Regulated Mis-Expression of PDX1 and NKX6.1 during Pancreatic Differentiation of Pluripotent Stem Cells

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Table of Contents

Chapter 2
Materials and Methods

Chapter 3
Definitive Endoderm Differentiation

Chapter 4
Tet-On Inducible Expression System

Chapter 5
Definitive Endoderm Differentiation

Chapter 6
Definitive Endoderm Differentiation

Appendices

Abstract

Human pluripotent stem cells represent a potential source of pancreatic beta cells for the treatment of type 1 diabetes. Methods for generating pancreatic endoderm from pluripotent stem cells are becoming increasingly robust, although the general applicability of any given method to a broad range of stem cell lines remains a challenge. Therefore, a deeper understanding of the molecular mechanisms controlling the commitment of cells to pancreatic endoderm is desirable. A key step in this process is the co-ordinated up-regulation of two transcription factors, PDX1 and NKX6.1; factors that together identify cells fated to become pancreatic endoderm and to later give rise to endocrine cells. In this thesis, we examined the effects of over-expressing PDX1 and NKX6.1 during the course of PSC differentiation. We employed the use of a number of transgenic systems, namely the doxycycline inducible Tet-On expression system, Destabilisation Domains (DD) and Estrogen Receptor (ER) fusion proteins. Our experiments demonstrated a potential for each of the 3 expression systems, however we encountered drawbacks within each one preventing us from establishing a definitive conclusion as to the affects of over-expressing PDX1 and NKX6.1 during PSC differentiation. We conclude that only once both the expression systems and differentiation protocols are optimised will it be possible to determine the effects of co-expressing these two factors during the course of pancreatic differentiation.

List of Publications

Tan, G., Elefanty, A.G., and Stanley, E.G. (2014). Beta-cell regeneration and differentiation: how close are we to the 'holy grail'? Journal of Molecular Endocrinology *53*, R119–R129.

PART A: General Declaration:

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in peer reviewed journal. The core theme of the thesis is is the regulation of mis-expression of PDX1 and NKX6.1 in pancreatic differentiation of pluripotent stem cells. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the department of Anatomy and Developmental Biology under the supervision of Ed Stanley.

All of the data presented in this thesis, with the exception of two expression vectors constructed by Tanya Labonne, is my own work.

Therefore, I assess my contribution to the work included in this thesis as greater than 90%.

Signed: ……  ……

31.7.15

Date: ……

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Throughout my Masters I have been extremely fortunate to have had an army of people supporting me and encouraging over the past 3 years.

I would first like to whole-heartedly thank my supervisors Ed Stanley and Andrew Elefanty. Their guidance and support has been unfailing throughout my time with them. In particular, Ed, has shown incredulous patience with me, giving me the opportunity to experience “the life of science”, taking me from a young padawan all the way to a young Jedi (I hope). He has endured my endless rants (on occasion, suggesting a career change to radio talk show host), my neurotic pessimism for life and my multitude of questions (which tend to come all at once) over the last 3 years. I don’t think I could have envisioned or hoped to have a better supervisor. Andrew on the other hand has provided me with endless help with “heavy machinery” within the lab. Learning how to use the FACS machine and dealing with “user friendly” zen programs would have left me without any hair by now if it wasn’t for his help.

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“Do. Or do not. There is no try”
-Yoda

Chapter 1

Introduction

1

Chapter 1 Introduction

1.1 Human Pluripotent Stem Cells

Pluripotent stem cells (PSCs) are immortal cells that can be differentiated into any cell type found in the body. Because of this, much interest has been focused on the possibility of deriving insulin producing β cells from PSCs for the treatment of diabetes. There are two types of PSCs: Embryonic stem cells (ESCs) derived from blastocyst stage human embryos and induced pluripotent stem cells, derived by reprogramming somatic cells to an embryonic stem cell like state. Human ESCs were first cultured by Thomson and colleagues (Thomson, 1998) and paved the way for the subsequent derivation of iPSCs - cells that grow under the same conditions as PSCs and have the similar properties. The method by which somatic cells are reprogrammed to iPSCs was first described by Takahashi and Yamanaka (2006). Using mouse and human somatic cells, often dermal fibroblasts, they found that the retroviral-mediated introduction of four embryonic stem cell associated factors, OCT3/4, SOX 2, c-MYC and KLF4 induced pluripotency (Narita, Ichisaka, Tomoda, & Yamanaka, 2007; Takahashi & Yamanaka, 2006; Yu et al., 2009). Subsequently, further research found that other combinations of factors, small molecules and RNAs could be used to achieve an equivalent outcome (Desponts, C & Ding S, 2010; Li et al., 2011). The differentiation potential, proliferative capacity, morphology and gene expression profiles of iPSCs are highly similar to that of embryonic stem cells (ESCs), but the former avoids the ethical complications of deriving PSCs from human blastocysts (Takahashi & Yamanaka, 2013) (Koh & Piedrahita, 2014). In spite of their similarities, iPSCs hold slightly different promise than ESCs, namely the use iPSCs for patient-specific therapy. Human iPSCs are a genetic match to the person from whom they were generated, theoretically circumventing the issue of immune rejection of iPSC derivatives. However, in the case of type 1 diabetes, it would not be

unreasonable to suppose that iPSC derived β cells would be rejected by the same autoimmune mechanism that led to the disease in the first place. Therefore, at least for type 1 diabetes, it is questionable whether iPSCs would offer any advantages over ESCs as a source of new β cells for transplantation therapies. Lastly, the amount of work and cost required to derive, validate and ensure safety for any given cell line means that individualised iPSCs may not be economically practical as a source of new β cells. In this sense, a generalisable “off the shelf” stem cell derived product is likely to be more viable, particularly in the short term.

1.2 Development of the pancreas

In order to differentiate PSCs *in vitro* towards a pancreatic cell fate, it is necessary to understand the process of pancreatic organogenesis. Extensive studies involving mouse models have lead to a better understanding of the processes underlying early development of the embryo and the steps leading to the formation of complex tissues. Using these processes as a roadmap, researchers have been able to guide PSCs through analogous stages of development toward the formation of desired cell types. The pancreas is a derivative of definitive endoderm, one of the three primordial germ layers generated during the process of gastrulation. Once generated, definitive endoderm folds into a primitive gut tube which is then further regionalised into subdomains, a process that occurs under the influence of growth factors secreted by juxtaposed tissues. The pancreas arises from two patches of epithelium that evaginate dorsally and ventrally from the foregut endoderm, situated between the stomach and duodenum (Murtaugh & Melton, 2003). The dorsal bud receives signals from the notochord and dorsal aorta and establishes a permissive environment for dorsal and pancreatic specification within the gut endoderm (Hebrok, Kim, & Melton,

1998). Conversely, the ventral bud relies on signals from the adjacent cardiac mesenchyme and lateral plate mesoderm (Kumar, Jordan, Melton, & Grapin-Botton, 2003). Following budding, the pancreatic primordia undergo a phase of considerable growth and branching, culminating in fusion of the two buds into a single organ (Murtaugh & Melton, 2003; Slack, 1995). Over this period, expansion of the pancreatic epithelium is principally driven by mesenchymal cells, which secrete proliferative growth factors such as FGF10 (Bhushan et al., 2001). In the mouse, pancreatic development is accompanied by the branching of epithelial tubules; a stage sometimes referred to as the "secondary transition". During this time, acini form and begin to differentiate whilst mesenchymal derived growth factors continue to drive epithelial growth and new acini formation (Landsman et al., 2011). Concomitant with this process, endocrine progenitors delaminate from the epithelium and aggregate to form islets (reviewed in (Slack, 1995)). Maturation of endocrine cells within these islets generate glucagon, insulin, somatostatin and pancreatic polypeptide producing cells. Studies in the mouse suggest that endocrine precursors continue to differentiate throughout fetal development and for up to three weeks after birth. After this stage, endocrine tissue is maintained through a low frequency of replication (Bonne-Weir et al., 2006; Kaung et al., 2005).

Although the process of human pancreatic development is thought to resemble that documented for the mouse, there are a number of points of difference that are pertinent to the discussion of pluripotent stem cell differentiation towards pancreatic lineages. First, it is unclear whether the human pancreas has developmental stages that can be equated with those documented for the mouse (Sakar et al., 2008). A key issue relating to the similarities of mouse and human pancreatic development centres around the appearance and function of cells expressing multiple hormones.

Experiments in the mouse suggest that insulin and glucagon expressing cells have distinct developmental origins (Herrera et al., 2000). As such, it is unclear how and/or whether cells which express multiple hormones during early mouse development (Teitelman et al., 1993) contribute to adult mouse endocrine system. In human development, cells expressing multiple hormones range from 5-20% of hormone positive cells, a fraction that remains relatively stable between weeks 10 and 20 of fetal life (Jeon et al., 2009). At this stage, it is still unclear if such cells ever give rise to fully functional β cells or whether they represent a fetal cell type that makes no contribution to the adult endocrine organ (Hrvatin et al., 2014). Until recently, polyhormonal cells were a common feature of many PSC to pancreatic differentiation protocols (reviewed in (Nostro and Keller, 2012)). However, newer methods of differentiation reported by and (Pagliuca et al., 2014; Rezania et al., 2014) appear to have rectified this issue, raising the possibility of generating fully functional β cells *in vitro*.

1.3 Signalling Pathways Involved in Pancreatic Development

Extrinsic signals are cues sent across the extracellular space that allow cells and tissues to control complex processes such as organogenesis. These pathways regulate developmental process through the binding of a ligand to an extracellular receptor, transducing an intracellular response. Often, these morphogens activate pathway specific transcription factors which in turn lead to a cascade of changes in the expression of genes involved with directing developmental decisions of the cell. Three key signalling pathways involved in pancreas development are the Notch, FGF and sonic hedgehog (shh) signalling systems. In the case of the last, experiments show the inhibition of shh signalling in the pre-pancreatic endoderm is necessary for pancreatic specification (Hebrok et al., 1998). Similarly, a complex interaction

between notch and FGF signalling is required to balance proliferation and differentiation definitive pancreatic progenitors.

The pancreas is an organ highly dependent on mesenchymal-epithelial interactions. Understanding the interactions that induce the proliferation of pancreatic progenitor cells during development provides insight into potentially ways of expanding hESCs towards a pancreatic fate. One such interaction is the production of FGF10 from the mesenchyme and its interaction with the epithelium to elicit growth, branching morphogenesis and differentiation as the pancreas develops. FGFR2IIIb, a receptor of the FGF signalling pathway, when activated by FGF10 has been found to stimulate exocrine differentiation and proliferate endocrine progenitor cells in *in vitro* cultures of rat pancreatic EBs (Elghazi et al., 2002).

Murine models nullizygous for FGF10 were established to investigate the role of the protein in the development of the pancreas. Results from those studies revealed regular evagination of the epithelium with the dorsal and ventral buds of the pancreas forming normally as indicated by Pdx1 expression (Bhushan et al., 2001). However, studies also discovered that the pancreatic epithelium was greatly reduced during development, with a potential cause being a defect in the formation of epithelial progenitor cells. These cells are distinguished by the expression of Pdx1 and it was noted that in the $FGF10^{-/-}$ mice, Pdx1 expression decreased over time. Significantly, the differentiation of endocrine and exocrine cells within the pancreas was not directly affected, as only the reduction in numbers of the progenitor pool led to the reduction in differentiation. Conversely, as the proliferation of pancreatic progenitors did not occur in the absence of FGF10, the pool of progenitors available for differentiation reduced, ultimately leading to the insufficient number of cells to generate the pancreas. These results suggest that the role of FGF10 during pancreogenesis is

primarily to regulate the proliferation of pancreatic progenitors and thereafter the size of the pancreatic primordium.

Further investigation into the mechanisms of pancreatic progenitor proliferation found that Notch signalling was induced by FGF10 during development. The Notch pathway mediates short range signalling limited to adjacent cells through its receptors (the Notches) and ligands (Deltas and Jaggeds) (Fiúza and Arias, 2007). Upon binding to the ligand, the Notch receptor is cleaved to release an intracellular portion that then migrates to the nucleus. Once bound to the Notch transcriptional mediator Rbpj, the complex is converted from being a repressor to become an activator (Cleaver and MacDonald, 2010).

Notch signaling has been known to have two different activator properties. In some cases it can control the decision of cells in a population to choose one differentiation pathway or another through a process of “lateral inhibition”, but more importantly, it has been observed to promote the expansion of precursor populations by suppressing signals sent for cell differentiation. In the case of the Jagged ligands, it is suggested that when the ligand activates the Notch receptors, it acts to suppress exocrine differentiation and promote proliferation of the progenitors. In addition, production of the Delta ligands similarly activates the Notch receptors to initiate a negative feedback loop of the Neurogenin3 (Ngn3), which drives progenitors to favour an endocrine fate.

To understand the process of FGF10 and Notch signalling in the proliferation of pancreatic progenitors, studies were developed to either overexpress or be deficient in different components of the pathway. In support of the suppressive maintenance

model, a study by Hart and colleagues found that persistent expression of FGF10 and FGFR2IIIb enhanced and prolonged proliferation of pancreatic epithelial cells. It was also identified that the effects exerted by FGF10 reduced the expression of Delta ligand 1 (Dll1) and NGN3 whilst maintaining the expression of Notch 1 and Hes 1 in the pancreatic epithelium, perturbing lateral inhibition (Hart et al., 2003). In other studies involving transgenic mice deficient for Dll1 and Rbpj, endocrine differentiation was augmented as premature differentiation of progenitors was detected; similarly this took place in mice overexpressing NGN3. Although differentiation took place, the resultant pool of cells with the ability to differentiate reduced, resulting in mice that were insulin-deficient with endocrine and exocrine hypoplasia (Fujikura et al., 2006; Apelqvist et al., 1999).

The interaction between FGF10 and the Notch signalling pathway (fig 1.1) plays an important role in the development of the pancreas as it enables the proliferation of the original pool of progenitors whilst inhibiting premature differentiation.

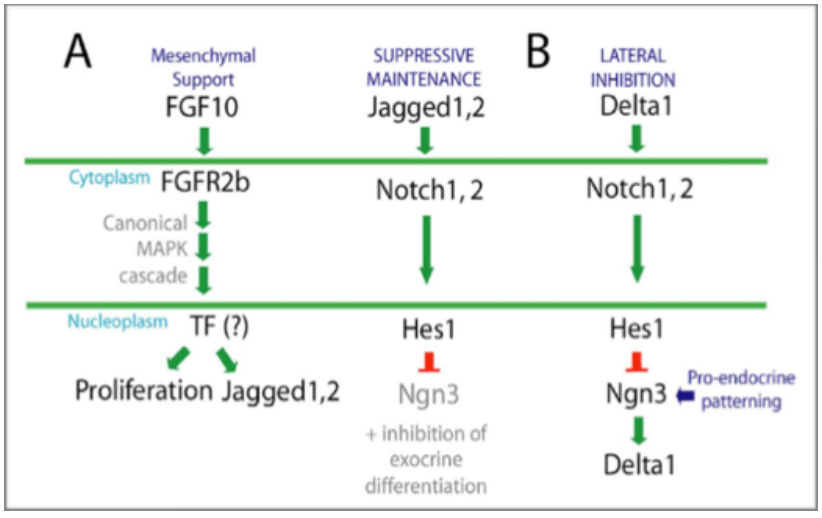

***Figure 1.1**. Model of Notch signaling. A) Suppressive Maintenance – Fgf10, normally produced by the mesenchyme leads to the induction and proliferation of Jagged 1 and 2, which activate Notch 1 and 2, blocking differentiation through Hes1. B) Lateral inhibition – in which endocrine differentiation mediated by Nn3 leads to expression of delta, activation of notch and repression of Ngn3 through the activation of Hes1. Thus a progenitor that has undergone endocrine differentiation inhibits its neighbour from differentiating (Norgaard et al., 2003).*

1.4 Differentiation of PSCs to Pancreatic cells

Although numerous groups have published protocols for the differentiation of PSCs towards either pancreatic progenitors and endocrine cells, the most influential methods come the US biotech company, Viacyte Inc. In a protocol developed by D'Amour et al., combinations of growth factors involved in pancreatic development were used in a stage specific manner to guide PSCs from their undifferentiated state into insulin expressing cells through a series of obligatory intermediate cell types identified through developmental studies (D'Amour et al., 2005; 2006; Kroon et al., 2008; Schulz et al., 2012). Since 2006, many other laboratories have published their own pancreatic differentiation protocols which often include elements of D'Amour et al.'s approach (for example, (Jiang et al., 2007; Nostro and Keller, 2012; Rezania et al., 2014; Xu et al., 2011).

Although there are a large number of variations between individual pancreatic differentiation protocols, a number of common themes are evident. All methods are based on the ontogenetic framework described above that is used to rationalise the use of particular factors/treatments at specific stages of differentiation. To successfully differentiate PSCs to a pancreatic fate, it is recognised that multiple crucial developmental steps need to be accurately modelled; this includes the induction of definitive endoderm, the patterning and specification of endoderm to a pancreatic fate and the generation of endocrine/exocrine cells (Nostro et al., 2011). The first step invariantly employs Activin A to induce definitive endoderm, a TGFβ family member that mimics the action of Nodal, the ligand used by the embryo to drive development of the mesoderm and definitive endoderm (Jiang et al., 2007; Kroon et al., 2008; Kubo et al., 2004; Lowe et al., 2001; Osada and Wright, 1999).

Following this, definitive endoderm cells are treated with retinoic acid (RA) to induce foregut (pancreatic endoderm). This treatment was initially used in mouse embryonic stem cells for pancreatic differentiation (Micallef et al., 2005) and was rapidly adopted to PSC systems (D'Amour et al., 2006). In both the cases of Activin A (Nodal) and retinoic acid, the key experiments defining the role of these factors were performed in model organisms such as xenopus, zebrafish and the mouse (Chen et al., 2004; Conlon et al., 1994; Esni et al., 2001; Stafford and Prince, 2002). Following the emergence of pancreatic endoderm, many methods treat cells with the Bone morphogenetic protein (BMP) antagonist, noggin; a factor found to promote the development of pancreatic progenitors defined by the expression of PDX1 (Bose et al., 2012; Jiang et al., 2007; Kroon et al., 2008). The final steps that take pancreatic endoderm through to genuine functional endocrine cells are still unclear. For this reason, many researchers transplanted cells at the pancreatic progenitor stage, allowing the final steps of differentiation and maturation to occur *in vivo*. In instances in which the final stages of differentiation were carried out *in vitro*, factors such as Nicotinamide, IGF1 and HGF were used to induce β cell maturation, generating insulin and glucagon expressing cells (D'Amour et al., 2006; Jiang et al., 2007; Mfopou et al., 2010). Despite the fact that the actual signals required for final endocrine differentiation are unclear, a number of groups have taken a empirical approach to defining combinations of molecules eliciting this step. As previously mentioned, two such studies were undertaken by (Hrvatin et al., 2014) and (Rezania et al., 2014). These studies developed a strategic method to define and differentiate glucose response hESC-derived β cells. Through the comparison of the transcriptome of hESC-derived pancreatic cells to their *in vivo* counterpart, (Hrvatin et al., 2014) was able to validate hESC-derived β cells and in addition generate a catalog of genes involved in their differentiation. The manipulation of these genes

may in future aid in converting hESCs to functioning and responsive β cells. On the other end of the spectrum, (Rezania et al., 2014) built upon their previously published protocols to enrich their population of hESC-derived β cells. Their improved protocol generated highly differentiated cells marked by genes associated with mature β cells which rapidly reversed diabetes within 40 days. Taken together, these two studies may forge a way to improve the differentiation and yield of glucose responsive β cells.

1.5 The Role of *PDX1* and *NKX6.1* in Pancreas Development

Pancreas formation is dependent on the co-ordinated expression of several transcription factors. One such transcription factor is pancreatic and duodenal homeobox 1, PDX1. Originally identified as a transcriptional activator of insulin and somatostatin expression in pancreatic endocrine cells (Leonard et al., 1993; Ohlsson et al., 1991), embryological studies showed that PDX1 was one of the earliest markers of pancreatic specification (Ahlgren et al., 1996; Offield et al., 1996). In the mouse, *Pdx1* expression can be observed as early as embryonic day (E) 8-8.5 in the ventrolateral domains of the developing foregut. By E9.5 dorsal expression of *Pdx1* clearly identifies regions within the foregut that will give rise to the developing dorsal and ventral pancreatic buds

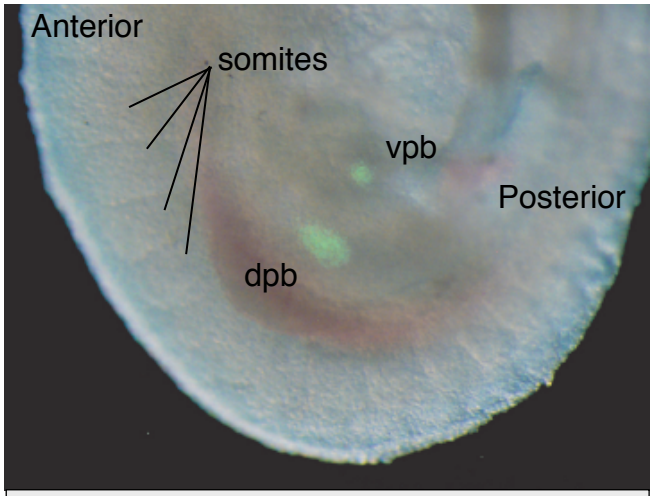

***Figure 1.2**. Bright field GFP overlay image of the trunk region of a E9.5 mouse embryo in which GFP is expressed from the Pdx1 gene. The position of GFP⁺ dorsal (dpb) and ventral (vpb) pancreatic buds are indicated.The anterior-posterior axis of the embryo is marked, as are 4 somites anterior to the pancreatic primordium. Picture taken from (Holland et al., 2006)*

(Holland et al., 2006)(Figure 1.2). Expression of *Pdx1* is maintained in the pancreas throughout development. In adults, *Pdx1* is detected in the nuclei of islet cells (predominantly β cells) and in immature acinar cells containing amylase (Guz et al., 1995; Holland and Góñez, 2004). *PDX1* expression has also been found in pancreatic endoderm as early as 8 weeks in humans (Lyttle et al., 2008).

Mouse knockout models indicate that *Pdx1* is indispensable for the development of the pancreas and *Pdx1* null neonates are born without a pancreas (apancreatic). Although formation of the pancreatic buds appeared to be normal within *Pdx1* null (-/-) embryos, subsequent expansion of the pancreatic primordium did not occur. As a consequence, *Pdx1* null neonates succumb to fatal hyperglycaemia shortly after birth (Stoffers et al., 1997;Jonsson et al., 1994). Furthermore, neonatal mice heterozygous for a *Pdx1* (*Pdx1*$^{+/-}$) were found to have an altered glucose response, with animals remaining hypoglycaemic for longer periods following glucose challenge. This compromised response is thought to partly result from the impaired expression of Insulin (*Ins1*) and Glucose Transporter 2 (*Glut2*) (Ahlgren et al., 1998), two genes directly under the control of *Pdx1*. In addition, the architecture of islets differed between the *Pdx1*$^{+/-}$ and wildtype mice, with *Pdx1* heterozygote islets appearing smaller and containing large regions devoid of β cells, suggesting that reduced *Pdx1* expression may drive differentiation to favor β cells (Dutta et al., 1998). Lastly, mutations in *PDX1* have also been identified in human neonates with diabetes indicating the role and function of this transcription factor is well conserved across species (Schwitzgebel et al., 2003).

Previous studies with mouse ES cells indicate that timely ectopic activation of *Pdx1* during the course of differentiation *in vitro* can up-regulate the expression of

pancreatic genes, including *Insulin 1* (*Ins1*) and *Insulin 2* (*Ins2*). To observe the temporal effects of *Pdx1* on pancreatic differentiation, *Pdx1* expression was regulated by using the Tet-on (Kubo et al., 2011) or Tet-off (Miyazaki et al., 2004) system. In the Tet-on system, gene expression is activated by the addition of Doxycycline (DOX). Conversely, with the Tet-off system, the continuous presence of DOX represses expression of the transgene. In this case, activation of the transgene occurs when DOX is removed. Following induction, cells were analysed for expression of pancreatic lineage marker genes.

In the study by Miyazaki and colleagues, up-regulation of *PDX1* lead to a substantial increase in the expression of Ins2, somatostatin and the pancreatic transcription factor Nkx2.2. However, expression of INS1, glucagon, PPY, or Glut2, which are all specific to the endocrine pancreas *in vivo* was not detected, even within the condition that up-regulated PDX1. This result indicates that enforced expression of *PDX1* alone was insufficient to drive the pancreatic differentiation program. Alternatively, these results might suggest that although *PDX1* expression is required for initial specification of pancreatic progenitors, continuous expression could suppress some endocrine-specific genes during the final maturation stage (Miyazaki et al., 2004).

Kubo et al. (Kubo et al., 2011) used the Tet-on system to over express either *Pdx1* or *Pdx1* in combination with Ngn3, a transcription factor required for endocrine lineage differentiation. In their studies, enforced expression of *Pdx1* did appear to activate expression of the insulin 1 gene, but only when Ngn3 was over-expressed at the same time.

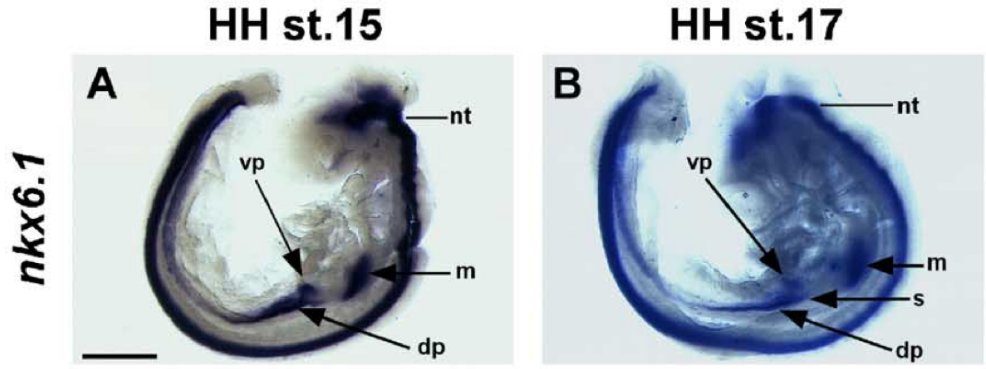

Figure 1.3. *Pancreatic morphogenesis in chick embryos at (A) HH stage 15 and (B) stage 17. Nkx6.1 expression is divided in a dorsal domain and two ventro-lateral domains. A thickening of the dorsal pancreatic epithelium is observed with Nkx6.1 expression also beginning to become restricted to the two ventral pancreatic thickenings of the endoderm (Pederson et al., 2005). nt, neural tube; dp and vp, dorsal and ventral pancreas; m, stomach mesenchyme; s, posterior stomach.*

The above results suggest that pancreatic endocrine differentiation requires the expression of *Pdx1* in combination with other genes. Another transcription factor associated with pancreatic β cell formation is the homeobox protein *NKX6.1*. Analysis of developing mouse embryos revealed that expression of *Nkx6.1* paralleled that of *Pdx1*. The first signs of *Nkx6.1* expression within the foregut were noted as early as embryonic day (e) 10.5, where it was found to be broadly expressed within the epithelial cells of the pancreatic bud (figure 1.3) (Watada, 2000). At this point, *Nkx6.1* was detected in multi-potent pancreatic progenitors which were also positive for *Pdx1*. As development continued, expression of *Nkx6.1* persisted in common progenitor cells for ductal and endocrine cell lineages. Eventually *Nkx6.1* expression became restricted to the β cell lineage (Rudnick et al., 1994).

To further understand the role of *NKX6.1* in the development of the pancreas, a mouse knockout model carrying a null mutation of the *Nkx6.1* gene (*Nkx6.1*$^{(-/-)}$) was developed. In this study carried out by Sander et al. (2000), mice deficient for *Nkx6.1* displayed a dramatic reduction in β cell numbers but otherwise showed normal development of all other endocrine and exocrine cell types (Sander et al., 2000). They also noted that the inactivation of *Nkx6.1* lead to the disruption of β cell

development, especially during the secondary transition; a phase of pancreatic development marked by a massive wave of β cell differentiation.

These results prompted researchers to test whether over-expression of *NKX6.1* could stimulate β cell proliferation or enhance β cell function. Schaffer et al. (2011), investigated this hypothesis by over-expressing *Nkx6.1* in normal mice or in mice that had been depleted of β cells (Schaffer et al., 2011). Their experiments showed that, regardless of the model, over-expression of *Nkx6.1* failed to elicit an increase in β cell proliferation nor did it affect or improve glucose tolerance or clearance (Schaffer et al., 2013). From these results it was deduced that *NKX6.1* over-expression alone was insufficient to promote β cell expansion nor improvement of glucose metabolism *in vivo*.

When taken as a whole, these results suggest that *NKX6.1* does plays an integral role in β cell specification but its function is likely to modulated by additional factors and to be dependent on the developmental context.

