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DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO PANCREATIC ENDOCRINE PROGENITORS

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DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO PANCREATIC ENDOCRINE PROGENITORS

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ABSTRACT

Diabetes is becoming increasingly prevalent in North America, highlighting the necessity to create a renewable source of pancreatic β-cells for therapeutic purposes. The current study was undertaken to explore the hypothesis that ectopic expression of lineage-determining transcription factors could generate a source of proliferative, homogenous pancreatic progenitor cells capable of subsequent differentiation to functional endocrine cell types, including β-cells. We validated transgenic systems for transgene delivery in HESC and subsequently generated and analyzed gene expression patterns in transgenic cell populations engineered to constitutively express pancreatic transcription factors PAX4, PDX1, or NGN3. We demonstrate that ectopic expression of either PAX4 or PDX1 is not sufficient to specify pancreatic cells from human embryonic stem cells or definitive endoderm cells. In contrast, ectopic NGN3 expression specifies pancreatic endocrine cells from definitive endoderm. In conclusion, this study provides proof of principle of the ability to generate pancreatic endocrine cells using transcription factor overexpression in human embryonic stem cells.

Keyword: Diabetes, human embryonic stem cells, transcription factor, SOX17, PDX1, NGN3, PAX4, differentiation

CO-AUTHORSHIP STATEMENT

SOX17-ER^{T2}-PURO HESC were generated by Dr. Cheryle Séguin.

CA1 SOX17-ER^{T2}-PURO PB-TET-PDX1 HESC were generated by Courtney Brooks.

Genotyping and LacZ staining of CA2 SOX17-ER^{T2}-PURO PB-TET-PDX1 HESC in Figure 3.15 was done by a Scholar's Electives student Mike Sattin.

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ABBREVIATIONS AND SYMBOLS

AMY	Amylase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
βgeo	β-galactosidase/neomycin resistance fusion protein
BMP	Bone morphogenic protein
bZip	Basic leucine zipper
CAG	CMV enhancer combined with chicken β-actin promoter
cDNA	Complementary deoxyribonucleic acid
CER1	Cerberus 1
CK19	Cytokeratin 19
CXCR4	C-X-C chemokine receptor type 4
DE	Definitive endoderm
DLX5	Distal-less homeobox 5
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxycycline
E	Embryonic day
EB	Embryoid body
ER^{T2}	Estrogen receptor ligand binding domain
ES	Embryonic stem
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
Fl	Floxed
FOXA2	Forkhead box A2
G418	Geneticin 418
GATA4	GATA binding protein 4
GATA6	GATA binding protein 6
GCG	Glucagon
GFP	Green fluorescent protein
GLUT2	Glucose transporter 2
HESC	Human embryonic stem cell
HEX	Hematopoietically expressed homeobox
HFP	Human fetal pancreas
HMG	High mobility group
HNF1β	Hepatocyte nuclear factor 1 homeobox β
HNF4α	Hepatocyte nuclear factor 4 homeobox α
HNF6	Hepatocyte nuclear factor 6
IA1	Insulinoma associated antigen 1
IgG	Immunoglobulin G
INS	Insulin
IRES	Independent ribosomal entry site
ITR	Inverted terminal repeats
Klf4	Krueppel-like factor 4
MAFA	v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B

MCS	Multiple cloning site
MEF	Mouse embryonic fibroblast
MGC	Mammalian gene collection
MODY4	Maturity onset diabetes of the young 4
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NEUROD1	Neurogenic differentiation 1
NGN3	Neurogenin 3
NKX2.2	NK2 homeobox 2
NKX2.5	NK2 homeobox 5
NKX6.1	NK6 homeobox 1
NLS	Nuclear localization sequence
OCT4	Octamer-binding transcription factor 4
ORF	Open reading frame
PAX4	Paired box gene 4
PAX6	Paired box gene 6
PB	PiggyBac
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.1% Triton X-100
PBX1	Pre B-cell leukemia transcription factor
PCR	Polymerase chain reaction
PDX1	Pancreatic and duodenal homeobox gene 1
PP	Pancreatic polypeptide
PURO	Puromycin resistance gene

RNA	*Ribonucleic acid*
rtTA	Reverse tetracycline transactivator
SHH	Sonic hedgehog
SOX2	Sry-related HMG box 2
SOX3	Sry-related HMG box 3
SOX7	Sry-related HMG box 7
SOX9	Sry-related HMG box 9
SOX17	Sry-related HMG box 17
SOX18	Sry-related HMG box 18
SSEA3	Stage specific embryonic antigen 3
SSEA4	Stage specific embryonic antigen 4
SST	Somatostatin
TAM	Tamoxifen
TBP	TATA binding protein
TE	TRIS EDTA
TGF-β	Transforming growth factor β
TIE2	TEK tyrosine kinase
α	Alpha
β	Beta
δ	Gamma
ε	Epsilon

1.0 INTRODUCTION

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic, metabolic disease of insulin insufficiency, affecting millions of people worldwide. During the disease state, there is a high level of glucose in the blood that results from the autoimmune destruction of insulin secreting β-cells in the islets of Langerhans, or from β-cell dysfunction. As a result of high blood glucose, patients present with frequent urination, increased thirst and increased hunger. Long-term effects of diabetes are cardiovascular disease, renal failure, and retinal damage (Group, 1993). Recently, diabetes mellitus has become more prevalent, with 2.5 million Canadians having the disease in 2010. This rise in incidence can be attributed to factors such as increased obesity rates, and high rates of cardiovascular disease (Hotamisligil et al., 1993).

Despite the prevalence of diabetes, and the large financial burden to society, there is no renewable source of β-cells for therapeutic replacement. Currently, therapies are limited to insulin injection regimens and whole pancreas or pancreatic islet transplantation; however there is an insufficient source of donor tissue to keep up with demand for treatment (Bonner-Weir and Weir, 2005; Shapiro et al., 2000). This insufficient source of donor tissue has stimulated researchers to seek methods to generate a renewable source of functional β-cells.

1.1.1 Types of Diabetes Mellitus

There are three predominant types of diabetes: type 1 diabetes, type 2 diabetes, and gestational diabetes. Type 1 diabetes affects 10% of the diabetic population. This

type of diabetes occurs due to a T-cell mediated autoimmune destruction of pancreatic islet β-cells, leading to insulin insufficiency (Bedossa et al., 1989). Individuals with Type 1 diabetes are commonly diagnosed at an early age with a sudden onset.

Type 2 diabetes is the most common form of the disease, affecting approximately 90% of patients diagnosed with diabetes. Type 2 diabetes is characterized by resistance to insulin action due to dyslipidemia, and subsequent reduction in insulin secretion with disease progression (Boden, 1996). This form of diabetes has classically presented in adults, however there is now an increased prevalence of children and adolescents that are being diagnosed. The prevalence of type 2 diabetes can be attributed to increased obesity rates within the population (Hotamisligil et al., 1993).

Gestational diabetes is defined as glucose intolerance with onset or first recognition during pregnancy (Metzger and Coustan, 1998). Gestational diabetes is present in 2 to 5% of pregnant women and is most commonly diagnosed in the third trimester. If left untreated, gestational diabetes can affect the health of both the mother and the baby. Women that have gestational diabetes are at a much higher risk of developing type 2 diabetes and cardiovascular disease following pregnancy, while their offspring tend to be large for gestational age and are at a greater risk for developing childhood obesity and resultant type 2 diabetes (Boney et al., 2005; Carr et al., 2006).

1.1.2 Treatments for Diabetes Mellitus

Currently, the predominant therapy for both type 1 and type 2 diabetes mellitus is insulin injection regimens. This treatment consists of subcutaneous injections of

biosynthetic insulin. There are different types of exogenous insulin: rapid acting, short acting, intermediate acting, long acting and biphasic acting. These variations in exogenous insulin allow for patient-specific insulin injection regimes. Rapid acting insulin is used immediately prior to a meal, as it begins to work within 15 minutes. Short acting insulin is injected up to an hour prior to a meal, and lasts up to 8 hours following injection. Intermediate acting insulin must be injected at least an hour prior to a meal, and lasts for up to 16 hours. Intermediate acting insulin is commonly coupled with short acting insulin for complete daily coverage. Long acting insulin only requires one injection per day either in the morning or before bed, as it lasts for up to 24 hours. In addition to subcutaneous injections, the use of an insulin pump has become common, as it is capable of providing a constant drip of insulin throughout the day (Group, 1993). Though proven to be effective, insulin injections are not as sensitive as endogenous insulin and can result in unfavourable patient outcomes. In addition, patients can still have episodes of hypoglycemia, which can lead to unconsciousness, seizures, and brain damage. Additionally, multiple, daily injections can be a financial burden on the population.

An alternative to insulin injection regimens is whole pancreas or pancreatic islet transplantation (Ballinger and Lacy, 1972; Kelly et al., 1967; Shapiro et al., 2000). Transplantation is an option for therapy, as diabetes affects a single cell type. According to the Edmonton Protocol developed in 2000, 600,000 islet equivalents or 1 billion β-cells are required per transplant (Shapiro et al., 2000). The Edmonton Protocol built on these strategies to optimize islet transplantation in humans. This technique involves the transplantation of islets into the hepatic portal vein to allow them to lodge in a safe area.

Patients undergoing transplantation require constant immunosuppression to prevent immune rejection. Unfortunately, longitudinal studies concluded that only 10% of patients were insulin independent 5 years following their initial transplant (Ryan et al., 2005). Approximately 80% of patients had islet graft survival at this point, however most demonstrated impaired function. This outcome has been postulated to be due to the inflammatory response that occurs immediately after transplantation, continued autoimmune destruction, in addition to the immunosuppressive drugs which are toxic to β-cells at high concentrations (Bell et al., 2003; Moberg et al., 2002; Ryan et al., 2005). At the moment, the greatest limitation to this therapy is availability of donor tissues for transplantation. However, the Edmonton Protocol provides the proof-of-principle that cell based therapy can effectively restore insulin-independence in patients (Shapiro et al., 2000).

1.2 Pancreatic Development

The mature pancreas is comprised of an exocrine compartment and an endocrine compartment (Figure 1.1). The exocrine compartment of the pancreas constitutes 98% of the total area and is made up of ductal and acinar cells that are responsible for secreting enzymes such as trypsinogen, chemotrypsin and pancreatic amylase to allow for digestion of carbohydrates, proteins and fatty acids (Rutter et al., 1968). Conversely, the endocrine compartment of the pancreas constitutes 2% of the total area and is comprised of functional units called the islets of Langerhans. Once the pancreas is formed, it is limited

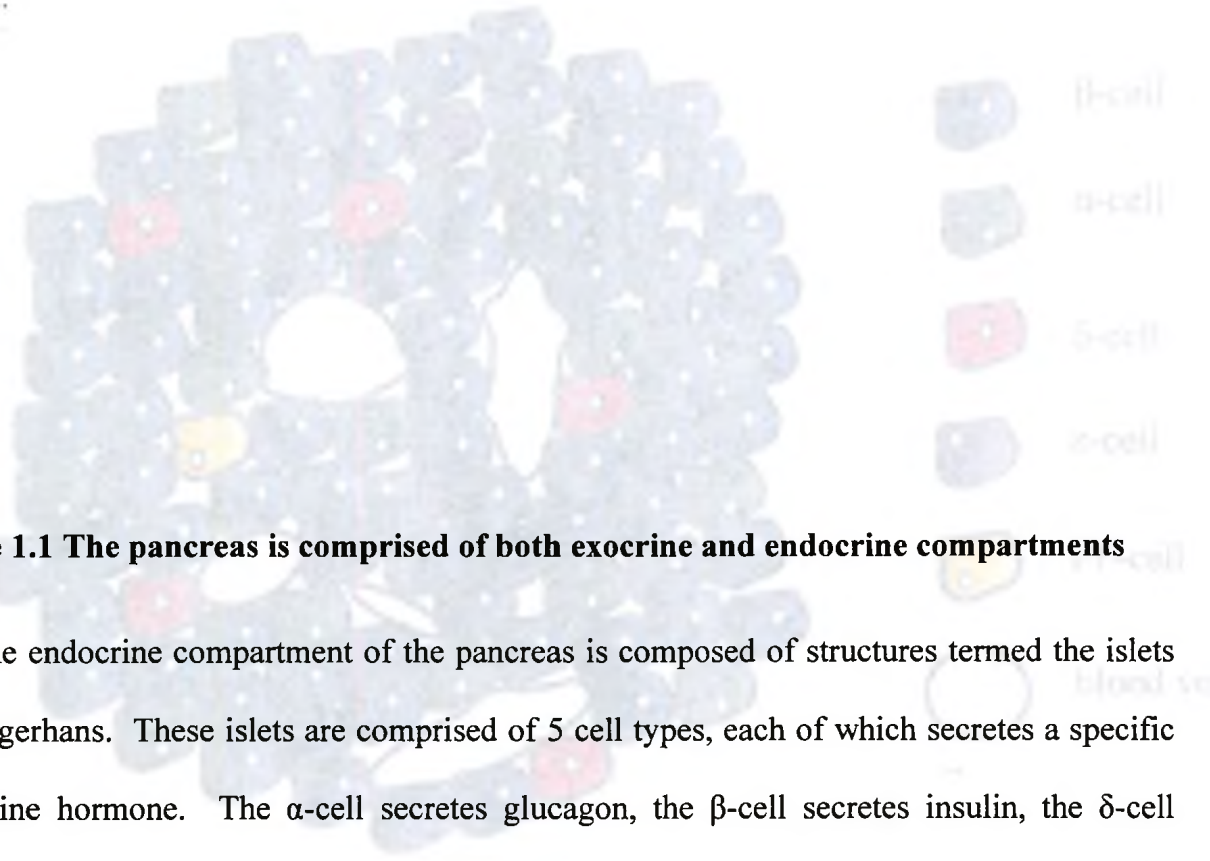
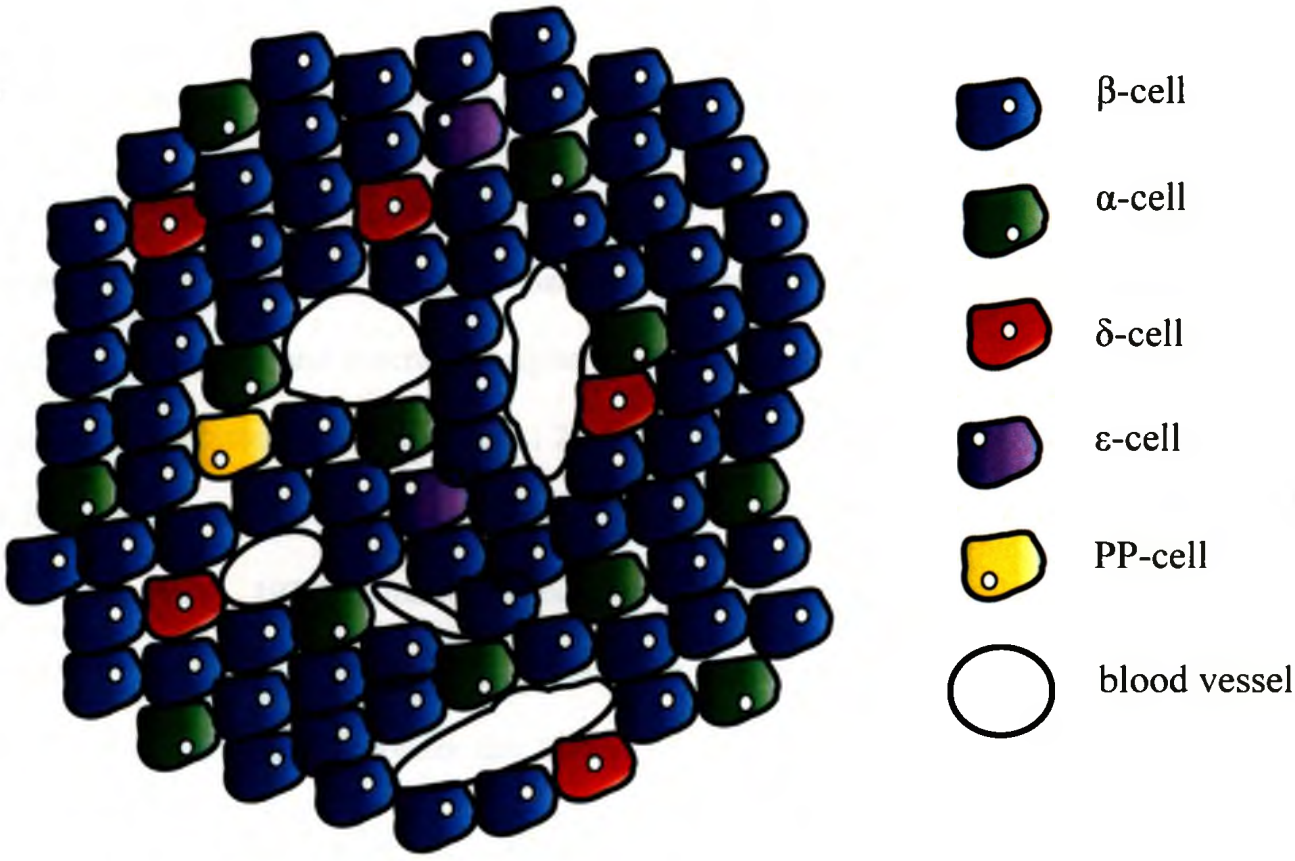


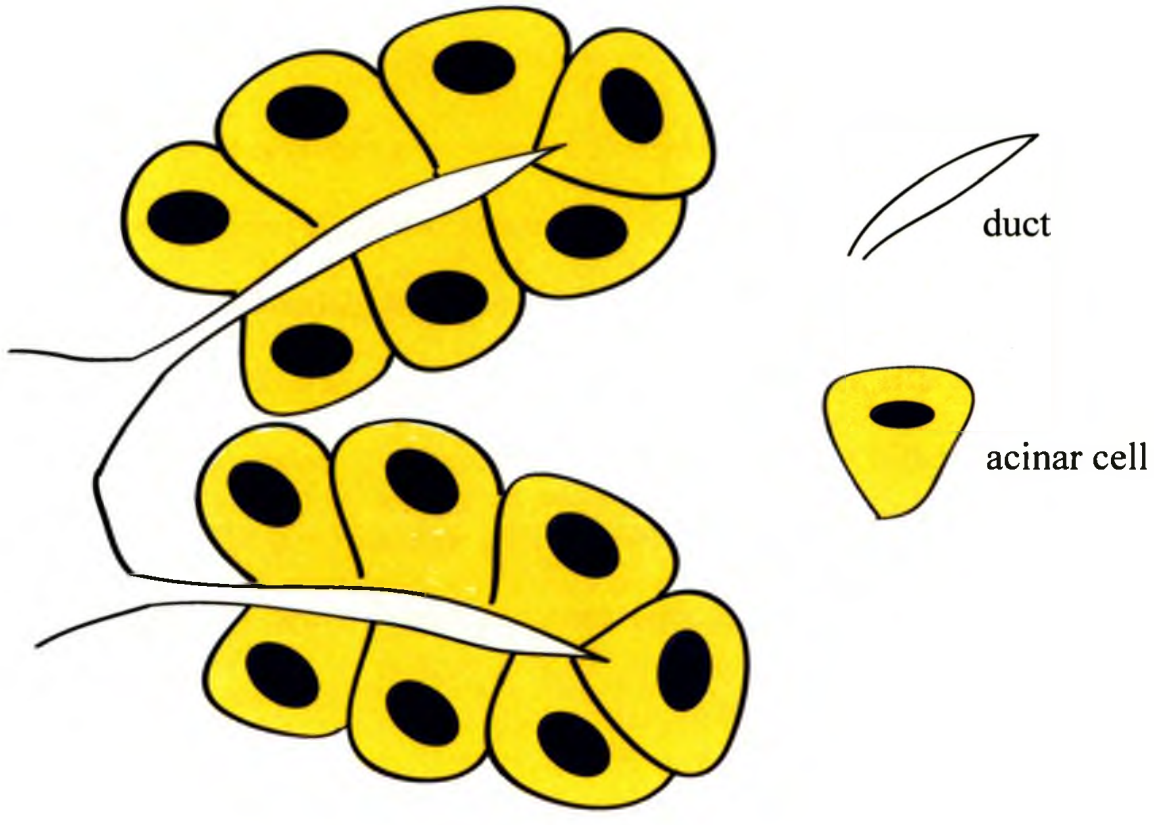
Figure 1.1 The pancreas is comprised of both exocrine and endocrine compartments

(A) The endocrine compartment of the pancreas is composed of structures termed the islets of Langerhans. These islets are comprised of 5 cell types, each of which secretes a specific endocrine hormone. The α-cell secretes glucagon, the β-cell secretes insulin, the δ-cell secretes somatostatin, the ε-cell secretes ghrelin, and the PP cell secretes pancreatic polypeptide. Cells are aggregated around capillaries, which allow for the hormones to enter the vasculature and act on target tissues. (B) The exocrine compartment of the pancreas is composed of pancreatic acinar cells and ductal cells. Acinar cells secrete enzymes necessary for digestion, while ductal cells provide the ducts for these enzymes to travel through.

A


β-cell
α-cell
δ-cell
ε-cell
PP-cell
blood vessel

B


duct
acinar cell

in its ability to regenerate, as there is a restricted pool of progenitor cells that do not readily differentiate upon pancreatic cell loss (Stanger et al., 2007).

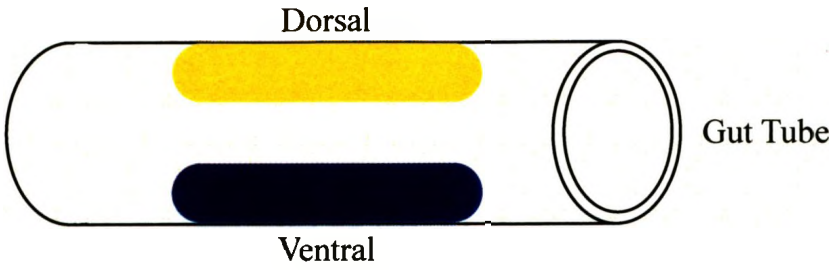
Pancreatic development begins at day 26 of human gestation when the definitive endoderm germ layer (DE), the layer that forms the embryonic gut and associated organs such as liver, lungs, and pancreas, evaginates into the surrounding mesenchyme, forming the dorsal pancreatic bud (Amit et al., 2000) (Figure 1.2). The ventral pancreatic bud arises from the caudal aspect of the hepatic/biliary bud by a similar mechanism six days later (Pictet et al., 1972). After both pancreatic buds have emerged, basal regions of each bud begin to elongate, forming stalks. Meanwhile, the apical region of each bud begins to branch at acute angles. Between day 37 and 42, the ventral and dorsal buds come into contact with each other due to gut rotation, resulting in the fusion of the ventral bud duct with the distal portion of the dorsal bud duct, forming the duct of Wirsung. The remaining portion of the dorsal bud duct is termed the duct of Santorini (Pictet et al., 1972).

At day 40 to 60 of human gestation, or E13.0-14.0 in the mouse, there is a change in the cellular composition of the pancreas in what is known as the secondary transition. Prior to the secondary transition, the pancreas is comprised of predominantly immature α-cells (Pictet et al., 1972). During the secondary transition, there is a change in cell architecture due to expansion of endocrine cell numbers concurrent with exocrine acinar cell differentiation. The new endocrine cells assemble in a linear pattern along the ducts. Shortly after birth, the newly formed endocrine cells aggregate to form the islets of Langerhans (Pictet et al., 1972).

Figure 1.2: Schematic Representation of Pancreatic Development

(A) In humans, at 26 days of gestation, pancreatic buds are formed on both the dorsal and ventral aspect of the gut tube. (B) This occurs when the definitive endoderm evaginates into the surrounding mesenchyme. (C-D) Following bud formation, branching and elongation begins, with the dorsal bud forming a larger structure than the ventral bud. By day 42, the ventral and dorsal buds fuse due to gut rotation. (E) This fusion generates the duct of Wirsung, and the accessory duct of Santorini.

A



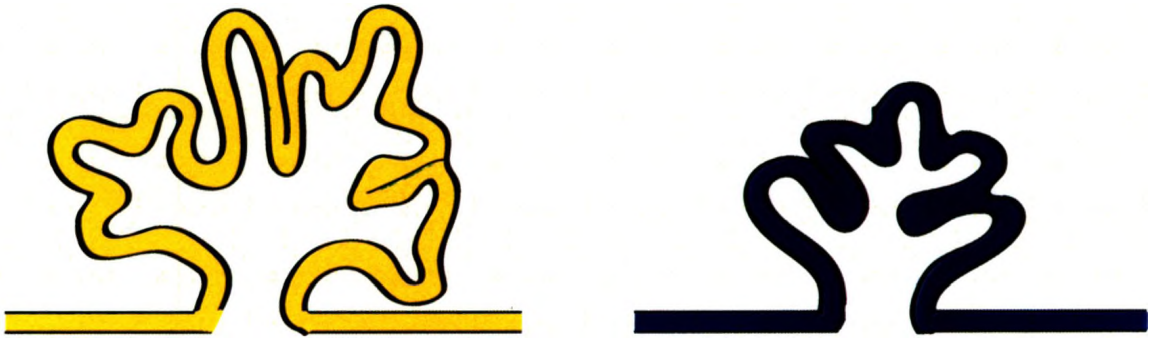
B

Pancreatic Bud

C

Pancreatic Bud Elongation

D



Pancreatic Bud Branching

E

1.2.1 Pancreatic Endocrine Cells

Within the islets of Langerhans, there are five endocrine cell types. The most predominant endocrine cell type, comprising 65-80% of the islets of Langerhans, is the β-cell (Pictet et al., 1972). In response to high blood glucose concentrations, glucose enters the β-cells through the GLUT2 glucose transporter and is metabolized to ATP. The resultant ATP induces β-cell membrane depolarization and insulin release. The insulin hormone then enters the circulation and eventually binds the insulin receptor on hepatocytes and myocytes, promoting the uptake of glucose, which is stored as glycogen in the liver and skeletal muscle. After insulin receptor binding, insulin is either released and able to act on other insulin receptors, or is degraded and cleared through the liver or kidney (Duckworth et al., 1998). β-cells also secrete C-peptide, a byproduct of insulin biosynthesis. To form mature insulin, proinsulin is cleaved by prohormone convertases 1 and 2, excising C-peptide from the center of the proinsulin peptide. The resultant insulin peptide fragments are then joined by disulfide bonds, forming insulin (Steiner and Oyer, 1967). In rodents, β-cells are localized to the center of the islets of Langerhans, while in humans, β-cells are dispersed randomly throughout the islets.

The second most predominant endocrine cell type in the adult pancreas is the glucagon-secreting α-cell, comprising between 10-15% of the endocrine compartment (Pictet et al., 1972). In response to low blood glucose, α-cells secrete glucagon, a peptide hormone that acts on hepatocytes to promote glycogenolysis. The resultant effect is increased blood glucose levels. In rodents, α-cells are localized to the outside of the islets, while in humans, α-cells are dispersed throughout the islets.

The third most abundant endocrine cell type, comprising 3-10% of the pancreas is the δ-cell. Delta cells secrete the peptide hormone somatostatin. The role of somatostatin within the endocrine pancreas is to inhibit the release of insulin and glucagon. In rodents, δ-cells are located at the periphery of the islet, while they are dispersed throughout in humans. Approximately 3-5% of the endocrine pancreas is comprised of PP cell that secrete pancreatic polypeptide. Pancreatic polypeptide (PP) is responsible for regulating hepatic glycogen levels by altering the insulin receptor found on the membrane of hepatic cells. In response to hypoglycemia, PP is released to promote glycogenolysis. Lastly, ε-cells comprise less than 1% of the endocrine compartment of the pancreas. Epsilon cells are responsible for secreting ghrelin, a hormone responsible for stimulating the feeling of hunger by acting on the hypothalamus (Klok et al., 2007).

1.2.2 Growth Factor Signaling Pathways in Pancreatic Development

During pancreatic development, there are many signaling pathways that are required for proper organ patterning. Wnt signaling is an important, and temporally regulated process required for the formation of the pancreatic endoderm. Generally, Wnt3a is localized within the pancreatic mesenchyme, with overexpression leading to pancreatic hypoplasia affecting the islets of Langerhans, as well as pancreatic acinar cells (Heller et al., 2002).

Mammalian transforming growth factor (TGF) -β1, -β2, and -β3 are detected in the developing pancreatic epithelium at E12.5 in the mouse, and later co-localize within acinar cells (Crisera et al., 1999). By E18.5, these TGF-β isoforms are specific to

pancreatic ducts, concurrent with expression of TGF-β receptor type I and type II (Tulachan et al., 2007). Transgenic mice expressing TGF-β1 under the rat insulin promoter have shown that TGF-β signaling is required for the ability of endocrine cells to form islets in the mouse pancreas (Lee et al., 1995; Sanvito et al., 1995). Inhibition of TGF-β signaling in mice through a dominant-negative form of TGF-β receptor type II results in ductal hyperplasia and acinar atypia (Bottinger et al., 1997). Taken together, TGF-β signaling is required to recruit duct cells to the endocrine lineage.

