 weeks, followed by a 4- or 12-week “chase” period without Dox (Figure 1A). We have previously found extremely low (0.1%-0.2%) non-specific labeling of YFP+ cells in control mice when doxycycline is given after birth (Thorel et al., 2010). Loss of Arx protein in ziAKO mouse α cells was confirmed by immunostaining (Figures S1B and S1C). We did not detect differences in glycemia during ad libitum feeding or after overnight fasting in control and ziAKO mice (Table S4). After 0, 4, or 12 weeks without Dox, we sacrificed mice and immunostained the pancreas to assess islet cell fates. By 4 weeks, 50% of YFP+ cells without Arx showed evidence of failure to maintain α cell identity. This included loss of α cell gene products like glucagon or MafB (Figures 1F, 1I, 1K, and S2F–S2T). Notably, the majority of cells (60%) co-expressing glucagon or MafB also produced gene products not observed in normal α cells, like insulin, Pdx1, or Nkx6.1 (Figures 1F–1I and S2); somatostatin; and, rarely, ghrelin or pancreatic polypeptide (Figures 1K, S3A–S3C, and S3E–S3G). Quantification of YFP+ cell phenotypes revealed that 20% co-expressed α cell (Gcg and MafB) and β cell gene products (Ins, Pdx1, and Nkx6.1; Figures 1G–1J, S1C). We did not detect changes of YFP+ cell proliferation after 12 weeks off Dox, this population of YFP+ Ins+ GcgNeg cells increased to 29% of YFP+ cells, but none expressed the mature β cell marker MafA (Figures S3D and S3H). By contrast, YFP+ cells remained 99.8% Gcg+ InsNeg in control mice (Figures 1C and 1J). In control and ziAKO mice, we scored production of the proliferation marker Ki67 and did not detect changes of YFP+ cell proliferation after 4 and 12 weeks off Dox (Figure 1J; Table S2). However, this does not exclude the possibility that α cell proliferation occurred at earlier times after Arx deletion. We also did not detect induction of Neurogenin3 (Neurog3), which encodes a bHLH transcription factor expressed in fetal pancreatic endocrine progenitor cells (Gradwohl et al., 2000; Gu et al., 2002). Thus, loss of Arx led to failure of adult mouse α cells to maintain their differentiated fates, leading to time-dependent adoption of alternate islet cell fates, mainly a population of polychromal cells, and a smaller fraction resembling islet β, δ, ε, and pancreatic polypeptide (PP) cells. These findings and approaches differ from those reported by Courtney et al., 2013, who constitutively inactivated Arx in adult Gcg+ cells and observed islet hyperplasia, with a large increase in the Ins+ GcgNeg cell number accompanied by reactivation of Neurog3 and without durable increases of polychromal Gcg+ cells expressing Sst, ghrelin, or PP (see Discussion).

Figure 1. Loss of α Cell Identity after Deletion of Arx

(A) Schematic showing experimental design for Dox treatment of knockout and control animals.

(B–I) Immunostaining showing expression of α and β cell markers (B and F) MafB, (B, C, and E–G) Ins, (C, D, and G–I) Gcg, (D and H) Nkx6.1, and (E and I) Pdx1, with YFP in control and ziAKO mice 4 weeks after Dox treatment. Yellow boxes show the specific area of the islet, which is enlarged and represented by arrows on the right to demonstrate gene expression within specific cells or sets of cells. Scale bars, 25 μm.

(J–L) Quantification of α to β cell conversion in control mice at the end of 3 weeks of Dox treatment (time 0) (J) and ziAKO mice at the end of a 4-week chase and 12-week chase (K and L).