1.6 Co-expression of *PDX1* and *NKX6.1*

In vitro differentiation of PSCs towards pancreatic cells generates a mixture of cell types that include pancreatic endoderm (PE) and polyhormonal endocrine cells. Transplantation experiments suggest that polyhormonal cells eventually give rise to glucagon expressing cells, but do not contribute to the generation of functional β cells (Basford et al., 2011; Kelly et al., 2011). Conversely, there is growing evidence that pancreatic endoderm, marked by the co-expression of *PDX1* and *NKX6.1* includes progenitors of the definitive endocrine system, including β cells. These *NKX6.1*$^{+}$/

PDX1^{+} cells, which also expressed another pancreatic transcription factor, PTF1A, formed islet like clusters, including ductal and endocrine tissue upon transplantation (Kelly et al., 2011). Analysis of these newly formed islet like clusters revealed cells that they co-expressed *NKX6.1*, *PDX1* and insulin. By contrast, polyhormonal cells found within the graft were devoid of *NKX6.1* and *PDX1* expression (Kelly et al., 2011).

Further studies have correlated the varying levels of expression of *NKX6.1* with cellular phenotype. Work by Rezania et al. showed that PSC derived pancreatic progenitors contained two distinct populations: (1) *NKX6.1*-high cells, which were enriched for pancreatic endoderm and contained a low number of endocrine cells, (2) *NKX6.1*-low cells that were enriched for polyhormonal endocrine cells and expressed high levels of PTF1A. Both cell populations were loaded into a Theracyte device and subsequently transplanted into mice to test for their ability to differentiate and mature into functional endocrine cells. Although both populations differentiated into pancreatic endocrine cells, *NKX6.1*-high derived grafts contained a higher proportion of insulin positive cells and exhibited an advanced maturation state compared to the *NKX6.1*-low grafts. In addition, hyperglycaemia was reversed within three months in mice implanted with *NKX6.1*-high graft (Rezania et al., 2013). Collectively, these studies suggest show that *PDX1* and *NKX6.1* are important for the development and differentiation of pancreatic endoderm. In addition, they suggest that up-regulation of these factors is associated with differentiation to the endocrine lineage. Lastly, co-expression of *PDX1* and *NKX6.1* appears to be a critical event during the generation of precursors that give rise to functional pancreatic endocrine cells.

1.7 Expression Systems - Inducing Co-expression of *PDX1* and *NKX6.1*

The co-expression of *NKX6.1* and *PDX1* is a defining milestone during pancreatic development *in vivo* and *in vitro*. In this study we set out to test the the effect of enforcing the co-expression of these factors during the course of PSC differentiation *in vitro*. The following sections contain a brief description of systems that potentially allow for inducible expression of transgenes during the course of PSC differentiation.

The TET-on/off system has previously used by other laboratories for the doxycycline (Dox) dependent induction of *PDX1* expression during PSC differentiation. This system encompasses two related methods of control - the Tet-on and the Tet-off system. In a Tet-on system, the reverse tetracycline activator (rtTA) protein is a sequence specific transcriptional activator that is able to bind to its target sequence (Tet operator) only if it is bound by a tetracycline or by one of its derivatives, usually Doxycycline or Dox (Gossen et al., 1995). Thus addition of Dox to cultures initiates transcription of gene downstream of the Tet operator sequence. The Tet-off system works in a similar but opposite fashion. In this case, the tetracycline transactivator (tTA) binds to the operator in the absence of tetracycline (Gossen and Bujard, 1992). Addition of Dox in this case prevents binding of the rtTA to its target sequences, leading to abatement of expression from the Tet responsive promoter. In comparing the two systems, the Tet-on system is the preferred choice if one wishes to keep the gene of interest inactive and to only “switch it on” occasionally.

Another system for controlling the expression of transgenes involves the use of destablisation domain, derived from *E.Coli* dihydrofolate reductase (ecDHFR). In this

system, the stability of fusion proteins between a domain from DHFR and the gene of interest is regulated a cell-permeable small molecule, the DHFR inhibitor trimethoprim (TMP). This system was developed by Iwamoto et al. who conducted a screen for ecDHFR mutants destablisation domains (DD), that caused almost complete removal of the ecDHFR-fusion proteins (Iwamoto et al., 2010). As previously mentioned, TMP stabilises the DD target protein fusion and inhibits protein degradation (Iwamoto et al., 2010). As a result of this screen, Iwamoto et al. identified domains whose relative stability was finely regulated by different concentrations of the stabilising ligand TMP. Stabilisation of the fusion protein was found to be reversible and upon the removal of TMP, the protein was rapidly degraded (Schrader et al., 2010). In addition to these attributes, the affinity of TMP for bacterial DHFR is much greater than for mammalian DHFR, potentially reducing side effects of using this drug *in vitro* and *in vivo* (Kuyper et al., 1985).

The third system for controlling gene expression also involves the creation of a fusion protein comprising the gene of interest and the hormone binding domain of the estrogen receptor (ER). The specificity of the system is enhanced by point mutations within the hormone binding domain that greatly increases its affinity for synthetic hormones (such as tamoxifen) compared to that of endogenous ligands (Littlewood et al., 1995). In the absence of the hormone, the fusion protein is kept inactive as it is complexed with heat shock protein 90 (HSP90) (Aumais et al., 1997; Devin-Leclerc et al., 1998) maintaining the ER fusion protein in an inactive state. The addition of tamoxifen or its metabolite 4-hydroxytamoxifen (4-OHT), causes a conformational change leading to the dissociation from HSP90. Once free of HSP90, the fusion protein is able to participate in its normal cell function. The advantage of this system

is that induction is very rapid. The disadvantage is that the bulky ER hormone binding domain may interfere with the function of some proteins.

The following pages describe our attempts to engineer pluripotent stem cells that co-expressed *NKX6.1* and *PDX1* in an inducible fashion during the course of differentiation. We discovered that constructing such a system was extremely challenging and that an ideal methodology for conducting these kinds of experiments may well require new technologies.

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CHAPTER 2

MATERIALS AND METHODS

2

2.1. Reagents

Cell Lines

- RM3.5 iPSC (referred throughout as iPS Parent) (unpublished)
- NKX6.1 #D3/A1 ΔNeo (referred throughout as NKX6.1 Parent) (unpublished)
- SOX17 H9$^{w/mCherry}$ (unpublished)

Tissue Culture Reagents

Table 2.1

PSC and iPSC Media

Component	Stock	Final Volume per 100 ml
DMEM/F12 (Invitrogen, USA)	1x	80 ml
Knockout Serum Replacement (Gibco, USA)	1x	20 ml
MEM non-essential amino acids (NEAA) (Invitrogen, USA)	100x	1 ml
Penicillin/ Streptomycin Solution (Pen/Strep) (Invitrogen, USA)	200x	500 μl
GlutaMAX I (Invitrogen, USA)	100x 100x	1 ml
2-mercapthoethanol (2-ME) (Sigma, USA)	50nM (100x)	200 μl
basic Fibroblast Growth Factor (bFGF) (StemRD, USA)	10ng/ml	100 μl

**iPSC Media - requires 5 times the concentration of bFGF, therefore final concentration will be 50ng/ml.*

E8 Essential Media: 500ml Basal Medium, 10 ml Supplement (Life Technologies, USA, cat# A1517001)

Geltrex Medium - Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Life Technologies, USA cat# A1413202) and DMEM.

Feeder Media: DMEM, 10% FBS (Fetal Bovine Serum, JRH Biosciences, USA), 50unit/mg Pen/Strep, 1% GlutaMAX I

EDTA-NaCL: Ultra pure 0.5M EDTA pH 8.0 (Invitrogen, USA), 500ml PBS (Invitrogen, Life Technologies, AUS), 200mg/ml NaCl (Sigma-Aldrich, USA)

Table 2.2
ITSX

Component	Stock	Final Volume per 10 ml
Sodium Selenite (Sigma, USA)	1mg/ml	10 μl
Rh Insulin (Sigma, USA)	5mg/ml	2 ml
Rh Holotransferrin (Sigma, USA)	20mg/ml	275 μl
PBS (Invitrogen, USA)	1x	7.95 ml

Table 2.3
AEL Media
(Ng et al., 2008)

Component	Stock	Final Volume per 100 ml
Iscove's Modification of Dulbecco's Medium (IMDM) (Invitrogen, USA)	1x	46.98 ml
HAMS F12 Nutrient Mix (Invitrogen, USA)	1x	46.98 ml
Albucult (Novozymes, UK)	10mg/ml	2.50 ml
Synthecol (Invitrogen, USA)	7 200x	13.90 μl
Linolenic Acid (Sigma, USA)	100x 100x	10.00 μl
Linoleic Acid (Sigma, USA)	100x 100x	10.00 μl
α-Monothioglycerol (α−MTG) (Sigma, USA)	13μl/ml	3.90 μl
ITS-X	100x	1.00 ml
Ascorbic acid 2-phosphate (AA2P) (Sigma, USA)	100x	1.00 ml
GlutaMAX I (Invitrogen, USA)	100x	1.00 ml
Pen/Strep (Invitrogen, USA)	100x	0.50 ml

Table 2.4
Differentiation Reagents

Component	Company	Cat #
Activin A	R&D systems	338-AC-010
PI-103 Kinase Inhibitor	Cayman Chemical, USA	371935-74-9
CHiR99021	Sigma-Aldrich, USA	252917-06-9
TTNPB	Sigma-Aldrich, USA	71441-28-6
DMH-1	Sigma-Aldrich USA	1206711-16-1
4-Hydroxytamoxifen (4-OHT)	Sigma-Aldrich USA	68047-06-3
Trimethoprim (TMP)	Sigma-Aldrich USA	738-70-5
Doxycycline	Sigma-Aldrich USA	24390-14-5

RNA Micro Synthesis Kit: Isolate II RNA micro kit, Bioline cat# BIO-52075

RNA Mini Synthesis Kit: Isolate II RNA mini kit, Bioline cat# BIO-52072

cDNA Synthesis Kit: Superscript III First-Strand Synthesis System, Life Technologies cat# 18080-051s

Table 2.5
Primers - realtime-PCR Applied Biosystems

GAPDH	Hs02758991_g1
SOX17	Hs00751752_s1
CXCR4	Hs00607978_s1
FOXA2	Hs00232764_m1
OCT4 (POU5F1)	Hs01895061_u1
MIXL1	Hs00430824_g1
EpCAM	Hs00901885_m1
PDX1	Hs00236830_m1
NKX6.1	Hs00232355_m1
INS	Hs02741908_m1
GCG (glucagon)	Hs01031536_m1

Universal Master Mix: Taqman Fast Universal PCR Master Mix (2x) (Life techonolgies, USA cat#4304437

Table 2.6

Antibodies - FACS and Immunofluorescence Staining

Conjugated	Cat #	Dilution	
PE/Cy7 anti-human CD184 (CXCR4)	Biolegend #306514	1:20	FACS
APC anti-human CD326 (EpCAM)	Biolegend #324208	1:100	FACS
APC anti-DYKDDDDK Tag	Biolegend #637308	1:100	FAC/ Primary (Immunofluorescence)
PE mouse anti-PDX1	BD #562161	1:40	FACS
PE mouse anti-NKX6.1	BD #563023	1:20	FACS
PE anti-human CD117 (c-kit)	BD #561682	1:25	FACS
APC anti-human IgG Fc	BD #550931	1:100	FACS
PE anti-human IgG Fc	Biolegend #409304	1:100	FACS
PE/Cy7 anti-human IgG Fc	Biolegend #306514	1:100	FACS
Anti-Estrogen Receptor Antibody	Abcam #Ab37438	1:100	Primary (Immunofluorescence)
Goat anti Rabbit IgG (H+L) Alexa Fluor 568	Life Technologies #A-11011	1:300	Secondary (Immunofluorescence)
Donkey anti Goat IgG (H+L) Alexa Fluor 568	Life Technologies #A-11057	1:300	Secondary (Immunofluorescence)
Goat anti Mouse IgG (H+L) Alexa Fluor 568	Life Technologies #a-11011	1:300	Secondary (Immunofluorescence)

Unconjugated	Cat #	Dilution	
goat Anti-PDX1	Abcam #ab47383	1:1000	Primary (Immunofluorescence)
mouse anti-NKX6.1	Developmental Studies Hybridoma Bank University of Iowa	1:50	Primary (Immunofluorescence)

Intracellular Staining Kit: Human intracellular cytokine staining kit cat# 559302

Live/Dead Stain: FACS wash, Propidium Iodide (1ug/ml) (Sigma, USA)

FACS Wash: PBS, 2% FBS

FACS Block: PBS, 1% rabbit serum, 1% goat serum, 2% FBS

Fix/Perm Cell Immunofluorescence Staining: 4% PFA, 0.5% Triton-X

TBS-tween: 10x TBS, dH_2O, Tween-20

DAPI staining: DAPI(1ug/ml), PBS

Mounting Media: Dako Immunofluorescence Mounting Medium cat# S302308-2

2.2 Targeting Vector Assembly for GTi-PDX1 (-T2A NKX6.1)

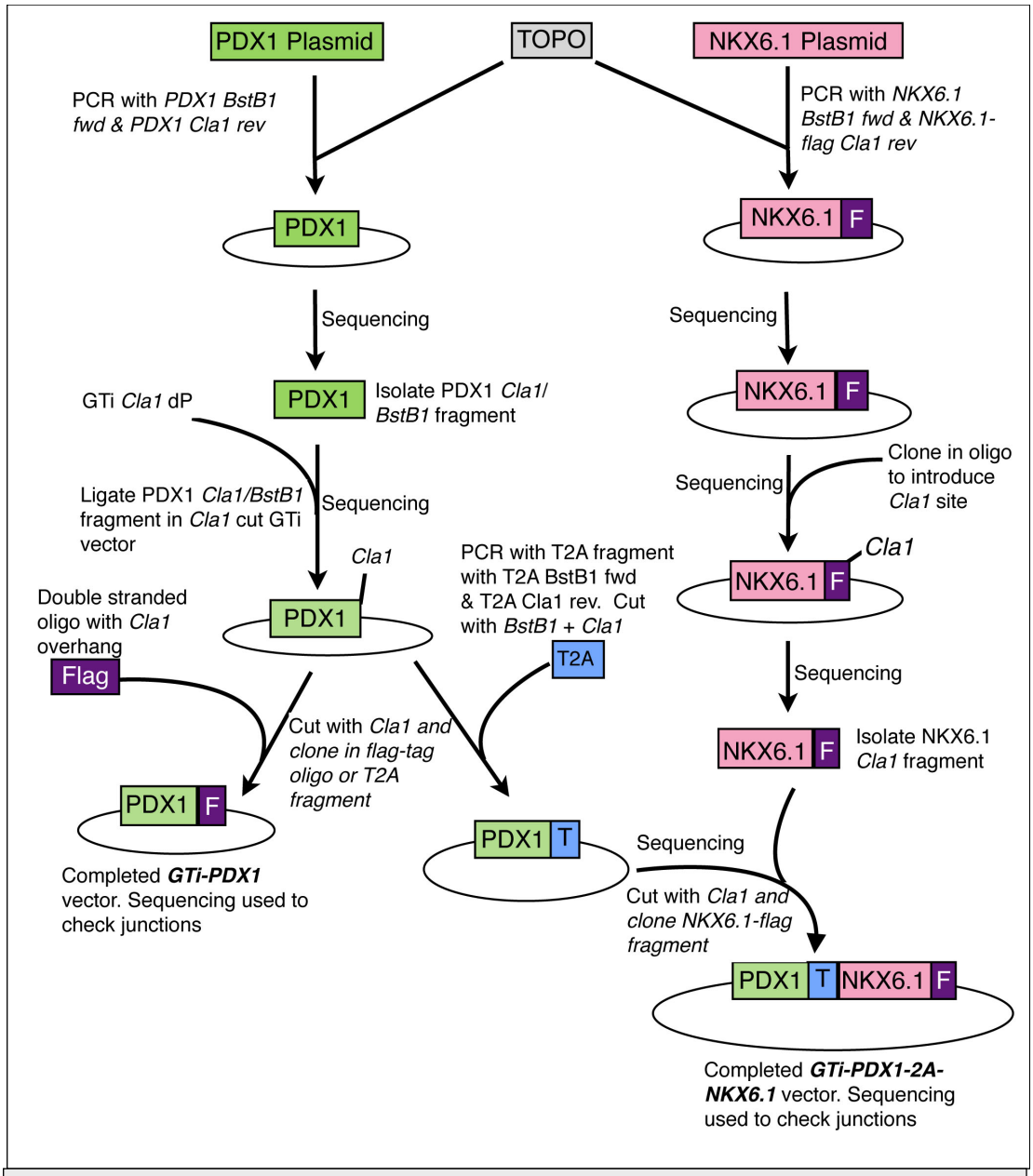

Figure 2.1. *Assembly plan for construction of GAPTrap vectors expressing PDX1 and PDX1 -T2A-NKX6.1 cDNAs. Plasmids encoding the cDNAs were obtained from Addgene (pWPT-PDX1 and pMXs NKX6.1). Following PCR, the cDNAs were cloned into the ClaI site of GTi expression vector. Genes in this position of the vector are placed downstream of the doxycycline responsive promoter (see structure of the GTi vector shown in Figure 2.2). The T2A sequence between the PDX1 and NKX6.1 cDNAs allows for co-translation of the two proteins from the single transcript. In order to enable expression of the cDNAs to verified, sequences encoding a flag-epitope tag were added to the 3' of either PDX1 or*

Bacterial stab cultures (Addgene) for the plasmids pWPT-PDX1 and pMXs-NKX6.1 were streaked and single colonies picked into LB-broth and cultured overnight at 37ºC. Plasmid DNA was prepared using a Qiagen kit according to the manufacturers instructions. DNA fragments representing the PDX1 and NKX6.1 cDNAs were amplified by PCR using the primers PDX1 BstB1 fwd, PDX1 Cla1 rev and Nkx6.1 Cla1 fwd, Nkx6.1 flag Cla1 rev, respectively (Table 2.7).

Table 2.7

PCR primers used to generate cDNAs encoding PDX1 and NKX6.1:

Primer Name	Sequence
PDX1 BstB1 fwd	caGGtTCGAaCCACCatgaacggcgaggagcagtac
PDX1 Cla1 rev	GATCACATCGATtcgtggttcctgcggccg
Nkx6.1 Cla1 fwd	GAGGCGATCGATatgttagcggtgggggcaatg
Nkx6.1 flag Cla1 rev	CCCATCGATtcaCTTGTCGTCGTCGTCCTTGTAGTCggatgagctctccggctc

The restriction endonuclease sites within the primers used for subsequent cloning steps are shown in blue text.

Each sample was run on a 1% agarose gel and DNA fragments were purified using a Qiagen gel purification kit as per the manufacturers instructions. Subsequently, each fragment was cloned into a TOPO 2.1 vector (Invitrogen) and transformed into competent bacteria. The next day, 10 colonies representing each construct were picked into LB-broth and subsequently, plasmid DNA was prepared as above. Plasmid DNA was digested with Cla 1 (NKX6.1) or Cla1 and BstB1 (PDX1), to identify plasmids containing the appropriately sized insert. At this stage, TOPO vectors containing the PDX1 cDNA (refer to figure 2.1) were sent for sequencing analysis (The Gandel Charitable Trust Sequencing Centre (Monash Health Translation Precinct, Melbourne, Australia).

From this point, expression vectors were assembled as illustrated in figure 2.1. The key components of the vector are described in the figure legend, as is the order in which the components were introduced into the GAPTrap targeting vector. In order to verify the structure of the completed vectors, cloning junction points were analysed by DNA sequencing. Plasmid DNA was cut with Pac1 before electroporation.

2.2.1 Targeting Vectors GT-DD.NKX6.1 and GT-PDX1.ER

Construction of the GT NKX6.1 DD and GT PDX1 ER vectors was performed by Tanya Labonne. As such, only the completed vectors of both constructs are shown below. A pictorial explanation of how these vector systems are predicted to work is also shown. Each construct was cut with Pac1 before electroporation.

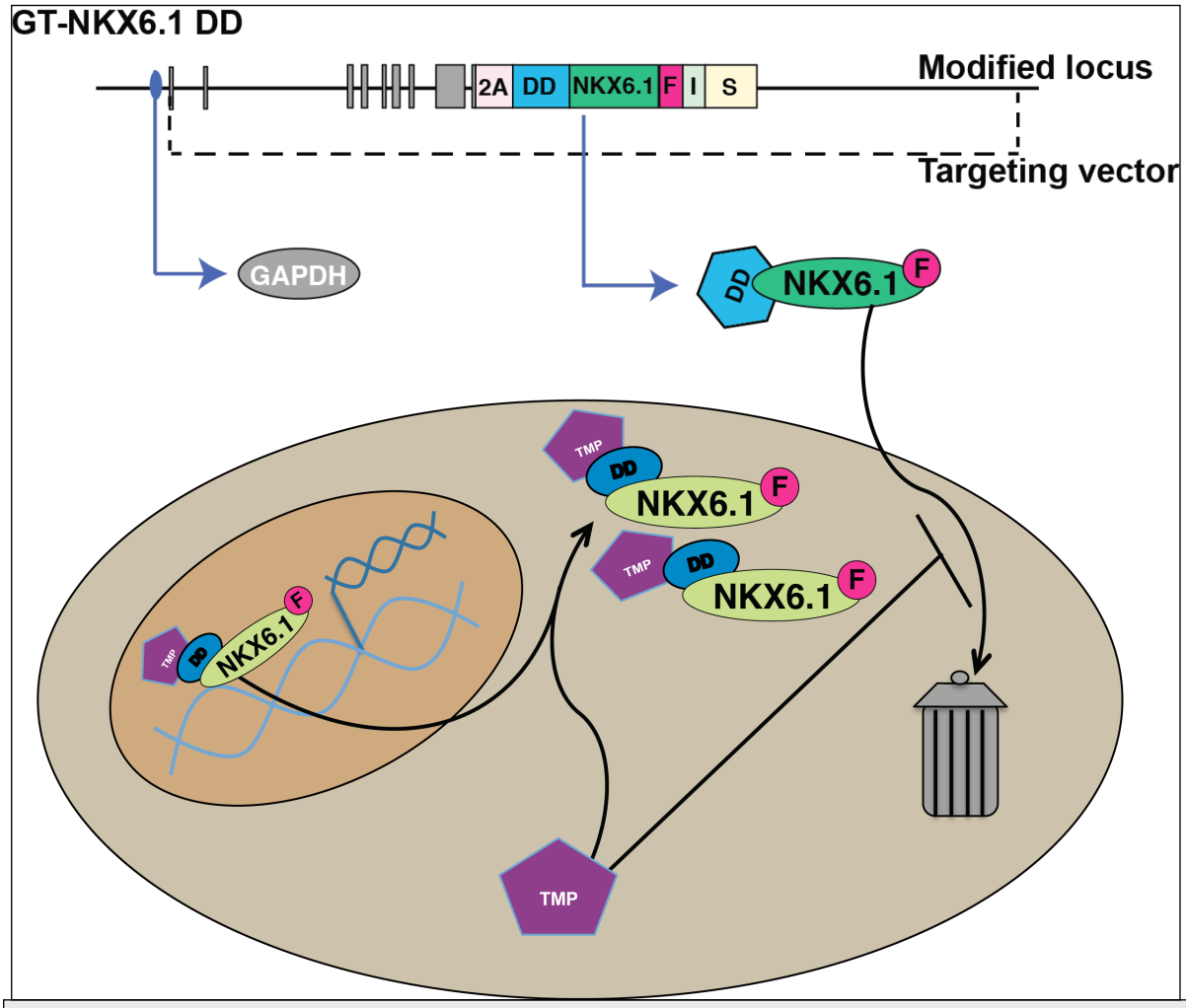

Figure 2.2. *Completed GT-DD.NKX6.1 vector. In this system NKX6.1 is expressed as fusion protein with a destablisation domain (DD) from dihydrofolatereductase (DHFR). This fusion protein also contains a flag epitope located at the c-terminus of NKX6.1. 3' of the fusion protein is an internal ribosomal entry site (I), which enables the selectable marker (S) to be translated from the GAPDH transcript. In this system the target protein NKX6.1, although constitutively made, is constantly degraded via the destablisation domain. In order to stabilise the DD.NKX6.1 fusion protein, a ligand, trimethoprim (TMP) is added. This ligand binds to DD, altering its conformation such that it is no longer a target for*

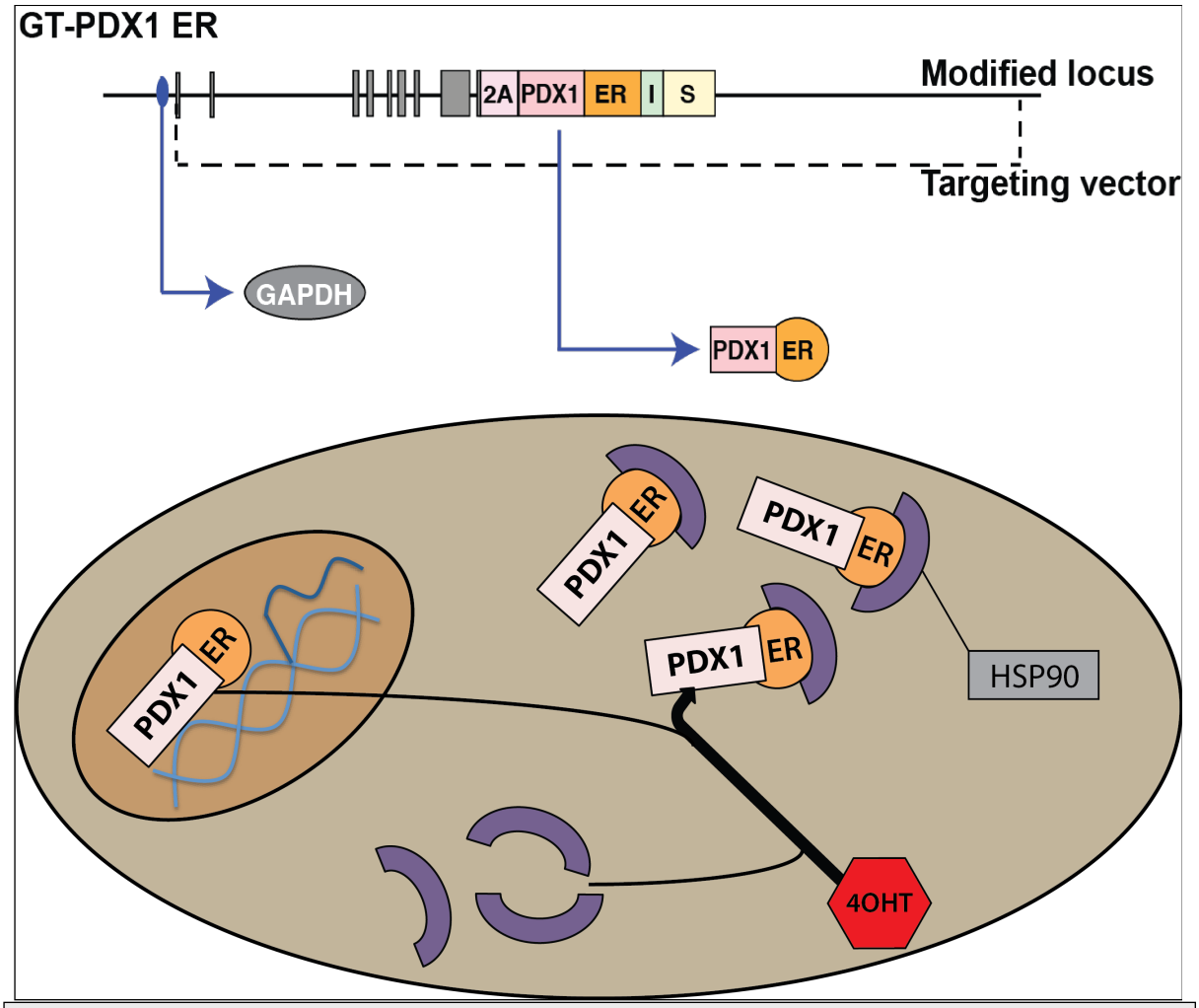

Figure 2.3. *Completed GAPTrap PDX1.ER vector. In this vector PDX1 is expressed as fusion partner of the hormone binding domain of the oestrogen receptor (ER). 3' of the fusion protein is an internal ribosomal entry site (I), which enables the selectable marker (S) to be translated from the GAPDH transcript. In this system, the fusion protein is constitutively made but remains inactive as it is complexed with heat shock protein 90 (HSP90). However with the addition of 4 hydroxytamoxifen (4OHT), the fusion protein is released, allowing it to translocate into the nucleus and participate in cellular functions.*

2.3. Electroporation, culturing and picking of PSC and iPSC

Pluripotent stem cells were electroporated essentially as described by Costa et al. (2007)(Costa et al., 2007). In the lead up to the electroporation, cultures of PSC and iPSCs were expanded and then, the day prior to electroporation, passaged onto tissue culture flasks pre-seeded with mouse embryonic fibroblasts (MEFs) at low density ($1x10^4$ per cm^2). Also, in preparation for plating out of electroporated cells, 12 x $6cm^2$ dishes were seeded with $0.42x10^6$ MEFS per plate (12 plates per electroporation, 10 plates for electroporated cells, 1 plate for non-electroporated cells and another plate for MEFS only).