Activin signaling is required to specify definitive endoderm, as well as distinct stages throughout pancreatic development for proper patterning, but fine control of this pathway is essential. The notochord is a structure that forms during gastrulation and is responsible for patterning the axis of the developing embryo. The pancreas is situated adjacent to the notochord during development. In order for proper pancreas formation, sonic hedgehog (SHH) must be inhibited in the endoderm of the pancreatic region of the gut tube. The notochord secrets activins, which are inhibitors of SHH, resulting in areas of the gut tube that are capable of undergoing pancreatic bud formation (Hebrok et al., 1998). In chick embryos, the removal of the notochord results in the absence of pancreatic gene expression (Kim et al., 1997; Melton et al., 1998). Activin A and B are expressed in developing rat and mouse pancreatic endocrine cells, especially α-cells (Furukawa et al., 1995; Maldonado et al., 2000). The addition of exogenous activin A to monolayer cultures of human fetal pancreas cells, results in increased number of insulin-positive cells and subsequent increases in insulin content (Demeterco et al., 2000). Conversely, inhibition of activin by follistatin, which is found within the pancreatic

mesenchyme, results in exocrine differentiation (Miralles et al., 1998). These studies suggest that activin A is required for the differentiation of pancreatic endocrine cells.

Fibroblast growth factors (FGFs) play an important role in branching morphogenesis of the developing pancreas (Hogan, 1999). FGF10 expressed in the mesenchyme is required for proper pancreatic formation by controling mesenchymal-to-epithelial signaling. Mice lacking FGF10 have restricted pancreatic growth (Bhushan et al., 2001). Conversely, ectopic expression of *Fgf10* in vivo under the control of the *Pdx1* promoter in mice results in increased proliferation of the pancreas at the expense of cell differentiation, as cells maintained a pancreatic progenitor phenotype and were unable to undergo terminal differentiation (Edlund et al., 2003). It is believed that FGF signaling in the pancreas is mediated by Notch signaling. This has been shown in studies where overexpresion of FGF10 in transgenic mice results in the maintenance of Notch pathway activation, preventing pancreatic progenitor differentiation (Norgaard et al., 2003). In the pancreas, inhibition of Notch signaling results in robust commitment of cells to the endocrine lineage (Edlund et al., 1999).

1.3 Pancreatic Transcription Factors

1.3.1 SOX17

Sry-related HMG box genes encode a family of transcription factors that possess a highly conserved high mobility group (HMG) responsible for DNA binding in the minor groove (Schepers et al., 2002; Wegner, 1999). This HMG box was originally identified in SRY, the sex determining gene on the Y chromosome. In general, SOX transcription

factors have weak binding specificity in addition to low affinity for DNA (Wegner, 1999). Sox transcription factors are encoded by 20 genes in human and mouse.

Sry-related HMG-box 17 (SOX17) is a 414aa transcription factor that plays a role in embryonic development and cell fate determination through the regulation of Wnt3a signaling (Figure 1.3 A). SOX17 is classified into the F group of SOX genes along with SOX7 and SOX18, transcription factors that share similarity outside the DNA-binding domain (Dunn et al., 1995; Schepers et al., 2002; Taniguchi et al., 1999). Importantly, *Sox17* has been found to play an essential role in endoderm differentiation: it is expressed concurrent with DE formation and is downregulated upon DE specification (Kanai-Azuma et al., 2002). *Sox17* null mice are embryonic lethal, due to apoptosis of the foregut endoderm and failure of mid- and hindgut expansion. Using chimera assays, *Sox17* $^{-/-}$ embryonic stem (ES) cells were shown to contribute to all aspects of gut DE, however they undergo incrased apoptosis in the foregut, and fail to differentiate in the posterior regions (Kanai-Azuma et al., 2002).

1.3.2 PDX1

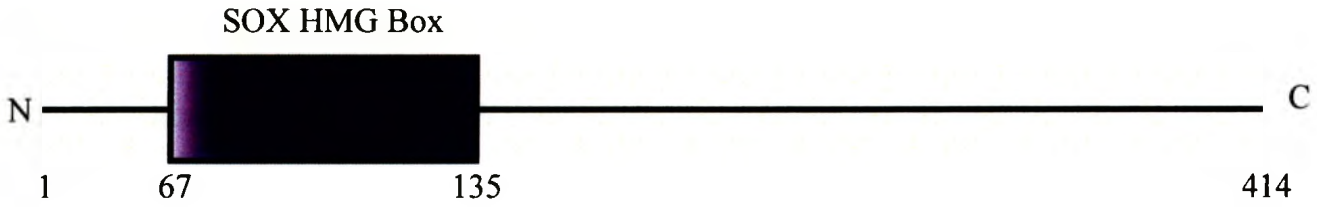
Pancreatic and duodenal homeobox gene 1 (PDX1) is a 283aa transcription factor that regulates the expression of insulin, somatostatin, islet amyloid polypeptide, glucokinase, Glut2 as well as itself (Figure 1.3 B) (Hui and Perfetti, 2002). In mice and humans, all pancreatic endocrine and exocrine cells are derived from PDX1 positive progenitors (Gu et al., 2003). PDX1 is characterized by a central DNA binding homeodomain that possesss a signal for nuclear localization (Hessabi et al., 1999), Lu

Figure 1.3: Pancreatic Transcription Factor Structures

Schematic representation of pancreatic transcription factor structures. (A) SOX17 has a SOX HMG box domain at the N-terminus of the transcription factor. (B) PDX1 contains a transactivation domain at the N-terminal of the protein, a homeodomain that includes a nuclear localization signal in the center of the protein, and a phosphorylation site at the C-terminus. (C) NGN3 belongs to the basic helix-loop-helix family of transcription factors that contain a helix-loop-helix domain in the center of the protein. (D) PAX4 has a paired box domain at the N-terminus of the protein, in addition to a homeodomain that is found in the center of the protein.

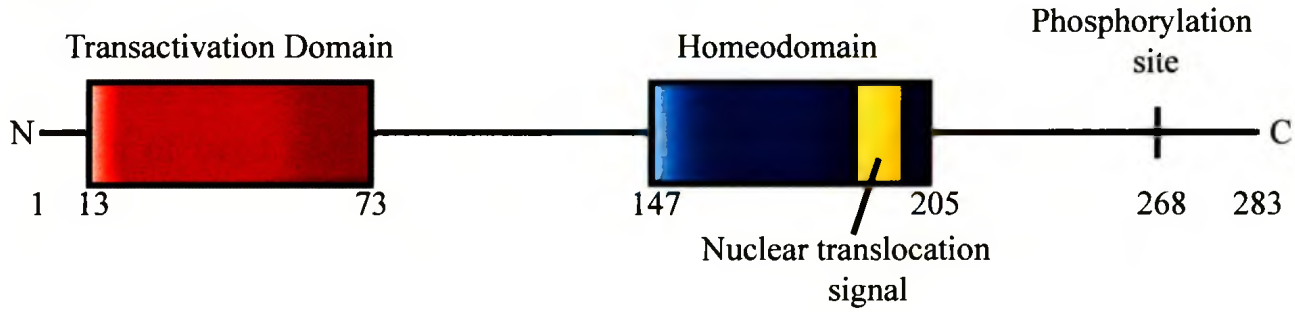
A

SOX17



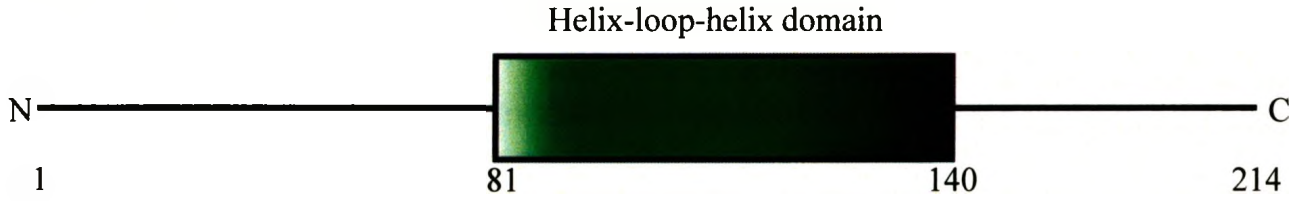
B

PDX1



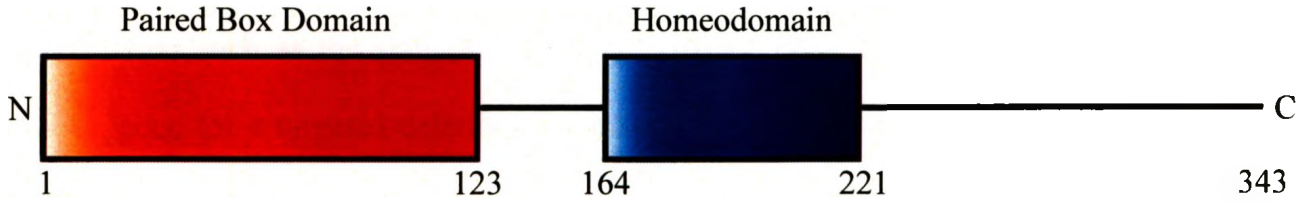
C

NGN3



D

PAX4



1996). The N-terminus of PDX1 contains a transactivation domain that allows the protein to interact with other transcription factors and transcriptional co-regulators such as hepatocyte nuclear factor 6 (HNF6), SOX9, hepatocyte nuclear factor 1 homeobox B (HNF1β), and forkhead box A2 (FOXA2) (Mosley and Ozcan, 2004; Oliver-Krasinski et al., 2009). Additionally, there is a proline-rich domain situated in the middle of the protein that has been shown to be involved in heterodimerization with the DNA binding protein Pre B-cell leukemia transcription factor (PBX1) and varying additional binding partners to form a transcriptional regulatory complex (Goudet et al., 1999). The C-terminus of PDX1 is not essential for DNA binding, however it is necessary for the activation of target gene promoters either by direct interaction, or by changing the conformation of other interacting regions (Oliver-Krasinski et al., 2009; Peers et al., 1994). There are many islet transcription factors such as *Pax6*, *MafA* and *FoxA2* that bind to PDX1 and activate it through conserved cis-acting elements between 1923bp and 2153bp. In addition, *Pdx1* binds to and regulates several promoters for downstream genes such as *Nkx6.1* and *Pax6* as well as preproinsulin (Svensson et al., 2007).

In the mouse, expression of *Pdx1* is restricted to the developing pancreas following pancreatic specification of the foregut endoderm. By E10.5, *Pdx1* expression is downregulated throughout the pancreatic compartment and remains low until E13.0 when it is upregulated upon β-cell differentiation (Ohlsson et al., 1993). *Pdx1* expression is maintained in adult β-cells (Ahlgren et al., 1998). Additionally, *Pdx1* is expressed in other tissues such as the skin (Mazur et al., 2010). *Pdx1* null mice, as well as mice homozygous for a targeted deletion of the homeodomain encoding exon 2 of the *Pdx1* gene lack a pancreas and consequently, die within a few days of birth (Ahlgren et al.,

1996; Jonsson et al., 1994). *Pdx1* haploinsufficient mice have smaller islets that contain fewer β-cells, leading to decreased insulin secretion and glucose intolerance that ultimately results in diabetes (Offield et al., 1996). In humans, a mutation at amino acid 178, located within the second helix of the homeodomain, results in neonatal diabetes with subclinical exocrine deficiency (Nicolino et al., 2010), while a homozygous frameshift mutation at amino acid 63 prevents translation of the homeodomain as well as the C-terminus, thereby causing pancreatic agenesis (Stoffers et al., 1997). Individuals that are heterozygous for PDX1 mutations associated with neonatal diabetes will later develop maturity onset diabetes of the young 4 (MODY4) (Nicolino et al., 2010).

Given its function during pancreatic development, several studies have tested the ability of *Pdx1* expression to induce endocrine differentiation. Mouse ES cells with ectopic tet-inducible *Pdx1* gene expression have been differentiated following a 5 step pancreatic differentiation protocol. At the end of the differentiation process, these cells express somatostatin, insulin2, *Nkx2.2*, *Pax4*, *Pax6* and *Ngn3*. Though these cells were c-peptide positive, they did not secrete insulin in response to glucose stimulation (Miyazaki et al., 2004). In contrast, rat small hepatocytes can successfully be differentiated into insulin-producing cells through overexpression of *Pdx1* by adenovirus transfection. These cells also expressed pancreatic markers *Ngn3*, *Pax6*, *Nkx2.2* and *Nkx6.1*. Unfortunately, small hepatocytes, the progenitor cell type in the liver, are difficult to isolate from the liver, and functional analyses have not been performed on these cells (Kawasaki et al., 2008). Stable PDX1 overexpressing telomerase-immortalized bone marrow derived mesenchymal stromal cells have also been generated. These cells demonstrate pancreatic gene expression and insulin promoter activity. However, these

cells were heterogeneous when stained for insulin and c-peptide, and did not respond to glucose at a biologically relevant level (Limbert et al., 2011).

1.3.3 NGN3

Neurogenin 3 (NGN3) is a 214aa basic helix loop helix transcription factor necessary for the development of hormone-producing islets (Figure 1.3C). In the developing mouse pancreas, *Ngn3* expression is initiated at E8.5 in the dorsal pancreatic bud, while expression begins at E9.5 in the ventral pancreas. From E9.5 to E12.5, expression of *Ngn3* is localized to the pancreatic endoderm where it is maintained until birth, and subsequently downregulated due to auto-repression (Gradwohl et al., 2000; Smith et al., 2004). In addition to pancreatic expression, *Ngn3* is expressed in the developing and mature hippocampus (Simon-Areces et al., 2010), as well as neuronal progenitor cells from E9.0 to E14.0 (Lee et al., 2001; Sommer et al., 1996). *Ngn3* positive cells give rise to pancreatic endocrine cells that express endocrine transcription factors such as *Nkx6.1* and *Pax6* (Jensen et al., 2000; Smith et al., 2004). It has been found that *Ngn3* binds to promoters of *NeuroD*, *Pax4* and insulinoma associated antigen 1 (*Ia1*) (Huang et al., 2000; Smith et al., 2003).

Mice lacking *Ngn3* lack the endocrine component of the pancreas, leaving an exocrine compartment that contains few glucagon expressing cells. These mice develop diabetes due to a lack of β-cells and die between 1 and 3 days after birth (Gradwohl et al., 2000). Ectopic expression of *Ngn3* in pancreatic ductal cells *in vivo* using an adenovirus results in the expression of endocrine specific genes, however this starting population is limited (Gasa et al., 2004). Ectopic NGN3 expression in human pancreatic ductal cells

produces insulin positive cells, however this occurs in only 13% of the cells (Heremans et al., 2002). In contrast, *in ovo* electroporation of *Ngn3* in chick embryos results in glucagon and somatostatin producing cells, however no insulin or c-peptide positive cells were reported (Grapin-Botton et al., 2001). Ectopic expression of *Ngn3* in conjuction with *Pdx1* in mouse ES cell-derived endoderm generates insulin and c-peptide positive cells, however these cells are not responsive to glucose (Kubo et al., 2011). Overexpression of *Ngn3*, *MafA*, and *Pdx1* is capable of transdifferentiating pancreatic acinar cells into insulin positive cells *in vivo*, however this strategy is only 20% efficient and has a limited starting population (Zhou et al., 2008).

1.3.4 PAX4

Paired box gene 4 (PAX4) is a 343aa nuclear transcription factor that is localized to the endocrine pancreas during embryonic development (Figure 1.3 D) (Sosa-Pineda et al., 1997). PAX genes encode regulators of embryonic organ development (Mansourie et al., 1996) and are characterized by a domain consisting of a highly conserved 128 amino acid motif that possesses DNA binding activities (Noll 1993). In particular, *Pax4* and *Pax6* share a homeodomain, as well as a highly conserved octapeptide (Dahl et al., 1997). Expression of *Pax4* begins at E10 in a small number of cells in the pancreatic bud (Sosa-Pineda et al., 1997). By E14.5, there is a significant increase in *Pax4* positive cells in the dorsal pancreas, and by E15.5 the number of *Pax4* expressing cells reaches its maximum (Dohrmann et al., 2000; Sosa-Pineda et al., 1997). *Pax4* is not expressed in any exocrine cell type (Sander 1997, St Onge 1997). *Pax4* is directly regulated by *Ngn3*, and serves to regulate expression of downstream targets including *Nkx6.1* (Smith et al., 2003; Tang et

al., 2006). It has been shown that *Pax4* is required to maintain proliferation and survival of pancreatic β-cells (Brun et al., 2004).

At birth, *Pax4* null mice have a normal pancreas with respect to size and weight; however, the pancreas lacks insulin-secreting β- and somatostatin-secreting δ- cells. To compensate for the lack of β and δ cells, there is an overabundance of α cells that are situated within the centre of an islet-like structure. Homozygous *Pax4*-deficient mice die 3-5 days after birth due to hyperglycemia (Sosa-Pineda et al., 1997). Overexpression of PAX4 in HESC following embryoid body-mediated definitive endoderm differentiation produces cells that exhibit functional properties that are typical of β-cells. These cells comprise 90% of the resultant cell population (Liew et al., 2008). Transgenic mice that express *Pax4* in the pancreatic endocrine tissue, or pancreatic epithelium die between 3 and 12 weeks after birth due to hyperglycemia. These mice had enlarged islets that contained mostly β-cells, as ectopic *Pax4* expression converted α-cell progenitors to β-cell progenitors (Collombat et al., 2009).

1.3.5 Additional Pancreatic Transcription Factors

V-Maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) is a transcription factor required for functional glucose-stimulated insulin secretion in the pancreas (Kataoka et al., 2002). In the pancreas, Maf proteins function by interacting with other bZip proteins, which are transcription factors that contain basic leucine zipper domains, forming heterodimers (Igarashi et al., 1994) that bind to the RIPE3b1 element in the promoter of genes such as insulin (Kataoka et al., 2002). During development,

expression of *MafA* is restricted to developing insulin positive cells, and is responsible for tissue specific insulin expression (Matsuoka et al., 2004). *MafA* is initially detected at E13.5 in mice, during the secondary transition of pancreatic development (Matsuoka et al., 2004). Conversely, *MafB* is present in developing α- and β-cells (Artner et al., 2006), but is restricted to α-cells following birth (Nishimuro 2006). In the development of the pancreatic β-cell, there is a transition between *MafB* expression and *MafA* expression that is required for proper glucose stimulated insulin secretion. Cells that are *MafB* positive and insulin positive are not functional until they are *MafA* positive and insulin positive (Kroon et al., 2008). $MafA^{-/-}$ mice have normal islet architecture at birth, however by 12 weeks of age, there is a change in the composition of pancreatic islets with an increase the ratio of α- to β-cells. Consequently, $MafA^{-/-}$ mice develop overt diabetes mellitus (Zhang et al., 2005). Overexpression of *MafA* in conjunction with *Pdx1* and *NeuroD1* can induce the expression of endogenous insulin in the liver (Kaneto et al., 2005). Alternatively, overexpressing *MafA* concurrently with *Pdx1* and *Ngn3* is capable of transdifferentiating pancreatic acinar cells into insulin positive cells (Zhou et al., 2008). *In ovo* electroporation of *MafA* in embryonic chick endoderm lead to the formation of insulin positive cells (Artner et al., 2008). Human placenta derived multipotent stem cells that overexpress MAFA were assessed by microarray analysis and were found to exhibit a gene expression profile consistent with pancreatic islets. Additionally, these cells were responsive to glucose and corrected hyperglycemia in immunocompromised mice that had streptozotocin-induced diabetes. A downfall to these cells is that they co-express glucagon and insulin (Chiou et al., 2011).

1.4 Human Embryonic Stem Cells

Human embryonic stem cells (HESC) are derived from the inner cell mass of the blastocyst, which is formed 4-5 days following fertilization. If maintained in the appropriate medium, containing basic fibroblast growth factor, HESC remain pluripotent and have the potential to form all 220 cell types of the body, including pancreatic β-cells (Thomson et al., 1998; Xu et al., 2005). Traditionally, HESC are cultured on mouse embryonic fibroblasts (MEFs) or in the presence of MEF-conditioned media. HESC have high levels of telomerase activity, allowing these cells to replicate indefinitely (Harley et al., 1992). HESC are characterized by the expression of the cell surface glycoproteins stage-specific embryonic antigen (SSEA)3 , SSEA4, TRA-1-60 and TRA-1-81, in addition to being alkaline phosphatase positive (Thomson et al., 1998). Additionally, given their pluripotent nature, HESC can produce cancerous and non-cancerous tumours called teratomas that contain tissues from each primary germ layer when injected into severe combined immunodeficient (SCID) mice.

Undifferentiated HESC have a distinct, unique morphology characterized by tightly packed colonies with no observable cell boundaries. The cells possess a high nuclear to cytoplasmic ratio with prominent nucleoli (Thomson et al., 1998). HESC are capable of spontaneous differentiation to all cell types, with first indication of HESC differentiation being change in cell morphology (Imreh et al., 2006).

1.4.1 Differentiation Strategies to Generate β-Cells from Human Embryonic Stem Cells

Several strategies have been developed for the generation of β-cells from pluripotent HESC. Growth factor-mediated approaches have yielded stepwise protocols to generate a population of pancreatic-like cells (D'Amour et al., 2005; D'Amour et al., 2006; Jiang et al., 2007a; Jiang et al., 2007b; Shim et al., 2007). Although these methods have been extensively characterized, they have limitations that are undesirable. Specification of DE using Activin A results in a heterogeneous, non-proliferative population of cells, thereby limiting the population that can further differentiate into pancreatic cell types. Additionally, these protocols produce a population of cells in which the β-cell phenotype comprises less than 15% of the final population. Furthermore, transplantation of these β-cells in mice does not generate functional cells *in vivo*, and the mice formed teratomas (D'Amour et al., 2005). Recent studies have optimized growth factor-mediated approaches by extending activin A treatment to specify DE, followed by inhibition of activin A, nodal, BMP and TGF-β following pancreatic progenitor formation in β-cell development, however resultant populations contain only 25% C-peptide positive cells (Nostro et al., 2011). These limitations have given rise to a need for a method to generate a homogenous population of pancreatic endocrine precursors that maintain a replicative capacity enabling the cell expansion required for eventual cell-based therapies.

1.4.2 Transgenic Strategies for Pancreatic Development from HESC

Pancreatic exocrine cells have been transdifferentiated into pancreatic endocrine cells by *in vivo* viral-mediated gene delivery of the transcription factors Mafa, Pdx1 and

Ngn3. The initial population of pancreatic exocrine cells is not self-renewable and the efficiency of transdifferentiation is very low (Zhou et al., 2008).

1.4.2 Genetic Manipulation of Human Embryonic Stem Cells

1.4.2.1 ER^{T2} System

The estrogen receptor ligand binding domain (ER^{T2}) has been used to to generate transgenes that are constitutively expressed but active only after the addition of 4-OH Tamoxifen to culture media (Hayashi and McMahon, 2002). This inducible construct allows for the control of the duration of transcription factor activity, which is beneficial when mimicking its developmental pattern of activity. The addition of 4-OH Tamoxifen allows the fusion protein to translocate into the nucleus, enabling it to bind DNA and regulate transcription (Figure 1.4).

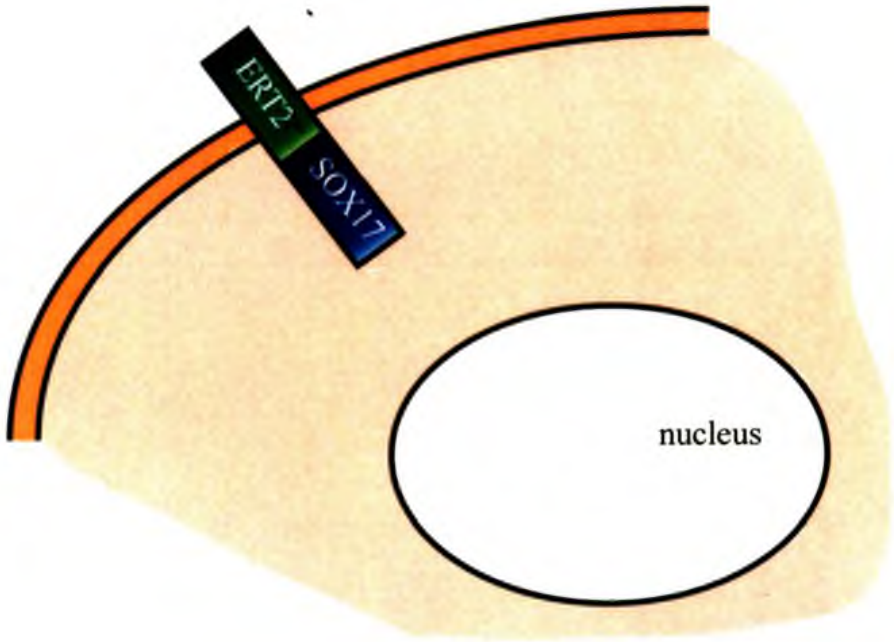
1.4.2.2 piggyBac System

The piggyBac (PB) transposase system is a non-viral vector system that uses the transient expression of a transposase enzyme and two inverted terminal repeats flanking the gene of interest to insert transgenes into the host genome at any TTAA site (Figure 1.5). This system is the most efficient transposon system characterized for use in mammalian cells and is unique in that transgenes can be removed leaving no genetic footprint behind using transient expression of PB transposase. The PB-TET system allows the gene of interest to be turned on/off by the addition/removal of doxycycline, which acts through the tetO2 promoter. PB-TET is co-electroporated with the PB

Figure 1.4: ER^{T2} System for Inducible Gene Delivery

(A) The ER^{T2} system allows for a gene of interest, fused in frame to the estrogen receptor ligand binding domain (ER^{T2}), to be constitutively expressed and held at the cytoplasmic membrane. (B) Following the addition of 4-OH Tamoxifen, an estrogen analog that shows a higher affinity for the mutated ER^{T2} receptor, the fusion protein translocates into the nucleus where it can become active.

A


ERT2
SOX17
nucleus

B

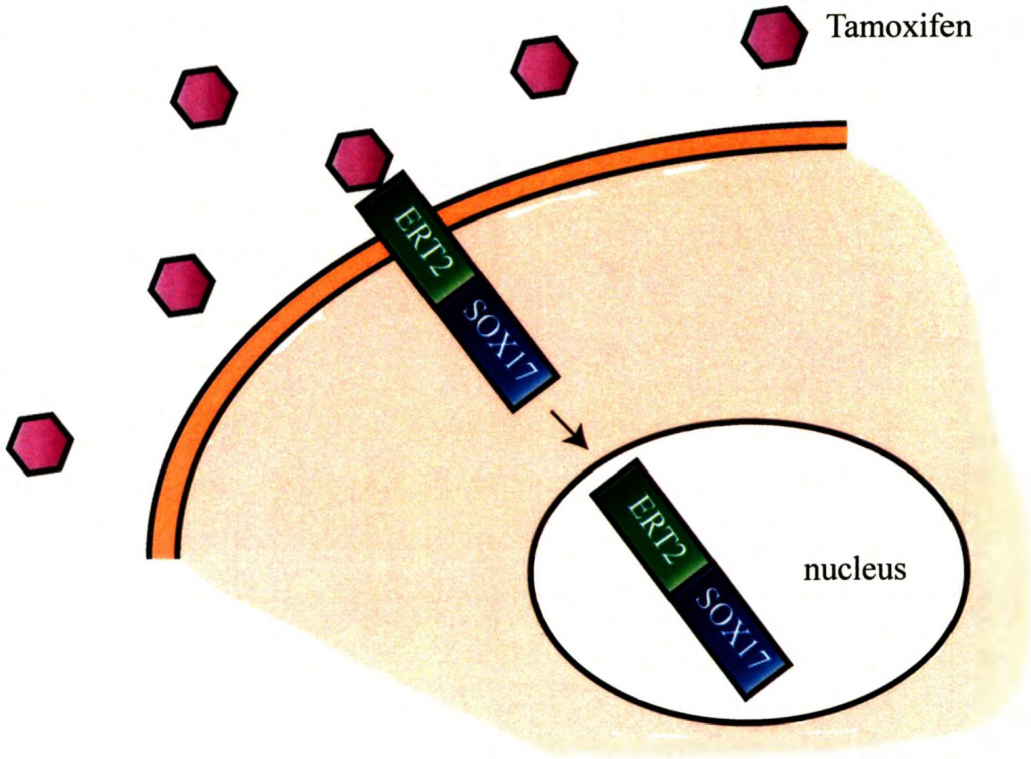
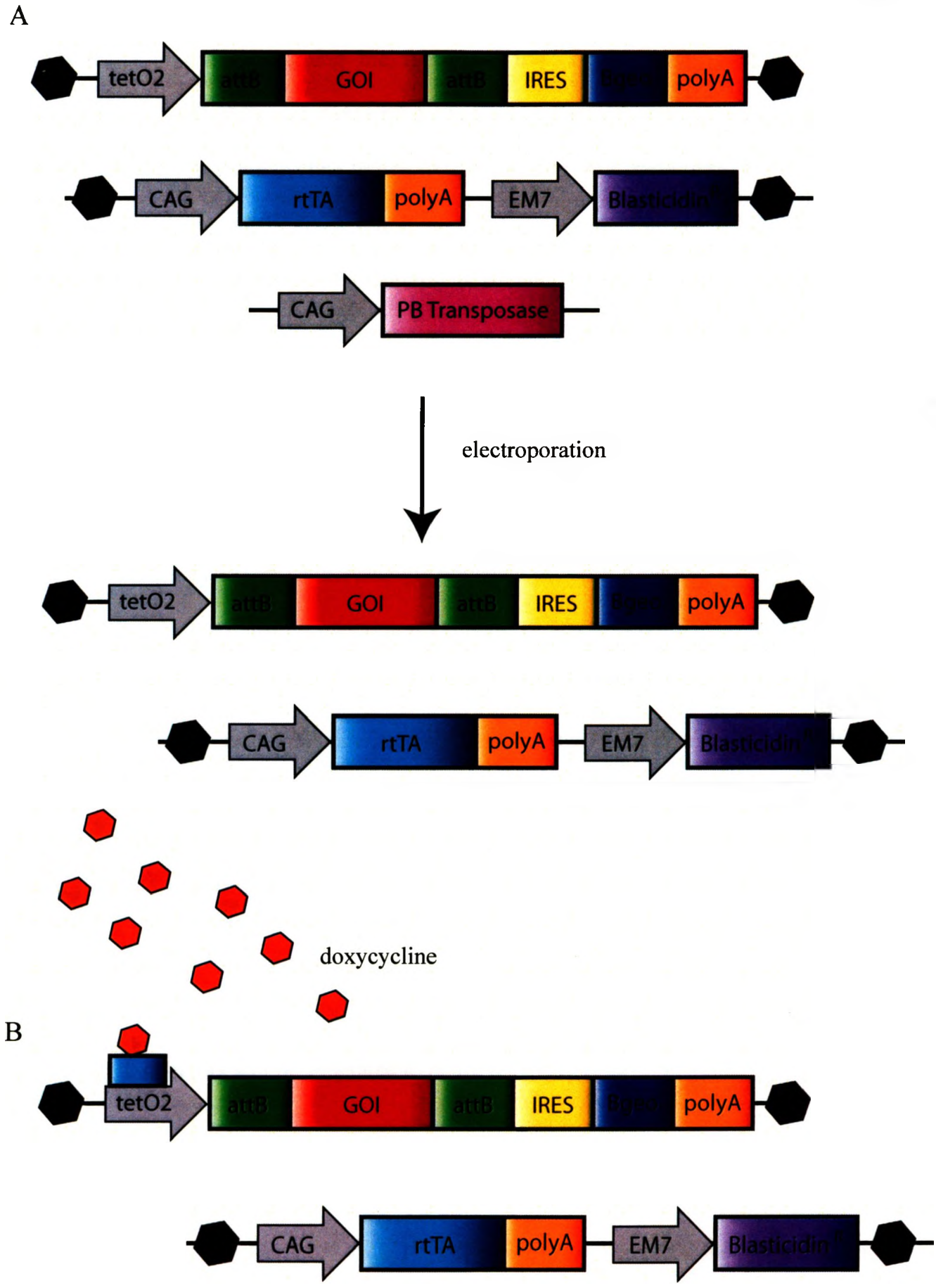

Tamoxifen
ERT2
SOX17
ERT2
SOX17
nucleus

Figure 1.5 Transposon Based Tools for Inducible Transcription Factor Expression in HESC

(A) The tetO2 piggyBac transposase system allows for the gene of interest (GOI), which is cloned into the PB-TET plasmid and flanked by inverted terminal repeats (ITR), to jump into the genome following transient expression of the piggyBac transposase enzyme. Entry occurs at TTAA sites and transgenic clonal populations selected for blasticidin resistance. (B) Transcription of the DNA of interest is induced by the addition of doxycycline to the culture media, which binds the reverse tetracycline transactivator (rtTA) protein, allowing it to bind the tet operator and initiate transcription. (Obtained by MTA from Dr. A. Nagy, Mount Sinai Hospital, Toronto). IRES – Independent ribosomal entry site, Bgeo – β-galactosidase/neomycin resistance fusion gene, polyA – polyadenylation tail, CAG – CMV enhancer combined with chicken β-actin promoter, EM7 – promoter for expression of blasticidin resistance.


