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A Study of the Role of Gata6 in Definitive Endoderm Specification and B-Cell Functionality by Genome Engineering of Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (PSCs) provide a powerful model system for the study of early human development, disease modeling and physiology. We chose to focus our studies on monogenic diabetes using this model system. Within the pancreas, β cells are one of the most critical endocrine cells as loss of this cell type disrupts blood glucose homeostasis, leading to diabetes. Due to the limited availability of primary human cells it is difficult to study them in vitro, especially in the context of genetic disease where patient material is even more difficult to obtain. Here, we characterize endodermal progenitor (EP) derived β-like cells as a model system for studying β cell development and function in vitro. EP cells are a population of endodermal stem cells, which can self-renew and be differentiated into multiple endodermal cell lineages. EP derived β-like cells are mono-hormonal for insulin and express a number of genes important for insulin processing and secretion. By use of both static stimulations and perifusion assays we show that EP derived β-like cells are responsive to both glucose and a number of other know secretagogues. Next, we demonstrate that genome editing with a zinc finger nuclease at the AAVS1 safe harbor locus can generate stable gene expression in PSCs during differentiation. We then combine these tools and describe the use of PSCs as an in vitro model system to study GATA6. Heterozygous mutations in this gene are the leading cause of pancreas agenesis while studies in mice do not replicate the human phenotype. Induced pluripotent stem cells were created from a pancreas agenesis patient with a heterozygous mutation in GATA6. Using genome editing technology, additional stem cell lines with mutations in both GATA6 alleles were generated and demonstrate a severe block in definitive endoderm induction. Re-expression of GATA6 or other GATA family members can rescue this endoderm phenotype. Partial rescue could also be achieved by treatment with a basic fibroblast growth factor. Using the EP cell culture system to bypass the developmental block at the endoderm stage, cell lines with mutations in one or both GATA6 alleles were differentiated into β-like cells. The mutant cells were shown to be functionally defective by failure to secrete insulin upon glucose stimulation. Decrease in retinoic acid concentrations used during the differentiation lead to decreased β-like cell differentiation efficiency of the heterozygous GATA6 mutants suggesting a possible mechanism for the patient phenotypes. These data show that GATA6 plays a critical role in endoderm specification and β-like cell functionality in humans while in mice it is dispensable, highlighting the importance of studying a human system.

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A STUDY OF THE ROLE OF GATA6 IN DEFINITIVE ENDODERM SPECIFICATION

AND β CELL FUNCTIONALITY BY GENOME ENGINEERING OF PLURIPOTENT

STEM CELLS

Amita Tiyaboonchai

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A STUDY OF THE ROLE OF GATA6 IN DEFINITIVE ENDODERM SPECIFICATION AND β CELL FUNCTIONALITY BY GENOME ENGINEERING OF PLURIPOTENT STEM CELLS

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List of Abbreviations

AAVS1	adeno-associated virus integration site 1
APC	allopycocyanin
bFGF	basic fibroblast growth factor
CA	chicken actin
CAS9	CRISPR associated protein 9
cDNA	complementary
CPE	carboxypeptidase E
CRISPR	clustered regularly-interspaced short palindromic repeats
DMEM	Dulbecco's minimum essential medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECC	embryonal carcinoma cells
ELISA	enzyme-linked immunosorbent assay
EP	endoderm progenitor
ES	embryonic stem
EpiSC	epiblast stem cells
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GCK	glucokinase
GFP	green fluorescent protein
GLUT1	glucose transporter 1 (also known as SLC2A1)
GLUT2	glucose transporter 2 (also known as SLC2A2)
HRP	horseradish peroxidase
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IMDM	Iscove's modified Dulbecco's medium
iPS	induced pluripotent stem
IRES	internal ribosome entry site
K_{ATP}	adenosine triphosphate sensitive potassium channel
Kir6.2	potassium channel (also known as KCNJ11)
KRBH	Kreb ringers buffer with HEPES
LIF	leukemia inhibitory factor
MEF	mouse embryonic fibroblast
MODY	maturity onset diabetes of the young
PCSK2	prohormone convertase 2
PCSK1/3	prohormone convertase 1/3
PCR	polymerase chain reaction
PDX1	pancreatic and duodenal homeobox 1
PE	phycoerythrin
PFA	parafolmaldehyde
PP	pancreatic polypeptide
PSC	pluripotent stem cells
qRT-PCR	quantitative real time PCR
RFP	red fluorescent protein
RNA	ribonucleic acid
ROCK	rho-associated kinase
rTTA	reverse tetracycline transactivator
shRNA	short hairpin ribonucleic acid
SFD	serum free differentiation media

SUR1	sulfonylurea receptor (also known as ABCC8)
TALEN	transcriptional activator-like effector nuclease
TEM	transmission electron microscopy
TRE	tetracycline response element
ZFN	zinc finger nuclease
$ZnSO_4$	zinc sulphate

CHAPTER 1

Introduction and Overview

1.1 Characteristics and Function of Pancreatic β-cells

β cells reside within the endocrine portion of the pancreas. They are important for the maintenance of blood glucose and a loss of their functionality leads to diabetes mellitus. As this thesis will first focus upon the characterization of an in vitro system for the study of β cells and then apply this system to the study monogenic diabetes caused by GATA6 mutations, we will first review the characteristics and functionality of β cells.

1.1.1 The Pancreas

The human pancreas is an organ consisting of exocrine, ductal and endocrine cells. Exocrine cells constitute the majority of the pancreatic tissue and function to secrete digestive enzymes such as amylase, proteases, lipases and nucleases to the duodenum via the pancreatic duct that runs through the middle of the pancreas. These enzymes function in nutrient digestion by catalyzing the breakdown of lipids, proteins and carbohydrates (Shih et al., 2013). Endocrine cells are located in small tight clusters in structures known as the Islets of Langerhans found scattered throughout the exocrine tissues. Within the islets of Langerhans in addition to the endocrine cells, there is also vasculature, neurons and mesodermal derived stromal cells (Pan and Wright, 2011). Endocrine cells function mostly independent of the exocrine pancreas. Each type of endocrine cell within the islet produces a specific type of hormone. α-cells secrete glucagon, β-cells secrete insulin, δ-cells secrete somatostatin, ε-cells secrete ghrelin and γ-cells secrete pancreatic polypeptide (PP) (Cleaver and Melton, 2004). The α-cells and β-cells in the endocrine pancreas function to maintain glucose homeostasis by secretion

of glucagon when blood glucose is low and secretion of insulin when blood glucose is high from their respective cell types. δ-cells, ϵ-cells and γ-cells function to regulate nutrient metabolism. Human islets are composed of 55% β-cells, 35% α-cells and 10% δ-cells with all cell types intermixed within the islet architecture. An adult pancreas can contain between 300,000 and 1,500,000 islets (Brissova et al., 2005).

1.1.2 Development of the Pancreas

In vitro differentiation protocols for pluripotent stem cells (PSCs) to β-like cells are informed by the events of in vivo development. By mimicking known signaling pathways that are important at each stage of development in vivo, PSCs can be directed to differentiate toward a specific lineage. To determine whether a stage of development has been achieved through in vitro differentiation, markers that are expressed in vivo are often assayed. Throughout this thesis, different stages of β-cell development will often be referred to along with their marker expression. Thus, we present here a summary of pancreatic development.

During early development, the region of the foregut endoderm from which the pancreas will arise is prepatterned. This region has a lack of expression of sonic hedgehog and requires the expression of retinoic acid. The presumptive pancreatic endoderm is characterized by the expression of Pdx1, Ptf1a and Sox9. At this stage the pancreatic epithelium is tightly surrounded by mesenchyme which highly expresses FGF10. FGF10 regulates expression of Ptf1a and Sox9 and is important for cell proliferation and expansion of the early pancreatic buds once they form (Pan and Wright, 2011; Shih et al., 2013).

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The pancreas begins to develop by forming as two epithelial buds, a ventral and a dorsal pancreatic bud at opposite sides of the foregut endoderm by evagination into the surround mesenchyme. In both mouse and human, the dorsal bud forms first from the dorsal foregut endoderm. The emergence of the dorsal bud appears to be regulated by extrinsic cues from the surrounding vascular endothelial cells. The evagination of the ventral pancreatic bud along with the liver from the ventral foregut endoderm follows. The ventral pancreatic bud receives cues form the cardiac mesoderm and the vitelline veins (Gittes, 2009; Pan and Wright, 2011). At this stage of development, the majority of the buds are composed of multipotent pancreatic progenitor cells that express the key transcription factors Nkx6.1, Nkx6.2 and Ptf1a. Also present in the buds are a small subset of cell from the 'first wave' of endocrine cells which are either glucagon positive or double positive for insulin and glucagon. These cells eventually contribute to a small subset of the mature α cell population (Herrera, 2000).

As the multipotent pancreatic progenitor cells continue to proliferate there is a segregation and change in morphogenesis of the pancreatic buds (Shih et al., 2013). The buds elongate and as the gut tube rotates, the ventral and dorsal buds come into contact and fuse together. Multiple small protrusions then begin to form from the edge of the of the pancreatic bud (Gittes, 2009). Cells that are located toward the edge of these protrusions are in the tip domain and express Ptf1a, c-Myc and Cpa (Shih et al., 2013). Tip domain cells are fated to become acinar cells. As development progresses acinar cells will continue to proliferate and increase in cell number by duplication (Pan and Wright, 2011). Cells that are located toward the inside of the buds are in the trunk domain and are characterized by the expression of Nkx6.1, Nkx6.2, Sox9, Tcf2,

Oncecut-1, Prox1 and Hes1. Trunk domain cells are bipotential and give rise to endocrine and ductal cells (Shih et al., 2013).

Endocrine progenitor cells in the trunk domain form the primitive duct (or also known as the epithelial cord), which is characterized by tubules lined by a single layer of polarized epithelial cells. At this stage a subset of cells within the primitive ducts express transient high levels of Neurogenin3 (Ngn3), which indicates the onset of endocrine cell differentiation. Cells that lack the expression of Ngn3 will become the ductal cells. Ngn3 positive endocrine precursors then delaminate from the primitive ducts, converting to non-epithelial cells through a process thought to involve an epithelial-to-mesenchymal transition (Pan and Wright, 2011; Shih et al., 2013). These cells migrate into the surrounding area and coalesce into aggregates that become the islets of Langerhans (Gittes, 2009). Each endocrine precursor cell will further differentiate into one of the five types of hormone expressing cells. The mature endocrine cells that the endocrine precursor will become is temporally determined and is dependent on the developmental stage at which their differentiation occurs. At this stage a large numbers of β cells and α cells begin to appear. α cell fate is determined by the expression of Arx while β cell identity is determined by Pax4, Pdx1 and Nkx6.1 (Shih et al., 2013).

1.1.3 Insulin Biosynthesis

β-cells function to produce, store and regulate the secretion of insulin (Rhodes et al., 2005). In experiments with in vitro differentiated β-like cells we examine the ability of the cells to process insulin as a method to determine the cells maturity and similarity to functional adult β cells.

Frederick Banting and Charles Best first discovered insulin in 1921 by treating diabetic dogs with extracts of the pancreas that did not contain any digestive enzymes (Banting et al., 1922). Upon treatment both blood glucose levels and sugar excreted in the urine of the dogs were lowered. Based upon these results, the first human diabetic patient was successfully treated with insulin in January of 1922 (Banting et al., 1922).

Insulin consists of two peptide chains, the A chain and the B chain that are connected by three disulphide bonds (Fu et al., 2013). Insulin is initially synthesized as the precursor molecule preproinsulin, consisting of an A-chain connected to the B-chain via a C-chain and a signal peptide located at the end of the B-chain peptide (Figure 1-1A). Preproinsulin is trafficked through the rough endoplasmic reticulum where the signal peptide is cleaved and the remaining proinsulin is folded into its three dimensional conformation (Figure 1-1B). Proinsulin is then loaded into immature granules and the conversion of proinsulin to insulin occurs in these granules. The immature granules are acidified as the prohormone processing enzymes prohormone convertase 2 (PCSK2) and prohormone convertase 1/3 (PCSK1/3) have optimal enzymatic activity at a pH of 5.0 to 5.5. Proinsulin is cleaved at two sites by PCSK2 and PCSK1/3 releasing the C-chain that is also known as c-peptide. Cleavage by the prohormone processing enzymes results in the exposure of the C-terminal basic amino acids on the insulin molecule. These residues are then cleaved by carboxypeptidase E (CPE) (Figure 1-1C) (Rhodes, 2004a; Rhodes et al., 2005). As the granule matures, the high concentration of insulin causes the insulin monomers to crystalize into hexamers associated with two zinc ions (Bilous and Donnelly, 2010; Fu et al., 2013). When viewed by electron microscopy mature insulin granules often appear as crystalline dense core granules surrounded by a

Figure 1-1

A

Pre-proinsulin

Signal Peptide   A chain

B chain

C-peptide

B

Proinsulin

PCSK2 +CPE

PCSK1+CPE

Signal Peptide

S——S   A chain

S–S

S–S   S–S

B chain

C-peptide

C

Insulin

S——S   A chain

S–S   S–S
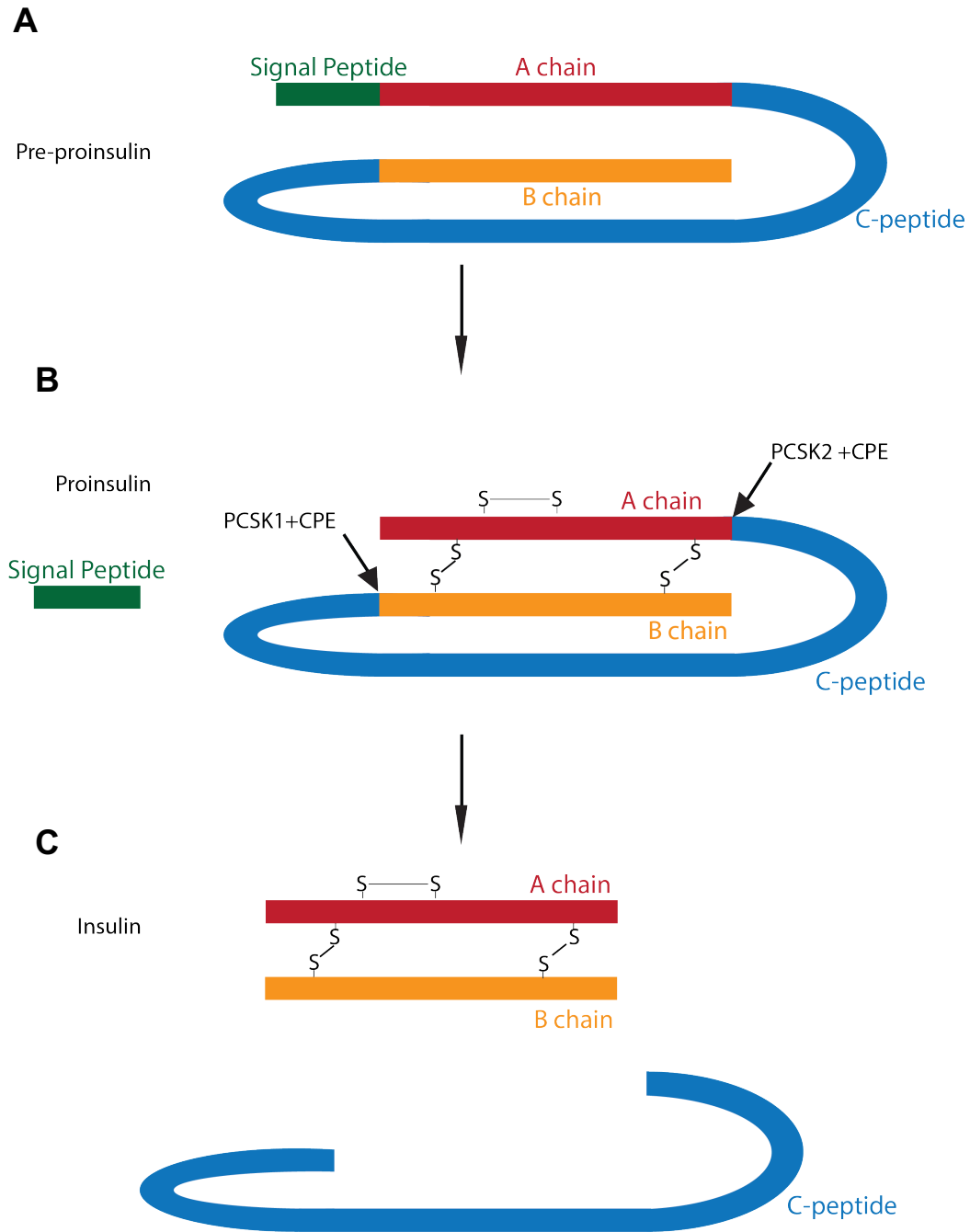
B chain

C-peptide

Figure 1-1. *Processing of the Insulin Molecule.* (A) Preproinsulin is synthesized with the signal peptide, A chain, Bchain and C-peptide connected. (B) The signal pepetide (C) Upon cleavage of insulin, mature insulin consists of the A chain and B chain connected via disulphide bonds. (PCSK1: prohormone convertase 1, PCSK2: prohormone convertase 2, CPE: carboxypeptidase E).

clear halo. These insulin granules are ready to be released by exocytosis from the β-cell upon stimulation (Bilous and Donnelly, 2010).

1.1. 4 Insulin Secretion

Glucose is one of the critical stimulants of insulin secretion. When human blood glucose levels are higher than 5mmol/L (90 mg/dL), β-cells begin to release insulin. To study β cell functionality, in vitro differentiated β cells should express the appropriate genes in order to sense increased glucose concentrations as well as respond with insulin secretion.

Glucose enters the β-cell through glucose transporters such as glucose transporter 1 (GLUT1 encoded by SLC2A1) or glucose transporter 2 (GLUT2 encoded by SLC2A2) (De Vos et al., 1995; McCulloch et al., 2011), where it is then phosphorylated by glucokinase (GCK). The glucose is then metabolized by glycolysis, causing an increase in the ratio of ATP relative to ADP. This causes the ATP sensitive potassium (K_{ATP}) channels which are normally open to close (Henquin, 2000). The K_{ATP} channel is an octamer composed of tetramers of the two subunits: the sulfonylurea receptor (SUR1 or ABCC8) and an inwardly rectifying K^+ channel (Kir6.2 or KCNJ11) (Koster et al., 2005). The closing of the K_{ATP} channel results in the depolarization of the cell surface membrane and the opening of the voltage gated calcium channels allowing calcium influx into the cytoplasm. The increased cytoplasmic calcium stimulates the exocytosis of insulin granules from the cell (Greeley et al., 2010). In addition to stimulating insulin release in β-cells, high glucose levels also induce proinsulin biosynthesis. Within 20 minutes of glucose stimulation, the increase in insulin biosynthesis can be observed. With prolonged stimulation, by 60 minutes the maximal rate of insulin biosynthesis is

Figure 1-2
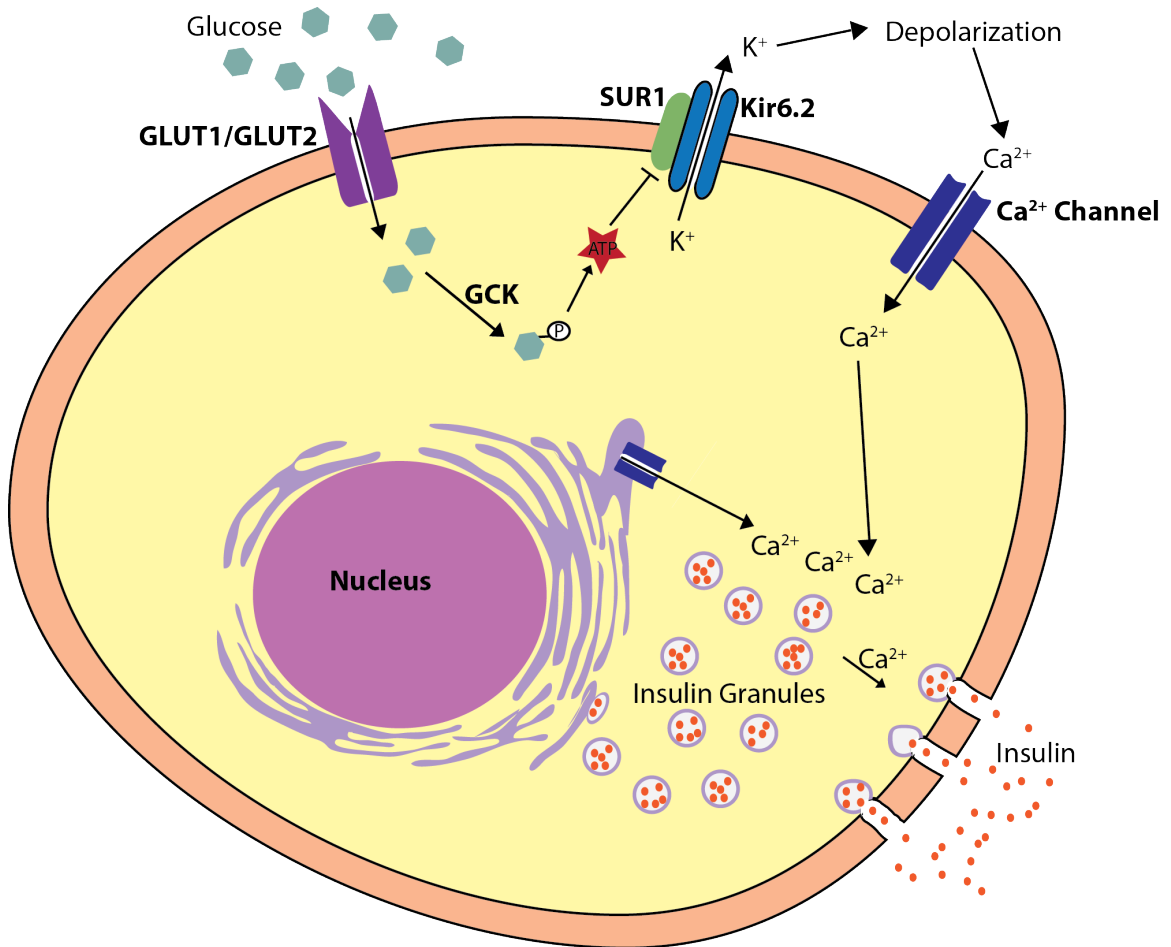
Figure 1-2. *Insulin Secretion*. Glucose enters the β cell via either GLUT1 or GLUT2 glucose transporters and is phosphorylated by GCK. This causes an increase in ATP to ADP ratio and results in the closure of the postassium channel (Kir6.2 and Sur1 are the subunits). Depolarization of the plama membrane then follows. This leads to the opening of the calcium channels and increase in cytoplasmic calcium concentrations triggering the exocytosis of insulin from the β cell.

reached. This can to 30 to 40 fold compared to basal levels (Alarcón et al., 2002; Rhodes, 2004b). In contrast, when unstimulated, β-cells are constantly releasing a basal level of insulin. The glucose that undergoes oxidative glycolysis is low enough that the ATP to ADP ratio does not change significantly and the K_{ATP} channels remain open (Henquin, 2009).

When β cells are stimulated in vivo or in vitro, insulin release is biphasic. During the first phase, large amounts of insulin are rapidly released from a 'readily releasable pool' of insulin granules. These granules are located near the cell surface membrane and can quickly undergo exocytosis. The first phase of insulin release lasts a few minutes from the time of stimulation. With prolonged stimulation, during the second phase of secretion, there is a decelerated rate of insulin secretion and the amount plateaus or gradually increases lasting as long at the stimulus is applied (Bilous and Donnelly, 2010; Henquin et al., 2006; Lacy et al., 1972). Insulin released during the second phase comes from a reserved pool of insulin granules that can account for up to 90%-95% of the total granules present within the β-cells. These granules are located further away and need to be modified and transported to the cell surface membrane in order to undergo exocytosis (Rorsman et al., 2000; Seino et al., 2009). In addition to high glucose levels, insulin secretion can also be stimulated by glucagon, gastric inhibitory peptides, epinephrine, and amino acids such as arginine (Cleaver and Melton, 2004).

Insulin functions to lower blood glucose levels by stimulating a 10 to 40-fold increase in glucose uptake by cells throughout the body with the liver, skeletal muscles, cardiac muscles, central nervous system and adipose tissue being important sites. In the liver and muscles, glucose either undergoes glycolysis to produce pyruvate, be oxidized to

generate ATP or is stored in the form of glycogen and tryiglycerides. In the adipose tissues, glucose is stored in the form of triglycerides (Saltiel, 2001).

Full functionality of β cells requires a combination of correct insulin synthesis and development of the appropriate mechanisms to respond to insulin secretion. During in vitro differentiation of PSCs, functionality of β-like cells is determined by insulin secretion in response to various stimuli, the most important being glucose. Furthermore, insulin granule morphology is often examined as an indicator

1.2 Diabetes Mellitus

Diabetes Mellitus is a disease that characterized by the dysregulation of insulin secretion and/or responsiveness in the body. Patients with this disease typically display abnormally high levels of circulating glucose (Rhodes et al., 2005; Saltiel, 2001). While type I and type II diabetes mellitus are the most common forms of diabetes and have been immensely studied (reviewed in Atkinson et al., 2014; Kahn et al., 2014; Lin and Sun, 2010; Nolan et al., 2011; Olokoba et al., 2012; Tuomi et al., 2014; Van Belle et al., 2011), this thesis will focus upon the less studied form of diabetes mellitus which results from monogenetic mutations in GATA6.

1.2.1. Characteristics and Types of Diabetes Mellitus

In humans, physiological levels of blood glucose prior to meals are typically 3.9 mmol/L to 7.2 mmol/L (or 70 to 130 mg/dL) and two hours after a meal should not be higher than 10 mmol/L (or 180 mg/dL). Blood glucose levels in the body are regulated by the release of the antagonistic hormones from α-cells and β-cells. During regular homeostasis, when blood glucose is low, α-cells secrete glucagon stimulating the hepatic conversion of

glycogen to glucose, which is then released into the blood stream. When blood glucose levels are elevated, β-cells respond by releasing insulin causing the uptake, utilization and storage of glucose. Furthermore, insulin also subdues the hepatic production of glucose resulting in the lowering of blood glucose levels (Rhodes et al., 2005; Saltiel, 2001).

Diabetes Mellitus can be categorized into four main categories, type I diabetes, type II diabetes, gestational diabetes and diabetes as a result of other causes. Other causes of diabetes include monogenic mutations, diseases of the exocrine pancreas and drug or chemical induced diabetes (American Diabetes Association, 2016).

1.2.2 Type I and Type II Diabetes Mellitus

Type I and Type II diabetes are the most common forms of diabetes. In 2014, an estimated 22 million people in the United States were diagnosed with diabetes. Approximately 5% of those diagnosed with diabetes have Type I diabetes while 90%-95% of those diagnosed have Type II diabetes (Centers for Disease Control Prevention, 2015). Type I diabetes is an autoimmune disease that causes the selective destruction and loss of β-cells. An absence of c-peptide occurs as the disease further progresses and eventually there is complete loss of β-cells within the islets of Langerhans. Type I diabetes is treated by rigorously monitoring glucose levels and daily insulin injections. (American Diabetes Association, 2016; Murphy et al., 2008). Type II diabetes is a complex metabolic syndrome with multiple causes that are still being elucidated. Risk factors for the development of type II diabetes include aging, obesity, a lack of exercise and a genetic predisposition. Management of type II diabetes initially includes regulating diet and increasing exercise (Bilous and Donnelly, 2010; Saltiel, 2001). While Type I and

type II diabetes continued to be immensely studied (Atkinson, 2014, 2012; Kahn et al., 2014; Lin and Sun, 2010; Nolan et al., 2011; Olokoba et al., 2012; Röder et al., 2016; Todd, 2010; Tuomi et al., 2014; Van Belle et al., 2011), this thesis will focus upon investigating monogenic diabetes, a rarer and less studied form of diabetes.

1.2.3 Monogenic Diabetes

Patients with monogenic diabetes have a mutation in a single gene involved in β-cell development or functionality. These mutations can be inherited as dominant or recessive, or arise spontaneously. Monogenic diabetes occurs approximately in 1 to 2 in 100,000 live births (Anik et al., 2015; Greeley et al., 2010) which accounts for 1%-4% of diabetes that are diagnosed in children (Rubio-Cabezas et al., 2014). Monogenic diabetes can be subcategorized into neonatal diabetes and maturity onset diabetes of the young (MODY) (American Diabetes Association, 2016).

A total of 13 types of MODY have been described in the literature. The four most common types of MODYs result from mutations in either HNF1α or MODY3 (30% to 50% of the cases), GCK or MODY2 (20% to 50% of the cases, HNF1β or MODY5 (5% of the cases) or HNF4α or MODY1 (5% of the cases) (Kim, 2015; Steck and Winter, 2011). Patients with MODYs typically do not have the presence of pancreatic or islet autoantibodies, have low insulin requirements and detectable c-peptide levels (Murphy et al., 2008; Rubio-Cabezas et al., 2014).

A distinguishing feature of patients with neonatal monogenic diabetes is that the majority of these patients are diagnosed with diabetes prior to 6 months of age (Edghill et al., 2006; Iafusco et al., 2002). Patients with neonatal diabetes can be divided into two

subcategories: those that have transient neonatal diabetes mellitus which resolves after a few weeks to months and those who have permanent neonatal diabetes mellitus (Steck and Winter, 2011). In addition to mutations that can result in non functional β cells, permanent neonatal diabetes can also be a result of pancreatic agenesis. Pancreatic agenesis is a rare congenital disease caused by heterozygous mutations in PDX1 (Schwitzgebel et al., 2003; Stoffers et al., 1997), PTF1A (Sellick et al., 2004; Weedon et al., 2014), GATA4 (Amato et al., 2010; Shaw-Smith et al., 2014) or most commonly GATA6 (Bonnefond et al., 2012; Chao et al., 2015; De Franco et al., 2013; Lango Allen et al., 2012; Nakao et al., 2013; Stanescu et al., 2014; Suzuki et al., 2014; Yorifuji et al., 2012).

1.3 GATA6

Human GATA6 heterozygous mutations are the most common cause of pancreatic agenesis. Due to discrepancies in the phenotype of currently available mouse models of a heterozygous loss of GATA6 and human patient phenotypes, this thesis focuses upon addressing this issue by development of an in vitro human based model system to study a loss of GATA6.

1.3.1 Characteristics of GATA6

GATA6 is a part of the six member GATA family of transcription factors that share a conserved DNA binding motif. GATA family members recognize and bind to the consensus sequence A/T-GATA-A/G. While GATA1, GATA2 and GATA3 are primarily expressed in the hematopoietic cell linages, GATA4, GATA5 and GATA6 are found in endodermal derived tissues, the heart and gonads (Molkentin, 2000; Viger et al., 2008). GATA6 consists of 7 exons with a total of 3770 base pairs, it contains two transactivation

Figure 1-3

GATA6
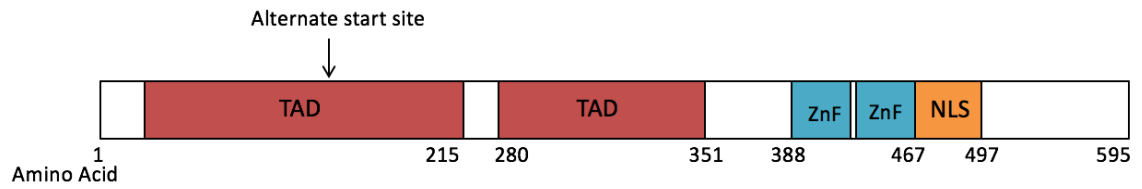
Figure 1-3. *GATA6 protein structure.* A schematic representation of GATA6 protein structure with the alternative transcriptional start site indicated. (TAD: Transcriptional activation domain, ZnF: Zinc Finger DNA binding domain, NLS: nuclear localization signal).

domains followed by a DNA binding domain and a nuclear localization signal (Figure 1-3). GATA6 has two transcriptional start sites, both located in the second exon resulting in the production of two isoforms of GATA6, one which is 595 amino acids in length and the other 449 amino acids long (Brewer et al., 1999). In mice, GATA6 is expressed in the primitive streak, heart, lung, intestine, gonads, adrenal and pancreatic tissues (Koutsourakis et al., 1999; Liu et al., 2002; Sartori et al., 2014).

1.3.2 The Function of GATA6

GATA6 is a transcription factor that is critically important for normal human pancreatic development. Heterozygous mutations in GATA6 are the most common cause of pancreatic agenesis (Amato et al., 2010; Shaw-Smith et al., 2014). Patients with a heterozygous mutation in GATA6 have been found with a range of phenotypes from no pancreatic defects to adult onset diabetes and pancreatic agenesis (De Franco et. al., 2013). The majority of GATA6 mutations in patients with pancreatic agenesis are de novo mutations. However, in rare cases where the mutation has been inherited, not all patients with the same mutation display the same phenotype suggesting incomplete penetrance. There have been studies of pancreatic agenesis patients and family members with identical GATA6 mutations who have adult onset diabetes or even no abnormalities of the pancreas (Bonnefond et al., 2012; De Franco et al., 2013; Yu et al., 2014). In addition to the pancreatic defects, GATA6 heterozygous patients often have a combination of other additional defects including gut abnormalities, intrauterine growth retardation and congenital heart defects (Chao et al., 2015).

In murine studies, mice with heterozygous loss of GATA6 are fertile and phenotypically normal (Carrasco et al., 2012; Morrisey et al., 1998; Xuan et al., 2012). Mice with

homozygous GATA6 null alleles are embryonic lethal by embryonic day 7.5 (Morrisey et al., 1998). Tetraploid complementation studies have shown that GATA6 is essential for extra embryonic endoderm formation and thus explains the embryonic lethality of GATA6 null mice However, it has also been shown that GATA6 null ES cells can form definitive endoderm and contribute to the primitive gut tube (Koutsourakis et al., 1999; Zhao et al., 2005). These studies were based upon a global loss of GATA6 within the mouse. As human patients with a heterozygous loss of GATA6 have phenotypes of the pancreas, mouse models with a pancreatic specific loss of GATA6 are beneficial in gaining an understanding of the function of GATA6 in the pancreas.

During the development of the mouse pancreas, GATA6 is found to be expressed in the pancreatic epithelium and eventually becomes restricted to the endocrine pancreas and ductal cells (Decker 2006). Mice with a homozygous conditional loss of GATA6 in the pancreas during embryonic development driven by either the PDX1 or the PTF1a promoter were born normal and had a normal life span. Furthermore, the loss of GATA6 in these mice did not affect glucose tolerance test or insulin levels when examined as adults (Carrasco et al., 2012; Martinelli et al., 2013; Xuan et al., 2012). Additionally, a conditional loss of GATA6 in adult β-cells leads to increased endoplasmic reticulum stress and minor β-cell death but does not have any effect on β-cell mass or glucose homeostasis (Sartori et al., 2014). This suggest that GATA6 is not required in the endocrine compartment in mice during the later stages of development and in the pancreas of adult mice, GATA6 plays a non essential role in β-cell functionality. However, GATA6 is required in the exocrine pancreas for the maintenance and functionality of acinar cells. In mice with a lack of GATA6, the acinar cells are gradually

lost resulting in the majority of the pancreas being replaced by fat (Martinelli et al., 2013).

In an alternative experimental system to study GATA family function in pancreas development, GATA6 was fused to an engrailed dominant negative repressor and driven by the PDX1 promoter. Expression of the GATA6-engrailed dominant negative protein lead to the majority of embryos having either the complete absence of a pancreas or the presence of a partial pancreas with disrupted morphology. Utilizing this more severe method to inhibit targets of GATA6 further demonstrates the importance of GATA family members in pancreatic development (Decker et al., 2006).

1.3.3 Functional Redundancy of GATA6

GATA4 and GATA6 have been demonstrated to have functional redundancy in the development of the heart (Zhao et al., 2008), gonads (Padua et al., 2015, 2014), adrenal glands (Tevosian et al., 2015) and intestine(Walker et al., 2014) in the mouse and in liver and pancreatic development in the zebra fish (Holtzinger and Evans, 2005). A conditional loss of both GATA4 and GATA6 during pancreatic epithelium development leads to mice being born with growth retardation and hyperglycemia. These mice died shortly after birth and upon examination were found to completely lack a pancreas suggesting functional redundancy between GATA4 and GATA6 (Carrasco et al., 2012; Xuan et al., 2012). This functional redundancy was further studied by substituting GATA6 cDNA in place of the GATA4 coding sequence. GATA6 was able to compensate for a loss of GATA4 during early development replacing the function of GATA4 and allowing the formation of the extra embryonic endoderm. Additionally, at embryonic day 10.5 the heart precursor cells could form and were comparable to wild type cells

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suggesting that at this stage GATA6 could compensate for a lack of GATA4. However, by embryonic day 12.5 the embryos displayed severe cardiac malformations. Furthermore, examination of the embryos revealed that there was agenesis of the liver and ventral pancreas indicating a necessity for GATA6 in the maturation of these organs (Borok et al., 2015).

In human patients a heterozygous loss of GATA6 results in more severe phenotypes in comparison to murine models. Additionally, in humans, there is incomplete penetrance of GATA6 mutations as patients have a range of phenotypes from adult onset diabetes to pancreatic agenesis with other organ deficiencies. Haploinsufficiency of GATA6 may be occurring in human patients while this is not the case in murine models. Additionally, GATA4 and GATA6 may have greater functional redundancy in the mouse as compared to humans and thus in mouse models, a heterozygous loss of GATA6 can be compensated for with GATA4. To study a loss of GATA6 in a human based system we chose to use the differentiation of pluripotent stem cells.

1.4 Pluripotent Stem Cells

Pluripotent stem cells (PSC) are cells that have the ability to indefinitely self renew in an undifferentiated state and have the potential to be differentiated into any cell type in the body. Embryonic stem (ES) cells can be derived from the inner cell mass of the blastocyst while induced pluripotent stem (iPS) cells are generated by introduction of pluripotency factors. PSCs have the potential to give rise to unlimited supplies of functional human cells that can be used in the study of developmental biology, disease mechanisms, drug discovery and therapeutics (Girlovanu et al., 2015; Irion et al., 2008; Murry and Keller, 2008). In this thesis, we describe the further development of a protocol

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for differentiation of human PSCs through an endoderm progenitor cell intermediate to β-like cells. We then utilize these cells as a model system to study human monogenetic diabetes and pancreas agenesis caused by heterozygous mutations in GATA6.

1.4.1 Mouse Embryonic Stem Cells

Pluripotent cells were first studied *in vitro* through the use of mouse teratocarcinoma stem cells or also known as embryonal carcinoma cells (ECCs) (Strickland, 1981). ECCs are formed by either grafting a 11 to 12-day old fetal germinal ridge or a 1 to 6-day old fertilized egg into the testes of adult of mice. The grafted cells then spontaneously proliferate and can be isolated as ECCs (Stevens, 1970). ECCs are pluripotent and single cells can be transplanted in vivo to form teratomas (Kleinsmith and Pierce, Jr., 1964). Teratomas are benign tumors that grow in vivo and contain tissues derived from all three germ layers, the ectoderm, mesoderm and endoderm. Due to these properties, teratoma formation is often used to prove the pluripotency of a cell (Zhang et al., 2012). In vitro, ECCs can also be differentiated to multiple cell lineages by formation of embryoid bodies or through use of chemical induction (Strickland, 1981). However, ECCs often have multiple chromosomal rearrangements, have an abnormal karyotype and in some lines have presence of only one X sex chromosome (Evans, 1981). Based on the pluripotency of ECCs it was hypothesized that ECCs were derived from a population of embryonic pluripotent stem cells and if these cells would be experimentally advantageous if they could be isolated (Evans, 1981).

Murine embryonic stem (ES) cells were first isolated in 1981 by Martin Evans, Matthew Kaufman and independently in the same year by Gail Martin. In their experiments, mouse ES cells were derived from the inner cell mass of either blastocysts that had

been delayed in implantation or the late mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). These cells form colonies that proliferate and could be maintained on a feeder layer of mouse embryonic fibroblasts. Alternatively, mouse ES cells can be maintained and propagated in culture without feeder cells in the presence of leukemia inhibitory factor (LIF) (Smith et al., 1988) or bone morphogenetic protein (BMP) signaling as these two factors can suppress differentiation (He et al., 2009) . Mouse ES cells have a normal karyotype that can be stability maintained during in vitro culture. They can form teratomas containing ectoderm, mesoderm and endoderm germ layers when transplanted into immune compromised mice, have high telomerase activity, stable karyotype and can contribute to the formation of mouse chimeras (Ginis et al., 2004). The discovery of the mouse ES cells laid the ground work for the genetic engineering of the mouse and derivation of human ES cells.

1.4.2 Human Embryonic Stem Cells

In 1998, James Thomson first described the derivation of primate and human ES cells. Human ES cells were derived from the inner cell mass of the human blastocyst and could be maintained as colonies on a feeder layer (Thomson et al., 1998). The optimal culture conditions for the long-term maintenance of pluripotency with basic fibroblast growth factor (bFGF) were established allowing the maintenance of human ES cells in in vitro cultures (Yamanaka, 2012). Similar to mouse ES cells, human ES cell lines were shown to have high telomerase activity, be karyotypically stable and displayed pluripotency as they are able to form all three germ layers in a teratoma assay. Human ES cells express the transcription factors NANOG, OCT4 and SOX2. They also express the extracellular markers SSEA-3, SSEA4, TRA-1-60 and TRA-1-81 (Thomson et al., 1998).

Mouse ES cells that are derived from the pre-implantation epiblast described to be in a naïve state of pluripotency while cells derived from the post implantation epiblast are termed epiblast stem cells (EpiSCs) described to have a primed state of pluripotency. Both ESCs and EpiSCs share the characteristics of the ability to form teratomas and express the same core markers of pluripotency, Oct4, Sox2 and Nanog. Naïve mouse ES cells can be incorporated into blastocysts to form chimeras and colonize the germ line, are amenable to gene targeting, can be cloned and propagated from single cells and in female cells do not have an inactivated X chromosome. In contrast, EpiSCs cannot contribute to germ line chimeras, are more difficult to genome engineering via homologous recombination, do not propagate well as single cells, have higher levels of DNA methylation and have one inactivated X chromosome. While mouse ES cells require LIF and BMP or GSK3 and MEK inhibition to remain pluripotent, mouse EpiSCs require FGF and Activin/TGF-β signaling. Although human ES cells are derived from the preimplantation blastocyst, their characteristics and methods of maintenance in culture are more similar to mouse EpiSCs leading to the interpretation that human ES cells are at a later stage of development than mouse ES cells (Davidson et al., 2015; Ginis et al., 2004).

