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The Role of Arx in Specification and Maintenance of Pancreatic Islet α-Cells

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Abstract

Correct specification and maintenance of pancreatic islet cells is an intricate process. Previous studies have demonstrated the essential role transcription factors play in this process. For islet glucagon-producing alpha-cells one such transcription factor is the aristaless-related homeobox gene (Arx). Previous studies have demonstrated that Arx is necessary and sufficient for alpha-cell specification where ablation of Arx results in complete loss of the alpha-cell lineage and misexpression leads to conversion into an alpha-cell phenotype. However, the role of Arx in maintenance of alpha-cell fate as well the impact non-null mutations of Arx have on alpha-cell development has not been explored. In this dissertation, I utilize mouse models and pancreatic analysis to address this question. My results demonstrate that Arx is necessary to maintain alpha-cell fate during development as well as in the adult. Furthermore, analysis of a non-null expansion mutation of Arx suggests dual roles for this factor during alpha-cell specification in activation of alpha-cell fate and repression of non-alpha-cell fate. These findings expand the role of Arx in the islet alpha-cell as well as provide an attractive avenue for future therapies for type II diabetes.

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THE ROLE OF ARX IN SPECIFICATION AND MAINTENANCE OF PANCREATIC ISLET α-CELLS

Crystal Wilcox

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Dedication

To my husband who has followed me on this journey and provided me with tireless love and support. Without him I would never have had the strength or courage to accomplish all that I have.

To my children, Christian and Bailey, who are the light of my life and make me laugh with their silly antics.

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ABSTRACT

THE ROLE OF ARX IN SPECIFICATION AND MAINTENANCE OF PANCREATIC ISLET α-CELLS

Crystal L. Wilcox

Catherine L. May, Ph.D.

Correct specification and maintenance of pancreatic islet cells is an intricate process. Previous studies have demonstrated the essential role transcription factors play in this process. For islet glucagon-producing α-cells one such transcription factor is the *aristaless-related homeobox gen*e (*Arx*). Previous studies have demonstrated that Arx is necessary and sufficient for α-cell specification where ablation of Arx results in complete loss of the α-cell lineage and misexpression leads to conversion into an α-cell phenotype. However, the role of Arx in maintenance of α-cell fate as well the impact non-null mutations of Arx have on α-cell development has not been explored. In this dissertation, I utilize mouse models and pancreatic analysis to address this question. My results demonstrate that Arx is necessary to maintain α-cell fate during development as well as in the adult. Furthermore, analysis of a non-null expansion mutation of Arx suggests dual roles for this factor during α-cell specification in activation of α-cell fate and repression of non-α-cell fate. These findings expand the role of Arx in the islet α-cell as well as provide an attractive avenue for future therapies for type II diabetes.

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CHAPTER I:

INTRODUCTION

The pancreas is a two-component organ responsible for maintaining glucose homeostasis and digesting nutrients.

The pancreas is an organ situated between the stomach and intestine composed of three tissue types: exocrine, endocrine, and ductal [1]. The exocrine portion of the pancreas makes up the majority of pancreatic mass [2]. It is comprised of acinar cells, which secrete digestive enzymes utilized in the duodenum of the digestive tract [2]. The endocrine portion of the pancreas, organized into small spheres called Islets of Langerhans, is responsible for producing and secreting hormones responsible for glucose homeostasis [1]. The ductal system of the pancreas is a series of tubes that connects the exocrine acinar cells to the duodenum of the intestine [1].

During embryogenesis dorsal and ventral evagination of the foregut endoderm occurs resulting in the formation of the dorsal and ventral pancreatic buds [3]. This event takes place at approximately embryonic day (E)9.5 for the dorsal bud and a day later for the ventral bud (Fig 1.1) [4]. Initially, the pancreas grows as two separate buds influenced by distinct signals from the surrounding tissues and transcriptional regulators [4]. During mid-gestation, these buds undergo branching morphogenesis and differentiation to form the mature pancreas containing the three major cell types [4]. Before birth, the ventral portion of the pancreas rotates around the gut to fuse with the dorsal portion, thereby forming the final pancreatic structure (Fig 1.1) [1].

Pancreatic development is organized into two transitions during embryogenesis (Fig. 1.1). The primary transition begins with the budding of the dorsal pancreas around

E9.5 [3]. The initial set-up of the pancreatic epithelium defines this period [3]. The secondary transition occurs between E13.5 and E15.5 and is marked by a peak of endocrine and exocrine proliferation and expansion [3]. During this time the exocrine acinar cells expand exponentially and begin to produce digestive enzymes [3]. In the endocrine compartment, the secondary transition is when the principal endocrine cells are formed and migrate to form the Islets of Langerhans [3].

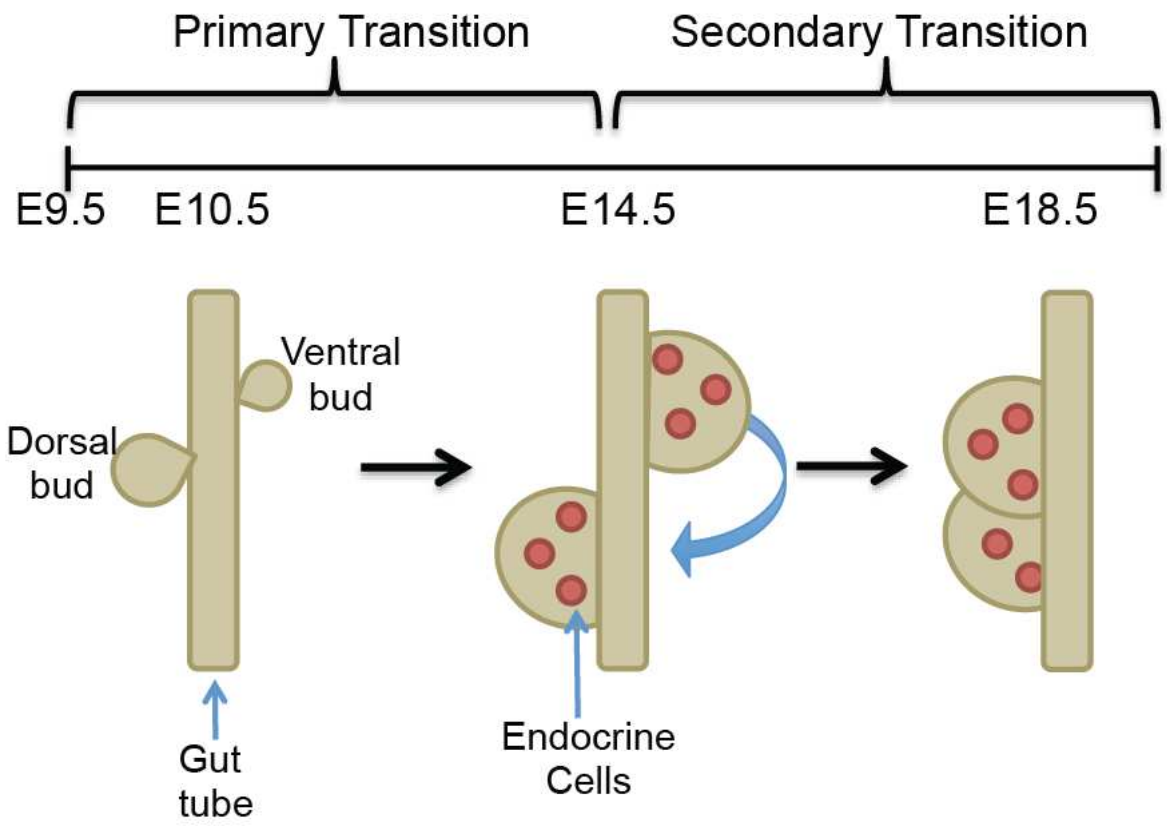


Figure 1.1: Primary and Secondary Transitions of Pancreatic Development

Figure 1.1: Primary and Secondary Transitions of Pancreatic Development. A schematic diagram depicting embryonic pancreas development. Around E9.5 dorsal and ventral pancreatic buds develop from the foregut/midgut junction of the definitive endoderm. The dorsal bud develops first, followed a day later by the ventral bud. The initial budding, proliferation, and set-up of the pancreatic structure define the primary transition. The secondary transition consists of a wave of exocrine and endocrine cellular proliferation. Finally, at ~E18, the ventral bud rotates around the gut and fuses with the dorsal bud to create the final pancreatic structure.

The endocrine Islets of Langerhans produce and secrete hormones responsible for glucose homeostasis.

The Islets of Langerhans contain five cell types: α, β, δ, PP, and ε [1]. Murine islets are comprised of a core of insulin producing β-cells with a surrounding mantle of α-, δ-, PP-, and ε-cells, which synthesize the hormones glucagon, somatostatin, Pancreatic Polypeptide, and ghrelin, respectively (Fig 1.2A) [1].

During the primary transition a few glucagon$^+$ and insulin$^+$ cells are present [5]. However, these cells do not contribute to the functional compartment of the endocrine pancreas [6]. The principal endocrine cells develop during the secondary transition. All endocrine cells are derived from a common endocrine progenitor population that is Neurogenin3$^+$ (Ngn3$^+$) [7]. Endocrine cells, first formed in the developing pancreatic ducts, undergo an endoderm-mesoderm transition to migrate into the surrounding acinar tissue and form the Islets of Langerhans [7]. This process begins during the secondary transition and is completed within the first two weeks of life [7].

The endocrine pancreas is responsible for glucose homeostasis, which is achieved through the converse actions of β- and α-cells secreting the hormones insulin and glucagon, respectively [8]. After ingestion of food, blood glucose levels rise and β-cells begin to secrete insulin [8]. Insulin signals to the skeletal muscle and adipose tissue to metabolize and store glucose, removing it from the blood stream, and consequently reducing blood glucose levels (Fig 1.2B) [8]. Conversely, during a period of fasting, such as sleep, blood glucose levels fall [9]. To maintain blood glucose levels during a

fasted state, glucagon is secreted from the pancreatic α-cells [9]. Glucagon signals to the liver to break down stored glucose and release it into the blood stream through the process of gluconeogenesis and glycogenolysis, resulting in a rise in blood glucose levels [10]. Together, these two hormones maintain proper blood glucose homeostasis.

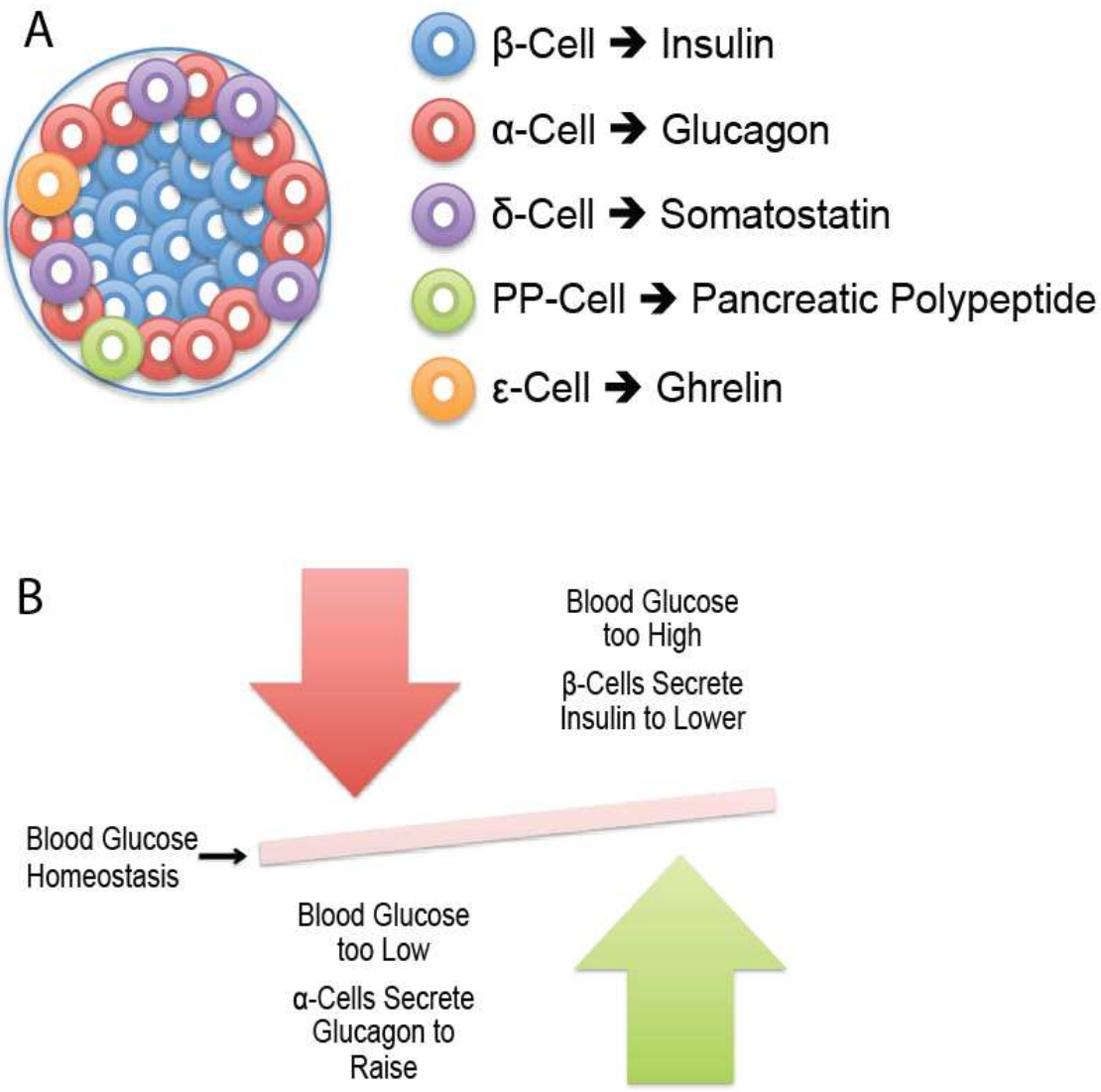


Figure 1.2: The Islets of Langerhans are composed of five different cell types and control blood glucose homeostasis.

Figure 1.2: The Islets of Langerhans are composed of five different cell types and control blood glucose homeostasis. (A): Murine Islets of Langerhans are comprised of five cell types: a core of insulin-producing β-cells (blue) with a surrounding mantle of glucagon-producing α-cells (red), somatostatin-producing δ-cells (purple), Pancreatic Polypeptide-producing PP-cells (green), and ghrelin-producing ε-cells (orange). **(B):** The converse actions of glucagon-producing α-cells and insulin-producing β-cells maintain glucose homeostasis. Under conditions of high blood glucose, β-cells secrete insulin, which causes uptake and storage of glucose and a concomitant decrease in blood glucose levels. Conversely, when blood glucose levels fall too low, α-cells secrete glucagon, resulting in the breakdown of stored energy and increased blood glucose levels.

Loss of normal glucose homeostasis results in the metabolic syndrome diabetes mellitus.

Diabetes mellitus is a metabolic syndrome characterized by the loss of normal glucose homeostasis leading to consistently elevated blood glucose levels, even during periods of fasting [11]. Clinically, there are two forms of diabetes, type I and type II. Type I, or juvenile diabetes, results from the loss of endocrine β-cells via an autoimmune attack [12]. Type II diabetes is the most common form of the disease and is the product of insulin resistance and β-cell dysfunction [12]. In type II diabetes, β-cells become exhausted and can no longer produce enough insulin to properly lower blood glucose levels [12].

In correlation with the rise in obesity, the incidence of type II diabetes over the past few decades has skyrocketed [13]. Currently, 8.3% of the United States population has been diagnosed with some form of diabetes, which translates into 25.8 million adults and children [13]. Furthermore, there are 1.9 million new cases each year [13]. Diabetes has a deleterious influence on overall health, including negative impacts on a patient's vision (glaucoma and cataracts), circulation (hypertension and neuropathy), skin (infections and inflammation), mental health, hearing, teeth (gum disease), stomach (gastroparesis), and kidneys (ketoacidosis and nephropathy), as well as dramatically increasing risk of stroke and heart attack [13]. Overall, diabetes and its related complications cost the United States $245 billion in 2012, making diabetes a global health crisis [13].

Recently, a new model underlying the clinical presentation of diabetes has been proposed. This new mechanism focuses on a bihormonal view of the disease, encompassing not only β-cell exhaustion and lack of insulin release, but also α-cell dysregulation and over secretion of glucagon [10]. In this model, β-cell exhaustion leads to loss of proper signaling to glucagon-producing α-cells [10]. This results in deregulation of α-cells, over secretion of glucagon, and further increases in blood glucose levels [14]. Additionally, continual glucagon release makes attempts to reduce blood glucose levels less effective, further compounding the problem [14].

The bihormonal view of diabetes suggests that loss of proper glucose homeostasis results from dysregulation of the entire endocrine pancreas, not just lost β-cell function and insulin secretion [10]. Thus, treatments aimed at increasing β-cell mass or function while decreasing α-cell mass/function would be extremely effective at obtaining blood glucose control [15]. Most therapies only focus on increasing insulin content, while largely ignoring the effect of glucagon in these patients, rendering current therapies largely ineffective [15]. Thus, novel treatments addressing both sides of the bihormonal theory of diabetes are essential.

Novel treatments for diabetes focus on *in vivo* replacement of β-cells.

Currently, exogenous insulin administration through daily subcutaneous injections is the most common treatment for Type I and II diabetes (Fig 1.3) [13]. While this treatment addresses the most pressing hallmark of diabetes, high blood glucose levels and reduced insulin secretion, it contains several inherent flaws. First, exogenous administration of insulin does not contain the same degree of control as endogenous β-cell sensing and insulin release. This lack of control requires constant blood glucose monitoring to ensure adequate insulin is administered to control blood glucose levels [13]. Second, exogenous insulin administration places patients at risk for severe and potentially life-threatening periods of hypoglycemia [16]. If insulin levels are forced too high this could cause blood glucose levels to fall dangerously low. Finally, exogenous insulin administration does not address the long-term problem of β-cell loss or dysfunction. Continual adjustment of insulin administration and dosage is necessary to combat insulin resistance and deteriorating blood glucose homeostasis [13]. New treatments that provide a finer degree of control and are more suitable for long-term use are critical, given the large percentage of the world population that suffers from some form of diabetes.

A novel treatment for type II diabetes has recently moved into clinical development: islet transplantation. Islets are isolated from multiple donors, pooled, and transplanted into the portal vein of the liver where the islets attach to the walls of the vein [17]. Clinical trials have demonstrated success at reducing blood glucose levels and improving diabetic patient outcomes utilizing this approach [17]. However, there are

multiple drawbacks to islet transplantation. Islets, especially β-cells, are sensitive to the immunosuppressants necessary for this procedure [17]. Furthermore, studies have demonstrated that transplanted islets have a finite life span of only five years before a patient requires either supplemental therapy or another transplant [17]. Additionally, multiple donors (usually 2-3) are necessary to obtain enough islets for a successful transplantation [17]. Overall, the current state of islet transplantation deems it unfeasible for large-scale use in treating Type II diabetes. New developments in prolonging islet life as well as improved harvesting methods might make this treatment a more attractive option in the future.

A different take on the concept of β-cell replacement is the use of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPS) to generate β-cells *in vitro*. ESCs are cells with unlimited differentiation potential originally derived from human blastocysts [18]. These cells are maintained in culture and, using the correct developmental signals, can be directed into any mature cell type [18]. Thus, ESCs can theoretically be used to create functional, mature β-cells [18]. In contrast to the finite supply available for islet transplantation, creation of new β-cells in a laboratory setting would yield a theoretically unlimited β-cell population and make transplantation procedures more feasible [18].

A variation on this method is the use of induced pluripotent stem cells (iPS cells). iPS cells are mature cells that are converted back into a stem-like fate [19]. These iPS cells can then be differentiated into a different mature cell type using the same technology as for ESCs [19]. The benefit of using iPS cells over ESCs is that iPS cells can be made from non-pancreatic tissue from the donor and thus be matched to the

recipient, eliminating the need for harmful and expensive immunosuppressive therapies (Fig 1.3) [19].

These stem cell based therapies have multiple obstacles, including lack of cellular homogeneity, inability to correctly differentiate, and questions regarding patient safety [19]. Neither the ESC or iPS fields have been able to produce mature β-cells that correctly respond to insulin in an *in vitro* setting [19]. Studies that have managed to make β-like cells report very low yield of potential β-cells from the starting stem cell population [20]. In fact, the majority of cells created are not β-cells, but rather are some other pancreatic or endoderm cell [20]. Finally, even if mature β-cells can be produced, they will require years of testing to ensure these cells do not become malignant once transplanted into recipient tissue [19]. Overall, while promising, these therapies are most likely decades away from being a treatment option for diabetic patients in a clinical setting.

Another very attractive therapy for type I and II diabetes is the use of transdifferentiation to create new β-cells *in vivo [19]*. This treatment involves altering the identity of other endogenous cells, typically pancreatic cell types, into a β-cell fate either through the loss or misexpression of specific proteins [19]. *In vivo* and *in vitro* studies have demonstrated that pancreatic cell types are relatively plastic and can be directed into a β-like-fate [21]. With the development of the bihormonal theory of diabetes, use of endogenous α-cells to create new β-cells *in vivo* has become a very appealing option [19]. Transdifferentiation of α-cells would address the bihormonal theory of diabetes by decreasing the number of glucagon-producing α-cells while

increasing the number of insulin-producing β-cells. This would provide an ideal long-term therapy for type I and II diabetic patients (Fig 1.3).

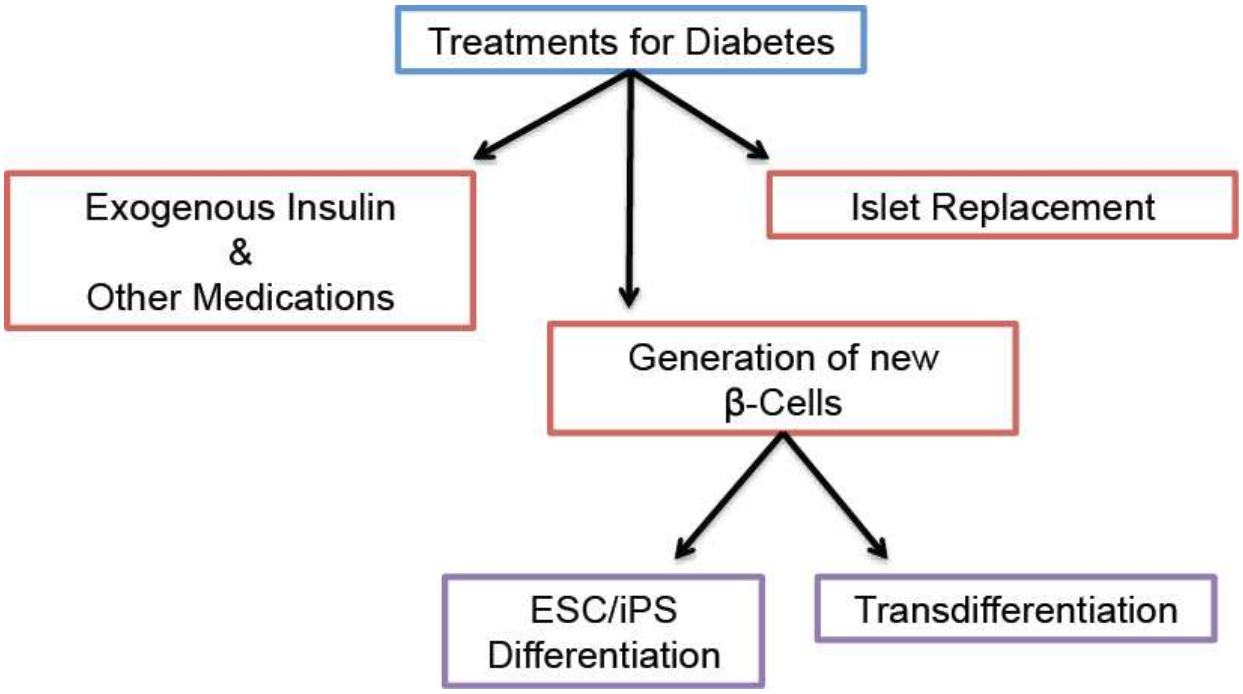


Figure 1.3: Current and novel treatment options for Type I and II diabetes

Figure 1.3: Current and novel treatment options for Type I and II diabetes. Flow chart depicting current (left most red box) and novel treatments (middle and right-most red boxes) for type I and type II diabetes. Current therapies focus on exogenous insulin replacement or boosting β-cell function (left most red box). New therapies currently in clinical development include islet transplantation (right most red box). Future therapies focus on the generation of new β-cells (center red box) either through ESC/iPS differentiation (left purple box) or transdifferentiation of other endogenous cell types (right purple box).