(M) Quantification of Ki67+ YFP+ cells at the end of a 4-week chase and 12-week chase compared to controls (N = 3 mice). Bar graph data are represented as mean ± SD. N = 4 mice per time point.
Our finding that over 60% of α cells failed to express insulin after Arx loss suggested that α cell fate was likely maintained by additional regulatory factors. Dnmt1 is an enzyme that functions to regulate genome-wide gene expression by methylating cytosine residues within regulatory regions of genes. Others have reported that Dnmt1 is important for islet cell fate maintenance across species (Dhawan et al., 2011, 2015; Bramswig et al., 2013; Anderson et al., 2009). Thus, we postulated that removal of Dnmt1 might compromise α cell fate. To test this, we constructed mice harboring the alleles Gcg-rtTA, Tet-O-Cre, Dnmt1<sup>fl/fl</sup>, and Rosa26-YFP (“αiDKO mice”; Figure 2A). After Dox exposure, we detected loss of Dnmt1 in YFP+ cells; however, YFP<sup>+</sup> cells produced no detectable insulin, Pdx1, or Nkx6.1, even 10 months after DOX removal (Figures 2B and 2C). Instead, YFP+ cells maintained expression of glucagon and MafB and did not produce detectable Sst or Ghr (Figures 2D–2F). Thus, unlike targeted Arx loss in α cells, targeted Dnmt1 inactivation did not discernably alter α cell fate.

**Figure 2. α Cells Maintain Their Fate in the Absence of Dnmt1**
(A) Schematic showing experimental design for Dox treatment of knockout and control animals.
(B–E) Immunostaining showing expression of α, β, δ, and ε cell markers (E) Gcg, (E) MafB, (B and C) Ins, (B and D) Pdx1, (C) Nkx6.1, and (D) ghrelin with YFP in αiDKO mice 10 months after Dox treatment. Yellow boxes show the specific area of the islet, which is enlarged and represented by arrows on the right to demonstrate gene expression within specific cells or sets of cells. Scale bars, 25 μm.
(F) Percentage of YFP<sup>+</sup> cells that express glucagon or insulin in knockout and control mice. Bar graph data are represented as mean ± SD. N = 4 mice per time point.
α Cells with Combined Arx and Dnmt1 Loss Resemble β Cells

To test whether simultaneous loss of Arx and Dnmt1 might alter the pattern of α cell conversion in adult mice, we intercrossed mice to produce progeny permitting Dox-dependent α cell inactivation of Dnmt1 and Arx combined with Rosa26-YFP lineage tracing (Experimental Procedures; Figure S1A). Mice with the six alleles Ggc-rtTA, Tet-O-Cre, ArxY′, Dnmt1′′, and Rosa26-YFP (hereafter, “iADKO mice”) and controls were exposed to Dox for 3 weeks, followed by 4 or 12 weeks without Dox (Figure 3A). Loss of Arx or Dnmt1 in α cells, labeled with over 90% efficiency by YFP, was confirmed by immunostaining (Figures S1B–S1E and S4U–S4Y).

After 4 weeks, immunostaining revealed that 50% of YFP+ cells failed to maintain α cell identity, showing either loss of α cell products like glucagon and MafB or co-expression of glucagon and MafB with gene products characteristic of β cells like insulin, Nkx6.1, and Pdx1 (Figures 3B–3D, 3G, 3I, and S4A–S4T) or δ cells (Sst; Figures 3F and 3J). At 4 weeks, 23% of YFP+ cells in iADKO mice co-expressed α and β cell gene products like glucagon and insulin, and 16% expressed β cell gene products like Pdx1 and insulin without detectable glucagon or MafB (Figures 3J and S4A–S4T). Only 14% of YFP+ cells produced Sst (Figure 3J). By contrast, we did not detect production of PP or ghrelin in YFP+ cells. After 12 weeks, immunostaining revealed that 82% of iADKO YFP+ cells had lost their α cell fate. In total, 50% of YFP+ cells had lost the expression of either Ggc or MafB or both and produced β cell factors, including insulin, Pdx1, and Nkx6.1 (Figures 3B–3G, 3K, and S4A–S4T). Like in iAKO mice, we did not detect changes in Ki67 or Neurog3 in iADKO YFP+ cells (Figures 3L and S4Z).

By 12 weeks, we also observed some YFP+ Ins+ GgcNeg cells expressing the glucose transporter Slc2a2 and MafA, markers and regulators of native β cells (Figures 3H and 3I). A total of 27% of YFP+ cells in iADKO mice produced Sst (Figures 3F and 3K), and 10% co-expressed Ggc and insulin (Figure 3K). Thus, using lineage tracing and conditional genetic inactivates to inactivate Arx and Dnmt1, we observed evidence of extensive direct α cell conversion into progeny resembling β cells.