1.4.3 Induced Pluripotent Stem Cells

In addition to the discovery of mouse and human ES cells, there are other major scientific breakthroughs that have together contributed to the development of induced pluripotent stem (iPS) cell technology. In 1962, John Gurdon demonstrated the first reprogramming of cells by somatic cell nuclear transfer. A nucleus from the intestinal epithelium cell of a swimming tadpole stage was transferred to an unfertilized egg and

shown that the egg could successfully develop into a tadpole demonstrating that development could be reverted to an earlier stage (Gurdon, 1962). Additionally, in 1989 Harold Weintraub described the transcription factor myosin D as a master regular whose expression could convert the cell fate of multiple cell lines to muscle cells (Weintraub et al., 1989). This was a very early demonstration that the introduction and expression a transcription factor could change cell fate (Yamanaka, 2012).

Based upon these ideas, in 2006, Takahashi and Yamanaka described the generation of iPS cells from both embryonic and adult mouse fibroblast cells. They initially screened 24 transcription factors known to play roles in maintaining pluripotency by retroviral expression and then narrowed this list down to 4 necessary factors, Oct3/4, Sox2, c-Myc and Klf4. The iPS cells were demonstrated to express markers of pluripotency, they could form teratomas and contribute to adult chimeric mice (Takahashi and Yamanaka, 2006). Their work demonstrated that adult differentiated cells could be reprogrammed back to an embryonic stem cell state.

The following year, human iPS cells were derived from adult human fibroblast. While the Yamanaka group utilized the same four transcription factors that were used to generate mouse iPS cells (Takahashi et al., 2007), James Thomson and colleagues demonstrated that an alternate set of four transcription factors, replacing c-Myc and Klf4 with LIN28 and NANOG, could be utilized (Yu et al., 2007). In both studies human iPS cells were shown to be karyotipically normal and pluripotent. The discovery of iPS cells was a large advancement in the field, as it provides a potential for studying pluripotency without the ethical complications that may be associated with the derivation of human ES cells and also a method to generate patient specific stem cells.

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Multiple studies have now demonstrated that there is flexibility in the transgenes that can be used to induce pluripotency from both mouse and human somatic cells. In addition, chemical induction of pluripotency that has resulted from screens of compound libraries provides an alternative method for the generation of transgene free iPS cells. This may be beneficial and a safer alternative if iPS cells are to be used in a clinical setting (Theunissen and Jaenisch, 2014).

1.4.4 Human Pluripotent Stem Cells as a Model System

The differentiation of human PSCs is advantageous as they provide an in vitro human based model system. Upon differentiation to different cell lineages, PSCs can be utilized to study basic biology such as human development and the effects of a gain or loss of gene function. Differentiated cells can also be used to elucidate disease mechanism and to develop and test new drugs. Furthermore, human PSCs provide a source of unlimited cells that once differentiated could be used for cell replacement therapies (Irion et al., 2008).

One powerful model system that has been intensely studied (Johannesson et al., 2015) is the in vitro differentiation of β cells. Differentiation of this cell type is important for a better understanding of the development and functionality of β cells as well as for their potential use in therapeutics. In humans, β cells have negligible proliferation with the exception of the first year of life where β cells proliferate at a rate of 1% to 3% and during pregnancy (Wang et al., 2015). β cells are difficult to maintain in vitro and cannot be cultured long term as they quickly lose functionality (Pellegrini et al., 2016).

Over the years a number of attempts to generate a β cell line have been made, however the majority of human β cell lines that were generated have low insulin content and are not glucose responsive (Skelin et al., 2010). To date, a total of 3 functional human β-cell lines have been generated from human fetal pancreases. These cells are glucose responsive, express β-cell specific markers and can be cultured for extended periods of time. The first β-cell line, EndoC-βH1 was generated by transducing fetal pancreatic buds with lenti viruses expressing SV40LT and human telomerase transcriptase (hTERT) followed by in vivo transplantation for expansion of the cells (Ravassard et al., 2011). Subsequent β-cell lines, EndoC-βH2 and EndoC-βH3, were generated by a similar method with the added capability to CRE-excise the SV40LT and hTERT resulting in enhanced functionality and β-cell specific gene expression (Benazra et al., 2015; Scharfmann and Pechberty, 2014). While these cells contain many properties of a β cell, there are limitations for the utilization of these cells because they are immortalized cell lines and may not perfectly mimic real beta cells. Furthermore, they are unsuitable for therapeutics.

Based upon these limitations the differentiation of PSCs to β cells is advantageous as it provides an unlimited supply of β cells for studies of β cell biology as well as the potential to be used in the clinic for treatment. Additionally, any genome editing or genetic manipulation that may be needed can be readily performed in PSCs prior to differentiation overcoming the difficulty posed by the slow proliferation rate of terminally differentiated cells.

1.4.5 Differentiation of Pluripotent Stem Cells

In vitro differentiation of PSCs is achieved by sequential exposure of PSCs to growth factors based on known signaling pathways and mechanisms of development. During in vivo embryonic development the process of gastrulation leads to the formation of the primary germ layers, ectoderm, mesoderm, and endoderm (Tam and Behringer, 1997). Tissues and organs in the body are then derived from one of the three germ layers. In the mouse embryo, the primitive streak forms during gastrulation in the posterior side of the embryo at the border between the epiblast and extraembyronic tissue. Uncommitted epiblast cells that will eventually become the mesoderm and definitive endoderm germ layer undergo an epithelial to mesenchymal transition and migrate through this structure. Epiblast cells that migrate through the more posterior region of the primitive streak will form the mesoderm while cells that migrate through the more anterior region of the primitive streak will form definitive endoderm. Ectoderm cells are derived from the most anterior region of the epiblast and do not migrate through the primitive streak (Gadue et al., 2005; Murry and Keller, 2008).

1.4.6 Definitive Endoderm and Endodermal Progenitor Cells

As development progresses, the endodermal germ layer of cells forms as a sheet of cells and folds into a primitive gut tube along an anterior to posterior axis. The primitive gut tube can be divided into three main regions: the foregut, the midgut and the hindgut. The thyroid, esophagus, lung, liver, pancreas and stomach are derived from the foregut, the small intestine develops from the midgut and the hindgut will eventually give rise to the colon (Zorn and Wells, 2009). In vitro primitive streak differentiation is first established by the addition of Wnt3a. Cells are then sequentially differentiated to definitive endoderm by the activation of Nodal signaling through the addition of high

concentrations of the Nodal surrogate Activin A. Activin A acts by binding to the Nodal receptor (D'Amour et al., 2005; Gadue et al., 2006; Kubo et al., 2004).

The generation of an expandable PSC-derived endodermal cell population is desirable as it would create cells with a greater restricted differentiation potential and would provide a pure population of cells that do not have contamination with cells from other germ layers (Cheng et al., 2013). One such cell population that has been described by our lab is the endodermal progenitor (EP) cell (Figure 1-4). EP cells are a population of endodermal stem cells that are derived from human ES or iPS cells. EP cells have the ability to self-renew and be maintained for many months in culture with trillions of fold expansion potential. They express markers of endoderm including SOX17, FOXA1, FOXA2, GATA4 but low levels of GATA6. Upon transplantation into immune compromised mice, EP cells do not form teratomas and can differentiate spontaneously endodermal derived tissues such as the gut epithelial and liver. In vitro, EP cells cannot be differentiated in mesodermal or ectodermal lineages and have been demonstrated to differentiate into multiple endoderm derived lineages including the pancreas, liver and intestine (Cheng et al., 2012).

1.4.7 Pluripotent Stem Cell Derived β Cells

As development of the endodermal germ layer proceeds, the primitive gut tube forms organs buds which will proliferate and eventually differentiate into the mature organs. One of these organs is the pancreas that initially forms as two distinct buds in the ventral foregut endoderm and eventually join together (Zorn and Wells, 2009). By mimicking the signaling pathways that occur in vivo, multiple protocols have been established to differentiate PSCs to β cells in vitro. Although many of these early protocols claim to

Figure 1-4
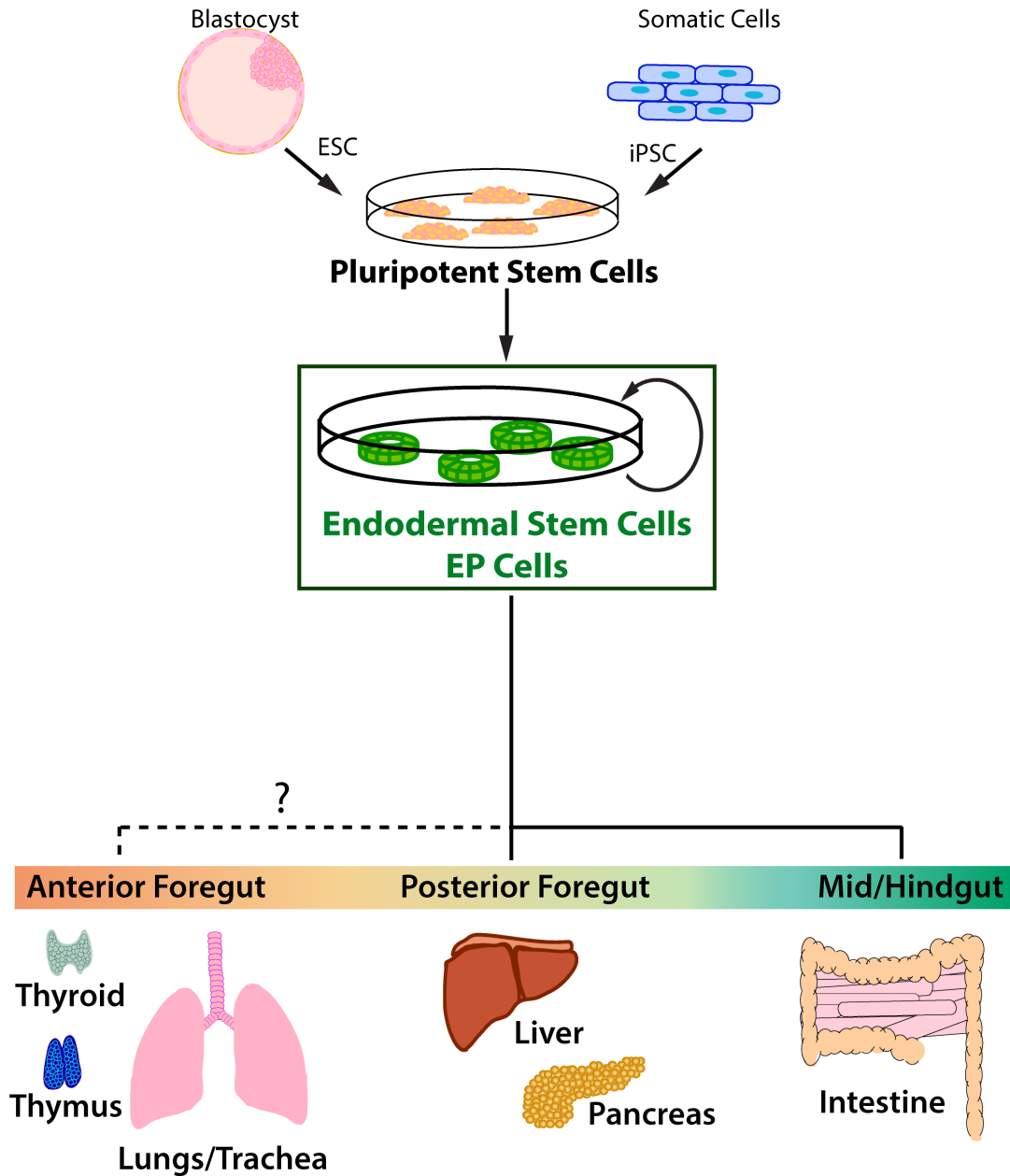
Figure 1-4. *Endodermal Progenitor Cells.* A schematic illustration of the generation of EP cells from pluripotent stem cells is shown. EP cells are capable of differentiating into cells of many endodermal organs including liver, pancreas and intestine that in vivo are derived from the foregut, midgut and hindgut. It is currently unknown if EP cells have the potential to give rise to all endodermal derivatives including the thymus, thyroid and lungs (ESC: embryonic stem cell; iPSC: induced pluripotent stem cell; EP cell: Endodermal progenitor cell).

generate β cells, these cells often failed to respond to glucose stimulation with insulin secretion. Analysis of gene expression revealed that there was expression of ARX, a gene that is typically expressed in α cells, and a lack of NKX6.1, a β-cell specific transcription factor important in β cell maturation and maintenance. Additionally, these cells are also polyhormonal expressing a combination of insulin and glucagon or insulin, glucagon and somatostatin (Basford et al., 2012; D'Amour et al., 2006; Micallef et al., 2012; Nostro et al., 2011). Studies in mouse embryos show that during early endocrine cell development in vivo in the 'first wave' of endocrine cell development, cells co-expressing insulin and glucagon have been observed. These polyhormonal cells may contribute to a small proportion of the mature α cells but are not the precursor of the mature monohormonal β cells (Herrera, 2000; Herrera et al., 1994; Nostro and Keller, 2012). Only during the 'second transition' of endocrine development do mature β cells develop and these cells are distinguished by their expression of NKX6.1. This suggest that the endocrine cells that were differentiated are more reminiscent of the first transition of endocrine cell development rather than a mature β cell (Nostro and Keller, 2012). In these earlier protocols, the only way to generate functional β cells was to transplant pancreatic progenitors into an immune compromised mouse and allow 3 to 4 months for their maturation in vivo to become glucose responsive (Kroon et al., 2008; Rezania et al., 2012; Schulz et al., 2012).

Our lab was the first to describe a differentiation protocol to generate monohormonal, glucose responsive β-like cells by utilizing an EP cell intermediate. EP cells were differentiated toward β-cells utilizing a previously established protocol designed for PSC differentiation starting from the primitive gut tube stage. EP derived β-like cells express

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insulin and do not express glucagon. Furthermore, upon glucose stimulation, the kinetics of insulin secretion was comparable to that of an adult human islet (Cheng et al., 2012).

By further manipulation of pathways involved in β-cell development and utilization of a three dimensional culture system, recently three groups were able to differentiate PSCs to functional β cells in vitro. While two of the groups cultured and differentiated PSCs in suspension, the other aggregated cells at the pancreatic progenitor stage. The majority of the PSC derived β cells are monohormal for insulin and a subpopulation of the insulin positive cells co-express two β-cell specific transcription factors PDX1 and NKX6.1. The PSC derived β cells can respond to glucose stimulation with a 2 fold increase in insulin secretion over basal, similar to the primary islet controls that were used. The cells could respond to multiple, sequential glucose stimulations with the secretion of insulin, however, in these studies insulin response to stimulation was poor in perifusion systems and displayed abnormal calcium flux. Upon transplantation into nondiabetic mice, human c-peptide was detected within 2 weeks. Transplantation into streptozotocin induced or Akita diabetic mice could reduce the hyperglycemia more quickly than in previous publications (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015a).

The ability to differentiate PSCs to functional β cells in vitro is important for the study of the mechanisms of β-cell failure. PSCs with specific mutations can be obtained through genome editing of ES cells or from diabetic patient derived iPS cells. In vitro derived β cells can also be utilized to investigate β cell proliferation, maintenance, in drug testing and provide the potential for the generation of an unlimited supply of β cells if these cells are to be used in tissue replacement therapy.

1.5 Overview of Research Goals

The goals of the work presented in the following chapters are to use the human pluripotent model system to study human beta cell development and disease, optimize beta cell in vitro development and enhance genome engineering technologies to help achieve these goals. Chapter 3 establishes and further characterizes EP derived β-like cells as an in vitro, human based model system to study the development and function of β-cells. We confirm the functionality of EP derived β-like cells and explore the similarities and differences of these cells to adult β cells. Chapter 4 describes a proof of principal study for the utilization of the zinc finger nuclease at the AAVS1 safe harbor locus as a genome editing technique in pluripotent stem cells. We describe the creation of a GFP reporter line by utilization of a lineage specific promoter region as well as knock down of transcription factor by expression of short hairpins. The tools previously described are then utilized in Chapter 5 to study the role that GATA6 plays in human endoderm development and pancreatic β cells. We studied the role of GATA6 by use of a GATA6 patient derived iPS cell line with a heterozygous mutation and a genome edited ES cell line with a compound heterozygous mutation. We show that while a heterozygous loss of GATA6 does not affect definitive endoderm specification, PSCs with a complete loss of both alleles do not differentiate efficiently to definitive endoderm. The role of other GATA family members can compensate for this loss. We then utilize EP derived β-like cells to show that while a loss of GATA6 is not absolutely required to differentiate to β-like cells in vitro, the presence of GATA6 is required for the β-like cells to be glucose responsive. In these studies, we demonstrate that the differentiation of PSCs and EP cells can be utilized as a means to study human monogenetic diabetes in vitro.

CHAPTER 2

METHODS AND MATERIALS

2.1 Maintenance of Human Pluripotent Stem Cells

The H9 human ES cells (NIH code WA09) were obtained from Wicell Research Institute, Madison, WI. The Mel1-INS-GFP ES cells were obtained from Ed Stanley and Andrew Elafanty at the Murdoch Children's Research Institute (Micallef et al., 2012). The CHOP.Panagenesis1 (patient iPS$^{+/indel}$) iPS cells were generated from a lymphoblastoid cell line (Stanescu et al., 2014) by reprogramming using episomal vectors (Okita et al., 2011) by the Stem Cell core at the Children's Hospital of Philadelphia.

Human ES cells were maintained in human ES cell maintenance media in gelatin coated 6-well tissue culture plates (Thermo Fisher Scientific) with irradiated mouse embryonic fibroblast. The composition of the human ES cell media consist of: DMEM/F12 containing 15% knock-out serum replacement, 2mM glutamine, 50U/mL penicillin, 50µg/mL streptomycin, 100µM Non-essential amino acids, 0.075% sodium bicarbonate, 1mM sodium pyruvate (all from Invitrogen, Grand Island, NY), 10^{-4}M β-mercaptoethanol (Sigma, St. Louis, MO) and 5ng/mL or 10ng/mL human bFGF (Stemgent). The cells were passaged every 3 to 6 days by treating with TrypLE (Invitrogen) for 3 minutes and replating in ROCK inhibitor (R&D) or thiazovivin (R&D).

2.2 Primary Human Islets (Chapter 3)

Isolated non diabetic human islets were obtained from Prodo Laboratories and maintained on Prodo Islet Media- Standard for 1 to 3 days prior to use for experiments.

2.3 Vector Construction For AAVS1 Targeting

ZFNs targeting the AAVS1 loci (Hockemeyer et al., 2009) were synthesized and sub-cloned into a PGK promoter expression vector (ZFN-left and ZFN-right). The CD43 targeting construct was generated using the AAVS1-SA-2A-puro-pA donor plasmid (Addgene, Cambridge, MA). A 2136 base pair (bp) sequence from -1386 to +750 of the transcription start site of the human CD43 gene (transcript ID: ENST00000395389) was used. This region includes the first exon, first intron and the second exon up to the ATG of the CD43 transcript. The CD43 promoter construct was cloned out by PCR using the fusion kit. This promoter construct was followed by GFP and the rabbit globin poly-adenylation sequence to create AAVS1-SA-2A-puro-CD43 promoter-GFP-PA (Figure 1A.1) Generation of the shPU.1 set 1 and set 2 constructs were carried out by cloning pairs of shRNA against PU.1 (Open Biosystems) into a miR-30 backbone (Wang, Theunissen, & Orkin, 2007) into the AAVS1-CAGGS-eGFP plasmid (Hockemeyer et al., 2009). The expression of the hairpins are driven by the CA promoter to create AAVS1-SA-2A-puro-CApromoter-GFP-shPU.1-PA. (Figure 1A.2). ShPU.1 set1 contains 2 hairpins, one against exon 2 and one against exon 5. Both hairpins in shPU.1 set2 are targeted against two regions of PU.1 in exon 5.

2.4 Gene targeting of the AAVS1 locus

Six-well tissue culture plates (Thermo Fisher Scientific) were pre-coated with matrigel (1:3 dilution) (BD Biosciences) followed by plating of 1×10^6 irradiated puromycin-resistant mouse embryonic fibroblast cells. H9 ES cells were plated at a density of 1.2-1.8×10^6 cells per 6-well dish in human ES cell media containing 10 μM Rho-associated kinase (ROCK) inhibitor (Cayman Chemical, Ann Arbor, MI). The next morning, 3-4 hours prior to transfection, the medium was changed to human ES cell medium

containing 20 μg/ml bFGF (Stemgent), without penicillin and streptomycin. The targeting

vector plasmid (0.6 μg), the ZFN-left plasmid (0.2 μg) and the ZFN-right plasmid (0.2 μg)

were added to 100 μL of IMDM (Mediatech) followed by addition of X-tremeGENE9 (3

μL) (Roche Diagnostics). The transfection mixture was gently mixed and incubated at

room temperature for 20 minutes before it was added drop wise into one well of H9 ES

cells. The next day, the medium was changed to human ES cell maintenance medium

and 48-72 hours post transfection, puromycin (0.5 μg/mL) (Sigma) was added with each

media change. Approximately 7 to 10 days later, single clones were manually picked.

The clones were passaged and expanded before being screened by Southern blot

analysis.

2.5 PCR Screen For ZFN Construct Integration (Chapter 4)

PCR was carried out using the JumpStart AccuTaq LA DNA polymerase kit (Sigma). The

primers used were homologous to regions in the both the left ZFN and the right ZFN.

The forward primer sequence is 5'-GCTTTTCCCAAGGCAGTC-3' and the reverse

primer sequence is 5'-GGGTCGACTTGCAGGTCG-3'. The amount of plasmid used was

equivalent to the genomes of DNA present from the cells. The PCR products were run

on a 1% agarose gel at 100 volts for 45 minutes.

2.6 Genome Editing Using CRISPR/Cas (Chapter 5)

The gRNA was cloned into the vector (Addgene, #41824) as described by Mali et al.

(Mali et al., 2013). PSCs were plated onto gelatin coated 6-well plates with mouse

embryonic fibroblasts (MEF)s 24 hours prior to transfection and were transfected at 40%

to 60% confluency. 1 μg CAS9-GFP (Addgene, plasmid #44719), 1 μg gRNA and 3 μL

X-tremeGENE9 (Roche) was added to 100 μL IMDM and gently mixed together. The

mixture was incubated at room temperature for 20 minutes and added drop wise into 1 well of cells. 18 to 24 hours post transfection, cells were harvested with TrypLE and cell sorted for GFP positive cells. These cells were plated at low density in human ES cell maintenance media with ROCK inhibitor (Cayman chemicals) onto a 1:3 matrigel coated 10 cm tissue culture dishes containing MEFs. Approximately 10 to 12 days later single colonies were manually picked and screened. To screen for mutations, genomic DNA was extracted from the clones by resuspending the cells in 20 µL of Accutaq PCR buffer (Sigma-Aldrich) with 0.1 µg/mL proteinase K (Qiagen) and incubated at 55°C for 60 minutes followed by 95°C for 10 minutes. Cell debris was spun down and 5 µL of supernatant was used for PCR. PCR primers were designed to flank the region that the gRNA was targeted resulting in a 124 base pair product. The sequence of the primers can be be found in Table S1. Phusion High Fidelity DNA polymerase was used for PCR (New England Biolabs Inc.). The PCR products were analyzed by 3.5% agarose gel electrophoresis and mutations were confirmed by sub-cloning PCR products using the TOPO TA cloning kit (Life Technologies) following manufacturers instructions and sequencing.

To create a wild type isogenic line from the patient iPS$^{+/indel}$, a 200 base pair oligonucleotide with wild type sequence was synthesized with 5 silent mutations at the guide RNA site and two silent mutations to cause the loss of a PstI restriction site. The sequence of the oligonucleotide can be found in Table S1. GATA6$^{+/indel}$ iPS cells were treated as described above for the generation of the compound heterozygous GATA6 mutations with the addition of 3 µg oligonucleotide during the transfection. To screen for the incorporation of the oligonucleotide, clones were expanded and genomic DNA was isolated from clones using PureLink Genomic DNA kit (Invitrogen) following the

manufacturers protocol. PCR primers (Table 2-1) were designed to amplify a region of 1046 base pairs flanking the oligonucleotide. The PCR was run as described above and a PCR cleanup (Invitrogen) was done prior to digestion with PstI (New England Biolabs) overnight at 37°C. Samples were run on an agarose gel and clones that did not have digested PCR products were sent for sequencing to verify the correct incorporation of the oligonucleotide.

2.7 Differentiation Of Human Pluripotent Stem Cells

For the monolayer adherent hematopoietic differentiation, PSCs were feeder depleted by plating onto 1:3 matrigel (BD Biosciences, Bedford, MA) coated 6-well plates. The differentiation was started 24 to 48 hours later when the cells had achieved approximately 70% confluency. The procedure for differentiation has been previously described (Mills et al., 2013; Paluru et al., 2013). Hematopoietic progenitors were expanded in cytokine cocktails to drive expansion of myeloid cells: 10ng/mL IL-3, 100ng/mL SCF and 200ng/mL GM-CSF; erythroid cells: 2 units of EPO and 100ng/mL SCF; and megakaryocytes: 100ng/mL TPO and 100ng/mL SCF. All cytokines from R&D Systems (Minneapolis, MN).

For monolayer definitive endoderm differentiation, cells were plated into a 1:3 coated matrigel coated 6-well plate with human ES cell media and ROCK inhibitor approximately 24 hours prior to differentiation. The differentiation was started once cells reached 80% to 90% confluency. Cells were differentiated toward definitive endoderm utilizing the following protocol. Day 0: RPMI supplemented with 2mM glutamine, 50U/mL penicillin, 50µg/mL streptomycin, 2 µM Chir99021 (Cayman Chemicals) and 100ng/mL Activin A (R&D). Day 1 and Day 2: RMPI supplemented with 2mM glutamine, 50U/mL

Table 2-1: Primers utilized in generation of GATA6 compound heterozygous mutations and correction.

Purpose	Sequence
Screening and sequencing primers for GATA6 indel mutations	Forward: 5'-TCCATGCTGCCCGGCCTAC-3' Reverse: 5'-TGCCGTATGGAGGGCTGT-3'
Screening primers for GATA6 IPS[+/indel] correction	Forward: 5'-TCACGGCGGCTTGGATTG-3' Reverse: 5'-ACAGCGAGCTGTACTGG-3'
Sequencing primers for GATA6 GATA6 IPS[+/indel] correction	5'-AGGAGATGTACCAGACCC-3' 5'-TGCCGTATGGAGGGCTGT-3'
200 base pair oligonucleotide for GATA6 IPS[+/indel] correction	5'-CCAGCTCCCCGGTCTACGTGCCCACCA CCCGCGTGGGTTCCATGCTGCCCGGCCTA CCGTACCACCTCCAAGGGTCGGGCAGTGG GCCTGCGAATCATGCCGGCGGCGCGGGC GCGCACCCCGGCTGGCCTCAGGCCTCGGC CGACAGCCCTCCATACGGCAGCGGAGGCG GCGCGGCTGGCGGCGGGGCCGCGGGGC C-3'

penicillin, 50µg/mL streptomycin, 100ng/mL Activin A, 20ng/mL VEGF, 5ng/mL bFGF and 0.25ng/mL BMP4. Day 3 onward: SFD supplemented with 2mM glutamine, 50U/mL penicillin, 50µg/mL streptomycin, 100ng/mL Activin A, 20ng/mL VEGF, 5ng/mL bFGF and 0.25ng/mL BMP4. For experiments with the GATA6 mutant PSC lines (Chapter IV), the base media was switched to SFD on day 2 instead of day 3.

For the monolayer neuroectoderm differentiation, undifferentiated PSCs were plated onto 1:3 matrigel coated 12-well plates one day prior to starting the differentiation. Cells were differentiated for 8 days in media composed of: DMEM/F12 (Corning) supplemented with 1% B27 without retinoic acid (Gibco), 2mM glutamine, 50U/mL penicillin, 50µg/mL streptomycin, 100 µM non-essential amino acids, 10^{-4}M β-mercaptoethanol, 250ng/mL Noggin, 0.15 µM SB431542 (Stemgent) and 0.1 µM PD0325901 (Stemgent) (Greber et al., 2011). Neuroectoderm cells were harvested at day 4 and day 8 by trypsinization.

2.8 Endodermal Progenitor (EP) Cell Generation And Maintenance

EP cells were generated and maintained as previously described (Cheng et al., 2012). Briefly, EP cells were generated from the majority of the cell lines by differentiation to day 5 definitive endoderm and 1.5×10^6 cells were replated onto EP cell conditions. For cells that resulted in poor definitive endoderm differentiation, definitive endoderm cells were harvested on day 5 and cell sorted for double positive CXCR4 and CKIT. For cell sorting, 0.25% trypsin/EDTA was utilized to create a single cell suspension. Cells were then stained with conjugated antibodies on ice, in the dark for 30 minutes in presort media consisting of IMDM supplemented with 15% FBS and 1% DnaseI (Roche). Cells were sorted in presort media and plated into EP cell conditions. EP cells were

maintained on a matrix of undiluted matrigel with 300,000 MEFs per 10cm dish in media consisting of: 75% homemade IMDM (GIbco), 25% Hams F-12 (Corning), 0.5% BSA in DPBS, 1% Glutamine (Corning), 1% Penicillin/Streptomycin (Corning), 100ng/mL human BMP4, 10ng/mL human bFGF, 10ng/mL human VEGF and 10ng/mL human EGF. The cells were passaged every 6 days with 1mg/mL dispase (Gibco) followed by 0.25% trypsin/EDTA (Invitrogen) and replated with rock inhibitor (Cayman Chemicals).

2.9 Pancreatic β Cell Differentiation

Differentiation was started 5 days after the splitting of EP cells. Differentiation to pancreatic β-like cells was performed as described (Cheng et al., 2012; D'Amour et al., 2006) with modifications at the end stage of the protocol as follows: Day 12: SFD with 50ng/mL noggin, SB431542 (Stemgent), 50ng/mL IGF-1, 50ng/mL HGF, GLP-1, 1.25mg/mL nicotinamide (Sigma-Aldrich) and insulin (Sigma-Aldrich). Day 15 onward: SFD with 50ng/mL IGF-1, 50ng/mL HGF, GLP-1, 1.25mg/mL nicotinamide and insulin. At day 13 of the differentiation, cells were reaggregated by treatment with 1mg/mL dispase for 15 minutes. Cells were replated into 1 well of low adherent 6-well plates (Corning). Following the reaggreation, the aggregates were spun down and fed every other day with the appropriate media.

2.10 Glucose Stimulated Insulin Secretion Assay

Kreb Ringers Buffer with HEPES (KRBH) prepared as three separate stock solutions as follows: (A) 1.83M sodium chloride (Sigma-Aldrich) (B) 80mM potassium chloride, 384mM $NaHCO_3$ and 62.55mM $MgCl_2H_2O$ (C) 35.2mM $CaCl_2H_2O$. Prior to use, 62.5mL of each solution, 0.25% w/v bovine serum albumin (2.5g) and 10mM HEPES (2.38g) is

mixed together and the total volume is bought up to 1L with tissue culture grade H_2O (Corning), the pH is adjusted to 7.4 and the medium warmed to 37°C.

β-like cell aggregates were washed twice with KRBH and incubated in 1mL KRBH for 1 hour. Cells were placed into 500µL of 1mM glucose in KRBH for 20 minutes and stimulated in 500µL of 20mM glucose in KRBH for 20 minutes. For stimulation with secretagogues and inhibitors of insulin secretion, β-like cells were placed in 500µL of 1mM glucose in KRBH for 20 minutes then stimulated in 500µL of 10mM with the addition of the secretagogue for 20 minutes. The secretagogues and inhibitors included 10mM arginine, 100µM glibenclamide, 100nM xxtendin-4 and 500µM diaxozide. Following each condition cells were spun down at 150g for 90 seconds. Supernatant was collected and stored at -20°C. C-peptide ELISA was performed using the ultra sensitive c-peptide kit following the manufacturers instructions (Mercordia). For the ELISA, supernatant was thawed to room temperature and diluted 8 to 30 fold.

2.11 Perifusion Of β–Like Cells (Chapter 3)

β-like cells were re-aggregated by treatment with 1mg/mL dispase into suspension culture 16 to 24 hours prior to perfusion. The β-like cells were placed on a nylon filter in a plastic perifusion chamber (Millipore, Bedford, MA). The perifusion apparatus consisted of a water bath (37°C), a fraction collector (Waters Division of Millipore), and a computer-operated high-performance liquid chromatography system (Waters 625 LC System), which allowed programmable rates of flow and glucose concentration in the perifusate. The perifusate was a Krebs bicarbonate buffer (pH 7.4) containing 2.2 mmol/l Ca^{2+} and 0.25% BSA equilibrated with 95% O_2/5% CO_2. Cell aggregates were perifused with an increasing ramp of 1 mmol/L glucose per min. The maximal insulin secretion

response was tested at the end of each experiment with 30 mmol/l KCl after washout of glucose.

2.12 Electron Microscopy (Chapter 3)

β-like cells were re-aggregated by treatment with 1mg/mL dispase into suspension culture 16 to 24 hours prior to fixation. The aggregates were fixed in 2.5% glutaraldehyde, 2.0% paraformaldehyde, and 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. After subsequent buffer washes, the samples were postfixed in 2.0% osmium tetroxide for 1 hour at room temperature and rinsed in distilled H_2O prior to en bloc staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences, Fort Washington). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software.

2.13 Flow Cytometry and Cell sorting

A list of primary and secondary antibodies can be found Table 2-2, Table 2-3 and Table 2-4. Single cell suspensions were prepared by treating cells with 0.25% Trypsin/EDTA for 3 to 5 minutes. For intracellular staining, cells were fixed with 1.6% paraformaldehyde (Electron Microscopy Science) for 30 minutes at 37°C. Cells were washed, permabilized and stained with saponin buffer (Biolegend). Primary and secondary antibodies were diluted to the appropriate concentrations in 100uL of saponin buffer and cells were stained for 30 minutes at room temperature. Following the staining, cells were resuspended in FACS buffer (DPBS with 0.1% BSA and 0.1% sodium azide). For extracellular staining, cells were washed twice with FACS and re-suspended in 100ul of

Table 2-2: Conjugated Antibodies for Flow Cytometry

Antibody	Dilution	Source
SSEA3-Alexa 647	1:50	Biolegend
SSEA4-PE	1:2000	Biolegend
KDR-APC	1:100	R&D Systems
CD18-APC	1:20	BD Pharmigen
CD31-PECy7	1:100	BD Pharmigen
CD41a-PE	1:50	BD Pharmigen
CD41a-APC	1:80	Biolegend
CD42a-PE	1:20	BD Pharmigen
CD43-PE	1:50	BD Pharmigen
CD45-Pacific Blue	1:100	Biolegend
CD235	1:10000	BD Pharmigen
CXCR4-PE	1:50	Invitrogen
CD117-APC	1:50	Invitrogen
SOX17-PE	1:50	BD Pharmigen

Table 2-3: Unconjugated Primary Antibodies

Antibody	Species	Dilution	Source
GATA6	Rabbit IgG	1:800 for flow cytometry 1:1200 for immunofluorescence 1:1000 for western blot	Cell signaling
SOX17-biotinylated	Goat IgG polyclonal	1:50 for flow cytometry 1:50 for immunofluorescence	R&D Systems
FOXA1	Mouse IgG2a	1:100 for flow cytometry	Santa Cruz
FOXA2	Mouse IgG2a	1:100 for flow cytometry 1:50 for immunofluorescence	Santa Cruz
Eomesdermin	Mouse IgG2b	1:25 for flow cytometry	R&D Systems
C-peptide	Rabbit IgG polyclonal	1:100 for flow cytometry	Cell signaling
Insulin	Guinea Pig IgG	1:100 for immunofluorescence	Abcam
PDX1-biotinylated	Goat IgG polyclonal	1:50 for flow cytometry	R&D Systems
SST	Mouse IgG1	1:100 for flow cytometry	Santa Cruz
Proinsulin-biotynylated	Mouse IgG2a polyclonal	1:50 for flow cytometry	R&D Systems
Glucagon	Mouse IgG1	1:1000 for flow cytometry	Sigma-Aldrich
β-actin		1:600 for western blot	Santa Cruz

Table 2-4: Secondary Antibodies

Antibody	Dilution	Source
Goat-anti-Rabbit IgG- Alexa488	1:400	Jackson Immunoresearch
Goat-anti-Rabbit IgG- Phycoerythrin	1:400	Jackson Immunoresearch
Goat-anti-Rabbit IgG- Alexa647	1:400	Jackson Immunoresearch
Goat-anti-mouse IgG1-Alexa488	1:400	Jackson Immunoresearch
Goat-anti-mouse IgG1- Phycoerythrin	1:400	Jackson Immunoresearch
Goat-anti-mouse IgG2a-Alexa488	1:400	Jackson Immunoresearch
Goat-anti-mouse IgG2a-Alexa 647	1:400	Jackson Immunoresearch
Goat-anti-mouse IgG2b- Alexa647	1:400	Jackson Immunoresearch
Goat-anti-guinea pig IgG-Alexa488	1:400	Jackson Immunoresearch
Streptavidin- Pacific Blue	1:400	Invitrogen
Streptavidin- Alexa647	1:400	Invitrogen
Goat-anti-rabbit IgG-HRP	1:8000	Biorad
Goat-anti-mouse IgG-HRP	1:10000	Biorad

FACS buffer containing the conjugated primary antibody for 15 minutes at room temperature. The cells were washed twice with FACS buffer prior to analysis. All samples were analyzed on a FACSCantos II flow cytometer (Becton Dickinson) and FlowJo (Treestar) software program. Cell sorting was carried out on single cell suspension using BD FACSAria II (Becton Dickinson).

2.14 Reverse Transcription and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Cells were harvested by treatment with 0.25% Trypsin/EDTA (Invitrogen) for 3 minutes followed by addition of fetal bovine serum and storage in RLT lysis buffer at -80°C. Cellular RNA was isolated using the RNAeasy micro kit (Invitrogen) as described by the manufacturer's instructions. Random hexamers were used with the Superscript II Reverse Transcriptase (Invitrogen) to synthesize cDNA. PCR reactions were done in triplicate utilizing either SYBR-GreenER qPCR Master Mix (Roche) or SYBR select master mix (Invitrogen). Quantitative PCR was carried out on a LightCycler 480 II. The primers that were used in this study can be found in Table 2-5. Serial dilutions of H9 ES cell cDNA were used to generate a standard curve and TBP (Veazey and Golding, 2011) was used as a house keeping gene to determine relative gene expression levels.

Table 2-5: Quantitative real time PCR primers

	Forward Primer	Reverse Primer
ABCC8	5'-CTTGCCAATGCCTACGTC-3'	5'-AGTAGGAGGGCAAGGAAC-3'
ARX	5'-TCACTCAGCACCACTCAAGACCAAA-3'	5'-CCGCATCCAGACTGCTGTGAAG-3'
cFMS	5'-AAACTTGGGAAGGTGAGGGATTCAGG-3'	5'-ACGAGGCCAACACCATGAGAACAGTAG-3'
CPE	5'-GGTGATACTGGAGATTGCTTATACCTGGA-3'	5'-GGGCTGTAAGGAACTGCCACTTT-3'
eKLF	5'-AGCCTGTTTGGTGGTCTCTTCACA-3'	5'-TGGGTCCGTGTTTGATATTGGGTGG-3'
Endogenous		
GATA4	5'-GCTGTGCTGTGGTGGGTTAAGT-3'	5'-CGCCCTGCATCCCTAATACCAAATC-3'
EOMES	5'- ACTCAATCCCCACTGCCCACTACAA -3'	5'- TTGCCACAGGTCACCCATT -3'
FOXA1	5'-ACTACTCCTTCAACCACCCGTTCT-3'	5'-TATTGCAGTGCCTGTTCGTATGCCTTG-3'
FOXA2	5'-GCATTCCCAATCTTGACACGGTGA-3'	5'-GCCCTTGCAGCCAGAATACACATT-3'
GATA1	5'-CGAAACCGCAAGGCATCTGGAAA-3'	5'-GCCACCACCATAAAGCCACCA-3'
GATA4	5'-CCTCCATCCACCCTGTCCTCTC-3'	5'-CCAAGACCAGGCGTGTTCCAAGAGT-3'
GATA6	5'-GAGGCTTGCTGAAAGAGTGAGAGAAGA-3'	5'-TCCTAGTCCTGGCTTCTGGAAGTG-3'
GCG	5'-TTCCCAGAAGAGGTCGCCCATTGTT-3'	5'-CAACCAGTTTATAAAGTCCCTGGCGGG-3'
GCK	5'-CGGCAGGAGGAGGAACAGA-3'	5'-AGTGAGCAACTCCCTTCTGGGAAA-3'
GLP1R	5'-ACCTGTTTGCATCCTTCATC-3'	5'-GCTGTGCTATACATCCACTTC-3'
GSC	5'-GGTACTTGGTCTCCTGGAAGAGGTT-3'	5'-ATGCTGCCCTACATGAACGTGG-3'
HNF1B	5'-GGCCGTTGGTGAGAGTATGGAAGA-3'	5'-TGGTTCAGGCCTTGGAGGAGTTG-3'
ID2	5'-CCATTTCACAAGGAGGACAAGTTGA-3'	5'-ATCACAGTCCAAGTAAGAAGAGAACAC-3'
INSULIN	5'-TTTGTGAACCAACACCTGTGCGG-3'	5'-GCGGGTCTTGGGTGTGTAGAAGAA-3'
ISL1	5'-CAGAAGGAGGACCGGGCTCTAAT-3'	5'-GACTGGCTACCAGGCTGTTAGGTGTAT-3'
KCNJ11	5'-GACTGAGGTCCCTGAGGTGAAGAG-3'	5'-GGAGAAGGCAGAGTTCTAGGCAAGTT-3'
MAFA	5'-TGCAGCAGCGGCACATTCT-3'	5'-CGCCAGCTTCTCGTATTTCTCCTTGT-3'
MEIS2	5'-CGAGTGAGAGCGAGCGA-3'	5'-TGTTCAAAGAAAGCAGTGAGAA-3'
MNX1	5'-CTCAGAGACGTAAGCATAAACCTT-3'	5'-AGAAGGCGGAAACCCACAGTGTTA-3'
MSX2	5'-ACCTGTTGAGAGGAATTGATG-3'	5'-CCCACCCAAATCACCTT-3'
NANOG	5'-CCTGAAGACGTGTGAAGATGAG-3'	5'-GCTGATTAGGCTCCAACCATAC-3'
NEUROD1	5'-GTGGTGCCTTGCTATTCTAA-3'	5'-AAAGCGTCTGAACGAAGG-3'
NKX6.1	5'-AAGAAGCACGTCGCCGAGATG-3'	5'-CCGAGTTGGGATCCAGAGGCTTATT-3'
OCT4	5'-AACCTGGAGTTTGTGCCAGGGTTT-3'	5'-TGAACTTCACCTTCCCTCCAACCA-3'
PAX6	5'-TCGAAGGGCCAAATGGAGAAGAGAA-3'	5'-GGTGGGTTGTGGAATTGGTTGGTAGA-3'

Gene	Sequence
PCSK1	5'-TCCGTGGATCCAGAGGTGGTTATG-3'
PCSK1	5'-TCCGTGGATCCAGAGGTGGTTATG-3'
PCSK2	5'-ATCACGGTCAACGCAAC-3'
PDX1	5'-GCCGGCTCTTCAAAGACAATGGA-3'
PF4	5'ACTGGAAGGACAGCCGGGAATAAA-3'
PPY	5'-CCGCTGGACTTATAATGCCACCCTCT-3'
PU.1	5'-CGCCTGTACCAGTTCCTGTTGG-3'
SLC2A1	5'-CACTTGCCTTCTTCACCCAGCTAATC-3'
SLC2A2	5'-GCTCAGCCCACAGGCCAAA-3'
SLC30A8	5'-TGGGAGTCTTGCTGTTGTCACAGA-3'
SOX1	5'-CCTGTGTGTACCCTGGAGTTTCTGT-3'
SOX17	5'-AGGAAATCCTCAGACTCCTGGGTT-3'
SOX2	5'-ATGACCAGCTCGCAGACCTACA-3'
SST	5'-GAGAATGATGCCCTGGAACCTGAAGA-3'
T	5'-CAGTGGCAGTCTCAGGTTAAGAAGGA-3'
TBP	5'-TTGCTGAGAAGAGTGTGCTGGAGATG-3'
TBX3	5'-TTATGTCCCAGCGAGGGTGA-3'
UCN3	5'-TTCAACATCGCCAAGGCCAAGAAC-3'

Gene	Sequence
PCSK1	5'-TGTGGCTGAGAAAGGAGACAGGTATA-3'
PCSK1	5'-TGTGGCTGAGAAAGGAGACAGGTATA-3'
PCSK2	5'-TGGACTTGGTGCCCATA-3'
PDX1	5'-GGTCGCCCGAGTAAGAATGGCTTTAT-3'
PF4	5'-TCAGTGCTCAGTGCGATGGGAAA-3'
PPY	5'-TTTATTGAGCCTGTGTGGGAGCAG-3'
PU.1	5'-TCATCTTCTTGCGGTTGCCCTTCT-3'
SLC2A1	5'-CCATAGCCACCTCCTGGGATAGAA-3'
SLC2A2	5'-GACGGTTCCCTTATTGTTTCTGTTCAT-3'
SLC30A8	5'-TCGATGACAACCACAGGGAGAAGA-3'
SOX1	5'-TGCACGAAGCACCTGCAATAAGATG-3'
SOX17	5'-CCCAAACTGTTCAAGTGGCAGACA-3'
SOX2	5'-GGACTTGACCACCGAACCCA-3'
SST	5'-ATTCTTGCAGCCAGCTTTGCGT-3'
T	5'-CGCTACTGCAGGTGTGAGCAA-3'
TBP	5'-CGTAAGGTGGCAGGCTGTTGTT-3'
TBX3	5'-ACGTGGTGGTGGAGATCTTG-3'
UCN3	5'-CTTCTTCCTCCCAATTGCGCCAT-3'

2.15 Southern Blot (Chapter 4)

Genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). Genomic DNA (5–10 µg) was digested overnight with SPH1-HF (New England Biolabs, IPSwich, MA). The genomic DNA was separated by 0.8% agarose gel electrophoresis and transferred to an Amersham Hybond-N$^+$ membrane (GE Healthcare). The membrane was hybridized with a ^{32}P-α-dCTP labeled probe synthesized using the High Prime Random Labeling (Roche) following the manufacturers instructions. The probe used for Southern blotting was a 480 base pair BamH1 fragment from the 5' arm of homology in the vector plasmid AAVS1-SA-2A-puro-PA.