A
tetO2
attB
GOI
attB
IRES
polyA
CAG
rtTA
polyA
EM7
Blasticidin
CAG
PB Transposase
electroporation
tetO2
attB
GOI
attB
IRES
polyA
CAG
rtTA
polyA
EM7
Blasticidin
doxycycline
B
tetO2
attB
GOI
attB
IRES
polyA
CAG
rtTA
polyA
EM7
Blasticidin

transposase plasmid containing the functional transposase enzyme that permits the integration of PB-TET and CAG-rtTA-blasticidin plasmids into the genome, which contains the reverse tetracycline transactivator necessary for transcription of the gene of interest. When bound by doxycycline, the rtTA protein acts on the tetO2 promoter to drive transcription of the gene of interest (Cadinanos and Bradley, 2007; Ding et al., 2005; Wang et al., 2008). Previous studies have used this method of gene delivery for the expression of c-Myc, Klf4, Sox2 and Oct4 in mouse fibroblasts and reported functional transcription factor activities (Woltjen et al., 2009).

1.5 Rationale

Diabetes is becoming increasingly prevalent in North America. Currently the only treatment options are insulin injection reginmens, or whole pancreas and pancreatic islet transplantation. Unfortunately, there is a scarce source of donor tissues for transplantation, as each transplant requires approximately 1 billion β-cells according to the Edmonton Protocol. This project will assay the ability of three independent transcription factors (PDX1, NGN3, PAX4) to specify a pancreatic endocrine phenotype following ectopic expression in HESC-derived definitive endoderm. Additionally, to better recapitulate the transient and/or biphasic patterns of transcription factor expression during development, cells with inducible, sequential activation of lineage-determining transcription factors will be generated to better recapitulate developmental patterns.

1.6 Hypothesis

Following SOX17-mediated definitive endoderm specification, expression of pancreas-specific transcription factors (PAX4, PDX1 or NGN3) will specify pancreatic endocrine progenitors from human embryonic stem cells.

1.7 Objectives

The specific objectives of this research project are to:

1. Use constitutive SOX17 expressing HESC to screen for the ability of PAX4, PDX1 or NGN3 to specify pancreatic endocrine progenitors from definitive endoderm
2. Characterize definitive endoderm differentiation using transient activation of SOX17
3. Generate transgenic HESC for transient activation of SOX17 to specify definitive endoderm, followed by PAX4, PDX1 or NGN3 to specify pancreatic endocrine cells

2.0 MATERIALS AND METHODS

2.1 Cell Culture

CA1 and CA2 (O'Connor et al., 2008; Peerani et al., 2007), as well as CA2 pClip CAG-Fl-βgeo-Fl-SOX17-IRES-PURO (Seguin et al., 2008) and CA1 CAG-SOX17-ER^{T2}-PURO human embryonic stem cells (HESC) were maintained using conditions modified from established protocols for HESC derivation and culture (Thomson et al., 1998). Briefly, cells were grown on mitotically inactivated mouse embryonic fibroblast feeders (SickKids ESC Facility) in knockout Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 15% serum replacement, glutamax, penicillin/streptomycin, 1mM nonessential amino acids, 0.5mM mercaptoethanol and 10ng/mL FGF2. Transgenic cell populations were selected for by culturing in media supplemented with the appropriate antibiotic (0.6μg/mL puromycin, 1.0μg/mL G418). When cultures had attained 75% confluence, cells were passaged by enzymatic dissociation (0.05% Trypsin-EDTA), pelleted at 244 rcf/g for 3 minutes and split at a ratio of 1:9 (every 4-6 days).

2.2 Plasmid Construction

The open reading frame (ORF) corresponding to the human NGN3 gene was amplified by PCR from cDNA that was previously generated in our laboratory from HESC that were engineered to constitutively overexpress SOX17 and underwent a pancreatic growth factor differentiation protocol, resulting in a population containing insulin-positive cells (Seguin et al., 2008). The sequence corresponding to the PAX4 ORF was amplified by PCR from the full-length cDNA clone ID# HsCD00079789 (Arizona State University). Both genes were amplified using primers designed to incorporate 5'Xho1 and 3'Not1 restriction enzyme sites to allow for directional cloning

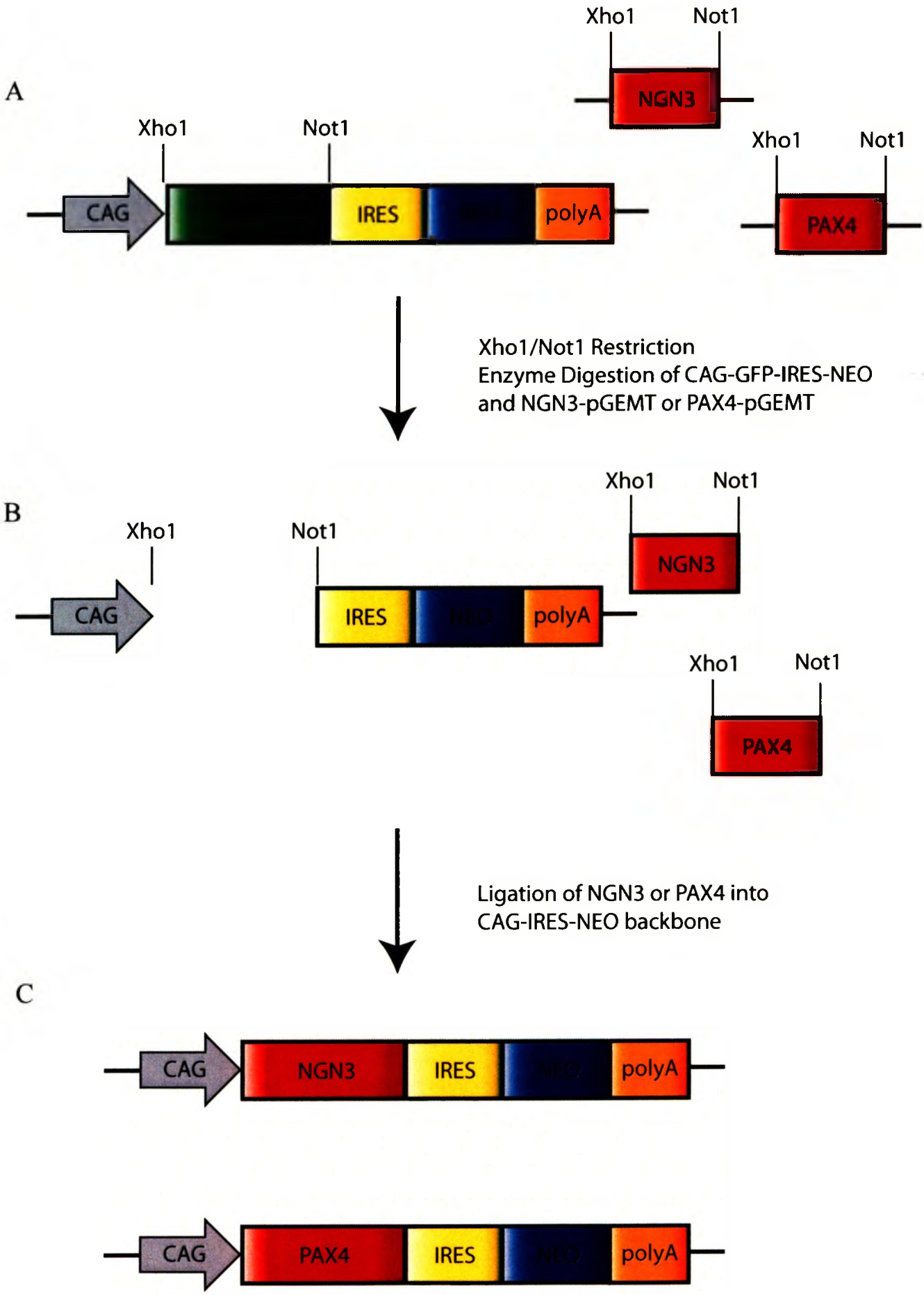
into the expression vector. The resultant amplicons were introduced into the pGEM-T vector by TA cloning. NGN3, and PAX4 ORFs were introduced independently into the CAG-MCS-IRES-NEO plasmid containing a CAG promoter (CMV enhancer combined with chicken β-actin promoter), a multiple cloning site (MCS), an independent ribosomal entry site (IRES) and a neomycin-polyA cassette (Figure 2.1) (generously provided by Dr. H Niwa, RIKEN Center for Developmental Biology). This construct confers constitutive expression of the gene of interest in addition to a neomycin resistance gene. To confirm correct integration and gene sequence, the MCS of the resultant plasmid was sequenced.

A PB-TET-PDX1 plasmid was generated through a Gateway cloning LR reaction using 100ng of the mammalian gene collection full-length cDNA clone ID# 40080648 (Invitrogen) PDX1-pDONR223.1, 150ng PB-TET plasmid, 5X LR Clonase II (Invitrogen), and TE buffer. Gateway cloning is a highly efficient method that uses site-specific recombination to move DNA sequences into vectors. The LR reaction allows a donor vector containing attL sites flanking the gene of interest to recombine with a destination vector containing attR sites flanking a ccdb sequence. The reaction mix was incubated at 37°C overnight and 1μL transformed into 50μL of One Shot OmniMAX 2T1 Phage-Resistant Cells (Invitrogen). Clonal populations were picked after ampicillin selection. Purified plasmids were sequence verified.

To visualize the activity of the PB-TET system, through green fluorescent protein (GFP), a PB-TET-GFP plasmid was obtained from Dr. Janet Rossant at the Hospital for Sick Children.

Figure 2.1: Schematic representation of construction of plasmids for constitutive transcription factor expression in HESC

(A) The open reading frames (ORF) for NGN3 and PAX4 were amplified using primers designed to incorporate Xho1 and Not1 restriction sites for directional cloning. (B) The CAG-GFP-IRES-NEO^R-PolyA plasmid was digested using Xho1 and Not1 to excise GFP. This plasmid contains a CAG promoter (CMV enhancer combined with chicken β-actin promoter), GFP (green fluorescent protein), IRES (independent ribosomal entry site), NEO^R (neomycin resistance gene), polyA (polyadenylated tail). (C) NGN3 and PAX4 ORFs were independently cloned into the CAG-IRES-NEO^R plasmid. The resultant plasmids were sequence verified.


A
Xho1
Not1
NGN3
Xho1
Not1
Xho1
Not1
CAG
IRES
polyA
PAX4
Xho1/Not1 Restriction
Enzyme Digestion of CAG-GFP-IRES-NEO
and NGN3-pGEMT or PAX4-pGEMT
B
Xho1
Not1
Xho1
Not1
CAG
IRES
polyA
NGN3
Xho1
Not1
PAX4
Ligation of NGN3 or PAX4 into
CAG-IRES-NEO backbone
C
CAG
NGN3
IRES
polyA
CAG
PAX4
IRES
polyA

2.3 Generation of Transgenic HESC

To generate DE cells, CA2 HESC that contained a single integration of the pClip CAG-Fl-βgeo-Fl-SOX17-IRES-PURO plasmid were used (Seguin et al., 2008). This plasmid contains a CAG promoter (CMV enhancer combined with chicken β-actin promoter), a floxed βgeo element (β-galactosidase/neomycin resistance fusion protein), the SOX17 ORF, an independent ribosomal entry site (IRES), and the puromycin resistance gene. These cells remain undifferentiated in culture, and are induced to express SOX17 following the introduction of Cre recombinase. The overexpression of SOX17 drives these HESC to a DE fate. To induce SOX17 expression, three confluent wells of a 6 well dish were enzymatically dissociated into single cells (0.05% Trypsin-EDTA) and centrifuged at 244 rcf/g for three minutes. The resultant cell pellet was resuspended in 400μL electroporation buffer (Gibco) and added to a 4mm electroporation cuvette containing 20μg of CAG-NLS-CRE plasmid. This plasmid contains a CAG promoter, a nuclear localization sequence (NLS) and the ORF for Cre recombinase. The electroporation cuvette was inserted into an electroporator (BioRad) and pulsed at 170V, 1050μF. Cells were then plated into a 6 well plate, and after 48 hours, transgene expression was selected for using 0.6μg/mL puromycin.

HESC were previously generated in the lab that allow for control of dose and duration of SOX17 expression. CA1 HESC were electroporated with a CAG-SOX17-ER^{T2}-IRES-PURO plasmid. This plasmid contains a CAG promoter, the ORF for SOX17 fused in frame to the estrogen receptor ligand binding domain (ER^{T2}), an independent ribosomal entry site (IRES), and a puromycin resistance gene. This transgene is

constitutively expressed, but the SOX17 transcription factor is only active following the addition of 4-OH Tamoxifen.

To generate HESC lines for pancreatic differentiation, NGN3, PAX4 or PDX1 were introduced into both CA2 and CA2 CAG-SOX17-IRES-PURO HESC. To allow for stable integration of the CAG-NGN3-IRES-NEO or CAG-PAX4-IRES-NEO transgenes, these plasmids were linearized prior to electroporation. Plasmids were linearized by restriction enzyme digestion using Sca1, which cuts once within the ampicillin resistance gene. Following digestion, the linearized plasmid was purified by ethanol precipitation, and resuspended in nuclease-free water to a concentration of 1μg/μL.

HESC with constitutive expression of NGN3 or PAX4 were generated by electroporating CA2 or CA2 CAG-SOX17-IRES-PURO HESC with 20 μg of linearized plasmid. To generate HESC with constitutive expression of PDX1, cells were electroporated with 4 μg of each of PB-TET-PDX1, PB-Transposase, and CAG-rtTA-blasticidin. For electroporation, three wells of a 6 well dish of HESC were enzymatically dissociated into single cells (0.05% Trypsin-EDTA) and centrifuged at 244 rcf/g for three minutes. The resultant cell pellet was resuspended in 400μL electroporation buffer and added to a 4mm electroporation cuvette containing the desired plasmid. The electroporation cuvette was inserted into an electroporator (BioRad) and pulsed at 170V, 1050μF. Cells were then plated into a 6 well dish and after 48 hours, transgene expression was selected for using 0.8 μg/mL neomycin.

To induce the expression of PDX1, 500ng/mL doxycycline was added to media daily, 24 hours after electroporation. Following 48 hours of growth, cells were treated with neomycin (1μg/mL) to select for expression of the PB-TET-PDX1 transgene.

To generate PB-TET-GFP HESC, 3μg of PB-TET-GFP, 5μg PB Transposase, and 5μg of CAG-rtTA-blasticidin plasmids were co-electroporated as described above into CA1 HESC. Cells were plated onto on Matrigel coated dishes (Invitrogen) in media that had been previously conditioned for 48 hours by mitotically inactivated mouse embryo fibroblast feeders (MEF-conditioned media). Matrigel was required because MEFs are not blasticidin resistant. After 48 hours, 5μg/mL blasticidin was added to culture media to select for expression of the CAG-rtTA-blasticidin plasmid. Clonal, blasticidin resistant colonies were picked each into one well of a four well dish on Matrigel in MEF conditioned media. Upon confluency, cells were enzymatically passaged 1:4 into four wells of a 12 well dish on Matrigel in MEF conditioned media. Once cultures were confluent, three wells were cryopreserved (90% FBS, 10% DMSO), and one well was expanded for experimental purposes. In total, 12 CA1 PB-TET-GFP clones were generated.

To generate CA1 CAG-SOX17-ER^{T2}-PURO PB-TET-PDX1 HESC, 3μg of PB-TET-PDX1, 3μg of PB Transposase, and 3μg of CAG-rtTA-blasticidin plasmids were introduced into SOX17-ER^{T2} HESC by electroporation, as described above. After 48 hours, 5μg/mL blasticidin was added to culture media to select for integration of the CAG-rtTA-blasticidin plasmid. Clonal blasticidin resistant colonies were picked each into one well of a four well dish on Matrigel in MEF conditioned media. Upon confluency, cells were enzymatically passaged 1:4 into four wells of a 12 well dish on

Geltrex in MEF conditioned media. Once cells were confluent, three wells were cryopreserved (90% FBS, 10% DMSO), and one well was expanded for experimental purposes. In total, 30 clonal populations of CA1 CAG-SOX17-ERT2-PURO PB-TET-PDX1 HESC were generated.

2.4 Gene Expression Analysis

CA1 CAG-SOX17-ERT2-IRES-PURO HESC were plated into 12 well plates and treated with 0.1μM 4-OH Tamoxifen in the absence of FGF2 for either two hours, 24 hours, two days, four days, or left untreated. At each timepoint, two wells of a 12 well dish were rinsed with PBS and 0.5mL Trizol Reagent © was added to each well. Cells were scraped and both wells were combined as one sample.

Following electroporation of CAG-NGN3-IRES-NEO, CAG-PAX4-IRES-NEO, or PB-TET-PDX1 into CA2 or CA2 pClip CAG-SOX17-IRES-PURO HESC, cells were maintained in HESC media with 1.0μg/mL G418. The day of electroporation was considered day 0. These cells were harvested at four days, eight days, 12 days, and 16 days following electroporation. At each timepoint, two wells of a 6 well dish were rinsed with PBS and scraped in 0.5mL PBS. Both wells were pooled as one sample and centrifuged at 244 rcf/g for 3 minutes. The resultant cell pellet was resuspended in 1mL Trizol Reagent ©.

Total RNA was isolated using Trizol Reagent © (Invitrogen) according to the manufacturer's instructions and quantified by spectrophotometry on a Nanodrop 2000. To generate cDNA, 1μg of RNA was reverse transcribed using the iScript cDNA

synthesis kit (BioRad) according to the manufacturer's instructions using reverse transcriptase and oligo(dT) and random primers. Gene expression was assessed by real-time PCR using the BioRad CFX384. PCR reactions were run in triplicate using 40ng of cDNA per reaction and 400μM forward and reverse primers with 2X Sso Fast Eva Green reaction mix (BioRad) and 40 cycles of amplification (95°C 2 min; 95°C 10sec; 60°C 30sec). Real-time PCR primers were designed to span intron-exon junctions, so that the PCR product from genomic DNA can be distinguished from the PCR product from cDNA through melt curve analysis. Primer sets were run on a standard curve to ensure an efficiency value between 90-110%. For every PCR experiment, specificity of primers was determined by performing a melt curve from 65°C to 95°C after each run. Gene expression was determined by relative quantification with values corrected for input using TATA-binding protein (TBP) and normalized relative to undifferentiated CA1 HESC. Analyses were performed in triplicate on three or four independent trials.

2.5 Immunostaining

Cells were fixed in 4% (w/vol) paraformaldehyde for 15 min at room temperature, rinsed twice in PBS, and stored at 4°C in PBS until use. For immunocytochemistry, cells were blocked for 1hr in PBST (PBS, 0.1% Triton X – 100) with 5% fetal bovine serum (FBS). Antibodies for SOX17 (R&D AF1924) 1:100, OCT3/4 (sc-5279) 1:400, NANOG (ab80892) 1:400, and SSEA3 (mc-631) 1:10 were diluted in PBST with 5% FBS and primary antibodies were incubated for one hour. Cells were washed 2x10 min with PBST, followed by incubation with species specific secondary antibodies Alexa Fluor 488 anti-goat IgG, anti-mouse IgG and anti-rabbit IgG 1:400 (Molecular Probes), and

Cy™5 anti-rat IgG 1:400 (Jackson ImmunoResearch) that were incubated for one hour at room temperature diluted in PBST with 5% FBS. Cells were washed 2x10 min with PBST, followed by nuclear staining using 1μg/mL Hoechst 33258 in PBST for 10 min. Cells were washed 2x10 min with PBS, and stored in PBS for imaging. Fluorescence images were taken with a Zeiss Imager using a 10X objective, as well as a Leica DM6000 inverted fluorescent microscope. Fluorescence imaging was performed on three independent trials.

2.6 LacZ Staining

To observe the functionality of CA1 SOX17-ER ^{T2}/PB-TET-PDX1 HESC through the expression of β-galactosidase, which is downstream of PDX1, cells were treated with 500ng/mL doxycycline for 4 days to induce the PB-TET-PDX1 transgene. Cells were then fixed in 0.25% glutaraldehyde for 15 min at room temperature, rinsed twice in PBS and covered in X-gal solution (0.2% X-gal, 2mM $MgCl_2$, 5mM $K_4Fe(CN)_6$•3H2O, 5mM $K_3Fe(CN)_6$ in PBS) and incubated overnight at room temperature. X-gal solution was removed, cells were rinsed twice with PBS, covered with PBS and incubated until blue staining was visible. Staining was imaged on a Nikon SMZ-1500 stereoscope.

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.00. Real-time PCR quantification was examined using a one-way ANOVA and Dunnett's post-test, as well as using a two-way ANOVA and Bonferroni post-test. $p<0.05$ was considered significant.

3.0 RESULTS

3.1 Constitutive expression of PAX4 in CA2 HESC does not change cell morphology.

To assess the ability of ectopic expression of PAX4 to specify pancreatic endocrine cells from pluripotent HESC, a transgene confering constitutive expression of pCAGS-PAX4-IRES NEO was introduced into CA2 HESC. Transgenic HESC were maintained as previously described and cells were imaged and RNA was isolated at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. There was no observable change in cell morphology following constitutive expression of PAX4 in CA2 HESC during this time period (Figure 3.1 A). Real time PCR analysis of PAX4 gene expression was performed (Figure 3.1 B). Expression of the PAX4 transgene showed a trend towards decreased expression over time in the resultant transgenic cell populations.

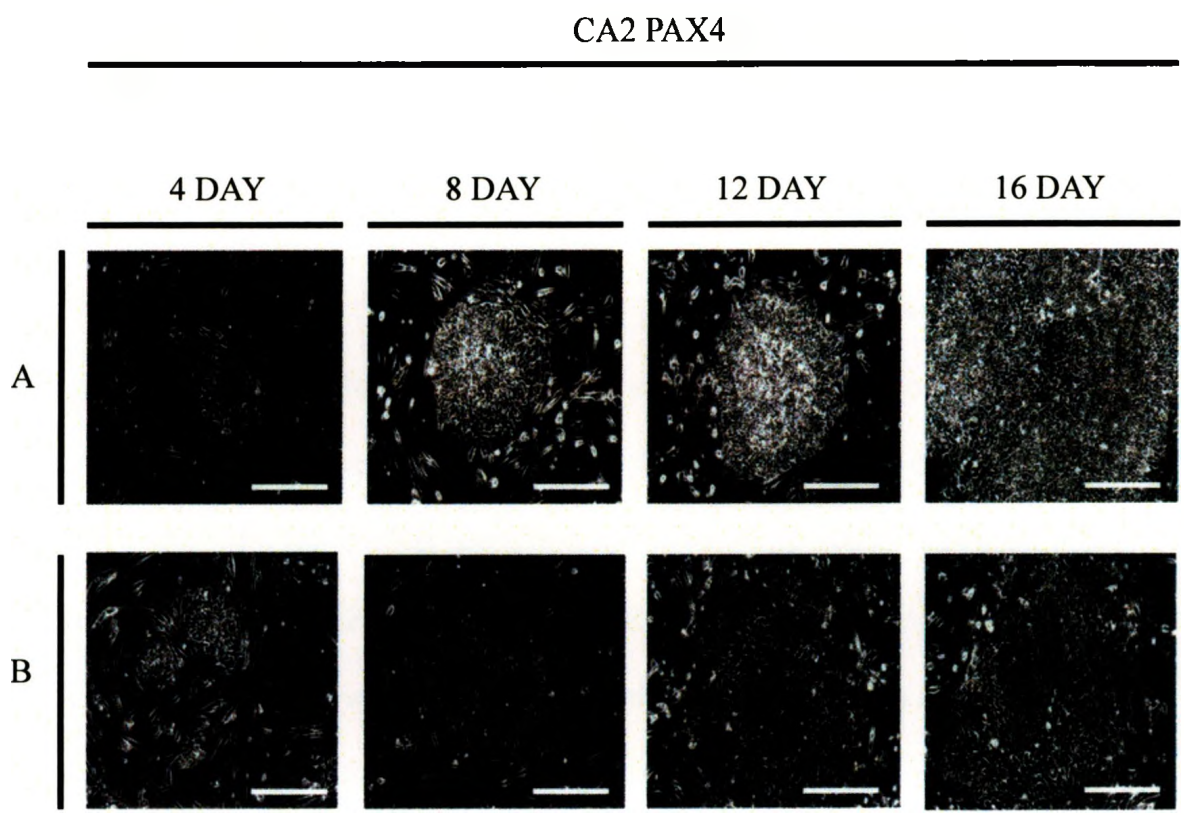
3.2 Constitutive expression of PAX4 in DE cells does not change cell morphology.

To assess the ability of ectopic expression of PAX4 to specify pancreatic endocrine cells from definitive endoderm, pCAGS-PAX4-IRES NEO was introduced into SOX17 expressing HESC (DE). There was no change in morphology of DE cells in the presence of constitutive PAX4 expression (Figure 3.2 A). Real time PCR analysis of SOX17 and PAX4 gene expression was performed (Figure 3.2 B). Expression of both SOX17 and PAX4 transgenes showed a trend towards decreased expression over time in the resultant transgenic cell populations. This finding is in keeping with the initial characterization of the SOX17 HESC lines (Seguin et al., 2008), which demonstrated decreased SOX17 expression with cell differentiation.

Figure 3.1 Constitutive expression of PAX4 in CA2 HESC does not change cell morphology.

(A) Photomicrographs of replicate populations of CA2 PAX4 HESC imaged at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. A transgene conferring constitutive expression of pCAGS-PAX4-IRES NEO was introduced into CA2 HESC (scale bars, 500 μm). There was no observable change in morphology following PAX4 expression in CA2 HESC. (B) Real time PCR quantification of PAX4 expresssion in resultant cell populations at 4, 8, 12 and 16 day time points. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC, (n=2). For PAX4, the SEM of technical replicates was not greater than 3.456.