Single-Cell RNA-Seq Reveals That Converted α Cells Closely Resemble Native β Cells

To investigate further the extent of α cell conversion toward fates resembling β cells, we performed single-cell RNA sequencing (scRNA-seq) after purifying YFP+ cells and control (YFPNeg) cells from iADKO mice and control mice by fluorescent-activated cell sorting (FACS). After Dox exposure, we obtained 127 YFP+ cells at 8 weeks (“early”); light gray bars, Figure 4A) and 44 YFP+ cells at 12 weeks (“late”); dark gray bars, Figure 4A), in addition to YFPNeg control native α cells, β cells, or δ cells (black bars, Figure 4A). All YFP+ cells expressed the YFP transgene and pan-endocrine genes like chromogranin A (CHGA) and chromogranin B, demonstrating the islet endocrine origin of these cells. These did not express endocrine precursor markers such as Ngn3 mRNA (Figure 4A), similar to prior findings with α cell conversion after β cell ablation (Thorel et al., 2010; Chera et al., 2014). Consistent with our immunohistological analysis, YFP+ cells from both early and late collections clustered into three major populations after t-distributed stochastic neighbor embedding (tSNE) dimensionality-reduction analysis: (1) cells that are similar to normal α cells, (2) cells that are similar to normal β cells, and (3) cells that express other islet hormones such as Sst (Figure 4B; data not shown).

Approximately 20% of the YFP+ cells at 8 weeks exclusively express insulin and other β cell genes. The majority of the YFP+ cells from this time point maintained mRNA expression of the α cell gene MafB (Figure 4A). In addition to these distinct populations, YFP+ cells that exhibit features of two or more islet cell types (including polyhormonal mRNA expression) occurred more frequently at 8 weeks than at later times. At 12 weeks, RNA-seq revealed that nearly 80% of the YFP+ cells expressed Ins1 and Ins2, and the majority of these did not express mRNAs encoding other islet hormones. Moreover, just 7% of YFP+ cells exclusively expressed Ggc at 12 weeks. Thus, α cells in iADKO mice appeared to preferentially convert toward β cell fates. Ingenuity Pathway Analysis (IPA) of these datasets identified pathways in converted α cells that are crucial for β cell identity and function, including maturity onset diabetes of the young (MODY) signaling factors (Hnf1a, Pdx1, and Gck) (Figures 4C and 4D). RNA-seq confirmed that a subset of the converted α cells expressed many of these regulators (like Pdx1, Nkx6.1, and Glis3) and their known downstream targets, such as Scn9a, Gck, and Scl2a2, which are established effectors of β cell function (Figure 4E). Together, scRNA-seq and our analysis provide unprecedented genome-scale evidence for the range, trajectory, and extent of gene expression changes in α cells directly converting toward β cells after conditional Arx and Dnmt1 inactivation.

Electrophysiological Resemblance of Converted α Cells and Native β Cells

In electrophysiological studies, mouse α and β cells have long been distinguished by characteristic differences in the voltage-dependent inactivation of Na+ channels (Göpel et al., 2000) and, more recently, by opposing glucose-dependent exocytotic responses to serial membrane depolarization (Ferdaoussi et al., 2015; Dai et al., 2014). Our scRNA-seq studies reveal upregulation of genes mediating the β cell Na+ current (Scn9a) and contributing to β cell glucose sensing (Slc2a2) in the converted α cells, but not in the unconverted α cells from iADKO mice (Figure 4E). We postulated that converted α cells lose the electrophysiological response features of α cells and acquire β cell responses. To assess this, we dispersed islets into single cells from iADKO mice after 12 weeks of Dox treatment and measured Na+ inactivation and glucose-dependent capacitance responses in YFP+ cells. We find that Na+ current inactivation is half maximal at ~−46 and ~−96 mV in α and β cells, respectively, from control mice (n = 13 and 9 cells; Figure 5A). Non-converted α cells from the iADKO mice (InsNeg,YFP+) maintained their right-shifted Na+ current inactivation, which was half-maximal at −49 mV (n = 21 cells; Figure 5B). However, Na+ current inactivation in converted α cells from the iADKO mice (InsNeg,YFP+), resembled that of native β cells, in which most channels (~70%) inactivated half-maximally at ~94.2 mV (n = 27 cells; Figure 5B).