2.16 Western Blot (Chapter 5)

A list of primary and secondary antibodies can be found in Table 1 and Table 3. Cells were harvested at day 4 of definitive endoderm differentiation with 0.25% Trypsin/EDTA and cell pellets were stored at -80°C. Briefly, protein from whole cell lysates was extracted using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher). Combined cell lysates were run on a 4%-12% Bis-Tris SDS-polyacrylamide gel (Invitrogen) and transferred to a PVDF membrane (Thermo Fisher) overnight. The membrane was then blocked in 5% nonfat dry milk in DPBS for 1 hour at 4°C. The membrane was probed with primary antibody diluted in 1% nonfat dry milk in DPBS with 0.1% Tween-20 (PBST) either overnight at 4°C or 1 hour at room temperature. The membrane was washed and placed in a horseradish peroxidase conjugated secondary antibody for 1 to 3 hours at room temperature. The membrane was detected by chemiluminescence (Thermo Fisher Scientific) and exposed onto HyBlot CL autoradiography film (Denville Scientific) for visualization.

2.17 Immunofluorescence staining (Chapter 5)

PSCs were cultured on 1:3 matrigel coated sterile round 18mM glass coversliPS (Thermo Fisher Scientific) and differentiated to definitive endoderm as described. On day 4 of definitive endoderm differentiation, cells were fixed in 4% parafolmadlehyde in DPBS for 15 minutes at room temperature. Cells were blocked for one hour (5% normal goat serum, 0.3% TritonX-100 in DPBS) and stained in primary antibody in staining buffer (1% BSA, 0.3% TritonX-100 in DPBS) overnight at 4°C. After washing, cells were stained in secondary antibody in staining buffer for 2 hours at room temperature. The cover slips were washed mounted onto glass slides and counterstained with VECTASHIELD containing DAPI mounting media (Vector Labs). Slides were viewed under a Leica DMI 4000B microscope and digital images were captured with Leica Application Suite software. A list of primary and secondary antibodies can be found Table 1 and Table 3.

2.18 Lentiviral vector generation and transduction (Chapter 5)

GATA1, GATA3, GATA4, GATA6, truncated GATA6 (containing the 4 base pair 'GTAC' patient duplication) cDNA was cloned into a lenti viral transfer vector. The lenti viral vector contains an inducible Tet-response element (TRE) driving the expression of the GATA factor cDNA. The cDNA is followed by an internal ribosome entry site (ires) driving expression of red fluorescent protein (RFP). Lenti virus was generated in HEK293T cells by transfection with 2M $CaCl_2$ and HEPES with the transfer vector, G protein of the vesicular stomatitis virus (VSV), Hgpm2, Tat and Rev. Virus was collected for 3 to 4 days following the transfection and concentrated by high speed centrifugation. PSCs were transduced at 40% to 60% confluency with lenti virus for 16 to 20 hours with 2µg/mL polybrene (Millipore). Two to three days following the transduction, PSCs were

split. Differentiation of these cells was performed for a maximum of 4 passages following the lentiviral transduction.

2.19 Microarray and Bioinformatics Analysis (Chapter 5)

Three replicates of each cell line were prepared by differentiating the cells for 24 hours toward definitive endoderm. Total RNA was extracted from the cells by use of the PureLink RNA mini kit (Invitrogen) following the manufacturers instructions.

2.20 Statistical Analysis

Results from multiple experiments are expressed as the mean ± standard error. An unpaired two-tailed Student's t-test was for groups with equal variance were performed to determine p values. In figures * P<0.05, **P<0.01 and ***P<0.001.

CHAPTER 3

The Characterization of Endodermal Progenitor Cell Derived β-like Cells

3.1 Introduction

The use of primary human β cells in research is limited by the availability of human tissue, which makes it difficult to study human β cell development, β cell proliferation and β cell related diseases such as diabetes using primary human cells. One potential model system of human β cells that is currently being explored is the *in vitro* differentiation of pluripotent stem cells (PSCs) including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells to β cells. PSCs can be directed to differentiate into various mature cell types *in vitro*, by mimicking the developmental events that occur *in vivo*. In an *in vitro* pancreatic differentiation, PSCs are specified through sequential stages that mirror *in vivo* development. The PSCs progress first through definitive endoderm, then pancreatic progenitors, then endocrine progenitors and eventually β cells in a stepwise fashion (Nostro and Keller, 2012).

Adult β cells should be monohormonal for insulin and secrete insulin in response to glucose stimulation. Initial approaches to generate β cells from PSCs *in vitro* were inadequate as they resulted in poly-hormonal endocrine cells that do not respond to glucose (D'Amour et al., 2006). In these early studies, glucose responsive β cells derived from PSCs could only been generated by the transplantation of PDX1+ endocrine precursors into mice for several months (Kroon et al., 2008; Rezania et al., 2012). More recently, a number of laboratories have described the generation of functional PSC derived β cells in vitro. The cells that are generated glucose responsive

in vitro and can rescue diabetic mice (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015a) .

One of the concerns with the use of PSC derived β cells is any undifferentiated PSCs at the end of the differentiation may have teratoma forming potential. This raises serious safety concerns if PSC derived endocrine cells are ever to be used as a cell replacement therapy. Furthermore, the protocols that have been described to generate functional β cells can take up to 40 days for PSCs to reach a terminally differentiated β cell with only limited functionality.

Our laboratory has been able to generate a population of endodermal progenitor (EP) cells that are self-renewing, do not form teratomas, and can be differentiated into multiple endodermal cell lineages. More importantly, these cells can be differentiated into pancreatic β-like cells that are mono-hormonal for insulin and can respond to glucose stimulation *in vitro* by utilizing a 14 to 18 day protocol (Cheng et al., 2012).

As EP derived β-like cells (henceforth referred to as β-like cells) are insulin expressing and glucose responsive in vitro we sought to determine whether β-like cells are a viable model system for the study of human β cells. By gaining an increased understanding of the similarities as well as the limitations of β-like cells when compared to adult β cells, we can recognize the scope of the conclusions that may be drawn from studies using these cells. Here we describe the optimization and further molecular and functional characterization of EP derived β-like cells.

3.2 Results

Differentiation of EP cells into β-like cells utilizes a differentiation protocol similar to that used to generate endocrine cells from ES cells (Nostro et al., 2011). However, in place of starting at the ES cell stage, differentiation from EP cells is initiated at the primitive gut tube stage. The method of differentiation is step-wise, first the EP cells are specified into posterior foregut , pancreatic endoderm and then β-like cells (Cheng et al., 2012). For these experiments we chose to use the H9 ES cell derived EP cells as these had been previously described by our laboratory.

3.2.1 Dispase Aggregation

At the final stage of differentiation, cells are harvested for analysis and further characterization. At this stage, the β-like cells are positive for c-peptide and negative for glucagon (Figure 3-1A). C-peptide is a small peptide that is produced at approximately at a 1:1 ratio to insulin during the cleavage of pro-insulin into insulin, thus it is a marker of endogenous insulin production (Rhodes, 2004b). Insulin is not used directly as a marker for β cells as insulin is present in the differentiation media that the cells are cultured in. To determine whether a three dimensional culture can improve the differentiation of β-like cells, the cells were transitioned from an adherent culture to a suspension culture on day 13 of the differentiation. The adherent β-like cells were treated with 1mg/mL dispase for a short period of time to partial disaggregate the cultures. In the suspension culture, the β-like cells form small aggregates (Figure 3-1B). Two days post aggregation in suspension culture, β-like cells remain c-peptide negative and glucagon positive, additionally it is observed that there is a small subpopulation of β-like cells which are double positive for c-peptide and somatostatin (Figure 3-1A). Although the suspension culture does not increase the absolute yield of c-peptide positive cells (Figure 3-1C), it

Figure 3-1
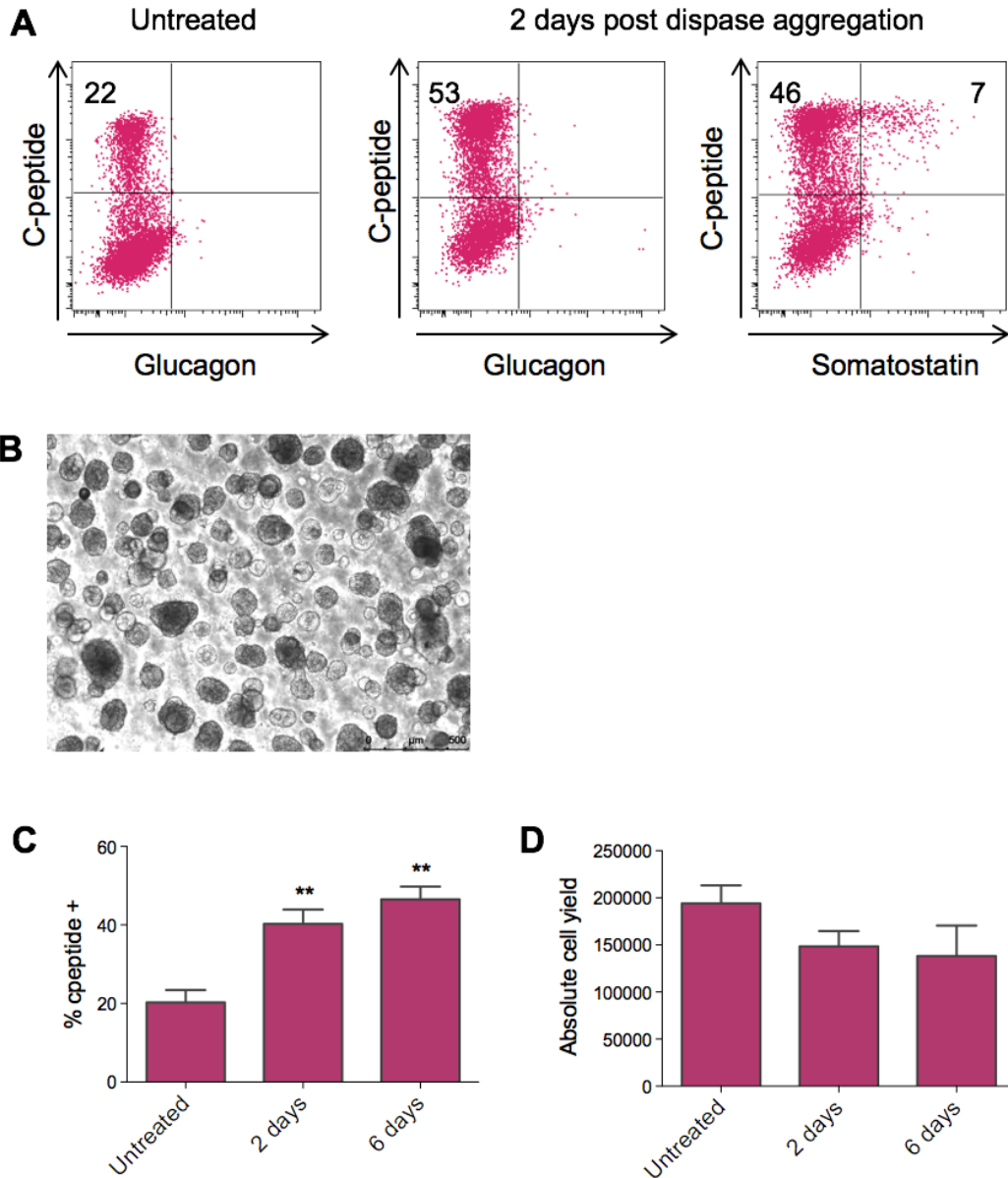
Figure 3-1. *Dispase aggregation enriches for c-peptide positive β-like cells.* EP cells were differentiated as an adherent culture to β-like cells. At day 13 of differentiation dispase treatment was use to reaggregate the cells into a suspension culture. Cultures were analyzed for c-peptide and glucagon expression prior to treatment with dispase. Two days post aggregation cells were analyzed for c-peptide, glucagon and somatostatin by intracellular flow cytometry. (B) Bright field image two days post aggregation of β-like cells from adherent culture. (C) Quantification of percentage c-peptide positive cells from untreated adherent cultures and aggregation cultures 2 days and 6 days post aggregation. (D) Quantification of absolute yield of c-peptide positive cells from untreated

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enriches for c-peptide positive cells. Two days post aggregation c-peptide positive cells increased by 2.1 ± 0.11 fold and 6 days post aggregation 2.7 ± 0.38 fold (Figure 3-1D), suggesting that there is a selective advantage for the survival or recruitment of c-peptide positive cells into the aggregates and offers a simple method for purification of the EP derived β-like cells.

3.2.2 Gene and Protein Expression

Gene expression profiles in EP derived β-like cells were compared to primary non-diabetic adult human islets to determine how similar EP derived β-like cells are to functional adult β cells (Figure 3-2). The gene expression levels of endocrine hormones correlated with the protein expression levels from flow cytometry analysis. Insulin (INS) was expressed within the EP derived β-like cells and there is low expression levels of somatostatin (SST). The EP derived β-like cells did not express glucagon (GCG) or pancreatic polypeptide (PPY), hormones that are expressed in other endocrine cells within the islet. To determine the maturity of β-like cells as compared to a human adult β cell, four β cell specific genes, NKX6.1, urocortin3 (UCN3), islet-1 (ISL1) and v-maf musculoaponeurtic fibrosarcoma oncogene homology A (MAFA), were examined (Blum et al., 2012; Hang and Stein, 2011; Schaffer et al., 2013). While EP derived β-like cells expressed UCN3 and ISL1, MAFA and NKX6.1 expression is low.

In β cells insulin is initially synthesized as proinsulin and must undergo a number of cleavages to become insulin by prohormone convertase 1 (PCSK1), prohormone convertase 2 (PCSK2) and carboxypeptidase E (CPE) (Rhodes, 2004b). In the β-like cells while PCSK1 is expressed at low levels, PCSK2 and CPE are expressed at

Figure 3-2
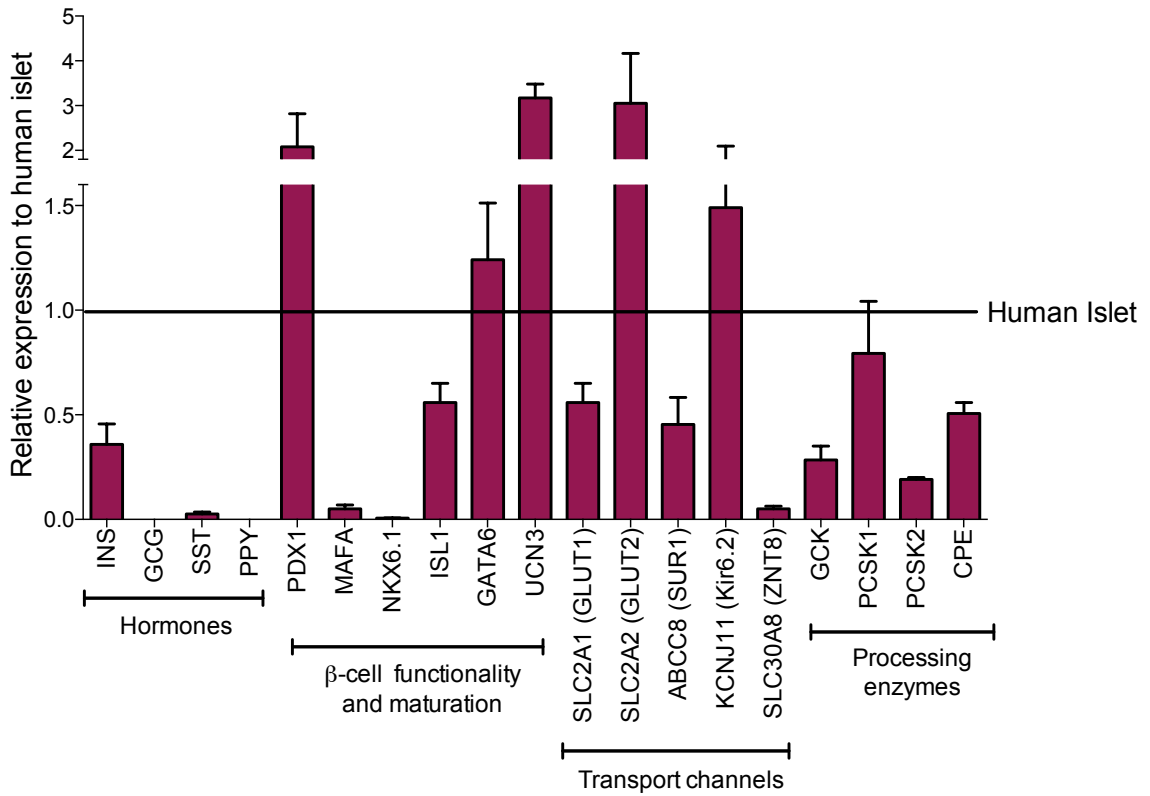
Figure 3-2. *Gene expression profile of β-like cells.* Quantitative real time PCR was done to examine gene expression of key hormones, transcription factors, channels and processing enzymes in β-like cells and primary human islets. Gene expression levels are normalized to an adult human islet.

moderate levels compared to human adult islets. When β cells are stimulated, insulin release in response to glucose begins with the uptake of glucose into the β cell via the GLUT1 or GLUT2 glucose transporter channels. Glucokinase (GCK) then breaks down the glucose into glucose-6-phosphate and through glycolysis and the citric acid cycle generates an increase in the ATP/ADP ratio. The high ATP/ADP ratio causes depolarization of the membrane through the ATP-potassium channel, of which Kir6.2 (also known as KJCN11) and SUR1 (also known as ABCC8) are the major subunits. Depolarization of the membrane causes the influx of Ca^{2+} stimulating insulin release (Hou et al., 2009). With the exception of GLUT2 which is expressed at high levels in β-like cells, GLUT1, SUR1 and Kir6.1 are all found to be expressed in β-like cells at comparable levels to adult human islets. GCK is expressed but at lower levels. Another important channel in β cell is the zinc transporter 8 (ZNT8 or also known as SLC30A8), which is involved in transport of zinc from the cytoplasm into insulin granules (Wijesekara et al., 2009). In β-like cells the expression of ZNT8 is very low compared to adult islets. Lastly, GATA6 expression level was examined as it is a gene of interest for us (see Chapter 5). GATA6 is expressed at similar levels in β-like cells when compared to adult islets. Based upon these analysis while the majority of genes specific for or involved in β cell functionality are expressed there still remains a few that have low expression levels in β-like cells.

3.2.3 Glucose Stimulation Insulin Secretion

The functionality of β-like cells can be examined using a static glucose-stimulated insulin secretion (GSIS) assay. In this assay, the glucose-responsiveness of the β cells is measured by incubating in a low glucose basal media followed by a high glucose

stimulation media. C-peptide secretion in response to glucose stimulation is then measured by ELISA.

To examine the functionality of the β-like cells, static GSIS was performed following aggregation of the β-like cells. The absolute amount of c-peptide released per c-peptide positive cell was found to be lower in β-like cells compared to per c-peptide positive cell in adult islets (Figure 3-3A). The fold change in c-peptide secretion between basal and glucose stimulation the stimulation index is 2.4 ±0.48 similar to the stimulation of human islets at 2.8 ±0.66 (Figure 3-3B). This data shows that while the amount of insulin secreted may be lower in β-like cells as compared to human islets, β-like cells do function similarly in terms of response to glucose when compared to an adult islet.

In addition to glucose, β-cells also respond to other secretagogues with increased insulin secretion. To examine the response of β-like cells to secretagogues, the cells were stimulated with either 10mM glucose or 10mM glucose with the addition of a secretagogue or inhibitor of insulin secretion for 20 minutes. To study the functionality of the potassium channel in β-like cells, glibenclamide and diazoxide was used. Glibenclamide is a sulfonylurea drug that binds to the SUR1 subunit and inhibits the K_{ATP} channel resulting in depolarization of the membrane (Kramer et al., 1995). Diazoxide is a K_{ATP} channel activator that inhibits insulin secretion by hyperpolarizing the membrane and increasing calcium flux into the cell (Alarcón et al., 2002; Grimmsmann and Rustenbeck, 1998). Addition of 100µM glibenclamide to β-like cells resulted in a a 2.7 ±0.35 fold statistically significant increase in stimulation while addition of diazoxide has no significant effect on the secretion of c-peptide from the β-like cells and the stimulation

Figure 3-3
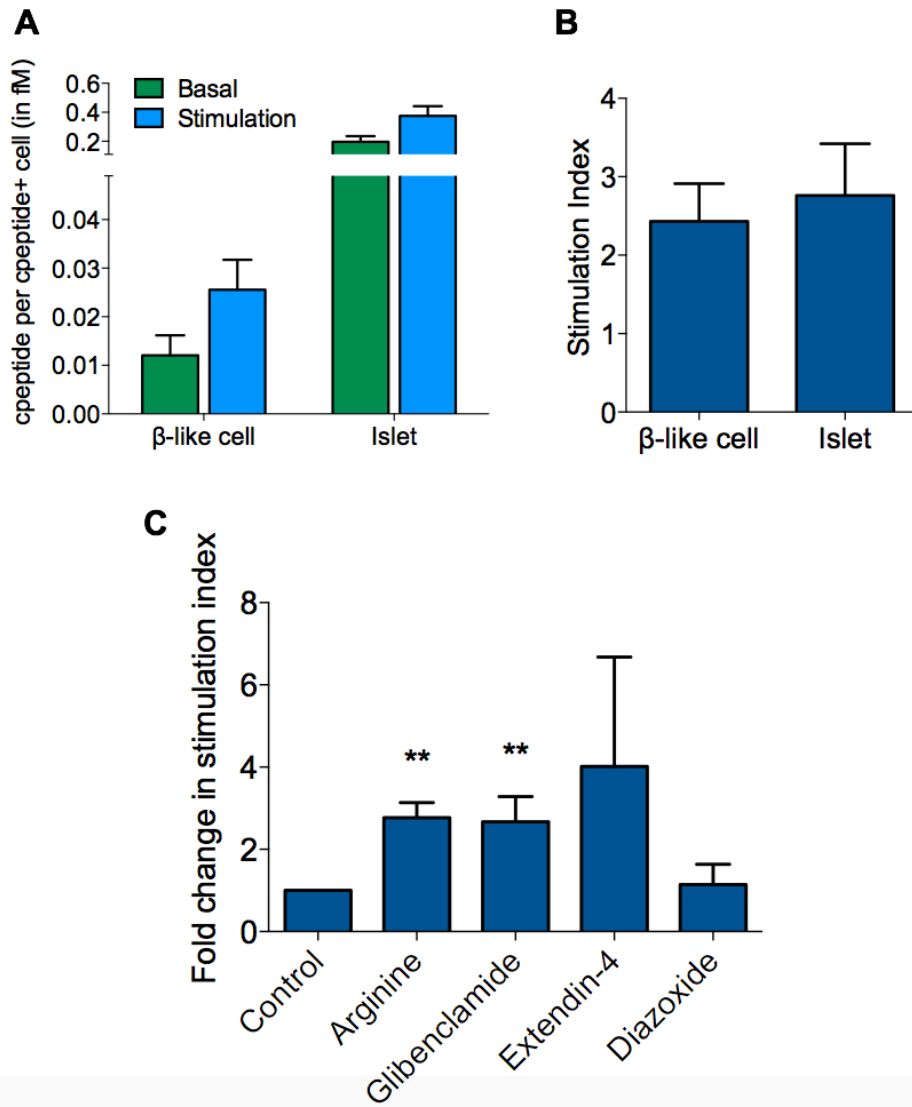
Figure 3-3. *Static stimulation of β-like cells* (A) Quantification of c-peptide secretion per c-peptide positive cell of β-like cells and primary human islets in basal glucose (1 mM) and stimulation glucose (20 mM) conditions. (B) Stimulation index of C-peptide secretion from A. (C) Stimulation index of β-like cells challenged with the secretagogues arginine, glibenclamide extendin-4 or the inhibitor diazoxide. Stimulation index are normalized to the control condition. For statistical analysis: * $P<0.05$, **$P<0.01$, ***$P<0.001$

index was 1.1 ±0.28 relative to the stimulation index of glucose stimulation alone (Figure 3-3C). These responses of the β-like cells to glibenclamide and diazoxide demonstrate that in β-like cells the K_{ATP} channel functions appropriately and can respond to drugs that inhibit or activate its function. Extendin-4 is an analog of glucagon like peptide 1 (GLP1) that binds to the GLP1 receptor and causes an increase in insulin secretion (Young et al., 1999). The addition of 0.1μM extendin-4 resulted in a 4.0 ±2.6 fold increase in c-peptide secretion compared to stimulation with glucose only. The amino acid arginine can also stimulate the release of insulin. Arginine is transported into the β-cell by the mCAT2A amino acid transporter leading to the depolarization of the plasma membrane and an increase in intracellular calcium concentrations (Newsholme et al., 2006). β-like cells stimulated with the addition of 10mM arginine lead to statistically significant increase in c-peptide secretion of 2.4 ±0.28 fold. Together these results demonstrate that β-like cells contain suitable mechanisms to respond to secretagogues in addition to glucose.

3.2.4 Perifusion

An alternative way to understand functionality of β-like cells is to utilize a perifusion system. Perifusion systems can challenge cells with glucose or other stimulants and measure dynamic insulin secretion. Insulin secretion *in vivo* is biphasic, when β cells are stimulated with high glucose insulin secretion peaks and with prolonged stimulation decreases and plateaus to a constant level until normoglycemia is reestablished (Gerich, 2002; Straub and Sharp, 2002). In order to determine whether β-like cells respond to glucose with a biphasic secretion of insulin and the glucose concentration where maximal insulin secretion is reached, β-like cells were perfused with a glucose ramp of

Figure 3-4
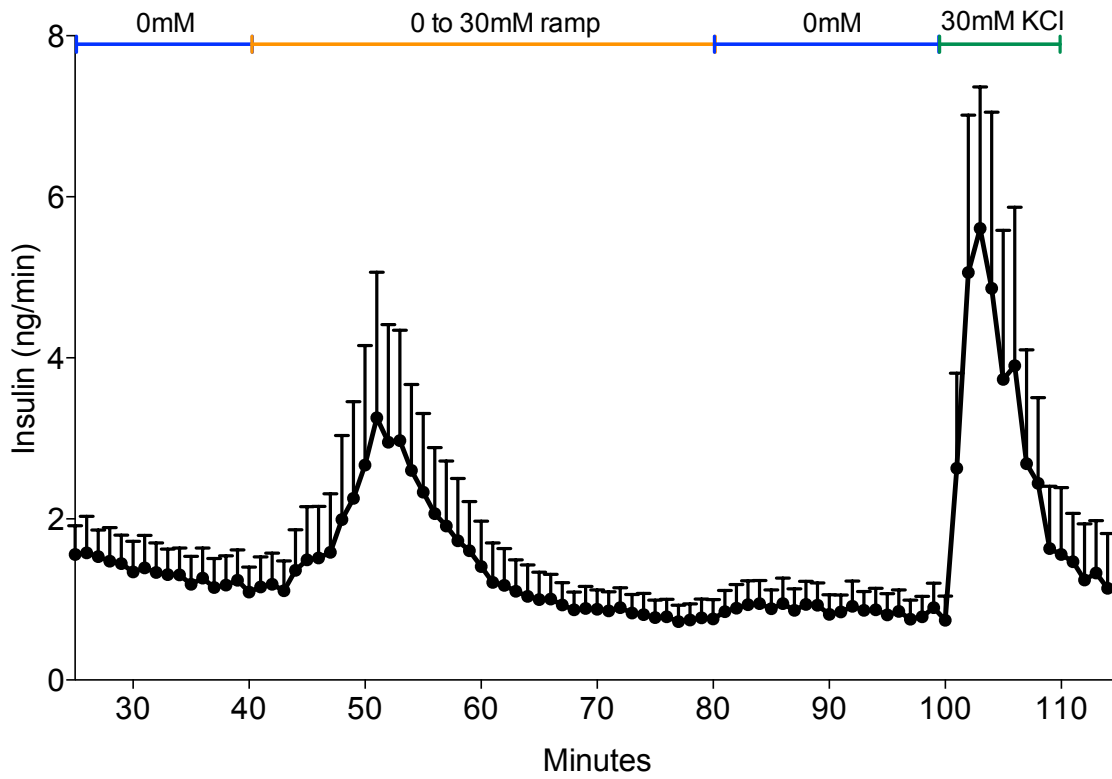
Figure 3-4. *Perifusion of β-like cells.* Quantification of insulin secretion from aggregated β-like cells perifused with a 0mM to 30mM glucose ramp for 40 minutes followed by a 30mM potassium chloride treatment at the end of the experiment to maximally stimulate the cells.

0mM to 30mM over a period of 40 minutes (Figure 3-4). At the end of every experiment, β-like cells were treated with 30mM potassium chloride. Samples were collected at 1 minute intervals and analyzed for the amount of insulin secreted.

Insulin secretion began at around 2mM glucose and maximal insulin secretion was at approximately 6mM glucose (Figure 3-4). During the glucose ramp there was an initial peak in insulin secretion indicating the first phase of insulin secretion was operative. However, following this peak, with prolonged stimulation, there was a decrease in insulin secretion to barely detectable levels demonstrating a lack of a robust second phase response in the β-like cells. Following the glucose stimulation, β-like cells were treated with 30mM potassium chloride and depolarization of the membrane was confirmed by secretion of insulin suggesting that there was not a complete depletion insulin present within the β-like cells. While functionality of EP cell derived β-like cells in the perifusion system did not completely mimic primary human islets, we are encouraged by these results considering that even the best published beta cell differentiation protocols only show functionality in static GSIS systems and fail to perform in the perifusion assay (Pagliuca et al., 2014; Rezania et al., 2014).

3.2.5 Ultrastructure

Electron microscopy can be used to visualize the intracellular ultrastructure of the cell. In adult human β cells, there is an abundance of mitochondria and insulin is found in dense core granules surrounded by a white 'halo' (Figure 3-5C) (Rhodes, 2004b). As β-like cells are glucose responsive, we questioned whether this correlates with the appropriate granule morphology and mitochondrial presence in the cells. Transmission electron microscopy (TEM) on β-like cells revealed the presence of a large number of

Figure 3-5
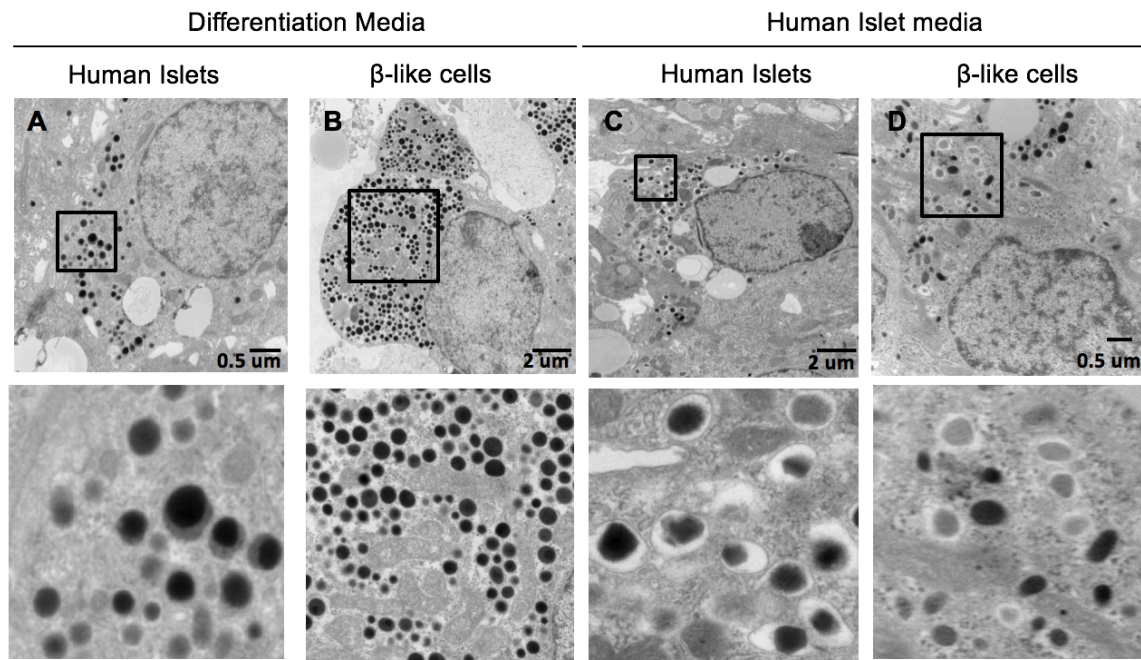
Figure 3-5. *Ultrastrucutre of β-like cells.* Transmission electron microscopy of (A) primary human islets cultured in differentiation media for 2 hours, (B) β-like cells cultured in differentiation media, (C) primary human islets cultured in islet media and (D) β-like cells cultured in human islet media for 24 hours. The bottom panel is an enlarged view of the boxed section from the top panel.

mitochondria. Dense core granules were observed, however these granules still had an abnormal morphology similar to an immature insulin granule (Rhodes, 2004b) and lacked the characteristic white halo (Figure 3-5B).

As a control, primary human islets were placed into the same media as the EP derived β-like cells for several hours. Unexpectedly, it was found that although dense core granules were present in the β cells in these islets, they did not contain the white halo (Figure 3-5A). This suggests that the presence of the white halo and correct morphology of the dense core granules may be dependent upon the components of the media that the cells are cultured in. To test this, we perform TEM on β-like cells that were cultured in the commercial islet media, which is normally used to culture primary human islets. Improvement of granule morphology was observed, with some of the granules displaying a white halo around the dense core (Figure 3-5D). Thus, it is likely that a component of the commercially available media is lacking from the differentiation media preventing formation of the appropriate dense core granule morphology.

3.2.6 Zinc Sulphate Treatment

Knockout ZNT8 mice have decreased zinc content in the islet but this is found to have modest effects on glucose homeostasis. Insulin granules present in these mice fail to crystallize to form a dense core granule (Davidson et al., 2015; Lemaire et al., 2009). Similarly, β-like cells are functional however have low expression of ZNT8 and a lack of dense core crystalline granules. Thus a lack of zinc in the β-like cells may be one explanation for why dense core granules do not form. As the commercial islet media contains zinc and the differentiation media does not, it was hypothesized that the lack of zinc in the differentiation media was the missing component preventing the correct

morphology of the insulin granules. To address this, end stage differentiated β-like cell media was supplemented with 100μM zinc sulphate (ZnSO$_4$).

The ultrastructure of the β-like cells was examined following 4 days of treatment with ZnSO$_4$ by TEM. The presence of zinc did not affect the morphology of the dense core granules present in the β-like cells and these granules still had similar morphology to the differentiation media control conditions. It was also observed that there small dark spots present throughout the β-like cells (some of which are indicated by the arrow head in Figure 3-6A) which had been cultured in the presence of ZnSO$_4$. The β-like cells appear to have taken up the ZnSO$_4$from the culture media but not into the insulin granule and thus this did not rescue the granule morphology.

In addition, following treatment gene expression analysis was performed every 2 days (Figure 3-6B). Treatment with ZnSO$_4$ did not causes changes in the expression of insulin in the β-like cells. Expression of the zinc transporter SLC30A8 which functions to transport zinc out of the cytosol was unaffected and compared to adult human islets levels were still low. SLC39A7, a zinc transporter channel that transports zinc into the cytosol from extracellular and from organelles was also unchanged with ZnSO$_4$ treatment. In both the treated and untreated conditions, prolonged culture appeared to have the most dramatic effect on increase in gene expression suggesting that prolonged culture may aid in the maturation of β-like cells and expression of the appropriate zinc transporter channels.

Figure 3-6
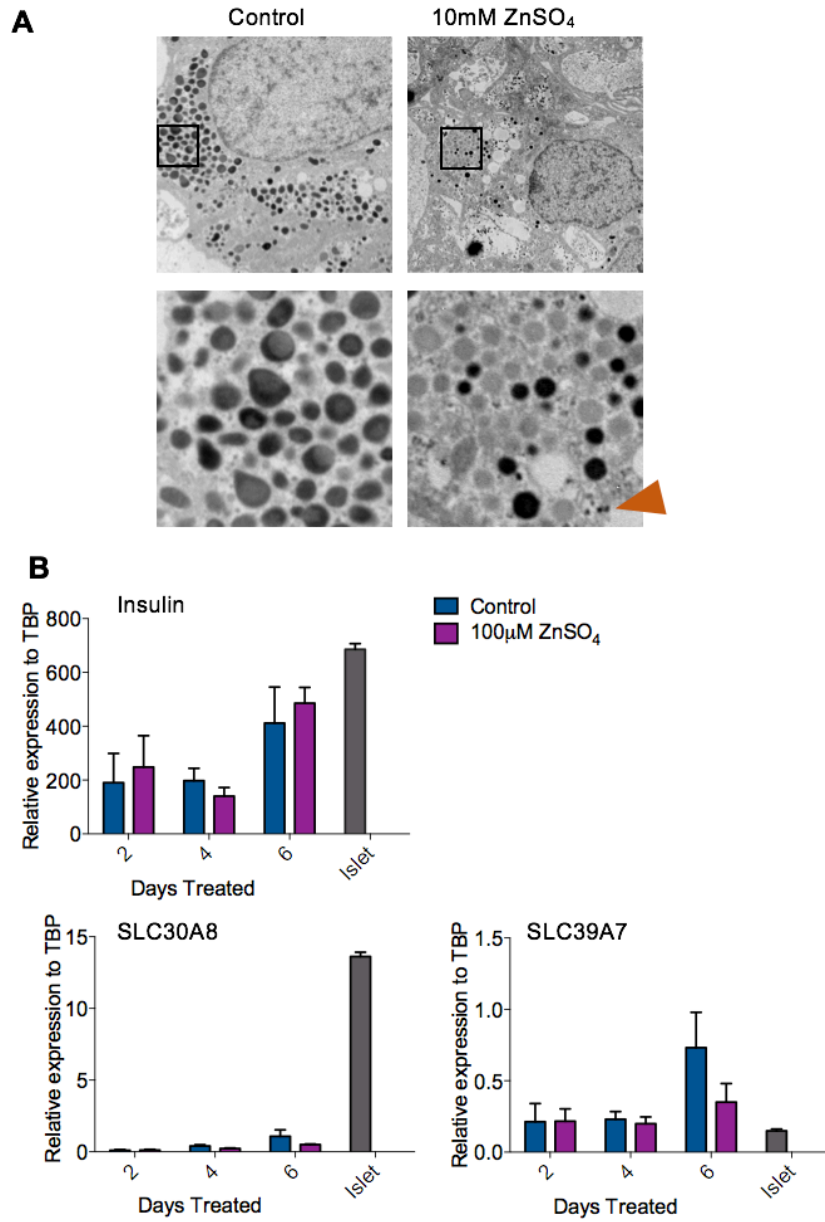
Figure 3-6. *Zinc sulphate treatment of β-like cells.* End stage β-like cell media was supplemented with 100 µM zinc sulphate. (A) Transmission electron microscopy of β-like cells following 2 days of zinc sulphate treatment. The lower panel is an enlarged view of the boxed section in the top panel. (B) Gene expression for insulin, SLC30A8 and SLC39A7 was examined every 2 days on control and zinc

3.3 Discussion

Here we have further examined EP derived β-like cells through both molecular characterization and functional assays. Gene and protein expression analysis of hormones have demonstrated that although low levels of somatostatin and insulin are co-expressed in a small population of the β-like cells, unlike PSC derived β cells, β-like cells never co-express glucagon and insulin. When examining gene expression, while a number of crucial β cell markers such as PDX1, ISL1 and UCN3 are expressed in the β-like cells, two other β cell maturation markers which are expressed at low levels are NKX6.1 and GLP1R. Furthermore, β-like cells were shown to express the majority of the genes involved with the processing and secretion of insulin thus corroborating the functionality of the cells. However, ZNT8, a gene important for zinc transportation into the cell is poorly expressed in β-like cells but is not required for beta cell functionality in vivo.