A


CA2 PAX4
4 DAY
8 DAY
12 DAY
16 DAY
A
B

B

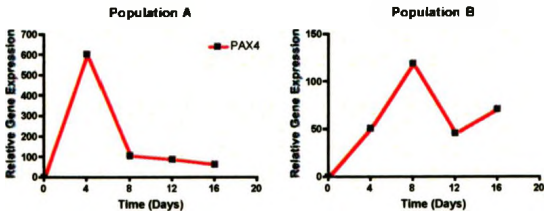
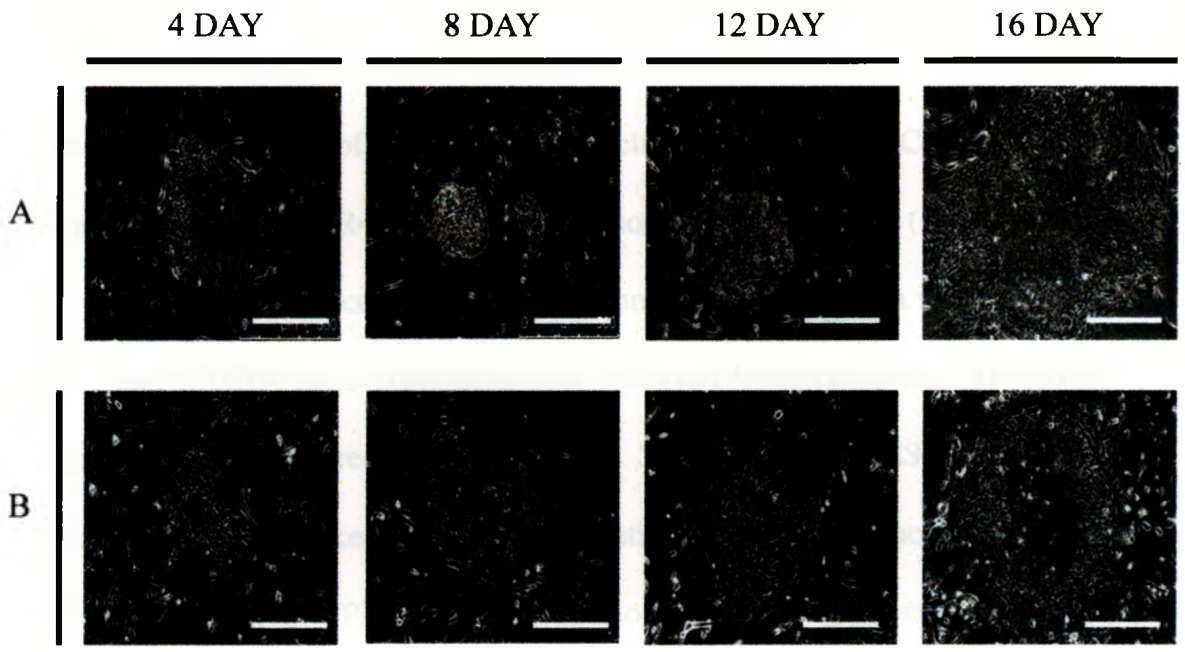

Population A
PAX4
Relative Gene Expression
Time (Days)
Population B
Relative Gene Expression
Time (Days)

Figure 3.2 Constitutive expression of PAX4 in DE cells does not change cell morphology.

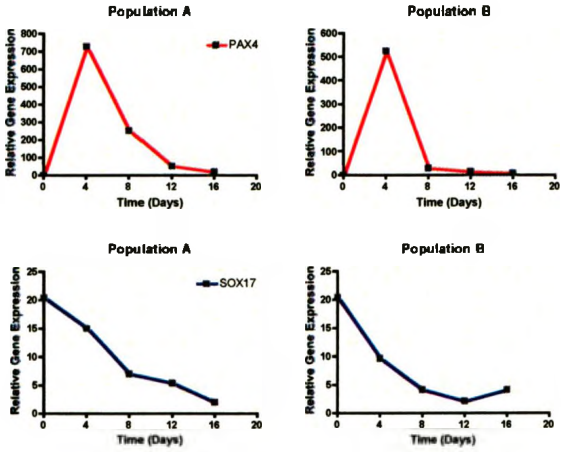
(A) Photomicrographs of replicate populations of SOX17 PDX1 HESC imaged at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. A transgene conferring constitutive expression of pCAGS-PAX4-IRES NEO was introduced into SOX17 expressing HESC (DE) (scale bars, 500 μm). There was no observable change in morphology following expression of PAX4 in DE cells. (B) Real time PCR quantification of PAX4 and SOX17 expression in resultant cell populations at 4, 8, 12 and 16 day time points. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Real time PCR analysis of SOX17 and PAX4 gene expression showed a decreasing trend in both transgenic cell populations, (n=2). For PAX4, the SEM of technical replicates was not greater than 2.459. For SOX17, the SEM of technical replicates was not greater than 0.8294.

A

CA2 SOX17D PAX4



B

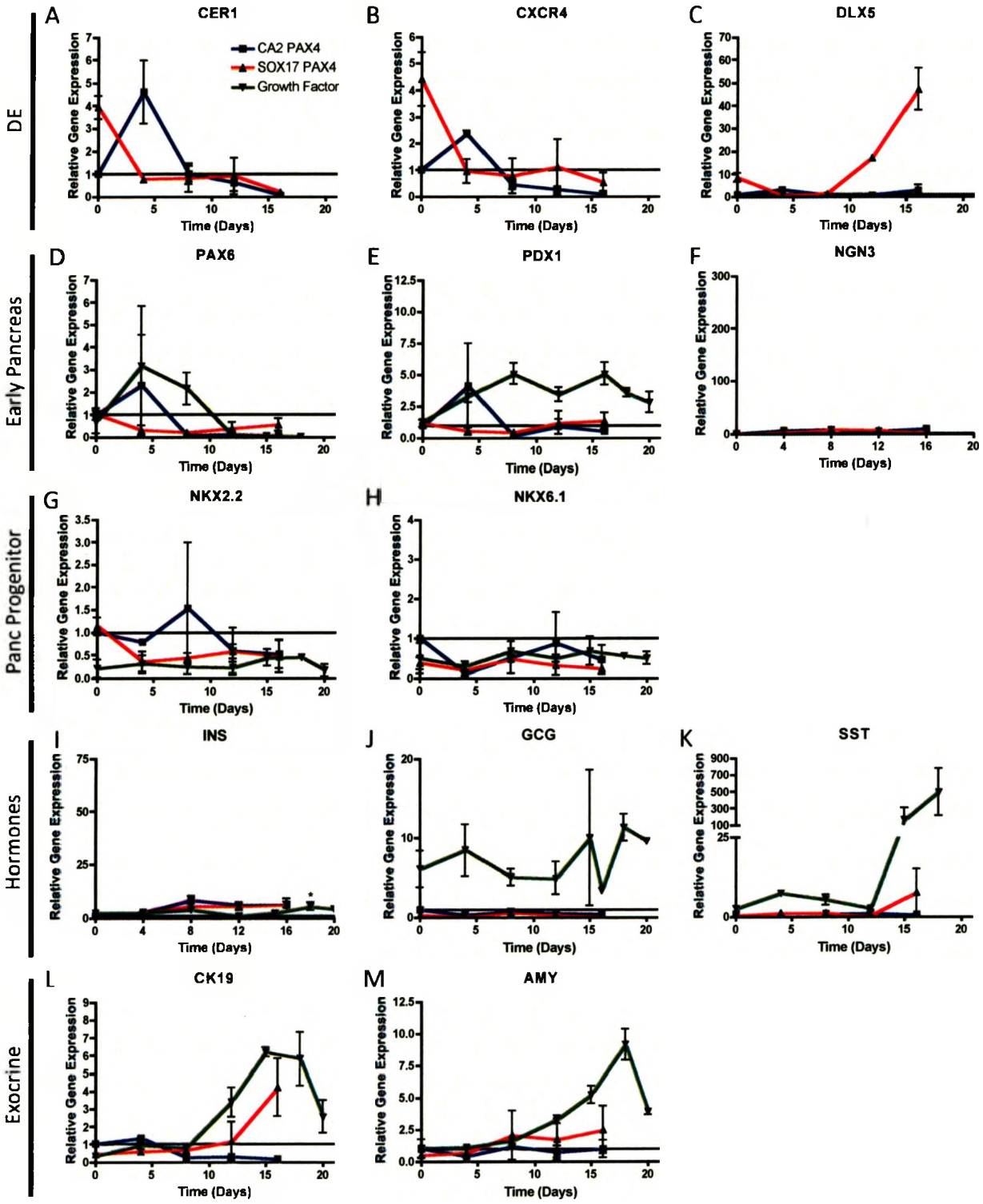


3.3 Constitutive expression of PAX4 in CA2 HESC and DE cells does not change cell phenotype.

To assess the gene expression profiles of cells following constitutive expression of PAX4, real time PCR was performed. To assess changes to cell phenotype following transgene expression, cells were directly compared to HESC subjected to growth factor mediated differentiation using previously established protocols (D'Amour et al., 2006). We first assessed the expression of markers of DE including Cerberus 1 (CER1), C-X-C chemokine receptor type 4 (CXCR4), and distal-less homeobox 5 (DLX5) (Figure 3.3 A-C). In both CA2 HESC and DE cells, PAX4 overexpression resulted in a trend towards decreased CER1 and CXCR4 gene expression over 16 days of differentiation. However, DLX5 gene expression was increased at day 16 in SOX17 PAX4 HESC. We next examined the expression of markers of early pancreatic differentiation including PDX1 and paired box gene 6 (PAX6). PDX1 and PAX6 showed decreased expression in both transgenic cell populations after 16 days of differentiation compared to unmodified CA1 HESC (Figure 3.3 D-E). Next, markers of pancreatic progenitors NK2 homeobox 2 (NKX2.2) and NK6 homeobox 1 (NKX6.1) were examined. There was no difference in NKX2.2 and NKX6.1 gene expression compared to unmodified CA1 HESC and growth-factor mediated pancreatic differentiation (Figure 3.3 G-H). To assess pancreatic hormone gene expression, we quantified mRNA levels of insulin (INS), glucagon (GCG) and somatostatin (SST) mRNA. Expression of INS showed a trend towards increased expression in both transgenic cell populations at day 12 and 16 compared to growth factor mediated pancreatic differentiation (Figure 3.3 I). Conversely, GCG and SST expression did not differ compared to unmodified CA1 HESC, levels below those detected in growth factor mediated pancreatic differentiation (Figure 3.3 J-K). Lastly, cytokeratin 19

Figure 3.3 Constitutive expression of PAX4 in CA2 HESC and DE cells does not change cell phenotype

Real time PCR quantification of gene expression in transgenic PAX4 populations at 4, 8, 12 and 16 day time points following electroporation. The day of electroporation was considered day 0. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Data are presented as the mean ± SEM from duplicate experiments (n=2). (A-C) To assess DE differentiation, expression of CER1, CXCR4 and DLX5 was quantified. (D-F) To assess early pancreatic differentiation, expression of PAX6, PDX1 and NGN3 was quantified. (G-H) To assess pancreatic progenitor differentiation, expression of NKX2.2 and NKX6.1 was quantified. (I-K) Expression of pancreatic hormones INS, GCG and SST were quantified. (L-M) To assess exocrine differentiation, expression of CK19 and AMY was quantified.


DE
Early Pancreas
Panc Progenitor
Hormones
Exocrine
A
CER1
CA2 PAX4
SOX17 PAX4
Growth Factor
B
CXCR4
C
DLX5
D
PAX6
E
PDX1
F
NGN3
G
NKX2.2
H
NKX6.1
I
INS
J
GCG
K
SST
L
CK19
M
AMY
Relative Gene Expression
Time (Days)

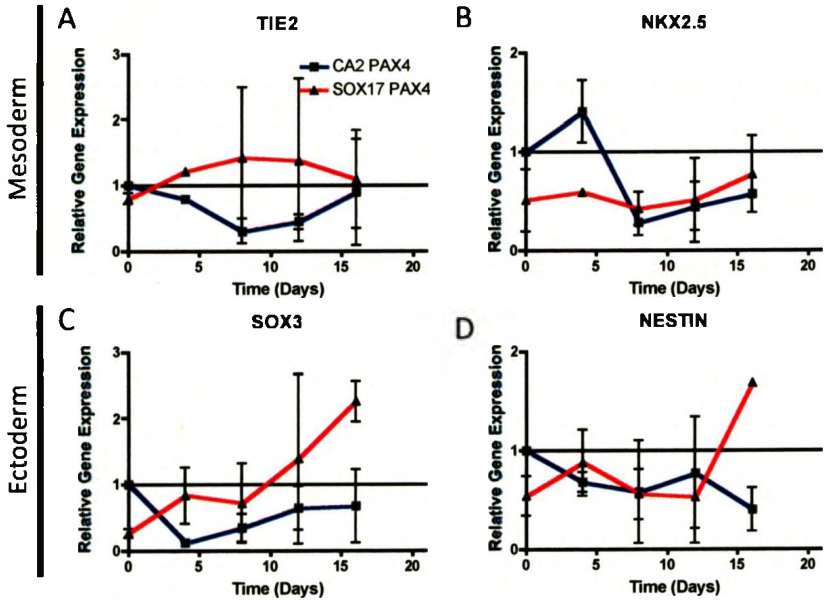
(CK19), and amylase (AMY) were investigated as markers of pancreatic ductal epithelium and exocrine differentiation respectively (Figure 3.3 L-M). Both transgenic cell populations had no change in CK19 expression compared to unmodified CA1 HESC until day 16 when SOX17 PAX4 HESC showed increased gene expression compared to CA2 PAX4 HESC and unmodified CA1 HESC (Figure 3.3 L-M). Throughout the timecourse, CK19 expression in these transgenic cell populations remained lower than in growth factor mediated pancreatic differentiation. AMY expression remained unchanged throughout the timecourse for both transgenic cell populations compared to unmodified CA1 HESC, while AMY gene expression was detected following growth factor mediated pancreatic differentiation. Taken together, these data suggest that overexpression of PAX4 does not promote pancreatic endocrine differentiation from HESC or DE.

3.4 Constitutive expression of PAX4 in CA2 HESC and DE cells does not result in mesodermal or ectodermal differentiation by day 16.

To determine if constitutive expression of PAX4 in HESC or DE cells resulted in differentiation to mesodermal or ectodermal lineages, selected marker genes were examined by real time PCR. To assess mesodermal differentiation, TEK tyrosine kinase (TIE2) and NK2 homeobox 5 (NKX2.5) were investigated (Figure 3.4 A-B). TIE2 gene expression was unchanged in both transgenic cell populations when compared to unmodified CA1 HESC. NKX2.5 gene expression decreased in both transgenic cell populations when compared to unmodified CA1 HESC. Next, SOX3 and NESTIN, markers of ectodermal differentiation were examined (Figure 3.4 C-D). There was no

Figure 3.4 Constitutive expression of PAX4 in CA2 HESC and DE cells does not result in mesodermal or ectodermal differentiation by day 16.

Real time PCR quantification of gene expression in transgenic PAX4 populations at 4, 8, 12 and 16 day time points following electroporation. The day of electroporation was considered day 0. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Data are presented as the mean ± SEM from duplicate experiments (n=2). (A-B) Expression of mesodermal markers TIE2 and NKX2.5 were quantified. (C-D) Expression of ectodermal markers SOX3 and NESTIN were quantified.


Mesoderm
Ectoderm
A
TIE2
CA2 PAX4
SOX17 PAX4
Relative Gene Expression
Time (Days)
B
NKX2.5
Relative Gene Expression
Time (Days)
C
SOX3
Relative Gene Expression
Time (Days)
D
NESTIN
Relative Gene Expression
Time (Days)

robust induction of either SOX3 or NESTIN gene expression observed when compared to unmodified CA1 HESC.

3.5 Constitutive expression of PDX1 in CA2 HESC results in cells with a flattened morphology after 16 days.

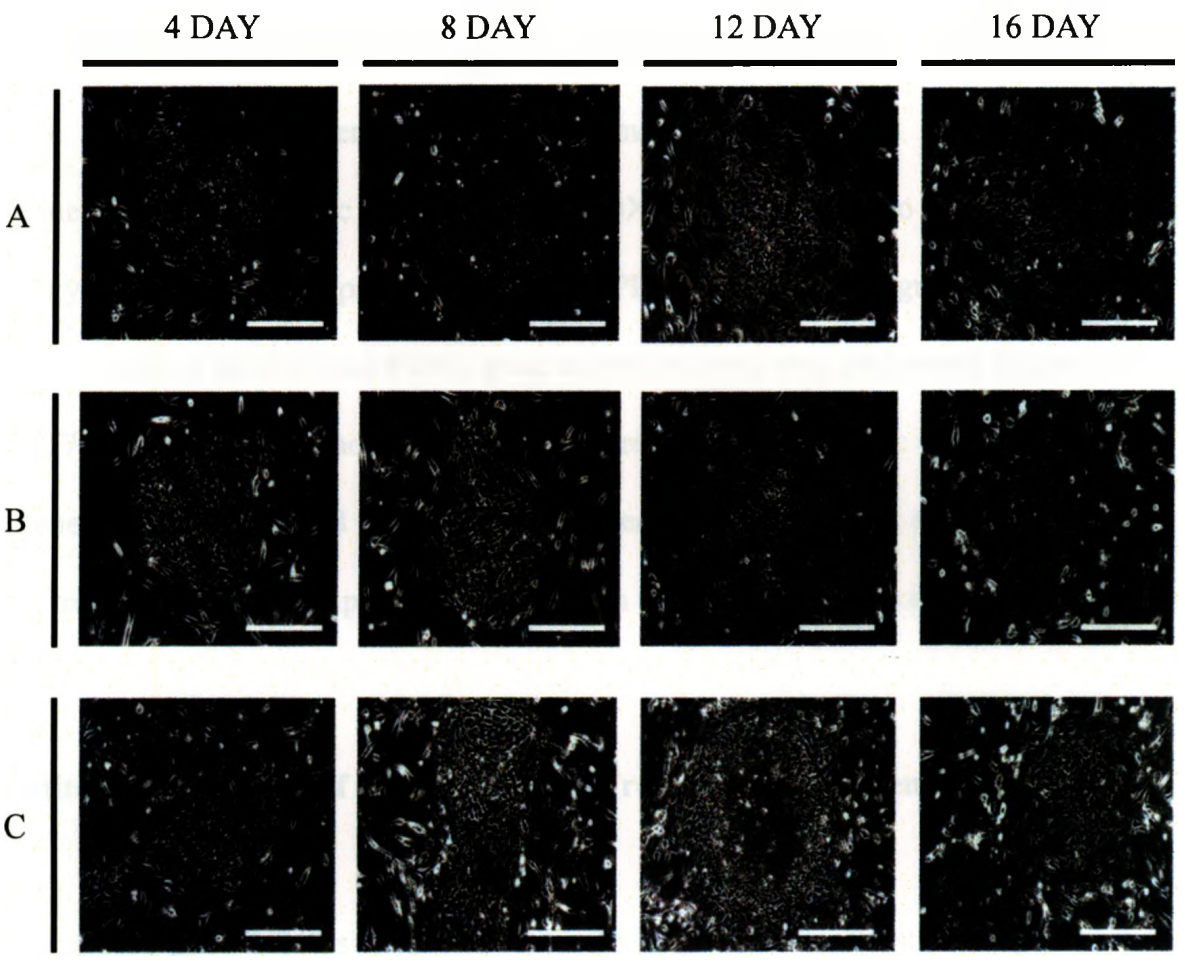
To assess the ability of PDX1 to specify pancreatic endocrine cells from pluripotent HESC, PB-TET-PDX1 was introduced into CA2 HESC. Transgenic HESC were maintained under standard conditions in media supplemented with 500ng/mL doxycycline to induce ectopic expression of PDX1. Cells were imaged and RNA was isolated at 4, 8, 12 and 16 day timepoints following electroporation (Figure 3.5 A). The day of electroporation was considered day 0. In transgenic populations A and B, cells adopted a more flattened morphology beginning at day 12, while transgenic population C adopted this morphology by day 16 (Figure 3.5 B). Real-time PCR analysis of PDX1 gene expression was performed in the 3 seperate replicate populations (Figure 3.5 C). Although all 3 populations had significantly increased PDX1 expression at day 4 compared to unmodified HESC, transgenic population C was shown to have the lowest amount of PDX1 transgene expression, demonstrating a 1402 fold increase vs HESC controls when compared to populations A and B which demonstrated relative expression values of 27071 fold and 24106 fold increase vs HESC controls respectively. However, PDX1 gene expression demonstrated a dramatic decrease over time in all three cell populations.

Figure 3.5 Constitutive expression of PDX1 in CA2 HESC results in cells with a flattened morpology after 16 days.

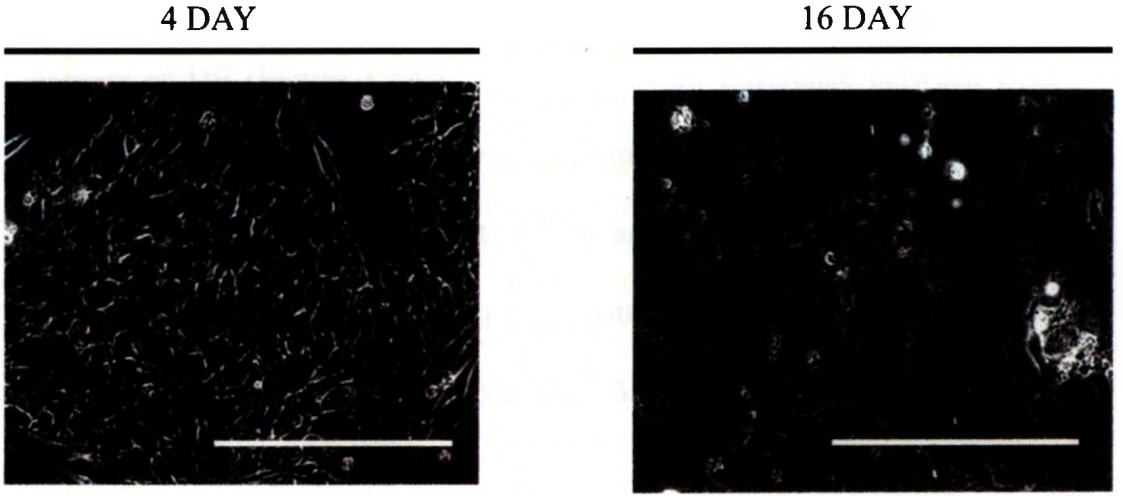
(A) Photomicrographs of replicate populations of CA2 PDX1 HESC imaged at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. A transgene conferring constitutive expression of PDX1 was introduced into CA2 HESC (scale bars, 500 μm). Following 16 days of constitutive PDX1 expression, cells adopted a flattened morphology. (B) Photomicrographs of CA2 PDX1 HESC at 4 days and 16 days demonstrating the flattened morphology of these cells at 16 days (scale bars, 500 μm). (C) Real time PCR quantification of PDX1 expresssion in resultant cell populations at 4, 8, 12 and 16 day time points. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC, (n=3). For PDX1, the SEM of technical replicates was not greater than 1.982.

A

CA2 PDX1



B



3.6 Constitutive expression of PDX1 in DE cells does not change cell morphology.

To assess the ability of PDX1 to specify pancreatic endocrine cells from definitive endoderm, PB-TET-PDX1 was introduced into SOX17 expressing HESC (DE). Transgenic SOX17 HESC were maintained in media supplemented with 500ng/mL doxycycline to induce ectopic expression of PDX1. There was no change in cell morphology of DE cells in the presence of ectopic PDX1 expression (Figure 3.6 A). Real time PCR analysis of SOX17 and PDX1 gene expression was also performed (Figure 3.6 B). SOX17 gene expression showed a decreasing trend in all transgenic cell populations. PDX1 gene expression varied substantially between the 3 replicate experiments and showed a trend of decreased expression over time in transgenic populations A and C.

3.7 Constitutive expression of PDX1 in DE cells results in insulin gene expression by day 16.

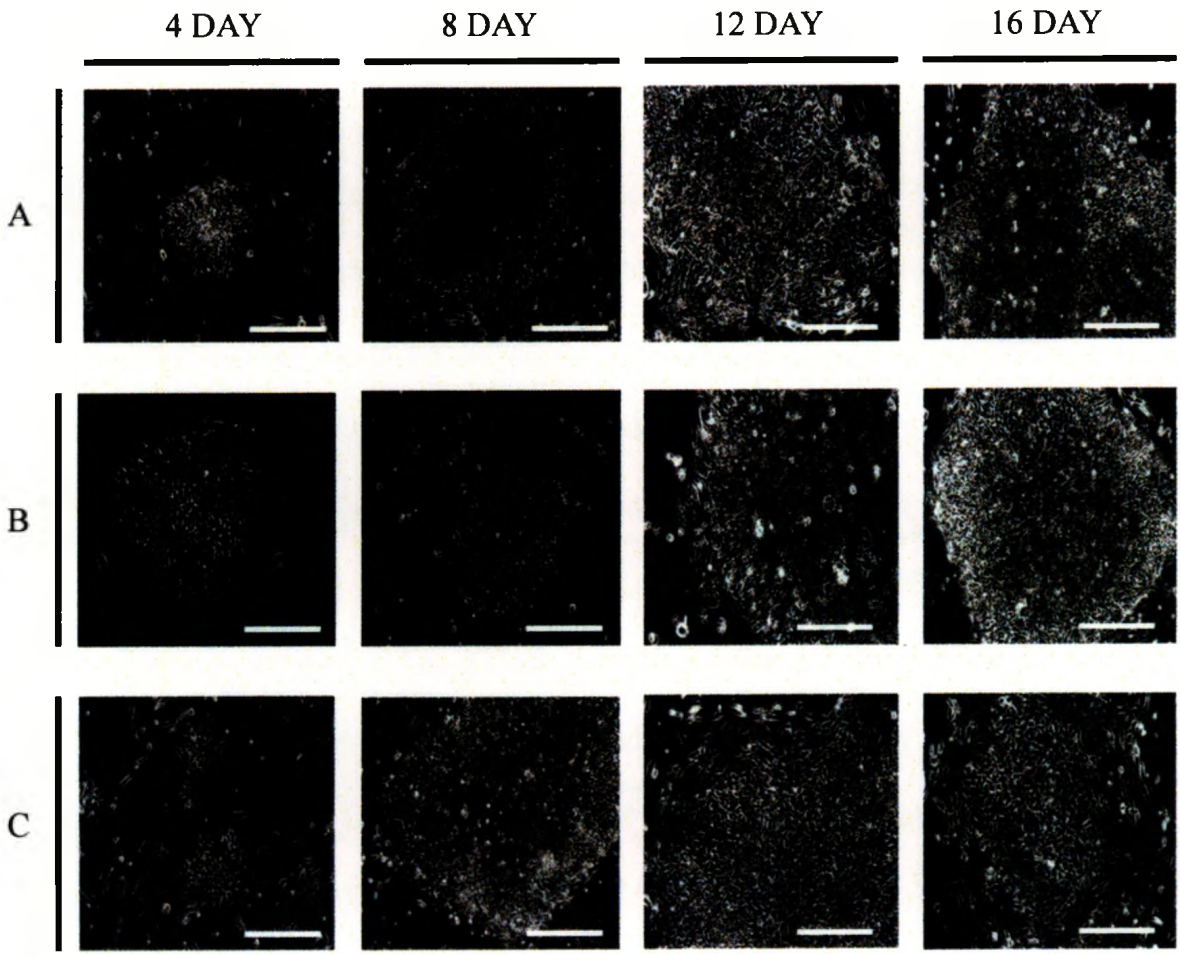
To assess gene expression profiles of cells engineered to constitutively express PDX1, we performed real time PCR. CER1, CXCR4, and DLX5 were once again examined as markers of DE (Figure 3.7A-C). There was no difference between CA2 PDX1 HESC and SOX17 PDX1 HESC at any of the timepoints examined. Markers of early pancreatic development including PAX4, PAX6 and NGN3 were also examined (Figure 3.7D-F). There were no differences in early pancreas gene expression in either transgenic cell population compared to unmodified CA1 HESC. Next, NKX2.2 and NKX6.1, markers of pancreatic progenitor differentiation, were assessed (Figure 3.7G-H). Following four days of ectopic PDX1 expression, both NKX2.2 and NKX6.1 expression showed a trend towards increased expression in SOX17 PDX1 HESC compared to CA2

Figure 3.6 Constitutive expression of PDX1 in DE cells does not change cell morphology.

(A) Photomicrographs of replicate populations of SOX17 PDX1 HESC imaged at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. A transgene conferring constitutive expression of PDX1 was introduced into DE cells (scale bars, 500 μm). (B) Real time PCR quantification of PDX1 and SOX17 expresssion in resultant cell populations at 4, 8, 12 and 16 day time points. Values were corrected for input using the expression TBP and expressed relative to undifferentiated HESC (n=3). SOX17 showed a trend towards decreasing gene expression in all transgenic cell populations. PDX1 gene expression was variable between the 3 replicate experiments. For PDX1, the SEM was not greater than 1.592. For SOX17, the SEM was not greater than 0.912.