Glucose stimulation amplifies the exocytotic response to membrane depolarization in β cells (Ferdaoussi et al., 2015) but suppresses the response in α cells (Dai et al., 2014).
Figure 3. Expression of β Cell Genes in Murine α Cells Lacking Dnmt1 and Arx

(A) Schematic showing experimental design for Dox treatment of knockout and control animals.
(B–I) Immunostaining showing expression of α, β, and δ cell markers (B and E) Ins, (B–D and F–I) Gcg, (C) MafB, (D and E) Nkx6.1, (F) Sst, (G) Pdx1, (H) Slc2a2, and (I) MafA with YFP in αiADKO mice 12 weeks after Dox treatment. Yellow boxes show the specific area of the islet, which is enlarged and represented by arrows on the right to demonstrate gene expression within specific cells or sets of cells. Scale bars, 25 μm.

(J and K) Quantification of α to β cell conversion in αiADKO mice at the end of a (J) 4-week chase and (K) 12-week chase.

(L) Quantification of Ki67+ YFP+ cells at the end of a 4-week chase and 12-week chase compared to controls (N = 3 mice). Bar graph data are represented as mean ± SD. N = 4 mice per time point.
Accordingly, exocytosis in α cells from the control mice is suppressed by a rise in glucose (n = 16 cells) and amplified by a drop in glucose (n = 21; Figures 5C and 5D), and this is also observed in the non-converted (InsNeg, YFP+) α cells from the αiADKO mice (n = 24 and 13 cells; Figures 5E and 5F). The converted α cells (Ins+, YFP+) from the αiADKO mice, however, again resembled native β cells, in which the exocytotic response was suppressed by a drop in glucose (from 20 to 2 mM; n = 14 cells) and amplified by a rise in glucose (from 2 to 20 mM; n = 23 cells; Figures 5G–5J). Together, these studies reveal a striking functional switch from α cell to β cell phenotypes in converted mouse β cells after conditional deletion of Dnmt1 and Arx.

Glucose-Dependent Insulin Secretion by Converted α Cells and Native β Cells

Our electrophysiological studies and findings suggested that converted α cells might also functionally resemble native β cells by (1) increasing their intracellular calcium ([Ca2+]i) upon glucose stimulation and (2) secreting insulin in response to glucose, two tightly coupled functions. To assess this, we dispersed islets into single cells from control (see Experimental Procedures) and αiADKO mice after Dox treatment, FACS purified the YFP+ cells, and then measured calcium influx and insulin secretion kinetics in response to glucose by relevant FACS-purified cells using a microfluidics perifusion system.
As expected, GFP+ β cells from mouse insulin promoter-GFP (MIP-GFP) mice secreted insulin, but not glucagon, in response to glucose stimulation, and Venus+ cells from glucagon-Venus mice secreted glucagon when challenged by glucose reduction (Figures 6A, 6B, S5A, and S5B). By contrast, converted α cells from αiADKO mice secreted insulin in response to high glucose (Figure 6C). Compared to native β cells, the level of insulin secretion by converted αiADKO α cells was lower but prolonged after glucose challenge (Figures 6A and 6C). Glucagon secretion by pooled YFP+ αiADKO α cells and Venus+ control α cells from glucagon-Venus mice was similar (Figure S5C), consistent with our finding that a subset of DOX-exposed αiADKO α cells maintain glucagon expression and electrophysiological features of native α cells.