Adult β cells can swiftly respond to elevated glucose levels with the fast release of insulin. The stimulation index of β-like cells in response to glucose during static stimulation is 2.4 (±0.48) fold, similar to the stimulation index of human islets which is reported to be in the range of 2 to 4 fold (Hilderink et al., 2015; Lamb et al., 2011; Street et al., 2004). In our hands the stimulation index of primary human islets was found to be 2.8 (±0.66) fold. In recently described ES derived β cell protocols, both PSC derived β cells and islets were shown to have a stimulation index of approximately 2 fold (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015a). While β-like cells can respond to glucose stimulation with a similar stimulation index to adult islets, some deficiencies still remain. The expression of insulin in β-like cells is 2 fold lower than adult islets, during

both basal and glucose stimulation the amount of insulin secreted per cell is several fold lower than in islets. This discordance could be due to other regulatory mechanism present within β cells controlling the translation of insulin or defects in the secretory mechanism of the β-like cells. Furthermore, β-like cells do not display a biphasic insulin secretion response to prolonged glucose stimulation. In order to be able to recapitulate the functionality of an adult islet, these two deficiencies must be addressed and improved in our β-like cell differentiation.

β-like cells have low levels of NKX6.1 expression. As NKX6.1 is known to be important for β-cell development (Henseleit et al., 2005; Schaffer et al., 2013) this may explain some of the deficiencies of the β-like cells. In adult mice a loss of NKX6.1 in the islets causes the mice to become diabetic and have decrease insulin content within the pancreas. In vitro static GSIS on islets derived from these mice demonstrated that they were functional in terms of glucose responsive but secreted a decreased amount of insulin when compared to wild type islets (Taylor et al., 2013). These phenotypes are similar to what has been observed in EP derived β-like cells suggesting that although the cells are glucose responsive, some of their deficiencies may result from the lack of NKX6.1. Additionally, a loss of NKX6.1 in the adult mouse islets lead to the decreased expression of GLP1R suggesting a regulatory mechanism between the two transcription factors (Taylor et al., 2013). In addition to low expression of NKX6.1, β-like cells also have low expression of GLP1R.

Addition of zinc in the form of $ZnSO_4$ to the β-like cell differentiation media did not enhance insulin granule crystallization in the β-like cells suggesting that there is a

component of the commercial media beneficial to insulin granule crystallization that remains to be identified. Through TEM it was observed that zinc was able to enter the β-like cell but failed to cause crystallization of the insulin granule indicating that zinc was unable to enter the insulin granule. Examination of ZIP7 (also known as SLC39A7), a zinc transporter channel involved in the movement of zinc into the cytoplasm from outside the cell or intracellular compartments (Wijesekara et al., 2009), revealed expression levels similar to that of human islets. Thus the presence of ZIP7 allowed entry of the zinc into the cytoplasm but a lack of ZNT8 lead to failure in transport of zinc into the insulin granules. In murine and guinea pig studies, global or β-cell specific knockout of ZNT8 have been studied with varying results. All studies agreed that a lack of ZNT8 lead to decreased overall zinc content within the islet. The insulin granules observed in these mice were diffuse and not crystallized. Results of in vitro GSIS and in vivo glucose tolerance test were discordant, with some studies reporting an improvement, some reporting no change while others suggest there was an impairment in β cell functionality (Davidson et al., 2014; Mitchell et al., 2016). These studies indicate that a lack of zinc in β cells lead to a lack of crystalline insulin granules but is inconclusive about the necessity of the granules for full β cell functionality.

In summary, while EP derived β-like cells do still have limitations in that they do no express β cell specific markers such as NKX6.1 and do not respond to prolonged glucose stimulation. Unlike PSC derived β cells, EP derived β cells do not pose any risk of contaminating PSCs as they are differentiated from a pure population of endodermal progenitor cells. While there remains some deficiencies of the functionality of β-like cells, in static stimulation β-like cells can respond to multiple secretagogues and in perifusion

assay perform better than any of the other currently available published protocols (Pagliuca et al., 2014; Rezania et al., 2014). The main function of a β cell is to secrete insulin in response to glucose stimulation and EP derived β-like cells can do so consistently.

CHAPTER 4

Utilization of the AAVS1 Safe Harbor Locus for Hematopoietic Specific

Transgene Expression and Gene Knockdown in Human ES Cells

4.1 Introduction

In human ES cells, introduction of stable genetic modifications by homologous recombination has been challenging for many laboratories. Most commonly, transgenes are introduced into ES cells via methods such as viral transduction or plasmid transfection. However, these methods lead to random integration within the genome (Tenzen et al., 2010). Although clones with stable transgene expression can be selected, most clones display transgene silencing upon ES cell differentiation. For example, even the very well characterized and strong beta globin promoter functions to appropriately induce transgene expression in only a minority of ES cell clones generated using a random integration strategy (Hatzistavrou et al., 2009).

The use of zinc finger nucleases (ZFNs) is one way in which transgenes can be introduced at specific locations within the genome and potentially avoid gene silencing. ZFNs cause a site-specific DNA double strand break and enhance homologous recombination efficiency to introduce a construct of interest using gene targeting (Carroll, 2011). One method to prevent silencing of transgenic constructs is to target genomic loci of constitutively expressed genes for gene targeting. One such site in the human genome is the AAVS1 "safe harbor" locus which encodes the PPP1R12C gene and when targeted with transgenes results in stable gene expression (DeKelver et al., 2010; Hockemeyer et al., 2009). It has been shown that transgenic constructs targeted to the AAVS1 locus maintain expression in hematopoietic cells differentiated from human

induced pluripotent stem (iPS) cells when using constitutive promoters (Garçon et al., 2013; Zou et al., 2011) or when using erythroid specific promoters (Chang & Bouhassira, 2012). Therefore, the AAVS1 locus offers a well-characterized site for transgene expression for use in human pluripotent stem cells.

Here we demonstrate several technical advancements for using the AAVS1 locus to generate both reporter and knockdown constructs in human ES cells. We generate a reporter for hematopoietic cells and a knockdown construct that inhibits myeloid cell formation. One construct is designed to express a green fluorescent protein (GFP) reporter driven by a human CD43 promoter fragment that accurately reflects endogenous CD43 gene expression in hematopoietic cells. The second construct is designed to express short hairpin RNAs driven by the constitutive chicken actin (CA) promoter. By using hairpins that target PU.1, the master regulator of myeloid development, we demonstrate an efficient and stable gene knockdown that leads to a dramatic inhibition of myeloid development. Finally, we show that a simple lipid transfection method can generate a large number of correctly targeted clones much more efficiently and easily than previously published reports using electroporation (Hockemeyer et al., 2009). These techniques provide quick and reliable tools for expressing transgenes and developing reporter and knockdown lines to study hematopoiesis using human pluripotent stem cells.

4.2 Results

4.2.1 Generation of a CD43 Hematopoietic Reporter Cell Line

To easily quantify hematopoietic development from human ES cells, we created a fluorescent reporter line. The pan-hematopoietic surface marker, CD43 (leukosialin/SPN), is expressed in all hematopoietic lineages beginning at the hematopoietic progenitor stage during in vitro human ES cell differentiation (Vodyanik et al., 2006). In the adult, CD43 is considered to be pan-hematopoietic; however, it is not expressed in mature circulating erythrocytes and a subset of B-cells (Remold-O'Donnell et al., 1987). CD43 was chosen over CD41 or CD45 as these markers are only expressed on subsets of hematopoietic progenitors derived from human ES cells (Vodyanik et al., 2006).

A construct containing the CD43 promoter region driving GFP was targeted to the AAVS1 locus by homologous recombination using the ZFN technology (Figure 4-1A). Rather than electroporation, which is known to be toxic to ES cells (Zwaka and Thomson, 2003), a lipid transfection reagent was used. This system is extremely robust with targeting efficiencies >90% and homozygous targeting in roughly 50% of the clones (Figure 4-1C). Approximately 25% of the clones had a single integration in one allele of the AAVS1 locus without additional random integrations as determined by Southern blot (Figure 4-1C and Figure 4-2). Furthermore, a PCR screen demonstrated that these clones are free of ZFN construct integration (Figure 4-3). These single integrant clones where chosen for further analysis.

Figure 4-1

A. Targetting Constructs

| AAVS1 Homology | Puro | CD43 promoter | GFP | PolyA | AAVS1 Homology |

| AAVS1-intron1 | AAVS1 |

| AAVS1 Homology | Puro | CA promoter | GFP | mir30 | mir30 | PolyA | AAVS1 Homology |

B. Targetting Schematic

Zinc Finger Nucleases

FOK1

FOK1

ES/iPS Cell

Selection of single clones

AAVS1 targetting construct

Homolgous Recombination into the AAVS1 locus
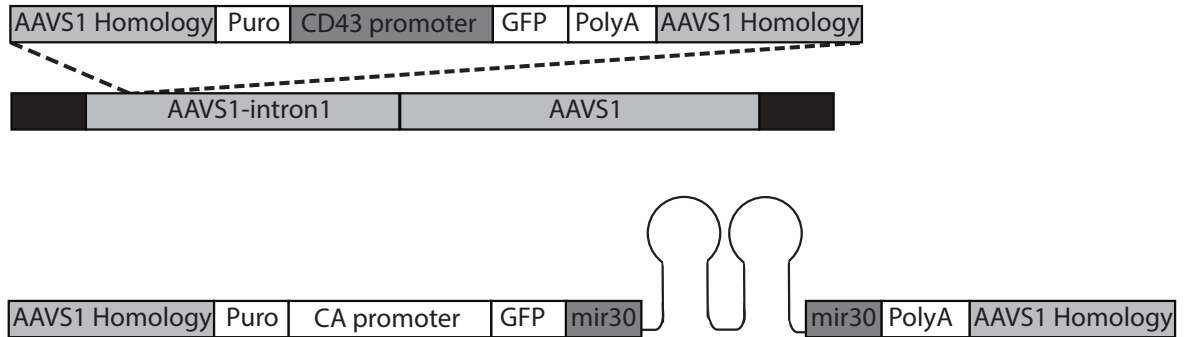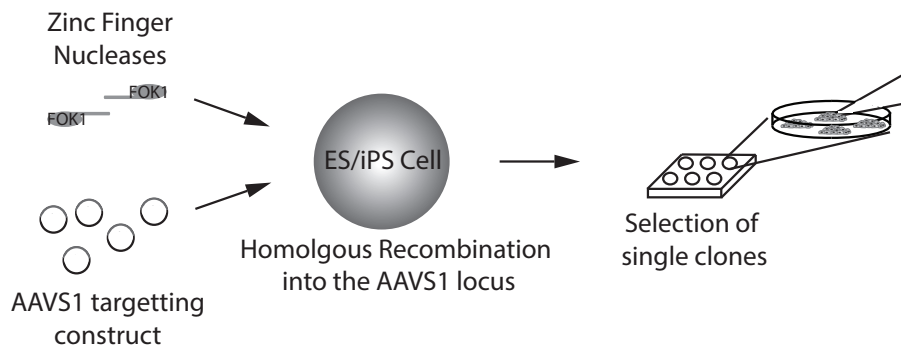
C. Targetting Efficiency

Line	Colonies per well of a 6-well dish	Colonies picked	Integration (PCR)	Het.	Homo.	Random Integrations	Single Int. w/o random integration
H9-CD43-GFP	x	6	6	3	3	3	2
H9-shPU.1 set1	103	9	8	2	6	3	2
H9-shPU.1 set2	202	4	4	2	2	1	1
Average of Total	x	19	18	7	11	7	5

Figure 4-1. *AAVS1 safe harbor gene targeting.* (A) Schematics of the constructs used in in gene targeting. Both constructs contain regions of homology to the AAVS1 locus in the first intron, a puromycin resistance gene (Puro) and a poly-adenylation sequence (PolyA). (i) The CD43-GFP reporter construct contains an approximately 2.1kb promoter from the human CD43 gene followed by GFP. (ii) The PU.1 knockdown construct contains the chicken actin promoter (CA promoter), GFP, and a pair of short hairpins against PU.1 in the context of the microRNA-30 (mir30) backbone. (B) Schematic of targeting the constructs into the AAVS1 locus. Cells were transfected with a high concentration of plasmids containing the construct and the FOK1 zinc finger nucleases. Transfection was done in 6-well plates with lipid transfection. Single clones were then picked and further characterized. (C) Table: Summary of the efficiency of the targeting for the CD43 reporter construct and the PU.1 knockdown constructs. (Het. Heterozygous; Homo. Homozygous; Single Int w/o random int. Single Integrant without random integration)

Figure 4-2
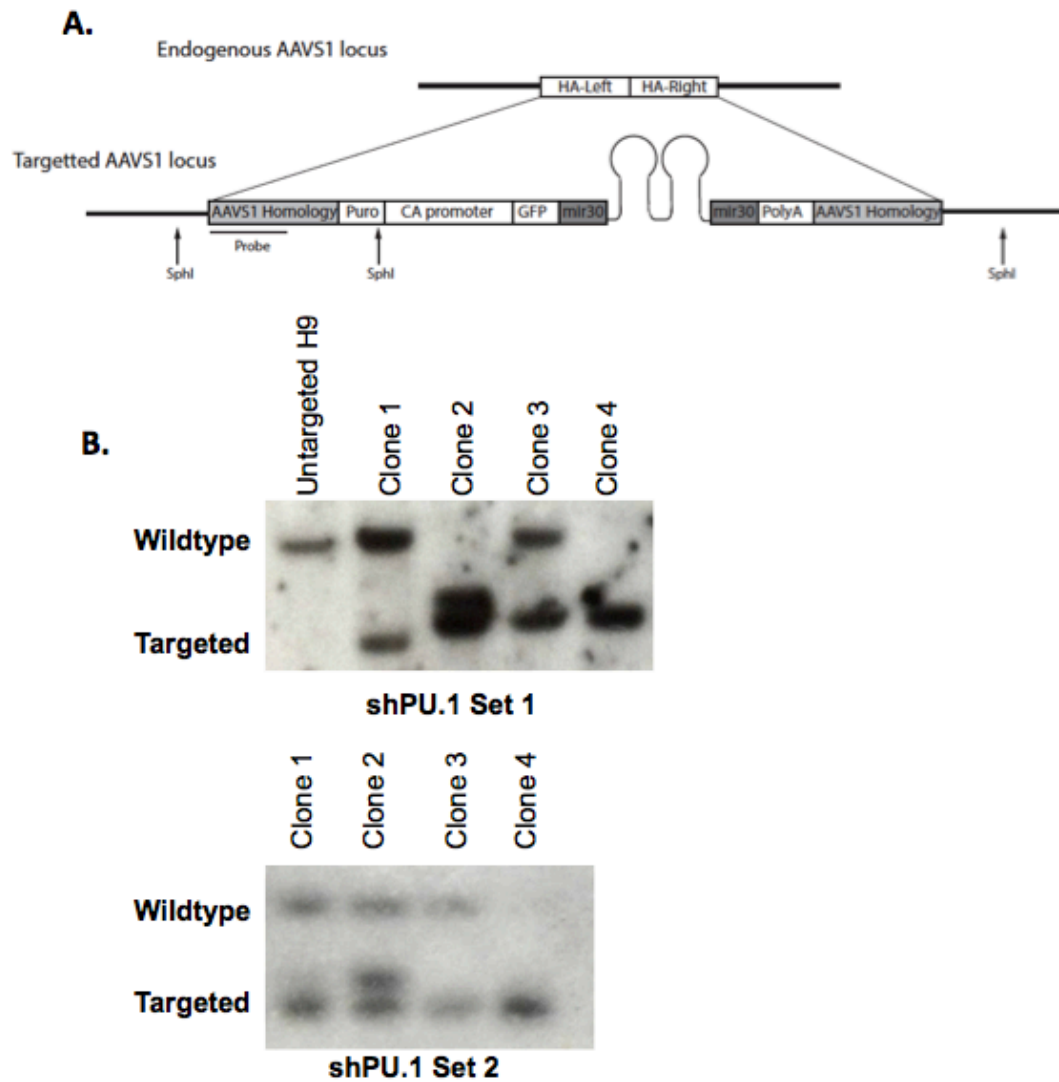
Figure 4-2. *Southern blot analysis of targeted clones.* (A) Schematic of AAVS1 locus with restriction enzymes sites and probe used in Southern blot analysis. (B) Southern blot analysis of SphI digested genomic DNA.

Figure 4-3
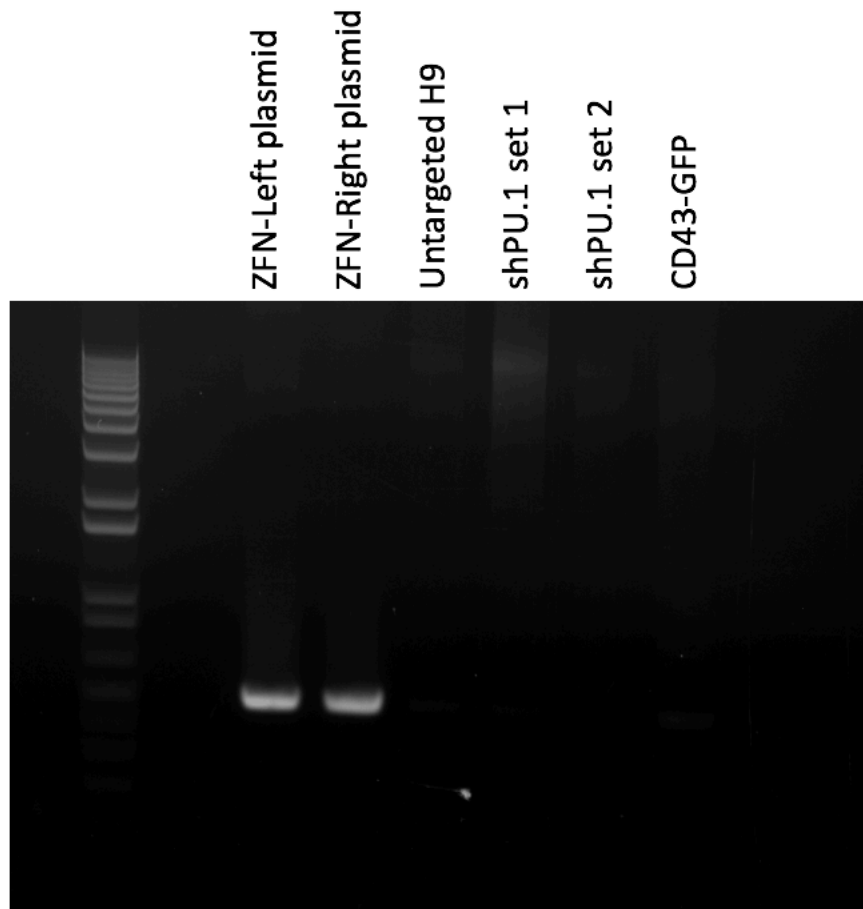
Figure 4-3. *The ZFN contruct is non integrating.* (A) PCR analysis of left and right ZFN constructs for integration into the genomic DNA of the AAVS1 targeted ES cells. (ZFN: zinc finger nuclease)

Figure 4-4
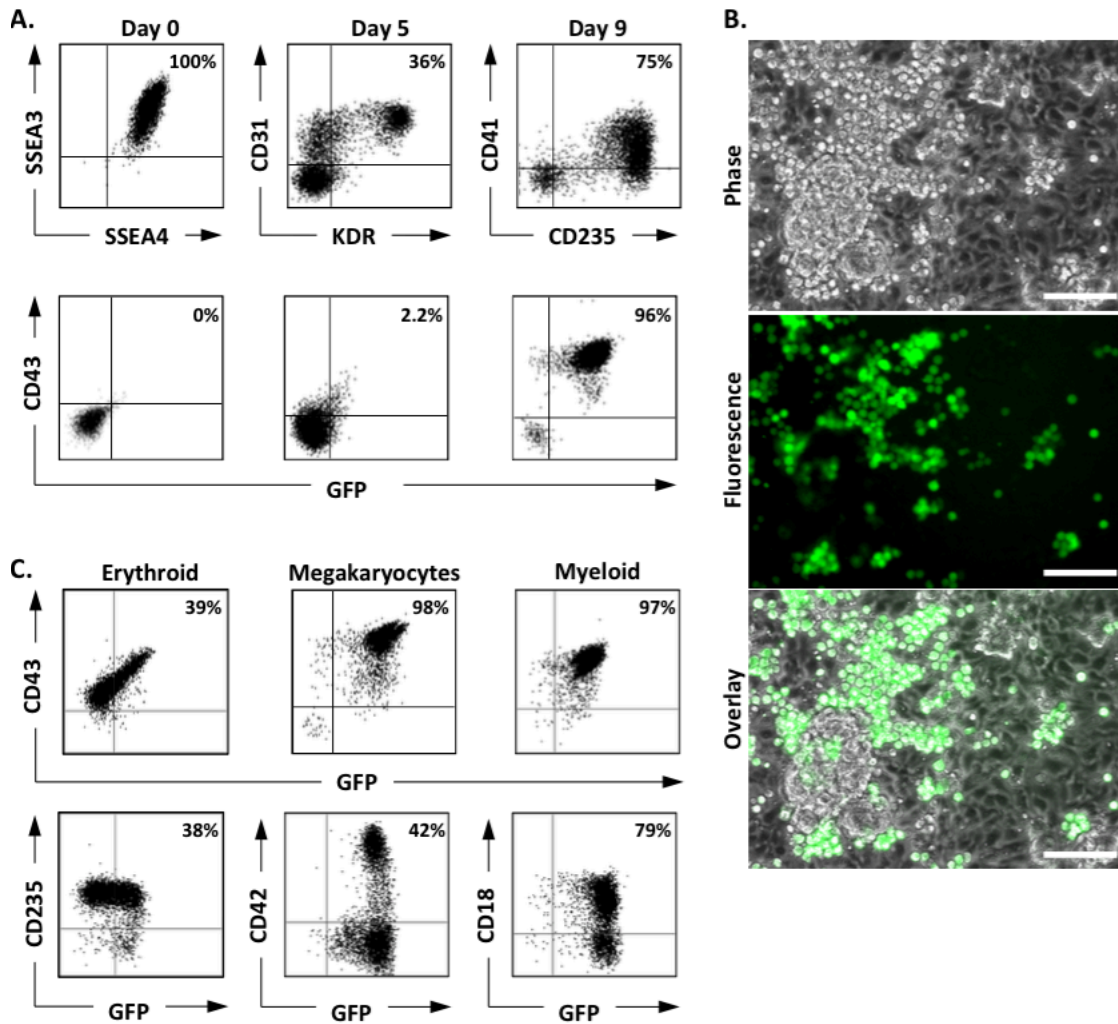
Figure 4-4. *Generation of a CD43-GFP reporter ES cell line*. (A) Flow cytometric analysis showing the differentiation of pluripotent CD43-GFP ES cells to hematopoietic progenitors. Cells were analyzed at days 0, 5, and 9 (non-adherent cells only) of differentiation respectively. (top) Flow cytometric analysis of SSEA3 versus SSEA4, CD31 versus KDR, and CD41 versus CD235. (bottom) Flow cytometric analysis of CD43 versus GFP expression. (B) CD43-GFP ES cells were differentiated as in A and examined by fluorescence microscopy. Bar is 100 um. (C) Day 9 non-adherent hematopoietic progenitors from the CD43-GFP ES cell line as described in A were cultured an additional 4 days in cytokine cocktails to promote erythroid, megakaryocytes, and myeloid development. (top) Flow cytometric analysis of CD43 versus GFP expression. (bottom) Flow cytometric analysis of CD235 versus GFP, CD42 versus GFP, and CD18 versus GFP

To test the specificity of the CD43 reporter construct, the CD43-GFP ES cells were differentiated in vitro into hematopoietic progenitors. Our in vitro differentiation protocol (Mills et al., 2013) generates mesoderm after 5 days as indicated by CD31 (PECAM-1) and KDR (VEGF-R2) co-expression (O'Donnell et al., 1987), and primitive hematopoietic progenitors appear in the supernatant by day 9 as demonstrated by CD235 and CD41 co-expression (Klimchenko et al., 2009; Vodyanik et al., 2006) (Figure 4-4A). Throughout the differentiation, a positive correlation is observed between endogenous CD43 expression, as determined by staining with an anti-CD43 antibody, and GFP expression from the CD43-GFP reporter construct (Figure 4-4A and 4-4C). In our monolayer differentiation system, the CD43-GFP expressing hematopoietic progenitors are easily visible as they bud off of the monolayer (Figure 4-4B).

To demonstrate the stability of transgene expression, the hematopoietic progenitor cells were expanded in liquid culture with the appropriate cytokine cocktails to induce maturation to erythroid (CD235+), megakaryocyte (CD42+), and myeloid (CD18+) lineages (Figure 4-4C). In all of the induced hematopoietic lineages, endogenous CD43 expression correlated with GFP expression levels. We observed that the CD235+ erythroid cells eventually expressed lower levels of CD43 and concurrently lost GFP expression. To evaluate the specificity in expression of the CD43 promoter, CD43-GFP ES cells were differentiated into neuroectodermal and definitive endodermal lineages (Figure 4-5A). Neither of these differentiated cells expressed GFP (Figure 4-5B). Together these data demonstrate that the CD43-GFP transgene is effective as a hematopoietic reporter with GFP expression corresponding to the endogenous expression of CD43.

Figure 4-5
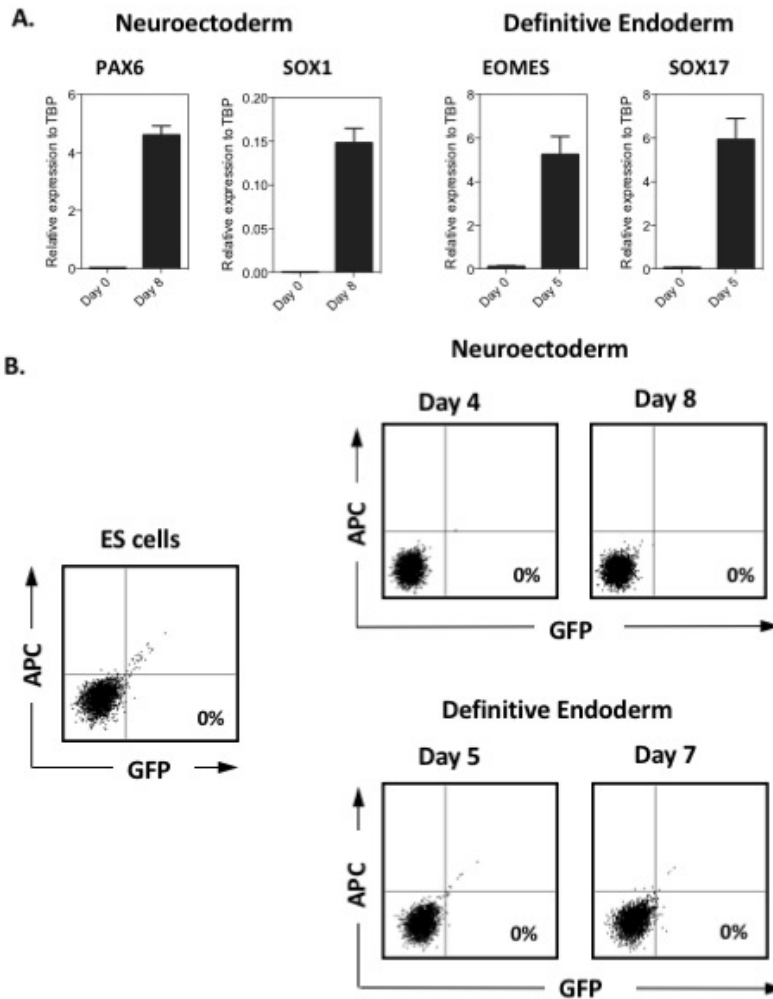
Figure 4-5. *CD43-GFP reporter line does not show mis-expression of GFP*. (A) Gene expression analysis of neuroectoderm and definitive endoderm CD43-GFP differentiated cells as compared to ES cells. (B) Flow cytometry analysis CD43-GFP ES cells for GFP expression when differentiated to neuroectoderm and definitive endoderm lineages.

4.2.2 Knockdown of the Myeloid Specific Transcription Factor PU.1

We next determined whether the AAVS1 targeting system could be used to stably knockdown hematopoietic genes of interest. As a proof of principle, the transcription factor PU.1 was chosen as it is expressed at high levels in monocytes, granulocytes and B lymphoid cells and plays a critical role in the regulation of the myeloid cell fate (Scott et al., 1997). We hypothesized that knockdown of the PU.1 gene would prevent the differentiation of ES cells into myeloid cells. To assure high levels of gene knockdown, we utilized a microRNA based system where two hairpins against a single target are present in the miR-30 backbone (Stegmeier et al., 2005; Sun et al., 2006; Wang et al., 2007). We generated two constructs each containing two short hairpin RNAs (shRNA set1 and set2) against PU.1. The constitutively expressed CA promoter was used to drive GFP followed by the shRNAs (Figure 4-1A.ii). ES cell lines expressing these two constructs (shPU.1 set1 and shPU.1 set2) were generated with efficiencies similar to that achieved with the CD43-GFP construct (Figure 4-1C). These data demonstrate the reliability and reproducibility of generating a variety of transgene constructs using this system.

To test the efficiency of gene knockdown, the ES cell lines expressing the shPU.1 constructs were differentiated into hematopoietic cells. The transcription factor PU.1 is not expressed in ES cells at day 0 of differentiation and becomes expressed by day 9 when hematopoietic progenitor cells are generated (Figure 4-6A). At this time point, the expression of PU.1 in the two knockdown cell lines was >80% lower than expression in the control GFP-expressing ES cell line. The day 9 hematopoietic progenitor cells were then put in liquid culture for an additional four days in a cytokine cocktail favoring myeloid cell generation. At day 13 of differentiation, the expression of PU.1 in the two

knockdown lines was >90% lower than expression in the control cell line (Figure 4-6A).

These data demonstrate that constitutively expressed AAVS1-targeted knockdown

constructs against PU.1 can successfully decrease expression levels throughout

differentiation.

To determine the effect of decreased PU.1 levels on hematopoietic cell development,

day 9 progenitor cells were put into liquid expansion cultures and analyzed for erythroid

(CD235+CD41-) and myeloid (CD18+CD45+) lineages.   Both knockdown cell lines

expressed almost undetectable levels of CD18 and CD45 compared to ~50% co-

expression of these markers on control GFP-expressing cells, indicative of a block in

myelopoiesis (Figure 3B). Furthermore, the absolute yield of myeloid cells in the two

knockdown lines was much lower compared to the GFP control cell line (Figure 4-6C).

These findings were confirmed by gene expression analysis of the myeloid marker

cFMS, which was also drastically reduced in the PU.1 knockdown cell lines (Figure 4-

6D).   Analysis of the erythroid lineage demonstrated an ~4-fold expansion in the

percentage of CD235+ cells (Figure 4-6B) with a concomitant increase in absolute yield

of cells, especially in the knockdown line set 2 (Figure 4-6C). These findings were

confirmed by an increase in gene expression of the erythrocyte transcription factor EKLF

in the two knockdown cell lines (Figure 4-6D). The pan-hematopoietic marker GATA1

was unaffected and there was a minor increase in the megakaryocyte marker PF4.

These data demonstrate that AAVS1-targeted knockdown of PU.1 specifically decreases

the myeloid lineage and skews the progenitor cells to an erythroid cell fate.

Figure 4-6
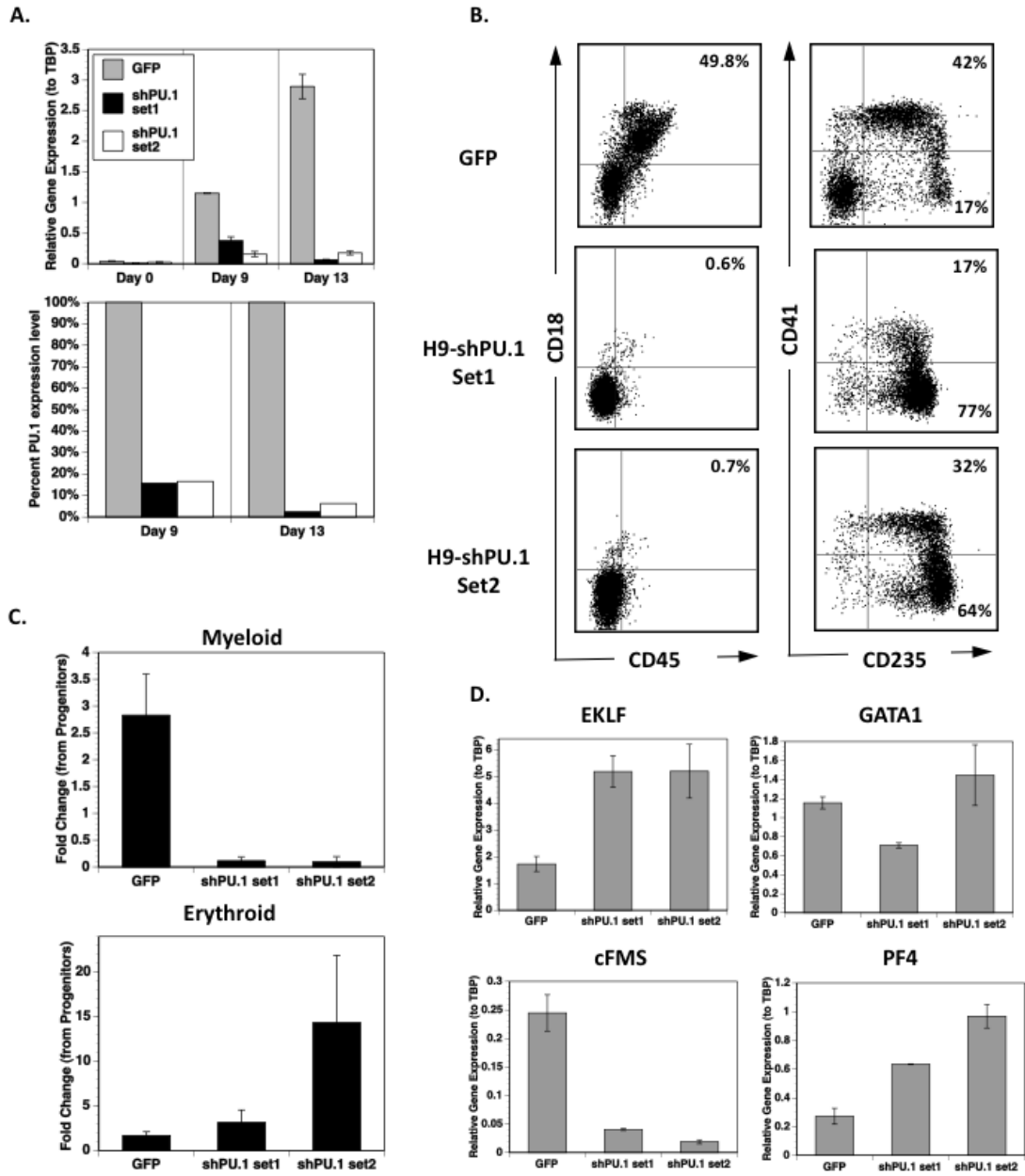
Figure 4-6. *Expression of PU.1 short hairpin RNAs inhibits myeloid development.* The PU.1 shRNA expressing ES cell lines (H9-shPU.1 set 1 and H9-shPU.1 set 2) along with a GFP expressing control line were differentiated into hematopoietic cells and at day 9, non-adherent progenitors were harvested and cultured an additional 4 days in a cytokine cocktail to promote myeloid development. (A) (top) Quantitative RT-PCR of PU.1. expression is reported relative to the housekeeping gene TBP. (bottom) Percentage of PU.1 expression relative to the control GFP construct in hematopoietic cells at day 9 and day 13 of differentiation. (B) Flow cytometric analysis (CD18 versus GFP and CD41 versus CD235) of H9-GFP and PU.1 knockdown cell lines at day 13 of differentiation. (C) Absolute cell counts of myeloid (CD18+CD45+) and erythroid (CD41-CD235+) cells in myeloid lineage conditions (SCF 50ng, GMCSF 200ng and IL3 10ng). Cell counts are expressed as fold change from the number of hematopoietic progenitors plated at day 9. (D) Quantitative RT-PCR of hematopoietic lineages genes. Gene expression levels are all reported relative to the housekeeping gene TBP.

4.3 Discussion

The CD43 promoter fragment we describe here allows tissue specific, controlled expression of a transgene specifically in cells of the hematopoietic lineages. During the differentiation of the CD43-GFP hematopoietic progenitors into the erythroid lineage it was observed that these cells began to lose the expression of GFP over time in culture. Since endogenous CD43 also decreases, this observation is most likely due to increased sensitivity of the CD43 antibody over GFP and not due to silencing of transgene expression. It has been shown that mature erythrocytes lose expression of CD43, hence this loss of expression is anticipated (Remold-O'Donnell et al., 1987). Importantly, myeloid cells, which maintain CD43 expression in the adult, do not lose GFP expression in the reporter lines with culture (Figure 4-6C). The CD43 reporter represents a valuable tool to easily follow hematopoietic specification from ESCs in live cultures with specificity that rivals CD41 or CD45 as all hematopoietic progenitors are CD43+ (Vodyanik et al., 2006).

In mouse models, a homozygous loss of PU.1 is embryonic lethal (Scott et al., 1994) or if mice are born alive, they die shortly after birth (McKercher et al., 1996). Mice that are born are found to lack any mature B and T cells, macrophages and neutrophils but have erythrocytes and megakaryocytes (McKercher et al., 1996) indicating that PU.1 is required for myeloid cell lineage development. A heterozygous loss of PU.1 is non-lethal, however it leads to a loss in the formation of multipotent myeloid progenitors (Scott et al., 1994). We demonstrate that a >80% knockdown of PU.1 in human hematopoietic cells results in a loss of myeloid lineage cells, phenocopying what occurs with a loss of PU.1 in the mouse. The differentiation of ES cells into primitive hematopoietic cells can

be considered to mimic human development thus our data suggests that during early human hematopoiesis PU.1 is also required for myeloid lineage development.

The advent of new technologies such as TALENs and CRISPRs offer extremely powerful methods for enhanced gene editing (Gaj et al., 2013). Indeed, these technologies have been shown to target the AAVS1 locus with similar efficiencies as ZFNs (Hockemeyer et al., 2011; Mali et al., 2013). However, targeted gene editing and transgene expression to a novel site will still require targeting vector construction, the development of screening protocols and the labor intensive screening of potentially many ES cell clones which will vary depending on the targeting construct and gene of interest. The advantage of the system we describe here is that the same targeting vector and screening methodology can be used for any construct, allowing the quick and easy generation of transgenic pluripotent stem cell lines with high efficiency. In addition, we have adapted the use of a simple lipid transfection reagent to introduce our targeting constructs which allows the generation of sufficient targeted clones from a single well of a six well dish.

We have shown that it is possible to have site-specific integration and stable expression of a transgene throughout hematopoietic differentiation. This system represents a powerful and flexible tool to study hematopoiesis, and for the case of gene knockdown to study the requirement of a particular gene in the context of any cell type where there is a differentiation protocol available. Both the constructs described here have the potential for use in future studies involving transgene expression in differentiated progeny from pluripotent stem cells. Together, these techniques allow the easy manipulation of gene

expression and will strengthen the use of ES cells as a model system for the study of early human development and disease.

CHAPTER 5

GATA6 Mutations Reveal a Critical Role in Human Definitive Endoderm

Development and Pancreatic β Cell Function

5.1 Introduction

Pancreatic agenesis is a rare congenital disease caused by a mutation in PDX1 (Schwitzgebel et al., 2003; Stoffers et al., 1997), PTF1A (Sellick et al., 2004; Weedon et al., 2014), GATA4 (Amato et al., 2010; Shaw-Smith et al., 2014) or most commonly GATA6 (Bonnefond et al., 2012; Chao et al., 2015; De Franco et al., 2013; Lango Allen et al., 2012; Nakao et al., 2013; Stanescu et al., 2014; Suzuki et al., 2014; Yorifuji et al., 2012). The majority of GATA6 mutations leading to pancreatic agenesis are de novo heterozygous mutations. Some GATA6 mutations have incomplete penetrance as determined by patients having identical mutations to pancreatic agenesis patients, but displaying either adult onset diabetes or an absence of pancreatic abnormalities (Bonnefond et al., 2012; De Franco et al., 2013; Yu et al., 2014). The majority of pancreatic agenesis patients also display a combination of other defects including congenital heart defects, gut abnormalities and intrauterine growth retardation (Chao et al., 2015).

GATA6 belongs to a family of transcription factors consisting of six members that bind to the consensus sequence (A/T)GATA(A/G). GATA1, GATA2 and GATA3 are mainly expressed in hematopoietic cell lineages, while GATA4, GATA5 and GATA6 are predominantly expressed in the heart, gonads and endodermal derived tissues (Viger et al., 2008). GATA6 is known to regulate endodermal gene expression and development of endoderm derived organs (Molkentin, 2000). In mice, GATA6 is expressed in the

primitive streak, heart, lung, intestine, gonads, adrenal and pancreatic tissues (Koutsourakis et al., 1999; Liu et al., 2002; Sartori et al., 2014). Within the adult pancreatic tissue, GATA6 is expressed in both the exocrine tissue and the islets of Langerhans (Sartori et al., 2014).