A

CA2 SOX17D PDX1



B

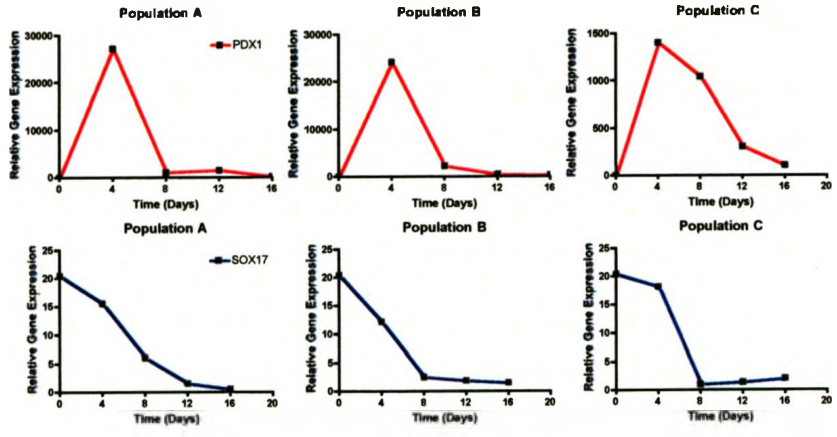
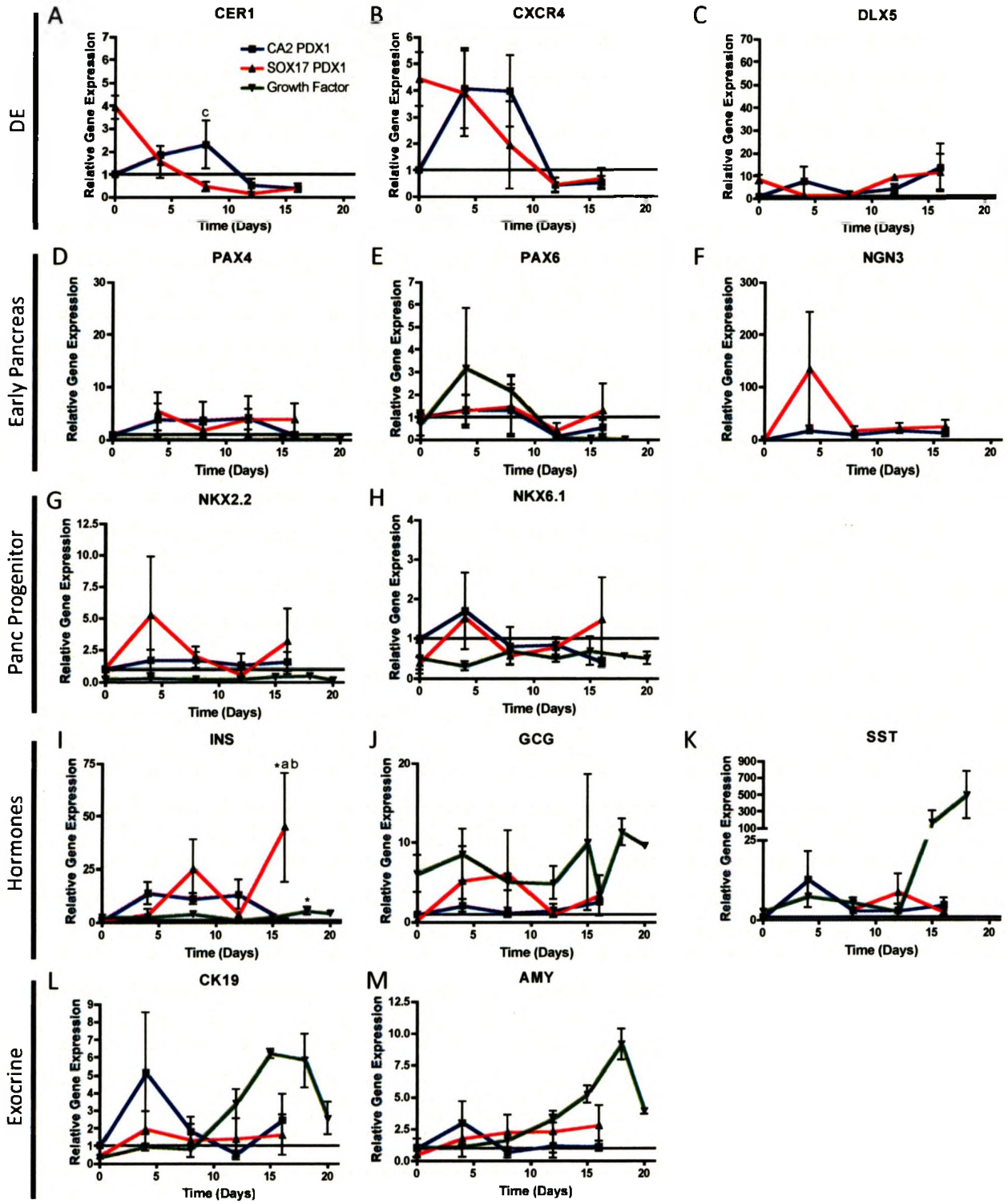


Figure 3.7 Constitutive expression of PDX1 in DE cells results in insulin gene expression by day 16.

Real time PCR quantification of gene expression in transgenic PAX4 populations at 4, 8, 12 and 16 day time points following electroporation. The day of electroporation was considered day 0. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Data are presented as the mean ± SEM from triplicate experiments (n=3). (A-C) To assess DE differentiation, expression of CER1, CXCR4 and DLX5 was quantified. (D-F) To assess early pancreatic differentiation, expression of PAX4, PAX6 and NGN3 was quantified. (G-H) To assess pancreatic progenitor differentiation, expression of NKX2.2 and NKX6.1 was quantified. (I-K) Expression of pancreatic hormones INS, GCG and SST were quantified. (L-M) To assess exocrine differentiation, expression of CK19 and AMY was quantified, (n=3). Statistical analysis was performed in two ways with $p<0.05$ considered significant. 1) One way ANOVA with a Dunnett's post hoc test to compare all time points to undifferentiated HESC. (* indicates significance from unmodified HESC) 2) Two-way ANOVA with a Bonferroni post hoc test to compare treatments within time points. (*a* indicats significance from CA2 PDX1 HESC, *b* indicates significance from growth factor mediated pancreatic differentiation, *c* indicates significance from SOX17 PDX1 HESC).


DE
Early Pancreas
Panc Progenitor
Hormones
Exocrine
A
CER1
CA2 PDX1
SOX17 PDX1
Growth Factor
B
CXCR4
C
DLX5
D
PAX4
E
PAX6
F
NGN3
G
NKX2.2
H
NKX6.1
I
INS
J
GCG
K
SST
L
CK19
M
AMY
Relative Gene Expression
Time (Days)

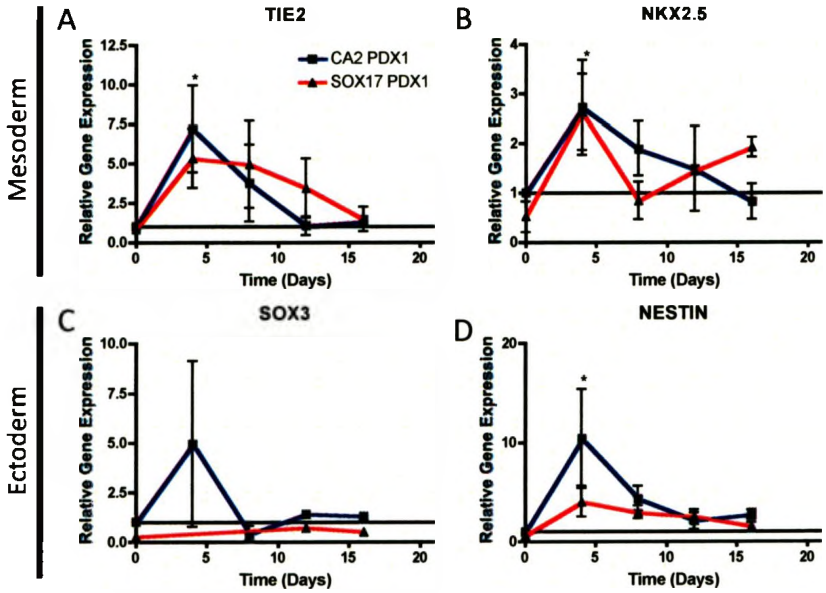
PDX1 HESC and growth-factor mediated pancreatic differentiation. We then quantified expression of the pancreatic hormones INS, GCG and SST (Figure 3.7I-K). By day 16, INS gene expression in SOX17 PDX1 HESC was significantly increased compared to both CA2 PDX1 and growth factor mediated pancreatic differentiation, while GCG and SST gene expression did not differ between SOX17 PDX1 and CA2 PDX1 HESC. Lastly, CK19 and AMY, markers of ductal epithelium and exocrine pancreas were examined. There were no changes in exocrine gene expression in SOX17 PDX1 and CA2 PDX1 HESC compared to unmodified CA1 HESC, however gene expression was lower than growth factor mediated pancreatic differentiation. Taken together, this data demonstrates that ectopic PDX1 expression in DE cells results in significantly increased insulin gene expression following 16 days of differentiation, however this is not accompanied by significant induction of any other markers of pancreatic endocrine differentiation examined.

3.8 Constitutive expression of PDX1 in CA2 HESC or DE cells does not result in mesodermal or ectodermal differentiation by day 16.

To determine if constitutive expression of PDX1 in HESC or DE cells resulted in differentiation to mesodermal or ectodermal lineages, selected marker genes were examined by real time PCR. To assess mesodermal differentiation, TIE2 and NKX2.5 expression were investigated (Figure 3.8A-B). TIE2 gene expression was significantly increased in both transgenic cell populations at the 4 day timepoint when compared to

Figure 3.8 Constitutive expression of PDX1 in CA2 HESC or DE cells does not result in mesodermal or ectodermal differentiation by day 16.

Real time PCR quantification of gene expression in transgenic PAX4 populations at 4, 8, 12 and 16 day time points following electroporation. The day of electroporation was considered day 0. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Data are presented as the mean ± SEM from duplicate experiments (n=3). (A-B) Expression of mesodermal markers TIE2 and NKX2.5 were quantified. (C-D) Expression of ectodermal markers SOX3 and NESTIN were quantified, (n=3). Statistical analysis was performed in two ways with $p<0.05$ considered significant. 1) One way ANOVA with a Dunnett's post hoc test to compare all time points to undifferentiated HESC. (* indicates significance from unmodified HESC) 2) Two-way ANOVA with a Bonferroni post hoc test to compare treatments within time points (*a* indicats significance from CA2 PDX1 HESC, *b* indicates significance from growth factor mediated pancreatic differentiation, *c* indicates significance from SOX17 PDX1 HESC).


Mesoderm
Ectoderm
A
TIE2
CA2 PDX1
SOX17 PDX1
Relative Gene Expression
Time (Days)
B
NKX2.5
C
SOX3
D
NESTIN

unmodified CA1 HESC. NKX2.5 gene expression was not significantly altered from undifferentiated HESC. Next, NESTIN, a marker of ectodermal differentiation was examined (Figure 3.8C). NESTIN gene expression was significantly increased at 4 day timepoints in both transgenic populations compared to unmodified CA1 HESC. Taken together, these results suggest that at early timepoints, there is mixed differentiation with ectopic PDX1 expression in CA2 and DE cells, producing early stages of mesendodermal and neurectodermal differentiation, however by day 16, there is no observable change in mesodermal and ectodermal markers compared to unmodified CA1 HESC.

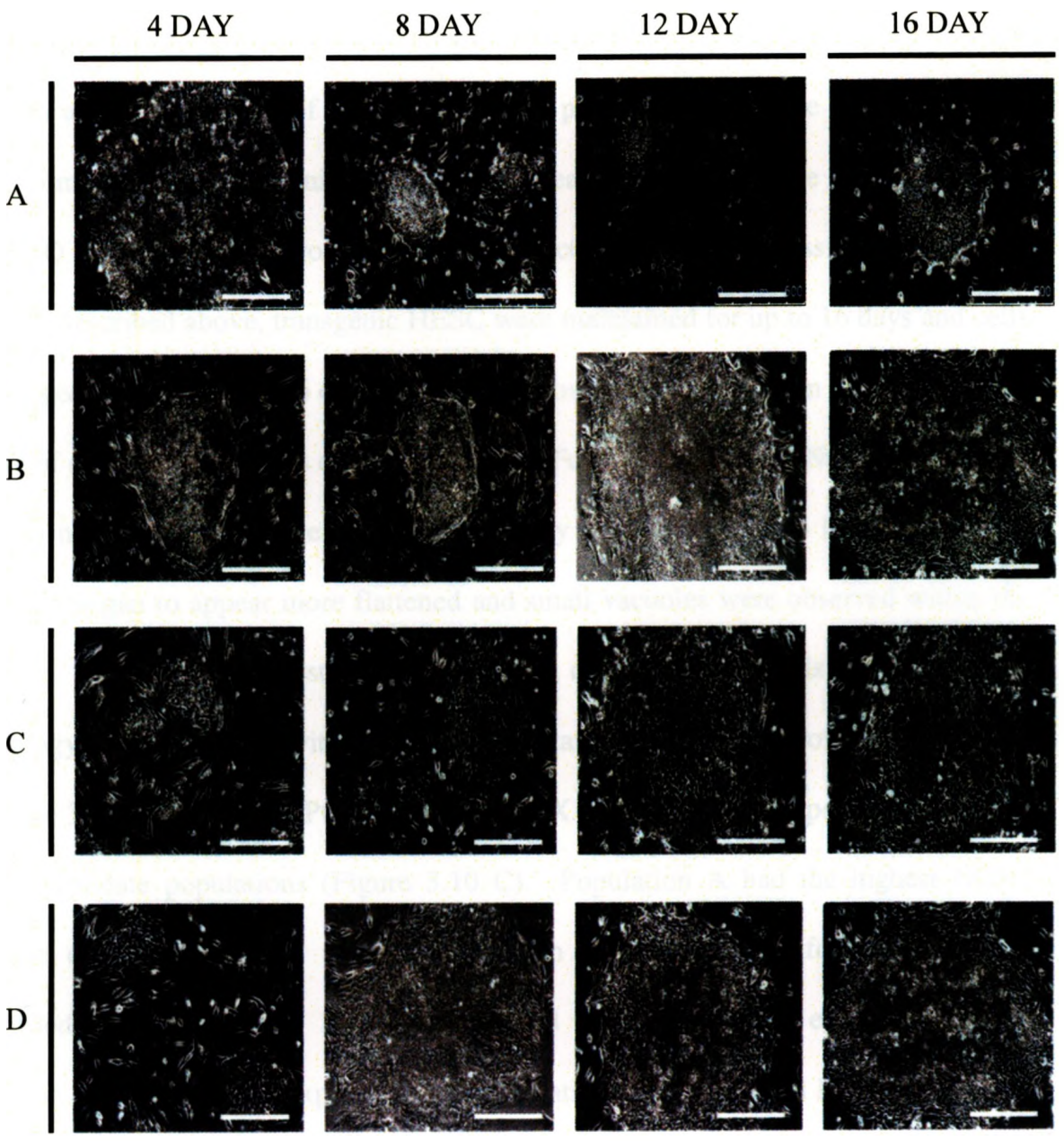
3.9 Constitutive expression of NGN3 in HESC does not alter cell morphology.

To assess the ability of ectopic expression of NGN3 to specify pancreatic endocrine cells from pluripotent HESC, a transgene conferring constitutive expression of pCAGS-NGN3-IRES NEO was introduced into CA2 HESC. Transgenic HESC were maintained under standard conditions in media supplemented with G418 to select for transgene expression for up to 16 days and cells were imaged and RNA was isolated at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. There was no change in cell morphology in CA2 HESC in the presence of constitutive NGN3 expression (Figure 3.9 A). Real-time PCR analysis of NGN3 gene expression was performed in the 4 separate replicate populations (Figure 3.9 B). Transgenic population A and C had relative expression values of 436526 and 75701- fold increase vs unmodified HESC controls respectively at day 4, compared to population B and D which had lower NGN3 transgene expression at 1061 and 340- fold increase vs unmodified HESC controls.

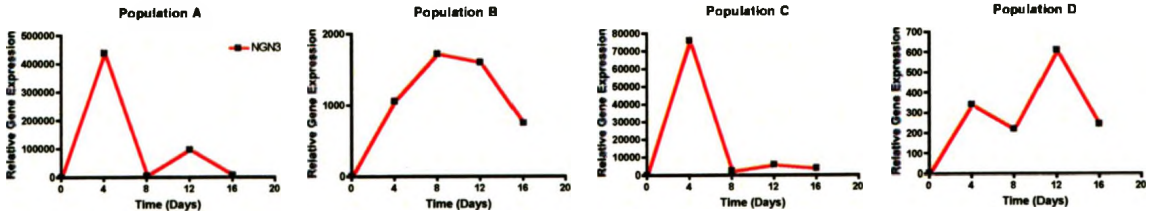
Figure 3.9 Constitutive expression of NGN3 in HESC does not alter cell morphology

(A) Photomicrographs of replicate populations of CA2 NGN3 HESC imaged imaged at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. A transgene conferring constitutive expression of pCAGS-NGN3-IRES NEO was introduced into CA2 HESC (scale bars, 500 μm). There was no observable change in morphology following NGN3 expression in CA2 HESC. (B) Real time PCR quantification of NGN3 expresssion in resultant cell populations at 4, 8, 12 and 16 day time points. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC, (n=4). For NGN3, the SEM was not greater than 3.402.

CA2 NGN3


4 DAY
8 DAY
12 DAY
16 DAY
A
B
C
D

B


Population A
NGN3
Relative Gene Expression
Time (Days)
Population B
Relative Gene Expression
Time (Days)
Population C
Relative Gene Expression
Time (Days)
Population D
Relative Gene Expression
Time (Days)

3.10 Constitutive expression of NGN3 in DE cells results in a flattened morphology and the presence of cytoplasmic vacuoles.

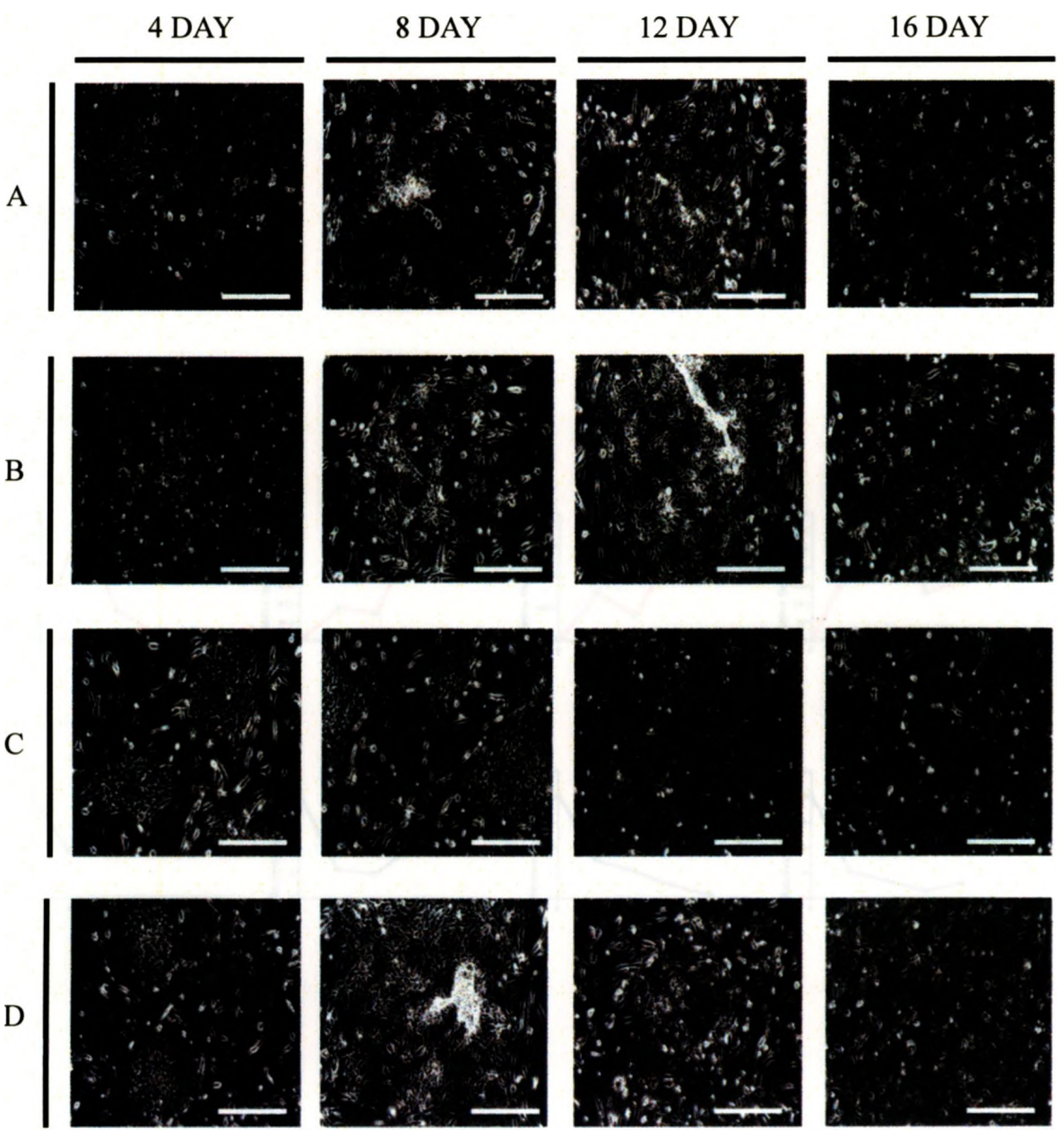
To assess the ability of NGN3 to specify pancreatic endocrine cells from cells already committed to the definitive endoderm lineage, the constitutive pCAGS-NGN3-IRES-NEO transgene was introduced into HESC constitutively expressing SOX17 (DE cells). As described above, transgenic HESC were maintained for up to 16 days and cells were imaged at 4, 8, 12 and 16 day timepoints following electroporation (Figure 3.10 A). The day of electroporation was considered day 0. Following 4 days of NGN3 expression, there was no detectable change in cell morphology when compared to DE cells. At day eight, cells began to appear more flattened and small vacuoles were observed within the cytoplasm. Throughout the rest of the timecourse, cells maintained this altered flattened morphology and spread out, with some cells maintaining the presence of vacuoles at day 16 (Figure 3.10 B). Real-time PCR analysis of SOX17 and NGN3 was performed in the 4 separate replicate populations (Figure 3.10 C). Population A had the highest NGN3 transgene expression with a relative expression value of 11565 fold compared to unmodified HESC controls. Population B had 892-fold NGN3 expression, while population C had 1482-fold expression and population D had 219 fold NGN3 expression compared to unmodified HESC controls. Additionally, each transgenic population showed a decreasing trend of SOX17 expression over 16 days.

Figure 3.10 Constitutive expression of NGN3 in DE cells results in a flattened morphology and the presence of cytoplasmic vacuoles.

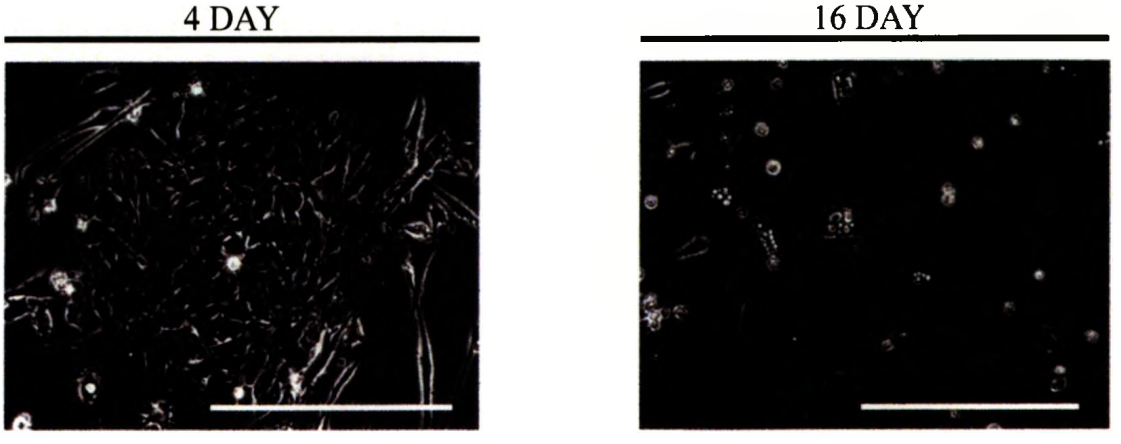
(A) Photomicrographs of replicate populations of CA2 NGN3 HESC imaged at 4, 8, 12 and 16 day timepoints following electroporation. The day of electroporation was considered day 0. A transgene conferring constitutive expression of NGN3 was introduced into CA2 HESC (scale bars, 500 μm). Following 8 days of NGN3 expression, cells adopted a flattened morphology with the presence of cytoplasmid vacuoles, with this morphology being maintained over the duration of the timecourse. (B) Photomicrographs of SOX17 NGN3 HESC at 4 days and 16 days demonstrating the flattened morphology and cytoplasmic vacuoles within these cells at day 16 (scale bars, 500 μm). (C) Real time PCR quantification of NGN3 and SOX17 expresssion in resultant cell populations at 4, 8, 12 and 16 day time points. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC, (n=4). SOX17 showed a trend towards decreased gene expression in all transgenic populations. NGN3 demonstrated variable gene expression between replicate populations. For NGN3, the SEM was not greater than 2.019. For SOX17, the SEM was not greater than 1.023.

A

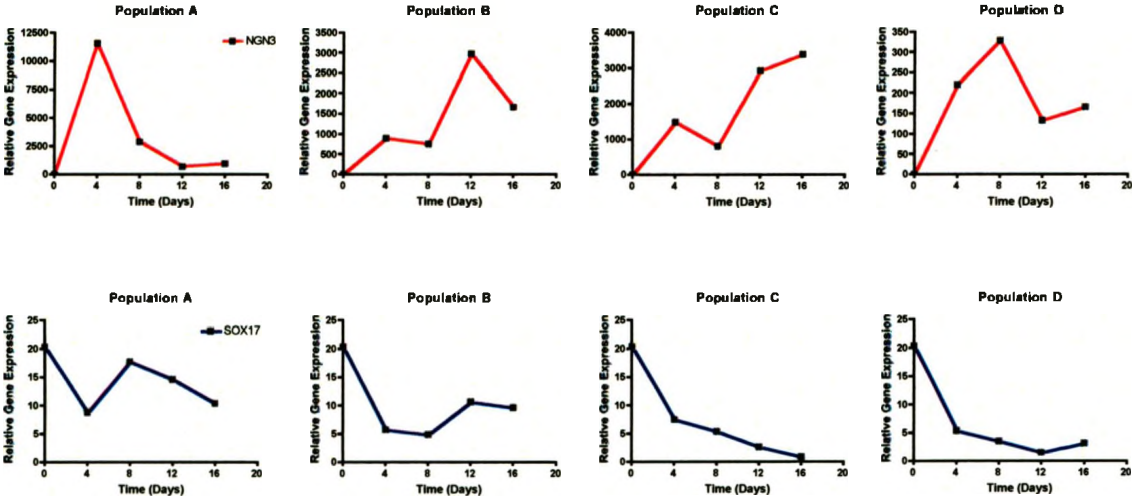
CA2 SOX17D NGN3



B



C

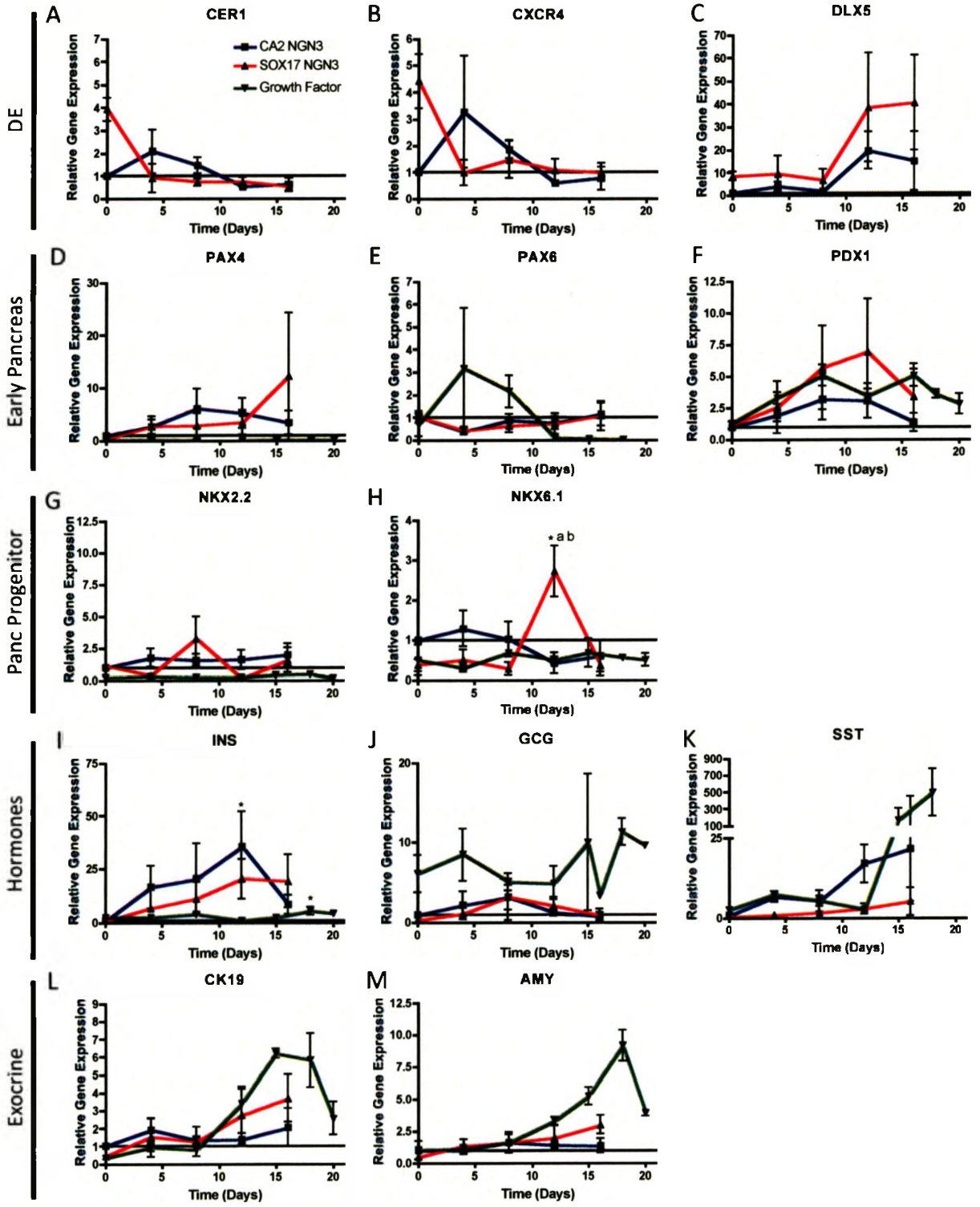

Population A
NGN3
Relative Gene Expression
Time (Days)
Population B
Relative Gene Expression
Time (Days)
Population C
Relative Gene Expression
Time (Days)
Population D
Relative Gene Expression
Time (Days)
Population A
SOX17
Relative Gene Expression
Time (Days)
Population B
Relative Gene Expression
Time (Days)
Population C
Relative Gene Expression
Time (Days)
Population D
Relative Gene Expression
Time (Days)

3.11 Constitutive expression of SOX17 and NGN3 in HESC drives cells to a pancreatic endocrine fate.

To assess the gene expression profiles of cells that constitutively expressed NGN3, we performed real time PCR. To assess the efficiency of differentiation, cells were directly compared to HESC subjected to growth factor mediated differentiation using previously established protocols (D'Amour et al., 2006). We first assessed the expression of markers of DE including CER1, CXCR4, and DLX5 (Figure 3.11 A-C). In both CA2 HESC and DE cells, NGN3 overexpression results in a trend towards decreased DE gene expression by 16 days of differentiation. We next examined the expression of markers of early pancreatic differentiation including PAX4, PAX6 and PDX1. By day 12, PAX4 expression is increased in cells that contain both SOX17 and NGN3 transgenes, in comparison to CA2 NGN3 HESC and growth-factor mediated pancreatic differentiation (Figure 3.11 D). Additionally, PAX6 expression was increased in both transgenic cell lines by day 16 compared to growth-factor mediated pancreatic differentiation (Figure 3.11 E). At day 8 and 12, PDX1 gene expression shows a trend towards increased gene expression in SOX17 NGN3 HESC compared to both CA2 NGN3 HESC in addition to growth-factor mediated pancreatic differentiation (Figure 3.11 F). Next, markers of pancreatic progenitors NK2 homeobox 2 (NKX2.2) and NK6 homeobox 1 (NKX6.1) were examined. At eight days of differentiation, NKX2.2 gene expression was increased in SOX17 NGN3 HESC in comparison to both CA2 NGN3 HESC and growth-factor mediated pancreatic differentiation (Figure 3.11 G). Additionally, NKX6.1 gene expression was significantly increased in SOX17 NGN3 HESC at day 12 in comparison to CA2 NGN3 HESC, growth-factor mediated pancreatic differentiation, and unmodified HESC controls (Figure 3.11 H).