Transcription Factors Are Essential for Proper Pancreatic Endocrine Specification and Maintenance.

In order to develop novel therapies to treat diabetes, knowledge of normal endocrine cell development and maintenance is critical. Decades of research have demonstrated that broadly expressed (i.e. Ldb1) and cell type specific (i.e. Pdx1, etc) transcription factors are essential for the proper specification and maintenance of pancreatic endocrine cells [2].

Transcription factors are proteins that bind to DNA and activate or repress specific transcriptional programs [22]. In the endocrine pancreas, these factors are first expressed in $Ngn3^{+}$ endocrine progenitors, in which they act to narrow and define cell fate [7]. In turn these transcription factors turn on other cell-type specific proteins, eventually leading to the correct transcriptional program for each discrete endocrine cell population (Fig 1.4A) [7]. The transcriptional control of pancreatic endocrine cell specification has been most extensively studied in β-cells. Three examples of β-cell specific transcription factors that are essential for correct specification and/or maintenance are Pdx1, Pax4, and MafA (Fig 1.4B).

Pdx1 is one of the first transcription factors expressed in the embryonic pancreas, starting at approximately E8.5 [23]. Expression originates in the entire pancreatic epithelium, but is eventually restricted to β- and δ-cells where it is expressed in the mature pancreas [24]. Ablation of Pdx1 early in development results in agenesis of the pancreas (Fig 1.4B) [23]. Additionally, a β-cell specific ablation of Pdx1 during

development results in a drastic reduction in the number of β-cells [24]. Finally, in the mature pancreas Pdx1 is essential for proper maintenance of β-cell function [25]. Ablation of Pdx1 in mature β-cells leads to loss of proper insulin signaling and eventually, apoptosis [25].

Another transcription factor important in β-cell specification and development is Pax4. Pax4 expression is seen at E9.5 in both pancreatic buds, but is restricted to β-cells after E15 [26]. Ablation of Pax4 in the developing pancreas results in loss of β- and δ-cells with a concomitant increase in α- and ε-cell fates (Fig 1.4B) [26].

One of the most important transcription factors for maintenance of endocrine β-cells is MafA. MafA is first expressed at E13.5 in insulin$^+$ cells, and persists into postnatal and adult stages [27]. Ablation of MafA during embryogenesis does not have a profound impact on β-cell specification, likely due to upregulation of the closely related protein MafB (discussed later) [27]. However, MafA is directly responsible for the transcriptional regulation of insulin and other glucose-responsive genes [27]. Ablation of MafA in the mature β-cell results in loss of insulin biosynthesis and secretion (Fig 1.4B). Furthermore, studies have demonstrated that MafA is a very accurate barometer of β-cell activity status [27]. When internal glucose levels are too high, MafA expression is reduced and eventually lost, indicating β-cell dysfunction and loss of glucose homeostasis [27].

While the transcription factors described above are generally β-cell specific, there are several other factors equally important to β-cells expressed in multiple cell types.

Specifically, Nkx2.2 is a transcription factor that is expressed in all endocrine cell types, except δ-cells, starting around E9.5 [28]. Ablation of Nkx2.2 results in loss of β-, α-, and PP-cells with a concomitant increase in ε-cells (Fig 1.4B) [29]. Interestingly, in Nkx2.2 null mice there is a population of immature β-cells that don't express Glut2 or glucokinase (mature β-cell markers), but do express other markers of β-cells, suggesting Nkx2.2 is crucial for proper β-cell maturation [29].

MafB, Brn4, and Arx are α-Cell Specific Transcription Factors Necessary for Correct Specification.

Similar to endocrine β-cells, transcription factors are also important in α-cell specification and maintenance. For many years the endocrine α-cell has not garnered as much attention as its neighbor, the β-cell. However, since the proposal of the bihormonal theory of diabetes, a higher degree of attention has focused on understanding the α-cell and transcription factors important in its specification and maintenance. Much is still unknown about the α-cell, including a list of important transcription factors similar to the list for β-cells. Currently, only three α-cell specific transcription factors necessary for specification and maintenance have been identified: MafB, Brn4, and Arx.

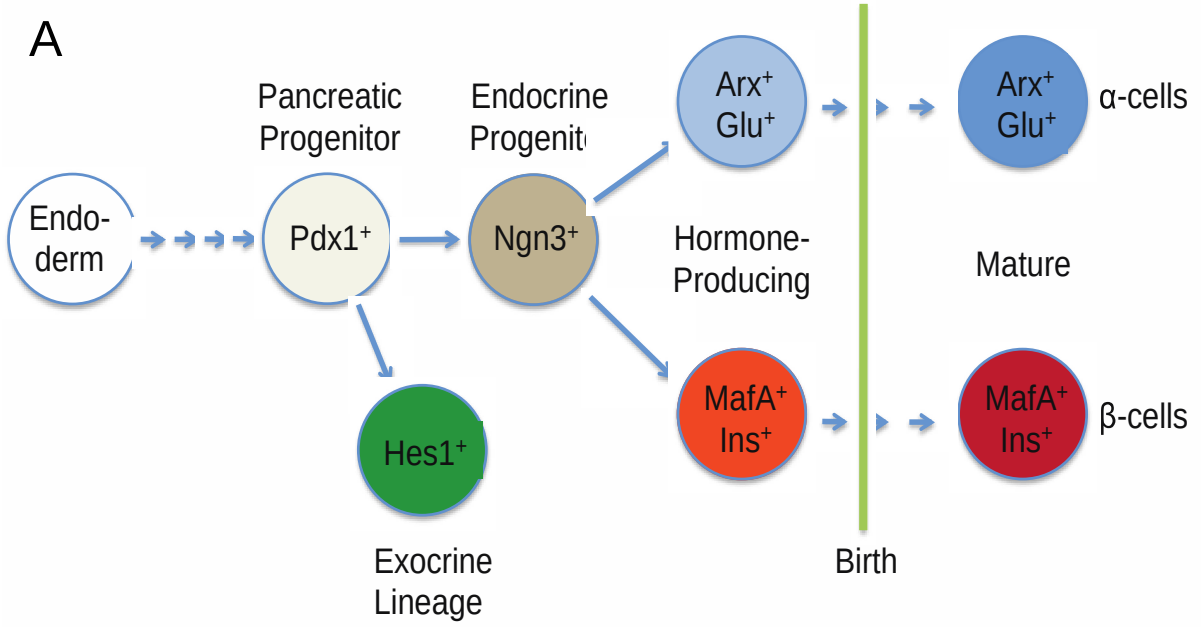
MafB is first expressed at E10.5 in hormone-producing endocrine cells (both insulin$^+$ and glucagon$^+$) [30]. It is expressed in both α- and β-cells during embryogenesis, but is restricted to α-cells after postnatal day (P)14 [30]. Ablation of MafB during embryogenesis results in α- and β-cell maturation defects [31]. In a mouse model of MafB ablation the number of glucagon$^+$ and insulin$^+$ cells is drastically reduced, although total endocrine mass is unchanged [31]. The authors demonstrate that while immature α- and β-cells do exist, these cells cannot become physiologically functional (Fig 1.4B) [31]. MafB continues to be expressed in adult α-cells from P14 onward, but a role for MafB in maintenance of α-cell fate has not yet been explored [30].

Another α-cell specific transcription factor is Brn4. Brn4 is exclusively expressed in endocrine α-cells beginning at E10 [32]. Ablation of Brn4 in mouse models does not

display a pancreatic phenotype, likely due to compensation by other factors [32]. Further studies have demonstrated that Brn4 binds to and regulates glucagon gene transcription in the endocrine α-cell (Fig 1.4B) [33].

The last transcription factor important in α-cell specification is the *aristaless-related homeobox gene* (*Arx*) [34]. Numerous studies have demonstrated that Arx is necessary and sufficient for proper specification of the endocrine α-cell, upon which I will expand further in the following section. Arx appears to be an attractive target for future transdifferentiation studies exploring novel therapies for diabetic patients.

A



B

Endocrine Transcription Factors	Embryonic Expression? Where?	Adult Expression? Where?	Ablation Phenotype
Pdx1	All	β, δ	Early: agenesis of pancreas β-cell specific: loss of β-cells
Pax4	β	β	Loss of β & δ, increas in α & PP
MafA	No	β	Loss of β-cell maintenance
Nkx2.2	α, β, ε, PP	α, β, ε, PP	Loss of β, α, and PP; increased in ε
MafB	β, α	α	Loss of α & β maturation
Brn4	α	α	None

Figure 1.4: Transcription Factors are Necessary for Proper Endocrine Specification and Maintenance.

Figure 1.4: Transcription Factors are Necessary for Proper Endocrine Specification and Maintenance. (A): Schematic depicting how transcription factors narrow endocrine fate through multiple developmental stages. Pdx1 expression defines pancreatic progenitor fate while Ngn3 expression restricts fate to the endocrine lineage. Cell specific transcription factors are expressed in $Ngn3^{+}$ endocrine progenitors and define fate for each endocrine cell lineage. Many factors continue to be expressed after birth and play a role in maintenance of cell fate. **(B):** Table outlining transcription factors important for endocrine specification and maintenance. Columns outline embryonic expression profile (2^{nd} column from left), adult/mature expression profile (2^{nd} column from right), and ablation phenotype (right most column).

Arx is necessary and sufficient for endocrine α-cell specification.

Arx is a paired homeodomain-containing transcription factor located on the X-chromosome at Xp22.13 [35]. Arx contains a variety of previously classified domains including an aristaless domain, an octapeptide domain, three nuclear localization sequences (NLS), a central acidic domain, and four polyalanine tracts [36]. Although Arx contains activation (aristaless) and repression (octapeptide and polyalanine tract 4) domains, several studies have demonstrated it mainly acts through a repressive mechanism (Fig 1.5A) [37]. Furthermore, Arx has been shown to co-localize with the co-repressors Groucho/Tranducin-like enhancer 1 (Tle1) and C-terminal binding proteins (CtBPs) [37,38]. This colocalization increases Arx's repressive ability in *in vitro* assays [37].

Previous studies have demonstrated that Arx is necessary and sufficient for α-cell specification. Global ablation of Arx results in complete loss of glucagon-producing α-cells with a concomitant increase in β- and δ-cells, maintaining normal pancreatic endocrine mass [34]. Ablation of Arx in pancreatic or endocrine progenitors demonstrates similar results, indicating this defect occurs in endocrine cell development (Fig 1.5B) [39]. Arx is Ngn3-dependent, and is first expressed in $Ngn3^{+}$ endocrine progenitors [34]. After specification, Arx becomes restricted to $glucagon^{+}$ cells, where it is maintained into postnatal life [34]. While it has been proposed that loss of Arx leads to respecification of presumptive α-cells into β- and δ-cell fates, lineage tracing has not been performed to validate this model. Arx is also sufficient to specify an α-cell fate. Forced misexpression

of Arx in the developing pancreas or islets results in the conversion of β- and δ-cells into an α- and PP-cell fate (Fig 1.5B) [40].

Contrary to pancreatic Arx ablation, loss of the β-cell transcription factor Pax4 in the developing pancreas results in loss of β- and δ-cells and a concurrent increase in α- and PP-cells (described above) [26]. Work examining the opposing roles of Arx and Pax4 in endocrine specification has demonstrated that these two factors negatively regulate each other through direct DNA binding and co-repression [34]. Loss of Arx results in a significant upregulation of Pax4 while in Pax4 mutant mice the converse is true [34]. These data suggests that Arx and Pax4 help control the proper specification of α- and β-cells through a dual repression model.

The interaction of Arx with other endocrine transcription factors has also been explored, including the interaction between Arx and Nkx2.2. Mastracci and colleagues demonstrated that loss of both Arx and Nkx2.2 causes an increase in ε-and δ-cells at the expense of α- and β-cell populations, which are completely lost in this model [41]. However, the increase in ε-cell number does not correspond to increased ghrelin transcript levels [41]. Furthermore, double mutants harbored a ghrelin^{+}/somatostatin^{+} endocrine population not observed in controls [41]. Finally, although ablation of Nkx2.2 alone results in a drastic decrease in the number of PP-cells, this cell population is restored in the double mutant mice [41]. Together these data demonstrates that there is a novel interaction between Arx and Nkx2.2 that helps regulate endocrine cell fate and specification. Future studies examining the interaction between different transcription

factors will help expand and clarify this as well as other interactions necessary for endocrine cell fate specification and maintenance.

Together these previous studies show that Arx is necessary and sufficient for proper α-cell specification in the developing pancreas. While the role of Arx in specification has been described, a possible role in cell fate maintenance has not yet been examined. Previous studies examining the role of the DNA methyltransferase, Dnmt1, in adult β-cell maintenance suggests that such a role could exist [42]. Loss of Dnmt1 results in progressive loss of DNA methylation, derepression of *Arx*, and transdifferentiation of β-cells into a glucagon^{+} α-cell fate [42]. These data suggest that similar to its role during embryogenesis, Arx is required for maintenance of α-cell fate in the adult pancreas.

Studies examining the role of Arx after specification are essential to determine a potential role for Arx in maintenance of endocrine α-cell fate. Additionally, these studies will be helpful in examining the possibility of using mature endogenous α-cells in transdifferentiation experiments for novel treatments for type I and II diabetes.

A

B

Organ	Model	Phenotype
Pancreas	Germ-line null, ablation in Pdx1^{+} cells, ablation in Ngn3^{+} cells	Complete loss of α-cells; increase in β & δ
Pancreas	Misexpression in developing pancreas/islets	Conversion of β- & δ-cells into α-cells
Pancreas	Loss of Dnmt1 in mature β-cells	Upregulation of Arx and conversion of β-cells into α-cell fate
Digestive Tract	Ablation in entire endoderm	loss of gastrin-, glucagon/GLP-1-, CCK-, and secretin-producing cell populations; increase in somatostatin-expressing cells
Muscle	Ablation of Arx in muscle progenitors	Delay in myogenesis
Testis	Ablation of Arx in developing testis	Extreme reduction in number of Leydig cells
Brain	Germ-line null	Defects in neuronal patterning, migration, differentiation, maturation, and axonal outgrowth and connectivity
Brain	Expansion of first polyalanine tract	Loss of GABA-ergic tangential migration but not radial; selective derepression of Arx targets

Figure 1.5: Arx is a homeodomain-containing transcription factor necessary for the correct development of multiple tissue types

Figure 1.5: Arx is a homeodomain-containing transcription factor necessary for the correct development of multiple cell types. (A): Diagram outlining the domain structure of the Arx protein. Arx contains an octapeptide domain, three nulclear localization sequences, four polyalanine tracts, an acidic domain, a homeodomain, and an aristaless domain. **(B):** Table outlining the role of Arx in the development of multiple tissue types. Columns depict organ, model utilized, and resulting phenotype (from left to right).

Arx plays diverse roles in the development of multiple tissues.

While the role of Arx in the developing endocrine pancreas has been described, Arx is also expressed in a variety of other tissues including the digestive tract, skeletal muscle, testis, and brain [43]. The role of Arx in these other tissues has also been examined (Fig 1.5B). In the digestive tract, ablation of Arx results in loss or severe reduction of several enteroendocrine cell populations including gastrin-, glucagon/GLP-1-, CCK-, and secretin-producing cell populations with an increase in somatostatin-expressing cells [44]. The role of Arx in the muscle and testis has also been described. During myogenesis (the development of muscle fibers) Arx acts as a positive regulator through its association with Mef2C and MyoD [45]. In testis development, ablation of Arx results in a drastic decrease in Leydig cell number due to defects in Leydig progenitor cells (Fig 1.5B) [46].

Finally, the role of Arx in neuronal development has been extensively described. Arx is first detected at the 3-somite stage [47]. After the 10-somite stage, it is confined to an area in the anterior neural plate where it is expressed throughout the telencephalic structures, including the ganglionic eminence, cerebral cortex, and hippocampus [48]. Arx null mice die within a day of birth and display severe neuronal defects [49]. Phenotypically, Arx null mice have smaller brains, olfactory bulbs, and testes [49]. There are multiple developmental problems that are responsible for the observed phenotypes. Defects exist in tangential migration from the medial ganglionic eminence to the cortex, in radial and tangential migration in the striatum, radial migration in the cortex, cholinergic neuronal differentiation, and development of GABAergic interneurons

[49]. These defects result in a thickened striatum, thinner cortical plate, lissencephaly, and severe loss of inhibitory GABAergic interneurons [49]. When taken together, these data demonstrates that Arx plays multiple roles in neuronal development, including patterning, proliferation, migration, differentiation, and axonal outgrowth and connectivity (Fig 1.5B) [47].

Mutation of Arx results in a spectrum of human mental disorders.

Similar to mouse models, mutation of ARX in human patients results in several neurological disorders [50]. X-linked mental retardation (XLMR) is a heterogeneous group of disorders that result from mutations in genes located on the X-chromosome [50]. XLMR dominantly affects male patients with some clinical defects reported for heterozygous females [50]. Mutations in ARX contribute significantly to this group of disorders. ARX mutations and the associated clinical disorders can be placed on a spectrum, where more severe mutations result in more severe clinical outcomes and vice versa [50]. This spectrum demonstrates a striking and unique genotype-phenotype correlation, suggesting that ARX is directly responsible for the clinical presentations of these patients [50].

The most severe ARX-related disorder, X-linked lissencephaly associated with abnormal genitalia (XLAG), is associated with null and missense mutations of *ARX* [47]. XLAG patients present with lissencephaly, agenesis of the corpus callosum, neonatal-onset intractable epilepsy, severe hypotonia, ambiguous genitalia, and death within the first few months of life [51]. The clinical presentation of XLAG patients closely mirrors that of Arx null mice, potentially allowing scientists to use Arx mouse models to develop treatments for patients affected by ARX-related disorders [52].

While XLAG mutations are on the most severe end of the ARX-disorder spectrum, the most common mutation is expansion of the first two polyalanine tracts of ARX [53]. Expansion of the first polyalanine tract by an additional seven alanines is

associated with the clinical disorder West syndrome, or infantile spasms (ISSX) [54]. Patients suffering from West syndrome present with infantile spasms, hypsarrythmia, and mental retardation; however, they do not usually have brain malformations (lissencephaly), and also have a longer life expectancy than XLAG patients [54].

To explore the mechanism behind the distinct differences in phenotype between XLAG-like mutations of ARX and the polyalanine expansion of ARX found in West syndrome, mice with a West syndrome expansion mutation were created [55,56]. Mice with an expansion mutation of Arx are born in normal Mendalian ratios and have brains that appear normal in size and gross morphology, unlike Arx null mice [55,56]. However, mutant mice do have seizures, and die within six months of birth [55]. Interestingly, GABAergic progenitor radial migration, which is absent in Arx null mice, appears normal in Arx expanded mice [55]. In contrast, tangential migration is lost, similar to the phenotype reported in the Arx null model [55]. Mechanistic studies behind these phenotypes demonstrate selective derepression of a subset of Arx targets in the polyalanine expansion mutant [57]. The authors attribute this selective derepression to loss of proper association with a subset of co-repressors [57]. Specifically, an expanded Arx demonstrated reduced, but not absent, association with Tle1 [57]. These studies demonstrate that expansion of Arx results in context specific defects due to selective loss of association with co-repressors.

Recently Itoh and colleagues explored the pancreatic phenotype in an ARX-null XLAG patient [58]. Interestingly, they demonstrated that similar to Arx null mice, human patients with null mutations in ARX have a complete loss of endocrine α-cells

[58]. This study demonstrates that, similar to neuronal studies, Arx null mice and XLAG patients display surprisingly similar pancreatic phenotypes. However, the question of whether, similar to the brain, there is a genotype-phenotype correlation associated with ARX/Arx mutation severity and the specification and maintenance of endocrine α-cells remains unanswered. Future studies using non-null Arx mutations are necessary to study this potential correlation. Furthermore, any impacts observed might lead to treatments for Arx-related disorders, as well as type I and II diabetes mellitus.

Summary and Specific Aims

Understanding endocrine α-cell specification and maintenance is essential to provide knowledge in designing novel treatments type I and II diabetes. The homeodomain-containing transcription factor, Arx, has been shown to be necessary and sufficient for α-cell fate specification [34,40]. However, Arx continues to be expressed in the mature α-cells and therefore could play an additional role in α-cell maintenance throughout life[34].

Additionally, neuronal studies on the role of Arx in proper migration and development of interneurons, and the human pathologies associated with different forms of ARX mutations, have demonstrated a unique genotype-phenotype correlation [47]. Arx null mice and XLAG patients demonstrate strikingly similar pancreatic phenotypes, namely a complete loss of endocrine α-cells [58]. Exploration of non-null Arx mutations, and the resulting impact on endocrine α-cells, will provide further information on proper endocrine specification and maintenance as well as whether a genotype-phenotype severity correlation exists in the pancreas as it does in the brain.

The following chapters in my thesis aim to expand the knowledge of the role of Arx in the endocrine α-cell. **The goal of these experiments is to (1) determine the role of Arx in maintenance of mature α-cell fate and (2) describe the effect a non-null *Arx* mutation has on α-cell specification and maintenance.** These results will demonstrate the role of Arx in maintenance of α-cell fate, as well as provide a better understanding of how a non-null Arx mutation impact α-cell development.

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CHAPTER II:

The Role of *Arx* in the Maintenance of Islet α-Cell Fate

Abstract

The specification and differentiation of pancreatic endocrine cell populations (α-, β-, δ, PP- and ε-cells) is orchestrated by a combination of transcriptional regulators. In the pancreas, *Aristaless-related homeobox* gene (*Arx*) is expressed first in the endocrine progenitors and then restricted to glucagon-producing α-cells. While the functional requirement of *Arx* in early α-cell specification has been investigated, its role in maintaining α-cell identity has yet to be explored. To study this later role of *Arx*, we have generated mice in which the *Arx* gene has been ablated specifically in glucagon-producing α-cells. Lineage-tracing studies and immunostaining analysis for endocrine hormones demonstrate that ablation of *Arx* in neonatal α-cells results in an α-to-β-like conversion through an intermediate bihormonal state. Furthermore, these *Arx*-deficient converted cells express β-cell markers including *Pdx1*, *MafA*, and *Glut2*. Surprisingly, short-term ablation of *Arx* in adult mice does not result in a similar α-to-β-like conversion. Taken together, these findings reveal a potential temporal requirement for *Arx* in maintaining α-cell identity.

Introduction

During development, the pancreas organizes into two distinct compartments: the exocrine acinar cells, which secrete digestive enzymes, and the hormone producing endocrine cells organized into islets of Langerhans [1]. These islets contain a core of insulin-producing β-cells with a surrounding mantle of α, δ, ε, and PP-cells, which produce the hormones glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively [2]. Islet β- and α-cells are the two key endocrine cell populations involved in maintaining glucose homeostasis [3]. Disruption of this homeostasis through β-cell loss or dysfunction leads to diabetes mellitus, a common metabolic disorder manifested at all ages.

Given the limited supply of functioning β-cells in diabetics, one potential treatment avenue is cell-replacement therapy [4]. Considerable effort has been invested in identifying alternative β-cell sources through either directed differentiation from embryonic/induced pluripotent stem cells or reprogramming from other differentiated cell types [5]. Due to the close lineage relationship between α- and β-cells, the reprogramming potential of an α-cell to adopt a β-cell fate has been recently investigated [3]. In one study, new β-cells were generated from glucagon-producing α-cells through a glucagon^{+}insulin^{+} bihormonal intermediate state after a near-total β-cell loss [6]. Moreover, an α-to-β-cell lineage conversion was observed when *Pax4*, a pro-β-cell transcription factor, was expressed in pancreatic endocrine progenitors or α-cells [7]. Similarly, forced expression of *Pdx1* in endocrine progenitors leads to an increase in β-cells and a decrease in α-cell number [8]. Although the α-cell population is mostly post-mitotic, these studies collectively illustrate that α-cell fate can be plastic and is able to be

reprogrammed to adopt β-cell fate. However, the extent of this plasticity during different stages of an animal's life is currently unknown.

One transcription factor capable of altering plasticity in endocrine cells is the *Aristaless-related homeobox* gene (*Arx*). In the mouse pancreas, *Arx* is expressed in a subset of endocrine progenitors and then restricted to glucagon-producing α-cells where it is expressed throughout the life of the animal [9,10]. When misexpressed in the developing pancreas, *Arx* is sufficient to force endocrine progenitors or β-cells to adopt an α-cell fate [11]. These results demonstrate that *Arx* is sufficient for β-to-α-cell reprogramming during development.