Two 50% confluent T150 flasks containing approximately $8x10^6$ cells per flask were prepared for each electroporation (which requires approximately $1x10^7$ cells). On the day of electroporation, PSCs were harvested using TryPle and counted, with the number of MEFs (estimated from the number seeded the previous day) subtracted from the total number of cells. The cell suspension was pelleted by centrifugation and subsequently re-suspended in cold PBS to give a final cell concentration of approximately 10.7 cells per 0.75mls. For targeting to the GAPDH locus, cells were electroporated with a mixture of 3 DNA constructs. In addition to the targeting vectors, two plasmids encoding TALENs that recognised the GAPDH locus were also included. TALENs are sequence specific nucleases that introduce a double stranded DNA break at the locus that is to be genetically modified. The cell then uses the targeting vector to repair this break, thus introducing the vector DNA into the cell's genome at the desired position. Experiments from our own and other laboratories indicated that inclusion of TALENs can substantially enhance the frequency of obtaining gene targeting events (Hockemeyer et al., 2011). The total volume of the electroporation cuvette was 800µl and cells were electroporated using parameters

250V and 500μF. Table 2.8 below displays the amount of linearised targeting vector DNA and GAPDH Talons used for each electroporation.

Once electroporated, cells were gently resuspended in warmed hESC or iPSC media, spun down at 1500 rpm for 3 minutes and plated onto the previously prepared 6cm^2 MEF plates. For the first 5 days, a media change was performed everyday due to the relatively high rate of cell death following electroporation. By this stage, each PSC colony on the plates contained approximately 30-60 cells. From this point on, media changes including antibiotic selection were performed everyday until colonies were ready to be picked. In addition, MEFs were topped up every week to ensure optimum growth conditions for the PSCs. Drug selection for each construct differed. Neomycin (G418) was used for GTi PDX1 Term and GTi PDX1 T2A 6.1 (Chapter 4), hygromycin (hygro) for NKX6.1 DD and puromycin (puro) for PDX1 ER (Chapter 5). Initially drug selection started at 50μg/ml for G418, 50μg/ml for hygromycin and 0.5μg/μl for puromycin. However as time progressed, drug selection for each of the constructs were increased to 100μg/ml G418, 100μg/ml hygromycin and 1μg/μl puromycin to ensure no background (non-drug resistant cells) remained.

Approximately two weeks from the date of electroporation, colonies could be seen macroscopically. At this point, colonies were picked for their expansion and for karyotyping. 2x48-well plates were prepared, each seeded with 2x10^4 MEF cells/cm^2. To pick PSC colonies, a grid was etched onto the colony and 2/3 was taken transferred to 1 well of a 48 well plate and 1/3 was transferred to the corresponding position on the second 48 well plate. The 48 well plate containing the majority of each colony was used for preparation of DNA in order to identify targeted clones. The

second 48 well plate was the stock plate used to recover targeted clones. Media changes were performed everyday until cells are able to be harvested for screening.

Table 2.8

Measurements of Linearised DNA and GAP s used in each electroporation (PSC and iPSC).

Component	Final (μg)	Final vol. (μl)
PDX1 Term/ PDX1 T2A 6.1/ NKX6.1 DD/ PDX1 ER	30	30
GAPDH Talon 1	10	5
GAPDH Talon 2	10	5

12 clones were chosen for DNA extraction from the 48 well plate designated for DNA analysis. Media was aspirated and cells were washed with PBS. 100ul of lysis buffer was added to wells for 24 hours at 37℃. Following this, DNA extraction was performed using a manifold vacuum DNA elution system. Samples were then screened for correct targeting by PCR. Screening involved the use of one primer bound to genomic DNA representing sequences 5' to 5' end of the targeting vector and another bound to the sequence corresponding to the T2A region within the targeting vector. PCR conditions were set up as follows -95º C for 2 minutes, then 30 x 95º C for 30 seconds, 60º C for 20 seconds, 68ºC for 3 minutes. PCR products were run on a gel and targeted clones yielded a DNA fragment of approximately 3.5kb.

Table 2.9
List of Reagents in PCR Screen

Volume (ul)	PCR components
1	Gapscreen2 Primer (100ng/ul) sequence: actgttctctccctccgcgcagc
1	T2Arev1 Primer (100ng/ul) sequence: CCGCATGTTAGAAGACTTCCTCTG
2	10x PCR Buffer
0.8	$MgSO_4$
1	DMSO
0.4	10nM dNTPs
1	DNA Sample
0.1	Platinum Taq HiFi
13	H_2O

TALENs against the GAPDH locus were designed and assembled by Cellectis Biosearch (Paris – France). The TALENs targeted sequences immediately 3' of the GAPDH stop codon as shown below. The coding sequences of GAPDH are shown in bold italicised text. The GAPDH ***stop codon*** is underlined. The left TAL Effector DNA-binding domain is in **red**, the right TAL Effector DNA-binding domain is in **blue**. Sequences cut by the TALEN pair are shown in **green** text.

tgacaactcttttcatcttctaggtatgacaacgaatttggctacagcaacagggtggtggacctcatggccca catggcctccaaggagtaagacccctggaccaccagccccagcaagagcacaagaggaagagagaccct cactgctggggagtccctgccacactcagtcccccaccacactgaatctcccctcctcacagtttccatgtagacccctt gaagaggggagg

2.4 PSC and iPSC Feeder Culture

PSCs were maintained in PSC and iPSC media, as required, and grown on primary mouse embryonic fibroblasts (MEFS) seeded at a density of $2x10^4$ cells/cm^2. Flasks were media changed either everyday or every second day depending on cell confluency. When cells reached approximately 80% confluency, cells were passaged onto new flasks seeded with MEFS. Cells were passaged using either Tryple select (for hESCs) or EDTA-NaCl PBS (iPSCs). One day prior to setup of differentiations or electroporation experiments, PSC cultures were passaged onto MEFa seeded at a density of $1x10^4$ per cm^2. As differentiation and electroporation requires single cell suspensions, iPSCs were harvested using TrypLE.

2.4.1 Geltrex Media Preparation

Geltrex LDEV-Free reduced growth factor basement membrane matrix (15mg/ml) was defrosted overnight on ice at 4℃. Subsequently, geltrex was aliquoted into 250ul and kept at -30℃. To prepare geltrex media, serumless Dubecco's modified eagle media (DMEM) was aliquoted into 25mls and kept on ice. 300ul of media was used to resuspend 250ul of geltrex (1:100). Once geltrex media was prepared, media was promptly distributed onto chosen culture surfaces. Pre-coated plates/flasks were either sealed in parafilm and kept at 4℃ or kept at 37℃. (If geltrex coated plates/ flasks were kept at 4℃, 20 minutes prior to use, plates/flasks would need to be taken out of 4℃ and placed at room temperature to ensure re-polymerisation of geltrex.)

2.4.2 PSC Feeder Free Culture

PSCs were transitioned from feeder culture to feeder free culture. Prior to passaging, 10cm tissue culture plates were pre-coated with geltrex media and PSCs were passaged with Tryple onto the Geltrex coated plates containing 50% PSC media and 50% essential 8 (E8) media. Plates were media changed everyday and cells were passages once they were ~80% confluent. Upon second passage, cells were harvested by incubating them with warmed EDTA-NaCl (kept in 37℃ incubator) for approximately 2 mins at room temperature. Sometimes cells were needed to be incubated with EDTA-NaCL for approximately 4-5 mins, and this was sometimes performed at 37ºC. Cells were then pipetted twice before being transferred onto a 10cm geltrex coated plate with 100% E8 media. Culture medium was changed everyday. Unlike PSCs grown on feeders, cell recovery increased when PSCs were harvested in smaller colonies, therefore differentiations were carried out without the need of single cell suspensions.

2.4.3 *In Vitro* Differentiations

PSC and iPSC cell lines were cultured on 1:100 Geltrex or feeders using E8 and PSC media respectively. In preparation for differentiation, 90% confluent cultures were washed with PBS (without Mg^{2+} and Ca^{2+}) and then incubated with either warm EDTA (geltrex) for 2-3 minutes at room temperature or TrypLE (feeders) for 3 minutes at 37ºC. PSCs harvested with EDTA were collected by centrifugation and resuspended in E8 media. Cell numbers were estimated by taking into account the surface area and confluence of the original cultures.Generally, one 90% confluent 10cm plate was transferred onto one 6-well tray (previously coated with Geltrex. Cultures were media changed everyday (E8 media) and differentiation was commenced when cells reached 70-80% confluency. PSCs harvested with TryPLE

were collected with PBS and centrifuged for 3 minutes at 1500 rpm. Cells were resuspended with PSC media and single cell suspensions were seeded at 5 x10^5 on geltrex coated surfaces. Cultures were media changed everyday and differentiation commenced when cells were 70-80% confluent.

Stage 1: Definitive Endoderm differentiation

Day 1, undifferentiated PSCs plated on geltrex coated surfaces were first exposed to AEL media supplemented with Activin A(100ng/ml), CHiR 99021 (2uM) and PI-103 kinase inhibitor (500nM). After 24 hours, cells were media changed to AEL supplemented with Activin A (100ng/ml) and DMH-1(1uM). Cells were exposed to this media for 48 hours.

Stage 2: Pancreatic endoderm, PDX1$^+$ and NKX6.1$^+$

From differentiation day 3 onwards, definitive endoderm was cultured in AEL media supplemented with DMH1 (1uM) and TTNPB (3nM) for a further 2 days. . On day 5 of differentiation, AEL medium was supplemented with 1mM TMP (DD system), 1uM 4OHT (ER system) or 1ug/ml DOX (TetO system). Cell analysis was performed at the 24 hour and 48 hour time point after cultures were treated with the inducing agents.

2.5 Flow cytometry; RNA synthesis; cDNA synthesis

2.5.1 Preparation of Cells for Flow Cytometry

Undifferentiated and differentiated PSCs were incubated with TryPLE at 37℃ for 3 minutes and collected by repeatedly pipetting the disaggregated cultures in FACS wash (PBS, 2% FBS). The cell suspension was passed through a filtered cap FACS tube to remove cell clumps. Cells were then pelleted by centrifugation for 3 minutes at 1500 rpm. Media was aspirated and cells were re-suspended in 100µl FACS wash supplemented with chosen antibodies (refer to reagents list for antibodies and dilutions). Tubes were kept on ice and in the dark for 20 minutes. Following labelling, cells were washed twice with 2mls of FACS wash and finally cell pellets were suspended in FACS wash containing 1ug/ml propidium iodide (PI). Cells were analysed using a BD LSRFortessa cell analyser. Flow cytometry gates were set using appropriate isotype control antibodies (ref. Table 2.6). When possible, 10,000 live events were recorded for each experiment. In general, 3 independent experiments were analysed for each specific differentiation condition. However, on some occasions where a condition displayed avert toxicity or clear negative result, these experiments were not repeated more than twice.

2.5.2 Preparation of Cells for Intracellular Flow Cytometry

Undifferentiated and differentiated PSCs were harvested and processed as described above. Following filtration and centrifugation, media was aspirated and cells were suspended in ~200-400µl Cytofix (BD) and kept on ice for 20 minutes. Cells were then washed twice with 2mls of Permwash (Cytoperm and dH_2O) and re-suspended in 100µl of permwash containing the chosen antibodies (refer to reagents list for antibodies and dilutions). Tubes were once again kept in the dark and on ice for 20

minutes. Following labelling, the cells were washed twice with 2ml of Permwash and finally re-suspended in FACS wash in preparation for flow cytometry.

2.5.3 RNA isolation, cDNA synthesis and Real Time PCR

RNA was isolated according using a Bioline micro or mini RNA kit according to the manufacturers instructions. RNA concentration was measured using a Thermo Scientific nanodrop. cDNA synthesis was performed using the Life Technologies First strand superscript III kit. Q-PCR was performed using the Taqman assays listed in table 2.5 were performed and analysed as described by (Pick et al., 2009).

2.5.4 Cell Immunofluorescence Staining of Undifferentiated PSCs

8 well premanox chamber slides were coated with Geltrex and cells were seeded at at an approximate density of between $5x10^4$ and $1x10^5$ cells/cm. Once cells were ~40-50% confluent, cells were exposed to media supplemented with TMP and 4-OHT for 24 hours. Following this, media was aspirated and cells were washed with PBS. 200ul of PBS containing 4% PFA and 0.5% Triton-X was then added to each chamber for 10-15 minutes. After washing cells 3 times with PBS, the fixed cells were then treated for 30 minutes with PBS containing 5% BSA. Primary antibodies were diluted in 1% BSA to the appropriate concentration of antibody solution added to each well of the chamber slide (refer to table 2.6 for antibody dilutions). Following 1 hour, the antibodies were removed and the cells were washed 3 times with Tris Buffer Saline and 0.05% tween (TBS-tween). The cells were then labelled with the appropriate secondary antibodies (refer to table 2.6 for antibody dilutions) for an hour at 4ºC. After incubation, cells were further washed 3 times with TBS-tween and cells were treated with a PBS solution of 1ug/ml DAPI for 2 mins. After another 3 washes with TBS-tween, cell chambers were detached and 3 drops of mounting media was

applied to the slide. Glass coverslips were then carefully placed onto the slide to ensure no air bubbles formed between the two layers. Once dried, the glass coverslip is then sealed to the slide with a nail varnish. Slides were analysed using a Zeiss LSM 780 confocal microscope alongside with Zeiss Zen Black and Blue microscope and imaging software.

2.6 Statistical Analysis

Most differentiation experiments were performed at least three times. Primary experimental data used for statistical analysis is presented in appendix 2. Where required, data from these independent experiments was analysed using GraphPad PRISM6. Analyses were confined to pair-wise comparisons of two conditions using a a student t-test. The results of these analyses are also provided in appendix 2.

2.7 Reference

Costa, M., Dottori, M., Sourris, K., Jamshidi, P., Hatzistavrou, T., Davis, R., Azzola, L., Jackson, S., Lim, S.M., Pera, M., et al. (2007). A method for genetic modification of human embryonic stem cells using electroporation. Nat Protoc *2*, 792–796.

Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat Protoc *3*, 768–776.

Pick, M., Azzola, L., Mossman, A., Stanley, E.G., and Elefanty, A.G. (2009). Differentiation of Human Embryonic Stem Cells in Serum-Free Medium Reveals Distinct Roles for Bone Morphogenetic Protein 4, Vascular Endothelial Growth Factor, Stem Cell Factor, and Fibroblast Growth Factor 2 in Hematopoiesis. Stem Cells *25*, 2206–2214.

CHAPTER 3

DEFINITIVE ENDODERM DIFFERENTIATION

3

3.1 Introduction

To successfully differentiate PSCs to definitive endoderm, it is generally accepted that key developmental pathways need to be accurately modelled. Current pancreatic differentiation protocols seek to activate or repress a limited number of developmentally important signalling pathways to generate definitive endoderm. Despite their differences, a common thread between these protocols involves the use of Activin A to induce definitive endoderm. Belonging to the TGFβ family, high concentrations of Activin A mimics the actions of Nodal, a ligand which promotes the development of the mesoderm and definitive endoderm within the embryo (Payne et al., 2011; Shiraki et al., 2008).

In a protocol developed by D'Amour et al. (2005), D'Amour et al. (2006) and Kroon et al. (2008), 3 day Activin A treatment was augmented by the brief addition of Wnt3a (1st day) to induce definitive endoderm by day 4. Jiang et al. (2008) used Activin A in the context of a chemically defined - serum free media (CDM), suggesting that exogenous Wnt3a was not required for the induction of definitive endoderm. Other signalling pathways potentially important for definitive endoderm induction involves another member of the TGFβ family, Bone morphogenic protein 4 (BMP4). BMP4 is a critical signalling molecule in early embryonic differentiation and acts upstream of Nodal as an initiator of mesendoderm formation, the key differentiation event during gastrulation. Studies involving the combination of high concentrations of Activin A alongside low concentrations of BMP4 have been observed to greatly improve the differentiation of definitive endoderm (D'Amour et al., 2005; Micallef et al., 2011a). Furthermore, studies have also shown a a role for Phosphoinositol-3 kinase (Pi3K)

signalling in definitive endoderm formation, demonstrating that its inhibition in combination with Activin A promotes the generation of definitive endoderm (Loh et al., 2014; Singh et al., 2014).

Despite the fact that most protocols generate large proportions of definitive endoderm, the overall yield of end stage differentiated insulin expressing β cells remains low. This is partly because incomplete differentiation at each stage over several stages gradually reduces the number of cells available to complete subsequent differentiation steps. Furthermore, the timing of each stage of differentiation varies significantly between protocols, regardless of growth factor combinations, with some methods taking up to 6 days to generate definitive

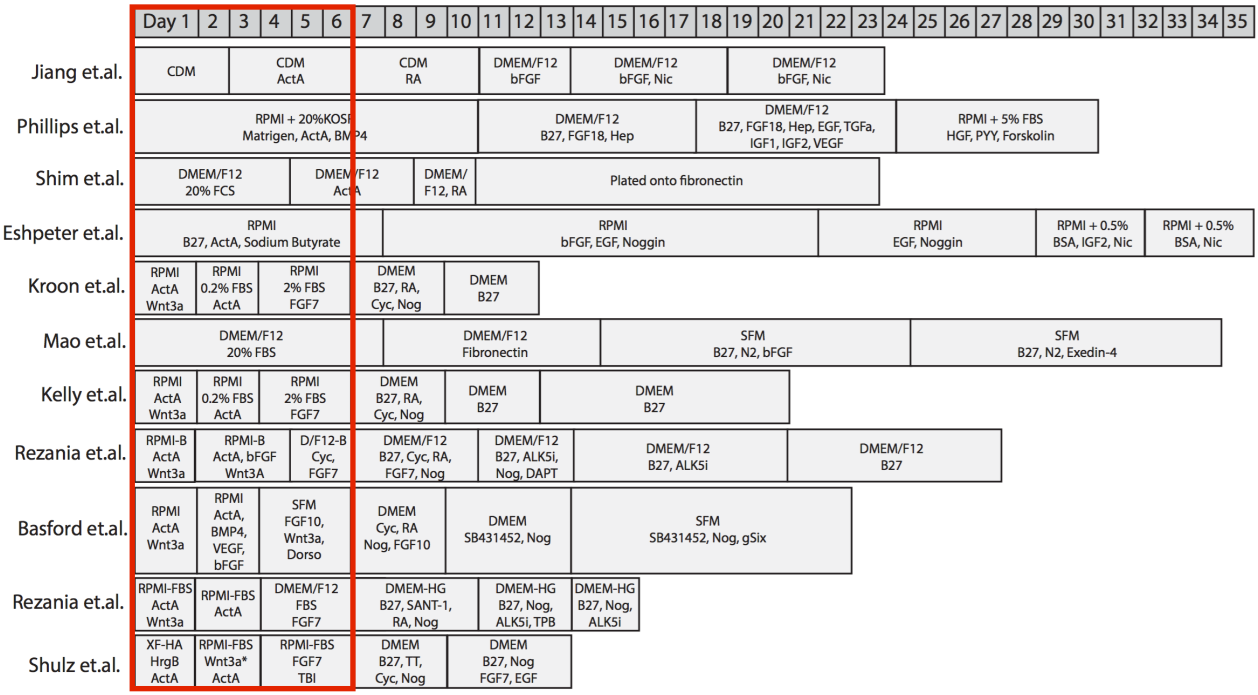

Figure 3.1. *Timeline of published pancreatic differentiation protocols. Sectioned in red, is various multistep procedures used to induced definitive endoderm. Abbreviations: CDM, Chemically Defined Medium; ActA, Activin A; FCS, Fetal Calf Serum; FBS, Fetal Bovine Serum; RA, Retinoid Acid; Cyc, Cyclopamine; Nog, Noggin; EGF, Epidermal Growth Factor; Nic, Nicotinamide; Alk5i. ALK5 inhibitor; BSA, Bovine Serum Albumin; Hep, Heparin; TTNPB, TGF-βR1 kinase inhibitor; FGF7(/18), Fibroblast Growth Factor 7(/18); bFGF, Basic Fibroblast Growth Factor; SFM, Serum Free Medium; HrgB, Heregulin; VEGF, Vascular Endothelial Growth Factor; RPMI-B, RPMI-BSA; IGF-1, Insulin-like Growth Factor 1; HGF, Hepatocyte Growth Factor; TGFa, Transforming Growth Factor a; D/F12-B, DMEM-F12 with BSA; DMEM-HG, DMEM-high glucose (Schiesser and Wells., 2014).*

endoderm. As such, we hypothesised that one contributing factor of the low yield of end-stage cells could be related to the length of time of each differentiation step - with longer times allowing for greater asynchrony in the cultures overall. In other words, the combination of incomplete differentiation and increasing culture asynchrony was likely to play a role in the inefficient production of the desired end cell product. For this reason, we tested a range of published differentiation conditions that promoted the first step in differentiation, the formation of definitive endoderm from PSCs.

In 2014, Loh et al. examined different definitive endoderm differentiation protocols in order to improve the efficiency of definitive endoderm induction through "logically directing signals controlling lineage bifurcations". Scrutinising different combinations of growth factors, their study found that early inhibition of endogenous BMP induction steered differentiation away from mesoderm and towards definitive endoderm. They discovered that the combination of high Activin A, ChiR (Wnt agonist) and Pi3K inhibition alongside with BMP inhibition yielded a 94 ± 3.1% $CXCR4^{+}PDGFR\alpha$

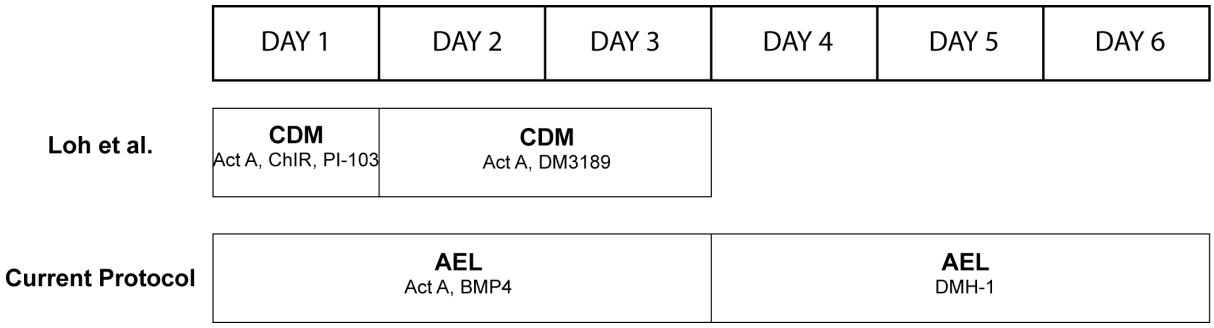

Figure 3.2. *Timeline of Loh et al. (2014) and Current Protocol for the Induction of Definitive Endoderm. In the Loh et al. (2014) protocol, day 1 involves the addition of Activin A (100ng/ml), CHIR99021 (2uM) and PI-103 (50nM) in chemically defined medium (CDM). On day 2, media is removed and supplemented with Activin A (100ng/ml) and DM3189 (250nM). Current protocol: Day 1-3 animal component and serum free media (AEL) is supplemented with Activin A (100ng/ml) and BMP4 (1ng/ml). Day 4-6, media is then supplemented with DMH-1(1uM).*

definitive endoderm population by differentiation day 3. This protocol generated definitive endoderm expressing markers such as SOX17, FOXA1, FOXA2, CER1 and FXD8 whilst repressing mesoderm, neuroectoderm and extraembryonic endoderm formation.

In this chapter, we compared Loh et al.'s approach to a version of our existing protocol to induce definitive endoderm (Micallef et al., 2011). Using defined, serum free media developed in our laboratory (Ng et al., 2008), we utilised a H9 SOX17 mCherry^{+} reporter line in order to compare four variations on endoderm differentiation protocols by monitoring SOX17 expression. In addition, we modified our current protocol to include Dorsomorphin Homologue 1 (DMH-1) in place of Noggin. This synthetic, small molecule BMP inhibitor blocks signalling from the BMP type 1 receptor. Compared to other BMP inhibitors such as Noggin, the synthetic nature of DMH-1 eliminates variation between batches. Compared to other small molecular weight inhibitors, DMH-1 has a higher and more specific affinity for the BMP type1 receptor (Ao et al., 2012). Furthermore, we re-examined if the presence and concentration of Activin A was an important factor in the new adapted protocol.

3.2 Results

Utilising a H9 SOX17 mCherry^{+} reporter line, cells were analysed by flow cytometry, QPCR and in addition, images of mCherry expression were captured using a fluorescence microscope over the time course of 7 days. Cells were differentiated using the Loh et al. definitive endoderm protocol as well as using variations to the protocol in which the concentration of Activin A was varied. Differentiations were set up according to section 2.4.3, chapter 2, and cell analysis was performed every day over the 7 day differentiation.

In the differentiations completely lacking Activin A, little expression of SOX17 was detected by mCherry fluorescence over the 7 day time-course (figure 3.3a-g). This was further validated by flow cytometric analysis which showed that cells maintained EPCAM expression throughout the differentiation but failed to induce CXCR4 expression (figure 3.3h) over the 7 days. Furthermore, QPCR analysis showed that SOX17 expression was absent at day 4 and day 7, despite the expression of another endoderm marker, FOXA2, on day 7 (figure 3.3 j).

Next, we wanted to determine whether low concentrations (10ng/ml) of Activin A were sufficient to differentiate PSCs towards a definitive endoderm fate. During the first 2 days of differentiation, no substantial SOX17 or CXCR4 expression was observed (figure 3.4a, b, h). However by differentiation day 3 (figure 3.4c) areas of mCherry expression could be observed within the cultures, a result further validated by flow cytometry which showed a large fraction of cells co-expressing EPCAM and CXCR4. By day 4, most cells robustly expressed SOX17, EPCAM and CXCR4 and maintained their expression throughout the remainder of the differentiation. This

result was reflected by QPCR analysis, which showed that at day 4, strong expression of SOX17, CXCR4 and EPCAM was detectable. Interestingly, by day 7, a sudden decline of SOX17 expression (figure 3.4j) was observed as assessed by both QPCR and fluorescence microscopy (figure 3.4g). Although the reason for this is unclear, we noted that at this stage cultures contained a lot of dead cells and in some areas of the plates cells had completely detached.

Figure 3.5 shows the results of differentiating PSCs with protocol of Loh et al. that was adapted such that the base medium was our recombinant protein based differentiation medium AEL (Loh et al., 2014; Ng et al., 2008). In contrast to the protocols either lacking Activin A or using a low concentration of Activin A, following 48 hours of differentiation, robust induction of EPCAM and CXCR4 was observed (figure 3.5b). By day 3, majority of the population expressed high levels of EPCAM and CXCR4 (figure 3.5c). For the remainder of the differentiation, high expression of these two genes was maintained as well as SOX17 expression. This conclusion was supported the results of QPCR analysis of day 4 and 7 of cultures, which showed day 4 cells expressed high levels of SOX17, EPCAM and CXCR4. However at day 7, expression of these three definitive endodermal markers begins to diminish (figure 3.5 j,k).

Finally we examined the conditions used in our current protocol that was adapted for adherent cells rather that floating aggregates. Unlike the results observed with the Loh et al. protocol, only marginal induction of EPCAM and CXCR4 was observed at differentiation day 2 (figure 3.6b). By differentiation day 3, half of the population was found to be expressing SOX17, EPCAM and CXCR4. Following another 24 hours of differentiation, expression of definitive endoderm markers was similar to that of Loh

et al. protocol and expression was maintained throughout the rest of the differentiation. Interestingly, from day 5 to 6 of differentiation, a noticeable increase was observed in mCherry(SOX17) expression (figure 3.6 f,g); a result supported by QPCR analysis of SOX17 expression at day 4 and 7 (figure 3.6j). Similarly, the increase in SOX17 expression was accompanied by the increase in CXCR4 and EPCAM expression from day 4 to 7.