Glucose-stimulated insulin secretion in native β cells is tightly coupled to glucose metabolism, membrane depolarization, and transient intracellular increases of \([\text{Ca}^{2+}]_i\). We assessed \([\text{Ca}^{2+}]_i\) changes in isolated control β and α cells and in YFP+ αiADKO cells during exposure to basal (2.8 mM) and high (14 mM) glucose concentrations or to potassium chloride (KCl), a general membrane depolarizer. Single-cell calcium imaging revealed that KCl provoked increased \([\text{Ca}^{2+}]_i\), in β cells from MIP-GFP mice, α cells from glucagon-Venus mice, and YFP+ cells from αiADKO mice (Figures 6D–6F). After exposure to 14 mM glucose, we observe an average increase of \([\text{Ca}^{2+}]_i\), in native β cells and YFP+ αiADKO cells, but not in native α cells (Figures 6D–6F, dark gray bars). Together, our physiological studies revealed that converted mouse α cells acquired multiple cardinal functional features of normal β cells, supporting our molecular findings.

**Evidence of Altered Glucagon+ Cell Fates in Pancreas from T1D Subjects**

Impaired glucagon responses to hypoglycemia in T1D (Cryer et al., 2003; Pietropaolo, 2013) have suggested that islet α cell fates may be altered in T1D. To determine whether changes, including loss of islet DNMT1 and ARX, might occur in human T1D, we used immunohistochemistry to analyze cell-enriched transcription factor and hormone expression in pancreata from control (Figures S6A–S6F) and T1D donors.
DISCUSSION

Dissecting and controlling the mechanisms governing cell fate is a central challenge for developmental and regenerative biology (Kim et al., 2016). We investigated α cells in mice affording conditional genetics, lineage-tracing, scRNA-seq, and functional analyses and in humans with T1D and β cell destruction. To determine the genetic mechanism by which insulin-producing cells might be spontaneously regenerated from α cells, we inactivated two genes, Arx and Dnmt1, in adult pancreatic β cells and found this was sufficient for direct, efficient conversion of islet α cells into progeny resembling β cells. We investigated islet cell identity in the human T1D pancreas and discovered changes of multiple regulators in glucagon+ islet cells, including loss of ARX and DNMT1. We speculate that such changes could underlie α cell dysfunction in T1D.

Directing effective conversion of non-β cells into insulin-producing cells could be crucial for achieving regenerative goals. Studies here revealed efficient formation of insulin-expressing cells within 3 months by 50%–80% of α cells after targeted inactivation of Arx and Dnmt1. Converted α cells resembled native β cells in their electrophysiology and ability to secrete insulin in response to glucose stimulation. Thus, our histology, lineage-tracing, scRNA-seq analysis, electrophysiological, and hormone studies provided an unprecedented assessment of the trajectory of cells undergoing α to β cell conversion. Formation of insulin-producing cells after targeted inactivation of Arx alone produced fewer glucagon"neg" insulin+ cells and more polyhormonal cell types, whereas Dnmt1 inactivation alone was insufficient to induce insulin+ α cells.

Prior studies postulated that effective somatic cell conversion may require at least two steps of re-programming: a “priming” step to poise genes for alternative expression and loss (or gain) of a cell-type-specific “master” regulator (Efe et al., 2011; Shu et al., 2013). The modest or ineffective α cell conversion following Dnmt1 loss alone or Arx loss alone is consistent with this hypothesis. We speculate that Dnmt1 loss could constitute a priming step, whereas loss of Arx, a master regulator of α cell fate, is a required concurrent step to achieve α to β cell conversion. The mechanisms by which deletion of Dnmt1 contributes to loss of α cell identity are not known. However, it seems likely that the reduction in promoter/enhancer methylation within transcriptional control sequences of key β cell genes, such as Insulin, Pdx1, and Nkx6.1 (Avrahami et al., 2015, Akinci et al., 2012; Park et al., 2008), combined with loss of Arx, permits activation of these genes (Papizan et al., 2011).
In iADKO mice, production of insulin-producing cells from α cells occurred without induction of embryonic islet regulators like Neurog3. In a prior study of Dox-induced Arx inactivation in mice (Courtney et al., 2013), lineage tracing reflected a schedule of constitutive Dox exposure and did not distinguish ductal cell from α cell progeny. In other work, continuous Arx inactivation from embryonic stages led to the development of polyhormonal cells (Wilcox et al., 2013). Future studies with complementary lineage-tracing methods would reinforce the findings and conclusions of our study.