In contrast to the severe disease phenotype found in humans having GATA6 heterozygous mutations, mice with GATA6 heterozygous mutations are fertile and phenotypically normal. Homozygous GATA6 null mice are embryonic lethal between E6.5 and E7.5 (Morrisey et al., 1998). Using tetraploid complementation, GATA6 has been shown to be essential for extra embryonic endoderm development explaining the embryonic lethality (Koutsourakis et al., 1999; Zhao et al., 2005); however, GATA6 null cells can contribute to the definitive endoderm that forms the primitive gut. Analysis of GATA6 in pancreas progenitors or adult β cells has demonstrated minimal impact on endocrine function, with normal numbers of β cells and no overt signs of diabetes even though a mild impact on ER stress was observed in one study (Carrasco et al., 2012; Decker et al., 2006; Martinelli et al., 2013; Sartori et al., 2014; Xuan et al., 2012).

Due to the major differences in phenotype between human and murine GATA6 disease models, human pluripotent stem cells (PSCs) offer an alternative system for the in vitro study of GATA6. With recent developments in the genome editing field, the use of the clustered regularly interspaced short palindromic repeats (CRiPSR)/CAS9 technology (Hsu et al., 2014; Ran et al., 2013) has enabled PSCs to become an even more powerful model system as mutant and control isogenic lines can be made to avoid confounding results due to differing genetic backgrounds.

Here, we study GATA6 mutant human PSCs. Induced pluripotent stem (iPS) cells were generated from a previously described pancreatic agenesis patient having a novel heterozygous GATA6 mutation resulting in a truncated protein (Stanescu et al., 2014). Using genome editing, PSC lines with mutations in both alleles of GATA6 were generated. These compound heterozygous GATA6 mutants failed to differentiate into definitive endoderm due to a block at the primitive streak stage of development. Re-expression of GATA6 or several other GATA family members restored this defect. Using endodermal progenitor (EP) cells as a tool to bypass the endoderm defect, pancreatic β cell differentiation was studied. We found that all mutant lines maintained the ability to differentiate into pancreatic β-like cells but that these cells were functionally defective in glucose responsiveness. These data suggest that human GATA6 plays a critical role in endoderm development and functionality of pancreatic β-like cells.

5.2 Results

5.2.1 Establishment of GATA6 PSC lines

To study the role of GATA6 in human development, mutant and control PSC lines were generated by established technologies for standard reprogramming and CRISPR/Cas genome editing. An iPS cell line was generated from cells of a previously described patient expressing a heterozygous GATA6 mutation (Stanescu et al., 2014). The four base pair duplication in the second exon of GATA6 caused a frame shift mutation resulting in a truncated protein (Figure 5-1A and 5-1B). This patient derived iPS cell line will be designated iPS$^{+/indel}$ (Table 5-1). To generate cell lines expressing mutations in both alleles of GATA6, CRISPR/Cas9 genome editing was performed using the patient iPS$^{+/indel}$ cell line and the Mel1-INS-GFP (Micallef et al., 2012) ES cell line (Figure 5-1C). The iPS$^{+/indel}$ cell line was used to maintain genetic background identity to the patient cells for experimental comparison. The Mel1-INS-GFP ES cell line (designated ES$^{+/+}$, Table 5-1) was used for two reasons in addition to confirming phenotypes in a second genetic background. First, the Mel1-INS-GFP line allowed easy purification of β-like cells as it contains a GFP reporter in the insulin locus. Second, to assist with inducible gene expression studies we generated a Mel1-INS-GFP sub-line that constitutively expressed the reverse tetracycline transactivator (rTTA) targeted to the AAVS1 safe harbor locus (Figure 5-2) using a previously described methodology (Hockemeyer et al., 2009; Tiyaboonchai et al., 2014). For CRISPR/Cas genome editing, the guide RNA strands (gRNAs) were designed to target the second exon of GATA6 near the patient mutation site (Figure 5-1B) creating frame shift insertion and/or deletion (Indel) mutations in both alleles of GATA6 (Table 5-1 and Figure 5-3A). The genome edited patient iPS cell line will be designated iPS$^{indel/indel}$ and the genome edited Mel-INS-GFP line will be

Figure 5-1
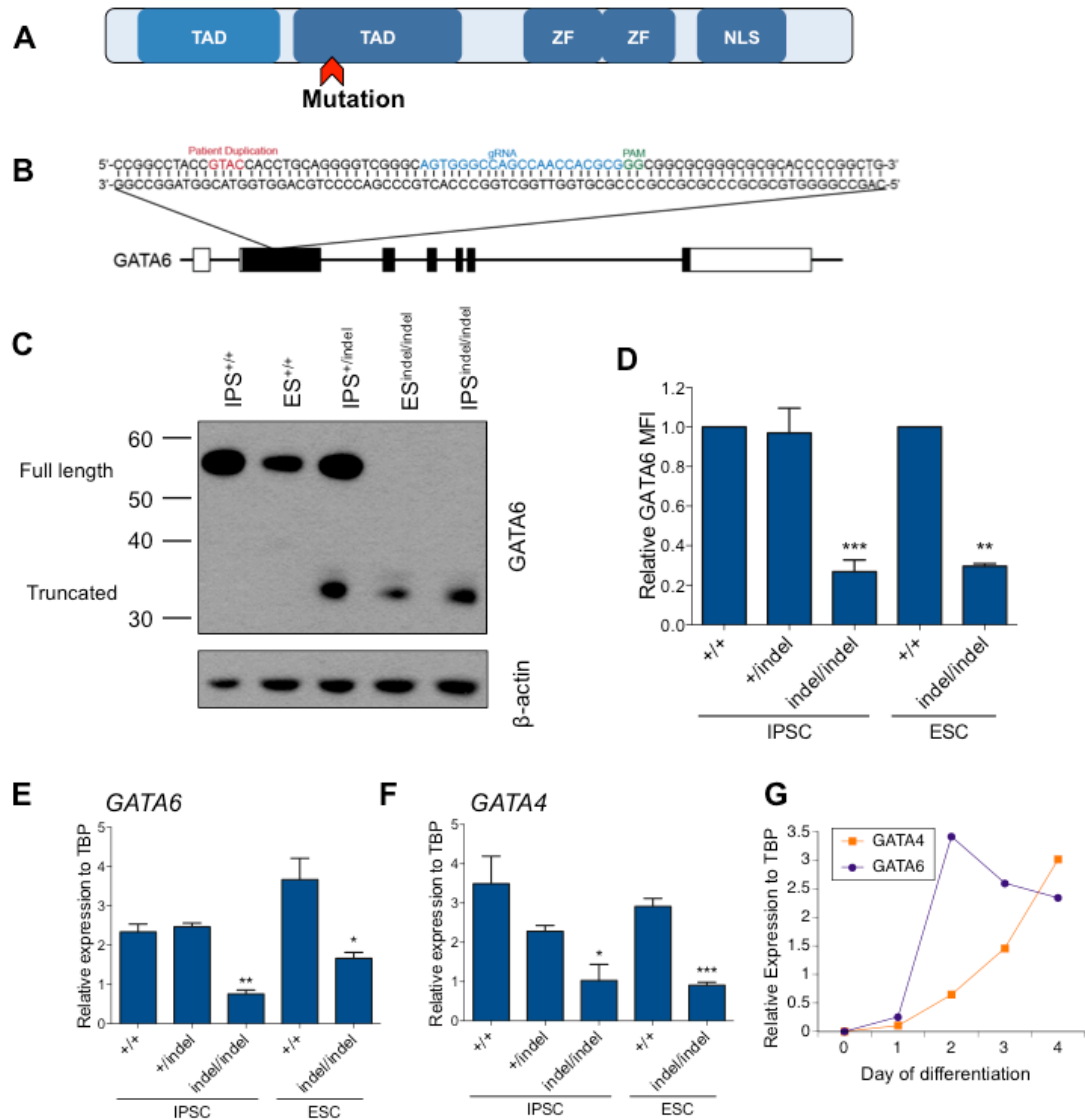
Figure 5-1. *Generation of pluripotent stem cell lines with GATA6 mutations.*
(A) Schematic of GATA6 protein with site of patient mutation. (B) Schematic of
GATA6 gene with location of GATA6 patient mutation and site of guide RNA with
the PAM sequence indicated. (C) Western blot of GATA6 protein on pluripotent
stem cell lines differentiated towards definitive endoderm for 4 days. (D)
Intracellular flow cytometric analysis of mean fluorescence intensity samples
examined in C. Expression levels were normalized to 1 in wild type (+/+) cells of
each genetic background. (E) QRT-PCR analysis of expression of GATA6 and
GATA4 in samples examined in C. (F) Time-course analysis of GATA4 and
GATA6 expression during definitive endoderm differentiation of GATA6^{+/+} cells by
qRT-PCR. For all statistical analysis: * P<0.05, **P<0.01, ***P<0.001

Table 5-1

Name	Genetic Background	Allele 1	Allele2
ES$^{+/+}$	Mel1-INS-GFP	Wild type	Wild type
iPS$^{+/+}$	CHOP.Panagenesis1	Wild type	Wild type
iPS$^{+/indel}$	CHOP.Panagenesis1	Wild type	4bp duplication
ES$^{indel/indel}$	Mel1-INS-GFP	1bp deletion	20bp insertion
iPS$^{indel/indel}$	CHOP.Panagenesis1	4bp duplication +39bp deletion	2bp deletion

Table 5-1. *Pluripotent Stem Cell Lines*. The name, genetic background and mutations in the two alleles of GATA6 for each of the pluripotent stem cell lines used in this chapter are described here.

Figure 5-2

A
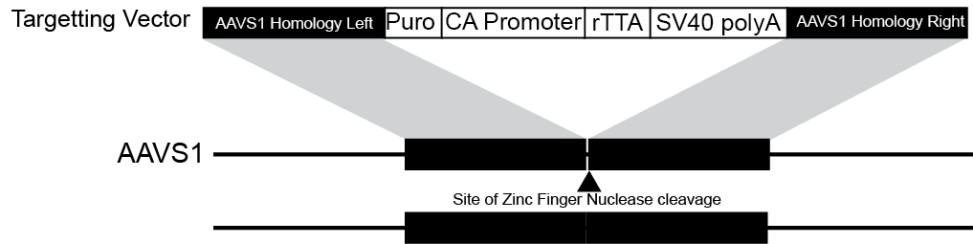
B
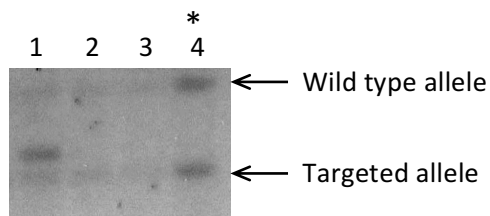
Figure 5-2. *Targeting the AAVS1 locus in Mel1-INS-GFP ES$^{+/+}$ cells.*
(A) Targeting of the AAVS1 locus with a construct containing a constitutive chicken actin (CA) promoter driving the expression of the reverse tetracycline transactivator (rTTA) by use of a pair of zinc finger nucleases. (B) Southern blot of targeted clones to confirm that a single allele has been integrated. The clone that is used throughout these experiments is indicated with an asterisk.

Figure 5-3

A

IPS+/+ (Corrected Patient Derived iPS)

```
CCGTACCACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACCACGCGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGGCCGAC   Reference
CCGTACCACCTCCAAGGGTCGGGCAGTGGGCCTGCGAATCATGCCGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGGCCGAC   Wild type
CCGTACCACCTCCAAGGGTCGGGCAGTGGGCCTGCGAATCATGCCGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGGCCGAC   Wild type
```

IPS+/indel (Heterozygous Patient Derived iPS)

```
CCTACCGTAC----CACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACCACGCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGG   Reference
CCTACCGTACGTACCACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACCACGCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGG   +4bp
CCTACCGTAC----CACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACCACGCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGG   Wild type
```

IPSindel/indel (Compound Heterozygous Patient Derived iPS)

```
CCGTAC----CACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACCACGCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCG   Reference
CCGTACGTACCACCTGCAGGGGTCGGGC-----------------------------------------CCCGGCTGGCCTCAGGCCTCG   +4bp and -39bp
CCGTAC----CACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACC--GCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCG   -2bp
```

ESindel/indel (Compound Heterozygous Mel1-INS-GFP)

```
CCGTACCACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACCACGCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGGCCG   Reference
CCGTACCACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACC-CGCGGGCGGCGCGGGCGCGCACCCCGGCTGGCCTCAGGCCTCGGCCG   -1bp
CCGTACCACCTGCAGGGGTCGGGCAGTGGGCCAGCCAACC--------------------CACCCCGGCTGGCCTCAGGCCTCGGCCG   -20bp
```

Figure 5-3 continued

B

IPS$^{+/+}$ (Corrected Patient Derived IPS): 46 XX

IPS$^{indel/indel}$ Compound Heterozygous Patient Derived IPS): 46 XX

ES$^{indel/indel}$ Compound Heterozygous Mel1-INS-GFP): 46 XY

Figure 5-3: Characterization of patient IPS$^{+/indel}$ CHOP.Panagenesis1 cells
(A) Generation of all three germ layers (ectoderm, mesoderm and endoderm) by teratoma assay to confirm pluripotency in the patient IPS$^{+/indel}$. (B) Patient IPS$^{+/indel}$ cells were subject to qRT-PCR analysis of common pluripotency markers: NANOG, OCT4, REX1, ABCG2, SOX2 AND DMNT3B. (C) Karyotype of patient IPS$^{+/indel}$ cells.

designated ES$^{indel/indel}$ (Table 5-1). To generate an isogenic control for the patient iPS$^{+/indel}$ line, genome editing was also used to correct the mutation and this line will be designated iPS$^{+/+}$ (Table 5-1). The iPS$^{+/indel}$ line was confirmed for pluripotency (Figure 5-4) and all genome edited PSC lines were confirmed to have a normal karyotype (Figure 5-3B).

Because GATA6 does not begin to be expressed until primitive streak induction and not in PSCs, protein and transcript levels were examined in control and mutant cells utilizing a protocol that induces definitive endoderm (Cheng et al., 2012; D'Amour et al., 2005; Kubo et al., 2004). By Western blot analysis, full length GATA6 protein was detected in the control lines, ES$^{+/+}$ and iPS$^{+/+}$, and in the patient line, iPS$^{+/indel}$, that expressed one normal allele of GATA6. The patient line, iPS$^{+/indel}$, also expressed one mutant allele of GATA6 that generated a truncated GATA6 protein of ~35kDa that was the only form of GATA6 detected in the compound heterozygous mutant lines, iPS$^{indel/indel}$ and ES$^{indel/indel}$ (Figure 5-1C). Using flow cytometry, GATA6 protein was also quantified by mean fluorescence intensity (MFI) normalized to wild type of each genetic background. We found that the truncated GATA6 protein in the mutant iPS$^{indel/indel}$ line was expressed at significantly lower levels (0.27 ± 0.06 fold) than the patient iPS$^{+/indel}$ (0.97 ± 0.13 fold) and control iPS$^{+/+}$ lines. The same results were observed in the mutant ES$^{indel/indel}$ line, with significantly decreased levels of GATA6 (0.30 ± 0.01 fold) compared to the control ES$^{+/+}$ line (Figure 5-1D).

Figure 5-4

A

Ectoderm	Mesoderm	Endoderm

B

C   IPS$^{+/indel}$ (Patient Derived IPS): 46 XX

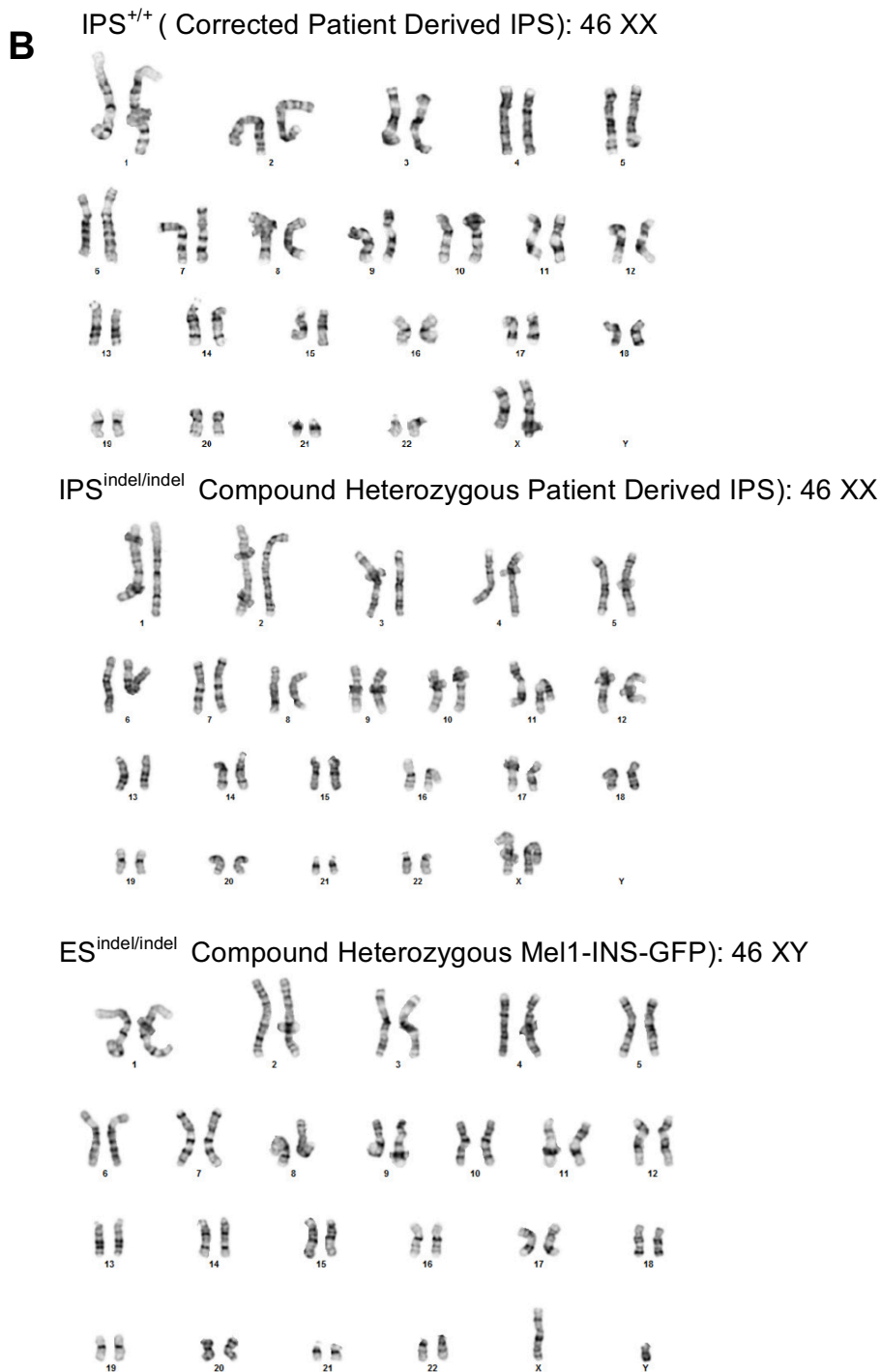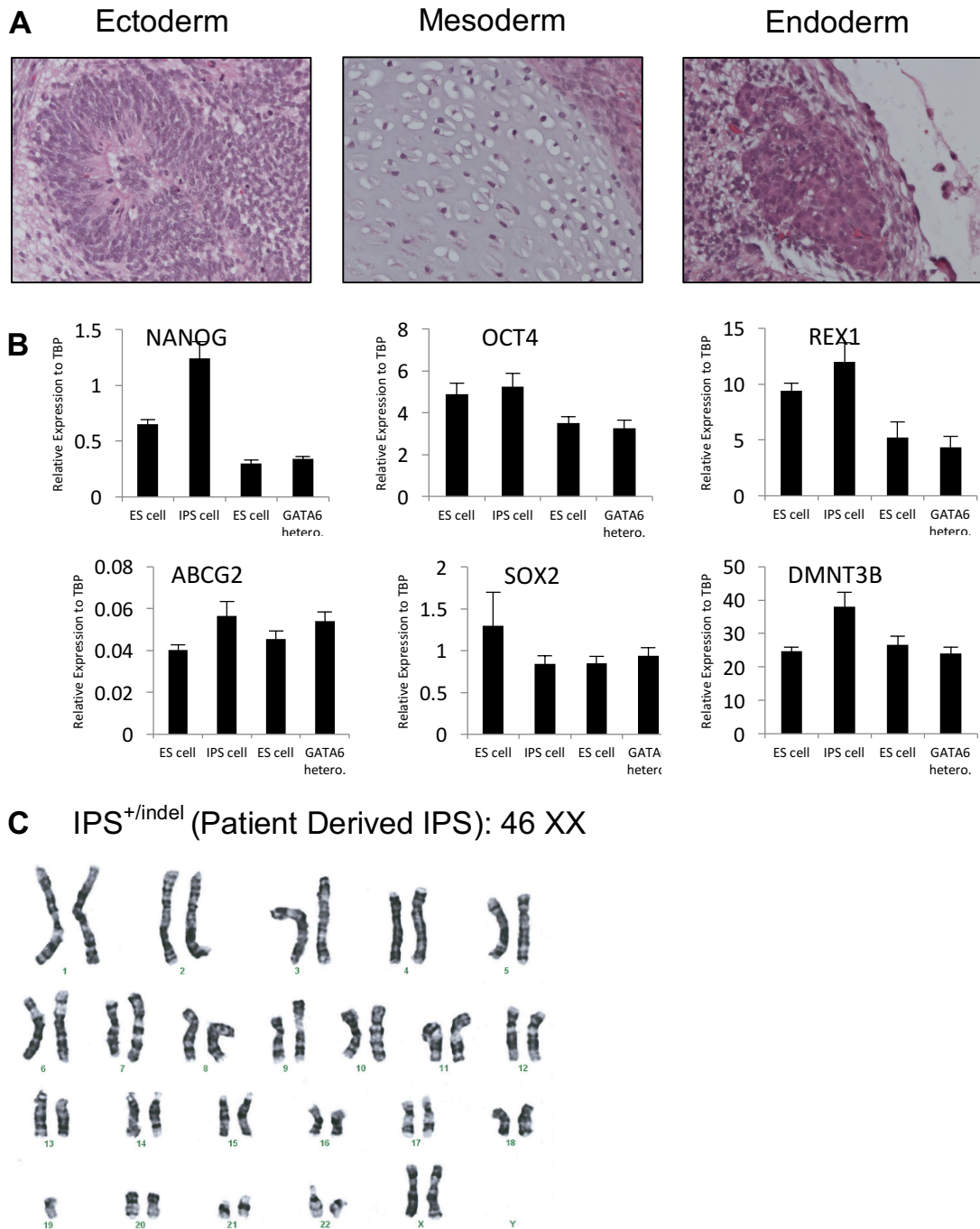Figure 5-4. *Characterization of patient iPS$^{+/indel}$ CHOP.Panagenesis1 cells.*
(A) Generation of all three germ layers (ectoderm, mesoderm and endoderm) by teratoma assay to confirm pluripotency in the patient iPS$^{+/indel}$. (B) Patient iPS$^{+/indel}$ cells were subject to qRT-PCR analysis of common pluripotency markers: NANOG, OCT4, REX1, ABCG2, SOX2 AND DMNT3B. (C) Karyotype of patient iPS$^{+/indel}$ cells.

In addition to GATA6, GATA4 is another member of the GATA family of transcription factors that is up-regulated during primitive streak and definitive endoderm induction (Arceci et al., 1993; Czysz et al., 2015). Both *GATA4* and *GATA6* transcript levels were measured by quantitative PCR in cells differentiated to definitive endoderm. *GATA6* transcript levels were significantly decreased in the mutant iPS$^{indel/indel}$ cell line as compared to the control iPS$^{+/+}$ cell line (Figures 5-1E and 5-1F) suggesting that GATA6 may be part of a self-regulatory feedback loop. *GATA4* was also significantly decreased in the patient iPS$^{indel/indel}$ line (Figure 5-1F). A time course analysis of both transcripts during endoderm differentiation in iPS$^{+/+}$ cells showed that GATA6 was expressed prior to GATA4 (Figure 5-1G), suggesting cross-talk between GATA4 and GATA6. These experiments were repeated in the control ES$^{+/+}$ and ES$^{indel/indel}$ cells and found to be consistent (Figure 5-1E and 5-1F). Together, these data suggest that GATA6 may be the initial GATA family member to be expressed maintaining both itself and GATA4 during endoderm induction.

5.2.2 Definitive Endoderm Differentiation

To analyze the impact of GATA6 on differentiation to definitive endoderm, a time course analysis was performed using an established protocol (D'Amour et al., 2005; Kubo et al., 2004). The expression of developmentally regulated markers at different stages of differentiation was analyzed using the corrected patient iPS$^{+/+}$, patient iPS$^{+/indel}$, and mutant iPS$^{inde/indel}$ lines (Figures 5-5A). These experiments were also repeated in the control ES$^{+/+}$ and mutant ES$^{indel/indel}$ lines (Figure 5-6A). In all lines, the pluripotency markers *NANOG*, *SOX2* and *OCT4* were down regulated by day 2 of differentiation and the primitive streak markers brachyury (*T*), goosecoid (*GSC)* and eomesodermin (*EOMES*) were expressed at the appropriate times during induction. The definitive

97
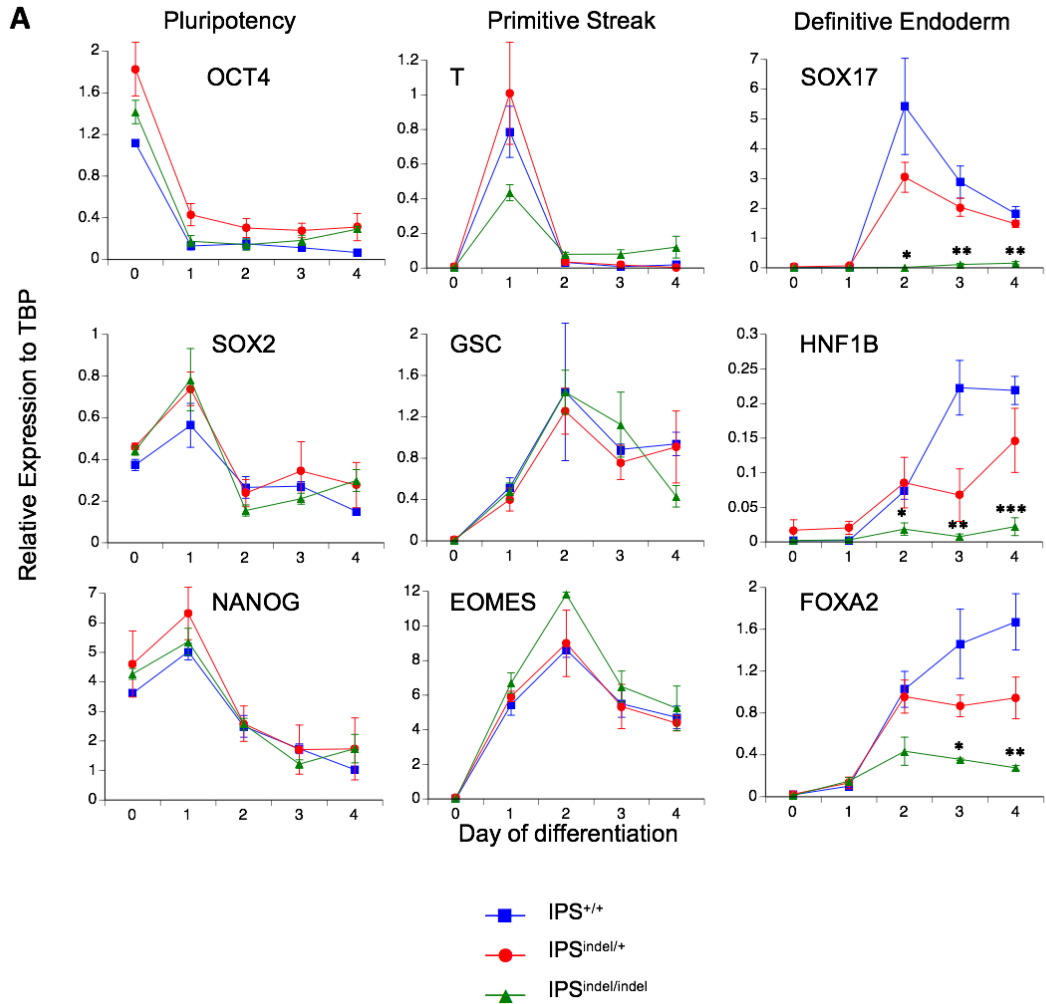
Figure 5-5

Figure 5-5 continued
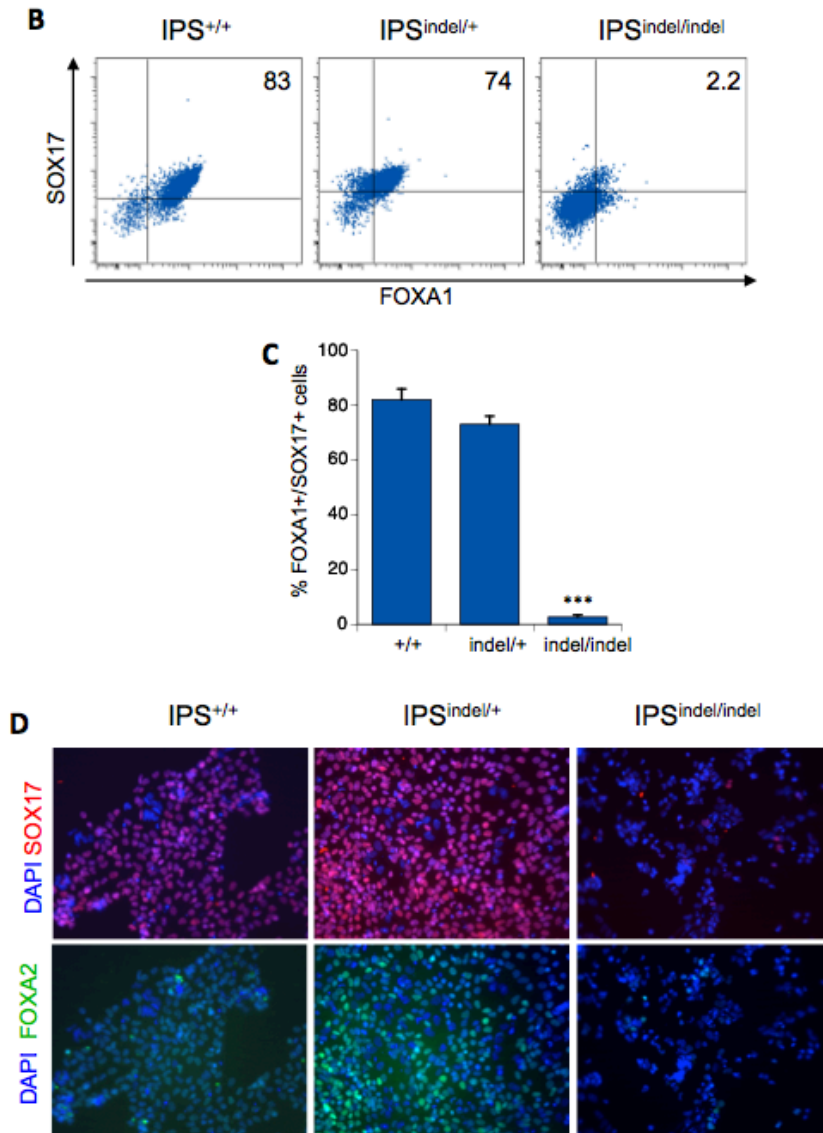
Figure 5-5. *GATA6 is required for definitive endoderm differentiation of pluripotent stem cells*. Control iPS$^{+/+}$, patient iPS$^{+/indel}$ and mutant iPS$^{indel/indel}$ cells described in Figure 5-1C were differentiated into definitive endoderm. (A) Time-course analysis of pluripotency markers (OCT4, SOX2, and NANOG), primitive streak markers (T, GSC, EOMES) and definitive endoderm markers (SOX17, HNF1B, and FOXA2) by qRT-PCR. (B) Analysis of the definitive endoderm markers FOXA1 vs. SOX17 at day 4 of differentiation by intracellular flow cytometry.(C) Quantitation of the percentage of FOXA1+SOX17+ cells in B. (D) Immunofluorescence analysis of SOX17 and FOXA2 at day 4 of differentiation. For all statistical analysis: * P<0.05, **P<0.01, ***P<0.001

Figure 5-6
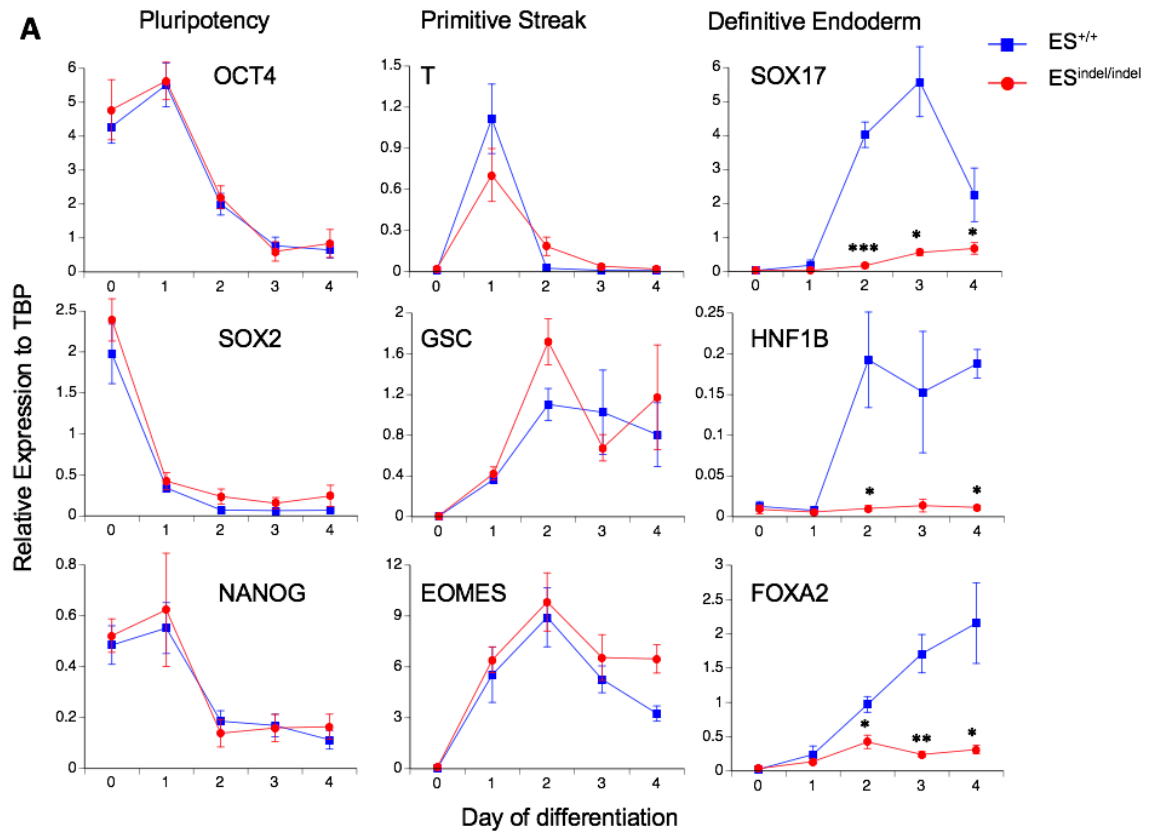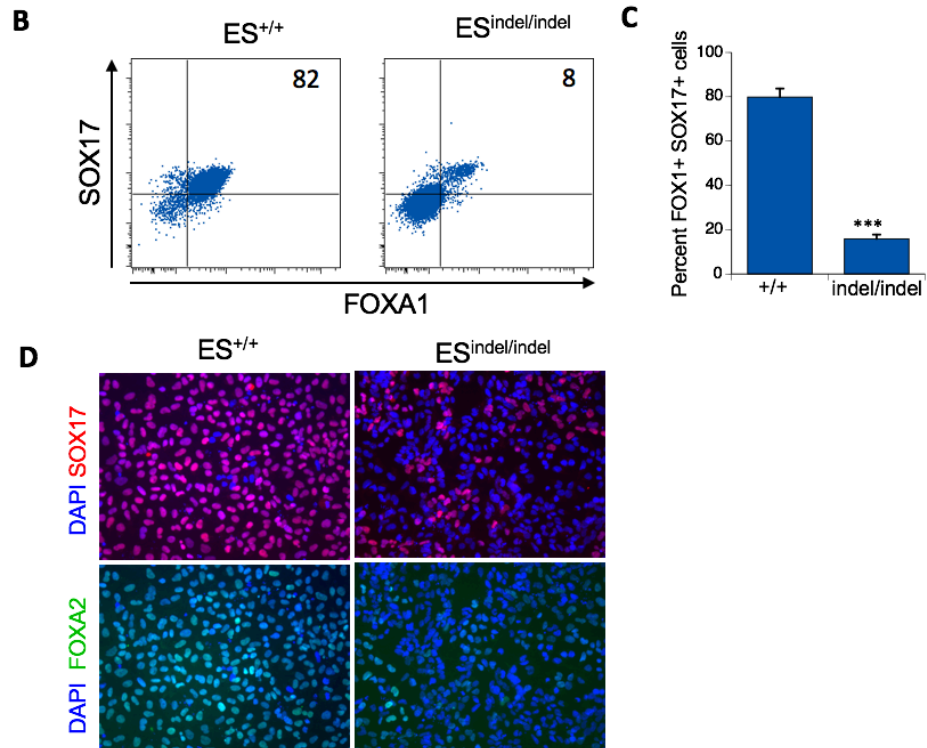
Figure 5-6. *Definitive endoderm differentiation experiments were repeated in the ES cell background..* (A) Time-course analysis of pluripotency markers (OCT4, SOX2, and NANOG), primitive streak markers (T, GSC, EOMES) and definitive endoderm markers (SOX17, HNF1B, and FOXA2) by qRT-PCR. (B) Representative analysis of the definitive endoderm markers FOXA1 vs. SOX17 at day 4 of differentiation by intracellular flow cytometry. (C) Quantitation of the percentage of FOXA1+SOX17+ cells described in B. (D) Immunofluorescence analysis of SOX17 and FOXA2 at day 4 of differentiation. For all statistical analysis:* P<0.05, **P<0.01, ***P<0.001

endoderm specification markers *SOX17*, *FOXA2* and *HNF1B* failed to upregulate in the mutant iPS^indel/indel and ES^indel/indel cell lines compared to the patient iPS^+/indel and control iPS^+/+ and ES^+/+ lines (Figures 5-5A and 5-6A). By using intracellular flow cytometry and examining the co-expression of the definitive endoderm markers, SOX17 and FOXA1, these data were confirmed and quantitated (Figures 5-5B, 5-5C and FIgure 5-6B, 5-6C). Robust co-expression of SOX17 and FOXA1 was observed in both control ES^+/+ (82% ±7%) and iPS^+/+ (82% ±4%) lines and patient iPS^+/indel (75% ±3%) lines, while an ~6-fold decrease was observed in the mutant ES^indel/indel (14% ±3%) and iPS^indel/indel (2.7% ±0.7%) lines. These data were confirmed in all cell lines by immunofluorescence staining for SOX17 and FOXA2 (Figures 5-5D and 5-6D). These data show that GATA6 is a critical transcription factor required during definitive endoderm specification in human cells.

To determine if GATA6 affected differentiation to the other two germ layers, previously established protocols were used to monitor differentiation of control ES^+/+, patient iPS^+/indel, and mutant ES^indel/indel and iPS^indel/indel lines (Greber et al., 2011; Mills et al., 2014; Paluru et al., 2013). All cell lines displayed similar differentiation efficiency to the mesoderm and ectoderm germ layers suggesting that GATA6 was not required (Figure 5-7).

5.2.3 Partial Rescue of Definitive Endoderm by the Addition of Growth Factor

A delay in proliferation during endoderm induction was observed with the mutant iPS^indel/indel and ES^indel/indel line as compared to the control iPS^+/+ and ES^+/+ as well as the patient iPS^+/indel lines (Figure 5-8A and 5-8B). Between day 1 and day 2 of the differentiation there was a decrease in cell number in the mutant iPS^indel/indel and

Figure 5-7
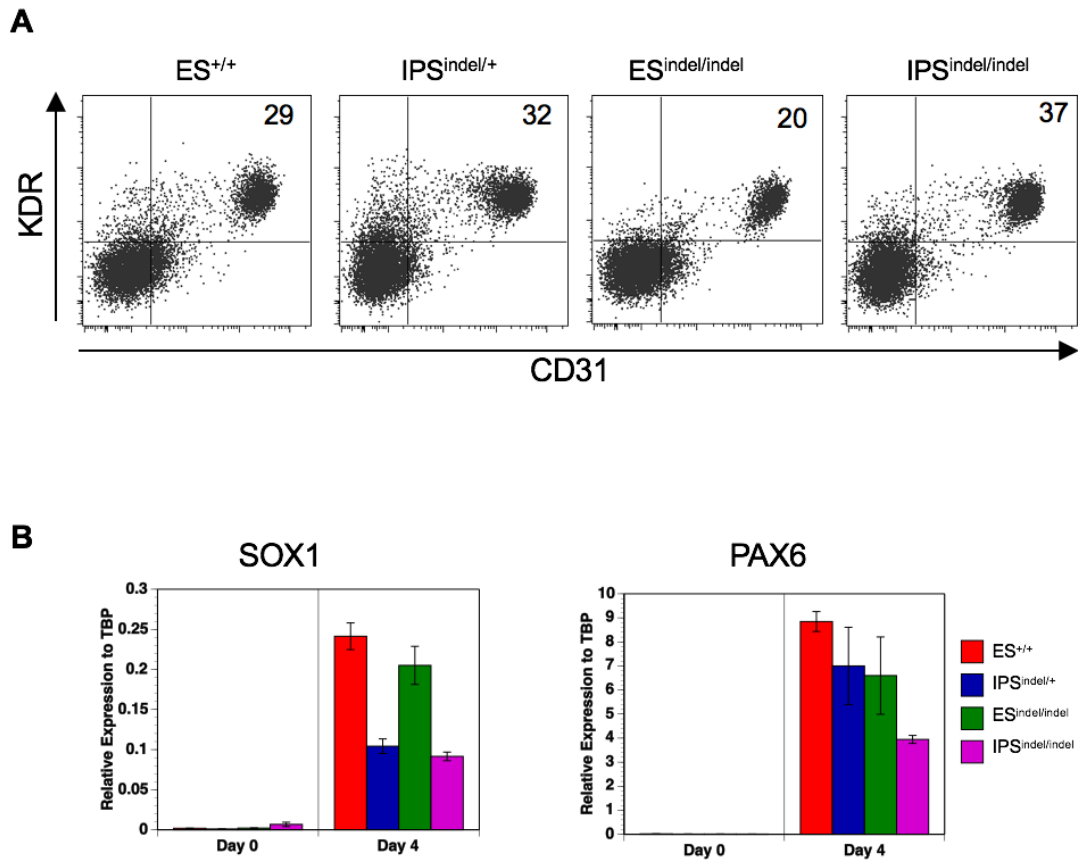
Figure 5-7. *Differentiation of GATA6 mutants to mesoderm and ectoderm lineages.* (A) Representative flow cytometry analysis of ES^{+/+}, iPS[+/indel], ES[indel/indel] and iPS[indel/indel] cells differentiated toward a mesodermal fate confirmed by the expression of surface antigens KDR and CD31. (B) qRT-PCR analysis of ectoderm markers SOX1 and PAX6 in ES^{+/+}, iPS[+/indel], ES[indel/indel] and iPS[indel/indel] cells differentiated toward an ectodermal fate.