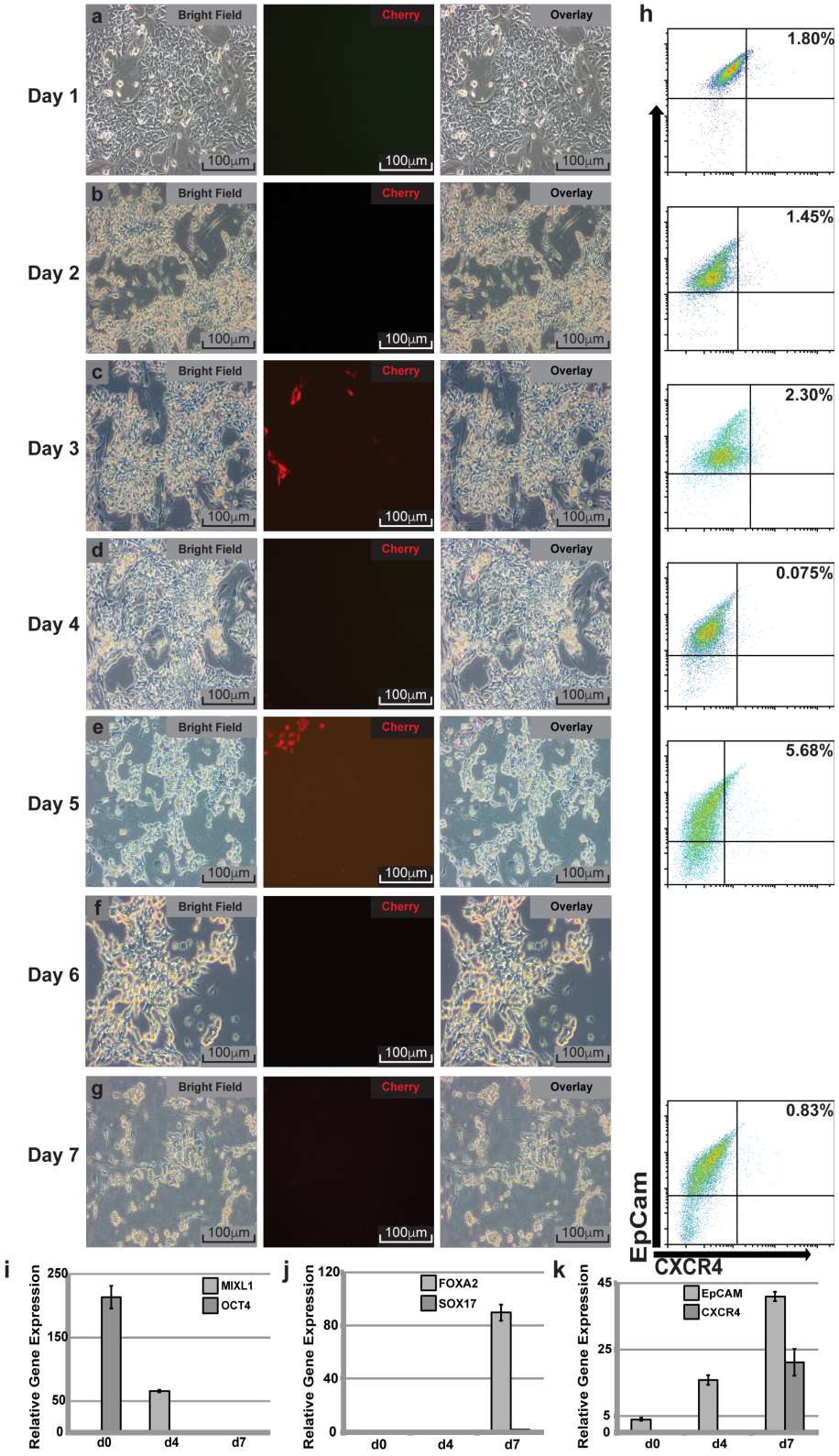

Figure3.3. *Time course of definitive endoderm differentiation using a version of the Loh et al. protocol lacking Activin A (Loh et al., 2014). During the 7 days of differentiation, little SOX17 expression was detected by fluorescence microscopy(a-g). Flow cytometric analysis of EPCAM vs CXCR4 expression over the course of the differentiation showed the cells remained EPCAM positive but CXCR4 negative throughout the time course (h). QPCR results for MIXL1; OCT4; FOXA2; SOX17; EPCAM; CXCR4 taken at day 0, 4 and 7 during differentiation indicate some differentiation did occur (loss of OCT4) and up-regulation of SOX17. Error bars represent SEM of 3 independent experiments. EpCAM refers to human EPCAM.*

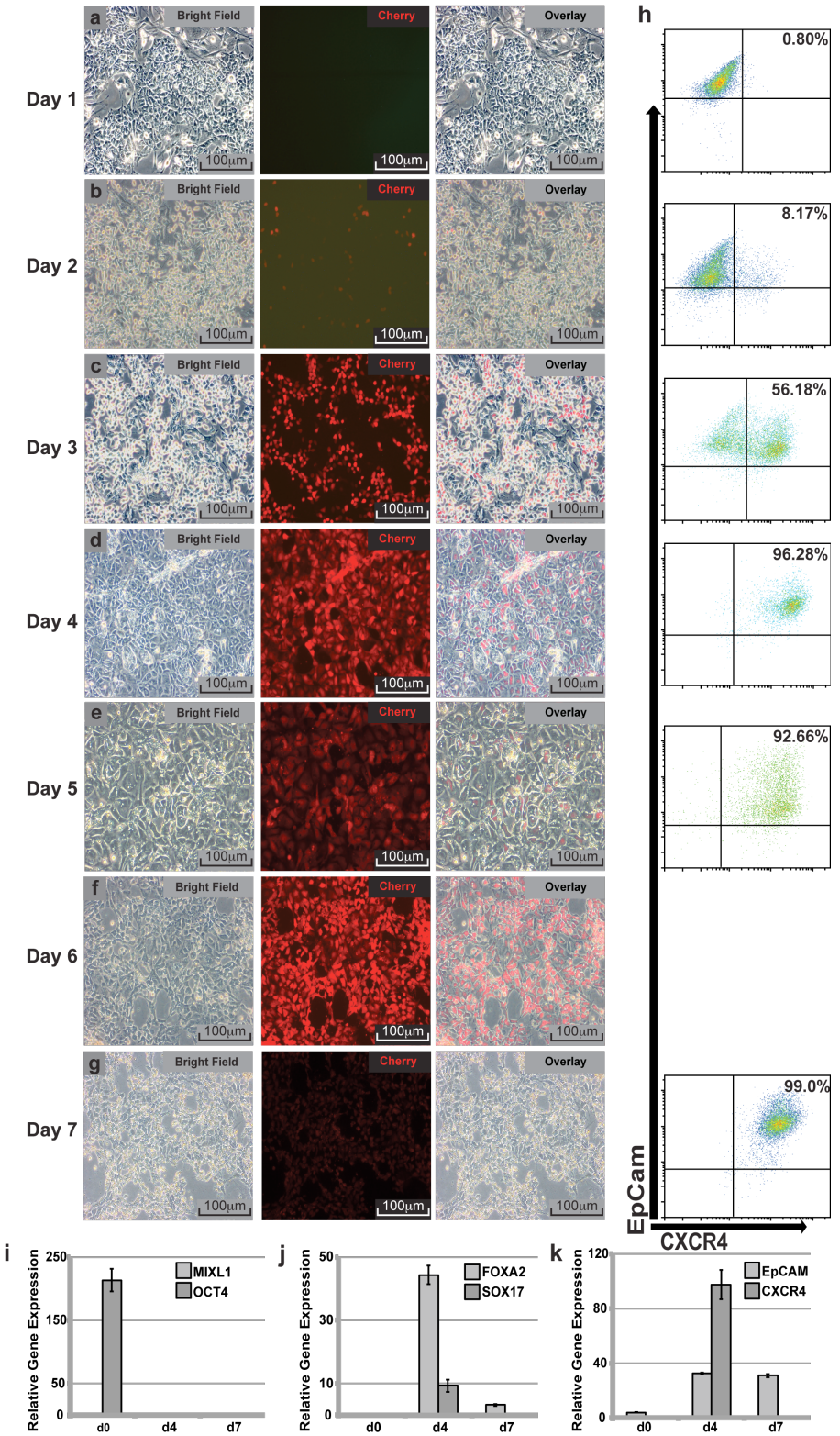

Figure 3.4. *Time course of definitive endoderm differentiation using a variation of the Loh et al., 2014 protocol that contained low levels of Activin A. SOX17 mCherry expression was observed over 7 days of differentiation and images were used using fluorescence microscopy. During the first 2 days of differentiation no SOX17 expression was detected (a,b) however by day 3, some cells robustly expressed mCherry (SOX17). Expression significantly increased by day 6 (d-f) but then rapidly declined by day 7 (g). (h) Flow cytometric analysis of EPCAM vs CXCR4 expression over the course of differentiation. (i-k) QPCR results of MIXL1; OCT4; FOXA2; SOX17; EPCAM; CXCR4 taken at day 0,4 and 7 during differentiation. Error bars represent SEM of 3 independent experiments. EpCAM refers to human EPCAM.*

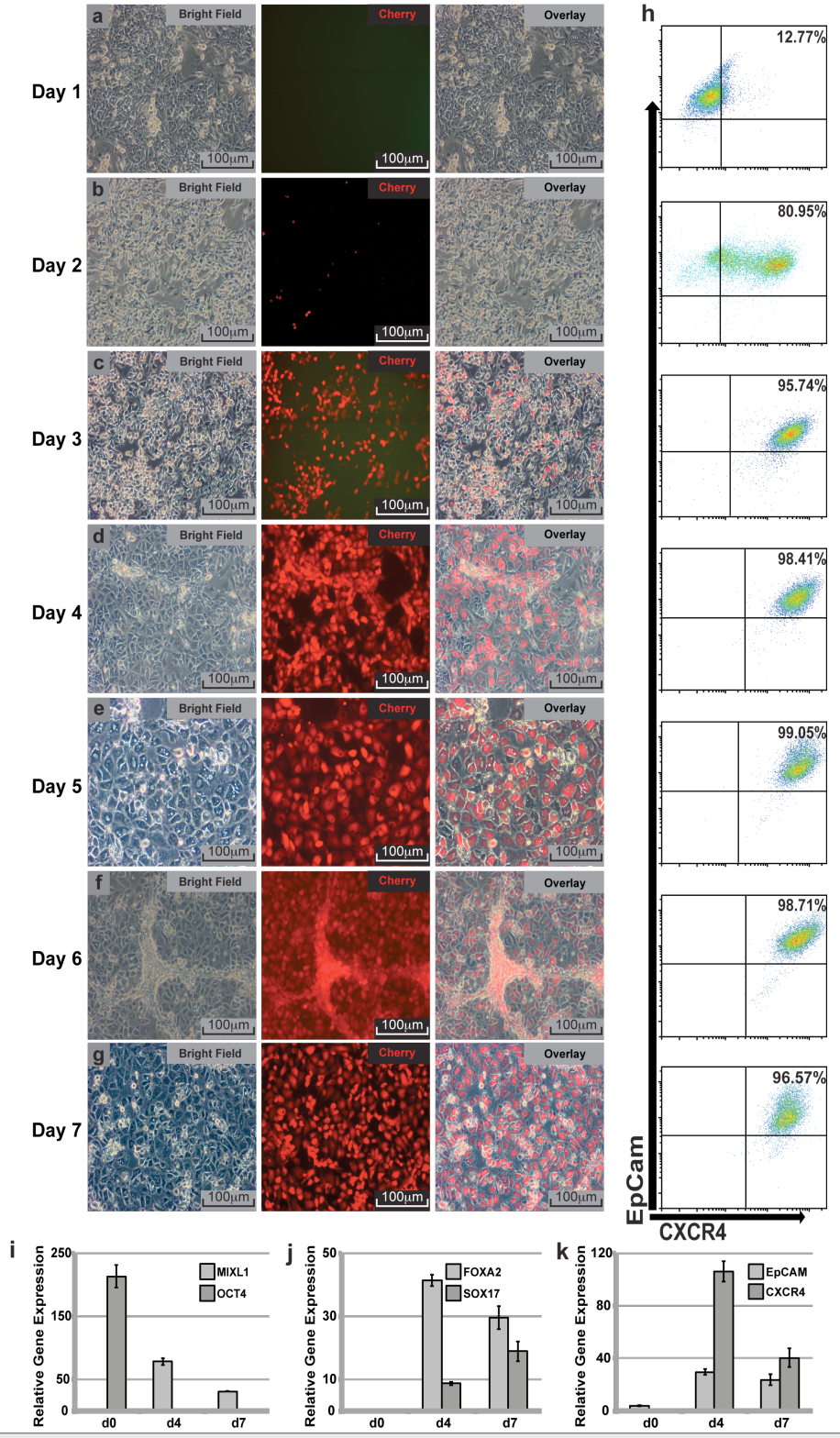

Figure 3.5. *Time course of Definitive Endoderm Differentiation Using Loh et al., 2014 Protocol. SOX17 mCherry expression was observed over 7 days of differentiation and images were captured using fluorescence microscopy. SOX17 expression was undetected on day 1 (a) however by day 2, few cells were expressing mCherry(SOX17) (b). By day 3 (c), areas of SOX17 expression were detected and expression significantly increased by day 4 (d). Expression plateaued and was maintained from day 5-7 (e-g). (h) Flow cytometric analysis of EPCAM vs CXCR4 expression over the course of differentiation. (i-k) QPCR results of MIXL1; OCT4; FOXA2; SOX17; EPCAM; CXCR4 taken at day 0, 4 and 7 during differentiation. Error bars represent SEM of 3 independent experiments. EpCAM refers to human EPCAM.*

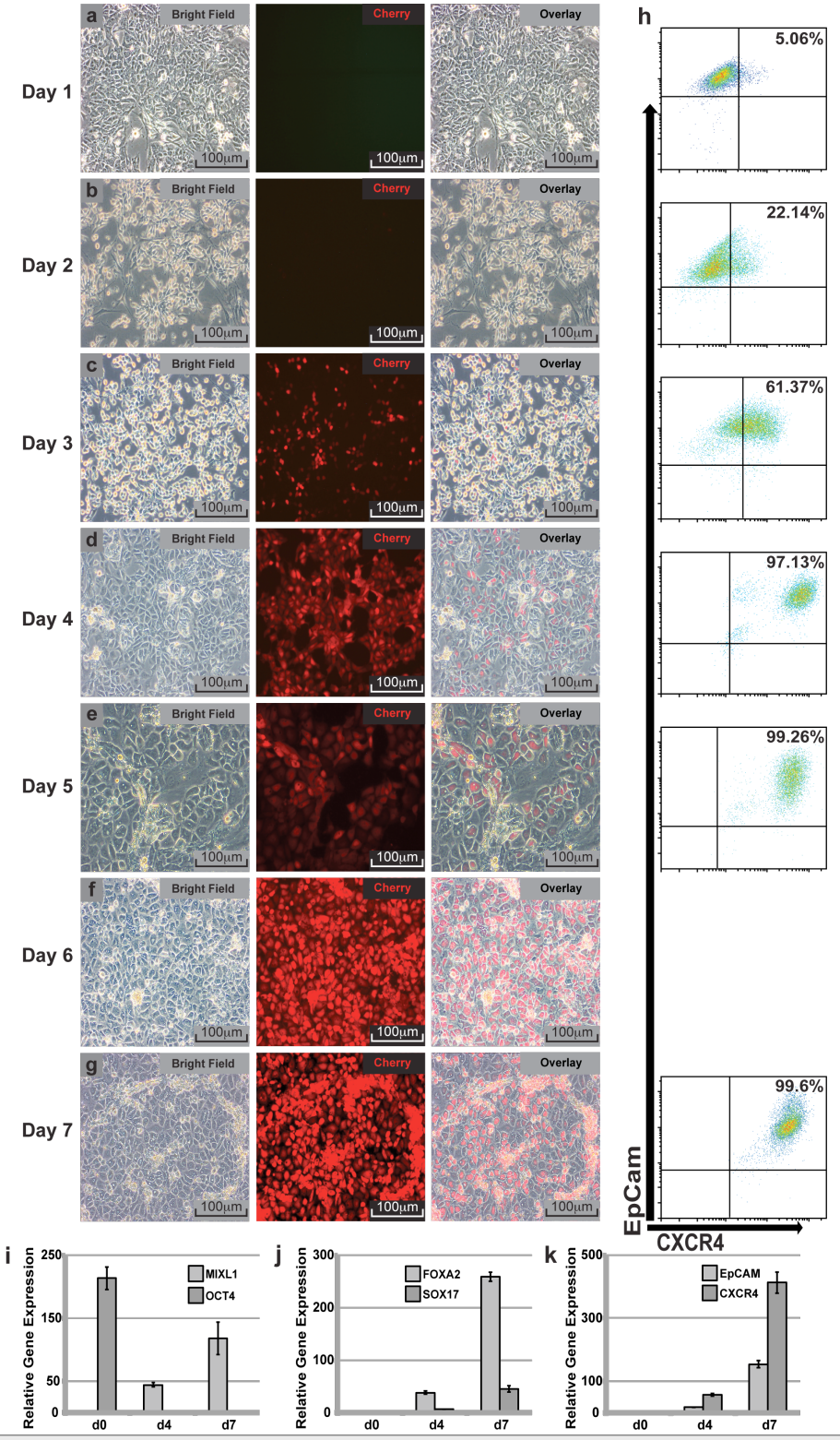

Figure 3.6. *Time course of Definitive Endoderm Differentiation Using Current Differentiation Protocol. SOX17 mCherry expression was observed over 7 days of differentiation and images were used using fluorescence microscopy. During the first 2 days of differentiation no SOX17 expression was detected (a,b) however by day 3, some cells were shown to be robustly expressing mCherry (SOX17). Expression significantly increased by day 6 (d-f) and was maintained at day 7 (g). (h) Flow cytometric analysis of EPCAM vs CXCR4 expression over the course of differentiation. (i-k) QPCR results of MIXL1; OCT4; FOXA2; SOX17; EPCAM; CXCR4 taken at day 0,4 and 7 during differentiation. Error bars represent SEM of 3 independent experiments. EpCAM refers to human EPCAM.*

3.3 Discussion

Our comparison of a number of differentiation methods indicated that a protocol based on the definitive endoderm differentiation method published by Loh et al. could be successfully implemented in our laboratory (Loh et al., 2014). This method generally yielded approximately 90-95% of EPCAM+ and CXCR4+ cells by day 3 and maintained high levels of expression following 7 days of differentiation. Compared to our current protocol which involved the use of Activin A and BMP4 for the first 3 days, the new adapted protocol utilised a combination of Activin A, the Wnt agonist Chir 99021 and a PI-103 Kinase Inhibitor. This combination of factors yielded approximately 80% of EPCAM+CXCR4+ cells following 48 hours of differentiation as opposed to 20% with our current protocol. In the Loh et al. method, after 24 hours, media was supplemented with Activin A and DMH1. This resulted in an increase in definitive endoderm markers, yielding 90-95% EPCAM+CXCR4+ cell population by day 3. At this stage, in the current protocol, a media change supplemented with DMH-1 for further definitive endoderm differentiation would only be introduced on day 3. Therefore by comparing EPCAM+CXCR4+ populations, our current protocol generated only 60% EPCAM+CXCR4+ cells on day 3 (as opposed to 95%) and only after a further 24 hours, the population of EPCAM+CXCR4+ cells was similar to that of the Loh et al. protocol.

QPCR analysis was also performed on day 4 and day 7 differentiated cells. Cells were assayed for the expression of the endoderm markers FOXA2, SOX17, CXCR4 and EPCAM. We found that in the Loh et al. protocol, at day 4, expression of all definitive endoderm markers were present with a greater expression of FOXA2 and CXCR4 compared to day 7. In contrast, despite having all definitive endoderm markers present at day 4, at day 7, expression of FOXA2 and CXCR4 was greater

than that of day 4. This result suggested that the Loh et al. protocol elicited the induction of definitive endoderm earlier than that of our current protocol, potentially enabling us to begin pancreatic endoderm differentiation by day 3 or 4 as opposed to day 6. In turn, we expected that this more rapid differentiation method would decrease asynchrony in the cultures and thus improve the overall efficiency of pancreatic differentiation.

In this chapter we also examined the role of Activin A in definitive endoderm differentiation. We tested two variations to the Loh et al. protocol, one being the absence of Activin A and the other using Activin A at a lower concentration (10ng/ml). Cells differentiated in the absence of Activin A maintained EPCAM expression over course of the 7 days but did not express CXCR4 (figure 3.3 h). This was confirmed by QPCR as EPCAM expression demonstrated a steady increase from day 0 time point to the day 7 time point (figure 3.3 k). Interestingly, FOXA2 expression was detected at day 7 (figure 3.3 j). It is unclear if this derived from a small population of endoderm cells or whether it signified the emergence of other non-endodermal cell types within the cultures.

In the presence of low concentrations of Activin A, the first two days of differentiation did not yield a substantial EPCAM+CXCR4+ cell population. However by day 3, mCherry (SOX17) expression was detected and this correlated with an increased proportion of cells expressing EPCAM and CXCR4 (56.18%) (figure 3.4, h). Despite the relatively slow differentiation during the initial stages, induction of mCherry expression substantially increased from day 2 to 3 (8.17% to 56.18%, respectively) and then further from day 3 to 4 (56.18% to 96.28%). Furthermore, EPCAM, CXCR4 and SOX17 expression was seen to be maintained from day 4 to day 6 yielding

approximately 90-95% EPCAM+CXCR4+ population. However, by day 7 mCherry expression notably diminished (figure 3.4 g) and QPCR analysis showed that SOX17, FOXA2 and EPCAM expression also reduced. Nevertheless, cell surface expression of CXCR4 and EPCAM assayed by flow cytometry was still robust at this stage. A possible explanation for this is the discrepancy is that reduction in the levels of CXCR4 and EPCAM mRNAs precedes that of proteins.

Overall, analysis of day 3 flow cytometry data showed that the method of Loh et al (2014) generated a significantly highly frequency of EPCAM+CXCR4+ cells (n=3, $p<0.0001$, see appendix 2) than any of the other conditions. This rapid induction of definitive endoderm is consistent with that observed by Loh et al (2014) in their original description of this method. Therefore, the results presented in this chapter show that we could successfully implement a more rapid definitive endoderm differentiation protocol. In the next chapters we used this protocol to examine the effect of conditionally expressing two key transcription factors required for differentiating PSCs towards a pancreatic fate.

3.4 References

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Chapter 4

Tet-On Inducible Expression System

4

4.1 Introduction

Protocols for the generation of pancreatic cells in vitro seek to recapitulate important signalling events required for embryonic pancreas development. By doing this, these methods aim to sequentially induce and repress sets of genes which make key stages of pancreatic ontogeny. As previously mentioned, two key transcription factors involved in the development of the pancreas is *PDX1* and *NKX6.1*. Of late, studies have demonstrated that these genes work in parallel to each other with their co-expression leading to the generation of a subset of progenitor cells which eventually give rise to functional pancreatic endocrine cells, namely β cells.

We sought to explore how the forced expression of these two factors, either individually or in combination, would affect differentiation towards pancreatic endoderm. To achieve this, we utilised a Tet-on system, in which expression of genes of interest can be regulated by doxycycline (dox). In the Tet-on system, transgenes remain silent until the dox is added to the culture medium. Using such a system would thus allow us to test how the timing of PDX1 or NKX6.1 expression influenced the course of pancreatic differentiation in vitro.

Several studies have used the Tet-on system as a “gene switch” in transgenic murine models. (Lottmann et al., 2001) examined the role of *Pdx1* during pancreatic endocrine development by generating a transgenic mouse in which the Tet regulator was expressed under the control of mouse insulin gene II. This mouse was crossed to a second mouse strain that contained a transgene in which a *Pdx1* anti-sense cDNA was located downstream of a Tet operator-promoter. In this system, addition of dox lead to expression of the antisense RNA which in turn inhibited translation of the

endogenous *Pdx1* protein. In the absence of dox, antisense RNA was virtually undetectable. However, significant induction was found after 24 hours in pancreatic islets (where insulin 2 is expressed) following administering of dox. Conversely, once dox was removed, antisense RNA was silenced. These experiments demonstrated that the down regulation of *Pdx1* compromised glucose tolerance, attesting to the essential role *PDX1* plays in β cell function. In addition, they also established that the Tet-on system offers the possibility of developing disease models in which genes of interest can be down-regulated in a temporally controlled manner, enabling the study of gene function at different developmental stages.

Instead of using the tet system to down-regulate a gene of interest during pancreatic development, (Cai et al., 2012), used this system to over-express it. Their study examined the co-ordinated development of islets and their associated vessels, whereby vascularisation allows β cells to quickly sense and respond to changes in blood glucose by secreting insulin. Their mouse model used the rat insulin promoter (RIP) to drive the β cell specific expression of the reverse tetracycline activator. This mouse was crossed to a second strain in which vascular endothelial growth factor was placed under the control of the tet responsive promoter. The study found that over-expressing VEGF-A throughout development to post-natal period progressively worsened islet formation. Furthermore, dox-induced over-expression of VEGF-A during islet development increased endothelial cell proliferation and vascularisation near insulin-positive cells whilst reducing β cell proliferation and mass, perturbing islet morphology. This alteration of islet morphology was even seen during brief periods of VEGF-A induction. Thus, applying use of the Tet-On induction system allowed Cai and colleagues to understand the role of VEGF-A during embryonic islet development and postnatal life.

The tetracycline inducible gene expression system has also be integrated into PSCs. (Zhou et al., 2006), introduced a lentiviral vector system for regulated transgene expression. In these experiments, PSCs were transduced with a tTs-expression vector (tTS is a version of the rtTA) and another lentiviral vector containing the transgene downstream of a promoter controlled by a high-affinity Tet-operator binding site. Within their system, the gene of interest remained inactive in the absence of dox. However with addition of dox, the tTS became active and initiated transcription from the promoter-Tet operator transgene. The key finding from this and later studies was that stable transgenic PSC lines harbouring the Tet-On induction system retained their pluripotency, normal karyotype, marker expressions and their differentiation potential (Szulc et al., 2006; Xia et al., 2008).

In this chapter we generated transgenic PSC lines in which PDX1 and NKX6.1 were placed under the control of the Tet-On regulatory system using the *GTi* expression vector system shown in figure 4.1. In experiments performed in our laboratory, we demonstrated dox dependent induction of GFP during PSC differentiation in vitro. The system works by targeting the expression vector to the ubiquitously expressed GAPDH locus using homologous recombination. In this vector, the rtTA is expressed from a T2A sequence fused in frame with the GAPDH coding sequences. The Dox responsive promoter is located 3' of the selectable marker, S (figure 4.1). Thus, this system had the added attraction that both the rtTA and TetO sequences were contained within a single vector and therefore only one targeting procedure was required to make transgenic lines. As described in Chapter 2, we replaced the GFP sequences with either the PDX1 or PDX1 and NKX6.1 coding sequences and subsequently generated and tested a number PSC lines harbouring these transgenes.

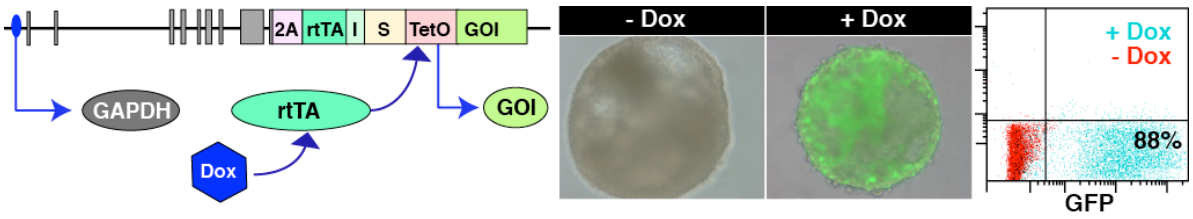

Figure 4.1. *GTi, an inducible system for expressing genes in pluripotent stem cells and their differentiated progeny. A schematic representation of the vector and it's key components is shown. 2A, peptide sequence allowing the reverse tetracycline activator (rTA) to be translated from the GAPDH transcript. I, internal ribosomal entry site enabling the selectable maker (S) to be translated from the GAPDH transcript. TetO, the tetracycline responsive promoter driving expression of the gene of interest (GOI). The middle two panels show differentiating PSCs (as embryoid bodies) that harbor a GTi-GFP vector in one GAPDH allele. The pictures show embryoid bodies formed in the absence or presence of 1 microgram/ml doxycycline. The right panel shows the results of flow cytometry analysis of GTi-GFP PSCs differentiated in the presence of absence of doxycycline. Figure courtesy of Ali Motazedian*

4.2 Results

The dox inducible PDX1 construct shown in figure 4.2 (a) was electroporated into RM 3.5 iPS cells and targeted clones were identified using a PCR based screening strategy (see section 2.3, chapter 2). Three correctly target clones were chosen for further analysis. We first tested induction of the dox responsive transgene in undifferentiated cells by treating cultures with dox for 48 hours. We then performed intracellular flow cytometry to examine expression of Flag epitope which we had incorporated into the c-terminus of the PDX1 protein. This analysis showed a robust induction of the Flag-tagged PDX1 in the iPS PDX1 cell line in the presence of dox

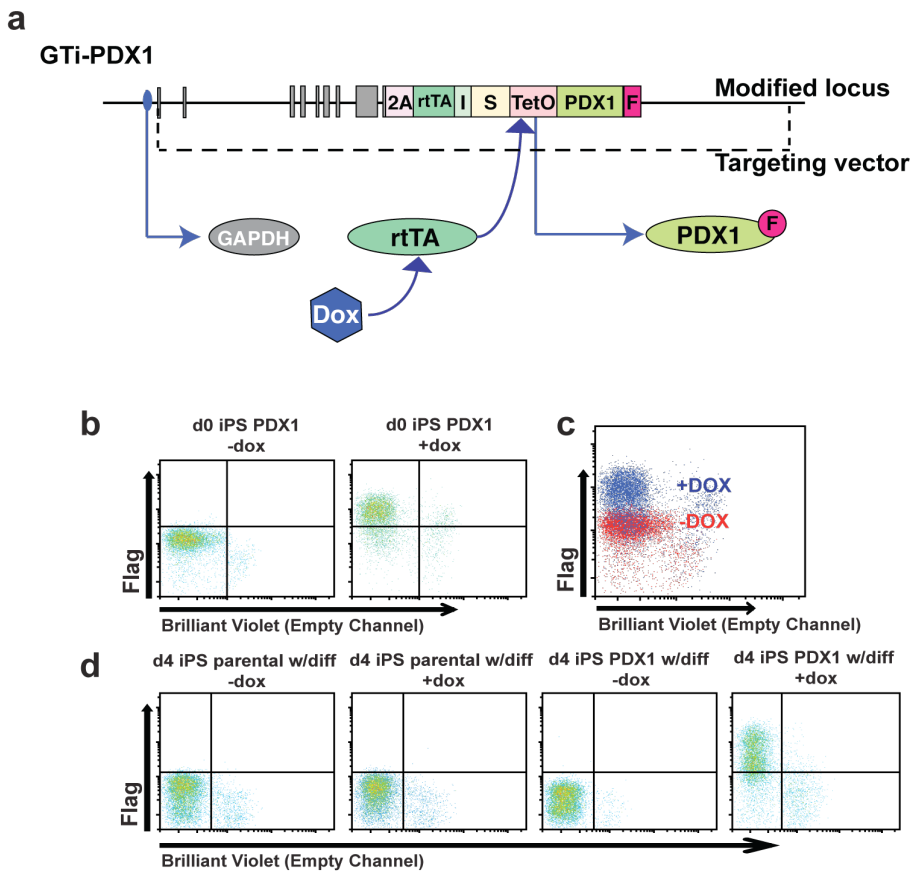

***Figure 4.2**. Analysis of iPS cells containing a dox inducible PDX1 transgene. (a) Schematic representation of the PDX1 expression construct (GTi-PDX1) and it's key components (as described in figure 4.1). Addition of dox, allows the rtTA to bind TetO-promoter sequences and initiate transcription of PDX1-flag. (b) Flow cytometric analysis undifferentiated iPS PDX1 cells (day 0) before and after induction with dox. (c) Overlay of the data in (b) showing the relative shift in PDX1-flag expression following addition of dox. (d) Flow cytometric analysis of PDX1-flag expression at differentiation day 4 (d4) in iPS Parental line and iPS PDX1 lines with and without dox treatment.*

(figure 4.2b). Next, we tested whether the PDX1 transgene was also able to be induced during differentiation. Utilising the definitive endoderm protocol (chapter 3), we observed that in the presence of dox, robust induction of Flag (PDX1) expression was detected, suggesting that transgene remained dox responsive during differentiation (figure 4.2). In addition to the above analyses, we also confirmed dox induction of the PDX1-flag transgene using immunofluorescence with an anti-flag antibody (Figure 4.3) and an anti-PDX1 antibody (Figure 4.4). Collectively, these experiments indicated that the PDX1-flag transgene could be induced with dox both in undifferentiated cells and during the initial stages of endoderm differentiation.