Although much is known regarding factors necessary and sufficient for endocrine development, the factors required to maintain the identity of mature α-cells during different stages are less clear. Mice with *Arx* null mutations in the germ-line, pancreatic progenitors, or endocrine progenitors all display a complete loss of α-cells with a concurrent increase in β- and δ-cells in the pancreas [9,10,12]. Moreover, α-cell loss has been reported in patients with null mutations in *ARX* [13]. However, none of the existing mouse models are suitable for determining the function of *Arx* in maintaining (as opposed to establishing) mature α-cell identity. Further, lineage-tracing experiments have not yet been performed to determine if loss of *Arx* leads directly to an α-to-β-cell conversion.

Here we show that *Arx* is required for α-cell lineage maintenance in the neonatal pancreas, but not in the adult pancreas. During the neonatal period, ablation of *Arx* results in loss of glucagon expression and activation of insulin and β-cell markers through an insulin^{+}glucagon^{+} bihormonal intermediate. In contrast, short-term *Arx*

ablation in the adult pancreas does not result in either a loss of glucagon expression or an activation of β-cell marker expression. These data suggest that *Arx* may act in a stage- and context-specific manner in maintaining α-cell identity and reveal potential differential plasticity between fetal and adult α-cells. When taken together, these findings have important implications for the potential use of α-cells for the purpose of β-cell replacement therapy.

Results

Arx removal in neonatal glucagon-producing cells.

Arx is expressed in endocrine progenitors, α-cell precursors, and mature glucagon-producing cells of the pancreas [9,10]. To investigate its role in the neonatal α-cell, we generated mice with *Arx* ablation in glucagon$^+$ cells ($Arx^{L}/_{Y}$ or $Arx^{L}/_{L}$;*Glucagon-Cre*; referred to as GKO hereon). First, a *Rosa-YFP* reporter was used to assess the Cre-mediated recombination efficiency in our GKO model. In P5 control mice (including $Arx^{+}/_{Y}$;*Glucagon-Cre*, *Rosa-YFP* and $Arx^{L}/_{+}$ or $^{+}/_{+}$; *Glucagon-Cre*;*Rosa-YFP*), Arx expression was found in all glucagon$^+$ cells; however, only 13% of glucagon$^+$ cells co-expressed Arx and YFP (Fig. 2.1A, C; white bar in control). All male and female control animals utilized in these experiments were phenotypically identical according to their islet morphology and were compared to their sex-matched GKO animals. These observations indicate a low Cre-mediated recombination rate, which is in agreement with what others have previously reported using this *Glucagon-Cre* transgenic mouse [19]. In GKO*;Rosa-YFP* mutant mice, Arx protein was removed in all glucagon$^+$YFP$^+$ cells, which equated to about 12% of glucagon$^+$ cells (Fig. 2.1B, C; grey bar in GKO). This result suggests that YFP expression faithfully marks cells that have undergone *Arx* ablation. The low Cre-mediated recombination frequency was also observed in P21 animals (Fig. 2.2 and data not shown). Real-time PCR analysis from P5 and P21 animals further showed *Arx* mRNA levels were decreased by 20% and 50% in GKO animals, respectively, although not significantly (Fig. 1D). In addition, in P5 control animals, while 90% of YFP$^+$ cells co-expressed glucagon, approximately 10% of the YFP$^+$ cells

were positive for insulin staining due to some leakiness of the Cre (see below and data not shown).

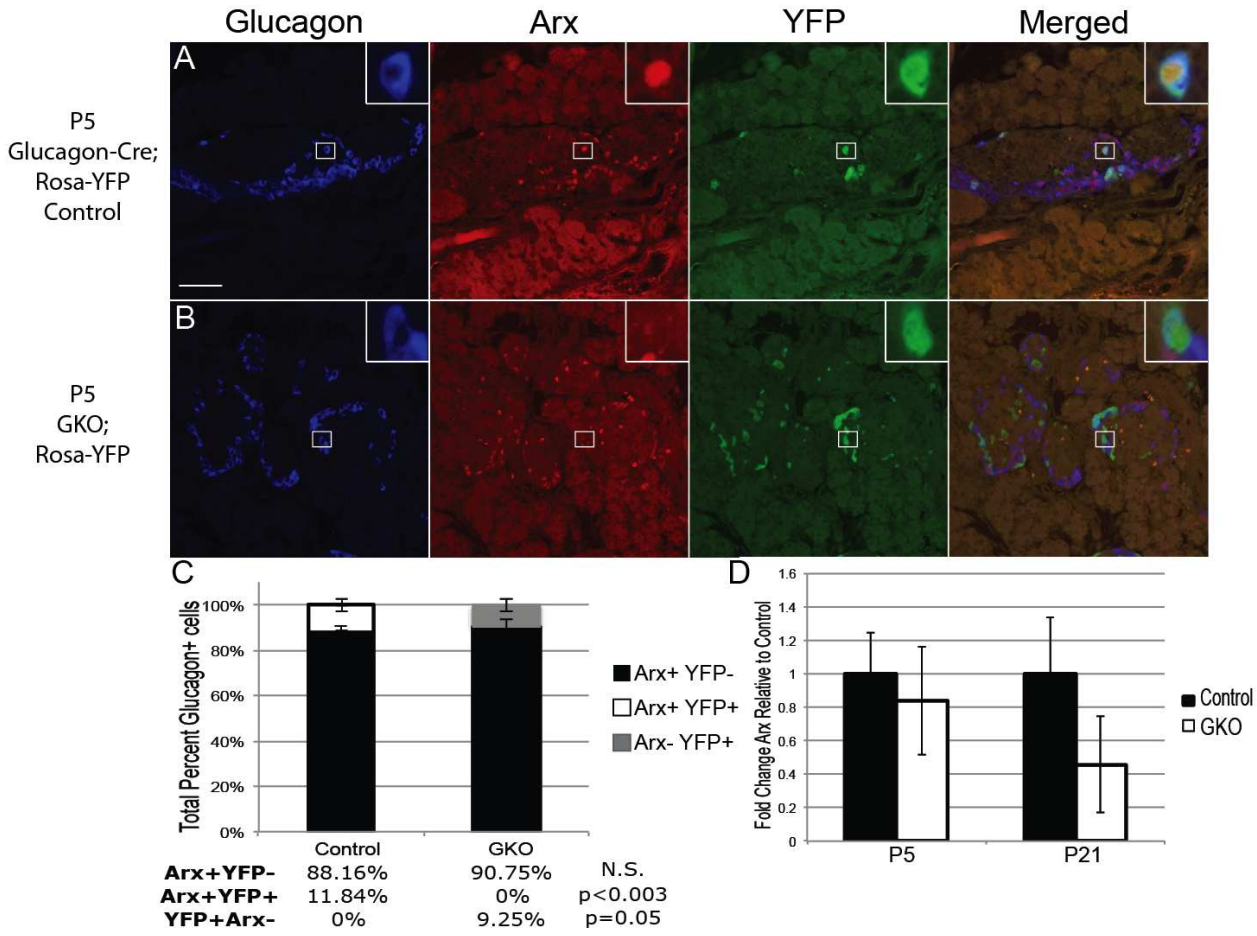

Figure 2.1: Arx is specifically ablated in YFP$^+$ α-cells of GKO;Rosa-YFP mice.

Figure 2.1: Arx is specifically ablated in YFP$^+$ α-cells of GKO;Rosa-YFP mice. P5 pancreatic sections were stained for glucagon (blue), Arx (red), and YFP (green). **(A):** Arx is expressed in all glucagon$^+$ cells in control;Rosa-YFP pancreata. A subset of glucagon$^+$Arx$^+$ cells is YFP$^+$. **(B):** In GKO;Rosa-YFP animals, there is a subset of glucagon$^+$ cells that express YFP. These YFP$^+$ cells have lost Arx expression. Scale bar represents 25μm. **(C):** Quantitative analysis of Arx and YFP expressing cells within glucagon$^+$ population in P5 animals. Over 500 total glucagon$^+$ cells were counted with three mice per group used. Error bars represent standard error of the mean with *p-value* indicated. N.S: not significant. **(D):** Quantitative PCR analysis for *Arx* mRNA in total pancreata at P5 and islets from P21 control and GKO animals. Control mRNA level was set at one fold ± standard error of the mean. Male and female control and GKO animals (n≥3) were sex-matched for all analyses.

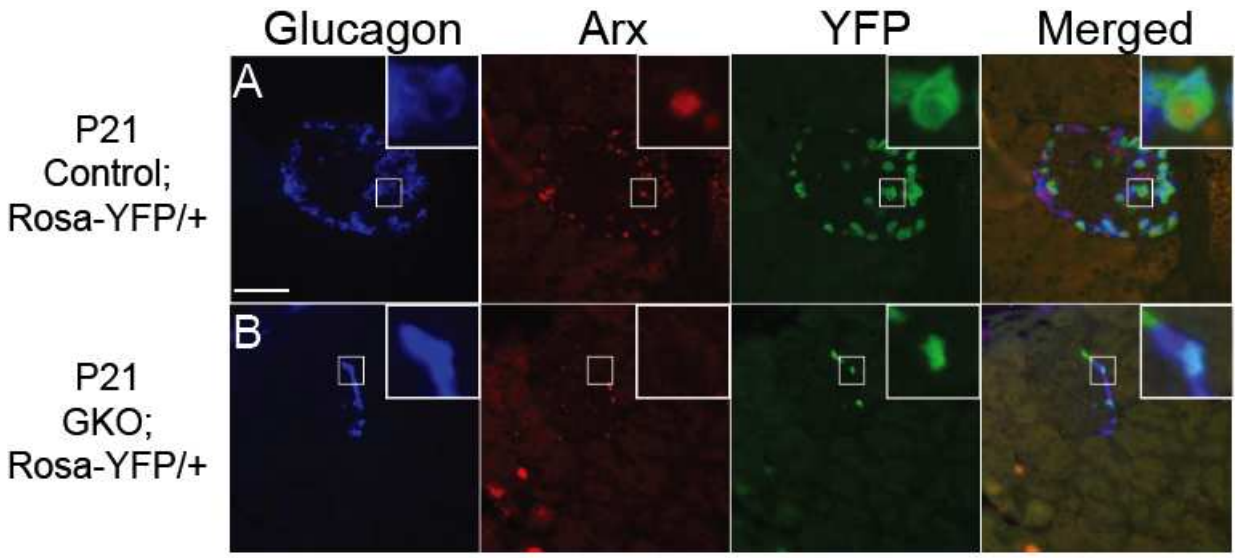

Figure 2.2: Arx is specifically ablated in P21 α-cells by *Glucagon-Cre* with *Arx*-deficient cells expressed YFP

Figure 2.2: Arx is specifically ablated in P21 α-cells by *Glucagon-Cre* with *Arx*-deficient cells expressed YFP. Pancreata were stained for glucagon (blue), Arx (red), and YFP (green). **(A):** Arx is expressed in all glucagon^{+} cells in control;*Rosa-YFP* pancreata. **(B):** In GKO;Rosa-YFP animals, Arx is ablated in all YFP^{+} cells. Male and female GKO mice (n≥3) were analyzed and compared to their sex-matched controls. Scale bar represents 50μm.

***Arx* ablation in the GKO mice results in an emergence of glucagon$^+$ insulin$^+$ co-expressing cells.**

To determine whether loss of *Arx* in the α-cells of GKO mice may have resulted in a change of cell fate in a small subset of cells, we performed double immunostaining for glucagon and other endocrine hormones. Given that only 12% of the glucagon-producing cells have lost Arx expression, we did not expect to observe any significant changes in the number of glucagon cells or the localization of these cells in P5 GKO mice. Indeed, immunostaining analyses confirmed this anticipated result (Fig. 2.3A-H). Real-time PCR analysis also revealed no significant changes in the mRNA levels of *glucagon* transcript between control and GKO mice (Fig. 2.3I). Although hormone cell numbers were not significantly altered, close examination revealed a small population of glucagon$^+$insulin$^+$ bihormonal cells in the pancreas of GKO mice (Fig. 2.3B). These bihormonal cells were only found in the GKO mice. There was no overlap or significant differences in the expression of glucagon with somatostatin or PP between P5 GKO and control mice (Fig. 2.3C-F). Endocrine cells expressing glucagon and ghrelin have been reported in the developing and neonatal pancreas [20,21,22]. The number and location of these glucagon$^+$ghrelin$^+$ cells were comparable between P5 control and GKO mice (Fig. 2.3G-H). Real-time PCR analysis did not reveal any significant changes in hormone expression between P5 control and GKO animals (Fig. 2.3I-L). Together, these data indicate that loss of Arx in glucagon$^+$ cells results in misexpression of the β-cell hormone insulin in glucagon$^+$ α-cells.

To determine the fate of this bihormonal population we evaluated glucagon, insulin, somatostatin, PP, and ghrelin expression in the pancreata of P21 control and GKO mice by immunostaining. Again, there was no significant change in the cell mass or distribution associated with these endocrine populations (Fig. 2.4A-B). Interestingly, while a few glucagon^{+}insulin^{+} cells could still be found in the P21 GKO mice, the frequency of this bihormonal population was dramatically reduced by this age relative to P5. Instead, many of the remaining bihormonal cells in P21 GKO mice have reduced glucagon staining in cells readily expressing insulin (Fig. 2.4A-B). These cells are likely in the later stage of their α-to-β-like cell fate conversion. These observations suggest that upon ablation of *Arx*, insulin expression is activated in the glucagon-producing α-cell, which then gradually loses glucagon expression. Finally, we evaluated mRNA levels for β-cell (*Pdx1* and *Nkx6.1*) and α-cell (*MafB* and *Brn4*) markers in the islets isolated from P21 control and GKO mice. From the real-time PCR analysis, we observed a significant upregulation of *Pdx1* mRNA and an upward trend of *Nkx6.1* levels (Fig. 2.4J). Conversely, we detected a significant reduction in *Brn4* with a small downward trend in *MafB* expression (Fig. 2.4J). While the significant changes in the mRNA levels of *Pdx1* and *Brn4* data are surprising in the context of the small changes in hormone expression, this result could be due to direct *Arx* regulation. *Arx* could potentially directly repress *Pdx1* and activate *Brn4*, which would result in a drastic increase of *Pdx1* (and resulting decrease of *Brn4*) upon *Arx* ablation. When taken together, these data suggest that glucagon-producing cells require *Arx* to maintain α-cell identity and repress β-cell markers during neonatal life.

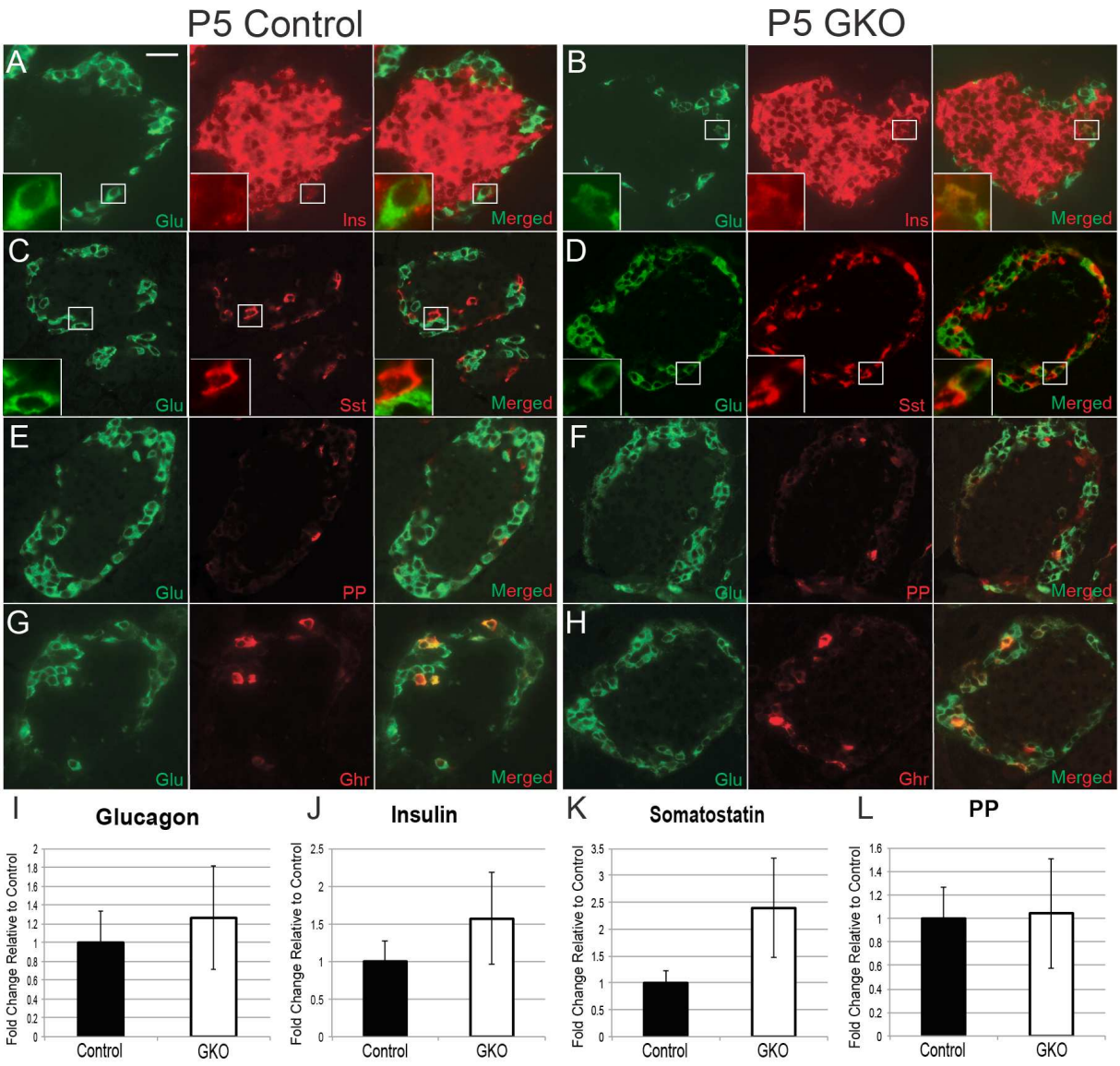

Figure 2.3: Loss of Arx in glucagon^{+} cells results in the appearance of a glucagon^{+}insulin^{+} population.

Figure 2.3: Loss of Arx in glucagon$^+$ cells results in the appearance of a glucagon$^+$insulin$^+$ population. (A-H): P5 control and GKO pancreata were stained for glucagon (green), insulin (red; A, B), somatostatin (Sst; red; C, D), PP (red; E, F), and Ghrelin (red; G, H). Glucagon$^+$insulin$^+$ cells are the only bihormonal population unique to GKO animals (B, D, F). Glucagon/ghrelin coexpressing cells are both found in control and GKO animals (G, H). Male and female control and GKO animals ($n \geq 3$) were sex-matched for all analyses. Scale bar denotes 25μm. **(I-L):** Quantitative PCR analysis examining glucagon (I), insulin (J), somatostatin (K), and PP (L) gene expression in P5 control and GKO animals. Control mRNA level was set at one fold ± standard error of the mean. For all GKO and control groups, at least 3 biologic replicates were performed.

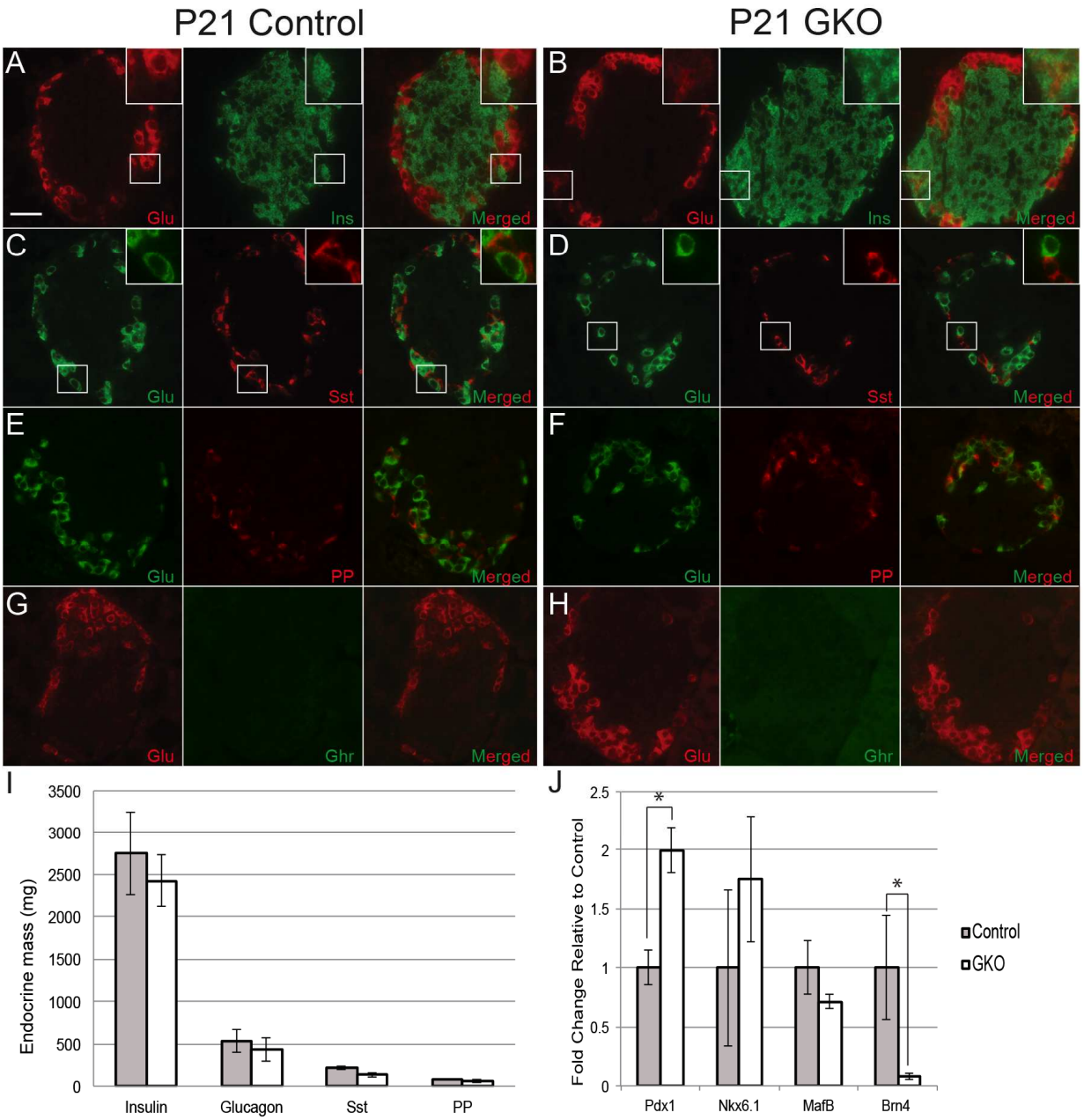

Figure 2.4: Loss of glucagon^{+}insulin^{+} cells in P21 GKO animals.

Figure 2.4: Loss of glucagon^{+}insulin^{+} cells in P21 GKO animals. (A-H): Immunostaining for glucagon (A-H), insulin (A-B), somatostatin (C-D), PP (E-F), and ghrelin (G-H) in P21 control and GKO animals with merged images shown. Glucagon positive cells do not overlap with insulin (A, B), somatostatin, (C, D) or PP (E, F). Ghrelin is no longer expressed at P21 (G, H). **(I):** Total insulin, glucagon, somatostatin, and PP cell mass in the pancreata of P21 control and GKO mice. Male and female GKO mice ($n \geq 3$) were analyzed and compared to their sex-matched controls. Scale bar denotes 25μm. **(J):** Quantitative PCR analysis for P21 control and GKO islets for β-cell markers Pdx1 and Nkx6.1 and α-cell markers MafB and Brn4. "*" denotes $p<0.05$. Error bars represents standard error of the mean.