scRNA-seq evaluation of converted α cells revealed rapid, extensive, and significant induction of gene expression networks known to regulate β cell fate and function, confirming our immunohistological findings (Arda et al., 2013; Maestro et al., 2007; Boj et al., 2010; Hunter et al., 2011) (Figure 5G). Our scRNA-seq analysis also revealed differences between these converted α cells and native β cells, reflecting the observed differences in hormone secretion. For example, only a subset of the converted α cells expressed β cell regulators like MafA, Pdx1, Nkx6.1, and Scl2a2. This heterogeneity of β cell gene expression within the population of converted α cells suggests that other β cell gene regulators may require activation to promote a more complete conversion toward a β cell fate.

Electrophysiological and hormone secretion assessments of cells undergoing α to β cell conversion confirmed our scRNA-seq predictions that a majority of the converted α cells are physiologically similar to normal β cells. We showed that Na+ channel inactivation, a classical electrophysiological marker for distinguishing mouse α cells versus β cells, shifts from an α cell phenotype to a β cell phenotype in converted α cells. Moreover, we showed that converted α cells, like normal β cells, secrete insulin and show a clear switch to high-glucose amplification of excytosis in contrast to unconverted α cells, which secrete glucagon and have exocytic responses blunted by high glucose (Gylfe, 2016). We postulate that loss of Arx and Dnmt1 in α cells triggers the upregulation of gene regulatory networks controlling metabolic sensing pathways that are intrinsic to β cells. Our findings suggest that additional extrinsic signaling modulation could additively enhance the pace and quality of α to β cell conversion achieved after targeted Arx and Dnmt1 inactivation. Thus, identifying signaling pathways that regulate Arx and Dnmt1 could be useful for directing α cells toward alternate fates.

Although mouse studies by us and others provide evidence that murine α cells can undergo conversion toward a β cell fate, the relevance of these findings to humans was unclear. Bi-hormonal human pancreatic cells, including glucagon+ insulin+ cells in subjects with diabetes (Piran et al., 2014; Yoneda et al., 2013) or in cultured islets (Bramswig et al., 2013), have been documented. However, the molecular or regulatory features underlying development of these abnormal glucagon+ cells in T1D have not been described, reflecting inherent difficulties of pancreas procurement in humans with specific diseases. The finding of ARX and DNM1L loss or reduction in glucagon+ insulin+ cells from two subjects with T1D corroborates prior studies in mice and human cell lines, suggesting that Arx or Dnmt1 establishes and/or maintains α cell fate and function (Colombat et al., 2003, 2007, 2009; Avrahami et al., 2015; Gage et al., 2015). Moreover, the abnormal expression of non-α cell factors, such as NKK6.1 and PDX1, in a subset of glucagon+ cells is consistent with prior reports of impaired α cell function in T1D (Cryan et al., 2003; Pietropaolo, 2013). Here, we found glucagon+ insulin+ cells in the pancreata of two T1D donors less than 10 years of age with 4–5 years of disease, but not in three samples from older donors with a longer disease duration. The possibility that formation or maintenance of bi-hormonal glucagon+ insulin+ Dnmt1f/f cells in humans with T1D might depend on subject age, duration of disease, or other variables requires additional studies with more sampling. A previous study has reported the presence of residual insulin C-peptide in subjects with T1D of several decades’ duration (Keenan et al., 2010). Because insulin or glucagon levels in our younger subjects are not known, the physiological relevance of these bi-hormonal cells remains unclear. Nevertheless, our findings suggest a potential molecular basis for compromised human α cell function in at least a subset of T1D patients. In summary, studies of human and mouse α cells here advance the understanding of α cell defects in T1D and promote the plausibility of targeted intra-islet cell conversion for regenerative goals.

**EXPERIMENTAL PROCEDURES**

**Human Tissues**

De-identified normal human pancreas specimens and pancreas specimens from type 1 diabetic donors were obtained from the International Institute for the Advancement of Medicine (IIAM) and the Network of Pancreatic Organ Donors (nPOD) (Table 1).