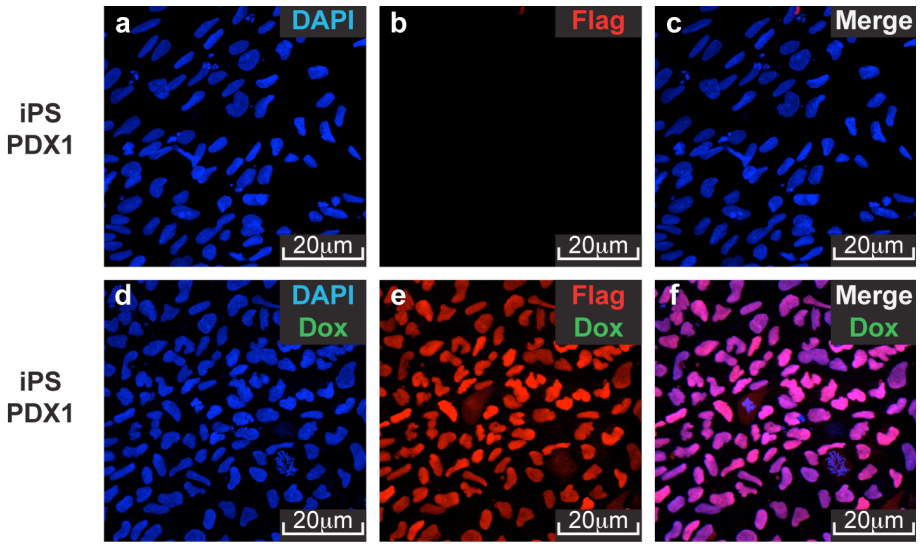

***Figure 4.3**. Immunofluorescence analysis of PSCs expressing a transgene for dox inducible PDX1. Undifferentiated cells were labelled with an APC conjugated anti-Flag (PDX1) antibody and images captured using confocal microscopy. In the absence of dox, iPS-PDX1 cells showed no flag expression (a-c). In contrast, dox treated iPS-PDX1 cells displayed intense nuclear staining with the anti-flag antibody following dox treatment, confirming robust induction of the PDX1 transgene (d-f).*

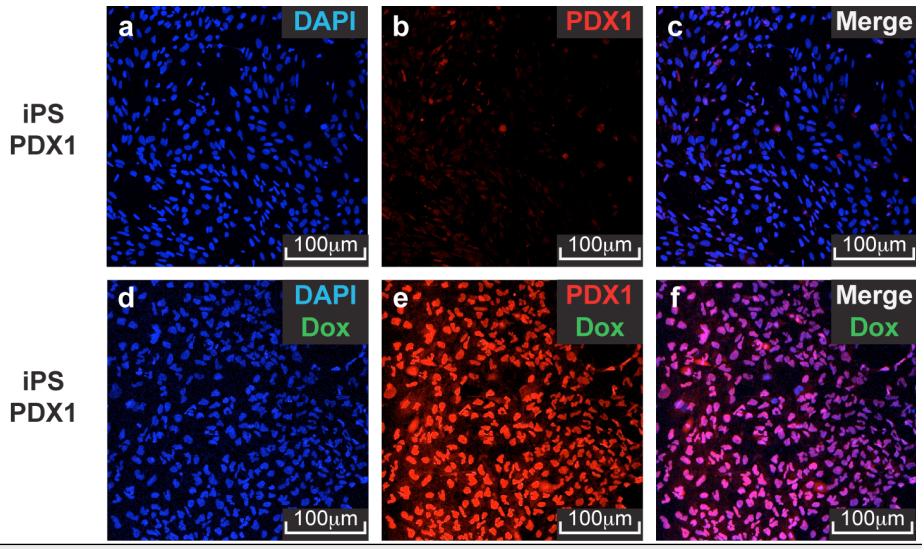

***Figure 4.4**. Immunofluorescence analysis of PSCs expressing a transgene for dox inducible PDX1. Undifferentiated cells were labelled with a primary unconjugated anti-PDX1 antibody and then subsequently with a secondary alexafluor 568 antibody. Images captured using confocal microscopy. In the absence of dox, iPS-PDX1 cells showed low levels of labelling with the anti-PDX1 antibody although it is unclear if this represents specific PDX1 staining (a-c). However, dox treated iPS-PDX1 cells displayed intense nuclear staining with the anti-PDX1 antibody (d-f), supporting observations made using the anti-flag antibody and flow cytometry.*

In addition to generating the iPS PDX1 transgenic cell line, we also constructed a double transgene vector designed to co-express PDX1 and NKX6.1. The construct is shown below in figure 4.5a. In this case, we engineered the NKX6.1 protein to incorporate the flag epitope tag at its c-terminus. This vector was also electroporated into RM 3.5 iPS cell line and targeted clones were again identified using a PCR based screening strategy (section 2.3, chapter 2). As was performed for the iPS PDX1 cell line described above, we used intracellular FACs on undifferentiated cells exposed to dox for 48 hours to observe the expression of Flag in lines carrying the PDX1-NKX6.1 transgene. Unlike the iPS PDX1 cell line (figure 4.5c), dox treatment produced only a very minor shift in population of cells expressing the flag epitope (figure 4.5d,e). We also examined the induction process using Q-PCR, looking for expression of both PDX1 and NXK6.1. This analysis gave results that were not entirely consistent with those obtained by flow cytometry, with PCR analysis showing

that the level of induction was only two fold less than that obtained with single PDX1 transgenic line (figure 4.5f). However, when cells were differentiated to endoderm, the level of induction in the iPS-PDX1-NKX6.1 cell line was substantially lower again, leading us to conclude that this transgene was undergoing silencing (data not shown).

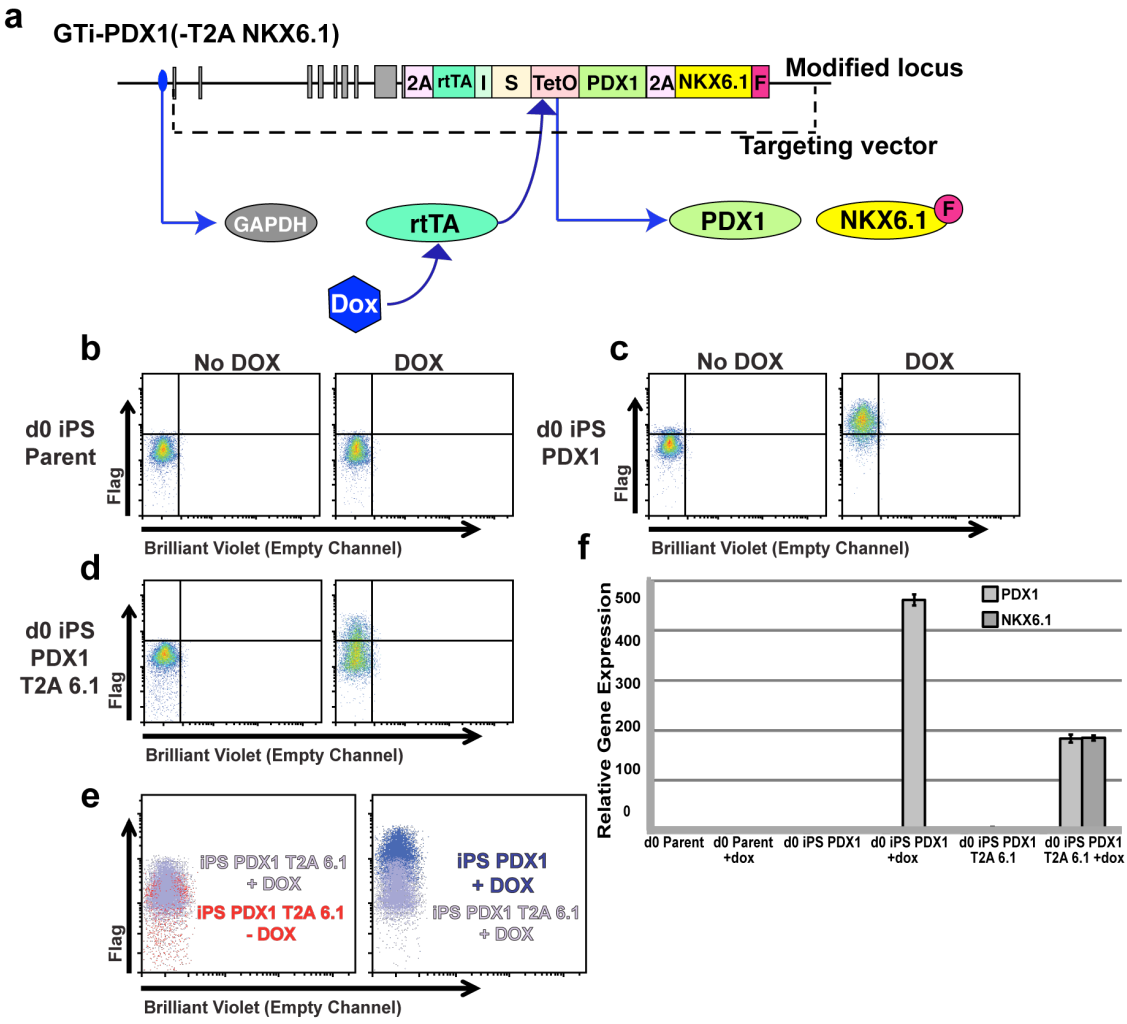

Figure 4.5*. Analysis of iPS cells containing a dox inducible PDX1-NKX6.1 flag transgene. (a) Schematic representation of the PDX1 expression construct (GTi-PDX1-T2A-NKX6.1) and it's key components (as described in figure 4.1). This transgene produces PDX1 and NKX6.1-flag as separate proteins. (b) Flow cytometric analysis of undifferentiated (day 0, d0) dox untreated and treated iPS Parental (b) iPS PDX1-flag (c), and iPS PDX1-T2A-NKX6.1 cells (d). (e) Overlay comparing dox induction in day 0 iPS PDX1-T2A-6.1 cells with the iPS PDX1-flag line demonstrating the relatively poor induction of the transgene in the former. (f) Results of QPCR analysis showing a comparison of PDX1 and NKX6.1 gene expression in iPS Parental, iPS-PDX1 and iPS PDX1-t2A-6.1 line in and without the presence of dox. Error bars represent SEM from 3 independent experiments.*

In addition to flow cytometry analysis, we performed immunofluorescence analysis on undifferentiated PSCs representing the single and double transgenic lines. Cells were seeded onto 8-well chamber slides and were exposed to dox for 48 hours. Similarly to that described above for the iPS-PDX1 cells, iPS PDX1 T2A 6.1 cells were labeled with APC conjugated anti-Flag antibody, an anti-PDX1 antibody and anti-NKX6.1 antibody. No expression of Flag and low levels of PDX1 was detected in the absence of dox (figure 4.6b and figure 4.7b, respectively). In addition, no expression of NKX6.1 was detected without the presence of dox (figure 4.8b). Contrastingly, in the presence of dox, robust nuclear staining was observed with all three antibodies (panel e in figures 4.6, 4.7 and 4.8). Taking into account the flow cytometry analysis, Q-PCR data and immunofluorescence analysis, we concluded that dox efficiently induced expression of both PDX1 and NKX6.1 in the iPS-PDX1-T2A-NKX6.1 transgenic cell line in undifferentiated cells. However, we found that dox failed to reliably induce expression of PDX1 and NKX6.1 when these cells were differentiated (data not shown). Tellingly, we also observed that over time in culture, this line

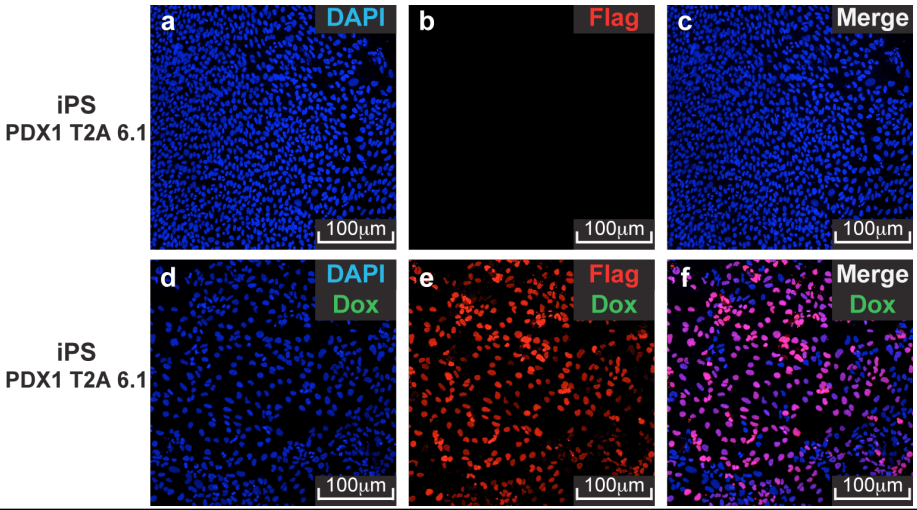

***Figure 4.6**. Immunofluorescence analysis of PSCs expressing a transgene for dox inducible PDX1 T2A NKX6.1(-6.1). Undifferentiated cells were labelled with an APC conjugated anti-Flag (PDX1) antibody and images captured using confocal microscopy. In the absence of dox, iPS-PDX1 T2A 6.1 cells showed no flag expression (a-c). In contrast, dox treated iPS-PDX1 cells displayed varying intensities of nuclear staining with the anti-flag antibody following dox treatment (d-f)*

gradually lost its capacity for induction. Because of this we decided to only conduct further experiments with the iPS-PDX1 lines.

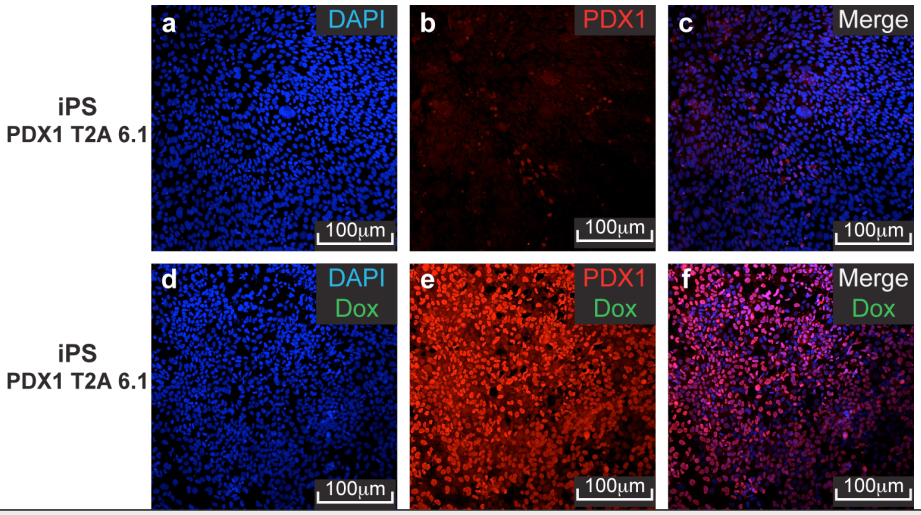

***Figure 4.7**. Immunofluorescence analysis of PSCs expressing a transgene for dox inducible PDX1. Undifferentiated cells were labelled with a primary unconjugated anti-PDX1 antibody and a secondary alexa fluor 568 antibody, images captured using confocal microscopy. In the absence of dox, iPS-PDX1 T2A 6.1 cells showed low levels of PDX1 expression (a-c). However, dox treated iPS-PDX1 cells displayed areas of intense nuclear staining with the anti-PDX1 antibody following dox treatment, validating induction of the PDX1 T2A 6.1 transgene (d-f).*

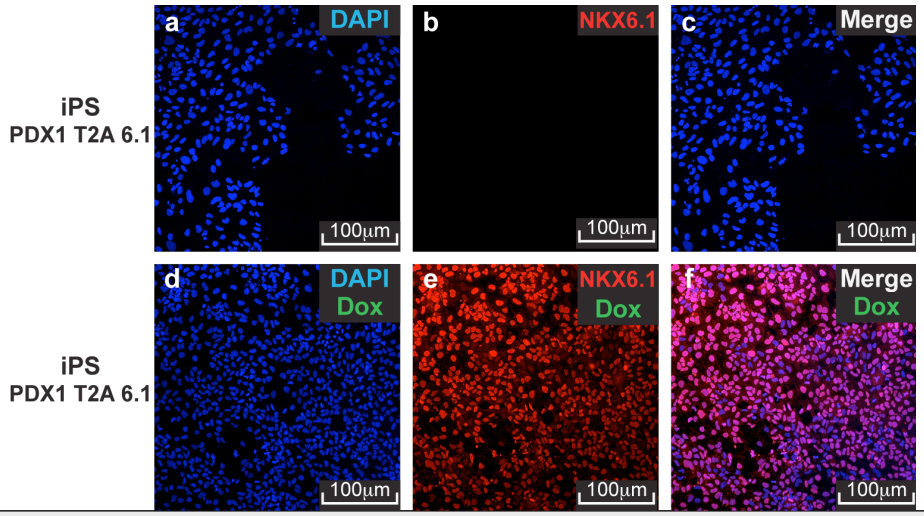

***Figure 4.8**. Immunofluorescence analysis of PSCs expressing a transgene for dox inducible PDX1. Undifferentiated cells were labelled with a primary unconjugated anti-NKX6.1 antibody and a secondary alexa fluor 568 antibody, images captured using confocal microscopy. In the absence of dox, iPS-PDX1 T2A 6.1 cells showed no NKX6.1 expression (a-c). Although, dox treated iPS-PDX1 T2A 6.1 cells displayed areas of varying intensities of nuclear staining with the anti-NKX6.1 antibody following dox treatment, validating induction of the PDX1 T2A 6.1 transgene (d-f).*

We tested the affect of PDX1 mis-expression on definitive endoderm differentiation. We differentiated cells for 4 days in the absence and presence of dox and analysed

cells by flow cytometry and QPCR at day 4 (figure 4.9a). In the absence of dox, both the parental iPS cell line and the iPS-PDX1 cell line differentiated well, with over 95% of cells co-expressing the definitive endoderm markers EPCAM and CXCR4 (figure 4.9b). However, in the presence of dox, CXCR4 expression was severely

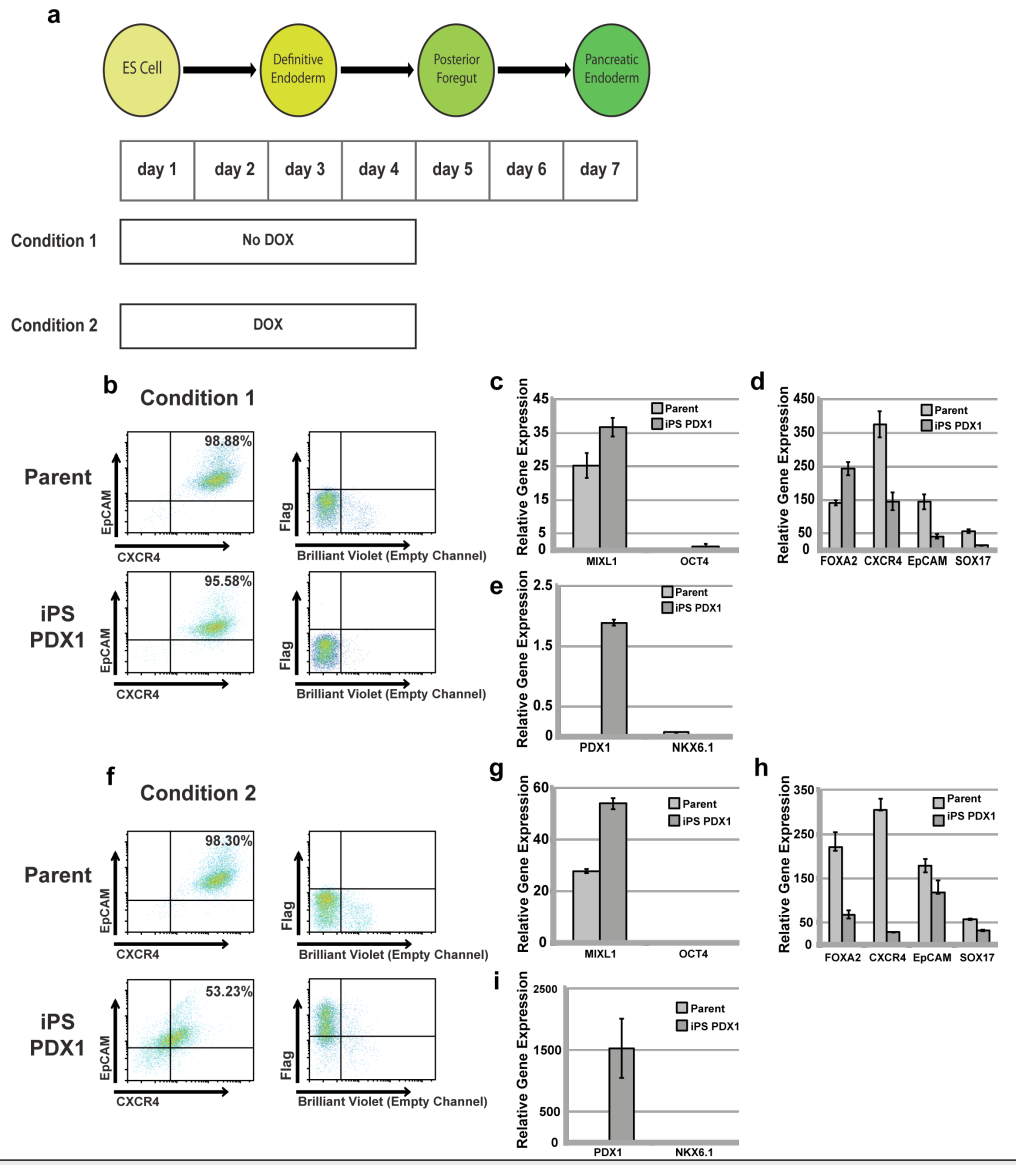

***Figure 4.9**. Analysis of untreated (condition 1) and dox treated (condition 2) iPS Parent and iPS PDX1 cells at differentiation day 4. (a) Schematic representation of timeline and conditions. (b) Flow cytometry analysis of CXCR4 and EPCAM and PDX1-flag expression on iPS Parent and iPS PDX1 transgenic lines in the absence of dox treatment. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 4 following differentiation in condition 1. (f) Flow cytometry analysis of CXCR4 and EPCAM and PDX1-flag expression on iPS Parent and iPS PDX1 transgenic lines treated for 4 days with dox (g-i) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 4 following differentiation in condition 2. (error bars represent SEM from 3 independent experiments). Student t-test indicates the mean expression of CXCR4 was reduced in DOX treated iPS PDX1 cells at differentiation day 4 ($p<0.01$, see table 3.5, appendix 2). EpCAM refers to human EPCAM.*

compromised in the iPS-PDX1 cell line, pointing to a disruptive effect exerted by the dox induced transgene (figure 4.9, f). This was supported by the QPCR results which showed that CXCR4 expression is noticeably less with the activation of the transgene compared to the parental line (figure 4.9, h). Furthermore, the early endoderm marker FOXA2 was also reduced in day 4 cells in which the PDX1 transgene had been induced with dox (compare figures 4.9d and h). Other markers analysed, including, OCT4, the mesendodermal marker MIXL1 the early endoderm marker SOX17 did not appear to be affected by PDX1 induction.

We next examined the effect of inducing PDX1 expression at varying times over 7 days of the pancreatic differentiation protocol. Cells were analysed by flow cytometry and QPCR at day 7. In agreement with the above results, flow cytometry analysis showed that induction of the transgene from day 1-4, retarded endoderm differentiation (figure 4.10, b), as measured by the frequency of cells co-expressing CXCR4 and EpCAM. In addition, this analysis showed that the removal of dox at differentiation day 4 resulted in the absence of Flag containing protein by day 7. These conclusions were again supported by QPCR analysis which showed *PDX1* expression in iPS-PDX1 cells was substantially reduced 3 days after dox removal (figure 4.10, e)

In contrast to the above results, flow cytometry analysis revealed that dox induction of the PDX1 transgene from day 4-7 yielded robust expression of the PDX1-flag protein but only marginally effected EPCAM and CXCR4 expression (figure 4.11b). The absence of an effect on the frequency of cells co-expressing CXCR4 and EPCAM most likely reflects the fact that endoderm induction is almost fully completed by differentiation day 4, prior to induction of the transgene. In contrast, cells treated

for 7 days with dox during differentiation resembled those exposed to dox from 1-4, with CXCR4 expression substantially repressed (Figure 4.11f and h). Under these conditions, FOXA2 expression was even further reduced, again suggesting endoderm formation had been severely compromised.