Arx-deficient cells fail to maintain α-cell identity

To directly determine the origin of the glucagon$^+$insulin$^+$ cells seen in P5 GKO mice, lineage-tracing studies were performed in P5 GKO mice. Triple-immunostaining for glucagon, insulin, and YFP were performed in the pancreas of control;*Rosa-YFP* and GKO;*Rosa-YFP* mice. YFP expression was detected in only a subset of glucagon-producing cells at P5 (Fig. 2.5A-B), due to the low frequency of the Cre-mediated recombination in the *Glucagon-Cre* transgenic mice (Fig. 2.1). The majority of YFP$^+$ cells in the P5 control mice expressed glucagon (Fig. 2.5A, E and F) though a very small number of insulin-producing cells positive for YFP expression were found (blue), demonstrating relatively high, though not 100%, fidelity of the Cre-mediated recombination (Fig. 2.5E and F). In P5 GKO mice, we noticed an emergence of glucagon$^+$insulin$^+$YFP$^+$ (purple) cells and an increase in the number of insulin$^+$YFP$^+$ (blue) cells while the number of glucagon$^+$YFP$^+$ (red) cells was reduced compared to controls (Fig. 2.5A-B, E and F).

To follow up with our previous observations that the glucagon$^+$insulin$^+$ cell number has dramatically reduced by P21, we evaluated the pancreata of control;*Rosa-YFP* and GKO;*Rosa-YFP* mice at P21 for glucagon, insulin, and YFP expression. Interestingly, corresponding to the previously described disappearance of bihormonal cells by P21 (Fig. 2.4A-B), the majority of YFP$^+$ cells in GKO;*Rosa-YFP* pancreata at this stage were insulin$^+$ (blue) with only a small percentage of YFP$^+$ cells expressing both insulin and glucagon (purple) or glucagon alone (red) (Fig. 2.5C-D and G). These data demonstrate that *Arx* loss in glucagon-producing α-cells leads to a failure in maintaining

α-cell identity and a conversion to a β-cell-like fate. Taken together, these lineage-tracing data indicate that the loss of neonatal *Arx* in glucagon-producing cells results in a cell fate conversion from a glucagon^{+} α-cell into an insulin^{+} β-cell-like fate through a bihormonal intermediate.

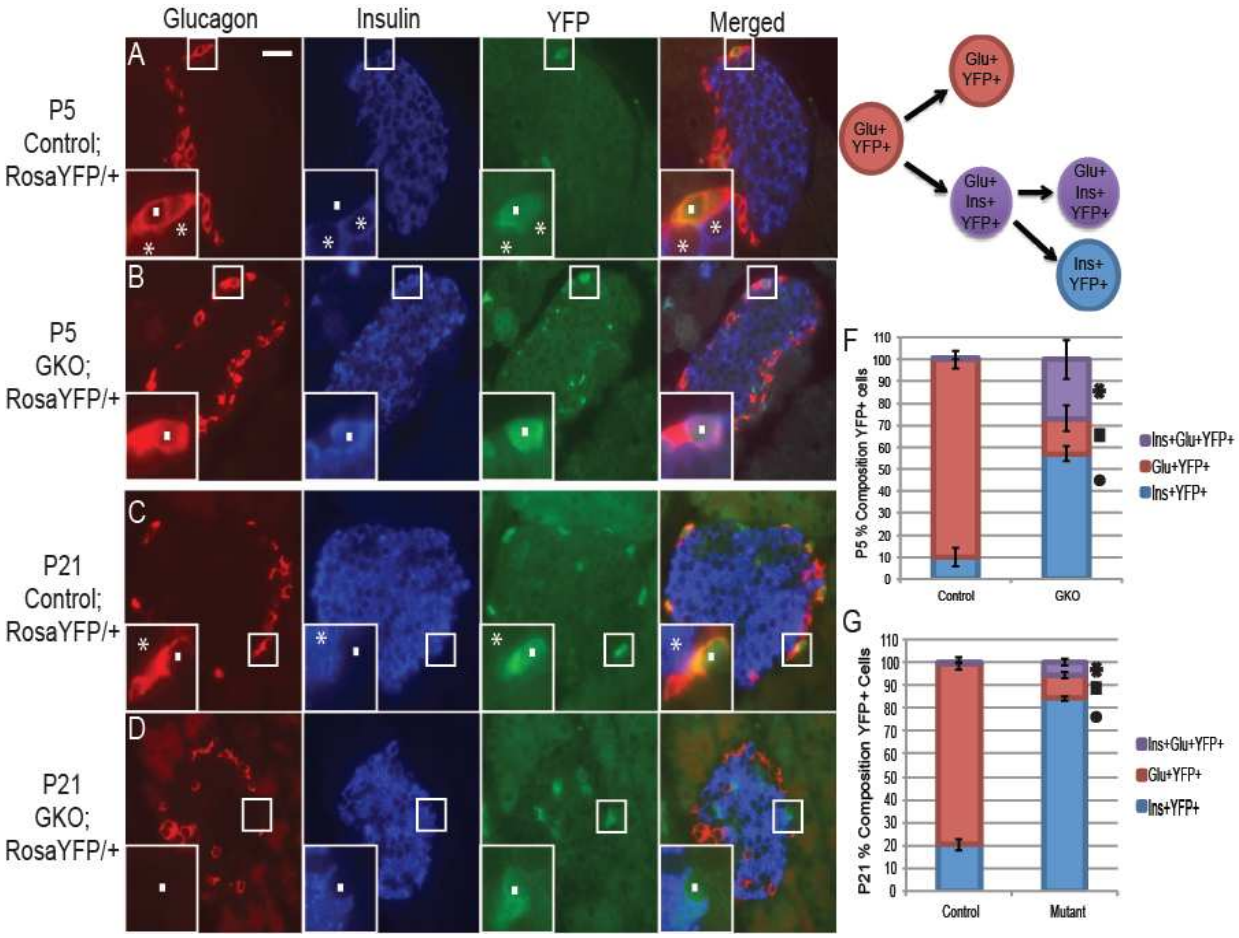

Figure 2.5: Lineage tracing studies demonstrate that Arx ablated α-cells become glucagon^{+}insulin^{+} at P5 then insulin expressing at P21.

Figure 2.5: Lineage tracing studies demonstrate that Arx ablated α-cells become glucagon$^+$insulin$^+$ at P5 then insulin expressing at P21. (A-D): Triple immunostaining for glucagon (red), insulin (blue), and YFP (green) in control;Rosa-YFP and GKO;Rosa-YFP pancreata at P5 and P21. YFP$^+$ cells in P5 or P21 control;Rosa-YFP animals are positive for glucagon (A and C;□). "*" denote insulin cells that are negative for glucagon or YFP expression (A and C; *) Glucagon$^+$insulin$^+$YFP$^+$ cells are found in P5 GKO animals (B; □), but rarely in controls (A). YFP$^+$ cells are positive for glucagon in control P21 pancreata (C; □) but insulin$^+$ in P21 GKO pancreata (D; □). **(E):** Schematic outlining cell populations resulting from lineage-tracing and immunostaining analysis. **(F, G):** Quantification of hormone expression in YFP$^+$ cells at P5 (F) and P21 (G). At P5 and P21, over a total of 10,000 cells were counted from 3-5 animals per group. Out of the 10,000 cells counted, approximately 1,000 cells were YFP$^+$. Each category was calculated and presented as a percentage of total YFP$^+$ cells per animal and then averaged. Error bars are denoted as standard error of the mean with significance ($p \leq 0.05$) between each color denoted with "*", "□", and "□". Male and female GKO mice ($n \geq 3$) were used for all analysis and compared to their sex-matched controls. Scale bar represents 25μm.

Markers associated with mature β-cells are activated in *Arx*-deficient YFP$^+$ cells.

To further examine how closely these newly converted β-like-cells were to true β-cells, expression of several known β-cell markers including Glut2, MafA, and Pdx1 were examined in control;*Rosa-YFP* and GKO;*Rosa-YFP* mice at P5 and P21. In P5 GKO;*Rosa-YFP* animals, there was a significant increase in the number of YFP$^+$ cells coexpressing Glut2, MafA, or Pdx1 (Fig. 2.6B, D, F, G, I, K). Similar increases were also seen in P21 mice with a further increase in the number of YFP$^+$ cells expressing Glut2 and Pdx1 in the pancreata of the GKO;*Rosa-YFP* mice (Fig. 2.6H, J, L, Fig. S3). As expected, due to the leakiness of the *Glucagon-Cre* transgene, we did find a small, but not significant, percentage of YFP$^+$ cells that coexpressed Glut2, MafA, or Pdx1 in P5 or P21 control;*Rosa-YFP* mice (Fig. 2.6A, C, E, G, I, K). Taken together, these data demonstrate that a subset of the converted cells in the GKO;*Rosa-YFP* mice activate β-cell markers as well as insulin expression in the absence of *Arx*.

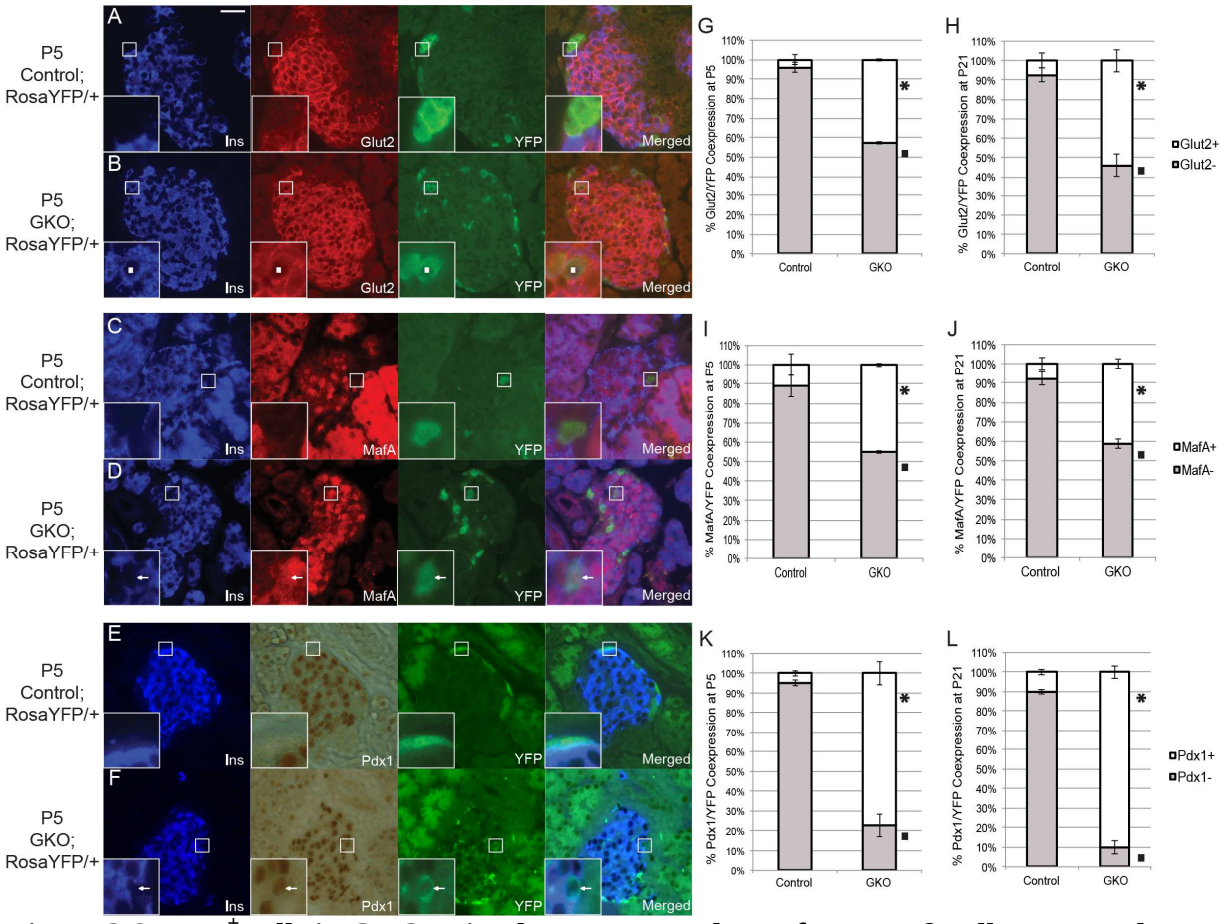

Figure 2.6: YFP^{+} cells in GKO animals express markers of mature β-cells at P5 and P21.

Figure 2.6: YFP$^+$ cells in GKO animals express markers of mature β-cells at P5 and P21. (A-F): Control;Rosa-YFP and GKO;Rosa-YFP P5 pancreata were stained for insulin (blue), YFP (green), Glut2 (A,B,red), MafA (C,D,red), Pdx1 (E,F,brown). YFP$^+$ cells in GKO animals are insulin$^+$Glut2$^+$ (B; □), insulin$^+$MafA$^+$ (D; □) and insulin$^+$Pdx1$^+$ (F; □). In control animals, the majority of YFP$^+$ do not express β-cell markers (A,C,E). The YFP$^+$ cells seen in exocrine tissue (panels E and F) is background due to the combined IHC, IF staining method used and not true signal. **(G-L):** Quantification of percentage of YFP$^+$ cells that express or do not express Glut2 at P5 (G) and P21 (H), MafA at P5 (I) and P21 (J), and Pdx1 at P5 (K) and P21 (L). Over 200 YFP$^+$ cells were counted for each stage with 3-5 animals per group. Error bars represent standard error of the mean with significance between each cell population ($p \leq 0.05$) denoted as "*" and "□". Male and female GKO mice ($n \geq 3$) were used for all analysis and compared to their sex-matched controls. Scale bar represents 25μm.

Short-term ablation of Arx in adult α-cells does not lead to loss of α-cell identity.

To explore the requirement for *Arx* in the maintenance of adult α-cell fate, we used a global, tamoxifen-inducible transgenic mouse model to ablate *Arx* in adult animals. Two-month-old control and $Arx^{L}/_{Y}$;*pCAGG-CreER* (IKO) mice were injected with tamoxifen for three consecutive days and the animals sacrificed two weeks later for tissue analysis (Fig. 2.7A). Efficiency of *Arx* removal was evaluated by immunostaining in control and IKO mice. While Arx expression was found in glucagon^{+} cells in control animals, all glucagon cells in IKO animals have lost Arx expression (Fig. 2.7B, B', C, C'; marked by arrows). To determine the impact of short-term *Arx* ablation in adult α-cells, gene expression and immunostaining for endocrine hormones were examined in control and IKO animals (Fig. 2.7D-I). Real-time PCR analysis revealed no significant changes in the mRNA levels of hormone genes between control and IKO islets (Fig. 2.7K). We also did not detect any significant changes in the numbers of glucagon-, insulin-, somatostatin-, and PP-producing cells in the IKO mice compared to controls (Fig. 2.7D-J). Unlike P5 GKO mice in which a large proportion of Arx^{-}glucagon^{+}insulin^{+} cells were found (Fig. 2.7A-B), we detected only a small number of bihormonal cells in adult IKO mice, which were not proportionally significant (less than 0.1%; data not shown). Additionally, analysis of α- and β-cell factors including MafB, Brn4, Glut2, and Pdx1 also did not reveal any significant changes in transcriptional profile of IKO animals (Fig. 2.7L). Since the pCAGG-Cre is globally expressed, the IKO animals develop an intestinal phenotype that excludes any meaningful analysis to explore the long-term impact of *Arx* on adult α-cell (data not shown). Taken together, using our current mouse

model with short-term Arx ablation, these findings demonstrate that *Arx* is likely dispensable in maintaining α-cell identity in adult mice. Future experiments utilizing an inducible α-cell specific Cre transgenic mouse will be required to study the long-term requirement for *Arx* in the maintenance of α-cell fate.

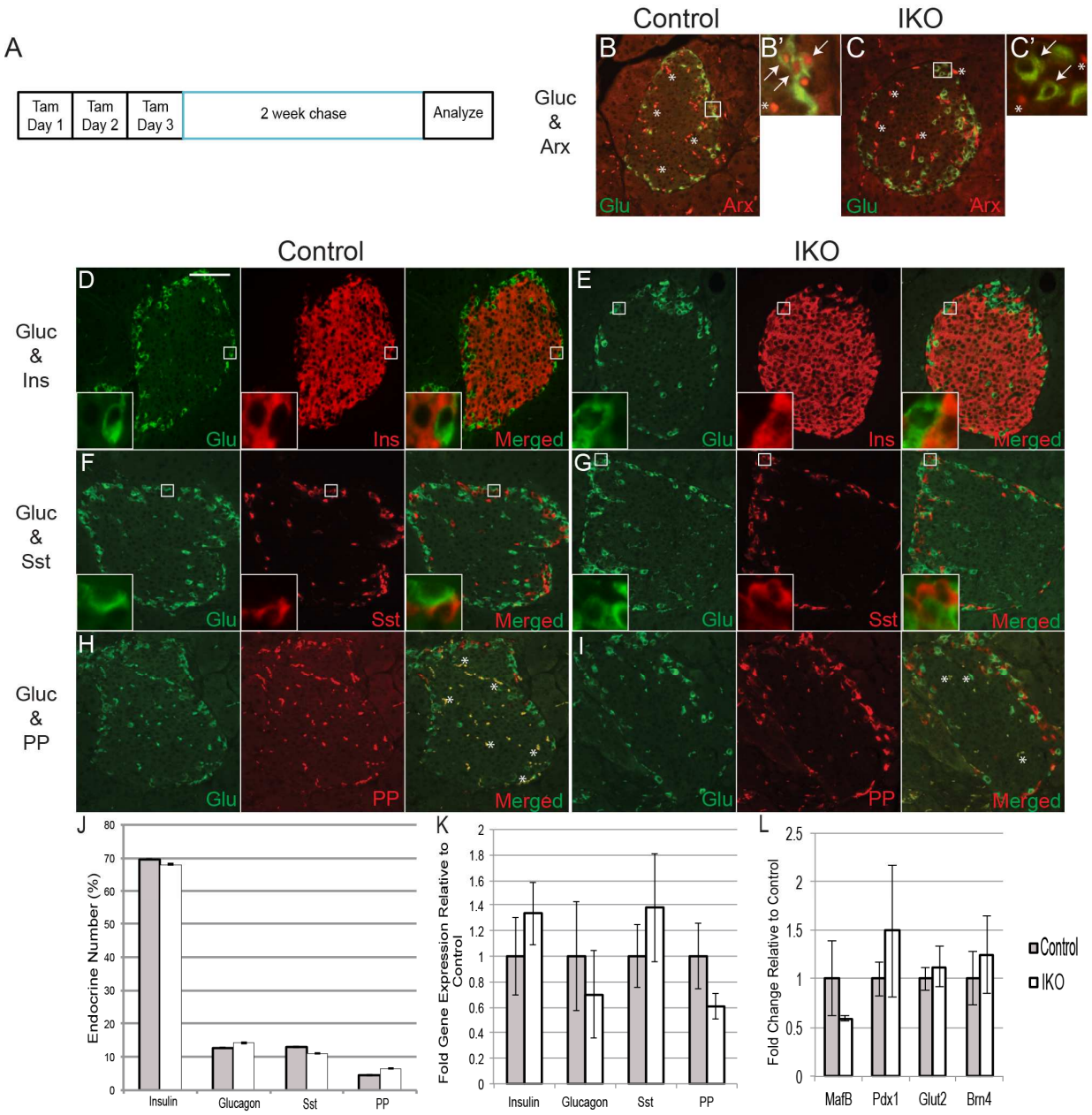

Figure 2.7: Short-term complete ablation of Arx in adult mice does not result in a loss of α-cells or changes in endocrine cell populations.

Figure 2.7: Short-term complete ablation of Arx in adult mice does not result in a loss of α-cells or changes in endocrine cell populations. (A): Diagram outlining experimental design. **(B-C):** Arx (red) is expressed in glucagon (green) cells in control (B,B'; arrows) but lost in IKO (C,C';arrows) animals. Asterisks (*) mark autoflourescent blood cells and are non-specific staining. **(D-I):** Control and IKO pancreata were stained for glucagon (green), insulin (red; D,E), somatostatin (Sst; red; F,G), and PP (red; H,I). No significant colocalization of glucagon with other hormones was seen in control or IKO mice. Male control and IKO mice were used for analysis though female control and IKO mice produced similar results. Scale bar denotes 75μm.

(J): Endocrine cell number quantification for insulin, glucagon, somatostatin, and PP in control and IKO animals (over 10,000 cells were counted from 3 animals per group). **(K-L):** Quantitative PCR analysis of gene expression in control and IKO islets. Results are displayed as fold change relative to control with error bars representing the standard error of the mean. For all analysis n=3.

Discussion

This study demonstrates a requirement of *Arx* in α-cell fate maintenance. Ablation of *Arx* in neonatal glucagon^{+} cells results in a loss of α-cell identity and conversion into an insulin-producing β-cell-like fate (Fig. 2.8). Conversely, short-term ablation of *Arx* in adult animals did not result in a significant loss or conversion of α-cells or an increase in β-cells or β-cell markers (Fig. 2.8). Our findings from neonates and adults expand the previously defined role of *Arx* in the specification of α-cells. When taken together, *Arx* plays a role during specification as well as during early maintenance of α-cell fate, but appears not to be required in adult animals for its fate maintenance.

Others have shown that ablation of *Arx* at any stage of specification results in a complete loss of the α-cell lineage with a concomitant increase of β- and δ-cells [9,10,12,23]. As there is no change in total endocrine mass reported in these studies, these α-cells likely undergo re-specification into β- and δ-cell lineages. Our findings add significant support to these observations by using lineage tracing to directly demonstrate that *Arx*-ablated α-cells convert to β-like cells in neonatal animals. Interestingly, our data, while showing coexpression of glucagon and insulin, does not show coexpression of glucagon and somatostatin. Our initial hypothesis was that *Arx*-deficient α-cells would give rise to both somatostatin and insulin populations. It is possible that the inefficiency of the *Glucagon-Cre* did not enable us to detect a rare population of glucagon^{+}somatostatin^{+} cells. Alternatively, as the animal ages, the plasticity of cells among different endocrine fates could be altered.

Previous studies have demonstrated that endocrine cell fate is relatively undifferentiated during gestation such that ablation of single transcription factors results in loss of cell fate [2]. As endocrine cells mature, however, this plasticity drastically decreases, and more extreme measures are needed to convert one endocrine cell type to another [4,6,8]. While *Pdx1* is normally restricted to β-cells, early overexpression in α-cells results in a postnatal loss of glucagon-expressing α-cells with a concomitant gain of insulin-producing β-cells demonstrating an α-to-β-cell fate conversion [8]. Conversely, overexpression of *Pdx1* in adult α-cells does not result in a similar conversion; instead, these cells maintain proper cell identity [8]. The potential temporal requirement of *Arx* closely parallels the results obtained through *Pdx1* overexpression in α-cells. Early in development, endocrine cell fate appears more plastic and subject to reprogramming. During later life, however, cell fate is more defined, and as a result, reprogramming is more difficult to achieve.

Interestingly, although overexpression of *Pdx1* in α-cells results in a gain of insulin-producing cells, those cells did not appear to lose all markers of α-cell fate [8]. Examination of immunostaining for the expression of β-cell-specific factors in the *Arx* ablated neonatal animals demonstrates that YFP^+ cells are at least partially reprogrammed with the expression of Glut2, MafA, and Pdx1. While *Arx* is necessary to maintain α-cell fate during development, loss of *Arx*, even immediately after specification, may not be sufficient to fully reprogram cells into a functional β-cell fate. Due to the low efficiency of the *Cre* utilized in our study, functional analysis of the insulin-producing cells derived from *Arx* ablation in α-cells was not feasible.

As the animal ages, there could be epigenetic changes that have occurred during the process of specification or maturation that inhibit these *Arx* deficient cells from becoming functional β-cells under homeostatic conditions. In fact, epigenetic modification has been shown to play important roles in the differentiation and maintenance of cell types. A recent study demonstrates that α-to-β-cell reprogramming could be promoted by manipulating the histone methylation signature in mammalian pancreatic islets [24]. Conditions of stress, however, may also make cell fate transitions more fluid. It has been shown that excessive loss of β-cell mass, induced by administration of a β-cell specific toxin, results in spontaneous reprogramming of α-cells into a β-cell fate [6]. Additionally, partial pancreatectomy in mice and rats can result in regeneration of β-cells through the conversion of duct cells or duct progenitor cells [25,26]. These studies demonstrate that while cell fate is more defined in adult animals, extreme conditions can force a non-β-cell into a β-cell fate. Future studies examining this possibility should be performed and will elucidate limits to cell fate maintenance in adult animals and how to overcome those limits. Particularly, the ability to utilize α-cells for conversion to functional β-cells could be a potential therapy for diabetes.