**Mouse Studies**

The Glucagon-crtTA, Tet-o-Cre, R26-YFP mouse has been described previously (Theorel et al., 2010). We generated four types of mice, all harboring the α cell lineage tracing system (Glucagon-crtTA, Tet-o-cre, R26-YFP): (1) control (with only the α cell lineage tracing system), (2) iAKO (with the addition of floxed alleles to inactivate Arx, either Arx<sup>F</sup> or Arx<sup>F</sup>), (3) iADKO (with the addition of floxed alleles to inactivate both Arx-Arxf/Y or Arxf/f), and (4) iADKO (with the addition of floxed alleles to inactivate Dnmt1-Dnmt1<sup>F</sup>). To achieve lineage labeling and inactivation of Arx, Dnmt1, or both, DOX (Sigma) was administered via drinking water, which was prepared freshly every 2 days at 2 mg/mL for a total exposure of 3 weeks. After DOX removal, mice were maintained for an additional 4 or 12 weeks without DOX treatment before sacrifice. Glucagon-Venus mice, in which cells that express proglucagon are labeled by the YFP Venus, have been described previously (Reimann et al., 2008). All animal experiments and methods were approved by the Institutional Animal Care and Use Committee (IACUC) of Stanford University.

**Immunohistology and Confocal Microscopy**

Human pancreas sections were stained with antibodies against a panel of endocrine, as described previously (Chen et al., 2011). In brief, slides were

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**Table 1. List of Human Pancreas Samples**

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Disease</th>
<th>Disease Duration</th>
<th>Number of Insulin+ Cells Scored</th>
</tr>
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<tbody>
<tr>
<td>27</td>
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<td>normal</td>
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<td>not applicable</td>
</tr>
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<td>7</td>
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<td>normal</td>
<td>not applicable</td>
<td>not applicable</td>
</tr>
<tr>
<td>9</td>
<td>female</td>
<td>T1D</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
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<td>T1D</td>
<td>4</td>
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<td>T1D</td>
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<td>T1D</td>
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<td>96</td>
</tr>
<tr>
<td>38.5</td>
<td>male</td>
<td>T1D</td>
<td>32.5</td>
<td>73</td>
</tr>
</tbody>
</table>

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overnight on 35-mm dishes as described previously (Dai et al., 2011). Cells were patch clamped in the whole-cell voltage-clamp configuration, and tyramide signal amplification (T30955; Thermo Fisher) were performed for specified antibodies (Table S5). For all mouse sections, in addition to the antibodies above, anti-YFP was used to detect YFP in lineage-marked α cells, and anti-Neurog3, K67, PFP, ghrelin, and Glut2 antibodies were used. Fluorescent secondary antibodies used were from Jackson ImmunoResearch or Molecular Probes (Table S5). Stained sections were mounted with VECTASHIELD Mounting Medium with DAPI (H-1200; Vector Laboratories) and visualized using a Leica SP2 inverted confocal laser scanning microscope (Supplementary Experimental Procedures).

Immuno-morphometry

For human sections, 60–100 islets were analyzed per staining and sample. Cells co-expressing GCG and a marker representative of another endocrine cell type were quantified as a percentage of the total number of GCG+ cells counted. For mouse sections, islets were counted from four mice per genotype (Tables S1–S3). These analyses were non-randomized. The number of α cells undergoing conversion into other endocrine cell types (where “conversion” is defined as the expression of β, δ, or other endocrine cell genes in YFP+ cells) was quantified as a percentage of total YFP+ cells counted. Two-tailed Student’s t test was used to determine whether the difference in the percentage of K67+ YFP+ cells between knockout and control mice was statistically significant (p > 0.05).

Statistical Analysis

Where indicated in the legends, graphed data in the figures are represented as mean ± SD for immuno-morphometry analyses.