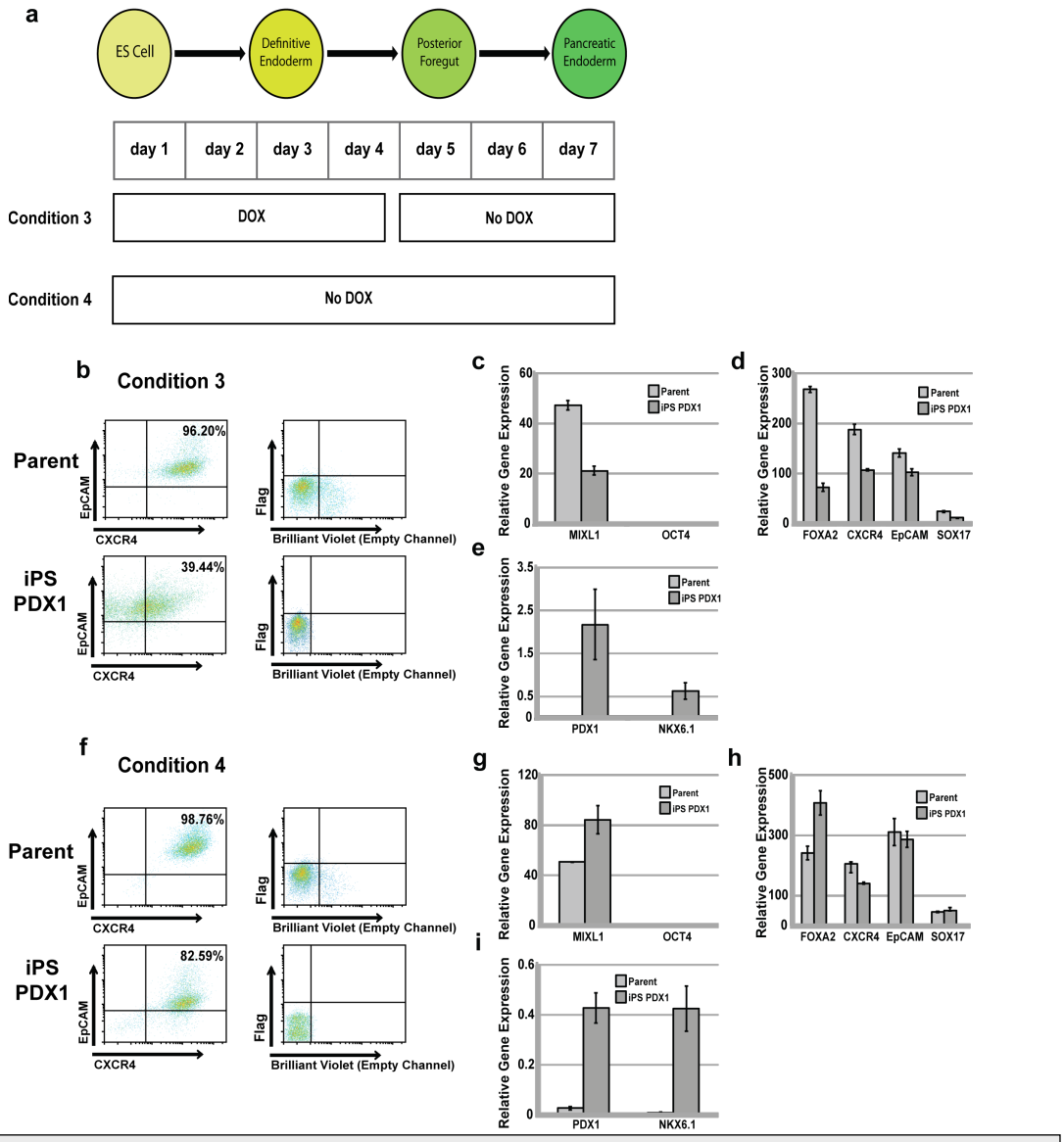

***Figure 4.10**. Day 7 Analysis of Condition 3 and 4 treatment of iPS Parent and iPS PDX1. (a) Schematic representation of timeline and conditions. Figure represents no. of days of treatment exposure during differentiation. (b) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 3. (f) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (g-i) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 4. (error bars represent SEM from 3 independent experiments). Student t-test indicates the mean expression of CXCR4 was reduced in dox treated iPS-PDX1 cells at differentiation day 7 ($p<0.01$, see table 3.6, appendix 2). EpCAM refers to human EPCAM.*

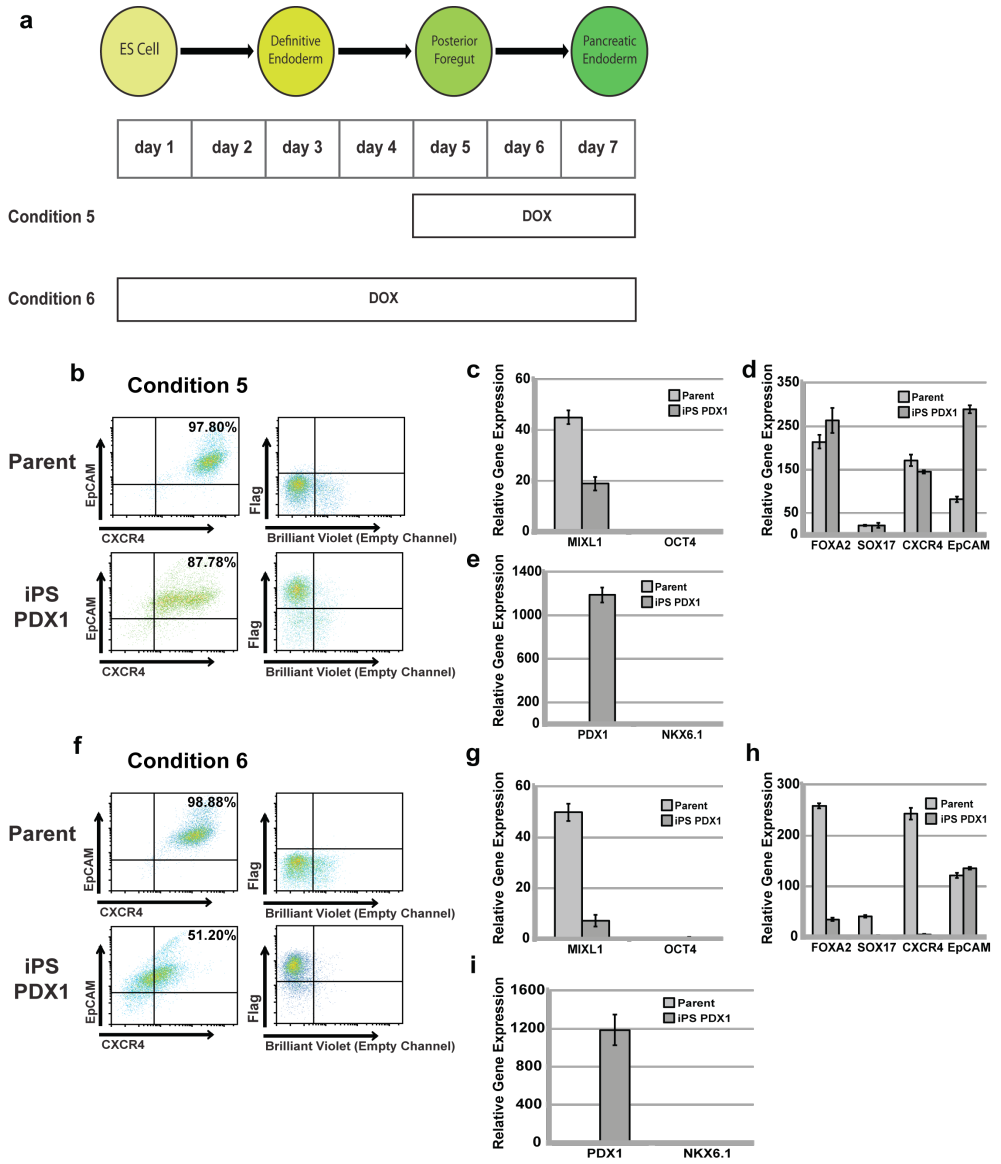

Figure 4.11. *Analysis of iPS Parent and iPS PDX1 cells treated with dox from day 4onwards (Condition 5) or for 7 days (Condition 6). (a) Schematic representation of timeline and conditions. (b) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic lines of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 5. (f) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (g-i) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 6. (error bars represent SEM from 3 independent experiments). Student t-test indicates the mean expression of CXCR4 was significantly reduced in dox treated iPSC-PDX1 cells at differentiation day 7 ($p<0.0001$, see table 3.7, appendix 2). EpCAM refers to human EPCAM.*

The above conditions solely examined the affect of PDX1 expression in un-patterned endoderm. However, induction of pancreatic endoderm requires retinoic acid (RA), usually applied to differentiating PSCs soon after endoderm induction. Therefore, we modified the endoderm induction protocol of Loh et al. to include an additional treatment with retinoic acid (RA) from day 4 onwards (figure 4.12).

We examined the effects of timed induction of the transgene for the first 4 days and subsequently treated the cells with RA for the remainder of the differentiation. Cells were analysed by flow cytometry and QPCR at day 7. Consistent with the results obtained above, the removal of dox after differentiation day 4 day resulted in no flag containing protein being detected at differentiation day 7. Also similar to above, expression of CXCR4 was still compromised (figure 4.12b). QPCR analysis of day 7 iPS parent and iPS-PDX1 cells revealed low level expression of PDX1 in both lines, consistent with previous observations that RA can induce the expression of PDX1 when applied during this temporal window. (figure 4.12i).

Lastly, we investigated the effects of inducing the transgene during RA treatment. Figure 4.13 compares conditions in which dox induction was performed during RA treatment from day 4-7 with cells that were subjected to dox and RA treatment for 7 days. Analysis of these cultures showed that robust expression of Flag containing proteins could be observed under both conditions (figure 4.13b and f). As expected from the results obtained above, induction of the transgene from day 4 onwards did not appear to affect CXCR4 expression, even in the presence of co-incidental RA treatment. However, dox and RA treatment for 7 days during differentiation noticeably suppressed CXCR4 expression (figure 4.13f).

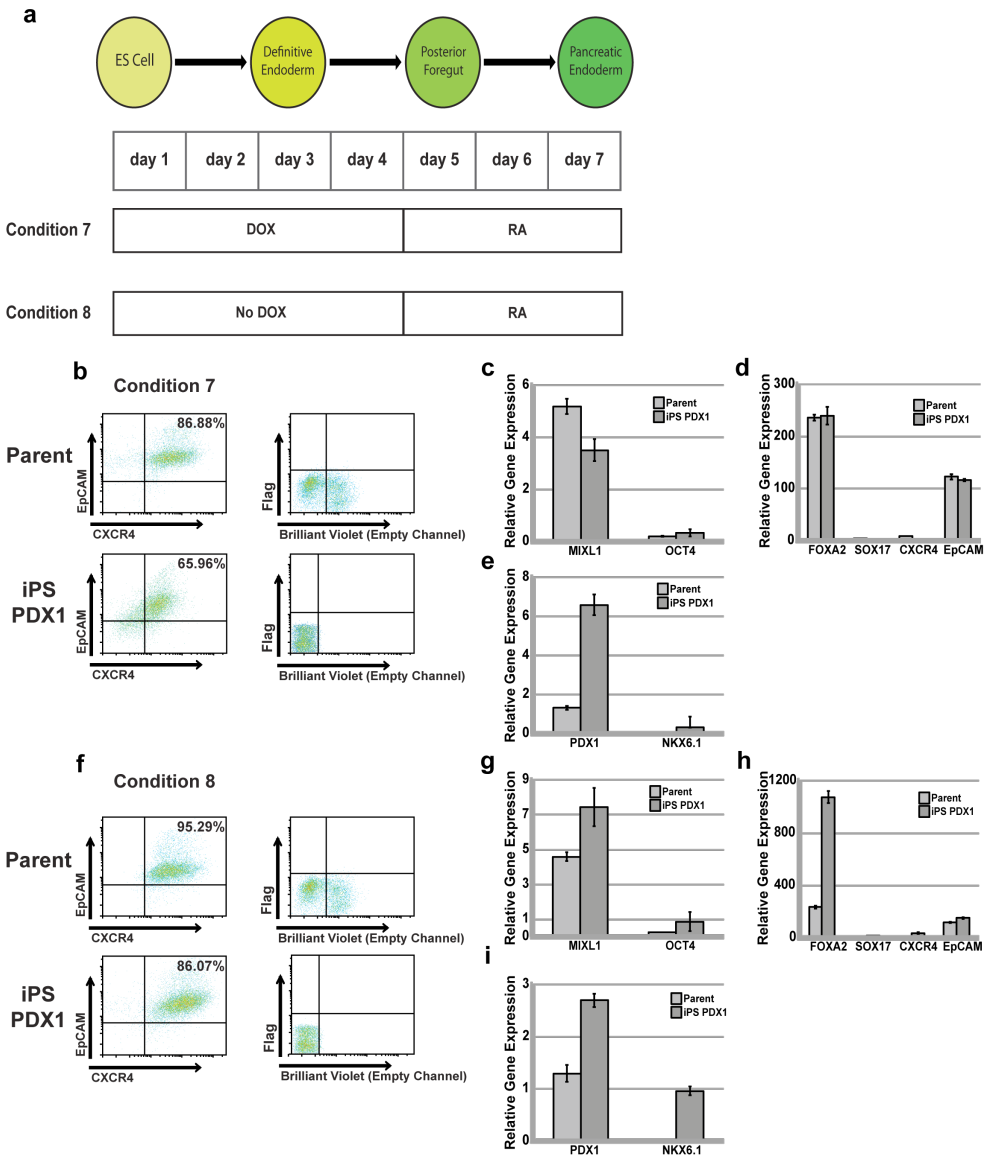

Figure 4.12. *Day 7 Analysis of Condition 7 and 8 treatment of iPS Parent and iPS PDX1. (a) Schematic representation of timeline and conditions. Figure represents no. of days of treatment exposure during differentiation. (b) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 7. (f) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (g-i) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 8. (error bars represent SEM from 3 independent experiments). Student t-test indicates the mean expression of CXCR4 was reduced in dox treated iPSC-PDX1 cells at differentiation day 7 (p=0.0135, see table 3.8, appendix 2). EpCAM refers to human EPCAM.*

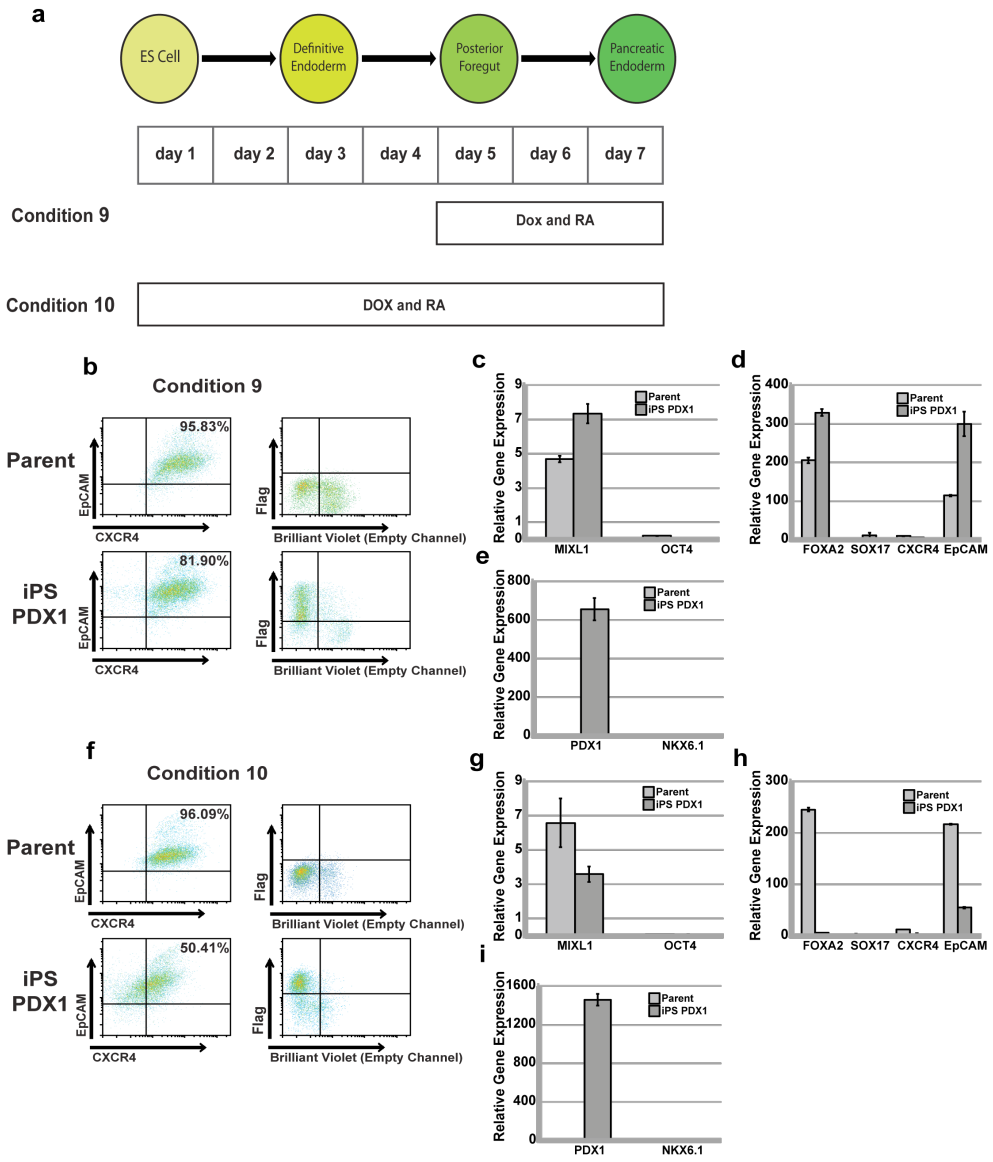

Figure 4.13. *Day 7 Analysis of Condition 9 and 10 treatment of iPS Parent and iPS PDX1. (a) Schematic representation of timeline and conditions. Figure represents no. of days of treatment exposure during differentiation. (b) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 9. (f) Flow cytometric analysis of iPS Parent and iPS PDX1 transgenic line of EPCAM vs CXCR4 expression and Flag (PDX1) expression. (g-i) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; SOX17; PDX1; NKX6.1 at day 7 following differentiation in condition 10. (error bars represent SEM from 3 independent experiments). Student t-test indicates the mean expression of CXCR4 was marginally reduced in dox treated iPSC-PDX1 cells at differentiation day 7 (p=0.2285, see table 3.9, appendix 2). EpCAM refers to human EPCAM.*

4.3 Discussion

Our goal in this chapter was to test a system that would allow us to co-express NKX6.1 and PDX1 in endodermal progenitors derived from differentiating PSCs. We chose to use a version of a doxycycline inducible vector that had previously been shown to allow for regulated expression of GFP during PSC differentiation (see introduction of this chapter). During testing, although vectors carrying PDX1 alone retained some inducibility during differentiation, vectors with both PDX1 and NKX6.1 appeared to progressively become silenced. Indeed, even in undifferentiated cells, induction of the vector containing the PDX1-T2A-NKX6.1 cassette was unimpressive figure 4.5e. Over time, even the PDX1 vector appeared to become silenced after subsequent passages of undifferentiated cell cultures. To combat this, we supplemented culture media with antibiotic (puromycin) which selected for cells that contained to transcribe the modified GAPDH allele. Interestingly, the cells were unaffected by the antibiotic, suggesting the modified GAPDH allele was still active and that silencing was restricted to the promoter associated with the Tet-operator sequences.

Transgene silencing is a common problem for vectors during differentiation and development (Toivonen et al., 2013). The Gaptrap expression system has been extensively studied with various genes (such as GFP and mCherry) and silencing had not been observed. However our vector differed from these prior versions as it contained additional gene regulatory elements and expression of the transgene required transcription to be initiated within the transgene rather than solely relying on expression from GAPDH promoter (figure 4.2a). Given the problems we saw with the

transgene containing both PDX1 and NKX6.1, further experiments were restricted to iPS PDX1 transgenic line.

First, we examined induction of the PDX1 transgene in undifferentiated PSCs. Cells were exposed to 1ug/ml of dox for 48 hours and analysed using an APC conjugated anti-Flag antibody. We found that the iPS PDX1 transgenic line exhibited robust induction of the transgene. To further validate this result we performed immunofluorescence staining on undifferentiated PSCs. Labelling cells with the same anti-flag antibody, we observed intense nuclear staining in the presence of dox (figure 4.3). In addition we further labelled PSCs with an anti-PDX1 antibody which showed high expression of the PDX1 transgene within the iPS PDX1 transgenic cell line as well (figure 4.4). In addition, efficient dox induction of the PDX1 transgene was demonstrated during the course of definitive endoderm differentiation,

Once we had validated the inducibility of the transgene, we wanted to determine the effects of inducing the PDX1 transgene during pancreatic endoderm differentiation. As mentioned in Chapter 3, we had implemented a new method of generating definitive endoderm using a flat culture system. For the induction of pancreatic endoderm, our current protocol introduces the treatment of retinoic acid (RA) into the differentiation culture after the emergence of the definitive endoderm. Therefore in the new protocol, we decided to begin RA treatment on day 4 of differentiation.

In the 10 conditions we investigated, we aimed to induce the transgene in conjunction with observing its interaction with effects of treatment with RA. Initially, we tested the induction of the transgene during the first 4 days of definitive endoderm differentiation. We found that at day 4, in the presence of dox, activation of the transgene retarded CXCR4 expression (figure 4.9f). Furthermore at day 7, induction

of the PDX1 transgene during the beginning of and throughout culture similarly saw a repression of CXCR4 expression in comparison to the parental cell line (figure 4.10). However in comparing the two day 7 cultures, the removal of dox at day 4 (figure 4.11d) seemingly allowed cultures to begin expressing more CXCR4 compared to cultures where dox was present throughout (figure 4.12h). In contrast, cultures where dox induction was introduced on day 4 of differentiation, only marginally affected CXCR4 expression (figure 4.11, 4.13).

Despite successful induction of PDX1 and the presence of definitive endoderm markers, no co-expression of NKX6.1 was detected in any of these conditions, suggesting that the activation of the PDX1 transgene alone was not enough to drive induction of pancreatic endoderm.

Cells were also subjected to dox induction during pancreatic endoderm differentiation which incorporated an RA treatment from differentiation day 4. As noted above, initial dox induction from day 1-4 reduced CXCR4 expression and a relative increase in the expression levels of FOXA2 and EPCAM (figure 4.9, 10 12). QPCR analysis of these cultures suggested that cultures derived from either the iPS parent or iPS-PDX1 lines sometimes expressed low levels of PDX1 and NKX6.1 (figure 4.10). In contrast, when dox and RA treatment was combined at day 4, or kept on throughout differentiation, no expression of NKX6.1 was detected. Finally, iPS-PDX1 cultures treated with 7 days of dox and RA met a similar fate to the condition where dox was alone was present throughout: that is, definitive endoderm differentiation was suppressed.

Overall, it is unclear whether the Tet-On expression system had a positive effect on pancreatic cell differentiation. Rather, our results suggested that premature induction of PDX1 repressed endoderm formation. Ideally, it would be interesting to see the effect of PDX1 induction at very late stages of differentiation, possibly even after endogenous PDX1 is activated. However, to do this, the expression system would need be validated for functionality at later times.

Despite its success in other settings, we observed that the Tet system containing two transgenes appeared to become silenced, preventing the transgene from functioning at its full capacity. In future experiments, this issue could be combatted by delivering the Tet system into a different locus such as AAVS1 (Qian et al., 2014; Sim et al., 2015; Tiyaboonchai et al., 2014). However it also may be prudent to seek out other alternative expression systems for the over-expression of these two proteins.

4.4 References

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CHAPTER 5

DESTABALISATION DOMAIN AND ESTROGEN RECEPTOR EXPRESSION SYSTEM

5

5.1 Introduction

Given our overall goal was to test the effect of co-induction of PDX1 and NKX6.1, we sought to examine other inducible systems that might allow for co-expression of these proteins. Two alternative inducible expression systems that hold promise for controlling transgene expression involve the creation of fusion proteins that incorporate a destabilising domain (DD) from bacterial dihydrofolate reductase (DHFR) or the hormone binding domain from the estrogen receptor (ER). In distinction to the Tet-on system, the transgenic protein of interest is continually produced but is rendered inactive until the inducing agent is added.

The DD regulatory system involves the generating a fusion between the protein of interest and a subdomain from *E.coli* dihydrofolate reductase (DHFR). This sub domain leads to the rapid proteosome degradation of the entire fusion protein. Thus the fusion protein is constitutively produced and destroyed (Banaszynski et al., 2006; Iwamoto et al., 2010). However, the cell-permeable small molecule DHFR inhibitor, Trimethoprim (TMP), can bind to the DD and alter its conformation such that it is no longer targeted for degradation. As such, levels of the fusion protein accumulate in the presence of TMP. Once removed, the system is reversed and the fusion protein is once again degraded. With it's ability to both express and deplete the fusion protein, DD systems allow for a tight regulation of genes of interest at the post translational level.

With few off-target effects, TMP is commercially available with good pharmacological properties, exhibiting a strong affinity for ecDHFR being commonly used as antibiotics. In addition, TMP is able to cross the blood-brain barrier enabling it to

augment the stability of proteins within the central nervous system (Quintino et al., 2013; Schrader et al., 2010; Tai et al., 2012). In light of these favorable properties, we chose to generate a PSC transgenic line in which NKX6.1 is under the control of the DD system.

Another inducible system we examined involved the creation of a fusion protein with a mutated variant of the estrogen receptor hormone binding domain. Unlike wild type estrogen receptor (ER), in the absence of its ligand, tamoxifen, this mutant variant is complexed with, and inactivated by, heat shock protein 90 (Hsp90) (Aumais et al., 1997). Similar to the DD system, this ER-fusion protein is constitutively produced, but remains in an inactive state. Upon addition of tamoxifen or its metabolite 4-hydroxytamoxifen (4-OHT), a conformational change occurs, releasing the fusion protein from HSP90 (Pratt, 1990; Scherrer et al., 1990). The fusion protein is then free to participate in the normal cell functions dictated by the fusion partner of the ER (Littlewood et al., 1995; Pratt, 1990; Scherrer et al., 1990; Whitesell and Cook, 1996).

Functional fusion proteins between transcription factors and the ER hormone binding domain have been reported by numerous studies [(Liu et al., 2005; Mandinova et al., 2009; Schebesta et al., 2007) and see http://sivelab.wi.mit.edu/grinformation/table1.html for an extensive list]. With this knowledge, we chose to generate a PSC transgenic line expressing an PDX1-ER fusion protein. Similarly, others have successfully employed destablisation domains to regulate the activity of transcription factors (Sui et al., 2014). Therefore, we chose to construct PSC line which expressed NKX6.1 as a DD fusion protein. Our aim was to test these independently and then, if they functioned as desired, combine them into a single expression vector. In these preliminary experiments, both proteins were expressed from the GAPDH locus using

modified versions of the GT expression vectors previously employed for the expression of fluorescent proteins.

5.2 Results

The construct shown in figure 5.1a was electroporated into NKX6.1 #D3/A1 △Neo PSC line and targeted clones identified using a PCR based screening strategy (see section 2.3, chapter 2). Firstly, we tested protein induction in undifferentiated cells by treating cultures with TMP for 48 hours. We then performed intracellular FACs to examine the expression of the NKX6.1-Flag transgene in the presence or absence of TMP. We tested a dilution of 1:1000 TMP (final concentration 0.1 mM), stock solution

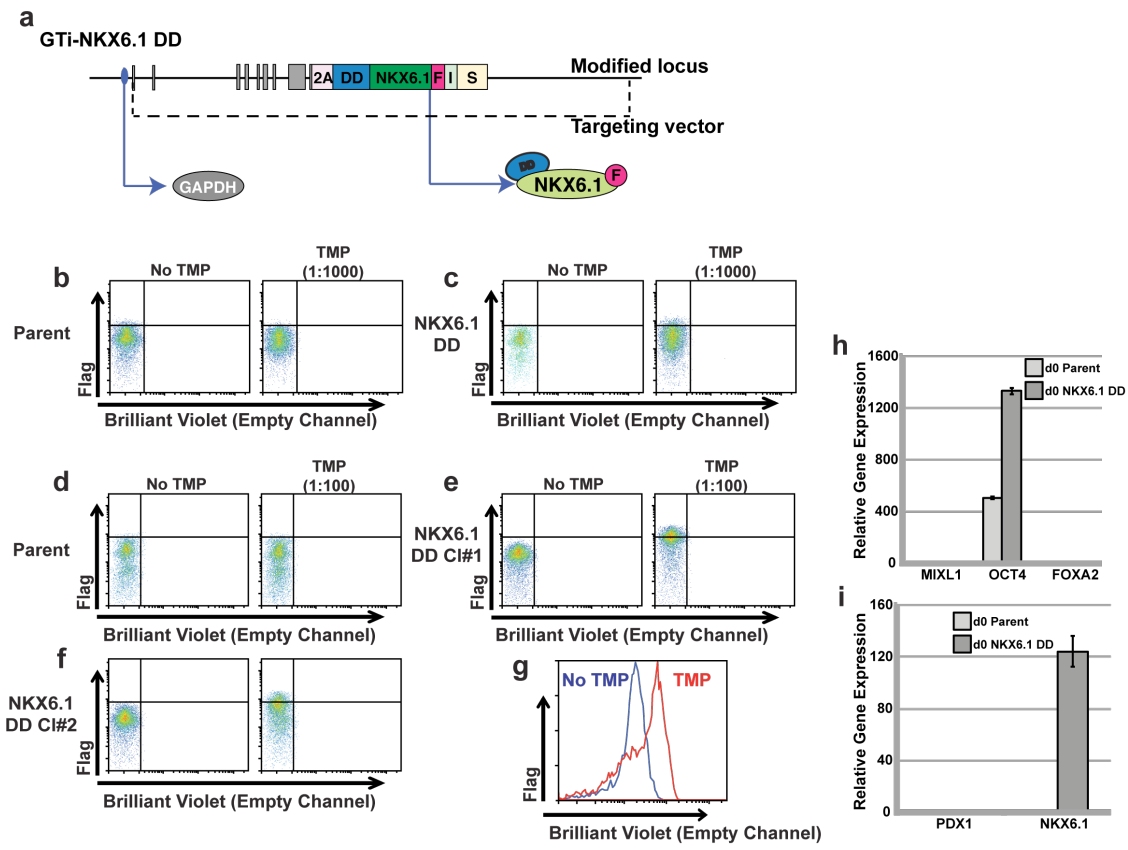

Figure 5.1. *Analysis of NKX6.1 protein induction in GTi NKX6.1 DD PSCs. (a) Schematic representation of the final construct of GTi-NKX6.1 DD. Flow cytometric analysis of Flag epitope expression in parental cell line (d) and in NKX6.1 DD cell line (e) with and without treatment with 0.1 mM TMP (1/1000). Flow cytometric analysis of Flag epitope expression in parental and NKX6.1 DD Clone #1 PSCs without and with treatment with 1 mM TMP (1/100). Flow cytometric analysis of Flag epitope (NKX6.1) expression in NKX6.1 DD Clone #2 cell line without and with TMP treatment (g). Histogram overlay showing the shift in NKX6.1-flag expression following treatment of NKX6.1 DD cultures with TMP for 24 hours (i). (j-k) QPCR analysis of MIXL1, OCT4, FOXA2, PDX1 and NKX6.1 expression at day 0. (error bars represent SEM from 3 independent experiments), validating the high levels of NKX6.1 transcript in the NKX6.1 DD PSC line.*

concentration being 100mM. At this concentration, no induction was observed in the NKX6.1 DD transgenic line (figure 5.1c). Therefore we tested the effect of TMP to at a concentration of 1 mM (1:100 dilution). At this new concentration, induction of the transgene was observed and expression of Flag was detected in both NKX6.1 DD clones (figure 5.1, e,f). Furthermore, we also validated the presence of high levels of NKX6.1 RNA in the transgenic line using QPCR (figure 5.1, i).