Finally, it is important to note that our current adult IKO mouse model does not allow for a complete investigation for the role of *Arx* in adult α-cells. *Arx* is required in early enteroendocrine cell development of the digestive tract [27]. Therefore mice with *Arx* removal in the intestine have alterations of specific enteroendocrine cell population, which lead to lipid malabsorption and diarrhea ([27]; and unpublished observations). Since the adult IKO mouse model was generated using a global inducible Cre transgenic

mouse, enteroendocrine cell populations were impacted (unpublished observations). It is important to note that we did notice a 0.1% increase in the number of bihormonal cells in IKO mice (data not shown). However, whether this small change is due to the direct impact upon *Arx* loss in α-cells or changes in the animal's physiology remains to be determined. An α-cell specific inducible *Arx*-deficient mouse model combined with lineage tracing studies will be required to precisely determine the role of *Arx* in adult α-cells.

In conclusion, the current study demonstrates a potential temporal requirement for *Arx* in maintenance of α-cell fate. Ablation of *Arx* in neonatal α-cells results in a loss of glucagon expression and a conversion of this cell population to adopt an insulin-producing β-cell-like fate. However, short-term loss of *Arx* in adult animals does not phenocopy this result but instead suggests that *Arx* is dispensable in maintaining α-cell fate in adulthood. These data expand the knowledge of the field not only related to the role of *Arx* in the endocrine α-cell but also in regards to global temporal restrictions for reprogramming endocrine cells. Future studies examining this temporal requirement, as well as perturbations to the cell that circumvent these restrictions, will help clarify this plasticity and bring understanding to endocrine cell fate specification, maintenance, and therapeutic potential.

Note: The data presented in this chapter has been published in PLOS ONE as an Original Research Article entitled,

Pancreatic α-cell specific deletion of mouse Arx leads to α-cell identity loss.

Crystal L.Wilcox, Natalie A. Terry, Erik R. Walp, Randall A. Lee, and Catherine Lee May (2013). PLOS ONE. 8(6):e66214.

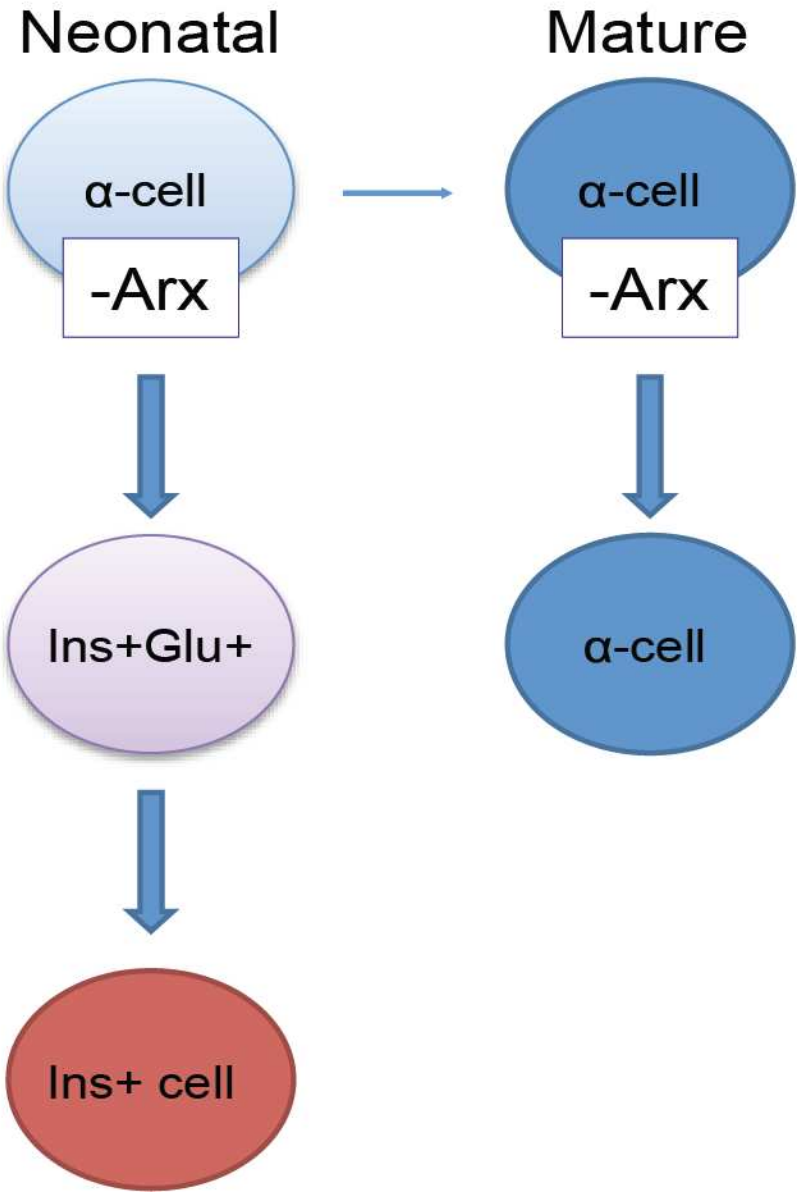

Figure 2.8: Proposed model showing that Arx is necessary to maintain α-cell fate in neonatal islets but not in mature α-cells in adult animals.

Figure 2.8: Proposed model showing that Arx is necessary to maintain α-cell fate in neonatal islets but not in mature α-cells in adult animals. Loss of neonatal Arx results in the conversion of glucagon^{+} α-cells into an insulin-producing β-like-cell through a bihormonal intermediate.

Materials and Methods

Ethics Statement

The Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee (IACUC) approved all animal experiments under the protocol number 2011-10-756. CLM monitored all animal studies.

Animals and Breeding Strategy

The derivation of the $Arx^{L}/_{Y}$ and *Glucagon-Cre* transgenic lines has previously been described [14,15,16]. To generate $Arx^{L}/_{Y}$*;Glucagon-Cre* mice, $Arx^{L}/_{+}$*;Glucagon-Cre* and $Arx^{L}/_{Y}$ mice were mated on a BL6 background. Male and female $Arx^{L}/_{Y}$ or $Arx^{L}/_{L;}$*Glucagon-Cre* mice were phenotypically indistinguishable in terms of their islet morphology, size, body size and weight. All mutants used in our analysis were compared to their sex-matched controls. $Arx^{+}/_{Y}$*;Glucagon-Cre*, $Arx^{+}/_{Y}$, $Arx^{L}/_{+}$ and $Arx^{L}/_{+}$*;Glucagon-Cre* mice were used for controls with no observable phenotypic differences in the islets between any of them. The reporter $Rosa26^{YFP}/_{YFP}$ was mated into this line in either heterozygosity or homozygosity for lineage tracing studies, which yielded the same result in all experiments [17]. The generation of *pCAGG-CreER* animals has been previously described [18]. $Arx^{L}/_{Y}$ or $Arx^{L}/_{L}$*;pCAGG-CreER* animals were generated by crossing $Arx^{L}/_{+}$*;pCAGG-CreER* females to $Arx^{L}/_{Y}$ males. Male and female mutants were phenotypically indistinguishable in the endocrine pancreas and both were used in this study.

Immunohistochemistry and Histology

All dissections were performed in cold 1X PBS and tail or toe snips collected for genotyping. Tissues were fixed in cold 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and 8µm sections collected. Antigen retrieval was performed in 10mmol citric acid buffer (pH 6.0) and endogenous peroxidase, avidin D, and biotin activity blocked with 3% H_2O_2 (Sigma) and Avidin/Biotin Blocking Kit (Vector), respectively. Endogenous protein was blocked with CAS-Block reagent (Invitrogen). Slides were incubated in primary antibody overnight at 4°C. Primary antibodies used were: Insulin (MS 1:400, Thermo Scientific and GP 1:1000, Abcam), Glucagon (1:3000, Millipore), Somatostatin (1:200, Invitrogen), PP (1:200, Invitrogen), Arx (1:250, gift from Dr. Kanako Miyabayashi at Kyushu University), GFP (1:250, Abcam), Ghrelin (1:200, Santa Cruz), Pdx1 (1:200, Santa Cruz), MafA (1:1000, Bethyl), Glut2 (1:1000, Millipore), and Chromogranin A (1:3000, DiaSornin). After rinsing in PBS, appropriate secondary antibodies were added for two hours at room temperature. Immunohistochemical detection was performed with the VECTASTAIN ABC kit (Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB) as the substrate. Immunofluorescence utilized secondary antibodies conjugated to Cy3, Cy2 or Cy5. All images were obtained using a Leica DM6000B microscope.

Real-Time PCR Analysis

Total RNA was extracted in TRIZOL (Invitrogen) using the protocol provided with reagent. Oligo-dT, Superscript II, and additional required reagents were used to

synthesize cDNA (Invitrogen). PCR reactions were performed using Brilliant SYBR Green PCR Master Mix (Agilent) in the Stratagene Mx3005P real-time PCR machine. All PCR reactions were performed in duplicate for each sample with at least 3 animals per group analyzed with reference dye normalization. Primer sequences are available upon request.

Hormone Cell Quantification

Hormone-positive cells from pancreatic sections were counted, averaged, and normalized to either total pancreatic area or total endocrine cell number. Three separate regions of each pancreas were used for quantification in both control and mutant mice. At least three animals for each group were used for quantification in all analyses. To determine hormone cell mass, hormone-positive area as well as pancreatic area was measured using the Aperio Image Analysis System. These areas as well as weight of the pancreas was used to determine hormone cell mass. For specific hormone cell number, hormone positive cells were counted and normalized to total endocrine cell number, which was determined by combining counts for all endocrine hormones (insulin, glucagon, somatostatin, and PP). Over 10,000 total endocrine cells were counted for each analysis consisting of over 5,000 insulin$^+$, over 1,000 glucagon$^+$ and somatostatin$^+$, and over 500 PP$^+$ cells.

Islet Isolation

For P21 and adult RNA analysis, islet isolation was performed by injecting 5 mL Collagenase P (Roche) in HBSS with 0.02% BSA (Sigma) into the clamped pancreatic

duct to inflate the pancreas. Once inflated and removed, pancreatic tissue was incubated in 15mL CollagenaseP/HBSS at 37°C at 50rpm for 16 minutes to digest exocrine tissue. After spin down and rinse, islets were isolated from remaining exocrine tissue in HBSS. Upon isolation, islets were placed in TRIZOL for RNA extraction.

Tamoxifen Induction

Two-month-old male and female mice, matched with littermate controls, were injected intraperitoneally (IP) with 50μg/g body weight of 10mg/ml tamoxifen (Sigma) solution, which consisted of 10% ethanol and 90% sunflower seed oil (Sigma). Injections were performed for three consecutive days followed by a two-week chase. After the chase period, pancreatic tissue was removed and processed for either immunostaining or RNA analysis.

Statistical Analysis

All values are presented as average ± standard error of the mean. Significance was determined using a two-tailed Student's t-test. *p*-values less than or equal to 0.05 were considered significant.

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Chapter III:

The Role of Arx in Maintenance of Adult α-cell Fate

Abstract

Type II diabetes is a global health crisis, affecting more than 25 million people in the United States alone. Diabetes is a metabolic disorder that stems from unregulated glucose homeostasis, which is normally maintained by glucagon-producing α-cells and insulin-producing β-cells in the endocrine pancreas. In order to develop novel treatments for type II diabetes, knowledge of normal endocrine cell development and maintenance is essential. Previous studies have demonstrated the importance of transcription factors in endocrine cell development.

An important transcription factor for endocrine α-cells is the *aristaless-related homeobox gene* (*Arx*). Previous studies have demonstrated that Arx is necessary for proper specification and maintenance of α-cell fate in the neonatal pancreas, but is dispensable for short-term α-cell fate maintenance in adult animals. However, the long-term impact of Arx ablation in mature α-cells has not been studied.

Here, we show that upon long-term ablation of Arx in mature α-cells, mice become more glucose tolerant and lose weight gradually over time. Phenotypic analysis demonstrated normal glucagon^{+} numbers; however, a majority of α-cells co-express insulin or PP. Finally, *in vitro* secretion assays demonstrate loss of glucagon secretion and content. Instead, Arx-ablated islets secrete insulin in response to both α and β stimuli. When taken together, these data demonstrate that long-term ablation of Arx in the mature α-cell results in loss of cell fate maintenance and conversion into an α-β-PP hybrid cell.

Introduction:

Type II diabetes is a metabolic disorder that affects over 25 million people in the United States [1]. The hallmarks of type II diabetes are inadequate insulin secretion combined with excess glucagon due to loss of proper glucose homeostasis [2]. Glucose homeostasis is largely maintained by the endocrine pancreas, which secretes insulin and glucagon in response to elevated and reduced blood glucose levels, respectively [3]. Thus, novel therapies to treat type II diabetes focus on the endocrine pancreas and seek to restore blood glucose homeostasis [4].

The pancreas is a two-component organ situated between the stomach and duodenum of the digestive tract [5]. The pancreas is comprised of an exocrine portion, which secretes digestive enzymes, and an endocrine component, which produces and secretes hormones involved in glucose homeostasis and metabolism [5]. The murine endocrine portion of the pancreas is organized into Islets of Langerhans, consisting of a core of insulin-producing β-cells with a surrounding mantle of α-, δ-, PP-, and ε-cells, which secrete the hormones glucagon, somatostatin, Pancreatic Polypeptide (PP), and ghrelin, respectively [5].

Glucose homeostasis is maintained by insulin-producing β-cells and glucagon-producing α-cells [3]. Increased blood glucose levels causes insulin secretion from β-cells, uptake and storage of excess glucose in skeletal muscle and adipose tissue, and lowering of blood glucose concentration [3]. Conversely, when blood glucose levels fall below baseline, insulin secretion is halted and amino acids are released, signaling α-cells

to secrete glucagon [3]. Glucagon signals to the liver to break down stored glucose through the processes of gluconeogenesis and glycogenolysis, resulting in an increase in blood glucose concentration [6]. The opposing processes induced by glucagon and insulin act to maintain proper blood glucose homeostasis.

In order to develop novel therapies for type II diabetes, knowledge of normal pancreatic endocrine cell development and maintenance is critical. Previous studies have demonstrated the crucial role transcription factors play in endocrine specification and maintenance [1]. During development, ablation of essential transcription factors leads to loss of cell fate specification and/or maintenance [1]. However, in adult animals pancreatic cell fate is more defined, and ablation of transcription factors is less likely to result in complete loss of cell fate [1]. Cell fate is maintained in mature endocrine cells through a combination of a complex transcriptional program and extensive chromatin modifications [7].

In endocrine α-cells, one such crucial transcription factor required for proper specification and maintenance is the homeodomain-containing protein *aristaless-related homeobox gene* (*Arx*) [8]. Ablation of Arx during development results in the complete loss of the α-cell lineage and reallocation of presumptive α-cells into β- and δ-cell fates [8,9]. Furthermore, forced misexpression of Arx in the developing pancreas or islets leads to conversion of β- and δ-cells into α- and PP-cell fates [10]. Thus, Arx is necessary and sufficient for α-cell specification.

Arx is also necessary for neonatal α-cell fate maintenance [9]. Arx is exclusively expressed in glucagon$^+$ α-cells at all time points examined after specification [8]. Loss of Arx in glucagon$^+$ α-cells results in conversion of Arx-ablated α-cells into a β-like cell fate through an insulin$^+$glucagon$^+$ bihormonal intermediate [9]. However, short-term ablation of Arx in mature α-cells does not result in loss of α-cells or misexpression of other endocrine hormones [9].

This previous study characterized the role of Arx in maintenance of neonatal α-cell fate and began to explore this role in mature α-cells [9]. Although short-term (2 weeks) ablation of Arx in mature α-cells does not result in a visible phenotype, the long-term (~8 weeks) impact of this ablation has not been explored. Here, we demonstrate that long-term ablation of Arx in mature α-cells results in improved glucose tolerance, gradual weight loss, and misexpression of insulin and PP, but not somatostatin, in glucagon$^+$ cells. *In vitro* secretion studies of Arx-ablated islets demonstrate loss of glucagon secretion in response to incubation with a mixed amino acid solution and instead insulin is secreted in response to α- and β-cell stimuli. Additionally, Arx-ablated islets display severely reduced glucagon content. These results suggest that long-term ablation of Arx in adult α-cells leads to loss of proper cell fate maintenance and conversion of α-cells into an α-β-PP hybrid cell that no longer secretes glucagon, but instead produces and secretes insulin in response to both α- and β-cell stimuli.

Results:

Ablation of Arx in adult animals leads to improved glucose tolerance

Short-term ablation (2 weeks) of Arx using a global, tamoxifen-inducible mouse model has been previously described [9]. To determine the long-term consequence associated with ablation of Arx in adult mice, the same mouse model was utilized. Two-month old control, $Arx^{L/Y}$;pCAGG-CreER male, and $Arx^{L/L}$;pCAGG-CreER female (IKO from here on) mice were induced with tamoxifen then analyzed by glucose tolerance tests (GTT) every two weeks to monitor changes in glucose homeostasis over time.

Two weeks post-induction, control and IKO mice demonstrated similar blood glucose levels after an overnight fast (Fig. 3.1A). However, IKO mice had significantly lower fasting blood glucose levels four and six weeks post induction when compared to controls (Fig. 3.1B-C). Interestingly at eight weeks post induction IKO mice no longer demonstrated significantly lower fasting blood glucose levels, though the data were approaching significance (Fig 3.1D).

Similar to the fasting blood glucose levels, IKO mice demonstrated a slightly, but not significantly, improved glucose tolerance starting at four weeks post induction (Fig. 3.1B-D). Two weeks post induction control and IKO mice demonstrated similar glucose tolerances (Fig. 3.1A). While control and IKO mice peaked at relatively the same blood glucose level during GTT, IKO mice returned to baseline faster.

Starting at 6 weeks post-induction, IKO mice began to display diarrhea, weight loss, and eventually rectal discharge (data not shown). On average, control mice gained

around 2g after induction (Fig 3.1E). Conversely, IKO mice began to lose weight starting at six weeks and weighed significantly less than controls by eight weeks post induction.

However, Arx is also required for proper enteroendocrine cell specification [11]. The model utilized in this study is a global ablation model resulting in ablation of Arx in the pancreas as well as the intestine. Thus, it is likely that the physiological data presented are due to ablation of Arx in the intestine as well as the pancreas. However, when taken together, these data suggest that while short-term ablation of Arx does not result in a visible phenotype, long-term ablation of Arx results in improved glucose tolerance and weight loss. To examine the pancreas specific defects associated with ablation of Arx in mature α-cells immunohistochemistry and *in vitro* assays were performed.

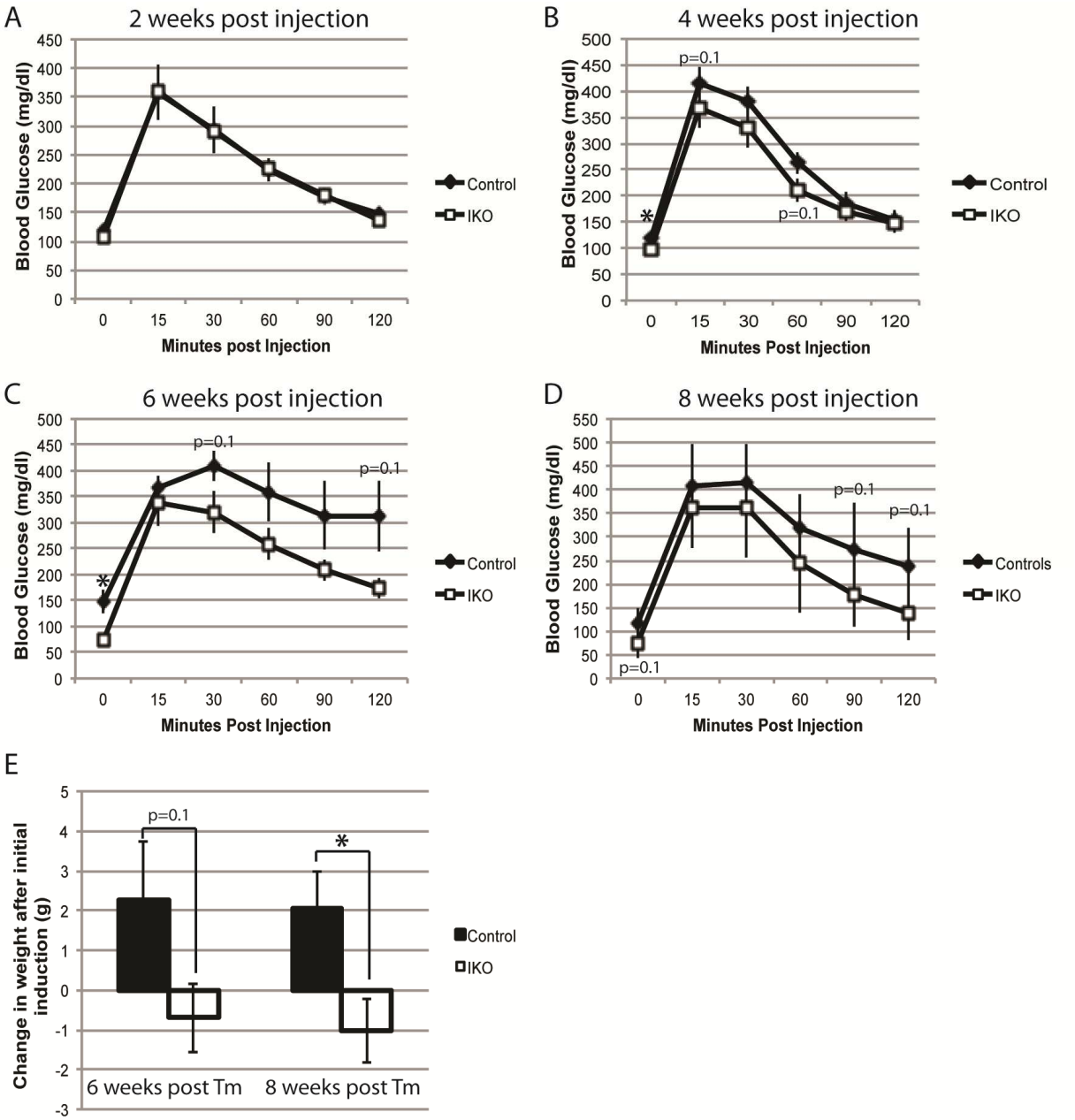

Figure 3.1: IKO animals display improved glucose tolerance and weight loss over time

Figure 3.1: IKO animals display improved glucose tolerance and weight loss over time (A-D): Glucose tolerance tests for control (black circles) and IKO mutant mice (white boxes) two(A), four(B), six(C), and eight(D) weeks after ablation of Arx. Results are graphed as blood glucose levels measured at 0, 15, 30, 60, 90, and 120 minutes after injection of a bolus of glucose. **(E):** Change in weight from initial induction to six and eight weeks post induction. Results are graphed as change in weight with controls represented by the black bars and IKO mutants represented with the white bars. All results are presented as ±SEM with (*) denoting significance where $p<0.05$. Non-significant data *p* values are indicated on corresponding charts. Five control and five IKO mice were utilized for each experiment.

IKO glucagon$^+$ α-cells misexpress insulin and PP, but not somatostatin, eight weeks after ablation of *Arx*

Previous studies have demonstrated that short-term ablation of Arx does not result in loss of α-cells or significant misexpression of other endocrine hormones even with complete loss of Arx expression [9]. To determine if a long-term ablation of Arx results in a pancreatic phenotype, control and IKO pancreata were examined nine weeks post induction for the presence, localization, and segregation of glucagon, insulin, somatostatin, and PP. Similar to the previous study, no apparent change in the quantity or localization of glucagon$^+$ cells was noted in IKO mice (Fig 3.2). However, in contrast to short-term ablation of Arx, long-term ablation resulted in the misexpression of insulin and PP in glucagon$^+$ cells (Fig 3.2 A, B, E, F). No co-expression was noted in control animals. Approximately 50% of glucagon$^+$ cells in IKO animals co-expressed the endocrine hormone insulin. Furthermore, a few α-cells were also noted to co-express PP and glucagon. Conversely, no misexpression of somatostatin was noted in control or IKO pancreata (Fig 3.2C-D). These data demonstrate that long-term ablation of Arx in adult animals does not result in loss of glucagon$^+$ α-cells, but does result in misexpression of the endocrine hormones insulin and PP in a subset of α-cells in IKO mice.