Flow Cytometry

Isolated mouse islets were dissociated into single cells and processed as described (Supplemental Experimental Procedures). We collected cells from a YFP+ lineage-traced population and a YFP+α, non-labeled population on a special-order five-laser FACS Aria II directly into a 96-well containing 4 μL lysis buffer with dNTPs37 for downstream scRNA-seq assays.

scRNA-Seq and Data Analysis

scRNA-seq libraries were generated as described (Picelli et al., 2014). Briefly, single cells were lysed, followed by reverse transcription, pre-amplification, DNA purification, and analysis for successful amplification products. Bar-coded sequencing libraries were prepared, and libraries were pooled and sequenced on the Illumina NextSeq instrument (Dobin and Gingeras, 2015; McKenna et al., 2010; Supplemental Experimental Procedures). Transcript counts were obtained using HT-Seq (Anders et al., 2015) and mm10 UCSC exon/transcript annotations. Pairwise distances between cells were estimated using Pearson correlation of overdispersed genes, as described (Fan et al., 2016). Subsequent hierarchical clustering was done using hclustfunction in R, and dimension reduction was used performing the TSNE method on pairwise distances (van der Maaten and Hinton, 2008). Data were also analyzed with QIAGEN IPA (www.ingenuity.com).

Electrophysiological Studies

Islets from control or αADKO mice were dispersed to single cells and plated overnight on 35-mm dishes as described previously (Dai et al., 2011). Cells were patch clamped in the whole-cell voltage-clamp configuration, and Na+ channels were activated by a depolarization to 0 mV following holding potentials ranging from −140 to 0 mV. Single-cell exocytosis was measured as described previously (Ferdaoussi et al., 2015). Briefly, cells were pre-incubated at either 2 or 20 mM glucose for 1 hr and transferred to a bath solution (Supplemental Experimental Procedures) with either 20 or 2 mM glucose ~10–30 min prior to patch clamping. Exocytosis was elicited by a series of ten 500-ms membrane depolarizations from −70 to 0 mV and monitored as increases in cell capacitance. Following the experiments, cells were immunostained for insulin and YFP to identify β cells (Ins+ only), α cells (YFP+ only), or converted α cells (Ins+,YFP+). Statistical analysis of exocytosis data was by two-way ANOVA, followed by Bonferroni post-test (p < 0.05 considered significant).

Hormone Secretion and Calcium Imaging

Hormone secretion and calcium imaging studies were performed as previously described (Adedwola et al., 2010; Xing et al., 2016; Supplemental Experimental Procedures). Briefly, islets from MIP-GFP, glucagon-Venus, and αADKO mice were dispersed into single cells, and GFP+, Venus+, or YFP+ cells were collected by FACS as described above. For calcium imaging, the sorted cells were incubated in Kreb’s ringer buffer (KRB) with 2 mM glucose and 5 μM Fura-2/AM (Molecular Probes) for 30 min, then loaded into a temperature-equilibrated microfluidic device mounted on an inverted epifluorescence microscope. KRB with 14 mM glucose or 2 mM glucose with 30 mM KCl was administered to the cells for 20 and 15 min, respectively. Dual-wavelength Fura-2/AM was excited at 340 and 380 nm (shift in excitation wavelength occurs upon binding Ca2+), and fluorescent emission was detected at 510 nm. Intracellular Ca2+ concentration was expressed as a ratio of fluorescent emission intensity (percent of F340/F380). The fluorescence signal was expressed as a change in percentage after being normalized to basal intensity levels established before stimulation. For hormone secretion studies, 5,000 GFP+ cells from MIP-GFP mice, Venus+ cells from glucagon-Venus mice, or YFP+ cells from αADKO mice were collected by FACS and loaded onto the microfluidic device. To measure insulin secretion, YFP+ cells were incubated in basal KRB with 2 mM glucose for 30 min and then stimulated with KRB containing 14 mM glucose for 30 min, followed by 2 mM glucose for 10 min. To measure glucagon secretion, YFP+ cells were incubated in KRB with 11.2 mM glucose for 30 min and then stimulated with KRB with 2 mM glucose for 30 min. Ultra-sensitive rodent insulin or glucagon ELISAs (Merdadia) were used to measure perfusate insulin or glucagon levels.

ACCESSION NUMBERS

The accession number for the scRNA-seq data reported in this paper is GEO: GSE79457.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with article online at http://dx.doi.org/10.1016/j.cmet.2017.01.009.

AUTHOR CONTRIBUTIONS


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