Next we examined the kinetics of TMP induction by treating cells with TMP at various time points over a 24 hours period. Cells were analysed by flow cytometry at 2, 4, 6, 8 and 24 hours post TMP treatment. Results showed that optimum induction occurred at the 24 hour time period (figure 5.2, a). Therefore, for all future experiments, we applied TMP treatment for at least 24 hours prior to any analyses.

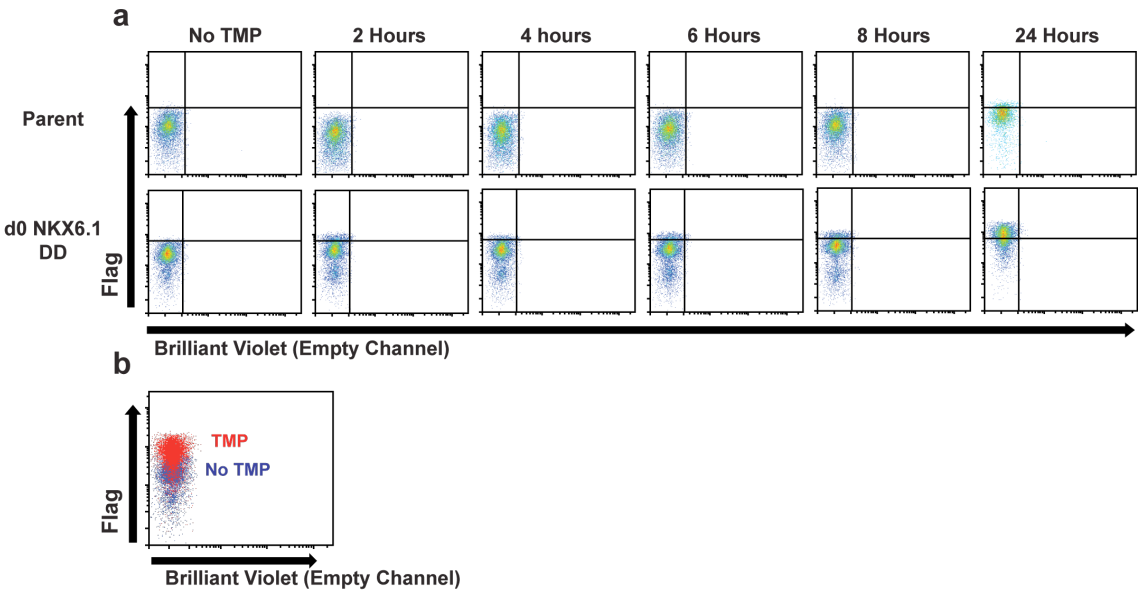

Figure 5.2. *Time course of TMP induction of Day 0 Parental and NKX6.1 DD PSCs. (a) Flow cytometric analysis of TMP induction marked by Flag (NKX6.1) expression at 2, 4, 6, 8 and 24 hours. (b) Overlay of TMP induction in NKX6.1 DD taken at 24 hour time point.*

To further characterise the NKX6.1 DD cell line, undifferentiated PSCs were labelled with a conjugated anti-Flag antibody and an anti-NKX6.1 antibody. Cells were seeded onto a 8-well chamber slide and treated with TMP for 24 hours. As the concentration of TMP was optimised in previous experiments, we chose to use TMP at a 1:100 dilution. Unfortunately, under these conditions, following a 24 hour TMP treatment, majority of the cells died (data not shown). Therefore we chose to use two lower dilutions of TMP, 1:300 (0.3 mM) and 1:1000 (0.1 mM). Using a confocal microscope, no expression of Flag was observed in the absence of TMP. However, cells treated with either 1:300 and 1:1000 TMP showed low levels of nuclear staining (figure 5.3). These experiments were repeated using an antibody directed at NKX6.1. In this case, some expression of NKX6.1 was observed in the NKX6.1 DD transgenic line in the absence TMP treatment (figure 5.4, d-f). This background level of expression was also detected using intracellular flow cytometry, with un-induced NKX6.1 DD PSCs showing slightly higher levels of staining than parental PSCs. When cells were treated with TMP, the anti-NKX6.1 antibody revealed intense nuclear staining with NKX6.1 DD PSCs, indicating robust induction (stabilisation) of the NKX6.1 protein in the NKX6.1 DD cell line.

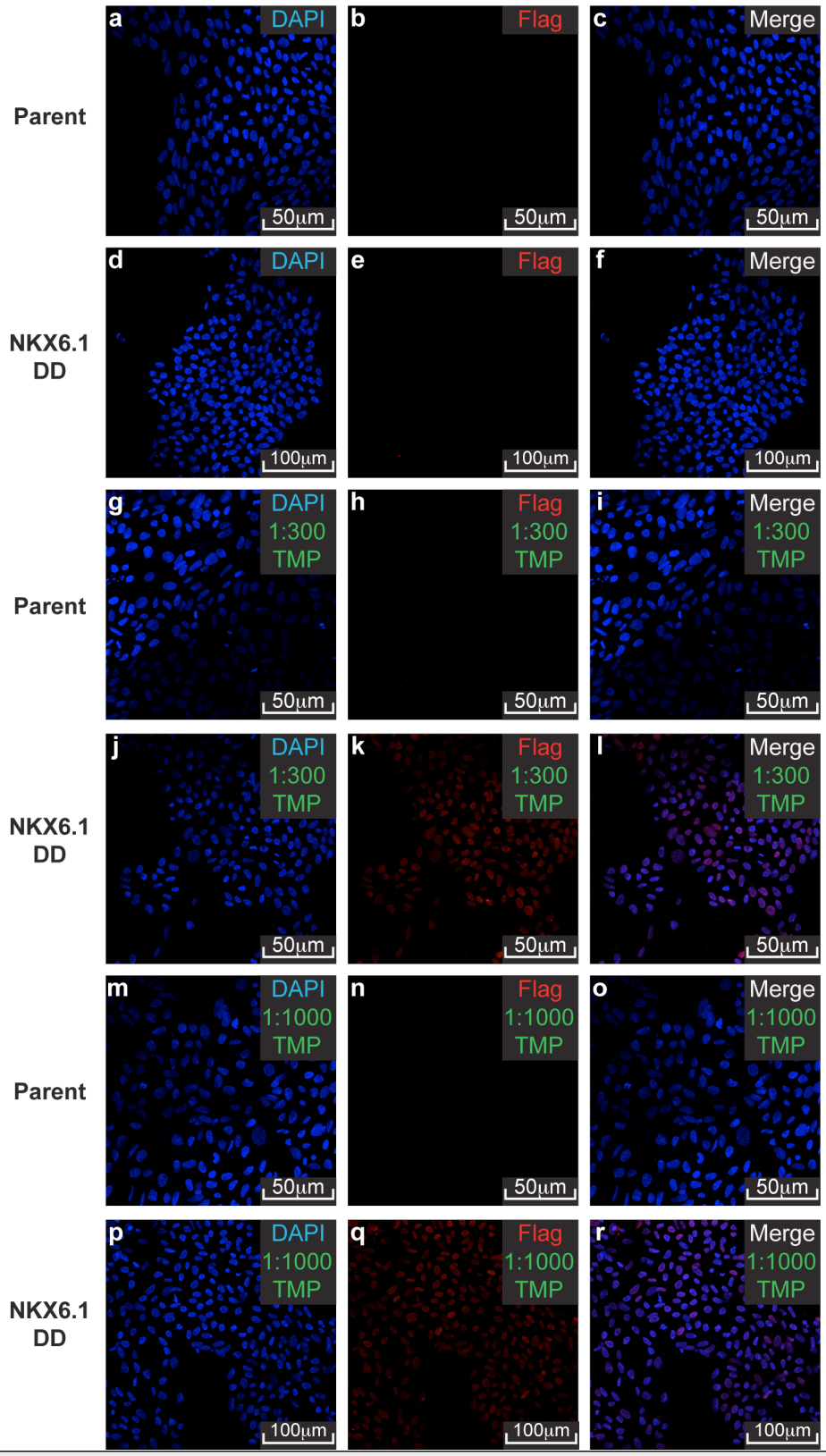

Figure 5.3 *Immunofluorescence analysis of PSCs expressing a transgene for TMP inducible NKX6.1. Cells were labelled with an APC-conjugated anti-Flag antibody and images were captured using confocal microscopy. In the absence or presence of TMP, no expression of Flag (NKX6.1) can be observed in the parental (a-c, g-i, m-o) PSC line. Flag expression was also absent from NKX6.1 DD PSCs that had not been treated with TMP (d-f). In contrast, Flag (NKX6.1) expression was clearly visible in the NKX6.1 DD PSCs at two dilutions of TMP, 1:300 (j-l) and 1:1000 (p-r), validating the induction of the NKX6.1 transgene.*

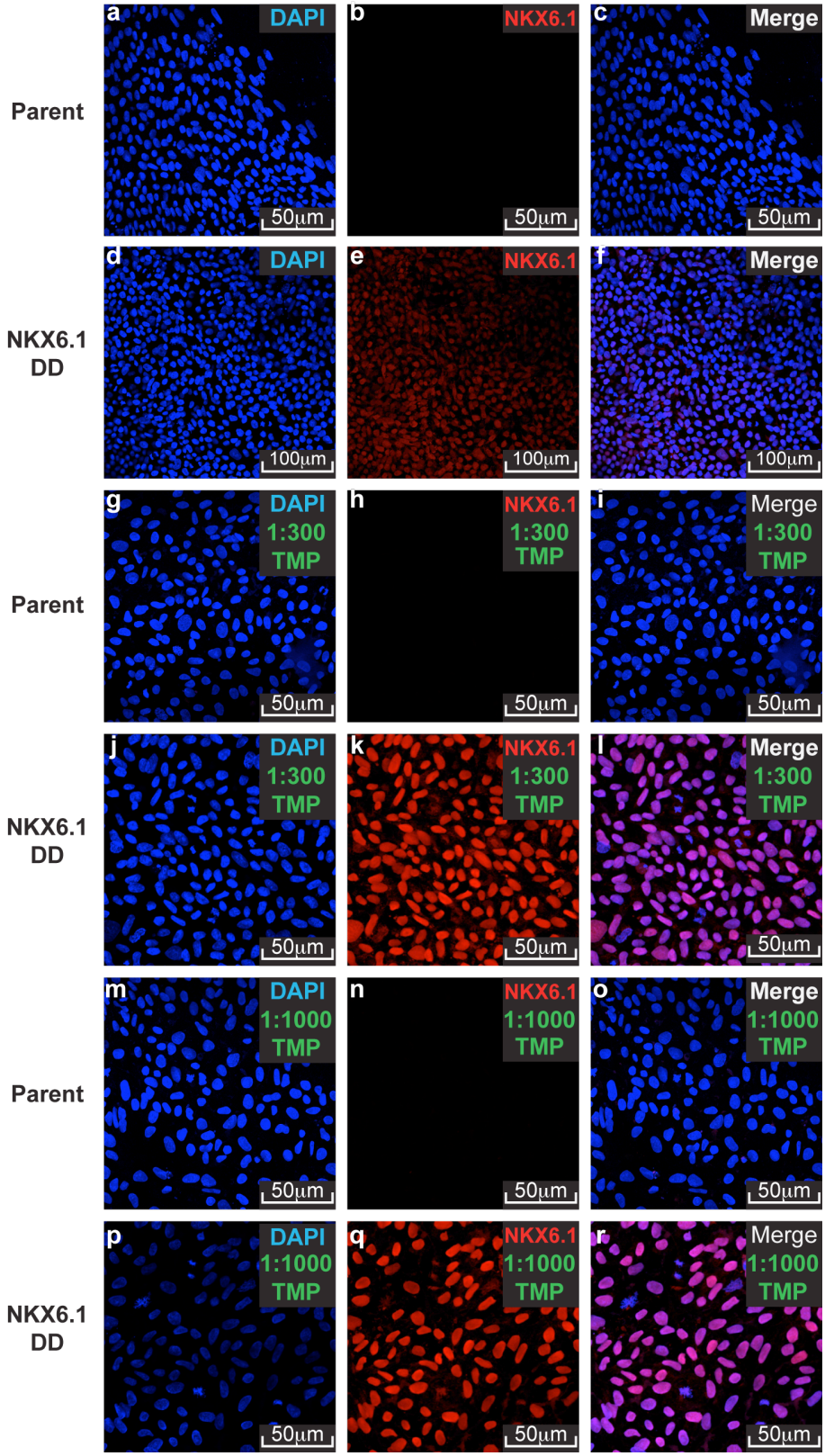

Figure 5.4 *Immunofluorescence analysis of PSCs expressing a transgene for TMP inducible NKX6.1. Cells were labelled with an anti-NKX6.1 antibody and subsequently with flurophore-conjugated anti-mouse secondary antibody and images captured using confocal microscopy. In the absence or presence of TMP, expression of NKX6.1 was observed in the parental (a-c, g-i, m-o) PSC line. Low level NKX6.1 expression was detected in NKX6.1 DD PSCs that had not been treated with TMP (d-f). In contrast, robust NKX6.1 expression was clearly visible in the NKX6.1 DD PSCs at two dilutions of TMP, 1:300 (j-l) and 1:1000 (p-r).*

We next examined TMP induction of the NKX6.1 transgene during endoderm differentiation. Cells underwent the two stage pancreatic endoderm differentiation outlined in section 2.4.3, chapter 2) and cells were analysed by flow cytomtery and QPCR at day 6 and 7 of differentiation. This analysis showed that at day 6 of differentiation, and following 24 hours of TMP treatment, robust induction of the transgenic protein could be detected with both and anti-flag antibody and with an anti-NKX6.1 antibody (figures 5.5 a, b and c). In addition, for both the parental cell line and the transgenic NKX6.1 DD PSCs, EPCAM, CXCR4 and CD117 expression was not affected by TMP treatment (figure 5.5, d,e, f, g). However, QPCR analysis revealed that the NKX6.1 DD transgenic line, in the absence and presence of TMP, expressed elevated levels of OCT 4 compared to the parental cells (figure 5.5, h). This persistence of OCT4 expression in these cells may reflect a peculiarity of this particular subclone or that the presence of the NKX6.1-DD protein blocked differentiation in a proportion of cells.

We repeated the analysis of parental and NKX6.1 DD PSCs at differentiation day 7. As above, uniform induction of the transgenic protein was detected in TMP treated NKX6.1 DD PSCs by intracellular flow cytometry using both and anti-flag and anti NKX6.1 antibody (figure 5.6 d,e, f, g). However, unlike day 6 cells, we observed that expression of both cKIT (CD117) and CXCR4 was reduced in the NKX6.1 DD line relative to parental PSCs. QPCR analysis for expression of the stem cell marker, OCT4, the mesendoderm marker MIXL1 and later endoderm markers was largely uninformative, perhaps except for the observation that OCT4 expression reduced in cells that were treated with TMP (figure 5.6, h). The significance of this observation is unclear.

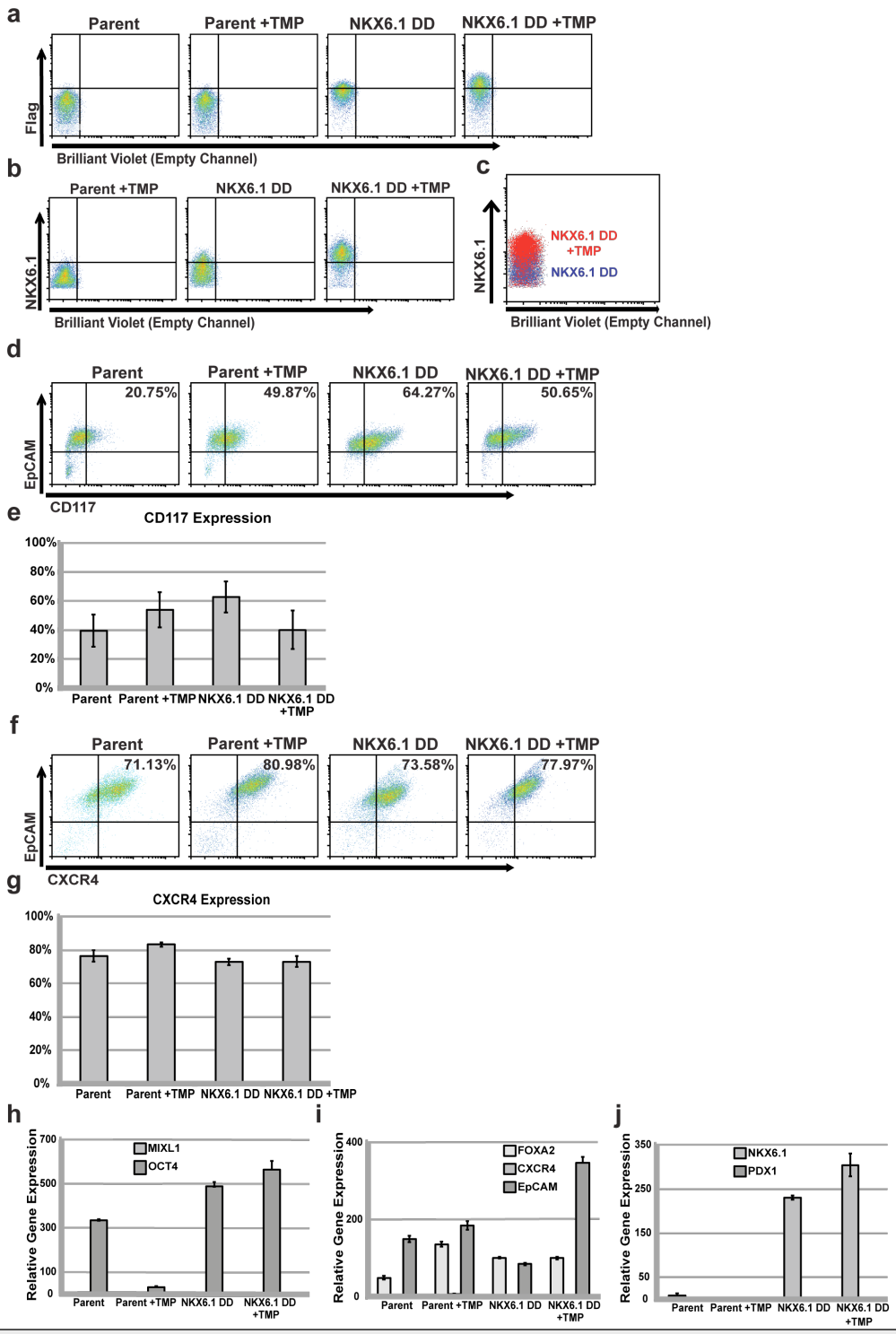

Figure 5.5. *Analysis of NKX6.1 DD PSCs at differentiation day 6. (a) Flow cytometric analysis of TMP induction of Flag (NKX6.1) expression in parent and NKX6.1 DD cell lines. (b) Flow cytometric analysis of TMP induction of NKX6.1 expression in parent and NKX6.1 DD cell line. (c) Overlay of TMP induction of NKX6.1 expression in NKX6.1 DD cell line. (d) Flow cytometric analysis of EPCAM vs CD117 expression of parent and NKX6.1 DD with and without the presence of TMP. (e) Percentage of CD117 expression, error bars represent SEM from 3 independent experiments. (f) Flow cytometric analysis of EPCAM vs CXCR4 in parent and NKX6.1 DD cell line with and without the presence of TMP. (g) Percentage of CXCR4 expressing cells under the conditions indicated. (h-j) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; PDX1 and NKX6.1 at day 6 following differentiation. In all cases error bars represent the SEM from 3 independent experiments. EpCAM refers to human EPCAM.*

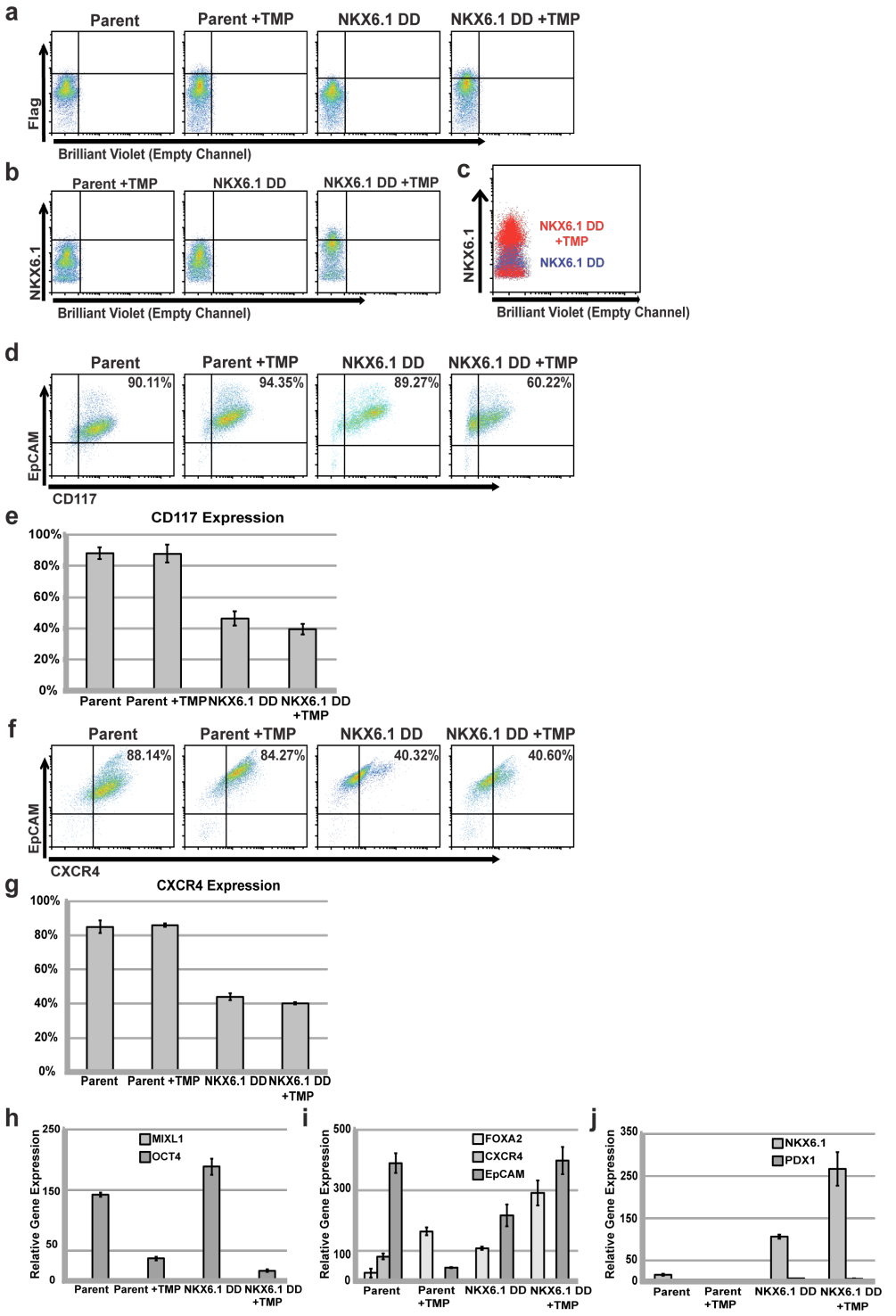

Figure 5.6. *Day 7 Analysis of NKX6.1 DD Following Differentiation. (a) Flow cytometric analysis of TMP induction of Flag (NKX6.1) expression in parent and NKX6.1 DD cell lines. (b) Flow cytometric analysis of TMP induction of NKX6.1 expression in parent and NKX6.1 DD cell line. (c) Overlay of TMP induction of NKX6.1 expression in NKX6.1 DD cell line. (d) Flow cytometric analysis of EPCAM vs CD117 expression of parent and NKX6.1 DD with and without the presence of TMP. (e) Percentage of CD117 expression, error bars represent SEM from 3 independent experiments. (f) Flow cytometric analysis of EPCAM vs CXCR4 in NKX6.1 parent and NKX6.1 DD cell line with and without the presence of TMP. (g) Percentage of CXCR4 expression, error bars represent SEM from 3 independent experiments. (h-j) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; PDX1 and NKX6.1 at day 6 following differentiation. Error bars represent SEM from 3 independent experiments. EpCAM refers to human EPCAM.*

Overall, our results with the NKX6.1-DD PSC lines failed to identify any dramatic consequences of NKX6.1 up-regulation. The potential reasons for this are unclear and are discussed at the end of this chapter.

In addition to the NKX6.1 DD PSCs, we also generated a PDX1 ER transgenic line by electroporating the vector shown in figure 5.7a into NKX6.1-GFP hESCs (Nostro et al., 2015). Again, target clones were picked using a PCR based strategy outlined in section 2.3, chapter 2. Three correctly targeted clones were picked for further analysis. The PDX1 transgene was not tagged with a flag epitope and therefore analysis using flow cytometry was not performed to examine the fidelity of the induction system. Instead undifferentiated cells were analysed by QPCR and using immunofluorescence microscopy. QPCR analysis indicated that PDX1 ER PSCs expressed robust levels of PDX1.