Figure 5-8
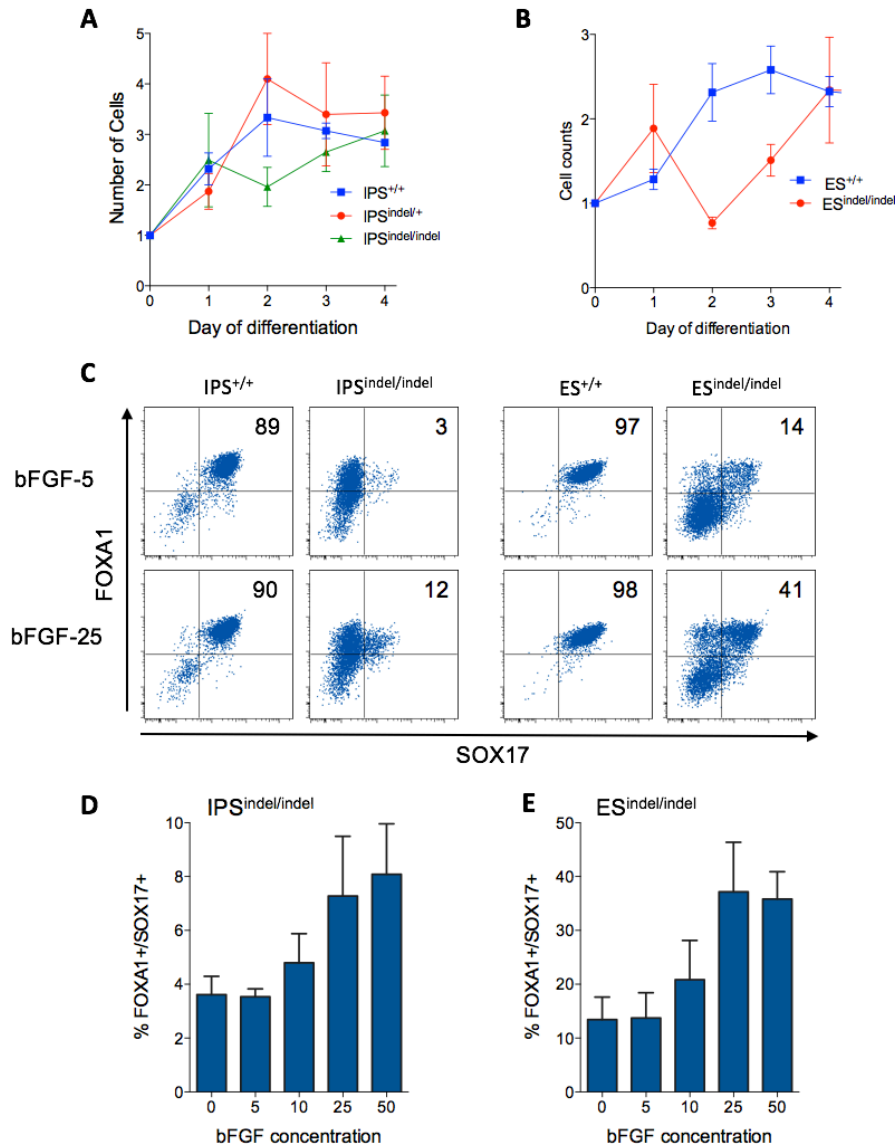
Figure 5-8. *Partial rescue of definitive endoderm differentiation by basic fibroblast growth factor.* All cell lines were differentiated to definitive endoderm. On day 1 of the differentiation cells were treated with varying concentrations of bFGF for 24 hours. (A) Quantitation of cell yield during differentiation without treatment in the control iPS$^{+/+}$, patient iPS$^{+/indel}$ and mutant iPS$^{indel/indel}$ cell lines. (B) As well as in the control ES$^{+/+}$ and mutant ES$^{indel/indel}$ cell lines. (C) Representative flow cytometry analysis of the cell lines at day 4 or day 5 of definitive endoderm differentiation with either 5ng/mL bFGF treatment or 50ng/mL bFGF treatment. (D) Quantification of of definitive endoderm cells co-expression SOX17 and FOXA1 in the mutant iPS$^{indel/indel}$ and (E) mutant ES$^{indel/indel}$ cell lines. For all statistical analysis:* $P<0.05$. (bFGF: basic fibroblast growth factor)

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ES$^{indel/indel}$ cell lines while the control iPS$^{+/+}$, ES$^{+/+}$ and iPS$^{+/indel}$ cells continued to proliferate and expand in number. As the differentiation progressed, mutant iPS$^{indel/indel}$ and ES$^{indel/indel}$ cells continued to proliferate and by day 4 had comparable cell numbers to the control and patient cell lines. To determine whether the defect in definitive endoderm differentiation of mutant iPS$^{indel/indel}$ and ES$^{indel/indel}$ cells was related to a proliferation defect, cells were treated with increasing concentrations of basic fibroblast growth factor (bFGF) for 24 hours from day 1 to day 2 of the differentiation. This time point was chosen as it was prior to the observed decrease in cell number. The co-expression of FOXA1 and SOX17 was examined at the end of the definitive endoderm differentiation. While there was no change in the efficiency of differentiation in the control iPS$^{+/+}$ and ES$^{+/+}$ cells with higher bFGF concentrations (Figure 5-8C) there is partial rescue of the definitive endoderm differentiation in both the mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ cells (Figure 5-8C, 5-8D and 5-8E). As a pro-survival signal can partially rescue the phenotype, this is suggestive that there is a proliferation or cell survival defect in the mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ cells.

5.2.4 Rescue of Definitive Endoderm by GATA6

To confirm that GATA6 was responsible for the ~6-fold decrease in endoderm induction from the mutant ES$^{indel/indel}$ line, GATA6 rescue experiments were performed. Because the mutant ES$^{indel/indel}$ line constitutively expressed rTTA, a lenti-viral vector containing the tet response element (TRE) was used to express GATA6/RFP in a doxycycline inducible manner (Figure 5-9A). By adding doxycycline on day one of the differentiation, robust co-expression of SOX17 and FOXA1 was observed in the mutant ES$^{indel/indel}$ line only after rescue with full-length and not truncated GATA6 (Figure 5-9B and 5-9C). Compared to the RFP alone vector, we observed that over expression of the truncated

Figure 5-9
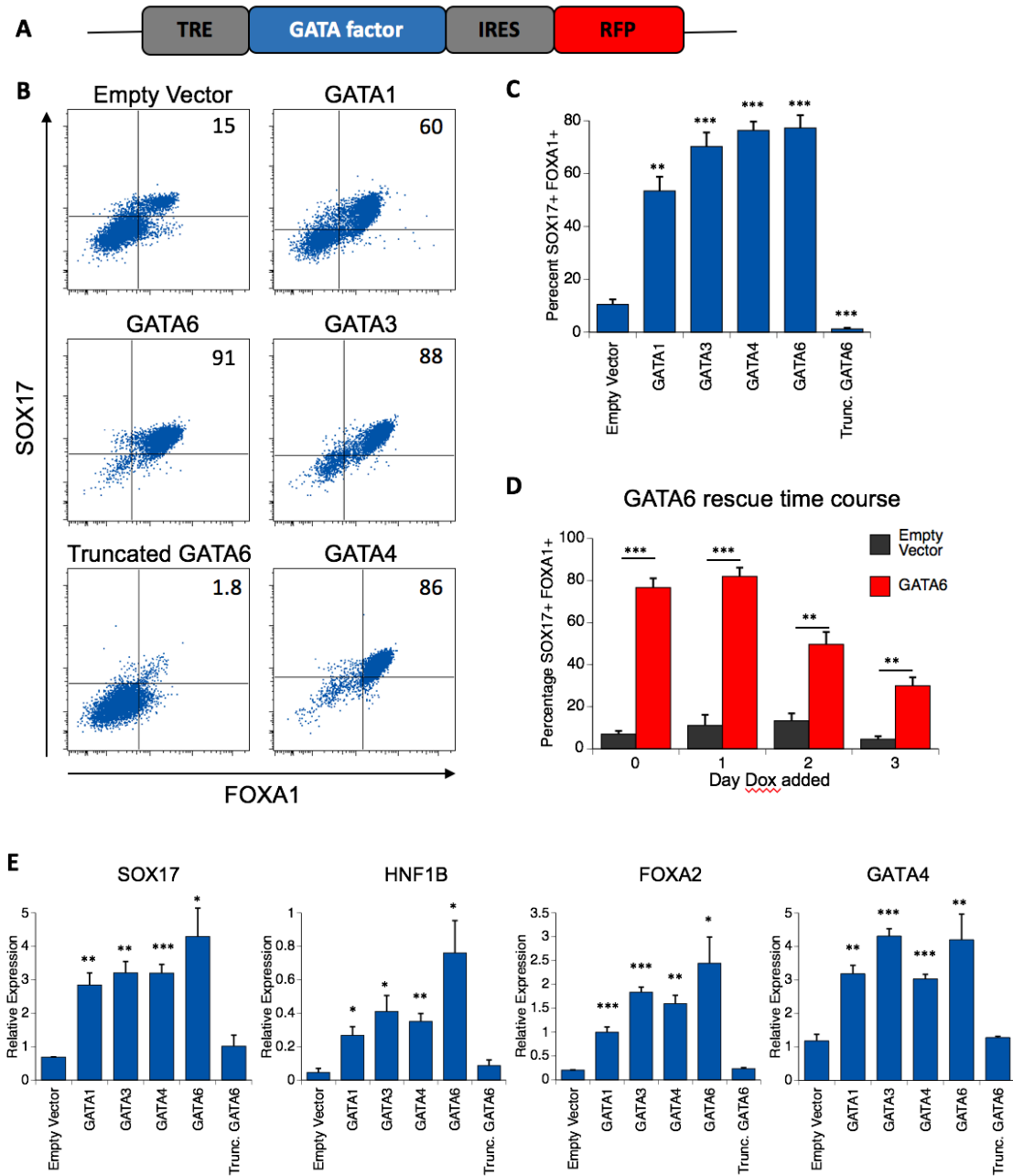
Figure 5-9. *Rescue of definitive endoderm differentiation by GATA factor re-expression.* ES[indel/indel] infected with a doxycycline inducible lentivirus transgenic system and differentiated into definitive endoderm. (A) Schematic of the doxycycline inducible lentivirus. (B) ES[indel/indel] cells were transduced with lentivirus harboring GATA1, GATA3, GATA4, GATA6, truncated-GATA6 transgenes or an empty vector and differentiated into definitive endoderm. Doxycycline was added on day 1 of the differentiation to induce gene expression. Representative intracellular flow cytometric analysis of FOXA1 vs. SOX17 expression at day 5 of differentiation. Data shown is gated on cells expressing RFP. (C) Quantitation of the percentage of FOXA1+SOX17+ cells in B. (D) Quantitation of the percentage of FOXA1+SOX17+ cells in cells transduced with GATA6 or a empty vector control lentivirus with doxycycline addition beginning at various days during the differentiation. Data shown is gated on cells expressing RFP at day 5 of differentiation. (E) Cells treated and differentiated as described in B, were cell-sorted for RFP expression at day 5 of differentiation and subjected to qRT-PCR analysis for SOX17, FOXA2, HNF1B and endogenous GATA4 expression levels. For all statistical analysis: * $P<0.05$, **$P<0.01$, ***$P<0.001$

GATA6 protein resulted in a decrease in the ability of the ES$^{indel/indel}$ cells to differentiate to definitive endoderm, suggesting a possible dominant negative activity of the truncated protein (Figure 5-9B and 5-9C). Gene expression analysis of other endodermal markers was also performed in cells expressing the GATA6 and truncated GATA6 transgene by sorting the RFP positive cells at day 5 of differentiation. The expression levels of *SOX17*, *FOXA2* and *HNF1B* in mutant ES$^{indel/indel}$ cells rescued with GATA6 were comparable to normal levels in control ES$^{+/+}$ cells differentiated to definitive endoderm (Figure 5-5A and Figure 5-6A). Expression levels of these markers in mutant ES$^{indel/indel}$ cells expressing truncated GATA6 were not statistically different to cells expressing the empty vector.

To address a temporal requirement of GATA6 during definitive endoderm specification, doxycycline was added at days 0, 1, 2, or 3 of differentiation and RFP+ cells were analyzed for the co-expression of SOX17 and FOXA1 at day five of differentiation (Figure 5-9D). These time points were chosen as they represent the primitive streak (days 0-1) and endoderm specification (days 2-3) stages of development (Figure 5-6A). The addition of doxycycline at days 0 and 1 of differentiation resulted in 77% ± 4.5% and 82% ± 4.2% of cells co-expressing SOX17 and FOXA1 while the addition of doxycycline at days 2 and 3 resulted in 50% ± 5.9% and 30% ±4.0% of cells co-expressing SOX17 and FOXA1. These data show that robust endoderm specification required GATA6 expression at the primitive streak stage of development.

5.2.5 Rescue of Definitive Endoderm by Other GATA Family Members

All members of the GATA transcription factor family share fairly conserved DNA activation and zinc finger DNA binding domains (Molkentin, 2000; Viger et al., 2008). To determine if other GATA family members can contribute to definitive endoderm

specification and compensate for a loss of GATA6, the same rescue experiments described above were performed using lenti-viral vectors expressing GATA1, 3, and 4. GATA4 was chosen because it is functionally redundant with GATA6 in animal models (Holtzinger and Evans, 2005; Xuan et al., 2012; Zhao et al., 2008). GATA1 and GATA3 are not typically expressed in definitive endoderm and were initially chosen as controls for the rescue experiments. As shown in Figure 5-9C, rescue with GATA1, GATA3, and GATA4 resulted in cells co-expressing 53% ±5.4%, 70% ±3.6%, and 76% ±3.3% SOX17 and FOXA1, respectively. The rescue of definitive endoderm differentiation with GATA4 was expected, but the ability of GATA1 and GATA3 to rescue definitive endoderm was not and suggested that any GATA family member, even those not typically expressed in endoderm, may compensate for GATA6. These data were confirmed by qRT-PCR analysis of *SOX17, FOXA2 and HNF1B* on RFP+ sorted cells (Figure 5-9E). As mutant ES$^{indel/indel}$ have significantly lower *GATA4* expression when compared to control ES$^{+/+}$ cells in definitive endoderm (Figure 5-6C), we questioned whether other GATA family members were rescuing the differentiation by inducing GATA4 expression. We show that any of the GATA factors can rescue expression of endogenous *GATA4* (Figure 5-9E), although further experimentation would be needed to formally demonstrate that the rescue seen with GATA1 and GATA3 was simply due to GATA4 induction.

5.2.6 Downstream Targets of GATA6 During Definitive Endoderm Specification

To determine possible targets of GATA6 during differentiation to definitive endoderm, a gene expression microarray was performed using the control ES$^{+/+}$ and mutant ES$^{indel/indel}$ lines. The cells were differentiated for 24 hours to the primitive streak stage of development when GATA6 but not SOX17 was upregulated (Figure 5-10A). The top candidates of differentially expressed genes between the two cell lines revealed a

Figure 5-10

A

ES$^{+/+}$ ES$^{indel/indel}$

GATA6

SOX17

B

Rank	Gene	Fold Change
1	ZNF594	-2.0
2	LINC00665	-4.7
3	ZNF738	-2.9
4	ZNF253	-5.3
5	PKIB	2.5
6	ZNF844	-2.6
7	IL6ST	-2.0
8	PRSS1	-1.7

C

ZNF844   ZNF594   ZNF253
ESC IPSC   ESC IPSC   ESC IPSC

IL6ST   ZNF738   linc000665
ESC IPSC   ESC IPSC   ESC IPSC
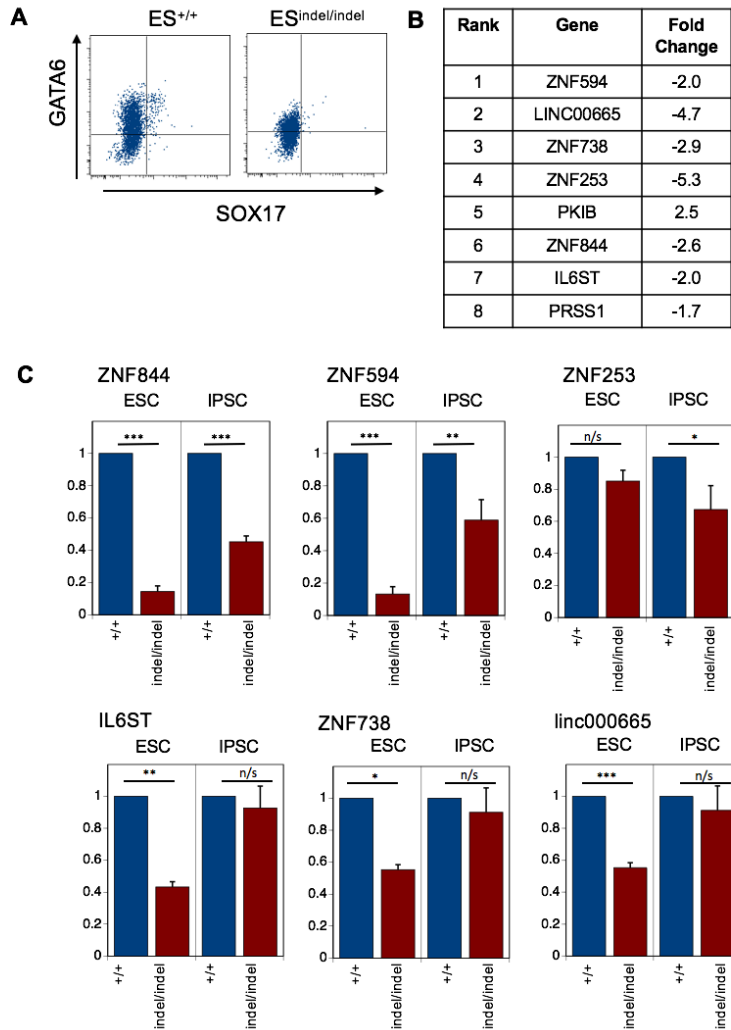
Figure 5-10. *Gene expression microarray to identify downstream targets of GATA6.* (A) Gene expression microarrays were performed on control ES$^{+/+}$ and mutant ES$^{indel/indel}$ cells differentiated toward primitive streak for 24 hours that have begun to upregulate GATA6 and prior to the upregulation of SOX17 as demonstrated by representative intracellular flow cytometric analysis. (B) Gene expression was analyzed by microarray as described in A. The top 8 genes that were enriched in ES$^{+/+}$ cells over ES$^{indel/indel}$ cells are shown. (C) Validation of genes from the microarray in mutant ES$^{indel/indel}$ and iPS$^{+/+}$ cells compared to control ES$^{+/+}$ and iPS$^{+/+}$ patient cells by qRT-PCR. Expression levels were normalized to 1 in wild type (+/+) cells of each genetic background. For all statistical analysis: * P<0.05, **P<0.01, ***P<0.001

number of zinc finger (ZNF) genes (Figure 5-10B). We chose to focus on genes that were down regulated in the mutant ES$^{indel/indel}$ line compared to the control ES$^{+/+}$ line as a loss of GATA6 prevented definitive endoderm specification (Figures 5-6). The down regulated genes were validated by qRT-PCR using both control iPS$^{+/+}$ and mutant iPS$^{indel/indel}$ lines (Figure 4C and S6). Two of the six genes, ZNF844 and ZNF594, were down regulated in both mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ lines (Figure 5-10C). The ZNF738, linc000665 and IL6ST were only validated in the mutant ES$^{indel/indel}$ line and ZNF253 was only validated in the mutant iPS$^{indel/indel}$ line (Figure 5-10C).

5.2.7 Functional Analysis of GATA6 Mutants as Endodermal Progenitor derived β-like Cells

The use of established differentiation protocols to study the role of GATA6 in pancreas development (D'Amour et al., 2006; Kroon et al., 2008; Pagliuca et al., 2014; Rezania et al., 2014, 2012; Russ et al., 2015b) was difficult due to the inability of the mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ lines to differentiate efficiently into definitive endoderm. To overcome this developmental block and generate enough cells for downstream applications, we utilized our protocol to establish endodermal progenitor (EP) cells from the corrected iPS$^{+/+}$, control ES$^{+/+}$, patient iPS$^{+/indel}$ and mutant ES$^{indel/indel}$ lines (Cheng et al., 2012). GATA6 is expressed at lower levels and GATA4 and GATA3 are expressed at higher levels in EP cells compared to definitive endoderm making the establishment of these lines feasible (Cheng et al., 2012). Cells were differentiated to definitive endoderm and CXCR+CKIT+ co-expressing cells were sorted for the generation of EP cell lines. All lines were characterized as EP cells displaying self-renewal capacity to greater than 20 passages (data not shown) and expression of appropriate markers including SOX17, EOMES, FOXA1, FOXA2, *TBX3, MSX2, MEIS2*, and *ID2* (Figure 5-11).

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Figure 5-11

A

B
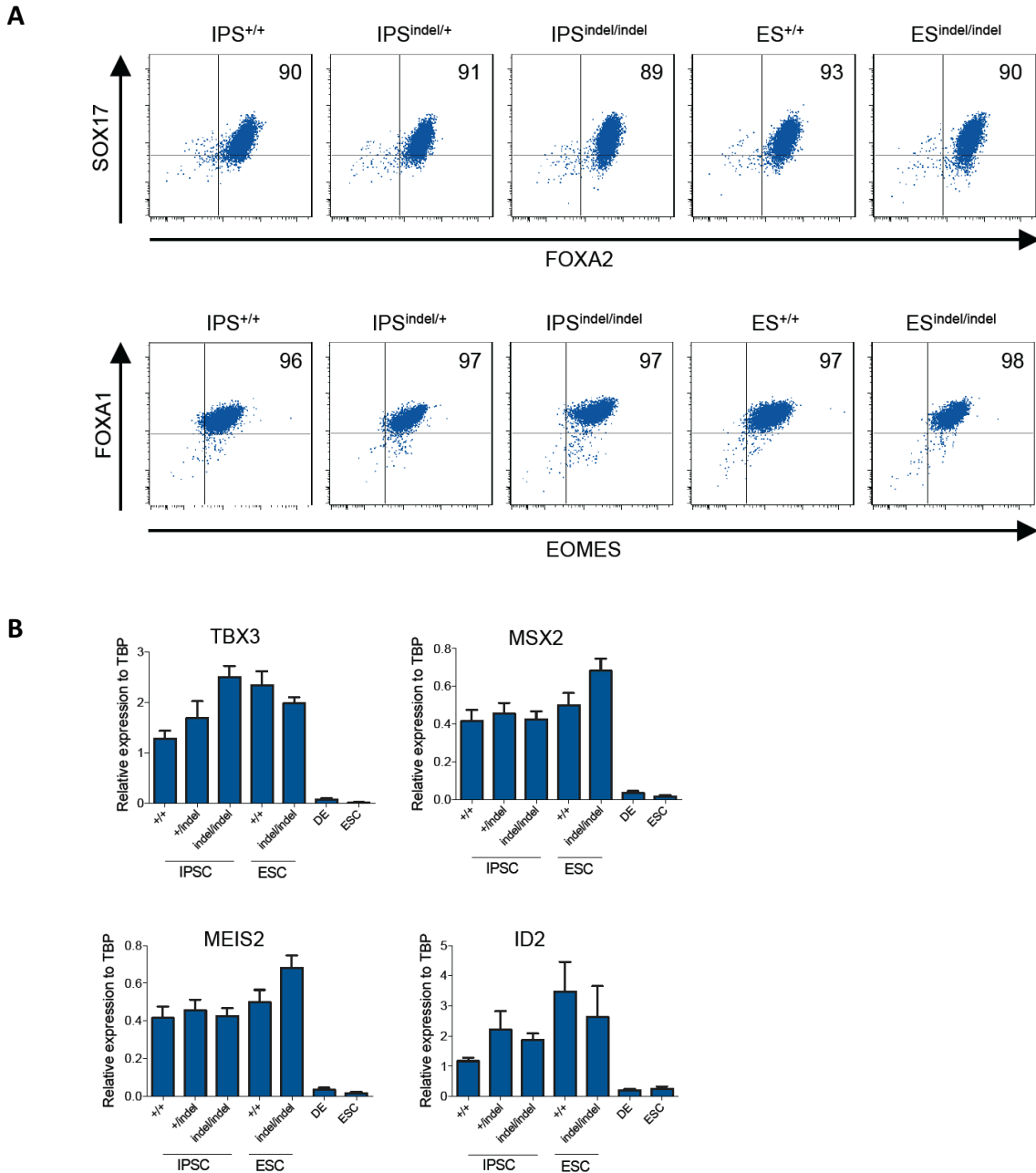
Figure 5-11. *Generation of endodermal progenitor cells.* (A) Representative flow cytometry analysis of SOX17, FOXA2, FOXA1 and EOMSEDERMIN in Endodermal Progenitor cells derived from control IPS$^{+/+}$, control ES$^{+/+}$, patient iPS$^{+/indel}$ and mutant IPS$^{indel/indel}$ and ES$^{indel/indel}$ cells (B) Endodermal Progenitor (EP) cells from A were subject to qRT-PCR analysis for markers that are known to be up-reguated in EP cells as compared to transient definitive endoderm (DE) and embryonic stem cells (ESC).

Figure 5-12
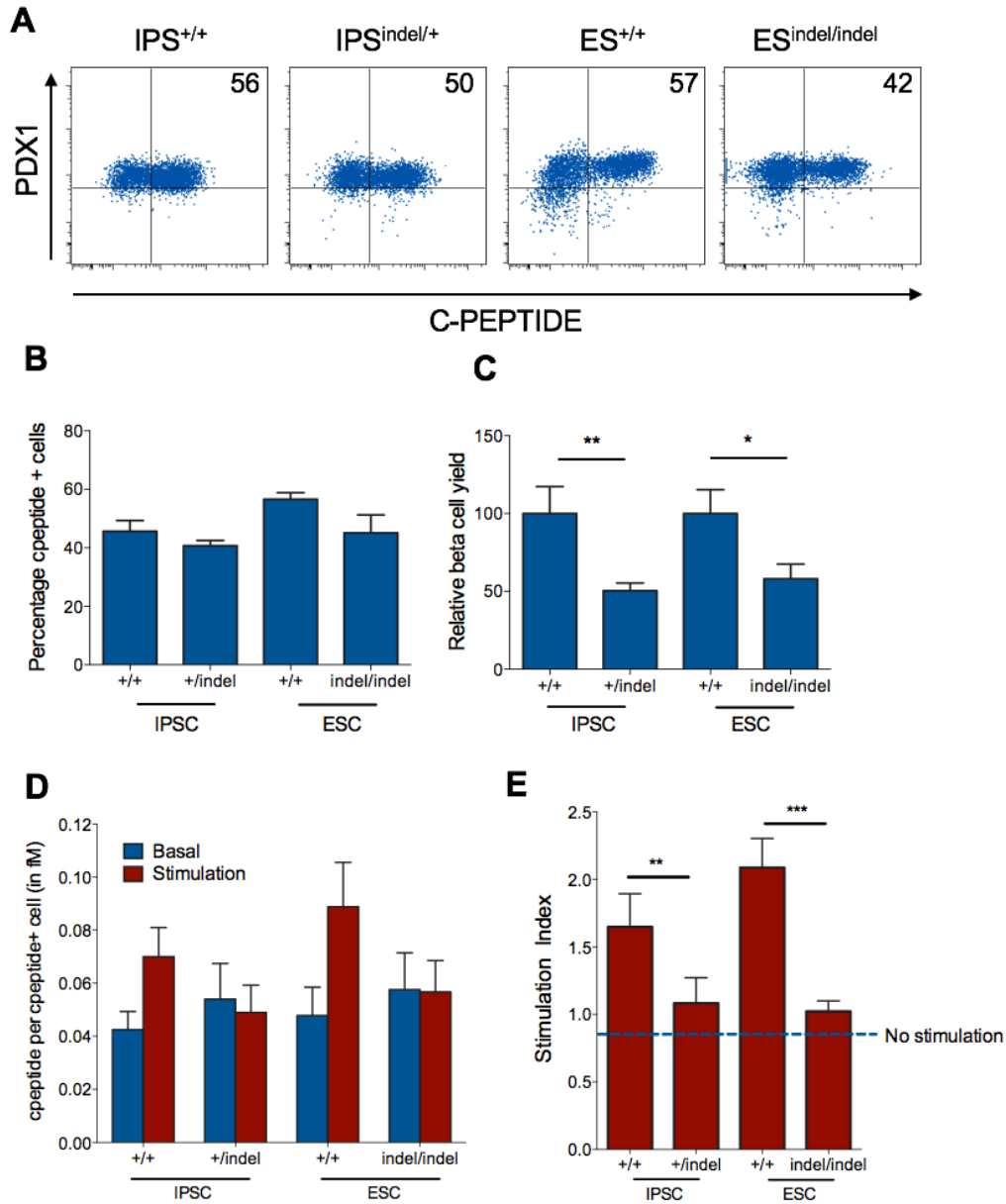
Figure 5-12. *GATA6 is dispensable for differentiation of pancreatic β-like cells but necessary for functionality*. EP cell lines were generated from control iPS^{+/+}, control ES^{+/+}, patient iPS[+/indel] and mutant ES[indel/indel] pluripotent stem cells and differentiated to β-like cells. (A) Representative intracellular flow cytometric analysis of C-Peptide vs. PDX1 at day 14 of differentiation. (B) Quantification of the percentage of C-peptide+ cells from A. (C) Absolute yield of c-peptide+ cells from A. (D) Quantification of C-peptide secretion per C-peptide positive cell on day 14 in basal glucose (1 mM) and stimulation glucose (20 mM) conditions. (E) Stimulation index of C-peptide secretion from D. For all statistical analysis: * P<0.05, **P<0.01, ***P<0.001

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To study pancreatic cell fate, the EP cell lines were differentiated into β-like cells following our published protocol (Cheng et al., 2012). All EP cell lines differentiated into β-like cells co-expressing c-peptide and PDX1 (Figure 5-12A). The efficiency of differentiation following aggregation of the cells into suspension culture, as calculated by the percentage of c-peptide$^+$ cells, was comparable between all lines (Figure 5-12B). However, when comparing absolute c-peptide$^+$ cell yields in the suspension culture of patient iPS$^{+/indel}$ to corrected iPS$^{+/+}$ and mutant ES$^{indel/indel}$ to control ES$^{+/+}$ there is decreased β-like cell yields in both GATA6 mutant lines (Figure 5-12C).

To analyze the functionality of these β-like cells, their responsiveness to glucose stimulation was determined. In unstimulated conditions, the corrected iPS$^{+/+}$, control ES$^{+/+}$, patient iPS$^{+/indel}$, and mutant ES$^{indel/indel}$ β-like cells secreted similar amounts of c-peptide when normalized for the absolute number of c-peptide+ cells per culture (Figure 5-12D). Upon glucose stimulation, the increase in c-peptide secretion of the corrected iPS$^{+/+}$ EP derived β-like cells was 1.7 ± 0.9 fold over basal levels and in control ES$^{+/+}$ EP-derived β-like cells was 2.1 ± 0.22 fold over basal levels (Figure 5-12E). The patient iPS$^{+/indel}$ and mutant ES$^{indel/indel}$ EP-derived β-like cells were unresponsive to glucose stimulation with stimulation indexes of 1.1 ± 0.16 and 1.1 ± 0.08 respectively (Figure 5-12D and 5-12E). These data demonstrate that the levels of GATA6 were critical in determining the functional responsiveness of pancreatic β-like cells to glucose stimulation.

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5.2.8 Gene Expression Analysis of GATA6 Mutant Endodermal Derived β-like cells

By cell sorting for GFP$^+$ β-like cells from the control ES$^{+/+}$ and mutant ES$^{indel/indel}$ lines, gene expression of insulin positive cells could be analyzed (Figure 5-13A). While a number of genes were not differentially expressed, *PDX1, PCSK1 GLUT1* and *HNF4α* were significantly lower in the mutant ES$^{indel/indel}$ cells compared to the control ES$^{+/+}$ cells (Figure 5-13B). Decreased levels of PDX1 in mutant ES$^{indel/indel}$ compared to control ES$^{+/+}$ were confirmed by flow cytometry. However, levels of PDX1 in the patient iPS$^{+/indel}$ was not different to levels in the control iPS$^{+/+}$ (Figure 5-13C). PCSK1 is a protease important in the processing of proinsulin to insulin (Steiner, 2004). To determine whether decreased levels of PCSK1 lead to defective proinsulin processing, levels of proinsulin were examined by intracellular flow cytometry. Patient iPS$^{+/indel}$ cells show a trend toward increased amounts of proinsulin within the β-like cell compared to control iPS$^{+/+}$ cells while mutant ES$^{indel/indel}$ have significantly decreased levels of proinsulin present (Figure 5-13D). Proinsulin secretion was also examined in the β-like cells. Control iPS$^{+/+}$ and patient iPS$^{+/indel}$ displayed no difference proinsulin secretion, however, compared to control ES$^{+/+}$ cells, ES$^{indel/indel}$ cells had significantly increased levels of proinsulin secretion (Figure 5-13E). These data suggest that while GATA6 does not affect the ability of EP cell differentiation into pancreatic β-like cells, it may be involved in maturation and insulin processing of the β-like cells.

5.2.9 Retinoic acid is the exogenous signal that allows patient iPS$^{+/indel}$ cells to differentiate in vitro

The patient from which the iPS$^{+/indel}$ cells were originally derived was born with pancreas agenesis, however the ability to generate β-like cells from the GATA6 mutant lines suggests that pancreas agenesis may be due to cell non-intrinsic mechanisms that can

Figure 5-13
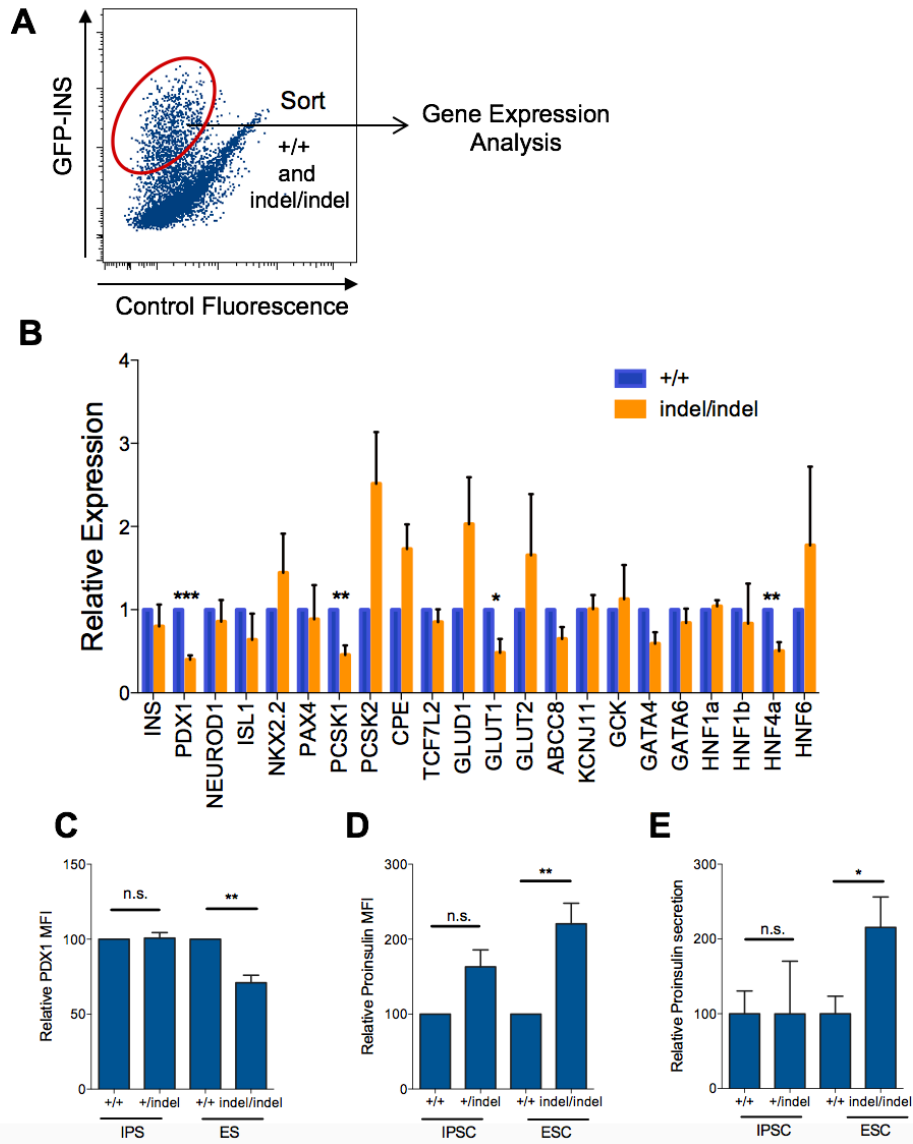
Figure 5-13. *Gene Expression Analysis of β-like cells.* (A) The Mel1-INS-GFP cells contain a GFP reporter in the insulin locus. β-like cells from $ES^{+/+}$ and $ES^{indel/indel}$ were purified by cell-sorting for GFP+ cells and analyzed for gene expression. (B) QRT-PCR analysis for expression of the indicated genes. Expression levels are normalized to $ES^{+/+}$. (C) The mean fluorescence intensity of PDX1 in c-peptide positive control $iPS^{+/+}$, control $ES^{+/+}$, patient $iPS^{+/indel}$ and mutant $ES^{indel/indel}$ cells. (D) The mean fluorescence intensity of proinsulin in c-peptide positive cells was determined by flow cytometry. (E) Quantification of proinsulin secreted per c-peptide positive cell. For these experiments, all values are normalized to the wild type of each genetic background. For all statistical analysis: * P<0.05, **P<0.01, ***P<0.001

be bypassed using a differentiation protocol supplying exogenous inductive signals. During differentiation of EP cells to β-like cells, exogenous signals which are applied include FGF signalling, inhibition of sonic hedgehog (SHH) and retionoic acid signalling. To address this, concentration so FGF10, cyclopamine and retinoic acid were titrated down during the differentiation. Differentiated cells were examined at the pancreatic progenitor stage for expression of PDX1 because patients who have pancreas agenesis should have a defect in the formation of the pancreatic progenitor. Furthermore, to determine the final outcome, the differentiation was examined the end stage in adherent culture for expression of c-peptide.

Lowering the concentrations of FGF10 and cyclopamine did not affect the differentiation (data not shown). However, lower retinoic acid concentrations resulted in a decrease in the differentiation efficiency of the patient iPS$^{+/indel}$ cells compared to the control iPS$^{+/+}$ cells. At the pancreatic progenitor stage there is a trend toward a reduced percentage of PDX1 positive patient iPS$^{+/indel}$ cells in low (0.025 μM) retinoic acid concentrations compared to the control condition (2 μM) retinoic acid (Figure 5-14A and 5-14C). However, lower concentrations of retinoic acid did not affect the percentage of PDX1 positive control iPS$^{+/+}$ cells. (Figure 5-14A and 5-14B). To determine whether GATA4 could compensate for a loss of GATA6, GATA4 expression levels were also examined. At control concentrations of retinoic acid, GATA4 expression was lowered in IPS$^{+/indel}$ cells compared to IPS$^{+/+}$ cells (Figure 5-14D). Both GATA4 and GATA6 can be induced by retinoic acid (Arceci et al., 1993; Mauney et al., 2010) and with decreased concentrations of retinoic acid there was decreased expression of GATA4 in both the IPS$^{+/indel}$ and IPS$^{+/+}$ pancreatic progenitor cells (Figure 5-14D). These findings further

Figure 5-14
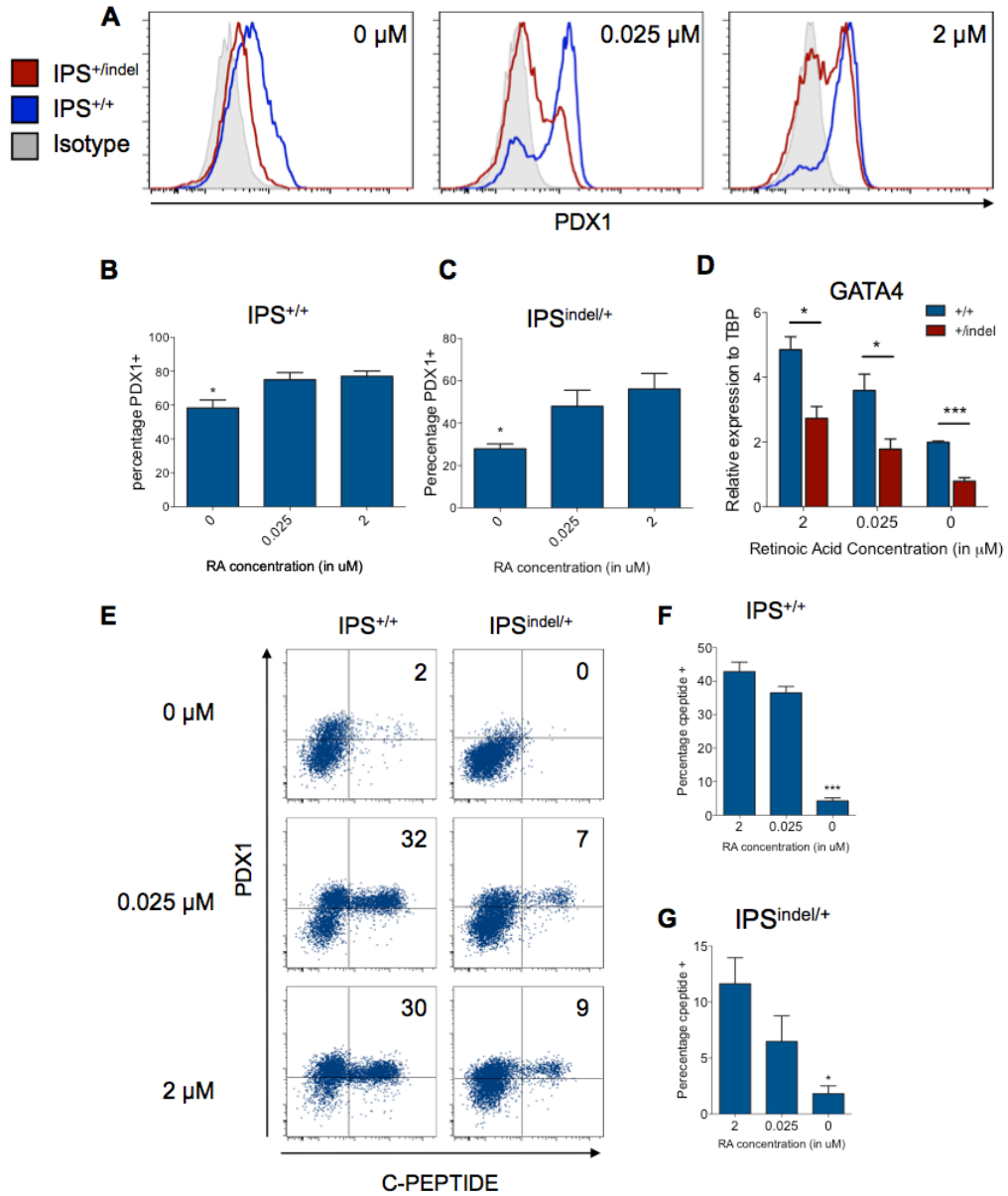
Figure 5-14. *Retinoic acid overcomes patient iPS*$^{+/indel}$ *cell deficiency during differentiation.* (A) Representative intracellular flow cytometry analysis of PDX1 in control iPS$^{+/+}$ and patient iPS$^{+/indel}$ at the pancreatic progenitor stage differentiated in different concentrations of retinoic acid. (B) Quantification of A for control iPS$^{+/+}$. (C) Quantification of A for patient iPS$^{+/indel}$. (D) Quantitative real time PCR for expression of GATA4 at the pancreatic progenitor stage. (E) Representative intracellular flow cytometry analysis of PDX1 and C-peptide of β-like cells in adherent culture differentiated in different concentrations of retinoic acid. (F) Quantification of D for control iPS$^{+/+}$. (G) Quantification of D for patient iPS$^{+/indel}$. For all statistical analysis: * P<0.05, **P<0.01, ***P<0.001. Statistical significant was calculated compared to 2uM of retinoic acid.

confirm the observations from the definitive endoderm stage that GATA4 may be downstream of GATA6.