Figure 3.11 Constitutive expression of SOX17 and NGN3 in HESC drives cells to a pancreatic endocrine fate.

Real time PCR quantification of gene expression in transgenic NGN3 populations at 4, 8, 12 and 16 day time points following electroporation. The day of electroporation was considered day 0. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Data are presented as the mean ± SEM from quadruplicate experiments (n=4). (A-C) To assess DE differentiation, expression of CER1, CXCR4 and DLX5 was quantified. (D-F) To assess early pancreatic differentiation, expression of PAX4, PAX6 and PDX1 was quantified. (G-H) To assess pancreatic progenitor differentiation, expression of NKX2.2 and NKX6.1 was quantified. (I-K) Expression of pancreatic hormones INS, GCG and SST were quantified. (L-M) To assess exocrine differentiation, expression of CK19 and AMY was quantified. Statistical analysis was performed in two ways with $p<0.05$ considered significant. 1) One way ANOVA with a Dunnett's post hoc test to compare all time points to undifferentiated HESC (* indicates significance from unmodified HESC). 2) Two-way ANOVA with a Bonferroni post hoc test to compare treatments within time points. (*a* indicats significance from CA2 NGN3 HESC, *b* indicates significance from growth factor mediated pancreatic differentiation, *c* indicates significance from SOX17 NGN3 HESC).


DE
Early Pancreas
Panc Progenitor
Hormones
Exocrine
A
CER1
CA2 NGN3
SOX17 NGN3
Growth Factor
B
CXCR4
C
DLX5
D
PAX4
E
PAX6
F
PDX1
G
NKX2.2
H
NKX6.1
* a b
I
INS
J
GCG
K
SST
L
CK19
M
AMY
Relative Gene Expression
Time (Days)

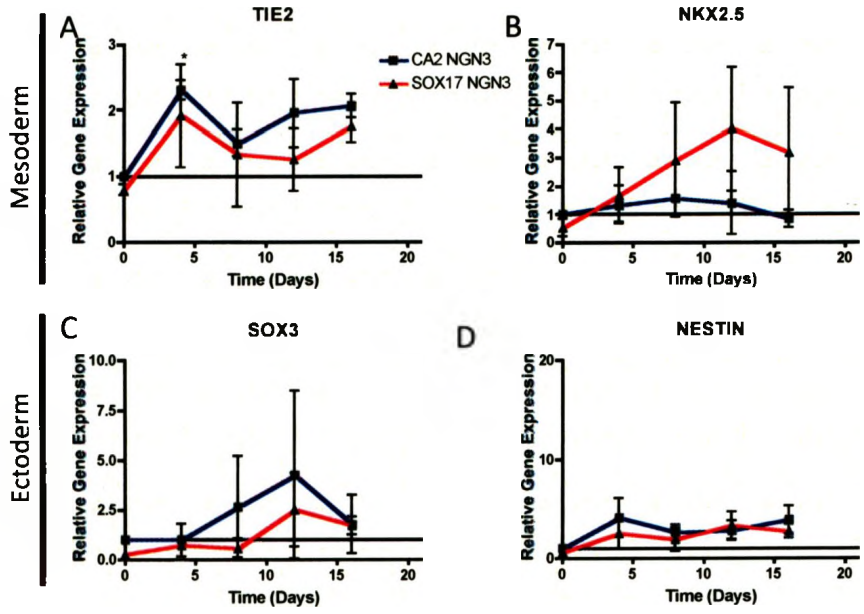
To assess pancreatic hormone gene expression, we quantified mRNA levels of INS, GCG and SST. Expression of INS was significantly increased in CA2 NGN3 HESC at day 12 compared to unmodified HESC controls, while SOX17 NGN3 HESC showed a trend towards increased INS gene expression throughout the timecouse (Figure 3.11 I). Conversely, GCG and SST expression did not differ compared to growth factor mediated pancreatic differentiation in both cell types until day 16, when CA2 NGN3 HESC had significantly increased SST compared to unmodified HESC controls (Figure 3.11 J-K). Lastly, cytokeratin 19 (CK19), and amylase (AMY) were investigated as markers of pancreatic ductal epithelium and exocrine differentiation respectively (Figure 3.11 L-M). In both transgenic cell populations, there was no change in ductal or exocrine gene expression, however they had lower gene expression compared to growth factor mediated pancreatic differentiation. Taken together, overexpression of NGN3 in DE cells suggests differentiation towards pancreatic endocrine progenitors.

3.12 Constitutive expression of NGN3 in CA2 HESC and DE cells does not result in mesodermal or ectodermal differentiation.

To examine the specificity of NGN3–induced differentiation in CA2 HESC and DE cells, we examined the expression of markers of both the mesodermal and ectodermal lineages. To assess mesodermal differentiation, TIE2 and NKX2.5 were investigated (Figure 3.12 A-B). At day 4, TIE2 was significantly upregulated in CA2 NGN3 HESC, however by day 16, there was no robust induction of mesodermal gene expression compared to unmodified CA1 HESC. Secondly, to investigate ectodermal gene expression, we determined the levels of SOX3 and NESTIN (Figure 3.12 C-D). No

Figure 3.12 Constitutive expression of NGN3 in CA2 HESC and DE cells does not result in mesodermal or ectodermal differentiation.

Real time PCR quantification of gene expression in transgenic PAX4 populations at 4, 8, 12 and 16 day time points following electroporation. The day of electroporation was considered day 0. Values were corrected for input using the expression of TBP and expressed relative to undifferentiated HESC. Data are presented as the mean ± SEM from quadruplicate experiments (n=4). (A-B) Expression of mesodermal markers TIE2 and NKX2.5 were quantified. (C-D) Expression of ectodermal markers SOX3 and NESTIN were quantified. Statistical analysis was performed in two ways with $p<0.05$ considered significant. 1) One way ANOVA with a Dunnett's post hoc test to compare all time points to undifferentiated HESC (* indicates significance from unmodified HESC). 2) Two-way ANOVA with a Bonferroni post hoc test to compare treatments within time points. (*a* indicats significance from CA2 NGN3 HESC, *b* indicates significance from growth factor mediated pancreatic differentiation, *c* indicates significance from SOX17 NGN3 HESC).


Mesoderm
Ectoderm
A
TIE2
CA2 NGN3
SOX17 NGN3
Relative Gene Expression
Time (Days)
B
NKX2.5
C
SOX3
D
NESTIN

significant induction of either NESTIN or SOX3 gene expression was induced by ectopic NGN3 expression in either transgenic cell population relative to unmodified CA1 HESC. Taken together, these findings suggest that constitutive expression of NGN3 in CA2 HESC and DE cells does not promote mesodermal or ectodermal differentiation.

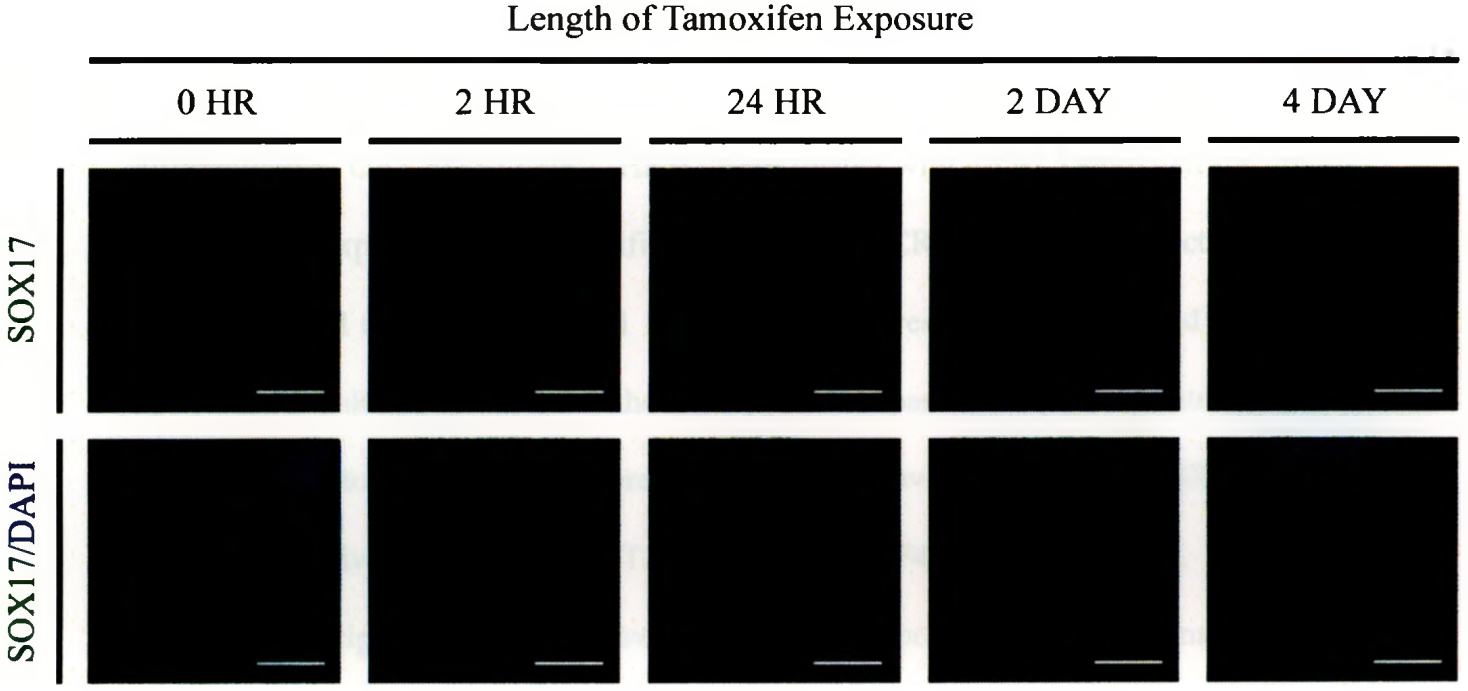
3.13 Characterization of DE differentiation using inducible SOX17 activation in HESC

3.13.1 4-OH Tamoxifen regulates SOX17 transgene cytoplasmic location.

During development, SOX17 is expressed during DE formation, and downregulated following formation of the gut tube (Kanai-Azuma et al., 2002). In order to better recapitulate this developmental pattern of transient expression, clonal HESC cell lines expressing a transgene conferring inducible activation of the SOX17 transcription factor were previously generated in the Séguin lab. The SOX17 coding sequence was fused in frame to the estrogen receptor ligand binding domain (ER^{T2}) to generate a protein that is constitutively expressed but active only following the addition of 0.1μM 4-OH Tamoxifen to culture media (Hayashi and McMahon, 2002). This inducible construct permits control of the duration of transcription factor activity, which is imperative when developing a differentiation strategy aimed at mimicking the developmental pattern of transcription factor activity. To visualize SOX17 localization, immunocytochemistry was performed on SOX17-ER^{T2} HESC that were either untreated (maintained under standard culture conditions), or treated with 0.1μM 4-OH Tamoxifen for 2 hours, 24 hours, 2 days

Figure 3.13.1 4-OH Tamoxifen regulates SOX17 transgene cytoplasmic location.

Immunocytochemistry demonstrating cellular localization of SOX17. SOX17-ERT2-PURO HESC were treated with 0.1μM 4-OH Tamoxifen for 2 hours, 24 hours, 2 days, 4 days, or left untreated. Cells were analyzed by standard immunocytochemistry protocols using a polyclonal antibody for SOX17. SOX17 is restricted to the cytoplasm until the addition of 4-OH Tamoxifen, when it undergoes nuclear translocation within 2 hours, (n=3), scale bars 200μM.


Length of Tamoxifen Exposure
0 HR
2 HR
24 HR
2 DAY
4 DAY
SOX17
SOX17/DAPI

or four days (Figure 3.13.1). Immunolocalization of SOX17 demonstrated that SOX17-ER ^{T2} was restricted to the cytoplasm prior to 4-OH Tamoxifen addition, however nuclear translocation is detected within 2 hours of 4-OH Tamoxifen addition, a cellular localization that is maintained in the continued presence of 4-OH Tamoxifen up to 4 days.

3.13.2 HESC adopt a DE phenotype following 4 days of SOX17 activation.

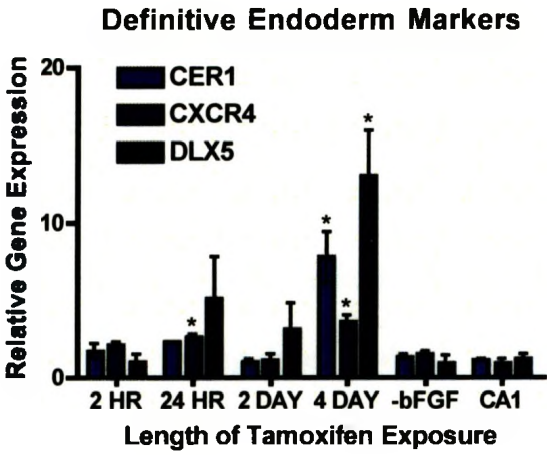
To determine the duration of SOX17 activation required to induce DE differentiation, CA1 SOX17-ER^{T2} HESC were treated with 4-OH Tamoxifen for up to 4 days. Gene expression was quantified by real-time PCR with values corrected for TBP and normalized to unmodified CA1 HESC. Gene expression was compared to SOX17-ER^{T2} HESC cultured in media without bFGF to compare transcription factor mediated differentiation to spontaneous differentiation. Genes investigated were, CER1, CXCR4, DLX5 (definitive endoderm), GATA4, GATA6, HNF4a (pan-endoderm), and OCT4, NANOG (pluripotency) using previously validated primer sets. DE markers were upregulated within 24 hours, and significantly expressed by 4 days of 4-OH Tamoxifen exposure (Figure 3.13.2A). The pan-endoderm markers GATA4 and GATA6 were significantly increased following 4 days of 4-OH Tamoxifen treatment (Figure 3.13.2B). The expression of pluripotency markers remain unchanged throughout the timecourse (Figure 3.13.2C), findings in keeping with the previous characterization of the constitutive SOX17 expressing HESC which maintain OCT4 and NANOG expression despite their DE phenotype (Seguin et al., 2008). Taken together, these data suggest that cells adopt a definitive endoderm phenotype following 4 days of SOX17 activation.

Figure 3.13.2 HESC adopt a DE phenotype following 4 days of SOX17 overexpression

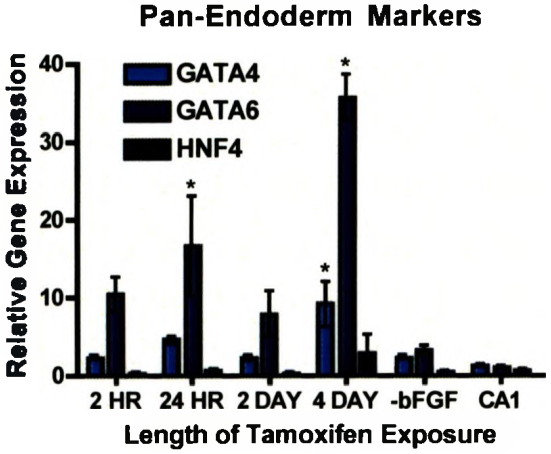
(A) Schematic representation of the SOX17-ER^{T2}-PURO transgene used for SOX17 delivery. (B-D) SYBR-based RT-PCR analysis of gene expression in SOX17-ER^{T2} HESC treated with 4-OH Tamoxifen for 2 hours, 24 hours, 2 days or 4 days. Expression of DE markers, pluripotency markers, and pan-endoderm markers suggests the robust induction of DE differentiation within 4 days. Gene expression normalized to TBP and expressed relative to untreated CA1 HESC (n=3). Furthermore, gene expression was compared to SOX17-ER^{T2} HESC cultured in media without bFGF to compare transcription factor mediated differentiation to spontaneous differentiation. Statistical analysis was performed using a one way ANOVA with a Dunnett's post hoc test to compare all time points to undifferentiated HESC. $P<0.05$ was considered significant (* indicates significance from unmodified HESC).


pCAGG
Promoter
SOX17
ERT2
IRES
PURO

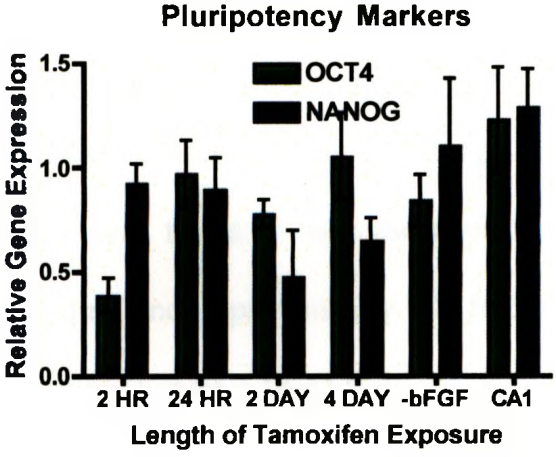
A


Definitive Endoderm Markers
CER1
CXCR4
DLX5
Relative Gene Expression
20
10
0
2 HR
24 HR
2 DAY
4 DAY
-bFGF
CA1
Length of Tamoxifen Exposure

B


Pan-Endoderm Markers
GATA4
GATA6
HNF4
Relative Gene Expression
40
30
20
10
0
2 HR
24 HR
2 DAY
4 DAY
-bFGF
CA1
Length of Tamoxifen Exposure

C


Pluripotency Markers
OCT4
NANOG
Relative Gene Expression
1.5
1.0
0.5
0.0
2 HR
24 HR
2 DAY
4 DAY
-bFGF
CA1
Length of Tamoxifen Exposure

3.14 Characterization of an inducible system for second transgene delivery.

3.14.1 The piggyBac system is induced in HESC by doses of doxycycline 100ng/mL or greater.

CA1 HESC were co-electroporated with a PB-TET-GFP plasmid (generated by J Rossant, SickKids), PB transposase plasmid, and PB-rtTA-blasticidin to generate novel clonal transgenic cell lines with doxycycline inducible GFP expression. To determine the dose of doxycycline required to induce transgene expression, two independent clonal cell lines were treated with increasing doses of doxycycline and imaged for GFP fluorescence up to 24hrs after doxycycline addition (Figure 3.14.1). The GFP transgene was induced by concentrations of doxycycline between 100ng/mL and 1000ng/mL, with no fluorescence detected at doses below 100ng/mL. Detection of fluorescence began 24 hours after the addition of doxycycline at concentrations of doxycycline at 100ng/mL or greater.

3.14.2 The piggyBac system is maximally induced by 500ng/mL doxycycline.

To verify our fluorescence findings quantitatively, PB-TET-GFP cells were exposed to the same concentrations of doxycycline for 24 hours prior to harvesting for RNA extraction for real-time PCR. The expression of GFP was examined. In PB-TET-GFP clone D, GFP was found to increase approximately 50 fold when treated with 100ng/mL doxycycline and approximately 10^4 fold when treated with 1000ng/mL and 500ng/mL doxycycline (Figure 3.14.2A). In PB-TET-GFP clone E, GFP increased

Figure 3.14.1 The piggyBac system is induced by doses of doxycycline 100ng/mL or greater

Fluorescence imaging of PB-TET-GFP HESC treated with increasing doses of doxycycline and imaged over 24 hours. Novel transgenic CA1 HESC containing the PB-TET-GFP transgene were subjected to increasing doses of doxycycline to visualize the activity of the PB-TET system in HESC. Tet-induced GFP expression was detected at concentrations of doxycycline 100ng/mL or greater, with detection beginning following 24hrs of doxycycline treatment (n=1), scale bars 100μM.

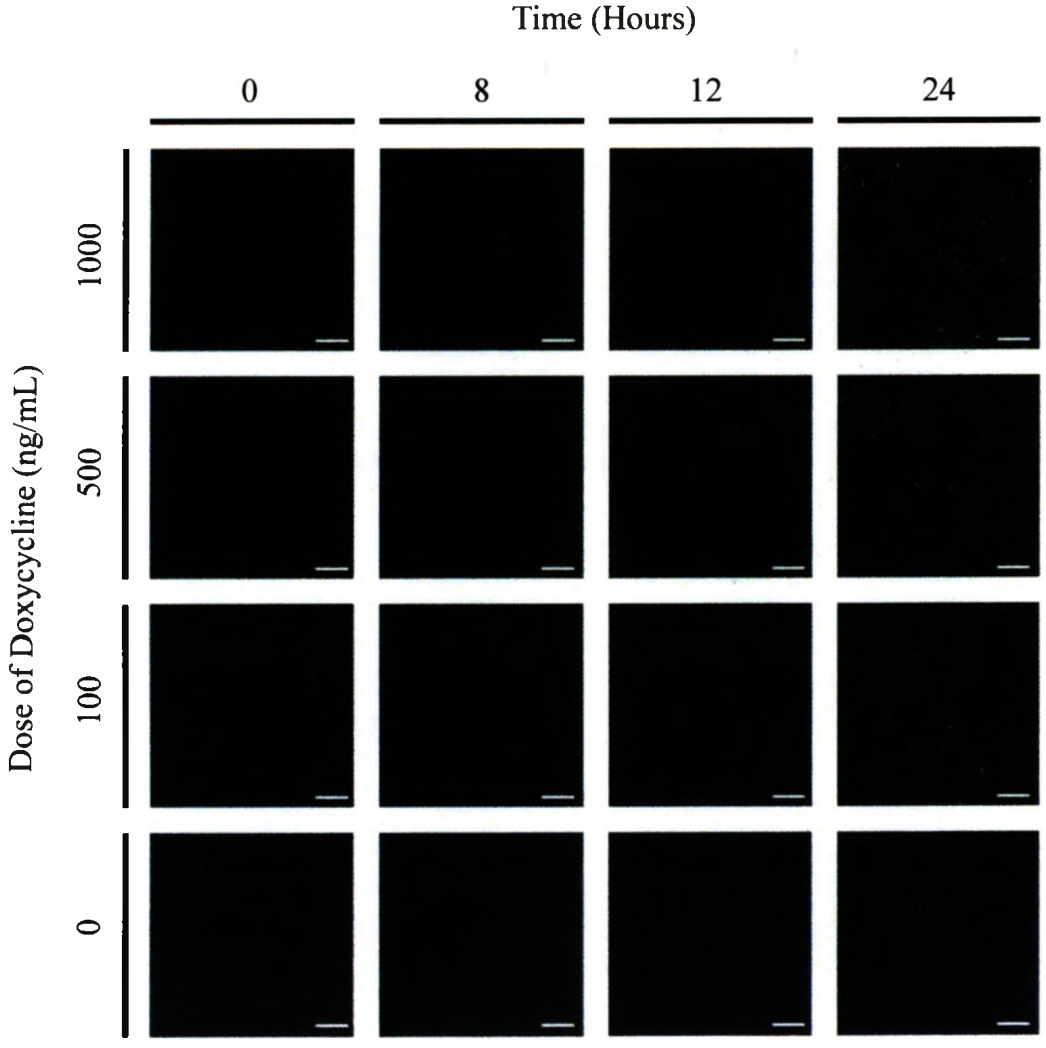
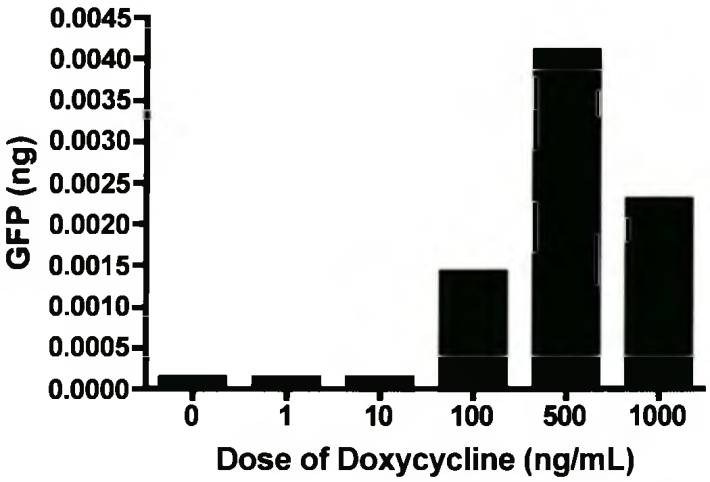
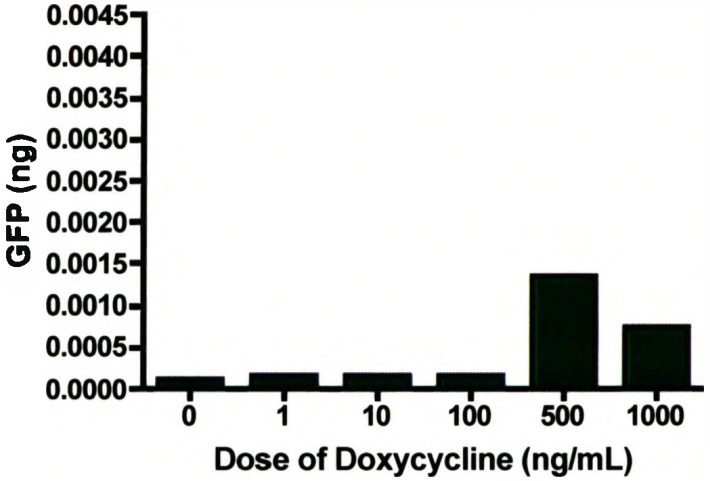

Time (Hours)
0
8
12
24
Dose of Doxycycline (ng/mL)
1000
500
100
0

Figure 3.14.2 The piggyBac system is maximally induced by 500ng/mL doxycycline. SYBR-based RT-PCR analysis of PB-TET-GFP cells. Two clonal populations of PB-TET-GFP HESC were treated with increasing concentrations of doxycycline for 24 hours to induce GFP expression. GFP is detected at doses 100ng/mL or greater, with maximal GFP at a dose of 500ng/mL. The amount of GFP was determined by absolute quantification (n=2).