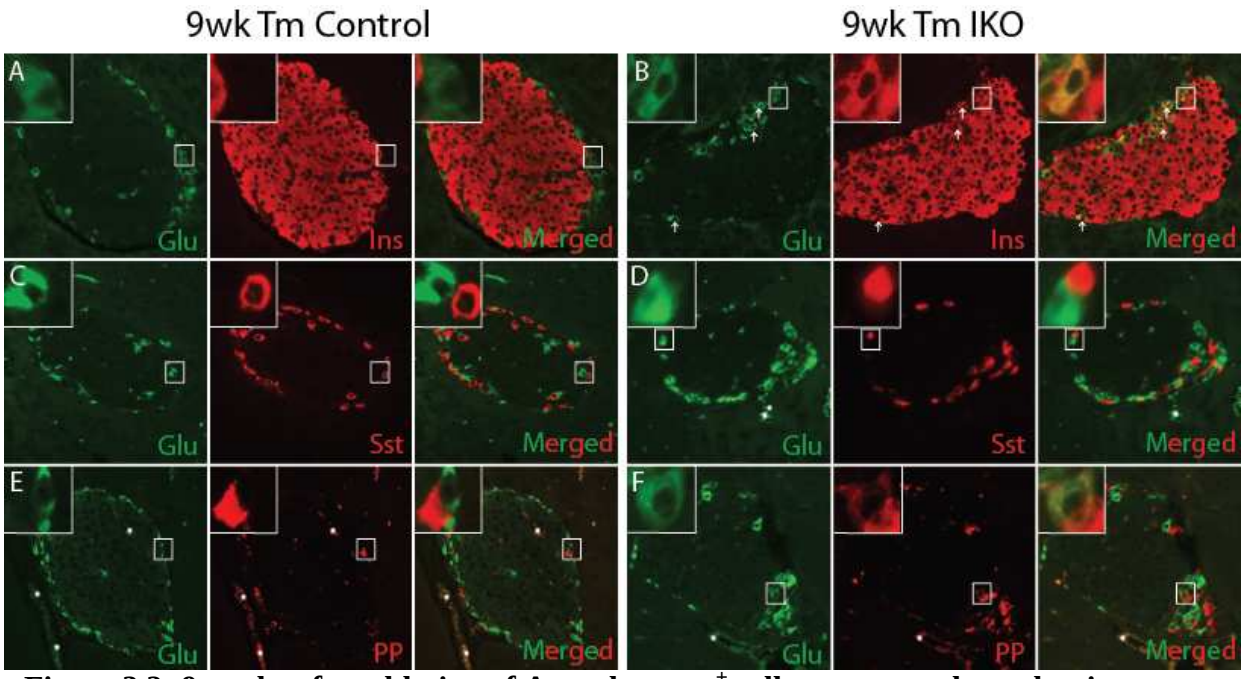

Figure 3.2: 9 weeks after ablation of Arx, glucagon^{+} cells coexpress the endocrine hormones insulin and PP, but not somatostatin

Figure 3.2: 9 weeks after ablation of *Arx* glucagon$^+$ cells coexpress the endocrine hormones insulin and PP, but not somatostatin. (A-F): Control (left) and IKO (right) pancreatic sections were stained for glucagon (green) and insulin (red, A-B), somatostatin (red, Sst, C-D), and PP (red, E-F). No change in the number of glucagon$^+$ cells was apparent in IKO animals. However, many glucagon$^+$ cells coexpressed the endocrine hormones insulin and PP, but not somatostatin. Images are representative from five controls and five IKO animals utilized for each experiment. (*) represent autoflourescing blood cells not real signal. Arrows point to copositive cells. Enlarged image in upper left hand corner represents boxed in cells in corresponding image.

IKO islets display loss of glucagon secretion and improper insulin secretion in response to α-cell stimuli

Given the striking bihormonal phenotype in α-cells upon Arx ablation in the adult pancreas, it was crucial to determine if these mutant islets still properly secrete hormones in response to glucose and amino acid stimulation. Further, since IKO animals demonstrate diarrhea and weight loss from an intestinal phenotype, we utilized *in vitro* secretion assays to isolate islet function from that of the intestinal phenotype. Control and IKO islets were incubated with either media (no stimulation), a 4mM mixed amino acid solution (stimulates glucagon secretion), or a 10mM glucose solution (stimulates insulin secretion). After stimulation, insulin or glucagon secretion was measured.

Glucagon secretion *in vitro* assays demonstrated that control islets secreted glucagon upon stimulation with a mixed amino acid solution, but not a glucose solution (Fig. 3.3A). However, IKO islets had a lower basal level of glucagon secretion and failed to secrete glucagon in response to a mixed amino acid solution (Fig 3.3A). This finding demonstrates loss of glucagon secretion upon long-term ablation of Arx.

To further characterize the secretion properties of IKO islets insulin secretion *in vitro* assays were performed. Control islets released insulin in response to a 10mM glucose solution, but not a mixed amino acid solution (Fig 3.3B). Conversely, IKO islets released insulin when stimulated with both a 10mM glucose and 4mM amino acid solution (Fig 3.3B). This result demonstrates that long-term ablation of Arx results in improper insulin secretion in IKO islets.

Finally, insulin and glucagon content was measured in control and IKO isolated islets. While insulin content was similar between control and IKO islets, glucagon content was drastically reduced in IKO islets when compared to controls (Fig. 3.3C-D). When taken together, these data demonstrate loss of glucagon content and secretion upon long-term Arx ablation in adult pancreata. Instead, Arx-ablated α-cells secrete insulin in response to both α- and β-cell stimuli.

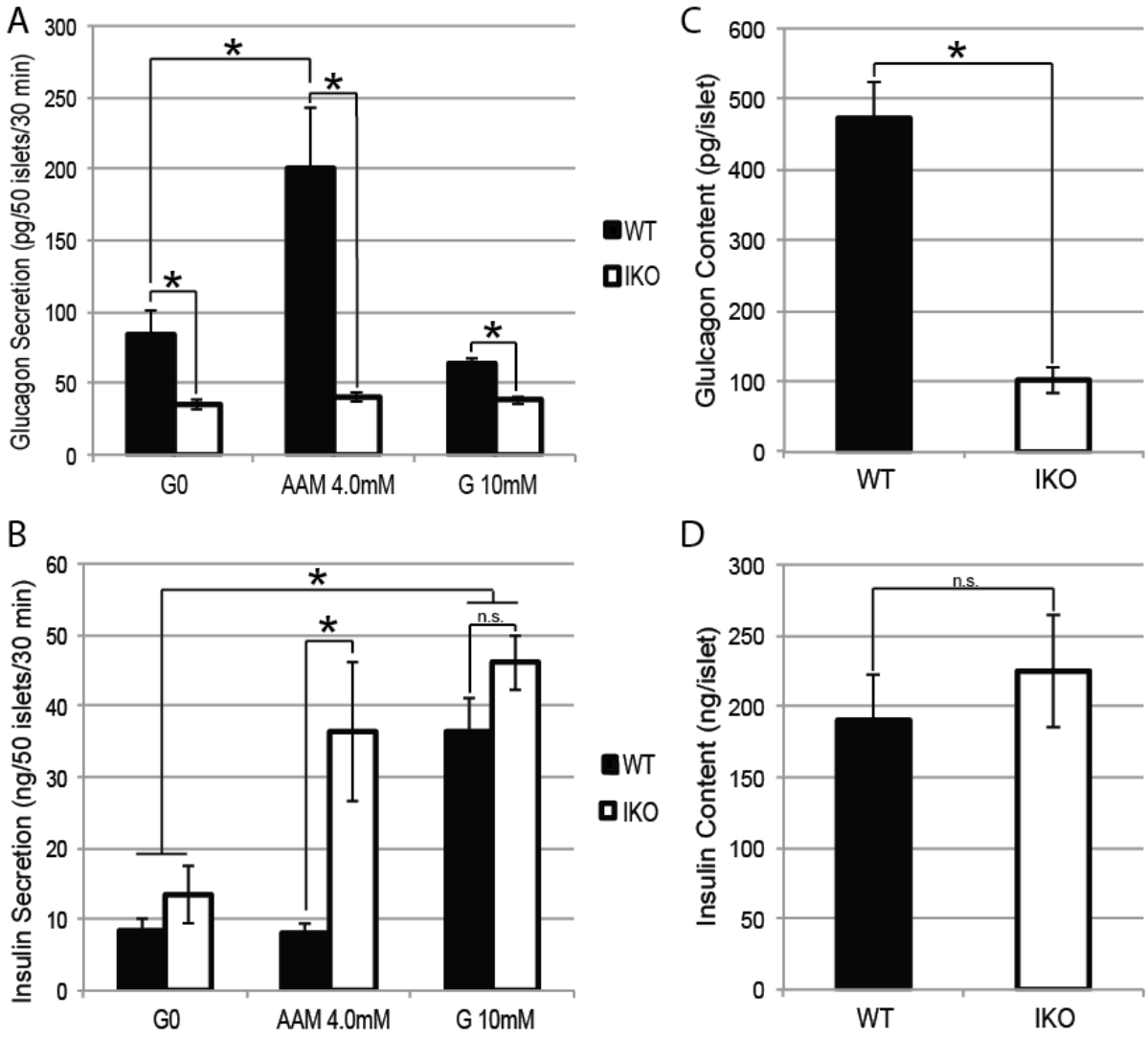

Figure 3.3: IKO islets have reduced glucagon secretion and content with improper insulin secretion

Figure 3.3: IKO islets have reduced glucagon secretion and content with improper insulin secretion. (A-B): *In vitro* insulin (A) and glucagon (B) secretion assay for control (black bars) and IKO (white bars) islets in response to media (G0, no stimulation), a 4.0 mM mixed amino acid solution (AAM, glucagon secretion), and a 10mM glucose solution (G, insulin secretion). Results are graphed as ng of insulin or pg of glucagon secreted per 50 islets in 30 minutes. **(C-D):** Insulin (C) and glucagon (D) content in control (black bar) and IKO (white bar) islets. Results are graphed as ng of insulin/pg of glucagon per islet. All results are graphed as ± SEM with (*) denoting significance where $p \leq 0.05$. n.s. indicates results that are not significant. Above studies were performed by Dr. ChangHong Li

Discussion:

The data presented here demonstrate loss of proper α-cell fate maintenance upon long-term ablation of Arx in adult mice. Eight weeks after tamoxifen induction, IKO mice demonstrate improved glucose tolerance, misexpression of insulin and PP, loss of glucagon secretion and content, and gain of insulin secretion in response to α-cell stimuli. These data suggest that ablation of Arx results in loss of α-cell fate and conversion of these cells into an α-β-PP hybrid cell that expresses multiple hormones, but exclusively secretes insulin in response to both α- and β-cell stimuli. Overall, these results expand the previously defined role for Arx, and demonstrate that Arx is necessary for cell fate maintenance in mature α-cells.

An α-cell specific ablation of Arx is essential to determine pancreas specific physiological defects.

The data presented here were obtained using a global, tamoxifen-inducible transgenic mouse model, resulting in ablation of Arx in the entire animal upon administration of tamoxifen. Prior studies have demonstrated that Arx is necessary for the proper development and maintenance of several different tissues, including the brain, digestive tract, testis, muscle, and pancreas [8,11,12,13,14]. Thus, the physiological defects described here, such as improved glucose tolerance, weight loss, and diarrhea, cannot be attributed solely to loss of Arx in the pancreas. Rather, it is likely that these observed phenotypes are due to the ablation of Arx in multiple tissue types, especially the

digestive tract. However, the immunohistochemistry and *in vitro* secretion assays performed demonstrate a clear pancreas specific defect separate from that of the intestine. These studies allowed pancreas specific analysis of the phenotype and demonstrate that long-term ablation of Arx in mature α-cells results in loss of cell fate maintenance. Future studies utilizing an α-cell specific model will clarify the physiological, pancreas specific, defects, as well as uncover other less severe phenotypes not observed in this global study.

Long-term ablation of Arx in mature α-cells results in loss of cell fate maintenance.

We first demonstrated that short-term (2 weeks) ablation of Arx in adult mice does not result in loss of α-cells or misexpression of other endocrine hormones [9]. We demonstrate that, using the same model, eight weeks post induction the majority of α-cells express multiple hormones, have lost glucagon secretion and content, and improperly secrete insulin in response to α-cell stimuli. These data demonstrate that Arx is necessary for long-term α-cell fate maintenance and function in mature α-cells.

While no apparent defects were noted 2 weeks post induction, Arx-deficient α-cells were likely beginning to lose control of cell fate maintenance. However, there are other cellular mechanisms in place to help maintain α-cell fate. Previous studies have demonstrated that mature α-cells have a vast landscape of activating and repressing chromatin marks [15]. This landscape aids in maintaining the proper transcriptional program in addition to expression of the correct transcription factors and proteins [15]. A

situation can be imagined where, upon ablation of Arx, other transcription factors and the remaining chromatin structure are able to maintain α-cell fate for a brief period of time. However, in the long term, these chromatin marks begin to erode and the necessary transcriptional programs begin to slip, resulting in loss of α-cell fate, misexpression of other endocrine hormones, and subsequent loss of correct signaling mechanisms. Future studies examining the role of histone modifications in mature α-cells, as well as a more detailed description of α-cell characteristics following ablation of Arx, will clarify this result, explore the above hypothesis, and begin to address how cell fate is maintained short-term and long-term in the mature endocrine pancreas.

Arx-ablated α-cells respond to both α- and β-cell stimuli by secretion of insulin.

In vitro stimulation of endocrine islets with glucose and amino acids is an accurate and sensitive way to examine insulin and glucagon secretion, respectively [16]. Control islets responded correctly to each stimulus, secreting insulin in response to a glucose solution and glucagon in response to a mixed amino acid solution. However, IKO islets improperly responded to both stimuli, failing to secrete glucagon in response to amino acids and instead secreting insulin in response to both glucose and amino acids.

This result indicates that upon ablation of Arx, α-cells begin to resemble an α-β-PP hybrid cell that is able to respond via insulin secretion to both α- and β-cell stimuli. Although ablation of Arx leads to loss of α-cell fate maintenance and glucagon secretion, it does not result in loss of glucagon^{+} α-cells as seen upon neonatal ablation [9]. Future

studies examining a longer chase period after ablation of Arx will be useful in determining if these hybrid cells eventually lose glucagon expression and acquire a pure β-cell phenotype. Alternatively, these cells could remain trapped in an intermediate fate and continue to secrete insulin in response to both α- and β-cell stimuli.

Additionally, these data will be useful in developing novel treatments for type II diabetes. Because type II diabetes results from inadequate insulin supply combined with excess glucagon secretion, therapies utilizing endogenous α-cells and transdifferentiating them into functional β-cells would present an ideal therapy [2,4]. If *Arx*-ablated α-cells eventually differentiate into a pure β-cell fate, this result can then be expanded as a possible novel treatment for diabetic patients.

Conclusion

The data presented here demonstrate an essential role for Arx in the maintenance of mature α-cell fate. Long-term ablation of Arx in adult animals leads to loss of α-cell fate maintenance. IKO mice display an improved glucose tolerance over time as well as diarrhea and weight loss. Phenotypic analysis determined co-expression of insulin and PP in glucagon^{+} cells. I*n vitro* secretion assays showed loss of glucagon secretion. However, the IKO islets were able to secrete insulin in response to amino acids which normally only stimulates glucagon secretion. These results demonstrate that Arx is necessary for α-cell fate maintenance. Future α- to β-cell transdifferentiation therapies

for type II diabetes focused on the role of Arx in α-cell fate may provide an avenue for endogenous restoration of normal blood glucose homeostasis.

Materials and Methods:

Animals and Breeding Strategy

The derivation of the Arx floxed and pCAGG-CreER transgenic lines has been previously described [17,18]. IKO mice were generated by crossing heterozygous $Arx^{L}/_{+}$;pCAGG-CreER females to $Arx^{L}/_{Y}$ males. IKO mutant mice consisted of $Arx^{L}/_{Y}$;pCAGG-CreER males and $Arx^{L}/_{L}$;pCAGG-CreER females. Control mice consisted of littermate sex-matched animals, both male and female, including $Arx^{+}/_{Y}$, $Arx^{+}/_{Y}$;pCAGG-CreER, $Arx^{L}/_{Y}$, $Arx^{L}/_{+}$, $Arx^{L}/_{+}$;pCAGG-CreER, and $Arx^{L}/_{L}$. No significant differences were noted between any control animals in terms of pancreatic and islet size, weight, or morphology. Male and female IKO animals were phenotypically identical and used interchangeably in this study.

Immunohistochemistry

Dissections were performed in cold 1X PBS. The entire pancreatic tissue was removed, weighed, and submerged in cold 4% PFA/PBS overnight at 4°C. Tissue was then rinsed, dehydrated, embedded in paraffin, and sectioned at 8 μm on charged glass slides. Immunohistochemistry was performed using 10 mmol citric acid buffer (ph 6.0) for antigen retrieval followed by blocking endogenous protein using CAS-Block reagent (Invitrogen). Primary antibodies were added and slides incubated overnight at 4°C. Primary antibodies used were: Insulin (MS 1:400, Thermo Scientific and GP 1:1000,

Abcam), Glucagon (GP 1:3000, Millipore and Rb 1:1000, Chemicon), Somatostatin (Rb 1:200, Invitrogen), and PP (Rb 1:200, Invitrogen)

Tamoxifen Induction

Two-month old IKO and matched littermate controls were injected with 50 µg/g body weight of 10 mg/ml tamoxifen solution to induce Cre expression. Tamoxifen was dissolved in a solution of 10% ethanol and 90% sunflower seed oil (Sigma). IKO and control animals consisted of both male and female mice. Injections were intraperitoneal and performed on three consecutive days followed by a specified chase period (2-8 weeks post induction).

Alternatively, induction was accomplished using an oral gavage system with the same concentration of tamoxifen solution and induction strategy previously described. Both methods resulted in similar ablation with no significant differences observed.

Glucose Tolerance Test

Glucose homeostasis was examined using an intraperitoneal glucose tolerance test. Mice were fasted overnight and injected with a bolus of glucose (2g/kg body weight, Sigma). Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes post injection using an automatic glucometer (One Touch Ultra; LifeScan).

Islet Isolation and Culture

Control and IKO islets were isolated via collagenase digestion. Once isolated islets were cultured in 10mM glucose RMPI 1640 media (Sigma) for three to four days. Culture media consisted of 2mM glutamine, 100units/ml penicillin, and 50µg/ml streptomycin. Islets were maintained at 37°C. Islet Isolation studies were performed by Dr. ChangHong Li of the Children's Hospital of Philadelphia.

***In vitro* Secretion Assays**

50 islets from each control and IKO animal were placed in 96-well plate, pre-incubated in warmed KRBB (115mM NaCl, 24mM $NaHCO_3$, 5mM KCl, 1mM $MgCl_2$, 2.5mM $CaCl_2$, pH 7.4) buffer for 30 minutes at 37°C, and than stimulated with either a 4.0mM mixed amino acid solution or a 10mM glucose solution for 30 minutes at 37°C. Supernatant was removed from well and glucagon secretion measured (Cisbio glucagon kit) or diluted 10X and insulin secretion measured (Cisbio insulin kit). Molecular Devices M5e plate reader was utilized to measure glucagon and insulin.

Insulin and Glucagon Content

Islets were isolated and washed 2X in cold 1X PBS in 96-well plate. 100µl homogenization buffer (10mM Trisma, 10mM Sodium Acetate, 10µM EDTA, 1% Triton X-100, pH 7.4) was added, islets were homogenized, and diluted 100X for glucagon

content or 2000X for insulin content. Molecular Devices M5e plate reader was utilized to measure insulin and glucagon content.

Statistical Analysis

All results are graphed as ± the standard error of the mean (SEM). In all calculations significance was determined using a two-tailed Student's t-test where $p \leq 0.05$ was considered significant. Other p values that did not reach significance, but were deemed meaningful are displayed on the corresponding graphs/charts.

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CHAPTER IV:

Arx polyalanine expansion in mice leads to reduced pancreatic α-cell specification and increased α-cell death

Abstract

ARX/Arx is a homeodomain containing transcription factor necessary for the specification and early maintenance of pancreatic endocrine α-cells. Many transcription factors important to pancreas development, including *ARX/Arx*, are also crucial for proper brain development. Although null mutations of *ARX* in human patients result in the severe neurologic syndrome XLAG (X-linked lissencephaly associated with abnormal genitalia), the most common mutation is the expansion of the first polyalanine tract of *ARX*, which results primarily in the clinical syndrome ISSX (infantile spasms). Subsequent mouse models of XLAG, ISSX and other human *ARX* mutations demonstrate a direct genotype-phenotype correlation in ARX-related neurologic disorders. Furthermore, mouse models utilizing a polyalanine tract expansion mutation have illustrated critical developmental differences between null mutations and expansion mutations in the brain, revealing context-specific defects. Although *Arx* is known to be required for the specification and early maintenance of pancreatic endocrine α-cell, the consequence of a polyalanine expansion has not been explored.

We report here that mice with an expansion mutation in the first polyalanine tract of *Arx* have impaired α-cell specification and maintenance with apoptosis playing a critical role in gradual α-cell loss. This finding is novel and distinct from what was reported in mice with germ-line *Arx* null mutation, in which α-cells were re-specified into β- and δ-cells. Overall, this analysis of an Arx polyalanine expansion mutation on pancreatic development further defines the critical nature of timing in α-cell specification and maintenance.

Introduction

Aristaless-related homeobox gene (*Arx*) encodes a homedomain containing transcription factor that is expressed in the brain, testis, muscle, pancreas, and digestive tract [1,2]. In the brain, *Arx* is essential for the proper development and migration of GABA-ergic interneurons and has a role in cortical ventricular zone proliferation [3,4]. In humans, various mutations of *ARX* result in a spectrum of neurologic disorders, the most severe clinical presentation being X-linked lissencephaly associated with abnormal genitalia (XLAG) [5]. XLAG, which has been linked to null and missense mutations in *ARX*, is characterized by a severe brain malformation, termed lissencephaly, corpus callosum agenesis, neonatal-onset intractable epilepsy, and early death [6]. *Arx* null mice phenocopy the clinical presentation of XLAG patients, displaying cortical brain malformations and agenesis with lethality within 24-hours of birth [3,4]. Histological and molecular analyses reveal a dual function for *Arx* in radial and tangential migration of GABA-ergic interneurons in mice [7].

Interestingly, polyalanine expansion mutations are the most common *ARX* mutations found in human disease [8]. ARX contains four polyalanine repeat tracts spaced throughout the protein [9]. It is the first two polyalanine repeats that are most often expanded in human disease [10]. Patients with these expansion mutations present with severe neurologic phenotypes, including seizures and mental retardation, but without brain malformations [11]. Expansion of the first polyalanine tract by an additional seven alanines has been associated with West Syndrome or infantile spasms (ISSX) [12].

Analyses using genetically modified mouse models have been performed to explore the impact different *ARX* polyalanine expansion mutations have on neuronal development and cognitive functionality; these models demonstrate a similar genotype-phenotype correlation to humans [13]. Specifically, mouse models with an expansion mutation of the first polyalanine tract of *Arx* reveal that only tangential migration of GABA-ergic interneurons is lost, with no significant impact to radial migration [14,15]. Thus, it appears that expansion of the first polyalanine tract of *Arx* results in context specific defects in neural development.

In addition to the profound effects *ARX/Arx* mutations have on the brain, it also has a severe impact on the development of other organs. Of note, Itoh and colleagues recently described complete loss of glucagon-producing α-cells in the pancreas of an *ARX*-null XLAG patient [16]. The mammalian pancreas contains an endocrine and exocrine compartment that functions to produce and secrete hormones and enzymes necessary for glucose homeostasis and digestion, respectively [17]. The endocrine compartment is organized into Islets of Langerhans with a core of insulin-producing β-cells and a surrounding mantle of α-, δ-, ε-, and PP-cells producing the hormones glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively [18]. *Arx* is expressed in $Ngn3^{+}$ endocrine progenitors and then restricted to the α-cell lineage where it is expressed throughout the life of the animal [2]. Loss of glucagon-producing α-cells in XLAG patients suggests that *ARX* is necessary for specification and/or maintenance of this endocrine cell population [16]. Similar observations in the pancreas were also reported in *Arx* null mice in which a complete loss of α-cells was detected [2,19].

Without Arx function, α-cells are lost while β- and δ-cells simultaneously increase without a change in total endocrine mass [2]. Recently, lineage tracing of these *Arx* ablated α-cells has demonstrated that removal of *Arx* in glucagon$^+$ cells results in conversion into an insulin$^+$ β-like fate through a bihormonal intermediate [20]. Interestingly, this conversion of α-cells into non-α-cell fates was only seen with loss of *Arx* during the neonatal period, not in adulthood [20].