At the β-cell stage, without retinoic acid c-peptide positive cells fail to develop in both control iPS$^{+/+}$ and patient iPS$^{+/indel}$ cells. Lowering the concentration of retinoic acid used during the differentiation, patient iPS$^{+/indel}$ cells lead to a downward trend of the percentage of c-peptide positive cells compared to the control condition (2uM retinoic acid) (Figure 5-14E and 5-14G). The differentiation of the control iPS$^{+/+}$ cells was unaffected (Figure 5-14E and 5-14F). These data suggest that iPS$^{+/indel}$ have increased sensitivity to retinoic acid and in vitro, under normal differentiation conditions, β-like cells can develop from iPS$^{+/indel}$ due to the supply of high concentrations of retinoic acid.

5.3 Discussion

This study established a human in vitro PSC model system to study the loss of GATA6 function. Considering that GATA6 null mice are embryonic lethal due to a requirement for extra-embryonic endoderm (Morrisey et al., 1998), PSCs are advantageous because extra-embryonic endoderm is unnecessary for maintenance and differentiation of these cells. We have shown that while patient iPS$^{+/indel}$ cells have no defects in definitive endoderm specification, mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ cells from different genetic backgrounds failed to efficiently differentiate into definitive endoderm. The down regulation of pluripotency markers and the induction of primitive streak markers in these cells remained normal, suggesting a defect in the transition of cells from the primitive streak stage of development to definitive endoderm. Transgene expression of GATA6 in the mutant ES$^{indel/indel}$ cells rescued endoderm differentiation demonstrating the importance of GATA6 in definitive endoderm specification and that off-target effects of genome editing were not responsible for the observed phenotype.

In chimeric mouse models, GATA6 null cells can contribute to the formation of the primitive gut tube (Koutsourakis et al., 1999). The inability of the mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ lines to generate definitive endoderm (Figure 5-5 and 5-6) suggested that there may be differences in the role of GATA6 during human versus mouse endoderm development. One explanation for this difference could be the timing of GATA6 induction in the human system. We observed the expression of GATA6 preceding GATA4 during endoderm induction from PSCs (Figure 5-1G). No other GATA factors were expressed at this time (data not shown). In mouse models, GATA4 and GATA6 were co-expressed in the primitive streak (Morrisey et al., 1997), therefore it is possible that in the mouse, GATA4 is compensating for GATA6 leading to the differences in phenotype.

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In the mutant ES$^{indel/indel}$ and iPS$^{indel/indel}$ cell lines, there was significantly decreased expression of both GATA6 and GATA4 (Figure 5-1E and 5-1G) suggesting that GATA6 may regulate its own expression in addition to GATA4. A similar observation had been made in the visceral and primitive endoderm of mouse GATA6 knockout embryos (Morrisey et al., 1998). However, when examining a loss of GATA6 in the heart in the embryo proper, there was no decrease in GATA4 expression levels in GATA6 mouse mutants (Zhao et al., 2005). These data are consistent with the possibility that GATA6 may differentially regulate GATA4 expression in definitive endoderm in human and mouse leading to the observed differences.

Subsequently, we show that transgenic expression of GATA4 rescued the definitive endoderm differentiation in the mutant ES$^{indel/indel}$ cells. GATA4 has been shown to compensate for a lack of GATA6 in zebrafish and murine model systems. In zebrafish, knockdown of GATA4 or GATA6 by morpholinos led to the formation of a small liver bud that did not expand; however, with a loss of both GATA factors the liver bud completely failed to form (Holtzinger and Evans, 2005). In mouse models, there have been multiple studies in different tissues including the ovary, intestinal epithelium and liver displaying similar compensation between GATA4 and GATA6 (Padua et al., 2014; Walker et al., 2014; Zhao et al., 2008). Thus, we corroborate that in the human system, GATA4 can compensate for a loss of GATA6.

It was unanticipated that GATA3 and GATA1 would rescue endoderm differentiation of mutant ES$^{indel/indel}$ cells as these genes are not normally expressed during definitive endoderm induction. While transgenic expression of the GATA factors rescued the

differentiation, GATA3 and GATA4 did so more robustly than GATA1. These experiments suggested that the presence of any GATA family member at the primitive streak stage was sufficient for definitive endoderm specification, but differences in efficiency were seen. GATA4 is normally expressed in endodermal derived tissues, GATA3 was reported to be expressed in endodermal progenitor cells (Cheng et al., 2013) and GATA1 is typically expressed in hematopoietic lineages (Moriguchi and Yamamoto, 2014). In addition to differences in tissue expression, there may also be tissue specific functions of particular GATA family members explaining efficiency variations in the rescue of endoderm induction.

Similar findings of compensation were reported in erythroid cell development. Transgenic expression of GATA2 or GATA3 could rescue erythroid cell development and prevent embryonic lethality in GATA1 mutant mice (Takahashi et al., 2000). Despite the fact that these two transcription factors were expressed in hematopoietic cells, they were not normally expressed in the same tissues as GATA1 (Bresnick et al., 2012; Orkin, 1995). Transgenic expression of GATA4 in GATA1 mutant mice prolonged the life span of the mice but did not prevent death due to anemia revealing that GATA4 could not completely replace GATA1 (Hosoya-Ohmura et al., 2006). These studies corroborate our own findings and suggest that hematopoietic and endoderm GATA family members may have distinct functions.

A truncated GATA6 protein was produced in the GATA6 mutant cell lines and transgenic expression of this protein in the mutant ES$^{indel/indel}$ line led to a further decrease in definitive endoderm differentiation (Figure 5-9). The expression of this protein had no impact in the control ES$^{+/+}$ line (data not shown). These data suggested that the

truncated GATA6 protein may have mild dominant negative activity. Examination of cells with GATA6 protein null alleles will be important to determine the stage and severity of the endoderm phenotype.

All of the GATA6 mutant EP lines differentiated into pancreatic β-like cells. During the differentiation high exogenous addition of retinoic acid may be the contributing factor that allows the differentiation to proceed despite any intrinsic deficiencies that may result from a loss of GATA6. Under the regular concentration (2 μM) of retinoic acid, patient iPS[+/indel] cells have a reduced expression of GATA4 compared to to control iPS^{+/+} cells, corroborating the findings of a decrease in GATA4 expression in cells with a loss of GATA6 at the definitive endoderm stage. In all cell lines examined, GATA4 is further decreased with lower concentrations of retinoic acid (Figure 5-14C). This was expected as the expression of both GATA4 and GATA6 can be induced by retinoic acid (Arceci et al., 1993; Mauney et al., 2010). One explanation for the differences between mouse and human phenotypes that result from a heterozygous loss of GATA6 may be lie in our findings that GATA4 is downstream of GATA6. In mouse models with a conditional loss of GATA6 during pancreatic development at the PTF1A stage, although absolute levels were not quantified, GATA4 remained express in the adult endocrine pancreas (Martinelli et al., 2013). Furthermore, a conditional loss of GATA6 in the jejunum of mice, revealed that a loss of GATA6 did not affect the expression of GATA4 and GATA4 expression levels remained comparable to wild type mice (Walker et al., 2014). In contrast to in vivo mouse models of a loss of GATA6, our findings suggest that in humans, GATA4 may be downstream of GATA6 and thus a loss of GATA6 cannot be compensated by GATA4.

Figure 5-15
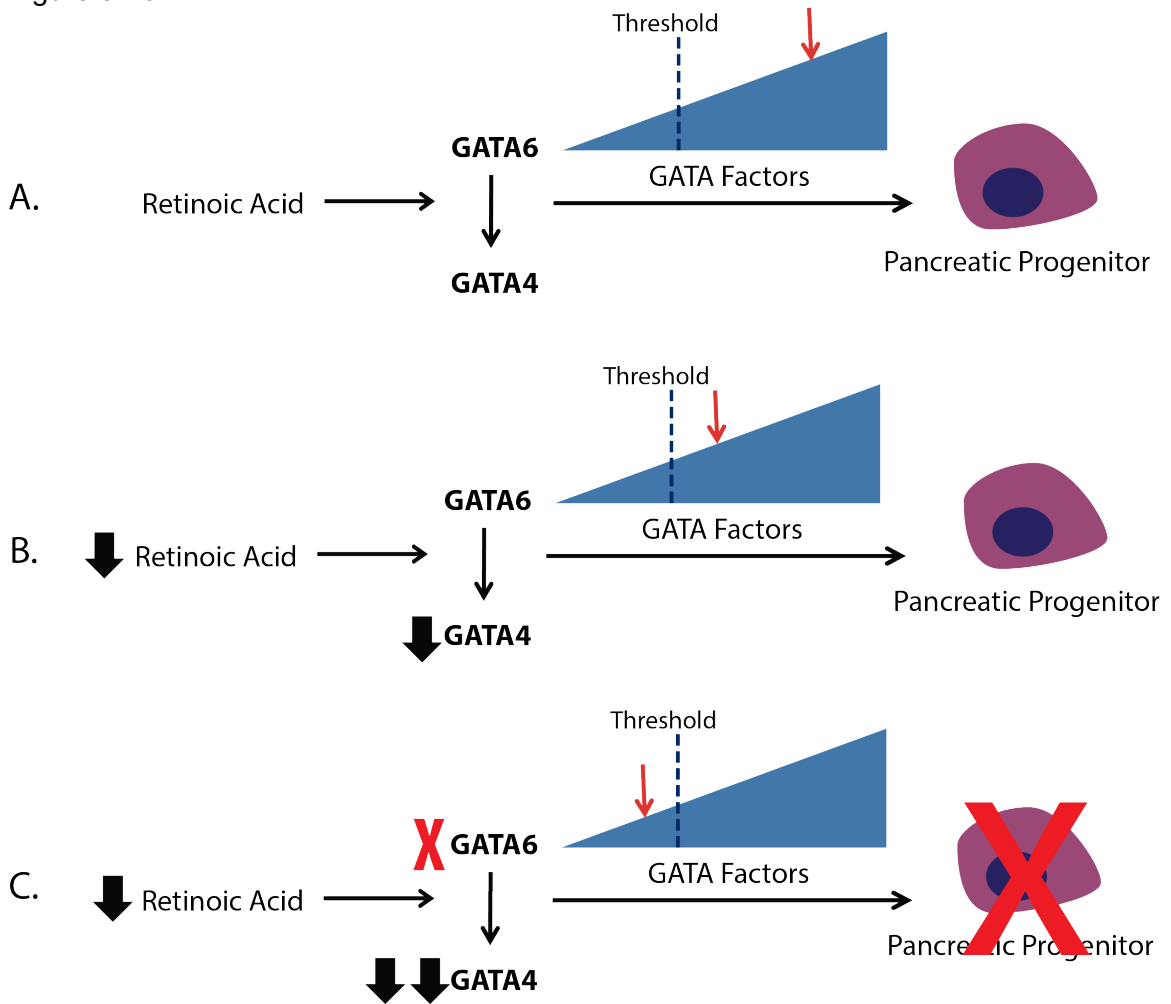
Figure 5-15. *A minimum threshold of GATA4 and GATA6 is must be met for pancreatic progenitor development.* (A) Retinoic retinoic acid can induce the expression of GATA6 and GATA4. GATA6 can also induce the expression of GATA4. Under normal conditions, combined expression levels of these GATA factors is above the minimum threshold for pancreatic progenitor development. (B) With decreased retinoic acid signaling there is a decrease in combine GATA factor expression. However, this remains above the minimum threshold required for pancreatic progenitor development. (C) A combination of reduced retinoic acid signaling and a haploinsufficiency in GATA6 may result in insufficient combined levels of GATA factors resulting in a failure in pancreatic progenitor development.

It is possible that in vivo, retinoic acid may be one inducer of GATA4 and GATA6 and a minimum combined expression level of GATA4 and GATA6 is necessary for pancreatic progenitor development (Figure 5-15A). Variability in endogenous retinoic acid signaling in different people can result from differences in genetic background. Some individuals may have slightly decreased retinoic acid and thus result in decreased expression levels of GATA4 and GATA6. This may not result in a phenotype as GATA4 and GATA6 expression may still remain about the minimum threshold for pancreatic progenitor cell specification (Figure 5-15AB). However, when there is a decrease in retinoic acid signaling combined with a haploinsufficiency in GATA6, the expression of GATA6 from the remaining allele and induction of GATA4 expression may be insufficient to meet the minimum threshold for combined GATA expression levels. Thus, this results in the inability to develop pancreatic progenitors and result in pancreas agenesis in the patient (Figure 5-15C). Furthermore, variability in vivo endogenous retinoic acid signaling may result in GATA6 mutations that have incomplete penetrance as displayed by different phenotypes in family members who have the same mutation (Bonnefond et al., 2012).

When examining the end stage β-like cell differentiation, the patient $iPS^{+/indel}$ and mutant $ES^{indel/indel}$ β-like cells had decreased β-like cell yield. Upon examination of functionality, the patient $iPS^{+/indel}$ and mutant $ES^{indel/indel}$ β-like cells did not secrete insulin in response to glucose stimulation. Gene expression analysis of mutant $ES^{indel/indel}$ β-like cells revealed a decreased expression of PSCK1. It was observed that these cells had an increase in levels of proinsulin within the cell and also during secretion suggesting defective insulin processing. These data in both the patient $iPS^{+/indel}$ and mutant $ES^{indel/indel}$ lines suggest that GATA6 is a critical transcription factor for the robust differentiation of human β cells and their full functionality. The role of GATA6 in the β-cell

functionality in the patient from which iPS cells were generated could not be determined as she was born with pancreatic agenesis (Stanescu et al., 2014). In mice, GATA6 knockout in adult β cells had no effect on β cell mass or function (Sartori et al., 2014) suggesting similarities and differences between the human and murine systems.

These cell lines described here also provide an important model for studying the subset of diabetic patients without agenesis who have GATA6 mutations. The in vitro differentiation of GATA6 mutant cells will provide a means to dissect the underlying disease mechanism at different stages of development. The PSC lines provided a human based model system to study the role of GATA6 in endoderm specification and pancreatic development. The utilization of an intermediate progenitor population was essential for defining the role of GATA6 in pancreatic β-cell functionality. A future interest will be examination of pancreatic β cell function in cells expressing heterozygous GATA6 mutations from patients who do not have pancreatic agenesis but present with adult onset diabetes.

In this study the use of PSCs has provided a powerful human based system to study GATA6 mutations. Important insights have been gained into the role of GATA6 in early human definitive endoderm development and pancreatic β-cell functionality.

CHAPTER 6

Summary and Speculations

6.1 Comparing ES based protocols to EP derived β-like cells

Due to the limited availability of primary human islets for use in therapeutics and research, the differentiation of β cells from PSCs that faithfully mimics an adult β cell has long been one of the goals in the field. One of the main concerns with the original protocols for ES cell differentiation to endocrine cells is that the resulting cells are polyhormonal, lack the expression of NKX6.1 and do not respond to glucose with insulin secretion (D'Amour et al., 2006; Kunisada et al., 2012; Nostro and Keller, 2012). These phenotypes are suggestive of a cell that may correspond to the first wave of endocrine cell that arises in vivo during the primary transition of fetal pancreas development. First wave endocrine cells arise prior to the expression of NKX6.1 and co-express a combination of insulin, glucagon and/or somatostatin (De Krijger et al., 1992; Riedel et al., 2012) but lineage tracing studies of these cells have shown that they do not develop into insulin expressing β cells (Herrera, 2000). Furthermore, unlike adult β cells, fetal β cells respond poorly to glucose stimulation with increased insulin secretion (Blum et al., 2012; Hayek and Beattie, 1997; Hrvatin et al., 2014). The phenotypes of the ES derived endocrine cells suggest that they are more similar to fetal β cells than an adult β cell.

EP cells are differentiated into β-like cells under the same protocols used in the original ES cell differentiation to endocrine cells, with differentiated initiated at the primitive gut tube stage. Differentiation of EP cells results in insulin monohormal and glucose responsive β-like cells. Although the functionality of the β-like cells did not completely

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replicate primary human islets, their response during perifusion is as good or better when compared to the functionality of ES derived β cells from any other published protocols, especially by perifusion assay (Pagliuca et al., 2014; Rezania et al., 2014). We have found that the passaging of EP cells is critical for this to occur. Differentiation of low passage EP cells results in a subpopulation of polyhormonal β-like cells co-expressing glucagon and insulin with poor glucose responsiveness. The percentage of co-expression gradually decreases and glucose responsiveness improves as the EP cells are passaged for longer periods of time (data not shown). We hypothesize that maintaining EP cells in culture and allowing their expansion could be permitting epigenetic changes to occur in the EP cells that are important for their later differentiation to glucose responsive β-like cells. To test this, chIP-sequencing can be done on the cells to examine active and repressive markers on chromatin. Additionally, bisulfide sequencing for observation of DNA methylation can also be performed. Using these techniques changes in low passage EP cells compared to high EP passage EP cells can be studied. These cells can also be compared to ES derived endocrine cells and ES derived β cells from the newer protocols.

6.2 Culturing of EP cells allows for their maturation

During human development, the first insulin positive endocrine cells begin to appear at approximately 7 weeks of fetal age (Jennings et al., 2015; Piper et al., 2004). The original ES differentiation protocols take approximately 18 days (D'Amour et al., 2006), a relatively condensed amount of time compared to in vivo development. The deficiencies that result in endocrine cells differentiated with this protocol may be due to the insufficient time available for the appropriate maturation of the cells. Although using the same differentiation protocol, EP cells may be able to overcome some of these

deficiencies as they have been expanded as an endodermal stem cell population, which may allow for the maturation of the endoderm.

More recently, newer protocols for the differentiation of ES cells have been described. These protocols take 30 to 45 days to achieve a β cell that has improved glucose responsiveness with a subset of the differentiated cells monohormonal for insulin with the co-expression of NKX6.1 (Pagliuca et al., 2014; Rezania et al., 2014). This longer differentiation protocol may allow more time for the cells to mature resulting in a functionally superior β cell compared to the original differentiation protocols. In vivo, adult β-like cells develop from a second wave of endocrine cell development that occurs once the cells have begun to express NKX6.1 (Nostro and Keller, 2012; Rieck et al., 2012). The newer ES based protocols may result in superior β cells compared to the original ES cell protocols as they more closely mimic what occurs during in vivo development. With the newer differentiation protocols, NKX6.1 and PDX1 cells are first derived then further differentiated to insulin positive cells more closely following what occurs in vivo. To improve upon the limitations of the current EP derived β-like cells, the adaptation and optimization of these newer protocols to EP cells may result in β-like cells with enhanced functionality and improved maturity.

6.3 The Lack of NKX6.1 Expression in EP derived β-like cells

It is interesting that EP derived β-like cells are glucose responsive despite a lack of NKX6.1 expression. Adult mice that have loss NKX6.1 expression become diabetic and have decreased insulin content, however isolated islets remain glucose responsive in vitro (Taylor et al., 2013). It is possible that NKX6.1 is not necessary for functionality in vitro but is necessary for in vivo for blood glucose homeostasis. To determine whether

this is the case, EP cells could be targeted in AAVS1 locus with a construct containing

NKX6.1 driven by an inducible promoter. β-like cells with and without NKX6.1 expression

could then be transplanted into the kidney capsule of immune compromised mice. Their

functionality in vivo can then be examined by glucose tolerance test. If shown to be

functional, EP derived β-like cells with and without NKX6.1 expression can be

transplanted into streptozotocin induced diabetic mice and their ability to maintain blood

glucose homeostatic in vivo can be tested. One current limitation that would first have to

be addressed in order to do this experiment, is that the current method of differentiation

does not allow for the generation of a sufficient number of EP derived β-like cells for in

vivo transplantation. Methods for scaling up of the differentiation would have to be

explored.

6.4 The Function and Relationship of GATA Family Members

While patient iPS$^{+/indel}$ cells can robustly differentiate to definitive endoderm, mutant

iPS$^{indel/indel}$ and ES$^{indel/indel}$ cell differentiation to definitive endoderm is inhibited. This is

consistent with the patient phenotype; as heterozygous patients have the presence of

other endodermal derived tissues suggesting no defects in endoderm formation (Chao et

al., 2015). We show that re-expression of GATA6 or expression of GATA4 in the

ES$^{indel/indel}$ cells can rescue the definitive endoderm differentiation. It has been shown in

other model organisms that GATA4 and GATA6 can compensate for the loss of one

another (Holtzinger and Evans, 2005; Xuan et al., 2012; Zhao et al., 2008). However, in

humans there have been reports of a few patients who have pancreatic agenesis as a

result of a mutation in GATA4 (Amato et al., 2010; Shaw-Smith et al., 2014) suggesting

that unlike other model systems, human GATA4 and GATA6 may have non-redundant

functionality. In our experiments, we observe that a loss of GATA6 leads to decreased

expression levels of GATA4 during definitive endoderm differentiation. To gain an understanding of the relationship between GATA6 and GATA4, PSCs with a heterozygous and homozygous loss of GATA4 can be generated by use of CRISPR-cas9 genome editing. Differentiation of loss of GATA4 mutant PSCs to definitive endoderm and observation of the impact on expression levels of GATA6 would provide a further understanding of their interactions. Additionally, if a loss of GATA4 leads to decreased efficiency of definitive endoderm differentiation, similar to a loss of GATA6, it could be examined whether expression of GATA6 in GATA4 mutants can rescue the phenotype.

In addition to GATA6 and GATA4, we found that GATA1 and GATA3 can also rescue the differentiation to definitive endoderm. It is possible that this rescue could be due to these GATA factors acting through GATA4 as there is up-regulation of endogenous GATA4. To investigate if GATA1 and GATA3 can directly rescue definitive endoderm differentiation, a cell line with the double knockout of both GATA4 and GATA6 could be created by using CRISPR-cas9 genome editing. We expect that with a double knockout, the cells will fail to differentiate to definitive endoderm. Expression of GATA1 or GATA3 can then be introduced during definitive endoderm differentiation and examined to see if they can rescue the differentiation in the absence both GATA4 and GATA6.

6.5 Discrepancies in Phenotypes of GATA6 Mutations

The phenotypes that we observed in our differentiation of human PSCs to definitive endoderm and β-like cells are different to those that are observed in mouse models with a heterozygous and homozygous loss of GATA6. In mouse models GATA6 null cells were shown to contribute to the endoderm and primitive gut tube by tetraploid

complementation (Koutsourakis et al., 1999; Zhao et al., 2005). One reason for this may be the differences of patient specific mutations compared to loss of function alleles in mouse models. While knockout mice are generated by deletion of multiple exons of GATA6 resulting in unstable mRNA which is lost (Morrisey et al., 1998; Sodhi et al., 2006), human patients often have point mutations or small insertions/deletions leading to the presence of a truncated proteins (Lango Allen et al., 2012). In some cases, the truncated protein may be non-functional and/or lost due to mRNA degradation and reflect the knockout mouse models. However, in other cases, the truncated protein may have a dominant-negative effect and thus mutant GATA6 mice may not be representative of the human condition. One way to address these discrepancies and determine whether it is a species specific difference is to generate mouse models with the same heterozygous GATA6 mutations as the human patient. It can then be observed if the same phenotypes occur with the same mutations across different species.

Alternatively, the phenotypes that we observe may be due to differences between the in vitro and in vivo systems. To study whether the phenotypes that are observed in human PSCs are due these differences, mouse PSCs with a heterozygous and homozygous loss of GATA6 that have previously been generated in other labs (Sodhi et al., 2006) can be obtained and differentiated in vitro following a similar protocol.

Utilizing the in vitro differentiation system patient $iPS^{+/indel}$ and $ES^{indel/indel}$ EP cells could be differentiated to β-like cells, however, compared to their respective wild type control cells from the same genetic background, absolute β-like cell yield from $iPS^{+/indel}$ and $ES^{indel/indel}$ was significantly lower. While these findings do not fully recapitulate the patient phenotype of pancreas agenesis, a decrease in β-cell mass hints at a defect in β-

cell development. We find that decreased concentrations of retinoic acid can lead to defective β-like cell differentiation in the patient iPS$^{+/indel}$ cells. During in vitro differentiation retinoic acid may be supplied at a much higher concentration than conditions that are found in vivo overcoming any deficiencies that result from a loss of GATA6. An alternate way to study the potential of GATA6 mutant cells is by transplantation of endoderm progenitor cells or pancreatic progenitor cells into immune compromised mice. By allowing spontaneous differentiation to occur without exogenous inductive signals driving the cells toward a β cell fate, it can be determine whether a loss of GATA6 affects the ability of these cells to differentiation to a functional β cell.

6.6 Down-regulation of HNF4α in GATA6 mutant β-like cells

Heterozygous mutations in HNF4α cause MODY1, one of the types of monogenic diabetes (Steck and Winter, 2011). It is a possible that the functional defects observed in GATA6 mutant β-like cells are due to the decreased expression levels of HNF4α. Embryoid bodies formed by spontaneous differentiation of mouse GATA6 homozygous null ES cells fail to express HNF4α which is one of the genes that is highly upregulated in wild type embryoid bodies (Morrisey et al., 1998). Moreover, in vitro studies in the mouse INS-1 cell line show that in the presence of GATA6 with patient specific mutations, GATA6 fails to bind the HNF4α promoter (Lango Allen et al., 2012) and the promoter is inactive (Chao et al., 2015). To address whether decreased levels of HNF4α is the cause of the functional defects in the GATA6 mutant β-like cells, HNF4α can be re-expressed in iPS$^{+/indel}$ and iPS$^{indel/indel}$ by targeting the AAVS1 safe harbor locus. If re-expression of HNF4α can rescue glucose responsiveness in the GATA6 mutant β-like

cells this would suggest that the loss of functionality in these cells is due to decreased levels of HNF4α.

6.7 Downstream targets of GATA6

To gain a better understanding of the mechanisms that underlie the functionality of human β cells, it would be beneficial to characterize and further explore the molecular pathways that function downstream of GATA6 in β cells. Genome-wide transcriptome analysis by RNA-seq of the differentiated β-like cells can be performed. β-like cells for can be purified by either utilization of the Mel1-INS-GFP reporter cell line or cell sorting of differentiated cells that have been paraformaldehyde fixed and stained for insulin. By comparison of genes that are differentially regulated in GATA6$^{+/+}$, GATA6$^{+/indel}$ and GATA6$^{indel/indel}$ β-like cells, we can investigate the mechanisms by which a loss of GATA6 leads to a functional defect.

6.8 Overcoming the limitations of EP derived β-like cells

The use of EP cells as a progenitor cell population for β-like cells is advantageous over other β cell differentiation protocols as it allows us to overcome the developmental block at the definitive endoderm stage in the ES$^{indel/indel}$ cells. However, one deficiency of EP derived β-like cells is that they do not express NKX6.1. Hence, a concern of using EP derived β-like cells is that we are studying a phenotype which results from the combined loss of both GATA6 and NKX6.1. To confirm that the phenotype observed in EP derived β-like cells is reliable, GATA6 mutant cells can be differentiated with other β cell differentiation protocols. However, without further genetic manipulation this would be

difficult with the ES^{indel/indel} and iPS^{indel/indel} cells due to the developmental block at the definitive endoderm stage.

Although lenti viruses could be used to rescue the phenotype during PSC to definitive endoderm differentiation, this strategy is not feasible for long-term differentiations as lenti viruses are silenced. One way to overcome the developmental block is to generate wild type PSCs with the endogenous GATA6 surround by loxP sites. The AAVS1 locus can be targeted with a CRE recombinase driven by the insulin promoter. Upon differentiation to insulin positive cells, GATA6 would then be loss through cre-mediated deletion. Alternatively, an inducible GATA6 construct could be targeted into the AAVS1 safe harbor locus of the ES^{indel/indel} and iPS^{indel/indel} cells. The expression of GATA6 can then be modulated to levels similar to wild type during early stages of the differentiation and removed as the differentiation progresses beyond the definitive endoderm stage. In addition, the differentiation of the GATA6 inducible ES^{indel/indel} and iPS^{indel/indel} cells using an alternative β cell differentiation protocol would also give us an opportunity to examine if GATA6 plays a role in regulating the expression of NKX6.1.

6.9 Rescue of Functionality in β-like Cells

In addition to the GATA6 inducible iPS^{indel/indel}, the introduction of an inducible GATA6 construct in the iPS^{+/indel} cell lines will also be useful. While the corrected iPS^{+/+} cells have improved glucose responsiveness confirming that defective functionality is due to a loss of GATA6, it would be beneficial to determine whether increased expression of GATA6 in the loss of function GATA6 mutants could also rescue the other observed phenotype. We would expect that with induced expression of GATA6, both the iPS^{indel/indel} and iPS^{+/indel} cell lines would have increased levels of PDX1 at the pancreatic

progenitor stage as well as normal glucose responsiveness and proinsulin processing upon differentiation to β-like cells. Additionally, inducing GATA6 expression at different stages of the differentiation would also allow us to study when GATA6 is needed. Utilizing this system and assessing for these outcomes, additional cell lines with inducible GATA4 could also be created. These cell lines would provide further insight into the degree of functional redundancy between GATA family members in β cells.

6.10 Exploring other GATA6 patient mutations and phenotypes

In this work, we explore one specific patient mutation. For future work additional patient mutations should be studied as there is incomplete penetrance of GATA6 heterozygous mutations. Patients with the same mutation have been shown to have a range in severity of phenotypes (Bonnefond et al., 2012; De Franco et al., 2013; Yu et al., 2014). One reason for this may be from differences in genetic background, with some genetic backgrounds resulting in a higher predisposition for a more severe phenotype. GATA4 is another gene which results in pancreas agenesis with varying penetrance (Amato et al., 2010; Shaw-Smith et al., 2014). Mouse model studies of a heterozygous loss of GATA4 in different strains examined for cardiac phenotypes had varying penetrance from no disease pathology to severe cardiac defects suggesting that different genetic backgrounds may play a role in penetrance (Rajagopal et al., 2007). To study whether genetic background variations affects penetrance, a greater number of patient samples could be obtained from heterozygous GATA6 patients with pancreas agenesis and also from adult patients who have diabetes or pancreas phenotype. Ideally, the same mutation with differing phenotypes in different patients would provide the most insight. From these samples iPS cells could be generated and differentiated to β cells. If genetic background did play a role, we would hypothesize that iPS cell lines generated from

samples with heterozygous GATA6 mutations that did not have a phenotype would be glucose responsive and express higher levels of PDX1 than those that did have a pancreatic phenotype.

Furthermore, an understanding of the mechanisms that regulate penetrance could be studied with these additional patient samples. We show that GATA4 is able to compensate for a loss of GATA6 during definitive endoderm differentiation. Expression levels of GATA4 could be examined in these additional patient samples at the pancreatic progenitor and the β-like cell stage. This would allow us to investigate whether GATA4 compensates for a loss of GATA6 in patients with milder pancreas phenotype. Additionally, variability of retinoic acid signaling could be examined across patient samples of different genetic backgrounds to determine whether this plays a role in the penetrance. By titrating different concentrations of retinoic acid during β-like cell differentiation, we would expect that patient samples from patients with pancreatic agenesis will be more sensitive to reduced concentrations.

In addition to pancreatic agenesis, GATA6 heterozygous patients often have other defects including biliary atresia and heart malformations (Chao et al., 2015). Further studies of these phenotypes can be undertaken by differentiation of PSCs to cholangiocytes and cardiomyocytes. These experiments will give us insight into whether in these other tissues GATA6 plays a cell intrinsic or extrinsic role in their specification and functionality. Additionally, it will provide an opportunity to examine the role of GATA6 during the development of these tissues.

BIBLIOGRAPHY

Alarcón, C., Wicksteed, B., Rhodes, C.J., 2002. Regulation of the production and secretion of insulin. Av. en Diabetol. 18, 168–174.

Amato, E., Giacopelli, F., Giannattasio, A., Annunzio, G., Bocciardi, R., Musso, M., Lorini, R., Ravazzolo, R., 2010. Genetic investigation in an Italian child with an unusual association of atrial septal defect, attributable to a new familial GATA4 gene mutation, and neonatal diabetes due to pancreatic agenesis. Diabet. Med. 27, 1195–1200. doi:10.1111/j.1464-5491.2010.03046.x

American Diabetes Association, 2016. Classification and Diagnosis of Diabetes. Diabetes Care 39(Suppl. , S13–S22. doi:10.2337/dc15-S005

Anik, A., Catli, G., Abaci, A., Bober, E., 2015. Maturity-onset diabetes of the young (MODY): An update. J. Pediatr. Endocrinol. Metab. 28, 251–263. doi:10.1515/jpem-2014-0384

Arceci, R.J., King, a a, Simon, M.C., Orkin, S.H., Wilson, D.B., 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol. Cell. Biol. 13, 2235–2246. doi:10.1128/MCB.13.4.2235.Updated

Atkinson, M.A., 2014. Losing a grip on the notion of beta-cell specificity for immune responses in type 1 diabetes: Can we handle the truth? Diabetes 63, 3572–3574. doi:10.2337/db14-1069

Atkinson, M.A., 2012. The Pathogenesis and Natural History of Type 1 Diabetes. Cold Spring Harb. Perspect. Med. 2.

Atkinson, M.A., Eisenbarth, G.S., Michels, A.W., 2014. Type 1 diabetes. Lancet 383, 69–82. doi:10.1016/S0140-6736(13)60591-7

Banting, F.G., Best, C.H., Collip, J.B., Campbell, W.R., Fletcher, A.A., 1922. Pancreatic extracts in the treatment of diabetes mellitus. Can. Med. Assoc. J. 12, 141–6. doi:10.1097/00005053-192401000-00013

Banting FG, Best CH, Collip JB, Cambell WR, Fletcher AA, Macleod JJR, N.E., 1922. The Effect Produced on Diabetes by Extractions of Pancreas. Trans. Assoc. Am. Physicians 37, 337–347.

Basford, C.L., Prentice, K.J., Hardy, a B., Sarangi, F., Micallef, S.J., Li, X., Guo, Q., Elefanty, a G., Stanley, E.G., Keller, G., Allister, E.M., Nostro, M.C., Wheeler, M.B., 2012. The functional and molecular characterisation of human embryonic stem cell-derived insulin-positive cells compared with adult pancreatic beta cells. Diabetologia 55, 358–71. doi:10.1007/s00125-011-2335-x

Benazra, M., Lecomte, M.J., Colace, C., Müller, A., Machado, C., Pechberty, S., Bricout-Neveu, E., Grenier-Godard, M., Solimena, M., Scharfmann, R., Czernichow, P., Ravassard, P., 2015. A human beta cell line with drug inducible excision of immortalizing transgenes. Mol. Metab. 4, 916–925. doi:10.1016/j.molmet.2015.09.008

Bilous, R., Donnelly, R., 2010. Handbook of Diabetes, 4th ed. Wiley-Blackwell, West Sussex.

Blum, B., Hrvatin, S.S.Š., Schuetz, C., Bonal, C., Rezania, A., Melton, D. a, 2012. Functional beta-cell maturation is marked by an increased glucose threshold and by expression of urocortin 3. Nat. Biotechnol. 30, 261–4. doi:10.1038/nbt.2141

Bonnefond, a, Sand, O., Guerin, B., Durand, E., De Graeve, F., Huyvaert, M., Rachdi,

L., Kerr-Conte, J., Pattou, F., Vaxillaire, M., Polak, M., Scharfmann, R., Czernichow, P., Froguel, P., 2012. GATA6 inactivating mutations are associated with heart defects and, inconsistently, with pancreatic agenesis and diabetes. Diabetologia 55, 2845–7. doi:10.1007/s00125-012-2645-7

Borok, M.J., Papaioannou, V.E., Sussel, L., 2015. Unique functions of Gata4 in mouse liver induction and heart development. Dev. Biol. 1–10. doi:10.1016/j.ydbio.2015.12.007

Bresnick, E.H., Katsumura, K.R., Lee, H.Y., Johnson, K.D., Perkins, A.S., 2012. Master regulatory GATA transcription factors: Mechanistic principles and emerging links to hematologic malignancies. Nucleic Acids Res. 40, 5819–5831. doi:10.1093/nar/gks281

Brewer, a., Gove, C., Davies, a., McNulty, C., Barrow, D., Koutsourakis, M., Farzaneh, F., Pizzey, J., Bomford, a., Patient, R., 1999. The Human and Mouse GATA-6 Genes Utilize Two Promoters and Two Initiation Codons. J. Biol. Chem. 274, 38004–38016. doi:10.1074/jbc.274.53.38004

Brissova, M., Fowler, M.J., Nicholson, W.E., Chu, A., Hirshberg, B., Harlan, D.M., Powers, A.C., 2005. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. J. Histochem. Cytochem. 53, 1087–97. doi:10.1369/jhc.5C6684.2005

Carrasco, M., Delgado, I., Soria, B., Martín, F., Rojas, A., 2012. GATA4 and GATA6 control mouse pancreas organogenesis. J. Clin. Invest. 122, 3504–3515. doi:10.1172/JCI63240DS1

Carroll, D., 2011. Genome engineering with zinc-finger nucleases. Genetics 188, 773–782. doi:10.1534/genetics.111.131433

Centers for Disease Control Prevention, 2015. Diabetes 2014 Report Card. Atlanta, GA.

Chao, C.S., McKnight, K.D., Cox, K.L., Chang, A.L., Kim, S.K., Feldman, B.J., 2015. Novel GATA6 Mutations in Patients with Pancreatic Agenesis and Congenital Heart Malformations. PLoS One 10, e0118449. doi:10.1371/journal.pone.0118449

Cheng, X., Tiyaboonchai, A., Gadue, P., 2013. Endodermal stem cell populations derived from pluripotent stem cells. Curr. Opin. Cell Biol. 25, 265–271. doi:10.1016/j.ceb.2013.01.006

Cheng, X., Ying, L., Lu, L., Galvão, A.M., Mills, J. a, Lin, H.C., Kotton, D.N., Shen, S.S., Nostro, M.C., Choi, J.K., Weiss, M.J., French, D.L., Gadue, P., 2012. Self-renewing endodermal progenitor lines generated from human pluripotent stem cells. Cell Stem Cell 10, 371–84. doi:10.1016/j.stem.2012.02.024

Cleaver, O., Melton, D.A., 2004. Development of the Endocrine Pancreas, in: Kahn, C.R., Weir, G.C., King, G.L., Jacobson, A.M., Moses, A.C., Smith, R.J. (Eds.), Joslin's Diabetes Mellitus. Lippincott Williams & Wilkins, Boston, pp. 21–40.