0.0045
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
GFP (ng)
0
1
10
100
500
1000
Dose of Doxycycline (ng/mL)


0.0045
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
GFP (ng)
0
1
10
100
500
1000
Dose of Doxycycline (ng/mL)

approximately 50 fold when treated with 100ng/mL doxycycline and approximately 10^3 fold when treated with 1000ng/mL and 500ng/mL doxycycline (Figure 3.14.2B). For both clones, there was little detectable GFP induction at doses below 100ng/mL

3.14.3 The piggyBac system turns off following the removal of doxycycline.

To ensure that transgene expression was reversable, doxycycline was removed from the culture media after 24 hours and cells were imaged for an additional 72 hours (Figure 3.14.3). The levels of GFP remained consistent at all doses of doxycycline until 48 hours after removal when the levels of GFP showed a marked decrease.

3.14.4 The dose of doxycycline used for piggyBac induction (500ng/mL) does not affect HESC phenotype.

To ensure that the dose of doxycycline used to induce the PB system did not alter the HESC phenotype, PB-TET-GFP HESC, cells were treated with 500ng/mL doxycycline for 24 hours, and immunocytochemistry was performed to examine levels of OCT 4, NANOG and SSEA3 as markers of pluripotency (Figure 3.14.4A). Following 24 hours of doxycycline treatment, there was no detectable change in the expression of these markers when compared to untreated HESC. To verify these findings quantitatively, four additional HESC lines were treated with 500ng/mL doxycycline for either 24 hours or four days and the expression of OCT4, NANOG and SOX2 was quantified by real-time PCR (Figure 3.14.4B). Following both 24 hour and four day doxycycline treatment, there

Figure 3.14.3 The piggyBac system turns off following the removal of doxycycline

Fluorescence imaging of PB-TET-GFP HESC exposed to increasing doses of doxycycline and imaged up to 72 hours following doxycycline removal. After 48hrs, levels of GFP decreased demonstrating that the system had been turned off, (n=1), scale bars 100μM.

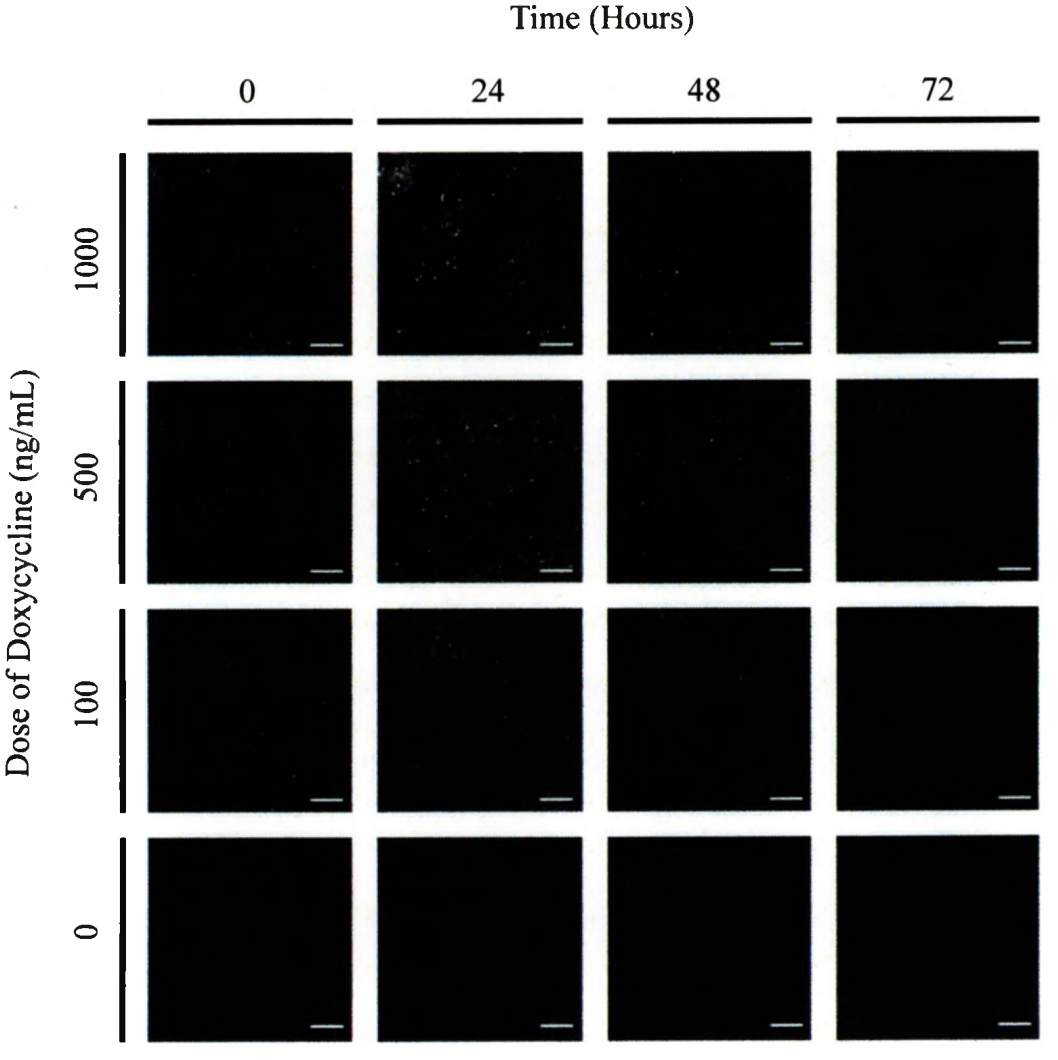
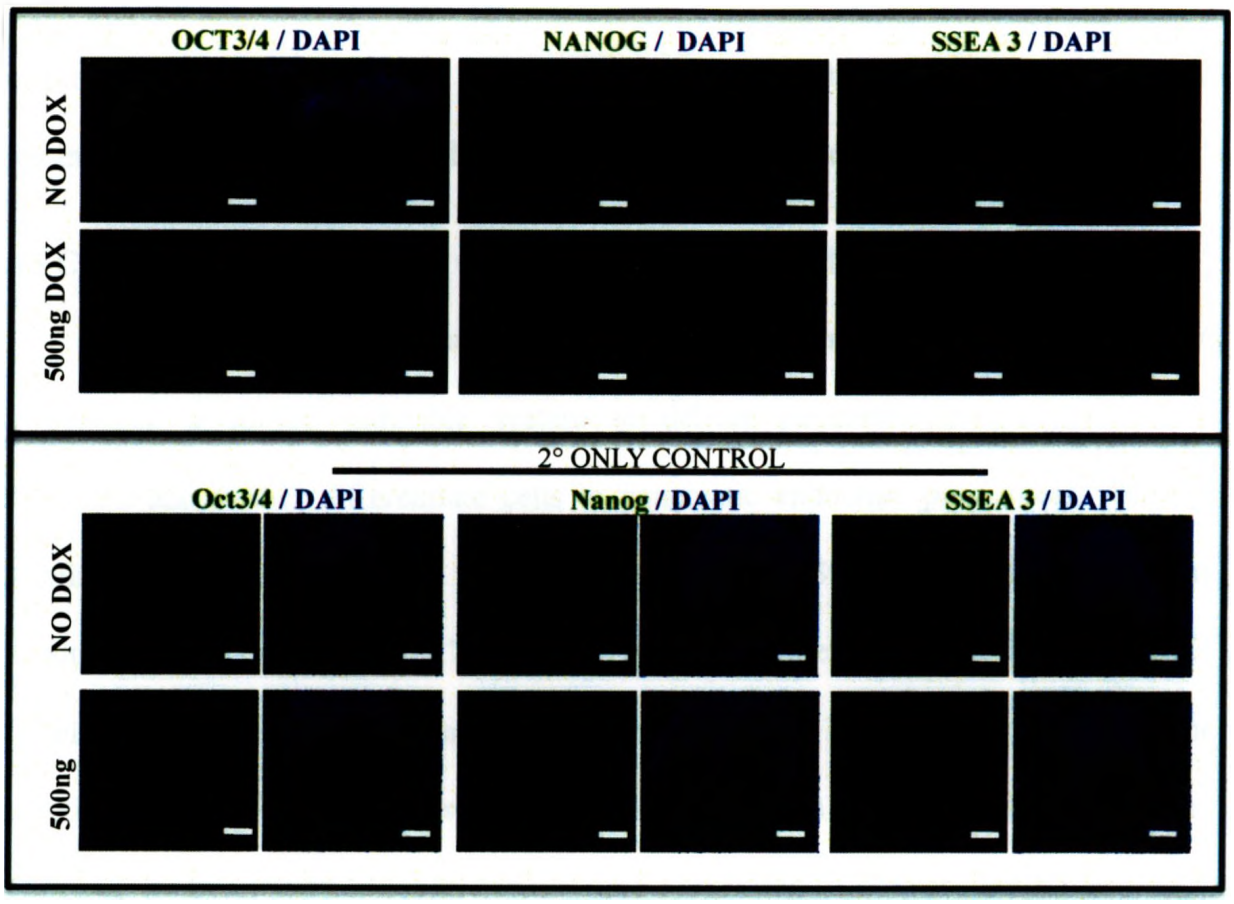

Time (Hours)
0
24
48
72
Dose of Doxycycline (ng/mL)
1000
500
100
0

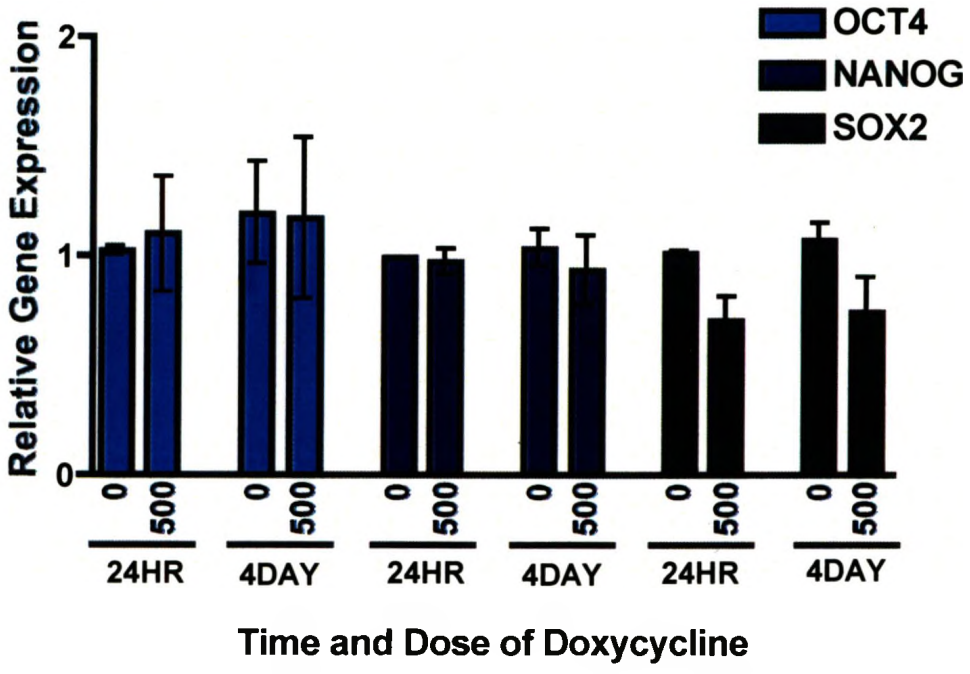
Figure 3.14.4 The dose of doxycycline used for piggyBac induction (500ng/mL) does not affect HESC phenotype

(A) Immunocytochemistry of PB-TET-GFP HESC exposed to 500ng/mL doxycycline for 24 hours express pluripotency markers OCT 3/4, NANOG and SSEA3 (n=4), scale bars 200μM. (B) SYBR-based RT-PCR analysis of gene expression in 4 pluripotent cell lines treated with 0 and 500ng/mL doxycycline. Gene expression was normalized to TBP and expressed relative to untreated CA1 HESC. There was no significant difference in OCT4, NANOG or SOX2 gene expression between HESC treated with doxycycline and untreated CA1 HESC (n=4, $p>0.05$).

A


OCT3/4 / DAPI
NANOG / DAPI
SSEA 3 / DAPI
NO DOX
500ng DOX
2° ONLY CONTROL
Oct3/4 / DAPI
Nanog / DAPI
SSEA 3 / DAPI
NO DOX
500ng

B


OCT4
NANOG
SOX2
Relative Gene Expression
2
1
0
0
500
24HR
4DAY
Time and Dose of Doxycycline

was no significant difference between treated and untreated HESC for the expression of markers of the pluripotent state. We therefore concluded that the dose of doxycycline used in these studies, 500ng/mL, does not alter the HESC phenotype.

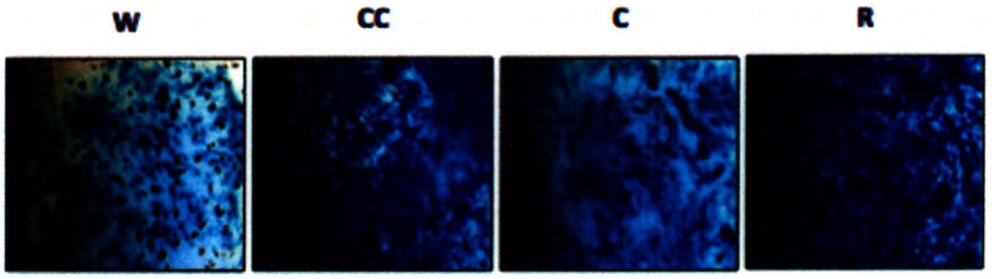
3.15 Characterization of double transgenic HESC with inducible SOX17 and PDX1.

Following the characterization of cells that allow for inducible SOX17 activity, we required a second inducible system to deliver pancreatic transcription factor expression and further differentiate cells to pancreatic endocrine progenitors. Having established the efficacy of the piggyBac system in HESC, we proceeded with the introduction of PDX1 into SOX17-ER ER^{T2} HESC. The presence of the PB-TET-PDX1 plasmid was verified by both LacZ staining and genomic PCR following doxycycline induction (Figure 3.15A-B). Preliminary characterization of clonal cell lines was performed by treating HESC with either 4-OH Tamoxifen or doxycycline for two days. Gene expression was assayed for SOX17 and PDX1 in each clone, and compared to gene expression levels in human fetal pancreas RNA (obtained commercially from Invitrogen) (Figure 3.15C). Two clones were identified that had SOX17 gene expression and PDX1 gene expression higher than in human fetal pancreas.

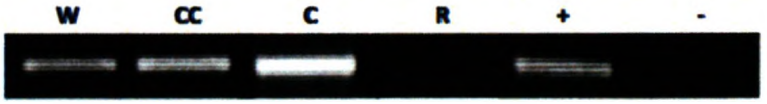
Figure 3.15 Characterization of double transgenic HESC with inducible SOX17 and PDX1

(A) β-galactosidase staining in clonal populations (W, CC, C, R) of CA1 SOX17-ER^{T2} PB-TET-PDX1 HESC after 2 days of doxycycline treatment (500ng/mL). β-galactosidase is expressed from the IRES-βgeo portion of the PB-TET expression construct. Positive staining indicates that the PB-TET plasmid has integrated into the genome and is being actively expressed. (B) Genotyping of genomic DNA extractions from double transgenic cell lines confirms the presence of the βgeo element. **(C)** SYBR-based RT-PCR analysis of a representative double transgenic cell line used to quantify expression levels of PDX1. Cells were untreated (U/T), or treated with either 0.1μM Tamoxifen (TAM) or 500ng/mL doxycycline (DOX), or both for 2 days. Gene expression normalized to TBP, expressed relative to CA1 HESC and compared to levels in human fetal pancreas (HFP).

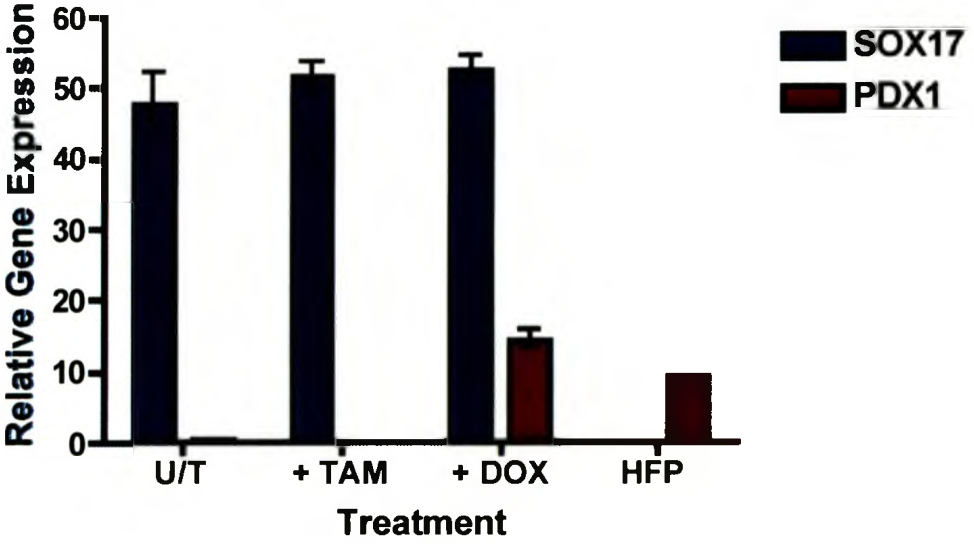
A


W
CC
C
R

B


W
CC
C
R
+
-

C


Relative Gene Expression
60
50
40
30
20
10
0
U/T
+ TAM
+ DOX
HFP
Treatment
SOX17
PDX1

4.0 DISCUSSION

4.1 Summary of Results

Pluripotent HESC are capable of differentiation into all 220 cell types present in the human body, providing tremendous potential for application in cell-based therapies. Diabetes mellitus is becoming increasingly prevalent in North America, highlighting the necessity to create a renewable source of pancreatic β-cells for therapeutic purposes. To date, differentiation strategies to generate pancreatic endocrine cells from various starting cell populations including HESC, pancreatic exocrine cells, and pancreatic ductal cells have generated small clusters of non-proliferative endocrine cells, of which only a small proportion are functional (Bernardo et al., 2009; Blyszczuk et al., 2003; Collombat et al., 2009; D'Amour et al., 2006; Heremans et al., 2002; Jiang et al., 2007a; Jiang et al., 2007b; Kawasaki et al., 2008; Kroon et al., 2008; Kubo et al., 2011; Lavon et al., 2006; Liew et al., 2008; Lin et al., 2007; Minami et al., 2005; Miyazaki et al., 2004; Nostro et al., 2011; Serafimidis et al., 2008; Shim et al., 2007; Thorel et al., 2010; Zhou et al., 2008). Therefore, there is a need to develop alternate strategies by which pancreatic endocrine cells can be obtained to satisfy the large number of cells required to restore insulin secretion in response to physiological stimuli as per the Edmonton Protocol (Shapiro et al., 2000).

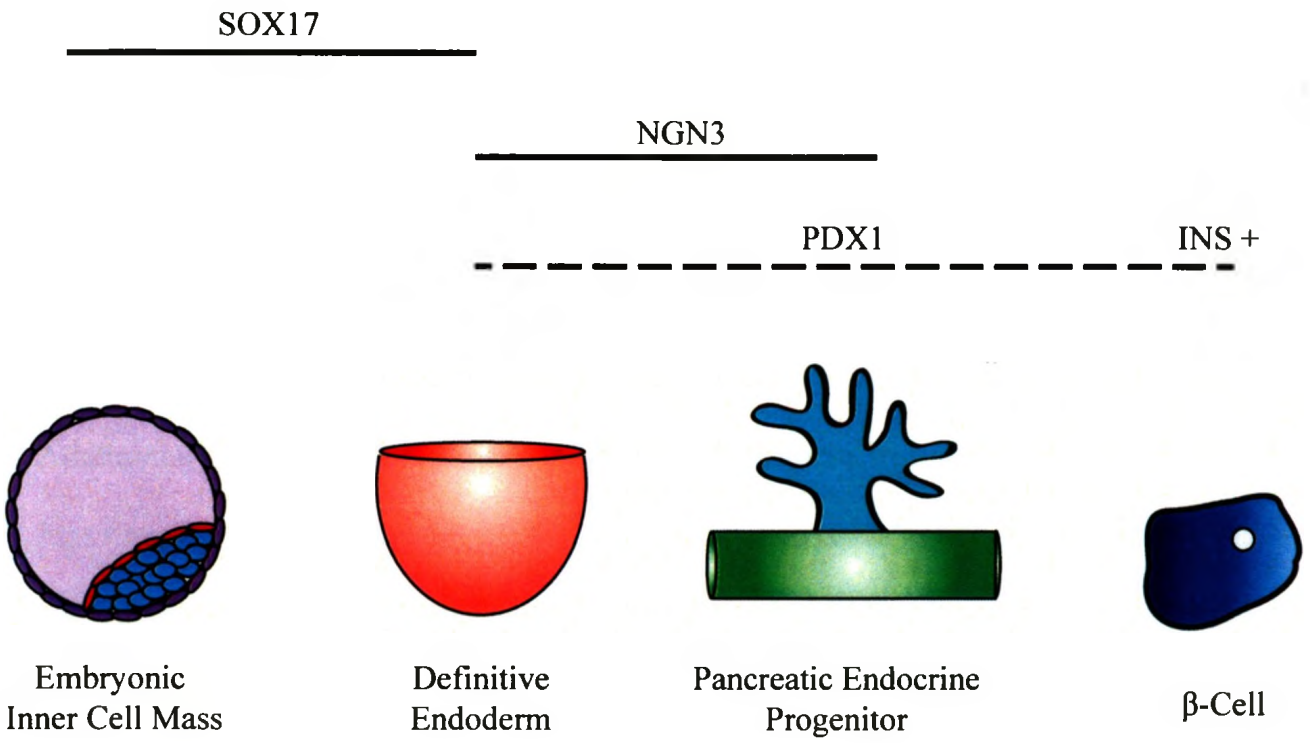
The current study was undertaken to explore the hypothesis that ectopic expression of lineage-determining transcription factors could be used to generate a source of proliferative, homogenous pancreatic progenitor cells capable of subsequent differentiation to functional endocrine cell types, including β-cells. Recent studies have shown that functional hepatocytes can be generated using inducible expression of SOX17, HEX and HNF4α, proving that transcription factor mediated differentiation is a viable

and successful differentiation strategy (Takayama et al., 2011). To address our hypothesis, transgenic cell lines were generated from both HESC and definitive endoderm cells (SOX17) that constitutively expressed the pancreatic transcription factors PAX4, PDX1 or NGN3. Populations were analyzed for gene expression and compared to standard growth factor-mediated protocols for HESC pancreatic differentiation. Given the ability of transcription factor expression to drive HESC differentiation, this strategy was further elaborated using transgenic strategies to confer inducible gene expression in HESC thereby better recapitulating the patterns of expression seen during embryonic development. As such, HESC with inducible activity of SOX17, as well as transgenic HESC with inducible activity of both SOX17 and PDX1 were generated and characterized.

We present evidence that ectopic expression of PAX4 is not sufficient to specify pancreatic cells from HESC or DE. PDX1 expression in DE promoted the expression of insulin, however did not induce the expression of any additional markers of endocrine differentiation. In contrast, we observed that expression of NGN3 could specify pancreatic endocrine cells from DE, as evidenced by the trend towards step wise induction of markers of endocrine specification (Figure 4.1). Furthermore, we demonstrated that HESC adopt a DE phenotype within 4 days of SOX17 activation. Lastly, we characterized the efficacy of the piggyBac transposon system for gene delivery in HESC and used this system to generate clonal double transgenic cell lines with inducible activation of both SOX17 and PDX1.

Figure 4.1 Schematic representation of our findings in relation to pancreatic differentiation

This study used a transcription factor based approach for the differentiation of pancreatic endocrine progenitors from HESC. Pancreatic differentiation requires the initial specification of DE prior to downstream differentiation. We demonstrated that differentiation of DE from HESC requires 4 days of SOX17 activation. Following DE specification, constitutive expression of NGN3 generates pancreatic endocrine progenitor-like cells after 16 days of differentiation. Furthermore, we demonstrated that constitutive expression of PDX1 generates insulin positive cells following 16 days of differentiation, however these cells do not express any additional markers of pancreatic development as indicated by the dotted line, therefore their status as β-cells remains to be determined.


SOX17
NGN3
PDX1
INS +
Embryonic
Inner Cell Mass
Definitive
Endoderm
Pancreatic Endocrine
Progenitor
β-Cell

4.1.1 Contribution of PAX4 expression to HESC differentiation

PAX4 is a transcription factor required for β-cell specification (Sosa-Pineda et al., 1997). Therefore, we hypothesized that ectopic PAX4 expression could promote differentiation of endocrine progenitors from pluripotent HESC and DE cells. However, gene expression analysis of PAX4 over-expressing transgenic cell populations in both CA2 HESC and DE cells revealed no significant induction of any pancreatic, mesodermal or ectodermal markers (Figure 4.1.1).

Previous studies have shown that over-expression of *Pax4* in mouse β-cells *in vivo* has a preventative effect against streptozotocin-induced hyperglycemia due to suppression of interleukin 1β and cytochrome C (Hu He et al., 2011; Lu et al., 2007). Additionally, in the neonatal rat pancreas, streptozotocin treatment induces *Pax4* and *Ngn3* expression in α-cells, resulting in dedifferentiation to endocrine progenitor cells that can contribute to pancreatic remodeling (Liang et al., 2011). In rat pancreatic β-cells, adenoviral-mediated expression of *Pax4 in vitro* increased cell proliferation (Brun et al., 2004). This correlated with induction of c-myc, a cell cycle regulator. Notably, though PAX4 has been implicated in the suppression of insulin and glucagon gene expression (Campbell et al., 1999), however the previously described method of overexpression does not inhibit pancreatic hormone gene expression. *Pax4* expression in mouse pancreatic epithelium and endocrine tissue resulted in death of the animals by 12 weeks due to progressive hyperglycemia (Collombat et al., 2009). This study also demonstrated the ability of *Pax4* expression to drive α-cells to a β-cell fate by generating transgenic mice that constitutively expressed *Pax4* in α-cells. Within 1 week after birth, these mice

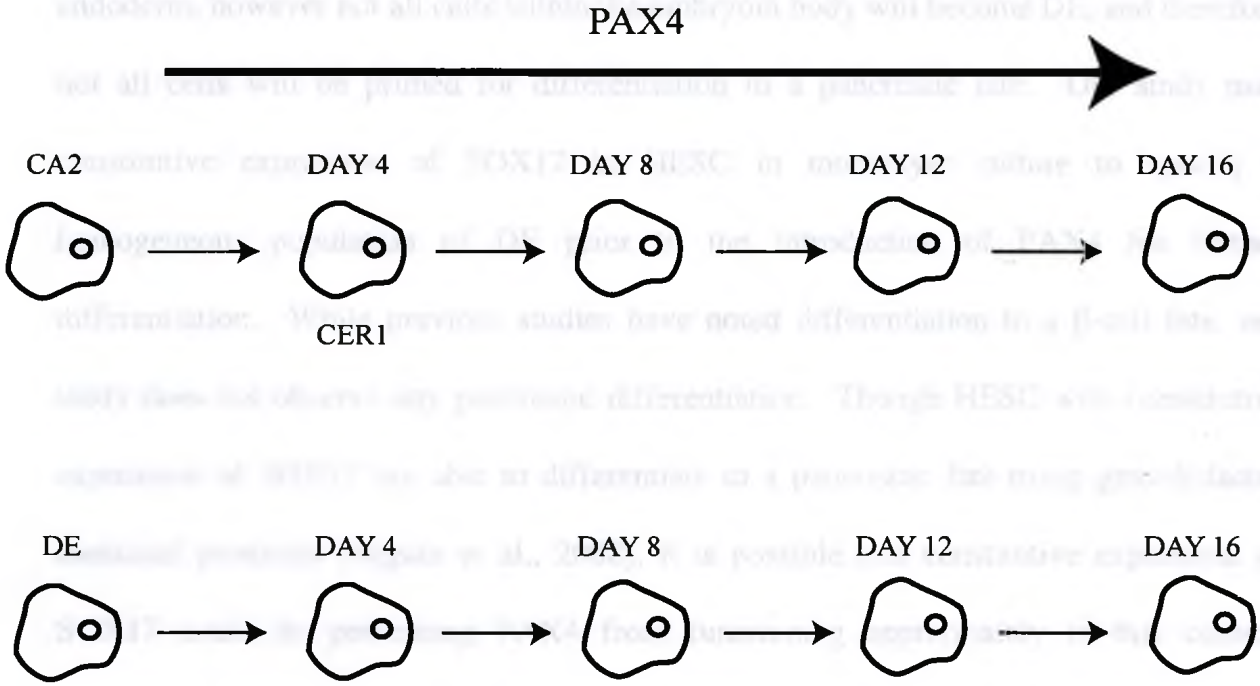
displayed a 77% decrease in α-cell mass, and an increase in *Pax4* positive, insulin positive cells that express β-cell markers *Nkx6.1*, *Pdx1* and *Glut2*.