Previous work has suggested a dual role for Arx in both specification of α-cells and repression of β- and δ-cell fate. However, no studies have investigated the effects of the more common polyalanine expansion mutation on endocrine pancreas α-cell specification and maintenance. Here we show that pancreatic defects associated with this *Arx* expanded mouse model (ArxE) are also context specific. Our results demonstrate a reduced number of glucagon-expressing α-cells in ArxE pancreata, suggesting impaired α-cell specification. However, a subset of α-cells is specified in ArxE mice and these cells do not express other hormones or β-cell specific transcription factors, indicating correct fate determination. Conversely, maintenance of this subset of α-cells is impaired, and these cells are gradually lost through apoptosis over time. Furthermore, unlike *Arx* null mutations, no change in β- or δ- cell mass is observed, suggesting that an expanded Arx protein is still capable of blocking other, non-α-cell fates.

These results describe a unique pancreatic phenotype associated with an *Arx* polyalanine expansion mutation and further illustrate the genotype-phenotype correlation associated with different forms of *ARX/Arx* mutations. Taken together, these findings

help elucidate our understanding of Arx-related syndromes outside of the brain as well as characterizing the different roles of *Arx* in α-cell specification versus maintenance.

Results

ArxE mice retain a subset of glucagon-producing α-cells at embryonic day (E) 15.5.

To determine the effect an *Arx* expansion mutation has on α-cell specification and maintenance, hemizygous $Arx^{Expanded}/_{Y}$ (referred to as ArxE from here on) mutant mice were obtained by crossing heterozygous $Arx^{Expanded}/_{+}$ females to wild-type $Arx^{+}/_{Y}$ males. ArxE mice were born in normal Mendelian ratios with birth weights similar to their littermate controls. However, ArxE mice displayed a slower growth curve and by postnatal day 6 (P6) were significantly smaller than control littermates (Terry and May, unpublished data). We did not observe any significant changes in pancreatic weight, morphology, or histology between ArxE mice and controls at any time point (data not shown).

To explore a possible pancreatic defect, immunostaining was performed in control and ArxE pancreata at E15.5 to examine the presence, localization, and segregation of each endocrine hormone. Immunostaining for glucagon demonstrated a dramatic reduction in the number of glucagon^{+} α-cells at E15.5 in ArxE mice (Fig. 4.1A-F). However, unlike *Arx* null animals, a subset of α-cells appeared to be specified in ArxE mice by glucagon staining [2,19]. Furthermore, these remaining glucagon^{+} cells did not coexpress insulin or somatostatin suggesting proper α-cell fate determination (Fig. 4.1A-D). Co-staining for glucagon and ghrelin did reveal 30% colocalization in both control and ArxE pancreata, similar to what has been previously described in wildtype pancreata (Fig. 4.1E-F) [21]. PP-cells are not normally present at E15.5 and immunostaining for

glucagon and PP did not reveal any precocious specification of this cell type in ArxE mice (data not shown) [17]. Consistent with our qualitative analysis, morphometric studies showed that while α-cell mass is significantly decreased to 20% of wild-type levels, no change in β- or δ-cell mass was observed (Fig. 1G). Furthermore, total endocrine mass was not significantly altered at this time point.

Although the endocrine mass of β- and δ-cells was not changed, we also measured the expression levels of the hormone genes in ArxE mice using quantitative PCR (qPCR). The expression level of glucagon was significantly reduced in ArxE pancreata, as expected (Fig. 1H). However, both insulin and somatostatin transcript levels were significantly upregulated in ArxE pancreata when compared to littermate controls (Fig. 4.1H). No significant difference in the expression level of PP was observed in ArxE mice, further indicating that there was no precocious expression of PP upon ArxE mutation (Fig. 4.1H). Since β- or δ-cell mass is not increased, our qPCR results suggest that there is a significant upregulation of hormone gene expression within the respective endocrine cells. These data demonstrate that the majority of α-cell specification is impaired in ArxE mice but, for a subset of α-cells that are specified, fate determination appears intact without any misexpression of other endocrine hormone populations within the α-cells, unlike Arx null mouse models.

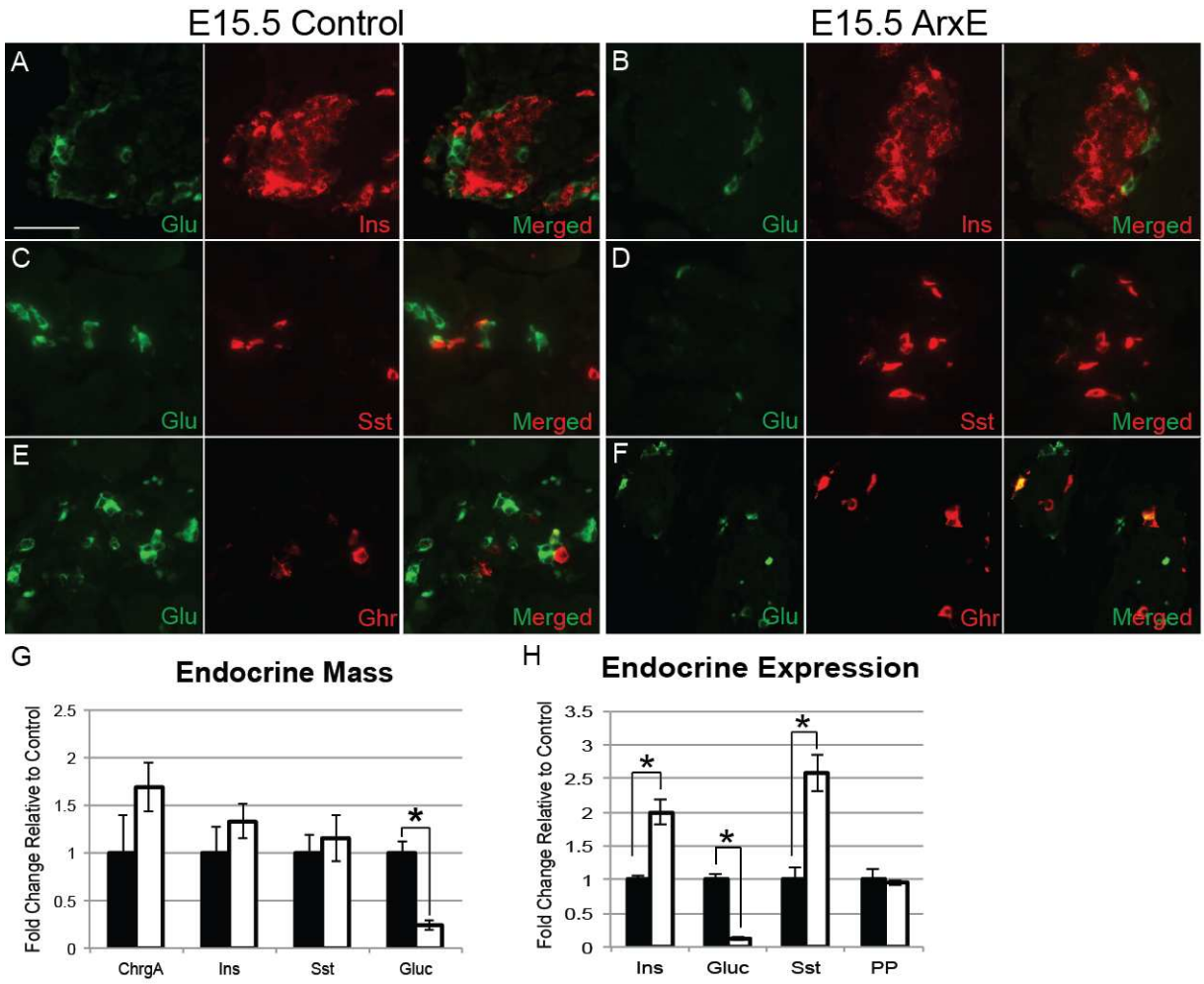

Figure 4.1: ArxE mice are able to specify a subset of α-cells at E15.5

Figure 4.1: ArxE mice are able to specify a subset of α-cells at E15.5. (A-F): Control and ArxE E15.5 pancreatic sections were stained for glucagon (green) and insulin (red; A-B), somatostatin (Sst; red; C-D), and ghrelin (Ghr; red; E-F). Scale bar denotes 50μm. **(G):** Quantification of total endocrine (ChrgA) and β-, δ-, and α-cell mass in control (black bar) and ArxE (white bar) pancreata. **(H):** Quantification of transcript levels for each endocrine hormone in control (black bar) and ArxE pancreata (white bar) at E15.5 using qPCR. All results are graphed as fold change relative to littermate controls ± standard error of the mean. Significance is denoted with (*) when $p \leq 0.05$. All analysis consists of 4-5 animals per group.

By P14, the α-cell lineage is profoundly lost in ArxE pancreata

To determine the fate of this remaining subset of α-cells in ArxE mice, control and ArxE P14 pancreatic sections were examined for the expression of each endocrine population. Morphometric analysis showed that α-cell mass is reduced by 99.5% with only a few single glucagon^{+} cells remaining (Fig. 4.2A-H, J). Immunostaining and quantification of β-, δ-, PP-, and ε-cell mass at P14 did not reveal any significant differences in quantity or localization of these endocrine cell types in ArxE mice (Fig. 4.2A-I). Finally, we observed a significant 30% reduction in total endocrine mass in P14 ArxE mice as measured by Chromogranin A staining (Fig. 4.2I). This reduction is likely the result of the drastic loss of α-cells in the ArxE mice at this age. These data suggest that α-cells are lost in ArxE mutant mice and not reallocated to a β- or δ-cell fate.

To examine when the loss of the α-cell lineage occurs, we measured α-cell mass in embryonic and postnatal pancreata, starting at E15.5 (Fig.4. 2J). As previously described, a subset of α-cells is properly specified in E15.5 ArxE pancreata; however, these cells are gradually lost over time. From our morphometric analyses of E15.5, E18.5, P0 and P14 pancreata, we conclude that this α-cell population is not maintained, and is lost in a temporal manner without reallocation to a β- or δ-cell fate, resulting in a significant reduction in total endocrine cell mass.

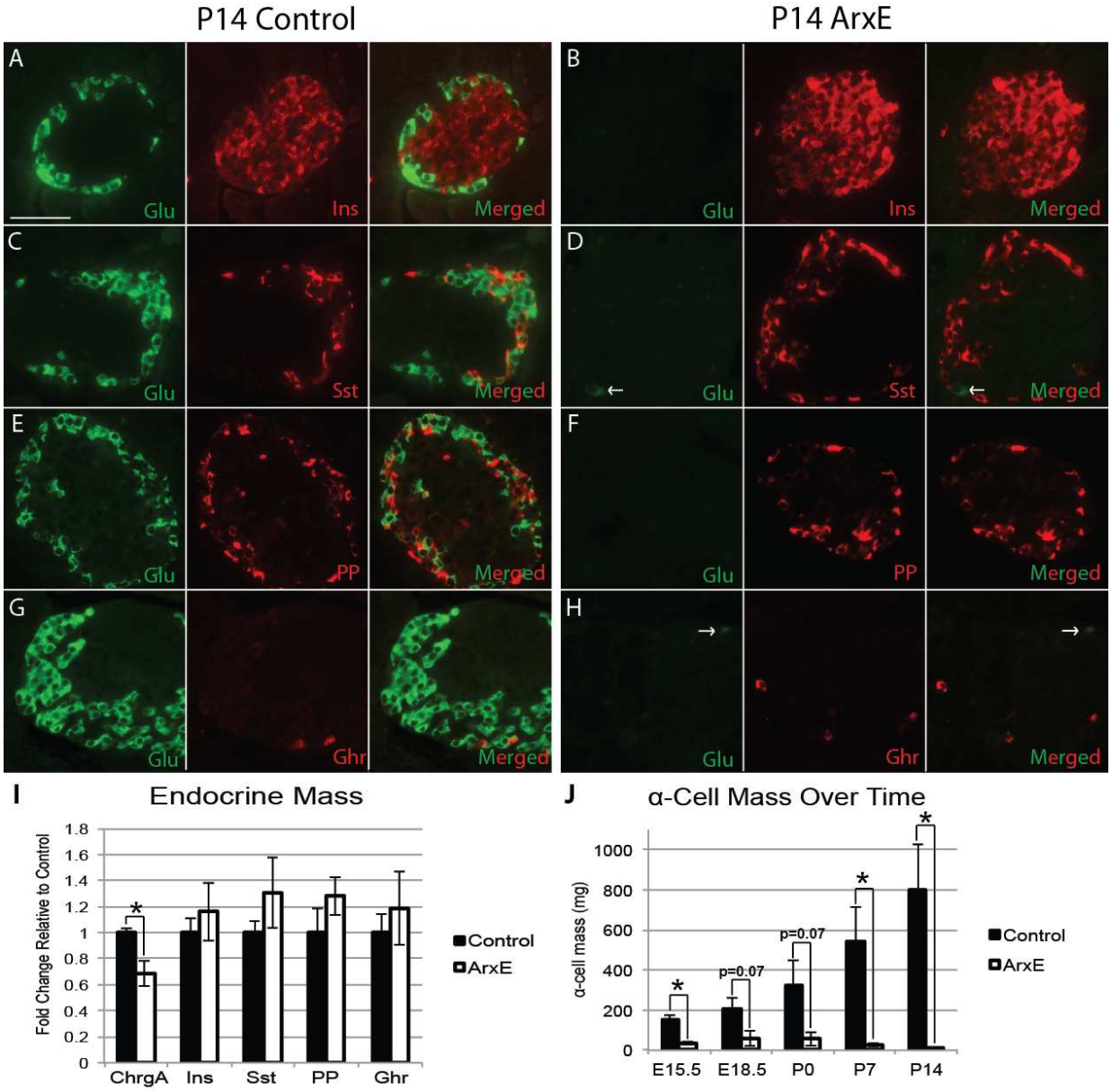

Figure 4.2: ArxE mice have almost complete loss of α-cell fate by P14 with a concomitant decrease in total endocrine mass, but no change in β- and δ-cell mass

Figure 4.2: ArxE mice have almost complete loss of α-cell fate by P14 with a concomitant decrease in total endocrine mass, but no change in β- and δ-cell mass. **(A-H):** P14 pancreatic sections were stained for glucagon (green) and insulin (red; A-B), somatostatin (Sst; red; C-D), PP (red; E-F), and ghrelin (Ghr; red; G-H). Scale bar denotes 50μm. **(I):** Quantification of endocrine hormone mass including total endocrine mass (ChrgA), insulin, somatostatin, PP, and ghrelin displayed as fold change in ArxE mice (white bar) relative to control (black bar). **(J):** Analysis of glucagon mass over time starting at E15.5 and ending at P14 in control (black bar) and ArxE (white bar) pancreata. Resulting p value is listed. (*) denotes significance where $p<0.05$. Error bars represent standard error of the mean (I, J). For all analysis 4-5 animals per group were analyzed with all ArxE mice being males and control mice consisting of male and female mice.

ArxE α-cells do not express β-cell specific transcription factors

To further examine whether α-cell specification is properly executed in ArxE pancreata, qPCR analysis was used to analyze the expression of α- and β-cell specific transcription factors in ArxE and control pancreata at E15.5. The α-cell specific transcription factors Arx and Brn4 were significantly downregulated in ArxE mice to approximately 30% of wild-type levels (Fig. 4.3A). This downregulation is similar to the reduction in α-cell mass and is likely the result of having fewer α-cells expressing these transcription factors. Strikingly, examination of β-cell specific factors MafA, Glut2, Pdx1, and Pax4 demonstrated a significant upregulation of MafA and Glut2 while Pdx1 and Pax4 levels were not altered (Fig. 4.3A).

To determine if the significant upregulation of MafA and Glut2 mRNA levels in ArxE mice results in misexpression of these factors in α-cells, immunostaining for glucagon, Pdx1, Glut2, and MafA was performed (Fig. 4.3B-G). Similar to control animals, no colocalization of glucagon with Pdx1, Glut2, or MafA was observed in ArxE pancreata at E15.5 (Fig. 4.3B-G). These data indicate that while an *Arx* expansion mutation results in upregulation of MafA and Glut2 transcript levels, this change does not lead to misexpression of the protein in ArxE E15.5 glucagon$^+$ α-cells. When combined with previous results, this result indicates that α-cell fate determination has occurred normally in the remaining glucagon$^+$ cells.

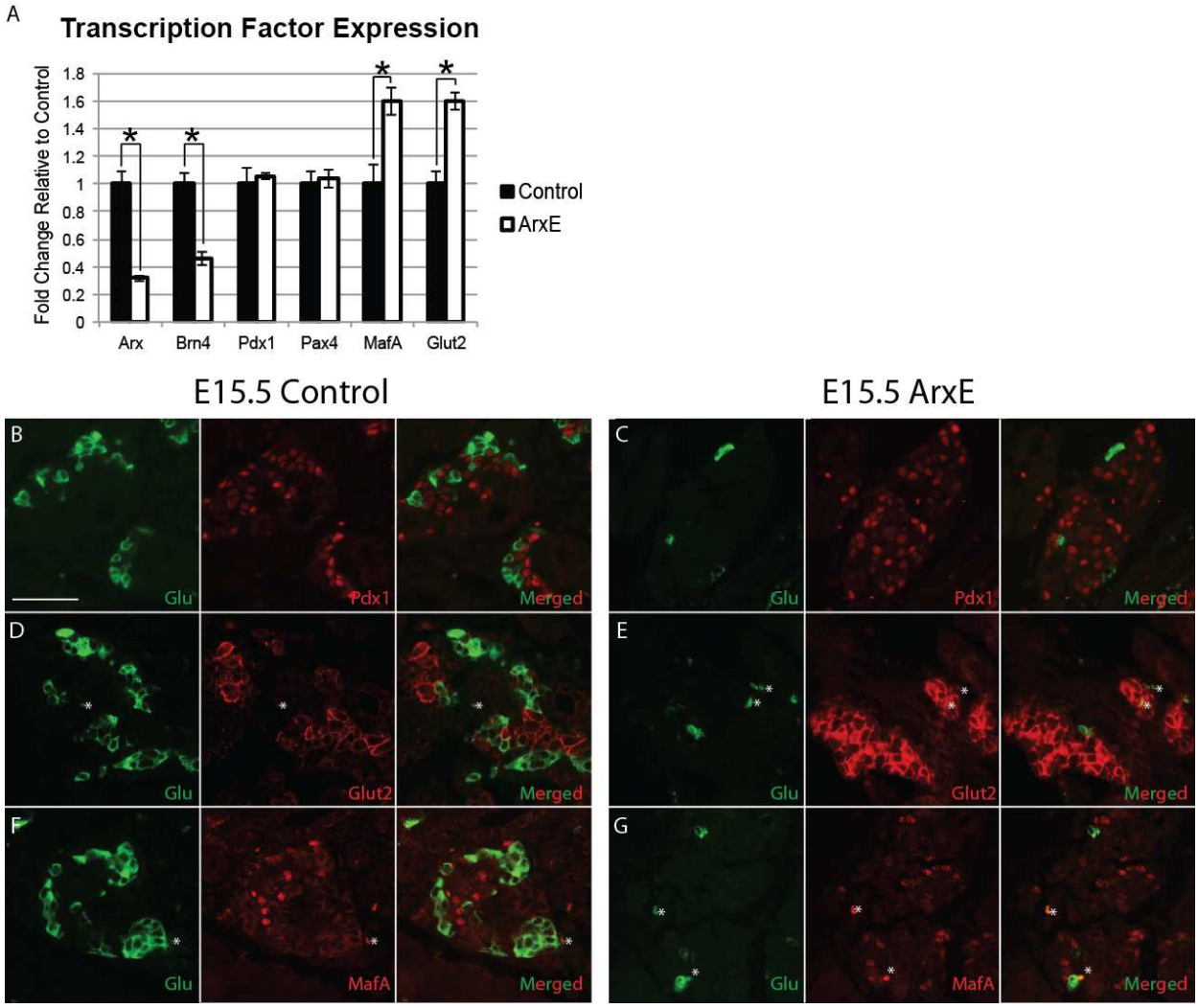

Figure 4.3: Specified α-cells in ArxE mice do not misexpress β-cell specific transcription factors at E15.5

Figure 4.3: Specified α-cells in ArxE mice do not misexpress β-cell specific transcription factors at E15.5. (A): Quantification of transcript levels for α- and β-cell specific transcription factors graphed as fold change in ArxE pancreata (white bar) relative to littermate control (black bar). Error bars represent standard error of the mean. Significance, when $p \leq 0.05$, is denoted with (*). **(B-G):** Control and ArxE E15.5 pancreatic sections were stained for glucagon (green) and Pdx1 (red; B-C), Glut2 (red; D-E), and MafA (red; F-G). Five specimens were analyzed for each group and representative images taken. (*) denotes autofluorescence of red blood cells. Scale bar denotes 50μm.

ArxE pancreata contain more apoptotic glucagon$^+$ cells

There are two possible causes for the apparent loss of α-cells in ArxE pancreata: reduced proliferation or increased apoptosis. Proliferation was measured by examining the localization and quantity of proliferating glucagon$^+$ α-cells using the proliferation marker Ki67. No change in the number of proliferating, Ki67$^+$ α-cells was noted in ArxE mice when compared to control littermates at E15.5 (Fig. 4.4A-B). Furthermore, expression analysis using qPCR for Ki67 and Birc5 (another proliferation marker) did not reveal any differences in the expression level between control and ArxE pancreata (Fig. 4.4C). These data demonstrates that α-cells proliferate normally in ArxE mice at E15.5.

To explore whether changes in the rate of apoptosis may have contributed to α-cell loss we performed TUNEL assays. Co-immunostaining for glucagon and TUNEL demonstrated an increase in the number of TUNEL$^+$/glucagon$^+$ cells in ArxE pancreata at E15.5 (Fig. 4.4D-E). Quantification of the percentage of glucagon$^+$ that were TUNEL$^+$ in control and ArxE pancreata demonstrated a significant and profound increase in the percent apoptotic α-cells in ArxE pancreata (Fig. 4.4F). Taken together, these results demonstrate that an increase in apoptosis is the major contributor to the temporal α-cell loss seen in ArxE pancreata.

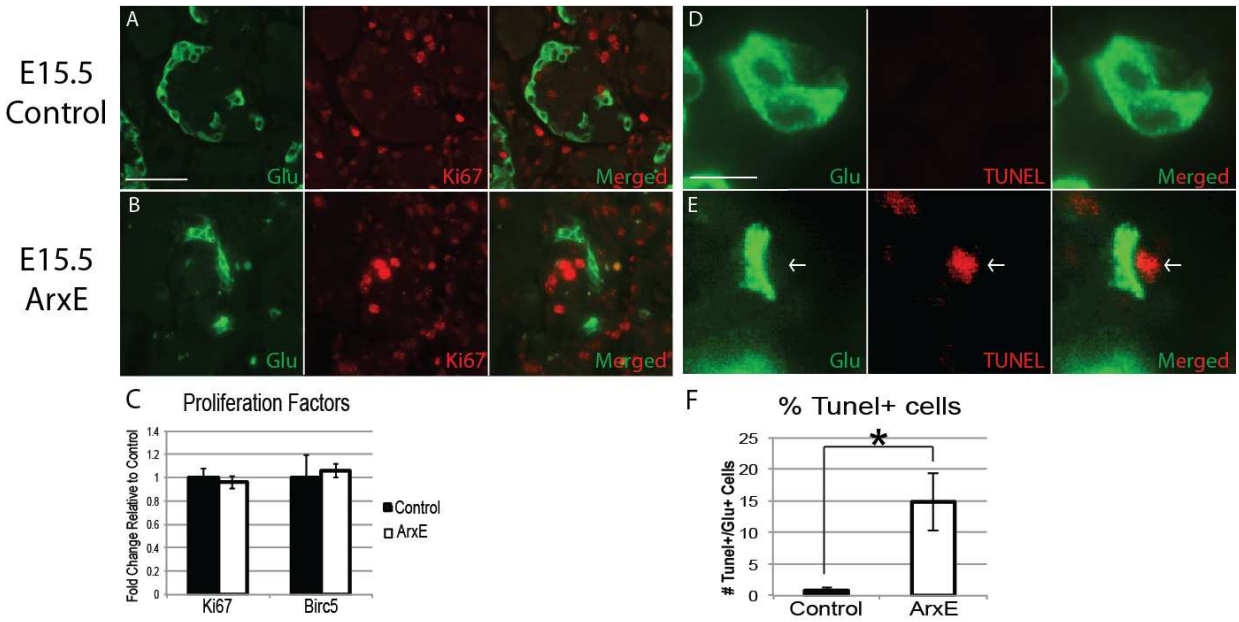

Figure 4.4: There is a drastic increase in the percentage of apoptotic glucagon^{+} cells in ArxE mice, but no apparent change in proliferation

Figure 4.4: There is a drastic increase in the percentage of apoptotic glucagon$^+$ cells in ArxE mice, but no apparent change in proliferation. (A-B): Control and ArxE E15.5 pancreatic sections were stained for glucagon (green) and Ki67 (red). Scale bar denotes 50μm. **(C):** qPCR analysis for two markers of proliferation, Ki67 and Birc5, in E15.5 control (black bar) and ArxE pancreata (white bar). Results are graphed as fold change relative to littermate control ± the standard error of the mean. **(D-E):** Control and ArxE E15.5 pancreatic sections were stained for glucagon (green) and TUNEL (red). Arrow points to TUNEL$^+$ cell. Scale bar denotes 10μm. **(F):** Quantification of the percentage TUNEL$^+$glucagon$^+$ cells over total counted glucagon$^+$ cells in control and ArxE E15.5 pancreatic sections. All glucagon$^+$ cells in five different pancreatic sections from both control (black bar) and ArxE (white bar) mice were counted and determined to be TUNEL positive or negative. The percentage of TUNEL$^+$glucagon$^+$ cells was calculated and graphed as ± standard error of the mean. (*) denotes significance where $p \leq 0.05$. Between 4 and 5 animals were examined per group for each analysis.