Czysz, K., Minger, S., Thomas, N., 2015. DMSO Efficiently Down Regulates Pluripotency Genes in Human Embryonic Stem Cells during Definitive Endoderm Derivation and Increases the Proficiency of Hepatic Differentiation. PLoS One 10, e0117689. doi:10.1371/journal.pone.0117689

D'Amour, K. a, Agulnick, A.D., Eliazer, S., Kelly, O.G., Kroon, E., Baetge, E.E., 2005. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat. Biotechnol. 23, 1534–1541. doi:10.1038/nbt1163

D'Amour, K. a, Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M. a, Kroon, E., Carpenter, M.K., Baetge, E.E., 2006. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat. Biotechnol. 24, 1392–401. doi:10.1038/nbt1259

Davidson, H.W., Wenzlau, J.M., O'Brien, R.M., 2014. Zinc transporter 8 (ZnT8) and β

cell function. Trends Endocrinol. Metab. 25, 415–424. doi:10.1016/j.tem.2014.03.008

Davidson, K.C., Mason, E. a., Pera, M.F., 2015. The pluripotent state in mouse and human. Dev. (Cambridge, UK) 142, 3090–3099. doi:10.1242/dev.116061

De Franco, E., Shaw-Smith, C., Flanagan, S.E., Shepherd, M.H., Hattersley, A.T., Ellard, S., 2013. GATA6 mutations cause a broad phenotypic spectrum of diabetes from pancreatic agenesis to adult-onset diabetes without exocrine insufficiency. Diabetes 62, 993–7. doi:10.2337/db12-0885

De Krijger, R.R., Aanstoot, H.J., Kranenburg, G., Reinhard, M., Visser, W.J., Bruining, G.J., 1992. The midgestational human fetal pancreas contains cells coexpressing islet hormones. Dev. Biol. 153, 368–375. doi:10.1016/0012-1606(92)90121-V

De Vos, A., Heimberg, H., Quartier, E., Huypens, P., Bouwens, L., Pipeleers, D., Schuit, F., 1995. Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. J. Clin. Invest. 96, 2489–2495. doi:10.1172/JCI118308

Decker, K., Goldman, D.C., Grasch, C.L., Sussel, L., 2006. Gata6 is an important regulator of mouse pancreas development. Dev. Biol. 298, 415–29. doi:10.1016/j.ydbio.2006.06.046

DeKelver, R.C., Choi, V.M., Moehle, E. a, Paschon, D.E., Hockemeyer, D., Meijsing, S.H., Sancak, Y., Cui, X., Steine, E.J., Miller, J.C., Tam, P., Bartsevich, V. V, Meng, X., Rupniewski, I., Gopalan, S.M., Sun, H.C., Pitz, K.J., Rock, J.M., Zhang, L., Davis, G.D., Rebar, E.J., Cheeseman, I.M., Yamamoto, K.R., Sabatini, D.M., Jaenisch, R., Gregory, P.D., Urnov, F.D., 2010. Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. Genome Res. 20, 1133–42. doi:10.1101/gr.106773.110

Edghill, E.L., Dix, R.J., Flanagan, S.E., Bingley, P.J., Hattersley, A.T., Ellard, S., Gillespie, K.M., 2006. HLA genotyping supports a nonautoimmune etiology in patients diagnosed with diabetes under the age of 6 months. Diabetes 55, 1895–1898. doi:10.2337/db06-0094

Evans, M., 1981. Origin of mouse embryonal carcinoma cells and the possibility of their direct isolation into tissue culture. J. Reprod. Fertil. 62, 625–631. doi:10.1530/jrf.0.0620625

Evans, M.J., Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156. doi:10.1038/292154a0

Fu, Z., Gilbert, E.R., Liu, D., 2013. Regulation of insulin synthesis and secretion and pancreatic β-cell dysfunction in diabetes. Curr. Diabetes Rev. 9, 25–53. doi:10.2174/157339913804143225

Gadue, P., Huber, T.L., Nostro, M.C., Kattman, S., Keller, G.M., 2005. Germ layer induction from embryonic stem cells. Exp. Hematol. 33, 955–964. doi:10.1016/j.exphem.2005.06.009

Gadue, P., Huber, T.L., Paddison, P.J., Keller, G.M., 2006. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 103, 16806–16811. doi:10.1073/pnas.0603916103

Gaj, T., Gersbach, C. a, Barbas, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 1–9. doi:10.1016/j.tibtech.2013.04.004

Garçon, L., Ge, J., Manjunath, S.H., Mills, J. a, Apicella, M., Parikh, S., Sullivan, L.M., Podsakoff, G.M., Gadue, P., French, D.L., Mason, P.J., Bessler, M., Weiss, M.J.,

2013. Ribosomal and hematopoietic defects in induced pluripotent stem cells derived from Diamond Blackfan anemia patients. Blood 122, 912–21. doi:10.1182/blood-2013-01-478321

Gerich, J.E., 2002. Is Reduced First-Phase Insulin Release the Earliest Detectable Abnormality in Individuals Destined to Develop Type 2 Diabetes? Diabetes 51, 117–121.

Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, a, Carpenter, M.K., Itskovitz-Eldor, J., Rao, M.S., 2004. Differences between human and mouse embryonic stem cells. Dev Biol 269, 360–380. doi:10.1016/j.ydbio.2003.12.034\rS0012160604000193 [pii]

Girlovanu, M., Susman, S., Soritau, O., Rus-Ciuca, D., Melincovici, C., Constantin, A.-M., Mihu, C.M., 2015. Stem Cells - biological update and cell therapy progress. Clujul Med. 88, 265. doi:10.15386/cjmed-483

Gittes, G.K., 2009. Developmental biology of the pancreas: a comprehensive review. Dev. Biol. 326, 4–35. doi:10.1016/j.ydbio.2008.10.024

Greber, B., Coulon, P., Zhang, M., Moritz, S., Frank, S., Müller-Molina, A.J., Araúzo-Bravo, M.J., Han, D.W., Pape, H.-C., Schöler, H.R., 2011. FGF signalling inhibits neural induction in human embryonic stem cells. EMBO J. 30, 4874–4884. doi:10.1038/emboj.2011.407

Greeley, S.A.W., Tucker, S.E., Worrell, H.I., Skowron, K.B., Bell, G.I., Philipson, L.H., 2010. Update in neonatal diabetes. Curr. Opin. Endocrinol. Diabetes. Obes. 17, 13–19. doi:10.1097/MED.0b013e328334f158

Grimmsmann, T., Rustenbeck, I., 1998. Direct effects of diazoxide on mitochondria in pancreatic B-cells and on isolated liver mitochondria. Br. J. Pharmacol. 123, 781–788. doi:10.1038/sj.bjp.0701663

Gurdon, J.B., 1962. The Developmental Capacity of Nuclei taken from Intestinal Epithelium Cells of Feeding Tadpoles. J. embrology Exp. Morphol. 10, 622–641.

Hang, Y., Stein, R., 2011. MafA and MafB activity in pancreatic beta cells. Trends Endocrinol. Metab. doi:10.1016/j.tem.2011.05.003

Hatzistavrou, T., Micallef, S.J., Ng, E.S., Vadolas, J., Stanley, E.G., Elefanty, A.G., 2009. ErythRED, a hESC line enabling identification of erythroid cells. Nat. Methods 6, 659–662. doi:10.1038/nmeth.1364

Hayek, A., Beattie, G.M., 1997. Experimental transplantation of human fetal and adult pancreatic islets. J. Clin. Endocrinol. Metab. 82, 2471–2475. doi:10.1210/jcem.82.8.4151

He, S., Nakada, D., Morrison, S.J., 2009. Mechanisms of stem cell self-renewal. Annu. Rev. Cell Dev. Biol. 25, 377–406. doi:10.1146/annurev.cellbio.042308.113248

Henquin, J., Nenquin, M., Stiernet, P., Ahren, B., 2006. In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: pattern and role of cytoplasmic Ca2+ and amplification signals in beta-cells. Diabetes 55, 441–451.

Henquin, J.C., 2009. Regulation of insulin secretion: A matter of phase control and amplitude modulation. Diabetologia 52, 739–751. doi:10.1007/s00125-009-1314-y

Henquin, J.C., 2000. Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes 49, 1751–1760.

Henseleit, K.D., Nelson, S.B., Kuhlbrodt, K., Hennings, J.C., Ericson, J., Sander, M., 2005. NKX6 transcription factor activity is required for alpha- and beta-cell development in the pancreas. Development 132, 3139–49. doi:10.1242/dev.01875

Herrera, P.L., 2000. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Dev. (Cambridge, UK) 127, 2317–2322.

Herrera, P.L., Huarte, J., Zufferey, R., Nichols, A., Mermillod, B., Philippe, J., Muniesa,

P., Sanvito, F., Orci, L., Vassalli, J.D., 1994. Ablation of islet endocrine cells by targeted expression of hormone-promoter-driven toxigenes. Proc. Natl. Acad. Sci. U. S. A. 91, 12999–3003. doi:10.1073/pnas.91.26.12999

Hilderink, J., Spijker, S., Carlotti, F., Lange, L., Engelse, M., van Blitterswijk, C., de Koning, E., Karperien, M., van Apeldoorn, A., 2015. Controlled aggregation of primary human pancreatic islet cells leads to glucose-responsive pseudoislets comparable to native islets. J. Cell. Mol. Med. 19, 1836–1846. doi:10.1111/jcmm.12555

Hockemeyer, D., Soldner, F., Beard, C., Gao, Q., Mitalipova, M., DeKelver, R.C., Katibah, G.E., Amora, R., Boydston, E. a, Zeitler, B., Meng, X., Miller, J.C., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., Jaenisch, R., 2009. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat. Biotechnol. 27, 851–7. doi:10.1038/nbt.1562

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., Zeitler, B., Cherone, J.M., Meng, X., Hinkley, S.J., Rebar, E.J., Gregory, P.D., Urnov, F.D., Jaenisch, R., 2011. Genetic engineering of human pluripotent cells using TALE nucleases. Nat. Biotechnol. 29, 731–4. doi:10.1038/nbt.1927

Holtzinger, A., Evans, T., 2005. Gata4 regulates the formation of multiple organs. Development 132, 4005–4014. doi:10.1242/dev.02227

Hosoya-Ohmura, S., Mochizuki, N., Suzuki, M., Ohneda, O., Ohneda, K., Yamamoto, M., 2006. GATA-4 incompletely substitutes for GATA-1 in promoting both primitive and definitive erythropoiesis in vivo. J. Biol. Chem. 281, 32820–32830. doi:10.1074/jbc.M605735200

Hou, J.C., Min, L., Pessin, J.E., 2009. Insulin granule biogenesis, trafficking and exocytosis. Vitam. Horm. 80, 473–506. doi:10.1016/S0083-6729(08)00616-X

Hrvatin, S., O'Donnell, C.W., Deng, F., Millman, J.R., Pagliuca, F.W., DiIorio, P., Rezania, A., Gifford, D.K., Melton, D.A., 2014. Differentiated human stem cells resemble fetal, not adult, β cells. Proc. Natl. Acad. Sci. U. S. A. 111, 3038–43. doi:10.1073/pnas.1400709111

Hsu, P.D., Lander, E.S., Zhang, F., 2014. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278. doi:10.1016/j.cell.2014.05.010

Iafusco, D., Stazi, M.A., Cotichini, R., Cotellessa, M., Martinucci, M.E., Mazzella, M., Cherubini, V., Barbetti, F., Martinetti, M., Cerutti, F., Prisco, F., 2002. Permanent diabetes mellitus in the first year of life. Diabetologia 45, 798–804. doi:10.1007/s00125-002-0837-2

Irion, S., Nostro, M.C., Kattman, S.J., Keller, G.M., 2008. Directed Differentiation of Pluripotent Stem Cells: From Developmental Biology to Therapeutic Applications. Cold Spring Harb. Symp. Quant. Biol. 73, 101–110. doi:10.1101/sqb.2008.73.065

Jennings, R.E., Berry, A.A., Strutt, J.P., Gerrard, D.T., Hanley, N.A., 2015. Human pancreas development. Development 142, 3126–37. doi:10.1242/dev.120063

Johannesson, B., Sui, L., Freytes, D.O., Creusot, R.J., Egli, D., 2015. Toward beta cell replacement for diabetes. EMBO J. 34, 841–55. doi:10.15252/embj.201490685

Kahn, S.E., Cooper, M.E., Del Prato, S., 2014. Pathophysiology and treatment of type 2 diabetes: Perspectives on the past, present, and future. Lancet 383, 1068–1083. doi:10.1016/S0140-6736(13)62154-6

Kim, S., 2015. Maturity-Onset Diabetes of the Young: What Do Clinicians Need to Know? Diabetes Metab. J. 6087, 468–477. doi:10.4093/dmj.2015.39.6.468

Kleinsmith, L.J., Pierce, Jr., G.B., 1964. Multipotentiality of Single Embryonal Carcinoma Cells. Cancer Res. 24, 1544–1551.

Klimchenko, O., Mori, M., DiStefano, A., Langlois, T., Larbret, F., Lecluse, Y., Feraud, O., Vainchenker, W., Norol, F., Debili, N., 2009. A common bipotent progenitor generates the erythroid and megakaryocyte lineages in embryonic stem cell-derived primitive hematopoiesis. Blood 114, 1506–1517. doi:10.1182/blood-2008-09-178863

Koster, J.C., Permutt, M.A., Nichols, C.G., 2005. Diabetes and Insulin Secretion The ATP-Sensitive K+ Channel (KATP) Connection. J. Natl. Med. Assoc. 54, 3065–3072. doi:10.2337/diabetes.52.1.1

Koutsourakis, M., Langeveld, a, Patient, R., Beddington, R., Grosveld, F., 1999. The transcription factor GATA6 is essential for early extraembryonic development. Development 126, 723–32.

Kramer, W., Muller, G., Girbig, F., Gutjahr, U., Kowalewski, S., Hartz, D., Summ -, H.D., 1995. The molecular interaction of sulfonylureas with beta-cell ATP-sensitive K+-channels. Diabetes Res. Clin. Pract. 28, S67–S80. doi:10.1016/0168-8227(95)01076-P

Kroon, E., Martinson, L. a, Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., Cunningham, J., Agulnick, A.D., D'Amour, K. a, Carpenter, M.K., Baetge, E.E., 2008. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat. Biotechnol. 26, 443–52. doi:10.1038/nbt1393

Kubo, A., Shinozaki, K., Shannon, J.M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H.J., Keller, G., 2004. Development of definitive endoderm from embryonic stem cells in culture. Development 131, 1651–1662. doi:10.1242/dev.01044

Kunisada, Y., Tsubooka-Yamazoe, N., Shoji, M., Hosoya, M., 2012. Small molecules induce efficient differentiation into insulin-producing cells from human induced pluripotent stem cells. Stem Cell Res. 8, 274–284. doi:10.1016/j.scr.2011.10.002

Lacy, P.E., Walker, M.M., Fink, C.J., 1972. Perifusion of isolated rat islets in vitro. Participation of the microtubular system in the biphasic release of insulin. Diabetes 21, 987–998.

Lamb, M., Storrs, R., Li, S., Liang, O., Laugenour, K., Dorian, R., Chapman, D., Ichii, H., Imagawa, D., Foster, C., King, S., Lakey, J.R.T., 2011. Function and viability of human islets encapsulated in alginate sheets: In vitro and in vivo culture. Transplant. Proc. 43, 3265–3266. doi:10.1016/j.transproceed.2011.10.028

Lango Allen, H., Flanagan, S.E., Shaw-Smith, C., De Franco, E., Akerman, I., Caswell, R., Ferrer, J., Hattersley, A.T., Ellard, S., 2012. GATA6 haploinsufficiency causes pancreatic agenesis in humans. Nat. Genet. 44, 20–2. doi:10.1038/ng.1035

Lemaire, K., Ravier, M. a, Schraenen, a, Creemers, J.W.M., Van de Plas, R., Granvik, M., Van Lommel, L., Waelkens, E., Chimienti, F., Rutter, G. a, Gilon, P., in't Veld, P. a, Schuit, F.C., 2009. Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. Proc. Natl. Acad. Sci. U. S. A. 106, 14872–7. doi:10.1073/pnas.0906587106

Lin, Y., Sun, Z., 2010. Current views on type 2 diabetes. J. Endocrinol. 204, 1–11. doi:10.1677/JOE-09-0260

Liu, C., Morrisey, E.E., Whitsett, J. a, 2002. GATA-6 is required for maturation of the lung in late gestation. Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L468–L475. doi:10.1152/ajplung.00044.2002

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013. RNA-guided human genome engineering via Cas9. Science (80-.). 339, 823–826. doi:10.1126/science.1232033

Martin, G.R., 1981. Isolation of a pluripotent cell line from early mouse embryos cultured

in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U. S. A. 78, 7634–7638. doi:10.1073/pnas.78.12.7634

Martinelli, P., Cañamero, M., del Pozo, N., Madriles, F., Zapata, A., Real, F.X., 2013. Gata6 is required for complete acinar differentiation and maintenance of the exocrine pancreas in adult mice. Gut 62, 1481–8. doi:10.1136/gutjnl-2012-303328

Mauney, J.R., Ramachandran, A., Yu, R.N., Daley, G.Q., Adam, R.M., Estrada, C.R., 2010. All-trans retinoic acid directs urothelial specification of murine embryonic stem cells via GATA4/6 signaling mechanisms. PLoS One 5. doi:10.1371/journal.pone.0011513

McCulloch, L.J., van de Bunt, M., Braun, M., Frayn, K.N., Clark, A., Gloyn, A.L., 2011. GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: Implications for understanding genetic association signals at this locus. Mol. Genet. Metab. 104, 648–653. doi:10.1016/j.ymgme.2011.08.026

McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, a J., Wu, G.E., Paige, C.J., Maki, R. a, 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. 15, 5647–58.

Micallef, S.J., Li, X., Schiesser, J. V, Hirst, C.E., Yu, Q.C., Lim, S.M., Nostro, M.C., Elliott, D. a, Sarangi, F., Harrison, L.C., Keller, G., Elefanty, a G., Stanley, E.G., 2012. INS(GFP/w) human embryonic stem cells facilitate isolation of in vitro derived insulin-producing cells. Diabetologia 55, 694–706. doi:10.1007/s00125-011-2379-y

Mills, J. a, Wang, K., Paluru, P., Ying, L., Lu, L., Galvão, A.M., Xu, D., Yao, Y., Sullivan, S.K., Sullivan, L.M., Mac, H., Omari, A., Jean, J.-C., Shen, S., Gower, A., Spira, A., Mostoslavsky, G., Kotton, D.N., French, D.L., Weiss, M.J., Gadue, P., 2013. Clonal genetic and hematopoietic heterogeneity among human-induced pluripotent stem cell lines. Blood 122, 2047–51. doi:10.1182/blood-2013-02-484444

Mills, J.A., Paluru, P., Weiss, M.J., Gadue, P., French, D.L., 2014. Hematopoietic differentiation of pluripotent stem cells in culture. Methods Mol. Biol. 1185, 181–195. doi:10.1097/00024382-200207000-00018

Mitchell, R.K., Hu, M., Chabosseau, P.L., Cane, M.C., Meur, G., Bellomo, E.A., Carzaniga, R., Collinson, L.M., Li, W.-H., Hodson, D.J., Rutter, G.A., 2016. Molecular Genetic Regulation of *Slc30a8* /ZnT8 Reveals a Positive Association With Glucose Tolerance. Mol. Endocrinol. 30, 77–91. doi:10.1210/me.2015-1227

Molkentin, J.D., 2000. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J. Biol. Chem. 275, 38949–52. doi:10.1074/jbc.R000029200

Moriguchi, T., Yamamoto, M., 2014. A regulatory network governing Gata1 and Gata2 gene transcription orchestrates erythroid lineage differentiation. Int. J. Hematol. 417–424. doi:10.1007/s12185-014-1568-0

Morrisey, E.E., Ip, H.S., Tang, Z., Lu, M.M., Parmacek, M.S., 1997. GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. Dev. Biol. 183, 21–36. doi:10.1006/dbio.1996.8485

Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S., Parmacek, M.S., 1998. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev. 12, 3579–90.

Murphy, R., Ellard, S., Hattersley, A.T., 2008. Clinical implications of a molecular genetic classification of monogenic beta-cell diabetes. Nat. Clin. Pract. Endocrinol. Metab. 4, 200–213. doi:10.1038/ncpendmet0778

Murry, C.E., Keller, G., 2008. Differentiation of embryonic stem cells to clinically relevant

populations: lessons from embryonic development. Cell 132, 661–80. doi:10.1016/j.cell.2008.02.008

Nakao, A., Takeda, T., Hisaeda, Y., Hirota, A., 2013. Pancreatic Agenesis with Congenital Diaphragmatic Hernia and Congenital Heart Disease : A Case Report. Am. J. Perinat. Reports 3, 119–121.

Newsholme, P., Brennan, L., Bender, K., 2006. Amino Acid Metabolism, beta-cell Function, and Diabetes. Diabetes 55, S39–S47. doi:10.2337/db06-S006

Nolan, C.J., Damm, P., Prentki, M., 2011. Type 2 diabetes across generations: From pathophysiology to prevention and management. Lancet 378, 169–181. doi:10.1016/S0140-6736(11)60614-4

Nostro, M.C., Keller, G., 2012. Generation of beta cells from human pluripotent stem cells: Potential for regenerative medicine. Semin. Cell Dev. Biol. 23, 701–10. doi:10.1016/j.semcdb.2012.06.010

Nostro, M.C., Sarangi, F., Ogawa, S., Holtzinger, A., Corneo, B., Li, X., Micallef, S.J., Park, I.-H., Basford, C., Wheeler, M.B., Daley, G.Q., Elefanty, a. G., Stanley, E.G., Keller, G., 2011. Stage-specific signaling through TGF family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Dev. (Cambridge, UK) 138, 1445–1445. doi:10.1242/dev.065904

O'Donnell, E.R., ZImmerman, C., Kenney, D., Rosen, F.S., 1987. Expression on Blood Cells of Sialophorin, the Surface Glycoprotein that is Defective in Wiskott-Aldrich Syndrome. Blood 70, 104–109.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H., Yamanaka, S., 2011. A more efficient method to generate integration-free human iPS cells. Nat. Methods 8, 409–412. doi:10.1038/nmeth.1591

Olokoba, A.B., Obateru, O.A., Olokoba, L.B., 2012. Type 2 diabetes mellitus: A review of current trends. Oman Med. J. 27, 269–273. doi:10. 5001/omj.2012.68

Orkin, S.H., 1995. Transcription factors and hematopoietic development. J. Biol. Chem. doi:10.1074/jbc.270.10.4955

Padua, M.B., Fox, S.C., Jiang, T., Morse, D. a, Tevosian, S.G., 2014. Simultaneous gene deletion of gata4 and gata6 leads to early disruption of follicular development and germ cell loss in the murine ovary. Biol. Reprod. 91, 24. doi:10.1095/biolreprod.113.117002

Padua, M.B., Jiang, T., Morse, D.A., Fox, S.C., Hatch, H.M., Tevosian, S.G., 2015. Combined loss of the GATA4 and GATA6 transcription factors in male mice disrupts testicular development and confers adrenal-like function in the testes. Endocrinology 156, 1873–1886. doi:10.1210/en.2014-1907

Pagliuca, F.W., Melton, D. a, 2013. How to make a functional β-cell. Development 140, 2472–83. doi:10.1242/dev.093187

Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., Melton, D.A., 2014. Generation of Functional Human Pancreatic β Cells In Vitro. Cell 159, 428–439. doi:10.1016/j.cell.2014.09.040

Paluru, P., Hudock, K.M., Cheng, X., Mills, J. a, Ying, L., Galvão, A.M., Lu, L., Tiyaboonchai, A., Sim, X., Sullivan, S.K., French, D.L., Gadue, P., 2013. The negative impact of wnt signaling on megakaryocyte and primitive erythroid progenitors derived from human embryonic stem cells. Stem Cell Res. 12, 441–451. doi:10.1016/j.scr.2013.12.003

Pan, F.C., Wright, C., 2011. Pancreas organogenesis: From bud to plexus to gland. Dev. Dyn. 240, 530–565. doi:10.1002/dvdy.22584

Pellegrini, S., Cantarelli, E., Sordi, V., Nano, R., Piemonti, L., 2016. The state of the art of islet transplantation and cell therapy in type 1 diabetes. Acta Diabetol. doi:10.1007/s00592-016-0847-z

Piper, K., Brickwood, S., Turnpenny, L.W., Cameron, I.T., Ball, S.G., Wilson, D.I., Hanley, N. a, 2004. Beta cell differentiation during early human pancreas development. J. Endocrinol. 181, 11–23. doi:10.1677/joe.0.1810011

Rajagopal, S.K., Ma, Q., Obler, D., Shen, J., Manichaikul, A., Tomita-Mitchell, A., Boardman, K., Briggs, C., Garg, V., Srivastava, D., Goldmuntz, E., Broman, K.W., Woodrow Benson, D., Smoot, L.B., Pu, W.T., 2007. Spectrum of heart disease associated with murine and human GATA4 mutation. J. Mol. Cell. Cardiol. 43, 677–685. doi:10.1016/j.yjmcc.2007.06.004

Ran, F., Hsu, P., Wright, J., Agarwala, V., 2013. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–308. doi:10.1038/nprot.2013.143

Ravassard, P., Hazhouz, Y., Pechberty, S., Bricout-Neveu, E., Armanet, M., Czernichow, P., Scharfmann, R., 2011. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. J. Clin. Invest. 121, 3589–3597. doi:10.1172/JCI58447

Remold-O'Donnell, E., Zimmerman, C., Kenney, D., Rosen, F.S., 1987. Expression on blood cells of sialophorin, the surface glycoprotein that is defective in Wiskott-Aldrich syndrome. Blood 70, 104–9.

Rezania, A., Bruin, J.E., Arora, P., Rubin, A., Batushansky, I., Asadi, A., O'Dwyer, S., Quiskamp, N., Mojibian, M., Albrecht, T., Yang, Y.H.C., Johnson, J.D., Kieffer, T.J., 2014. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat. Biotechnol. 32, 1121–1134. doi:10.1038/nbt.3033

Rezania, A., Bruin, J.E., Riedel, M.J., Mojibian, M., Asadi, A., Xu, J., Gauvin, R., Narayan, K., Karanu, F., O'Neil, J.J., Ao, Z., Warnock, G.L., Kieffer, T.J., 2012. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 61, 2016–29. doi:10.2337/db11-1711

Rhodes, C.J., 2004a. Processing of the Insulin Molecule, in: LeRoith, D., Taylor, S.I., Olefsky, J.M. (Eds.), Diabetes Mellitus: A Fundamental and Clinical Text. Lippincott Williams & Wilkins, Philadelphia, pp. 27–50.

Rhodes, C.J., 2004b. Processing of the Insulin Molecule, in: LeRoith, D., Taylor, S.I., Olefsky, J.M. (Eds.), Diabetes Mellitus: A Fundamental and Clinical Text. Lippincott Williams & Wilkins.

Rhodes, C.J., Shoelson, S., Halban, P.A., 2005. Insulin Biosyntheis, Processing and Chemistry, in: Kahn, C.R., Weir, G.C., King, G.L., Jacobson, A.M., Moses, A.C., Smith, R.J. (Eds.), Joslin's Diabetes Mellitus. Lippincott Williams & Wilkins, Boston, pp. 65–82.

Rieck, S., Bankaitis, E.D., Wright, C.V.E., 2012. Lineage determinants in early endocrine development. Semin. Cell Dev. Biol. 23, 673–684. doi:10.1016/j.semcdb.2012.06.005

Riedel, M.J., Asadi, A., Wang, R., Ao, Z., Warnock, G.L., Kieffer, T.J., 2012. Immunohistochemical characterisation of cells co-producing insulin and glucagon in the developing human pancreas. Diabetologia 55, 372–381. doi:10.1007/s00125-011-2344-9

Röder, P. V, Wu, B., Liu, Y., Han, W., 2016. Pancreatic regulation of glucose homeostasis. Exp. Mol. Med. 48, e219. doi:10.1038/emm.2016.6

Rorsman, P., Eliasson, L., Renström, E., Gromada, J., Barg, S., Göpel, S., 2000. The Cell Physiology of Biphasic Insulin Secretion. News Physiol. Sci. 15, 72–77.

Rubio-Cabezas, O., Hattersley, A.T., Njolstad, P., Mlynarski, W., Ellard, S., White, N., Chi, D.V., Craig, M.E., 2014. The diagnosis and management of monogenic diabetes in children and adolescents. Pediatr. Diabetes 15, 47–64. doi:10.1111/pedi.12192

Russ, H.A., Parent, A. V, Ringler, J.J., Hennings, T.G., Nair, G.G., Shveygert, M., Guo, T., Puri, S., Haataja, L., Cirulli, V., Blelloch, R., Szot, G.L., Arvan, P., Hebrok, M., 2015a. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. EMBO J. 34, 1735–1843.

Russ, H.A., Parent, A. V, Ringler, J.J., Hennings, T.G., Nair, G.G., Shveygert, M., Guo, T., Puri, S., Haataja, L., Cirulli, V., Blelloch, R., Szot, G.L., Arvan, P., Hebrok, M., 2015b. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. EMBO J. 1–14.

Saltiel, A.R., 2001. New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. Cell 104, 517–529. doi:10.1016/S0092-8674(01)00239-2

Sartori, D.J., Wilbur, C.J., Long, S.Y., Rankin, M.M., Li, C., Bradfield, J.P., Hakonarson, H., Grant, S.F. a, Pu, W.T., Kushner, J. a, 2014. GATA factors promote ER integrity and β-cell survival and contribute to type 1 diabetes risk. Mol. Endocrinol. 28, 28–39. doi:10.1210/me.2013-1265

Schaffer, A.E., Taylor, B.L., Benthuysen, J.R., Liu, J., Thorel, F., Yuan, W., Jiao, Y., Kaestner, K.H., Herrera, P.L., Magnuson, M. a, May, C.L., Sander, M., 2013. Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. PLoS Genet. 9, e1003274. doi:10.1371/journal.pgen.1003274

Scharfmann, R., Pechberty, S., 2014. Development of a conditionally immortalized human pancreatic β cell line. J. Clin. Invest. 124, 1–12. doi:10.1172/JCI72674.very

Schulz, T.C., Young, H.Y., Agulnick, A.D., Babin, M.J., Baetge, E.E., Bang, A.G., Bhoumik, A., Cepa, I., Cesario, R.M., Haakmeester, C., Kadoya, K., Kelly, J.R., Kerr, J., Martinson, L.A., McLean, A.B., Moorman, M.A., Payne, J.K., Richardson, M., Ross, K.G., Sherrer, E.S., Song, X., Wilson, A.Z., Brandon, E.P., Green, C.E., Kroon, E.J., Kelly, O.G., D'Amour, K.A., Robins, A.J., 2012. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One 7. doi:10.1371/journal.pone.0037004

Schwitzgebel, V.M., Mamin, A., Brun, T., Ritz-Laser, B., Zaiko, M., Maret, A., Jornayvaz, F.R., Theintz, G.E., Michielin, O., Melloul, D., Philippe, J., 2003. Agenesis of human pancreas due to decreased half-life of insulin promoter factor 1. J. Clin. Endocrinol. Metab. 88, 4398–4406. doi:10.1210/jc.2003-030046

Scott, E.W., Fisher, R.C., Olson, M.C., Kehrli, E.W., Simon, M.C., Singh, H., 1997. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. Immunity 6, 437–47.

Scott, E.W., Simon, M.C., Anastasi, J., Singh, H., 1994. Requirement of Transcription Factor PU.1 in the Development of Multiple Hematopoietic Lineages. Science (80-.). 265, 1573–1577.

Seino, S., Takahashi, H., Fujimoto, W., Shibasaki, T., 2009. Roles of cAMP signalling in insulin granule exocytosis. Diabetes, Obes. Metab. 11, 180–188. doi:10.1111/j.1463-1326.2009.01108.x

Sellick, G.S., Barker, K.T., Stolte-Dijkstra, I., Fleischmann, C., Coleman, R.J., Garrett, C., Gloyn, A.L., Edghill, E.L., Hattersley, A.T., Wellauer, P.K., Goodwin, G., Houlston, R.S., 2004. Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat. Genet. 36, 1301–1305. doi:10.1038/ng1475

Shaw-Smith, C., De Franco, E., Allen, H.L., Batlle, M., Flanagan, S.E., Borowiec, M.,

Taplin, C.E., Van Alfen-Van Der Velden, J., Cruz-Rojo, J., De Nanclares, G.P., Miedzybrodzka, Z., Deja, G., Wlodarska, I., Mlynarski, W., Ferrer, J., Hattersley, A.T., Ellard, S., 2014. GATA4 mutations are a cause of neonatal and childhood-onset diabetes. Diabetes 63, 2888–2894. doi:10.2337/db14-0061

Shih, H.P., Wang, A., Sander, M., 2013. Pancreas organogenesis: from lineage determination to morphogenesis. Annu. Rev. Cell Dev. Biol. 29, 81–105. doi:10.1146/annurev-cellbio-101512-122405

Skelin, M., Rupnik, M., Cencic, A., 2010. Pancreatic beta cell lines and their applications in diabetes mellitus research. ALTEX 27, 105–113. doi:20686743

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., Rogers, D., 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336, 688–690. doi:10.1038/336688a0

Sodhi, C.P., Li, J., Duncan, S.A., 2006. Generation of mice harbouring a conditional loss-of-function allele of Gata6. BMC Dev. Biol. 6, 1–6. doi:10.1186/1471-213X-6-19

Stanescu, D.E., Hughes, N., Patel, P., De León, D.D., 2014. A novel mutation in GATA6 causes pancreatic agenesis. Pediatr. Diabetes 4–7. doi:10.1111/pedi.12111

Steck, A.K., Winter, W.E., 2011. Review on monogenic diabetes. Curr. Opin. Endocrinol. Diabetes. Obes. 18, 252–258. doi:10.1097/MED.0b013e3283488275

Stegmeier, F., Hu, G., Rickles, R.J., Hannon, G.J., Elledge, S.J., 2005. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. 102, 13212–7. doi:10.1073/pnas.0506306102

Steiner, D.F., 2004. The Proinsulin C-peptide—A Multirole Model. Exp. Diabesity Res. 5, 7–14. doi:10.1080/15438600490424389

Stevens, L.C., 1970. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. Dev. Biol. 21, 364–382. doi:10.1016/0012-1606(70)90130-2

Stoffers, D. a, Zinkin, N.T., Stanojevic, V., Clarke, W.L., Habener, J.F., 1997. Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat. Genet. 15, 106–110. doi:10.1038/ng0197-106

Straub, S.G., Sharp, G.W.G., 2002. Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes. Metab. Res. Rev. 18, 451–463. doi:10.1002/dmrr.329

Street, C.N., Lakey, J.R.T., Shapiro, a M.J., Imes, S., Rajotte, R. V, Ryan, E. a, Lyon, J.G., Kin, T., Avila, J., Tsujimura, T., Korbutt, G.S., 2004. Implications for Predicting Long-Term Clinical Outcome. Diabetes 53, 3108–3114.

Strickland, S., 1981. Mouse Teratocarcinoma Cells: Prospects for the Study of Embryogenesis and Neoplasia. Cell 24, 277–278. doi:10.1016/0092-8674(81)90313-5

Sun, D., Melegari, M., Sridhar, S., Rogler, C.E., Zhu, L., 2006. Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. Biotechniques 41, 59–63. doi:10.2144/000112203

Suzuki, S., Nakao, A., Sarhat, A.R., Furuya, A., Matsuo, K., Tanahashi, Y., Kajino, H., Azuma, H., 2014. A case of pancreatic agenesis and congenital heart defects with a novel GATA6 nonsense mutation: Evidence of haploinsufficiency due to nonsense-mediated mRNA decay. Am. J. Med. Genet. Part A 164, 476–479. doi:10.1002/ajmg.a.36275

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell 131, 861–872. doi:10.1016/j.cell.2007.11.019

Takahashi, K., Yamanaka, S., 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126, 663–676. doi:10.1016/j.cell.2006.07.024

Takahashi, S., Shimizu, R., Suwabe, N., Kuroha, T., Yoh, K., Ohta, J., Nishimura, S., Lim, K.C., Engel, J.D., Yamamoto, M., 2000. GATA factor transgenes under GATA-1 locus control rescue germline GATA-1 mutant deficiencies. Blood 96, 910–916.

Tam, P.P.L., Behringer, R.R., 1997. Mouse gastrulation: The formation of a mammalian body plan. Mech. Dev. 68, 3–25. doi:10.1016/S0925-4773(97)00123-8

Taylor, B.L., Liu, F.-F., Sander, M., 2013. Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. Cell Rep. 4, 1262–75. doi:10.1016/j.celrep.2013.08.010

Tenzen, T., Zembowicz, F., Cowan, C.A., 2010. Genome modification in human embryonic stem cells. J. Cell. Physiol. 222, 278–281. doi:10.1002/jcp.21948

Tevosian, S.G., Jimenez, E., Hatch, H.M., Jiang, T., Morse, D.A., Fox, S.C., Padua, M.B., 2015. Adrenal development in mice requires GATA4 and GATA6 transcription factors. Endocrinology 156, 2503–2517. doi:10.1210/en.2014-1815

Theunissen, T.W., Jaenisch, R., 2014. Molecular control of induced pluripotency. Cell Stem Cell 14, 720–734. doi:10.1016/j.stem.2014.05.002

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science (80-.). 282, 1145–1147. doi:10.1126/science.282.5391.1145

Tiyaboonchai, A., Mac, H., Shamsedeen, R., Mills, J. a., Kishore, S., French, D.L., Gadue, P., 2014. Utilization of the AAVS1 safe harbor locus for hematopoietic specific transgene expression and gene knockdown in human ES cells. Stem Cell Res. 12, 630–637. doi:10.1016/j.scr.2014.02.004

Todd, J.A., 2010. Etiology of Type 1 Diabetes. Immunity 32, 457–467. doi:10.1016/j.immuni.2010.04.001

Tuomi, T., Santoro, N., Caprio, S., Cai, M., Weng, J., Groop, L., 2014. The many faces of diabetes: A disease with increasing heterogeneity. Lancet 383, 1084–1094. doi:10.1016/S0140-6736(13)62219-9

Van Belle, T., Coppieters, K., Von Herrath, M., 2011. Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies. Physiol Rev 91, 79–118. doi:10.1152/physrev.00003.2010.

Veazey, K.J., Golding, M.C., 2011. Selection of stable reference genes for quantitative RT-PCR comparisons of mouse embryonic and Extra-Embryonic stem cells. PLoS One 6. doi:10.1371/journal.pone.0027592

Viger, R.S., Guittot, S.M., Anttonen, M., Wilson, D.B., Heikinheimo, M., 2008. Role of the GATA family of transcription factors in endocrine development, function, and disease. Mol. Endocrinol. 22, 781–98. doi:10.1210/me.2007-0513

Vodyanik, M. a, Thomson, J. a, Slukvin, I.I., 2006. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. Blood 108, 2095–105. doi:10.1182/blood-2006-02-003327

Walker, E.M., Thompson, C. a., Battle, M. a., 2014. GATA4 and GATA6 regulate intestinal epithelial cytodifferentiation during development. Dev. Biol. 392, 283–294. doi:10.1016/j.ydbio.2014.05.017

Wang, J., Theunissen, T.W., Orkin, S.H., 2007. Site-directed, virus-free, and inducible RNAi in embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 104, 20850–20855. doi:10.1073/pnas.0710565105

Wang, P., Fiaschi-Taesch, N.M., Vasavada, R.C., Scott, D.K., García-Ocaña, A., Stewart, A.F., 2015. Diabetes mellitus—advances and challenges in human β-cell

proliferation. Nat. Rev. Endocrinol. 11, 201–212. doi:10.1038/nrendo.2015.9

Weedon, M.N., Cebola, I., Patch, A., Flanagan, S.E., De Franco, E., Caswell, R., Rodríguez-seguí, S. a, Shaw-Smith, C., Cho, C.H.-H., Lango Allen, H., Houghton, J. a L., Roth, C.L., Chen, R., Hussain, K., Marsh, P., Vallier, L., Murray, A., Ellard, S., Ferrer, J., Hattersley, A.T., Franco, E. De, Allen, H.L., Pancreatic, I., Consortium, A., 2014. Recessive mutations in a distal PTF1A enhancer cause isolated pancreatic agenesis. Nat. Genet. 46, 61–4. doi:10.1038/ng.2826

Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B., Miller, A.D., 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. U. S. A. 86, 5434–8. doi:10.1073/pnas.86.14.5434

Wijesekara, N., Chimienti, F., Wheeler, M.B., 2009. Zinc, a regulator of islet function and glucose homeostasis. Diabetes. Obes. Metab. 11 Suppl 4, 202–14. doi:10.1111/j.1463-1326.2009.01110.x

Xuan, S., Borok, M.J., Decker, K.J., Battle, M.A., Duncan, S.A., Hale, M.A., Macdonald, R.J., Sussel, L., 2012. Pancreas-specific deletion of mouse Gata4 and Gata6 causes pancreatic agenesis. J. Clin. Invest. 122, 3516–3528. doi:10.1172/JCI63352

Yamanaka, S., 2012. Induced pluripotent stem cells: Past, present, and future. Cell Stem Cell 10, 678–684. doi:10.1016/j.stem.2012.05.005

Yorifuji, T., Kawakita, R., Hosokawa, Y., Fujimaru, R., Yamaguchi, E., Tamagawa, N., 2012. Dominantly inherited diabetes mellitus caused by GATA6 haploinsufficiency: variable intrafamilial presentation. J. Med. Genet. 49, 642–3. doi:10.1136/jmedgenet-2012-101161

Young, A.A., Gedulin, B.R., Bhavsar, S., Bodkin, N., Jodka, C., Hansen, B., Denaro, M., 1999. Glucose-lowering and insulin-sensitizing actions of exendin-4: Studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). Diabetes 48, 1026–1034. doi:10.2337/diabetes.48.5.1026

Yu, L., Bennett, J.T., Wynn, J., Carvill, G.L., Cheung, Y.H., Shen, Y., Mychaliska, G.B., Azarow, K.S., Crombleholme, T.M., Chung, D.H., Potoka, D., Warner, B.W., Bucher, B., Lim, F.-Y., Pietsch, J., Stolar, C., Aspelund, G., Arkovitz, M.S., Mefford, H., Chung, W.K., 2014. Whole exome sequencing identifies de novo mutations in GATA6 associated with congenital diaphragmatic hernia. J. Med. Genet. 51, 197–202. doi:10.1136/jmedgenet-2013-101989

Yu, Vodyanik, Smuga-Otto, Antosiewicz-Bourget, Frane, Tian, Nie, Jonsdottir, Ruotti, Stewart, Slukvin, Thomson, 2007. Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. Science (80-.). 318, 1917–1920. doi:10.1126/science.1151526

Zhang, W.Y., de Almeida, P.E., Wu, J.C., 2012. Teratoma formation: A tool for monitoring pluripotency in stem cell research [WWW Document]. StemBook. doi:10.3824/stembook.1.53.1

Zhao, R., Watt, A.J., Battle, M. a, Li, J., Bondow, B.J., Duncan, S. a, 2008. Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Dev. Biol. 317, 614–619. doi:10.1016/j.ydbio.2008.03.013.Loss

Zhao, R., Watt, A.J., Li, J., Morrisey, E.E., Stephen, A., Luebke-wheeler, J., Duncan, S.A., 2005. GATA6 Is Essential for Embryonic Development of the Liver but Dispensable for Early Heart Formation GATA6 Is Essential for Embryonic Development of the Liver but Dispensable for Early Heart Formation. Mol. Cell. Biol. 25, 2622–2631. doi:10.1128/MCB.25.7.2622

Zorn, A.M., Wells, J.M., 2009. Vertebrate endoderm development and organ formation.

  Annu. Rev. Cell Dev. Biol. 25, 221–51. doi:10.1146/annurev.cellbio.042308.113344

Zou, J., Sweeney, C.L., Chou, B., Choi, U., Pan, J., Wang, H., Dowey, S.N., Cheng, L., Malech, H.L., 2011. Oxidase-deficient neutrophils from X-linked chronic granulomatous disease iPS cells : functional correction by zinc finger nuclease – mediated safe harbor targeting. Blood 117, 5561–5572. doi:10.1182/blood-2010-12-328161.

Zwaka, T.P., Thomson, J. a, 2003. Homologous recombination in human embryonic stem cells. Nat. Biotechnol. 21, 319–21. doi:10.1038/nbt788