The above-mentioned studies demonstrate a role for *Pax4* in late stage β-cell development and transdifferentiation of α-cells to β-cells in both rats and mice. Each of these studies induced *Pax4* expression in terminally differentiated cells, which is in contrast to our study which examined the effects of PAX4 expression in HESC-derived multipotent progenitors (Seguin et al., 2008). This may suggest that PAX4 requires binding partners and cofactors in order to be active that are not present prior to pancreatic cell specification. Additionally, PAX4 has been implicated as a transcriptional repressor of pancreatic hormones until the secondary transition at E13.5 (Smith et al., 1999). During development, PAX4 is expressed to specify β- and δ-cells, but is not expressed in the mature pancreas (Dohrmann et al., 2000). It is possible that constitutive expression of this transcriptional repressor in early cell types does not allow for differentiation to a pancreatic endocrine fate because it inhibits pancreatic hormone gene expression.

Transgenic mouse ES cells with constitutive *Pax4* overexpression cultured in embryoid bodies generate approximately 60% cells with a β-cell like phenotype, with the remaining cells differentiating into α-cells (Blyszczuk et al., 2003). Additionally, mouse ES cells subjected to embryoid body-mediated cell differentiation to specify a mesendodermal progenitor prior to *Pax4* nucleofection display expression of pancreatic endocrine markers *Pdx1*, somatostatin and produce insulin (Lin et al., 2007). In HESC, constitutive PAX4 overexpression during embryoid body differentiation for up to 21 days resulted in a small percentage of cells adopting a β-cell phenotype measured by

Figure 4.1.1 Contribution of PAX4 expression to directed cell differentiation

Schematic representation and summary of results obtained from PAX4 overexpressing cells. PAX4 expression does not induce any pancreatic, mesodermal or ectodermal markers in CA2 HESC or DE cells.


PAX4
CA2
DAY 4
DAY 8
DAY 12
DAY 16
CER1
DE
DAY 4
DAY 8
DAY 12
DAY 16

expression of *Pdx1* and insulin, in addition to release of C-peptide in response to tolbutamide (Liew et al., 2008).

Common to all of these studies is the combination of embryoid body-mediated differentiation with ectopic expression of *Pax4*. HESC require initial DE specification prior to further pancreatic differentiation (Wells and Melton, 1999). The above described studies have all relied on spontaneous embryoid body differentiation to specify definitive endoderm, however not all cells within the embryoid body will become DE, and therefore not all cells will be primed for differentiation to a pancreatic fate. Our study uses constitutive expression of SOX17 in HESC in monolayer culture to specify a homogeneous population of DE prior to the introduction of PAX4 for further differentiation. While previous studies have noted differentiation to a β-cell fate, our study does not observe any pancreatic differentiation. Though HESC with constitutive expression of SOX17 are able to differentiate to a pancreatic fate using growth-factor mediated protocols (Seguin et al., 2008), it is possible that constitutive expression of SOX17 could be preventing PAX4 from functioning appropriately in this context preventing transcription of its downstream targets. Additionally, by not culturing our HESC in embryoid bodies, we may be preventing cell-cell context that recapitulates how these cells differentiate *in vivo*.

4.1.2 Contribution of PDX1 expression to HESC differentiation

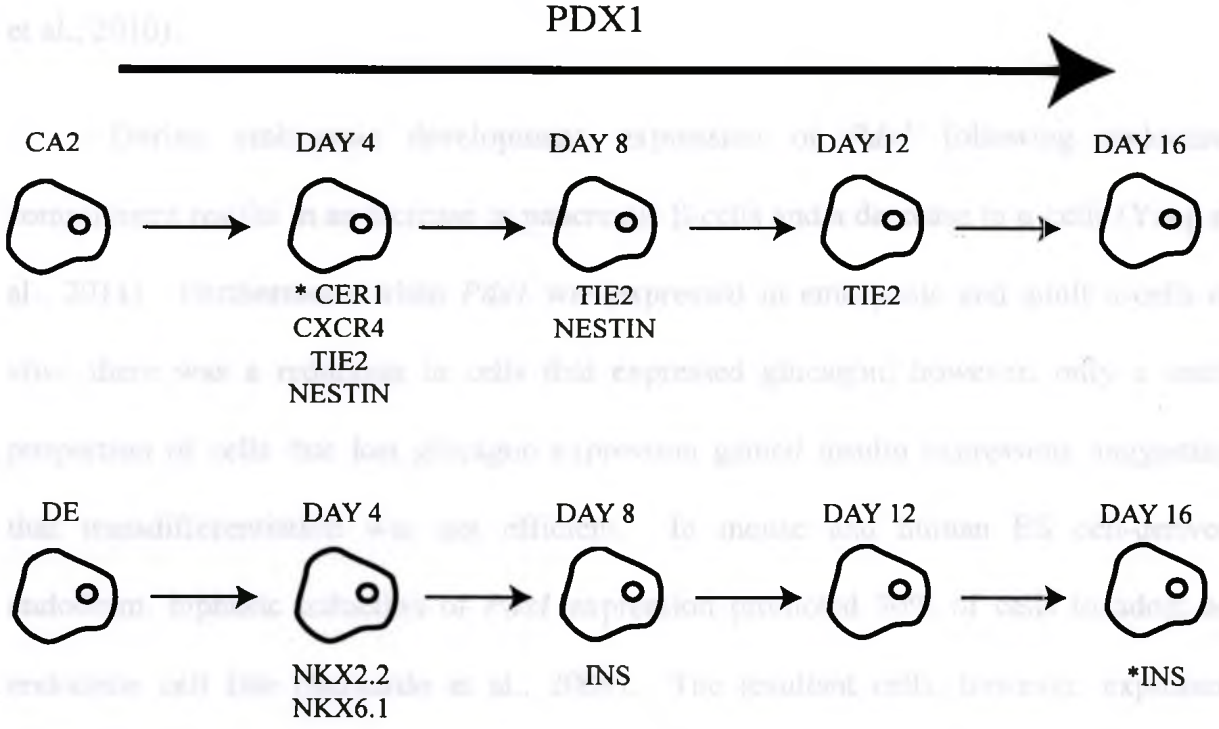
PDX1 is considered the master regulator of pancreatic development, as all pancreatic cells are derived from *Pdx1* positive progenitors (Gu et al., 2003). For this

reason, we assessed the ability of ectopic expression of PDX1 in pluripotent HESC and DE cells to specify the pancreatic endocrine fate. Our studies demonstrate that delivery of PDX1 in DE cells induced a significant upregulation of insulin gene expression within 16 days, however these cells did not demonstrate the induction of any other markers of pancreatic specification (Figure 4.1.2).

Previous studies have demonstrated that inducible expression of *Pdx1* in mouse ES cells induces insulin gene expression when transgenic cells are cultured in embryoid bodies and first exposed to Activin A for endodermal differentiation, followed by *Pdx1* activation (Kubo et al., 2011). In rat mesenchymal stem cells, *Pdx1* overexpression reportedly resulted in insulin positive cells, however insulin was only at low levels (Yuan et al., 2010). Both of these studies are limited in their interpretation since cell differentiation was only assessed by measuring insulin gene expression, and therefore it is not clear if resultant cells also expressed other markers of pancreatic development. In contrast, PDX1 overexpression in human bone marrow derived mesenchymal stromal cells induces expression of various pancreatic endocrine markers including *Pax4*, *Pax6*, *NeuroD1* and *Glut2* with some cells co-expressing insulin and c-peptide, however these cells did not secrete insulin at biologically significant levels (Limbert et al., 2011). Additionally, HESC with constitutive expression of PDX1 subjected to differentiation in embryoid bodies express pancreatic lineage markers such as *Pax4*, *Ngn3* and *Nkx2.2*, however did not express insulin or glucagon following 30 days of differentiation *in vitro* (Lavon et al., 2006). Following transplantation of transgenic HESC into the kidney capsule of immunocompromised mice and subsequent differentition for 1 month, *Pdx1*

Figure 4.1.2: Contribution of PDX1 expression to directed cell differentiation

Schematic representation and summary of results obtained from PDX1 overexpressing cells. Ectopic PDX1 expression in CA2 HESC induces significant induction of markers of several lineages. This induction of early and late markers at the same timepoint suggests that there is a mixed population. In DE cells, ectopic PDX1 expression induces late pancreatic endocrine markers by 4 days without the induction of any other pancreatic lineage markers. By day 16, these cells have significant insulin gene expression, suggesting that these are insulin producing cells. (* significantly induced)


PDX1
CA2
DAY 4
DAY 8
DAY 12
DAY 16
* CER1
CXCR4
TIE2
NESTIN
TIE2
NESTIN
TIE2
DE
DAY 4
DAY 8
DAY 12
DAY 16
NKX2.2
NKX6.1
INS
*INS

expressing cells expressed insulin mRNA, while non-transgenic HESC did not. These results suggest that differentiation of *Pdx1*-expressing HESC to a pancreatic endocrine fate requires signals present in the *in vivo* microenvironment. This requirement for appropriate *in vivo* microenvironment was also reported with mouse adipose tissue-derived stem cells engineered to express *Pdx1*; these cells did not express any pancreatic markers *in vitro*, however when injected into the tail vein of streptozotocin treated mice, these cells were able to correct hyperglycemia in 20% of transplanted animals (Kajiyama et al., 2010).

During embryonic development, expression of *Pdx1* following endocrine commitment results in an increase in pancreatic β-cells and a decrease in α-cells (Yang et al., 2011). Furthermore, when *Pdx1* was expressed in embryonic and adult α-cells *in vivo*, there was a reduction in cells that expressed glucagon; however, only a small proportion of cells that lost glucagon expression gained insulin expression, suggesting that transdifferentiation was not efficient. In mouse and human ES cell-derived endoderm, biphasic induction of *Pdx1* expression promoted 30% of cells to adopt an endocrine cell fate (Bernardo et al., 2009). The resultant cells, however, expressed multiple islet hormones. Transdifferentiation of mouse pancreatic exocrine cells to cells that resemble β-cells has been demonstrated following co-expression of *Pdx1*, *Ngn3* and *MafA* (Zhou et al., 2008). However, the efficiency of transdifferentiation was reported at only 20%.

It is important to note that while these studies have all reported the upregulation of insulin gene expression following ectopic PDX1 expression, only a subset of these studies also report the induction of pancreatic markers such as NGN3, NKX2.2 and during

differentiation (Lavon et al., 2006; Limbert et al., 2011). While our data demonstrate that constitutive PDX1 expression in DE cells induces insulin gene expression, we did not demonstrate induction of any additional genes involved in pancreatic development.

PDX1 is a known regulator of insulin gene expression, occupying three different regions in the human insulin promoter and two regions in the mouse insulin promoter (Melloul et al., 2002). This is an important consideration in studies where insulin gene expression is reported as a marker β-cell differentiation without examining a broader panel of markers of different stages of pancreatic development to assess if cells are following the appropriate developmental pattern. It is possible that many of these studies have generated insulin producing cells, but not cells that have a functional β-cell phenotype. This may explain why the majority of these studies produce cells with minimal or no response to physiological glucose stimulation.

Interestingly, when PDX1 was constitutively expressed in pluripotent HESC, we observed induction of neurectodermal and mesendodermal markers. It has been shown in mouse ES cells that markers of pancreatic differentiation *Pdx1*, *Ngn3*, and *Pax6* are expressed in ES-cell derived ectodermal cells (Roche et al., 2005). These ectodermal cells do not express insulin mRNA unless cultured in media conditioned by the rat insulinoma cell line INS-1. This is concurrent with our data where ectodermal markers SOX3 and NESTIN were upregulated during the early stages of PDX1-mediated differentiation during our differentiation process, suggesting some degree of neurectodermal differentiation. This demonstrates the importance of cell context prior to transcription factor introduction since DE cells are not competent to undergo ectodermal

differentiation and therefore show no induction of neural markers following PDX1 expression.

Analysis of transgene expression levels in SOX17 PDX1 HESC revealed considerable variation between transgenic populations. Population A demonstrated 100,000 fold increase in PDX1 expression vs undifferentiated controls at day 4, while population B had 1,200 fold induction and population C had a 4100 fold induction. Additionally, population A and C showed trends towards decreasing PDX1 expression over the time course, while population B demonstrated the opposite trend of increasing PDX1 expression. The inherent variation in transgene expression levels is likely due to both the number of integration events and the genomic site of PDX1 transgene insertion. Since the piggyBac system allows for transgene insertion at any TTAA site in the genome, it is possible that some copies of the transgene were inserted into heterochromatin and are therefore not actively expressed. Additionally, insertion site is important for the plasmid that confers rtTA expression, as this protein is required for PDX1 transcription. Some populations may have more rtTA expression, and therefore more protein for doxycycline to bind to, resulting in increased transgene expression. Importantly, these variations in PDX1 expression may have an effect on the resultant gene expression patterns in these cells in addition to cell morphology. Transgenic cell populations with higher PDX1 transgene expression may upregulate downstream PDX1 targets that populations with lower PDX1 gene expression do not. Specifically, transgenic population A which demonstrated a 100,000 fold upregulation of PDX1 transgene expression vs undifferentiated HESC may have overwhelmed the cell's transcriptional machinery and prevented any further upregulation of gene expression.

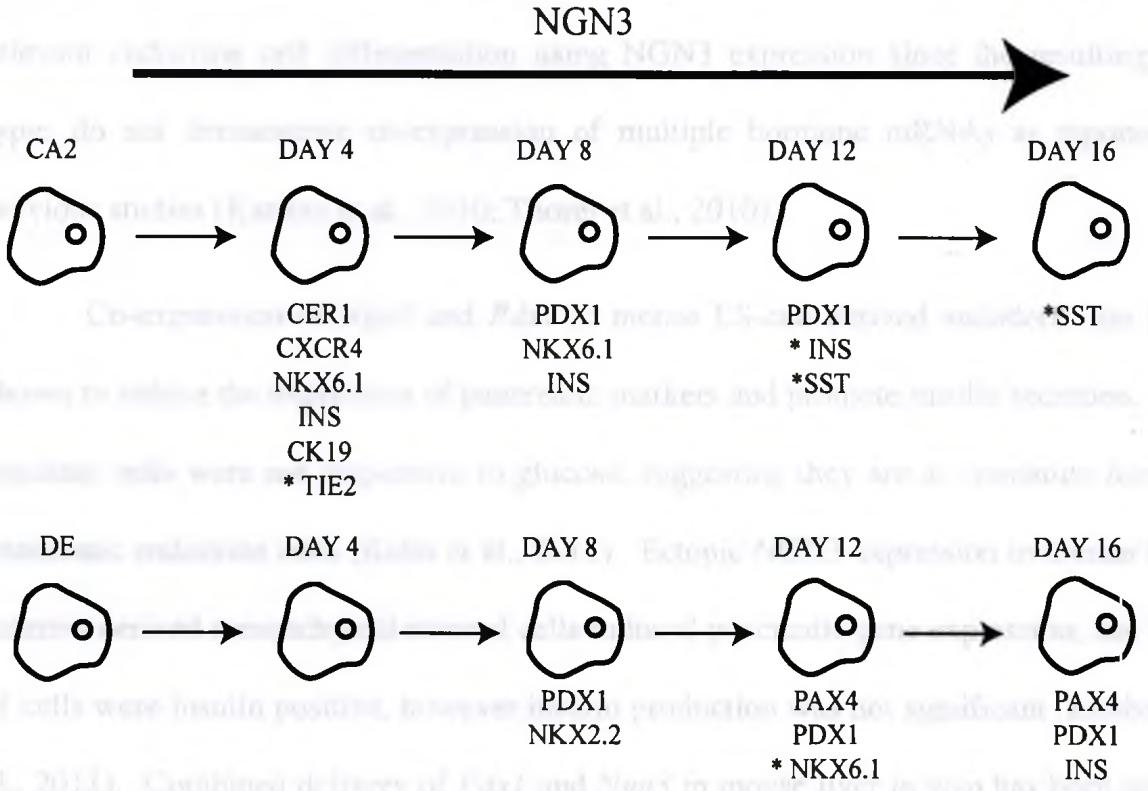
4.1.3 Contribution of NGN3 expression to directed cell differentiation

All pancreatic endocrine cells are derived from NGN3 positive progenitors (Jensen et al., 2000; Smith et al., 2004). Because of this, we hypothesized that constitutive expression of NGN3 could be used to generate pancreatic endocrine cells from DE cells. Ectopic NGN3 expression in DE cells resulted in an obvious change in cell morphology, increasing the cytoplasmic to nuclear ratio and promoting the accumulation of cytoplasmic vacuoles after 8 days of differentiation. The presence of cytoplasmic vacuoles suggests there is active endo- or exocytosis activities within in these cells (Burgoyne and Morgan, 2003). These cytoplasmic vacuoles have the potential to be insulin secretory granules (Russ et al., 2011), as insulin gene expression shows a trend towards upregulation at this time point. Further analysis by both immunocytochemistry and scanning electron microscope (SEM) would be required to characterize these subcellular structures.

Through gene expression analysis, we also demonstrate a trend toward initiation of a transcriptional cascade associated with pancreatic development that involved downregulation of DE markers and upregulation of PAX4, PAX6, PDX1, NKX2.2, NKX6.1 and INS (Figure 4.1.3). These genes were activated in a sequential manner that mimicked biological pancreatic endocrine cell development, presumably due to the presence of an appropriate DE context prior to NGN3 expression. Though we observed sequential gene activation, we are assuming that this is occurring within the same cell population. However, we acknowledge that since this analysis was done at the population level, different cells within the population may be expressing subsets of these markers. Our findings correlate with previous studies of where NGN3 expression in

Figure 4.1.3: Contribution of NGN3 expression to directed cell differentiation

Schematic representation and summary of results obtained from NGN3 overexpressing cells. In CA2 HESC, ectopic expression of NGN3 induces markers of DE, late pancreatic markers, as well as pancreatic exocrine markers at day 4. Ectopic expression of NGN3 in DE cells induces a cascade of pancreatic markers that are expressed in a sequential order mimicking pancreatic development. These genes demonstrated a trend toward increased gene expression, however did not reach significace. (* significantly induced)


NGN3
CA2
DAY 4
DAY 8
DAY 12
DAY 16
CER1
CXCR4
NKX6.1
INS
CK19
* TIE2
PDX1
NKX6.1
INS
PDX1
* INS
*SST
*SST
DE
DAY 4
DAY 8
DAY 12
DAY 16
PDX1
NKX2.2
PAX4
PDX1
* NKX6.1
PAX4
PDX1
INS

progenitor cell types was shown to initiate expression of factors associated with the endocrine differentiation cascade (Gasa et al., 2004; Grapin-Botton et al., 2001; Heremans et al., 2002; Kubo et al., 2011; Lee et al., 2001; Limbert et al., 2011). Interestingly, although NGN3 positive progenitors were capable of generating all endocrine cell types during development, in our studies constitutive expression of NGN3 in DE cells did not induce GCG or SST gene expression, suggesting the absence of α- or δ- cell differentiation. These findings further suggest the induction of developmentally relevant endocrine cell differentiation using NGN3 expression since the resulting cell types do not demonstrate co-expression of multiple hormone mRNAs as reported in previous studies (Katsuta et al., 2010; Thorel et al., 2010).

Co-expression of *Ngn3* and *Pdx1* in mouse ES-cell derived endoderm has been shown to induce the expression of pancreatic markers and promote insulin secretion. The resultant cells were not responsive to glucose, suggesting they are an immature form of pancreatic endocrine cells (Kubo et al., 2011). Ectopic NGN3 expression in human bone marrow derived mesenchymal stromal cells induced pancreatic gene expression, and 25% of cells were insulin positive, however insulin production was not significant (Limbert et al., 2011). Combined delivery of *Pdx1* and *Ngn3* in mouse liver *in vivo* has been shown to induce insulin gene expression (Kaneto et al., 2005). Taken together, these studies demonstrate that co-expression of PDX1 and NGN3 can generate immature pancreatic cells. An alternative to simultaneous expression of both of these transcription is sequential activation. We have demonstrated that following SOX17-mediated DE specification, activation of NGN3 can specify endocrine progenitors. It is possible that subsequent expression of PDX1 in these cells could generate functional pancreatic cells.

4.1.4 Comparison of transcription factor- and growth factor- mediated pancreatic endocrine differentiation

Early studies using HESC to generate pancreatic cell types *in vitro* established that the generation of pancreatic precursor cells requires the initial specification of DE (D'Amour et al., 2006). To date this has been accomplished using various methods to generate DE from HESC such as activin A-mediated growth factor differentiation (D'Amour et al., 2005), as well as embryoid body formation (Liew et al., 2008). Activin A is known to induce DE differentiation in vertebrates, (Stainier, 2002); however, is only 80% efficient in specifying DE from HESC (D'Amour et al., 2005). Conversely, culturing HESC in EBs results in spontaneous differentiation to all 3 primary germ layers and therefore has a lower efficiency than activin A–mediated differentiation. In general, these protocols results in the production of a heterogeneous population of cells, thereby limiting the number of cells that can go on to become pancreatic endocrine cells. Furthermore, while generation of DE using Activin A is more efficient than spontaneous EB differentiation, cell proliferation has been shown to be dramatically reduced with the onset of differentiation, thereby increasing the number of cells required for long-term differentiation. Alternatively, lineage-committed DE cells have been generated through constitutive expression of SOX17 in HESC (Seguin et al., 2008). This results in a homogeneous, proliferative population of cells receptive to signals for further differentiation to a variety of mature endoderm cell types including pancreatic cells.

Following DE specification, several strategies have been elucidated for the generation of β-cells. Stepwise protocols have been developed using sequential addition of developmentally relevant growth factors over long term *in vitro* culture to generate

pancreatic β-cells (D'Amour et al., 2006; Jiang et al., 2007a; Jiang et al., 2007b; Nostro et al., 2011; Shim et al., 2007). Although these methods have been extensively characterized, they have limitations that are undesirable. These protocols produce a population of cells in which the β-cell phenotype comprises less than 15% of the final population (D'Amour et al., 2005). Researchers have optimized these growth factor-mediated approaches by manipulating key pathways in β-cell development, however despite these efforts the resultant populations contained only 25% C-peptide positive cells (Nostro et al., 2011). Alternatively, pancreatic exocrine cells have been transdifferentiated into pancreatic endocrine cells by *in vivo* viral-mediated gene delivery of the transcription factors *MafA*, *Pdx1* and *Ngn3* (Zhou et al., 2008). The initial population of pancreatic exocrine cells is not self-renewable and the efficiency of transdifferentiation was only 20% (Zhou et al., 2008). Previous studies have also described the ectopic expression of the transcription factors PDX1 (Kawasaki et al., 2008; Kubo et al., 2011; Lavon et al., 2006; Limbert et al., 2011; Miyazaki et al., 2004), NGN3 (Kubo et al., 2011; Limbert et al., 2011; Serafimidis et al., 2008) and PAX4 (Blyszczuk et al., 2003; Liew et al., 2008) in various cell types including pancreatic ductal cells, pancreatic exocrine cells, and ES-derived endoderm cells in efforts to generate pancreatic endocrine cells.

4.1.5. Generation of definitive endoderm through transient expression of SOX17

Previous data has demonstrated that constitutive expression of SOX17 in HESC is sufficient to generate DE (Seguin et al., 2008). Additionally, it is known that during murine development *Sox17* is expressed concurrent with DE formation and is

downregulated upon DE specification (Kanai-Azuma et al., 2002). Therefore, this study sought to characterize HESC with inducible activation of SOX17 in order to better mimic developmental patterns. We present data demonstrating that following 4 days of induction, SOX17-ER^{T2} HESC adapt a DE phenotype, with significant expression of DE markers CER1, CXCR4 and DLX5. The timeframe of 4 days to specify DE from HESC is markedly shorter than 16 days to specify DE from the inner cell mass in a developing embryo. This may be due to the levels of SOX17 in the transgenic HESC, in addition to the monolayer culture that the cells are grown in permitting 4-OH Tamoxifen to bind to every cell resulting in subsequent differentiation. However, our current studies do not determine if 4 days of SOX17 expression is sufficient to irreversibly commit HESC to the DE lineage. To determine if cells are committed, the transgene would have to be removed and cell potential analyzed following transgene removal. Additionally, previous reports of HESC with constitutive SOX17 expression were shown to demonstrate a mesendodermal phenotype, generating both mesoderm and endoderm tissue in a teratoma assay (Seguin et al., 2008). To determine if DE cells generated from transient SOX17 activity are committed to the endoderm lineage, or if they adopt a mesendodermal phenotype like the HESC with constitutive expression of SOX17, a teratoma assay would have to be performed.

4.1.6 Characterization of the piggyBac transposase system in HESC and generation of double transgenic HESC for pancreatic endocrine differentiation

The piggyBac transposase system used in these studies is a tet-inducible system for transient and reversible transgene delivery in mammalian cells (Ding et al., 2005). This system has been used to express *c-Myc*, *Klf4*, *Sox2* and *Oct4* to reprogram mouse fibroblasts to induced pluripotent stem cells (Woltjen et al., 2009). Through generation of PB-TET-GFP HESC, we determined that 500ng/mL doxycycline maximally induces transgene activity without affecting cell phenotype. Doses of doxycycline greater than 500ng/mL may be cytotoxic to the cells, which could account for decreased gene expression. The piggyBac system is unique, as it allows for complete transgene removal, leaving no genetic footprint. Additionally, this inducible transgenic system does not require the generation of a fusion protein that could potentially affect gene function.

Once we characterized a second drug-inducible gene delivery system, we generated double transgenic HESC that had inducible expression of SOX17 and PDX1. Expression of both SOX17 and PDX1 during embryonic development follow biphasic patterns of induction (Hui and Perfetti, 2002; Kanai-Azuma et al., 2002; Offield et al., 1996), therefore an inducible model will allow us to better recapitulate developmental patterns of gene expression. We found clonal, double transgenic populations that express PDX1 at the level of, or greater than, human fetal pancreas.

4.2 Limitations of Research

For this study, we assayed the ability of three pancreatic transcription factors to drive specification of pancreatic endocrine cells from pluripotent cells, as well as cells

that are committed to the definitive endoderm lineage. To do this, we generated transgenic cell populations through electroporation-mediated plasmid delivery and observed changes at the population level. Population analysis allowed us to develop a protocol for rapid, high throughput screening to assay the ability of individual pancreatic transcription factors to specify pancreatic endocrine cells but real-time PCR. One important limitation to this approach is that analysis was not conducted clonal cell populations. Consequently, all phenotypic analysis was conducted on a mixed population of cells due to factors such as the variation in the number of transgene integration events in each cell, as well as cell-cell communication within a heterogeneous population. Furthermore, given the disparity between transgene expression levels across the population, we cannot assume that differentiation was homogeneous. Furthermore, during development, pancreatic genes are temporally regulated, and disruption of this regulation can cause adverse effects. Constitutive expression of SOX17 to generate DE, followed by constitutive PAX4, PDX1 or NGN3 expression is an effective method for initial screening of these pancreatic genes, however it limits the ability to recapitulate complex and temporal developmental patterns of transcription factor activity.

The primary method used in these studies for characterization of novel transgenic HESC lines was quantitative gene expression analysis. This provided us with the ability to screen for markers of multiple lineages in a high-throughput manner. However, mRNA transcript levels do not always accurately reflect cellular protein levels due to post-transcriptional and translational regulation. In order to validate the changes in mRNA expression observed, functional analysis should be undertaken by immunoblotting or immunocytochemistry for markers such as insulin, glucagon and somatostatin. In

addition, immunocytochemistry should be performed for the products of the transgenes PDX1, NGN3 and PAX4 to ensure that they are being translated into protein, and that the protein is localized to the nucleus for functional downstream transcriptional regulation.

4.3 Future Directions

The current studies present preliminary but promising evidence supporting the hypothesis that transcription factor expression can be used to efficiently direct the differentiation of HESC to a pancreatic endocrine phenotype. To further this study, I would generate clonal populations of HESC with inducible SOX17 activity using the ER^{T2} system (Hayashi and McMahon, 2002) and NGN3 activity through the use of the piggyBac transposase system (Woltjen et al., 2009). This would allow for control of dose and duration of both definitive endoderm and pancreatic transcription factor activity.

Following the generation of these transgenic HESC, I would perform differentiation experiments maintaining cells in both monolayer culture and 3D culture using embryonic bodies (EB). The use of an EB system may better recapitulate cell-cell contact during development thereby enabling a more developmentally relevant context. Real-time PCR would be performed to assay gene expression, while immunocytochemistry would be performed for transgenes in addition to pancreatic hormones INS, GCG and SST. To determine the efficiency of differentiation, flow cytometry would be performed. Once a homogeneous population of pancreatic endocrine cells is generated, I would perform a teratoma assay to assess if the cells are committed to the pancreatic endocrine lineage.

Once a source of pancreatic progenitors is obtained, I would transplant them into the tail vein in a mouse model of diabetes and track cell migration to see if these cells are functional and correct hyperglycemia. Injection in the tail vein allows access to vasculature and the cells are able to migrate to multiple tissues. A common problem with transplantation is the endogenous immune response that will destroy the transplanted cells. Commonly, cells are transplanted in immuno-compromised mice to avoid this problem. Alternatively, cells could be coated in alginate (Tuch et al., 2011), a semi-permeable material that will allow for insulin to pass through, but prevent immune cells from destroying cell transplants. Polyethylene glycol coatings have also been studied as a method for delivering islet cells (Kizilel et al., 2010). Interestingly, cells encapsulated with polyethylene glycol can secrete more insulin compared to control islets.

Lastly, I would determine if the transplanted cells end up in the pancreas, or if they migrate to alternative tissues. To do this, I would label the cells with iron nanoparticles so that the animals could be live-imaged using MRI techniques (Tai et al., 2006). It is known that iron nanoparticles do not affect functionality of islet cells (Kim et al., 2009).

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