Discussion

This study demonstrates that expansion of the first polyalanine tract of *Arx* results in impaired specification and maintenance of endocrine α-cells through a mechanism of programmed cell death, as opposed to α-cell fate re-specification. We show that only a subset (20%) of α-cells is present in E15.5 ArxE pancreata. However, these glucagon^{+} α-cells are not maintained over time and eventually undergo apoptosis, leading to a complete absence of the α-cell lineage and a significant decrease in total endocrine cell mass by P14. While cell proliferation is a major mechanism to expanding α-cell mass during development, we did not detect a significant change in the rate of proliferation in ArxE pancreata. These findings suggest that programmed cell death is the principal cause, after initial specification, for α-cell loss in ArxE mice (Fig. 4.5).

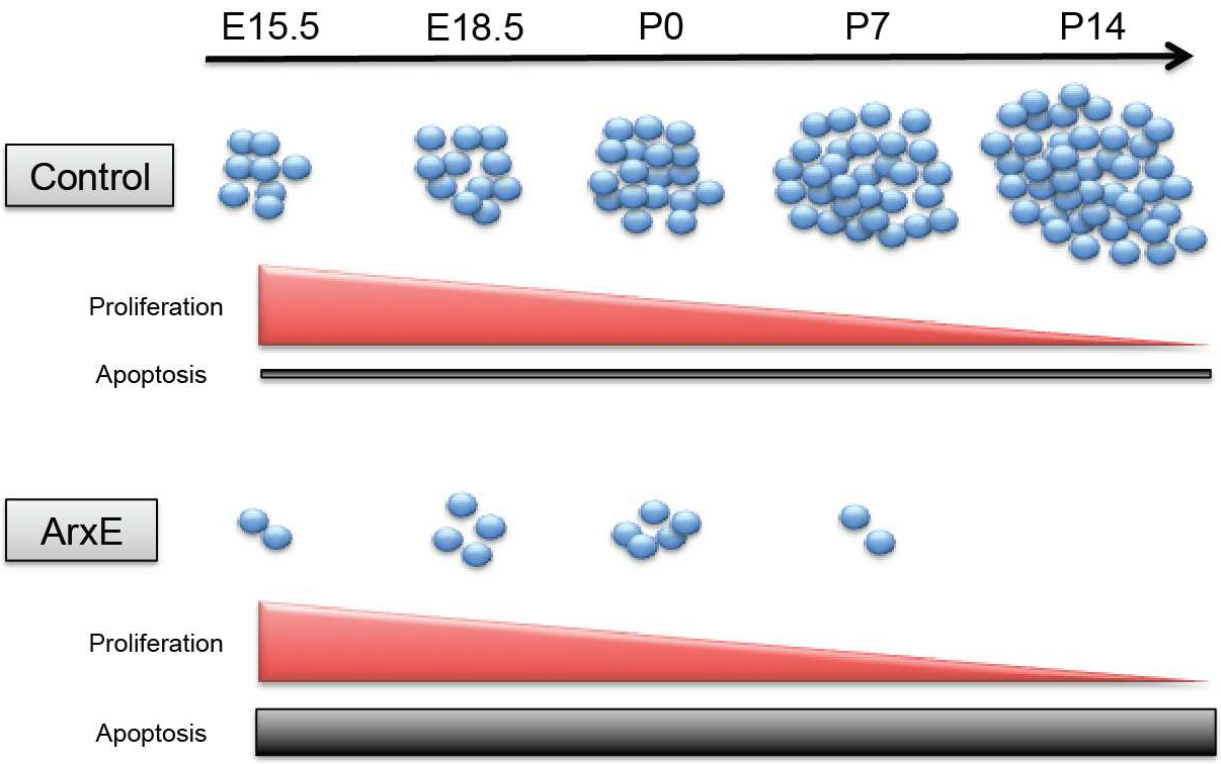

Figure 4.5: ArxE mice are able to correctly specify a subset of α-cells, but α-cells are gradually lost through apoptosis

Figure 4.5: ArxE mice are able to correctly specify a subset of α-cells, but α-cells are gradually lost through apoptosis. Model demonstrating normal proliferation, but increased apoptosis in ArxE mice. Normal proliferation during embryonic time points maintains the α-cell lineage by replacing cells lost to apoptosis. However, proliferation slows during the neonatal stage leading to loss of the α-cell lineage.

ArxE mice reveal context specific defects in α-cells that differ from Arx null mice.

Our study reveals a novel and unique impact of ArxE mutations on the pancreas. Phenotypes found in ArxE mice differ from those reported in the *Arx* null mouse models: 1) there is no change in β- and δ-cell mass, 2) there is a significant reduction in total endocrine cell mass, and 3) a small number of α-cells are present during embryogenesis [2]. *Arx* null mutations result in loss of α-cell specification in which glucagon^{+} cells are never present, even at embryonic time points [2]. Loss of *Arx* in these endocrine progenitors results in loss of specification and reallocation of these cells into a β- or δ-cell fate, thus maintaining total endocrine mass [2]. Conversely, ArxE mice are able to specify a small subset of α-cells and, more importantly, repress non-α-cell fates, including β and δ. This retained repressive ability results in normal β- and δ-cell mass even with the eventual complete loss of the α-cell lineage. Finally, α-cells in ArxE mice are not able to maintain their fate, as these α-cells are gradually eliminated over time through apoptosis, not reallocation or reprogramming, resulting in an overall decrease in endocrine cell mass.

ArxE mice display selective derepression similar to previous neuronal studies.

Cell culture based mechanistic studies have demonstrated that Arx associates with the groucho-family corepressor Tle1 [22]. This association is carried out through Arx's octapeptide domain and results in increased repressive activity [22]. Examination of this association in an expanded neuronal model demonstrates a decreased, but not complete

loss, of Arx-Tle1 protein binding [23]. Furthermore, neuronal studies have demonstrated that expansion of the first polyalanine tract of Arx results in selective derepression of a subset of Arx targets [23]. It is hypothesized that loss of association with specific co-repressors (if not Tle1 itself) results in this selective derepression [23]. Interestingly, we show here that ArxE mice do not misexpress either the endocrine hormones insulin and somatostatin or β-cell specific transcription factors in embryonic α-cells. Thus, it appears that at least part of Arx's repressive abilities are intact. Future studies using chromatin immunoprecipitation to determine direct targets of Arx in the pancreas will serve to clarify this finding. The increased expression of insulin, somatostatin, and β-cell markers are in the cells where Arx is not expressed, suggesting a secondary effect.

Comparing ArxE and Arx null mouse models demonstrate a direct genotype-phenotype correlation.

In neuronal studies, there appears to be a direct genotype-phenotype correlation associated with various *Arx* mutations [24]. Our study demonstrates that this correlation is likely to be applicable in the pancreas as well. In the brain, more severe phenotypes are attributed to null and missense mutations of *Arx* [8]. In the pancreas, the *Arx* null mutation has an earlier onset with complete loss of glucagon-expressing α-cells [2]. Although the α-cell population is almost completely lost by P14 in the ArxE mice, their survival curve compared to the global null mice is improved. It is difficult to determine if this improved survival can be attributed to the pancreatic phenotype without a tissue-

specific model. Although it has been suggested that hypoglycemia is the cause of early fatality, blood sugar levels cannot be attributed solely to the pancreas in the setting of diarrhea and lean body mass in the ArxE mouse model (Terry and May, unpublished data). Complete understanding at a molecular level of why this spectrum of disease exists in both mouse models and human patients will be essential in generating future treatments for patients with *ARX*-related disorders.

Conclusion

In conclusion, this study demonstrates dual functions for Arx in α-cell gene activation and β-cell gene repression during fate specification and maintenance. Utilizing a mouse model with an expansion mutation of the first polyalanine tract of *Arx*, we demonstrate present but impaired α-cell fate specification. Although α-cell number is dramatically reduced, proper fate determination is observed in the remaining α-cells. However, these cells eventually undergo apoptosis, which leads to complete loss of α-cell fate postnatally. β- or δ-cell mass is not increased, and there is a significant decrease in total endocrine cell mass, attributed to α-cell death. Our study begins to explore the more common polyalanine expansion, non-null *Arx* mutations and the effect they have on α-cell specification and maintenance. Being able to separate the dual function for gene activation and gene repression leading to fate specification and maintenance will lead to a better understanding of the clinical presentation of ARX-related disorders and help in designing future therapeutic treatments.

Materials & Methods

Ethics Statement

The Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee (IACUC) approved all animal experiments under the protocol number 2011-10-756. CLM monitored all animal studies.

Animals and Breeding Strategy

The derivation of mice with an expansion mutation in the first polyalanine tract of Arx has been described previously [15]. Since Arx is located on the X chromosome $Arx^{Expanded\ (E)}/_{+}$ females were bred to $Arx^{+}/_{Y}$ males to generate $Arx^{E}/_{Y}$ mutant males and $Arx^{E}/_{+}$, $Arx^{+}/_{+}$, and $Arx^{+}/_{Y}$ control females and males, respectively. Above controls, both males and females, were physiologically indistinguishable in all aspects examined and as such were used interchangeably. Mice were mated on a C67BL/6 background. The Children's Hospital of Philadelphia's Institutional Animal Care and Use committee approved all experiments.

Immunohistochemistry and Histology

All dissections were performed in cold 1X PBS. Entire pancreatic tissue was removed, weighed, and submerged in cold 4% PFA/PBS overnight. Tissues were rinsed in PBS, dehydrated, embedded in paraffin, and sectioned at 8μm. Antigen retrieval was performed in 10mmol citric acid buffer (pH 6.0) followed by blocking of endogenous peroxidase and avidin/biotin activity with 3% H_2O_2 (Sigma) and avidin/biotin blocking

kit (Vector), respectively. Slides were incubated in primary antibody overnight at 4°C. Primary antibodies used were: Glucagon (GP 1:3000, Millipore and Rb 1:1000, Chemicon), Insulin (MS 1:400, Thermo Scientific and GP 1:1000, Abcam), PP (Rb 1:200, Invitrogen), Sst (Rb 1:200, Invitrogen), Ghrelin (Gt 1:200, Santa Cruz), Pdx1 (Gt 1:200, Santa Cruz), MafA (Rb 1:1000, Bethyl), Glut2 (Rb 1:1000, Millipore), and Chromogranin A (Rb 1:3000, DiaSornin). Appropriate secondary antibodies were added at room temperature (Vector Laboratories). For immunofluorescence, secondary antibodies were conjugated to either Cy2 or Cy3 (Jackson Laboratories) while immunohistochemical detection was obtained with the VECTASTAIN ABC kit and diaminobenzidine tetrahydrocholoride (DAB) substrate (Vector Laboratories). All images utilized in this study were obtained using a Leica DM6000B microscope.

Real-Time PCR Analysis

For expression analysis, whole pancreatic tissue was dissected in cold 1X PBS and homogenized in 1mL TRIZOL reagent (Invitrogen). RNA was isolated using provided protocol and cDNA synthesized using Oligo-dT, Superscript, and additional necessary reagents (Invitrogen). All quantitative PCR (qPCR) analysis was conducted in duplicate for each specimen including at least three biologic replicates for control and mutant analyzed with reference dye normalization. qPCR analysis was performed using Brilliant SYBR Green PCR Master Mix (Agilent) in the Stratagene Mx3005P Real-time PCR machine. Changes in expression level were determined by calculating and graphing fold change relative to control. Primer sequences are available upon request.

Hormone Cell Quantification

Total endocrine and hormone cell mass was calculated through the use of Aperio Software. Two sections per animal were used with four or five animals per group (both control and ArxE) analyzed. Sections were stained for either hormone mass (glucagon, insulin, somatostatin, PP, and ghrelin) or total endocrine mass (ChrgA) using immunohistochemistry. After dehydration and mounting, slides were scanned into the Aperio software and positive hormone area and total pancreatic area determined. Hormone mass and total endocrine mass was than calculated using pancreatic weight. All ArxE mice were males while control specimens consisted of male and female mice (see animal breeding section above). No difference in hormone or total endocrine mass was seen between any of the controls utilized.

Statistical Analysis

Error bars were determined as ± standard error of the mean (SEM) and all values are displayed as ± SEM. Significance was determined using a two-tailed Student's t-test and results considered significant when $p \leq 0.05$.

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Chapter V:

Conclusions and Future Directions

Conclusions

Type II diabetes mellitus is a global health crisis [1]. Current therapies for this disease, the majority of which attempt to control blood glucose levels through exogenous insulin administration, only address the most immediate consequences of inadequate insulin signaling [1]. Long-term therapies that restore endogenous blood glucose homeostasis will provide patients with better control of their condition while vastly improving quality of life [1]. One of the most attractive long-term therapies is transdifferentiation of endogenous pancreatic endocrine α-cells into physiologically functional β-cells [2].

This treatment option stems from the novel bihormonal view of diabetes, which posits that both decreased insulin action and hyperglucagonemia contribute to hyperglycemia [3]. Transdifferentiation of α- to β-cells would alleviate both of these pathological issues by increasing β-cell numbers and insulin secretion while simultaneously decreasing α-cell numbers and glucagon secretion. This therapy will provide patients with long-term endogenous control of blood glucose levels and eliminate the need for exogenous insulin administration [3].

In order to develop novel α- to β-cell replacement therapies to treat diabetes, a complete understanding of normal α-cell development and maintenance is essential [3]. Previous studies have demonstrated the key roles transcription factors play in endocrine cell fate specification and maintenance [1]. Knowledge of the involvement of these factors can be utilized to manipulate α-cells in designing an effective protocol for future

therapies [1]. My dissertation attempted to broaden knowledge of normal α-cell specification and maintenance by examining the multi-faceted roles of the homeodomain containing transcription factor Arx. Previous studies have demonstrated that Arx is necessary and sufficient for α-cell specification [4,5]. However, the role of Arx in maintenance of α-cell fate, as well as the processes that are impacted by non-null Arx mutations during α-cell development, has not been examined.

In Chapter 2, I examined the role of Arx in maintenance of α-cell fate. I demonstrated that ablation of Arx in glucagon$^+$ cells during development results in loss of proper cell fate maintenance and conversion into a β-like-cell through a glucagon$^+$insulin$^+$ bihormonal intermediate. By P21 the majority of Arx-ablated α-cells were only insulin$^+$, suggestive of transdifferentiation to a β-cell fate. Many of these newly converted cells also expressed other β-cell markers, including MafA, Glut2, and Pdx1. Conversely, short-term ablation (2 weeks) of Arx in adult mice does not result in loss of endocrine α-cells or misexpression of other endocrine hormones. These data determined that Arx is necessary for neonatal α-cell fate maintenance, but not for short-term cell fate maintenance in the adult.

I expanded on these results in Chapter 3, in which I explored the impact of a long-term ablation of Arx in mature α-cells using a global, tamoxifen-inducible, transgenic mouse model. This long-term study demonstrated that eight weeks after Arx ablation, mutant mice had an improved glucose tolerance and had lost weight compared to littermate controls. Pancreatic analysis revealed that while normal numbers of glucagon$^+$ cells are present in mutant mice, many α-cells co-express other endocrine hormones,

including insulin and PP, but not somatostatin. Additionally, insulin and glucagon secretion studies demonstrated loss of glucagon secretion and content, as well as improper insulin secretion in response to both α- and β-cell stimuli. When taken together, these data determined that although Arx-ablated α-cells are capable of maintaining glucagon expression, over time these cells lose their identity and become an α-β hybrid cell. These hybrid cells expressed both α- and β-cell hormones, but only secreted insulin in response to α- or β-cell stimuli, indicative of a transdifferentiation-like process.

Finally, in Chapter 4 I explored the effect non-null Arx mutations have on proper α-cell specification and maintenance. Using a knock-in mouse model with an expansion mutation in the first polyalanine tract, I determined that pancreatic α-cell development is severely impaired in these mice. However, unlike Arx null mutations, a subset of α-cells are specified and do not misexpress other endocrine hormones or β-cell specific transcription factors, indicating correct α-cell specification and repression of non-α-cell fates. This subset of α-cells subsequently undergoes apoptosis, and the α-cell lineage is lost by P14, along with a significant reduction in total endocrine mass. Together, these studies suggest that Arx plays a dual role during α-cell specification through both repression of non-α-cell fates and activation of α-cell fate. An expansion mutation of Arx results in loss of activation of α-cell fate (only a subset of α-cells specified), but not repression of non-α-cell fates (no misexpression of β-cell-specific hormones or proteins).

Future Directions

What are the cellular differences between neonatal and adult endocrine cells?

Numerous studies show that complete maturation of β-cells occurs during the first two weeks of life [6]. Before and during this period insulin-producing β-cells are classified as immature and cell fate is more plastic [6]. After weaning (P21) and maturation of β-cells is complete, cell fate is more defined and cells are less likely to convert into other cell types [6]. The end of this period is marked by the loss of MafB and gain of MafA expression in the mature β-cell [7].

Since pancreatic endocrine cells have a common lineage, a similar maturation period seems likely in endocrine α-cells [8]. I demonstrated phenotypic differences between ablation of *Arx* in neonatal versus adult α-cells. These observed differences could be attributed to immature versus mature α-cells and the associated plasticity in cell fate. These data also correlate with a study examining misexpression of Pdx1 in α-cells during late development and in mature α-cells [9]. Misexpression of Pdx1 in "immature" α-cells led to transdifferentiation into β-cells [9]. However, misexpression of Pdx1 in mature α-cells did not result in such a conversion [9].

When taken together, these studies suggest that cell fate maintenance is different in neonatal versus adult α-cells. Future studies identifying differentially expressed transcription factors will begin to characterize each state. Further experiments ablating transcription factors at diverse time points will aid in mapping the maturation process for endocrine α-cells.

Likely, chromatin marks and histone modifications play an important role in this maturation process [10]. A situation can be imagined in which newly formed endocrine cells are largely devoid of lineage-specific histone modifications and thus more plastic. However, over time histone modifications are added, making cell fate more defined and the cell mature. Previous studies have demonstrated an extensive landscape of activating, repressing, and bivalent chromatin marks in mature α-cells [11]. Furthermore, loss of normal DNA methylation in mature β-cells leads to loss of cell fate, derepression of *Arx*, and conversion into an α-cell fate [12]. Experiments examining histone modifications and other chromatin marks will determine the nature of these marks, if there are differences throughout the maturation process, and if these differences correlate with maturing α-cells. α-cell specific characterization and ablation of each mark during development as well as in mature α-cells will greatly clarify this issue.

Does Arx play multiples roles in specification and maintenance of α-cells?

Using a mouse model with an expansion mutation in the first polyalanine tract of *Arx*, I demonstrated a reduced, but present, pancreatic α-cell population. α-cell specification appears correct, with no misexpression of other endocrine hormones or β-cell transcription factors. These data suggest that Arx plays a dual role during specification in which it not only activates α-cell fate, but also leads to repression of other non-α-cell fates. While an expanded form of Arx is not able to allocate normal numbers

of α-cells (activation of α-cell fate), it is able to repress non-α-cell fates, resulting in proper specification of a subset of α-cells.

Future experiments utilizing other non-null mutations of Arx that differentially affect α-cell function will help clarify and elucidate these roles. Human neuronal studies have classified a wide range of Arx mutations that differentially affect Arx's functional abilities during neuronal development [13]. Exploring these mutations and the resulting pancreatic phenotypes will further elucidate the role(s) of Arx during α-cell development. In addition, chromatin immunoprecipitation (ChIP) experiments are essential to identify direct targets of Arx. Since Arx predominantly functions as a repressor, and is also thought to repress β-cell fate, likely targets include β-cell specific transcription factors, such as Pdx1 and Pax4 [14,15]. These ChIP experiments will determine if each role of Arx is due to direct repression/activation of Arx at a gene promoter or the indirect result of Arx activity in endocrine α-cells. Finally, co-immunoprecipitation experiments will determine if Arx is interacting with other co-repressors to exert its transcriptional regulation in endocrine progenitors and newly created α-cells. Neuronal studies have demonstrated Arx association with the co-repressors Tle1 and CtBP, leading to increased transcriptional repression ability [14,16]. Studies examining these potential associations during endocrine development will determine their role in the specification of α-cells. Together, these experiments will clarify the function and mechanism(s) of Arx during α-cell development.

Once the role(s) of Arx during α-cell development is understood, this knowledge can be expanded to the role of Arx in maintenance of α-cell fate. My data demonstrate

that Arx is necessary for α-cell fate maintenance. It would be interesting to explore the targets and co-factors of Arx in the adult α-cell and determine if they are similar during embryogenesis and postnatal life, or if there are important changes. This knowledge would illustrate key differences between neonatal and mature α-cell fate maintenance. Factors expressed in mature α-cells, but not during development, are likely to be important in maintenance of cell fate. These studies will help expand the knowledge of Arx in α-cells as well as how the endocrine pancreas is specified and maintained in general.

Novel therapies for Type II Diabetes utilizing Arx in α-cells

During embryogenesis, endocrine cells are plastic and immediately lose cell fate upon disruption of the proper transcriptional program [15]. However, as these cells mature they become less plastic, and disruptions in the transcriptional program do not immediately result in striking phenotypes [17]. In line with this, ablation of *Arx* in mature α-cells does not result in a rapid or complete loss of α-cell fate. Instead, *Arx*-ablated α-cells appear to slowly lose cell identity and take on an α-β-cell hybrid fate that secretes insulin in response to both α and β stimuli. Other cellular processes may make it difficult for mature α-cells to quickly and completely abandon their determined cell fate. Previous studies have illustrated the extensive chromatin landscape in mature α-cells [18]. This landscape, combined with the normal transcriptional program, likely maintains cell fate even upon transcriptional disruptions.

Novel therapies for type II diabetes could emphasize decreased Arx expression combined with additional stimuli to transdifferentiate mature α-cells into functional, physiologically correct β-cells. Previous studies have demonstrated that upon total ablation of β-cells, mature α-cells can spontaneously convert into functional β-cells [19]. Future experiments combining ablation of *Arx* with other pancreatic stressors or ablation of chromatin marks would extend the observed adult phenotype and bring the field closer to innovative treatments for type II diabetes. Furthermore, these experiments will extend the field's knowledge of cell fate maintenance and its limits.

Summary

The goal of this dissertation was two-fold: (1) to determine the role of Arx in maintenance of neonatal and mature endocrine α-cells, and (2) to characterize the effect of a non-null polyalanine expansion mutation on normal α-cell specification and maintenance. Overall, these results expand the previously described role of Arx in the early specification of α-cells. The data presented here demonstrate that Arx is also necessary for maintenance of α-cell fate, and plays a dual role during specification through the activation of α-cell fate and repression of non-α-cell fates.

Together these studies significantly contribute to the field in multiple ways. First, I demonstrated a novel role for Arx in α-cell fate maintenance, which can be utilized for novel treatments of type II diabetes. Additionally, the discovery of dual roles for Arx during α-cell specification raises the possibility that additional transcription factors are

playing similar roles in other endocrine cell types. Finally, the loss of α-cell fate maintenance during long-term ablation of Arx demonstrates that while mature pancreatic islet cells may be able to function normally in the short-term upon loss of Arx, over time even mature endocrine cells can lose their cell identity. Overall, these data expand the field's knowledge regarding normal α-cell specification and maintenance, and provide an attractive avenue for future transdifferenation studies to develop novel treatments for type II diabetes.

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