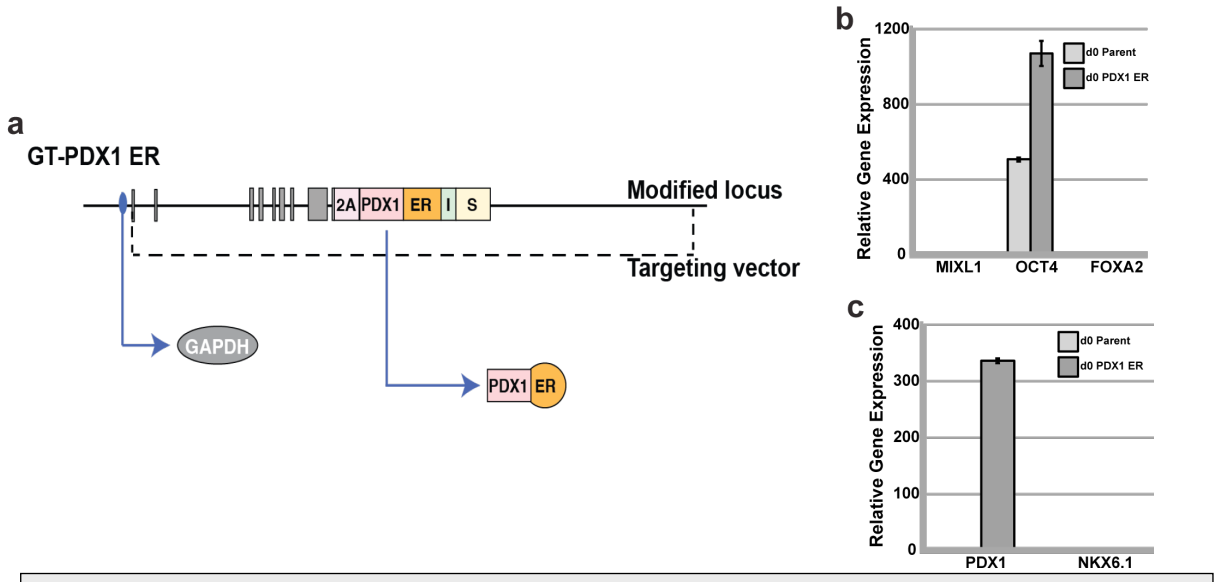

Figure 5.7. *Generation and analysis of PDX1-ER transgenic PSCs. (a) Schematic representation of PDX1 ER construct. (b-c) QPCR analysis of MIXL1; OCT4; FOXA2; PDX1; NKX6.1 in parental and PDX1 ER PSC lines in the presence of 4OHT. Error bars*

To examine PDX1 expression using immunofluorescence analysis, parental and PDX1 ER PSCs were seeded onto an 8 well chamber slides and then either treated or not treated for 48 hours with 4OHT. Fixed cells were then labelled with an anti-ER antibody or an anti-PDX1 antibody and images were captured using confocal microscopy. Interestingly, in the parental lines without the presence of 4OHT, low levels of staining were detected with both the ER and PDX1 antibodies (figure 5.8 a-c, figure 5.9 a-c). With the addition of 4OHT, a marginally brighter and more intense

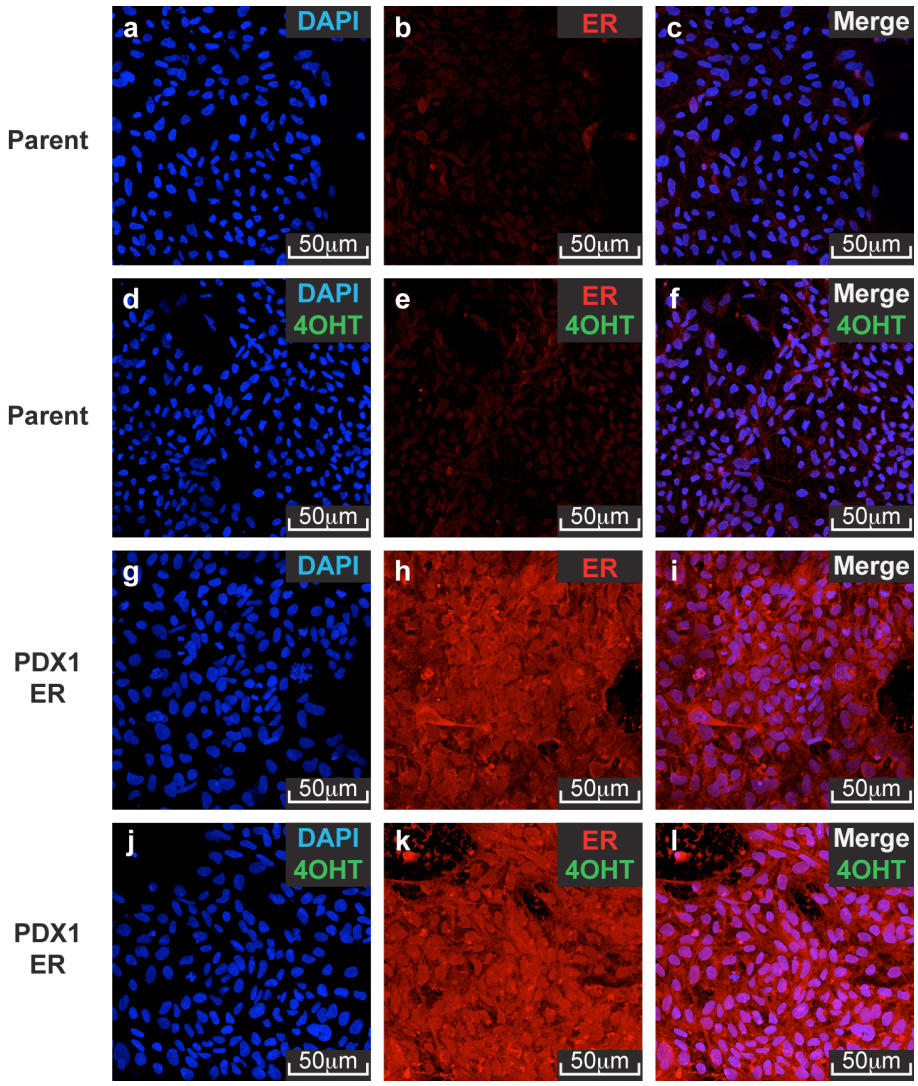

Figure 5.8. *Immunofluorescence analysis of PSCs expressing a PDX1-ER fusion protein. Undifferentiated cells were labelled with a primary un-conjugated anti-estrogen receptor (ER) antibody and a secondary alexa fluor 568 antibody, images were captured with confocal microscopy. In the absence and presence of 4OHT, low levels of ER expression was observed (a-c, d-f respectively). Intense staining was observed in the PDX1 ER cell line in the absence or presence of 4OHT (g-i, j-l).These results demonstrate the strong expression of the PDX1-ER fusion protein but also its failure to undergo a redistribution in sub-cellular location in the presence of 4OHT.*

expression of ER and PDX1 was observed (figure 5.8 d-e, figure 5.9, d-e, respectively). In contrast, we observed that PDX1 ER PSC line, in the absence of 4OHT, exhibited intense ER (figure 5.8 g-i) and PDX1 (figure 5.9 g-i) staining across the entire cell. Furthermore, following addition of 4OHT, both the ER (figure 5.8 j-l) and PDX1 (figure 5.9 j-l) proteins remained distributed across the entire cell. This result differed from that previously observed in our laboratory for other ER fusion proteins, where addition of 4OHT concentrated the fusion protein in the nucleus.

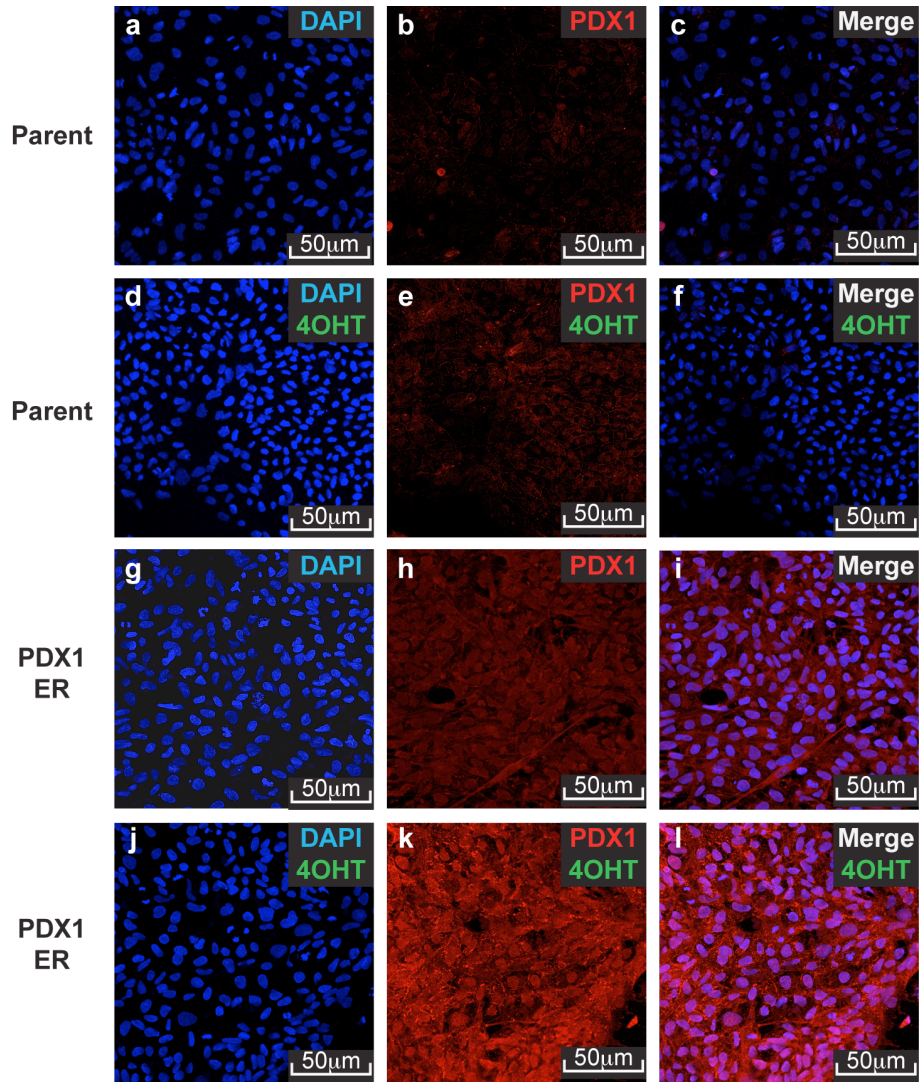

Figure 5.9. *Immunofluorescence analysis of PSCs expressing a transgene for 4OHT inducible PDX1. Undifferentiated cells were labelled with a primary unconjugated anti-PDX1 antibody and a secondary alexa fluor 568 antibody, images were captured using confocal microscopy. In the absence of 4OHT, the parental and transgenic PSC lines showed weak staining with the anti-PDX1 antibody. Staining was also low in the parental line following 4OHT treatment (d-f). In the presence of 4OHT, the PDX1-ER transgenic PSC line displayed intense staining of PDX1 expression across the cell, in a pattern that resembled that observed using the anti-ER antibody (figure 5.8).*

We also tested whether activation of the PDX1-ER fusion protein affected the course of pancreatic differentiation. Cells were differentiated using the two stage pancreatic endoderm differentiation outline in section 2.4.3, chapter 2 and were analysed by flow cytometry and QPCR at differentiation at days 6 and 7. Flow cytometry analysis at day 6 showed that a higher proportion PDX1-ER transgenic cells expressed CD117, irrespective of whether 4OHT was present in the medium (figure 5.10 a, b). Similarly, expression of the early endodermal marker, FOXA2, was also higher in the PDX1-ER PSC line than in parental PSCs (figure 5.10 d). Results obtained from the analysis of day 7 cultures paralleled those from day 6: the frequency of CD117 expressing cells was higher in PDX1-ER PSCs (flow cytometry) and FOXA2 levels were also elevated relative to the parental line (PCR) (figure 5.11). These analyses also showed that all cell types retained some expression of OCT4, perhaps pointing to presence of some undifferentiated cells within these cultures.

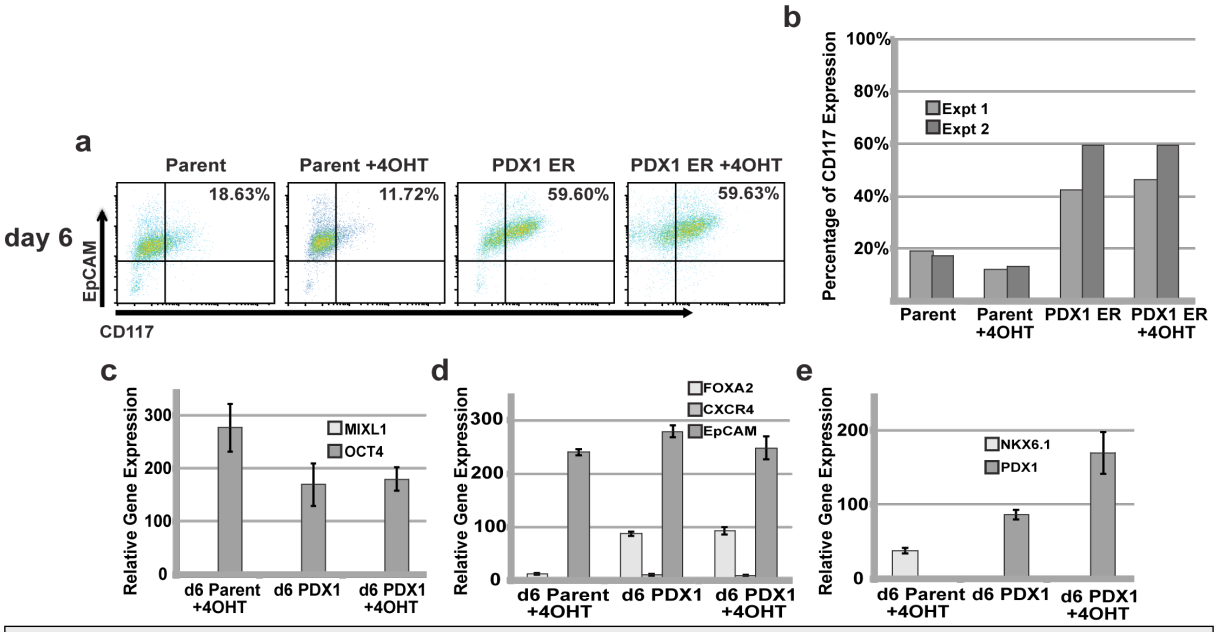

Figure 5.10. *Analysis of PDX1 ER PSCs at differentiation day 6. (a) Flow cytometric analysis of EPCAM vs CD117 (cKIT) expression in parental and PDX1 ER PSCs with and without the addition of 4OHT. (b) Comparison of fraction of CD117 positive cells in 2 independent experiments with and without the presence of 4OHT. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; PDX1; NKX6.1 at day 6 following differentiation. Error bars represent SEM from 3 independent experiments. Student t-test indicates the mean expression of NKX6.1 was significantly reduced compared to the PDX1 ER line in the presence and absence of 4OHT at day 6 of differentiation (p=0.0001 and p<0.0001 respectively, see table 3.11 and 3.12, appendix 2). EpCAM refers to human EPCAM.*

At both day 6 and day 7, endogenous PDX1 expression could be detected in parental PSCs, suggesting that the differentiation protocol we had used produced some amount of pancreatic endoderm (figures 5.10e and 5.11e). The level of endogenous PDX1 produced in differentiated transgenic PDX1-ER PSCs could not be assessed because the TaqMan QPCR probes we employed could not distinguish endogenous PDX1 from that originating from the transgene.

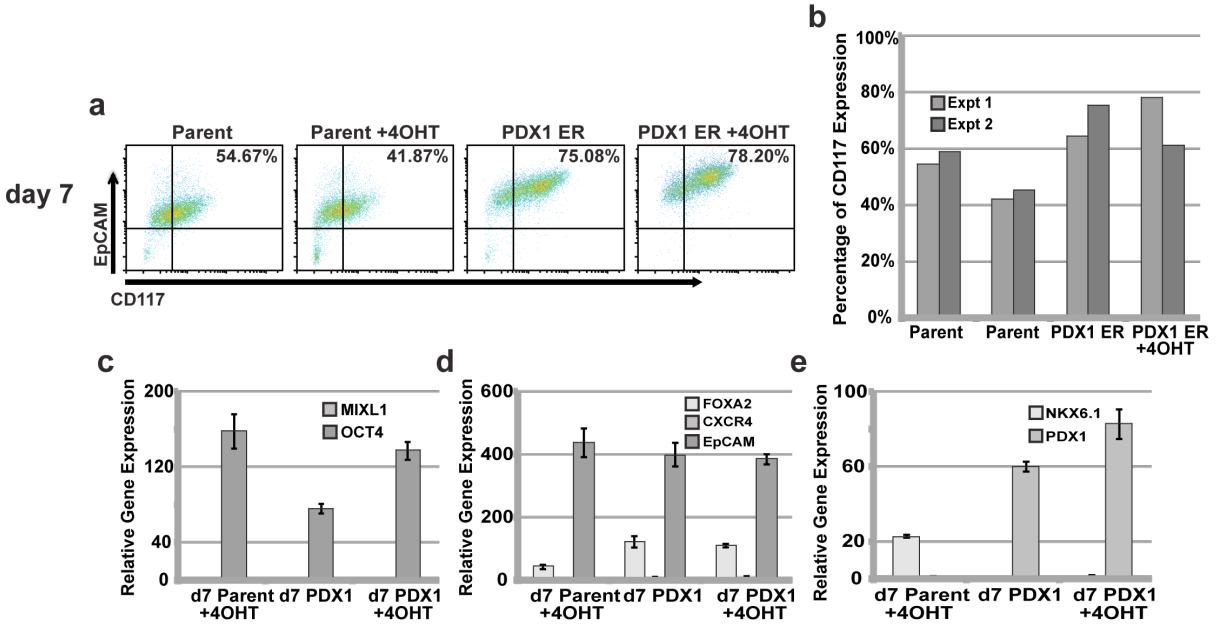

Figure 5.11. *Analysis of PDX1 ER PSCs at differentiation day 7 (a) Flow cytometric analysis of EPCAM vs CD117 (cKIT) expression in parental and PDX1 ER PSCs with and without the addition of 4OHT. (b) Comparison of fraction of CD117 positive cells in 2 independent experiments with and without the presence of 4OHT. (c-e) QPCR analysis of MIXL1; OCT4; FOXA2; CXCR4; EPCAM; PDX1; NKX6.1 at day 7 following differentiation. Error bars represent SEM from 3 independent experiments. Student t-test indicates the mean expression of NKX6.1 was significantly reduced compared to the PDX1 ER line in the presence and absence of 4OHT at day 7 of differentiation (p=0.0001 and p=0.0003 respectively, see table 3.13 and 3.14, appendix 2). EpCAM refers to human EPCAM.*

5.3 Discussion

Our aim in this chapter was to test two alternative expression systems in which our two proteins of interest, NKX6.1 and PDX1 could be expressed and regulated independently. Ultimately, if these systems worked, we would look into combining them into a single expression system. However, through our results, we found that both the DD and ER fusion protein systems encountered problems that would need to be solved before such a system could be constructed.

With the DD expression system, we observed that even without the presence of TMP, background expression of NKX6.1 was detected (for example, figure 5.4). During differentiation the NKX6.1 DD transgenic line, in the absence or presence of TMP, had reduced expression of CXCR4 and CD117 by day 7. Because of the leaky expression of the transgene, it is unclear if this effect was a consequence of the fusion protein or simply an example of line to line variation.

At differentiation day 7, we noted that parental cultures that had not been treated with TMP expressed low levels of NKX6.1, indicative of some degree of pancreatic differentiation. Addition of TMP abolished this expression, suggesting that TMP itself is not entirely neutral. We also observed that differentiated NKX6.1 DD cultures in the absence and presence of TMP expressed low levels of PDX1. Coupled with the changes in CXCR4 and CD117 expression, these results suggest that NKX6.1DD line was capable of transitioning from a definitive endoderm state towards a pancreatic fate. However, activation of the DD NKX6.1 fusion protein was not sufficient to drive this differentiation more completely towards a pancreatic progenitor fate.

With the ER system, expression of the PDX1-ER transgene was clearly evident by QPCR and immunofluorescence analysis. Of greatest concern was the sub-cellular distribution of PDX1-ER protein in the presence of 4OHT. We had anticipated that addition of 4OHT would lead to the fusion protein becoming concentrated in nucleus, but instead found this treatment accentuated the punctate pan-cellular distribution of the protein (see figure 5.12 below). Given PDX1 is primarily a transcription factor, it is extremely unlikely that a PDX1-ER fusion protein with the observed subcellular distribution would have been able to effect any normal functions of endogenous PDX1. Because of this, it is difficult to interpret the results obtained following 4OHT activation of fusion protein during differentiation. In future experiments, it would be necessary to determine whether this atypical distribution of protein was also present within differentiated cells.

Despite these reservations about the functionality of the transgene, differentiation of the PDX1 ER transgenic line differed from that of the parental line. For example, a greater proportion of cells expressed CD117 later in the differentiation. It is possible that this difference simply reflected clone-to-clone variation or that it resulted from adverse effects of the fusion protein. In either case, these differences did not appear to be 4OHT dependent and therefore were unlikely to result from a specific function of the transgenic fusion protein.

We commonly observed some expression of OCT4 by QPCR at day 6 and 7 in both parental and transgenic cultures. As outlined in chapter 2, we transitioned cells onto a feeder free system, meaning differentiations were set up in flat-adherent cultures rather than using our previous spin-embryoid method (Micallef et al., 2011). Differentiations were started when cells reached a confluency of 70-80%, and by day

6, cell cultures were over-confluent. A possible reason for the expression of OCT4 is that the highly packed PSC cultures failed to properly differentiate. Indeed, subsequent experiments in our laboratory have shown that endoderm differentiation is more efficient if cells are around 20-30% confluent at the start of differentiation.

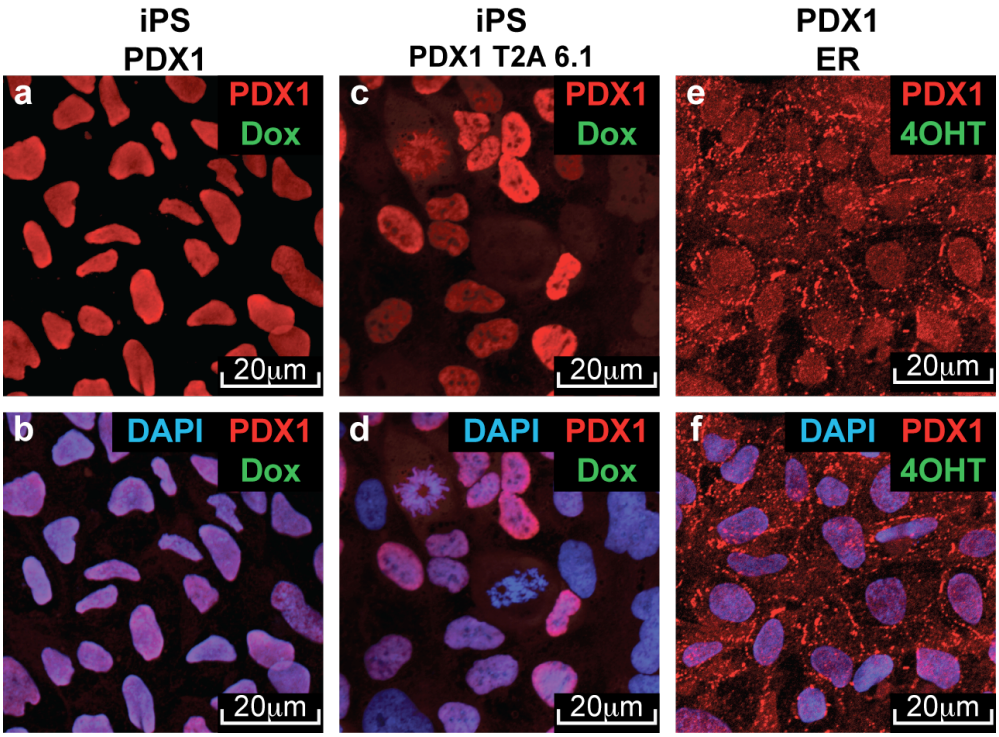

Figure 5.12 *Immunofluorescence analysis of PSCs expressing a transgene for the dox inducible and 4OHT activatible PDX1. Undifferentiated cells were labelled with a primary unconjugated anti-PDX1 antibody and a secondary alexa fluor 568 antibody, images captured using confocal microscopy. In the presence of dox, iPS PDX1 (a,b) and iPS PDX1 T2A (c,d) cell lines showed intense nuclear staining with the anti-PDX1 antibody. However, in contrast, the PDX1 ER cell line (e,f) in the presence of 4OHT exhibited lower levels of PDX1 expression distributed across the whole cell with some localised nuclear staining.*

In both systems, it is unclear whether the fusion proteins were able to perform their normal function. Given both systems have been successfully used to control gene expression (of other transcription factors), it could be that we were unlucky with the specific combinations used here. Therefore a potential future study could involve testing configurations of fusion proteins where the regulatory domains were at different ends of the protein, or involved different fusion partners (i.e NKX6.1-ER and

PDX1-DD). It is also possible that neither protein will function effectively as a fusion-protein, meaning the exploration of alternative expression systems would need to be undertaken.

5.4 References

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CHAPTER 6

GENERAL DISCUSSION

6

6.1 General Discussion

In this thesis, we set out with a goal to examine the effect of enforcing the expression of two pancreatic transcription factors, PDX1 and NKX6.1, during the course of pancreatic differentiation in vitro. We did this in two stages, first we started by implementing an improved endodermal differentiation protocol in an effort to eventually improve the overall yield of end stage differentiated insulin expressing β cells. Second, we constructed and tested a number of inducible transgenic expression systems to observe the effect of over-expressing our two proteins of interest in regulated manner.

To begin, we scrutinised various pancreatic protocols to pick out the common themes between them. One developmental pathway which was vital to the differentiation of pancreatic progenitors was the formation of the definitive endoderm. In a paper published by (Loh et al., 2014), they demonstrated a 3 day definitive endoderm differentiation protocol which appeared to improve the efficiency of definitive endoderm differentiation via a logical direction of signals controlling its lineage pathway. We replicated Loh et al.'s protocol, adapting it to our own differentiation media (AEL). We also took the opportunity to perform a side-by-side comparison of this protocol with a version of one we had used previously.

We tested 4 protocols, 2 of which were variations of the newly adapted definitive endoderm differentiation. These 2 protocols examined if the presence and concentration of Activin A was essential to the new adapted protocol. Consistent with the results of many prior reports, we confirmed that Activin A was essential to the induction of definitive endoderm. However, we also observed that at low

concentrations (10ng/ml), induction of definitive endoderm still occurred, but however reached its optimum induction at differentiation day 4 - 5.

Using the protocol of Loh et al, we observed rapid induction of a EpCAM+CXCR4+ population within 48 hours and by day 3, the majority of cells were EpCAM+CXCR4+. In our method, day 3 cells were still transitioning towards a definitive endoderm fate, with maximal co-expression of EpCAM and CXCR4 observed at days 5 and 6. Thus in terms of reducing the length of time cells were in culture (to potentially reduce asynchrony within the overall differentiation), the Loh et al protocol allowed us to being introducing growth factors associated with pancreatic endoderm differentiation at an earlier time point than our current method.

Given the above results, we amalgamated the new endoderm differentiation protocol with later stages of our existing pancreatic differentiation protocol. In practice, this meant starting retinoic acid treatment at differentiation day 4 rather than differentiation day 6. However due to our time constraints, we did not systematically optimise this amalgamated protocol, and thus we never explicitly tested if the the more rapid endoderm induction materially improved the final differentiation outcomes.

To tackle our second goal, we constructed a doxycycline inducible transgenic PSC line in which PDX1 and NKX6.1 were placed under the control of the Tet-on regulatory system. The first transgenic line consisted of a PDX1 coding sequences containing a Flag-tagged epitope at its c-terminus whilst the second co-expressed the two proteins (with the flag tag attached to NKX6.1). We chose to utilise this expression system as other members of our laboratory had previously tested the same dox inducible vector containing GFP. In their experiments, robust expression of

GFP could be induced at later stages of PSC differentiation. Whilst our GTi PDX1 vector showed promise, with robust expression following dox induction, the double vector containing PDX1 T2A 6.1 cassette induced relatively poorly, particularly once the cells were differentiated.

As noted in chapter 4, we attributed the poor induction of the transgene to the possible silencing of the Tet promoter. In addition, as iPS PDX1 cell line reached higher passages, induction of the transgene became poorer, suggesting that silencing of the vector was also occurring. To further investigate this, we put the iPS PDX1 cell line back on drug selection - a process that would select for cells that still expressed the modified GAPDH allele (chapter 2). However, we observed no cell death during selection and concluded that the silencing was not occurring within the GAPDH promoter but within the Tet responsive promoter located within the transgene. In future studies, this problem my be averted by inserting the dox inducible vector into a different locus (e.g. AAVS1). Recent studies have demonstrated the successful generation of stable and tightly controlled dox inducible expression systems after insertion into the AAVS1 locus (Qian et al., 2014; Sim et al., 2015; Tiyaboonchai et al., 2014).

Due to the silencing of the PDX1 T2A 6.1 vector, further analyses were conducted only the iPS PDX1 transgenic line. Despite the successful induction of the transgene, we found that induction of PDX1 alone was not enough to drive expression towards a pancreatic fate, evidenced by the absence up regulation of NKX6.1 and other pancreatic markers (including insulin and glucagon - data not presented). However, It is also possible that if we had further optimised the pancreatic differentiation protocol, that over-expression of PDX1 might have driven expression of later pancreatic

markers. Nevertheless it was also important to also seek out other alternative expression systems which may drive the over-expression of our genes of choice.

From past experience, our laboratory has reliably expressed protein coding sequences (i.e. GFP, Tandem Tomato, mCherry) from GAPDH promoter using the GAPDH expression system employed in this study. Therefore we speculated that more reliable expression might be achieved if we could eliminate the need for additional promoter sequences within a transgene whose expression was driven by the GAPDH locus. Thus, we generated a TMP inducible NKX6.1 destablisation domain (DD) expression system and a 4OHT inducible PDX1 estrogen receptor (ER) expression system. In the case of the DD systems, we observed reproducible up-regulation of the transgene. However, this system was prone to “leakiness” - meaning background expression of the transgene was detected in the absence of the inducing agent.

Another drawback of the DD system was the toxicity of high TMP concentrations during differentiation. Induction was not able to be detected via intracellular flow cytometry when TMP concentration was at a 0.1mM. Once concentration was increased to 1mM, adequate induction of the transgene was detected. However, as mentioned in chapter 5, when cells were at a low density were exposed to TMP at 1mM, many of the cells died. Hence for the preparation of cells for immunofluorescence staining, lower concentrations were used to induce expression of the NKX6.1 transgene.

For the ER expression system, immunofluorescence staining of undifferentiated cells lead to the finding that in the presence of 4OHT, the PDX1-ER fusion protein

appeared to form aggregates. Again in our laboratory, two other researchers have utilised the ER expression system to express other functional genes (i.e. PAX5 and NKX2.5) and did not observe this phenomenon. An explanation for the this could be due to protein misfolding or due to a peculiarity of fusion protein containing these proteins in combination.

In both systems it is unclear whether the fusion proteins were able to affect pancreatic differentiation. A way to possibly rectify some of the issues of the two systems might be to fuse the regulatory domains to different ends of the protein or to exchange the fusion partner.

6.2 Conclusion

This thesis examined the effect of over-expressing two key pancreatic transcription factors during the course of PSC differentiation in vitro. We employed a number of transgenic systems to engineer controllable induction of these two factors, NKX6.1 and PDX1. Our preliminary experiments indicated that, although these systems showed potential, all of them had drawbacks that prevented us from making a definitive conclusion concerning the affects of over-expressing these two factors. In addition future work should include a re-analysis of all these expression systems in the context of a robust and reliable pancreatic differentiation protocol. Only once it is clear that both the expression systems and the differentiation protocols are reliable will it be possible to definitively determine the effect that co-expression of these two factors will have on the course of pancreatic differentiation.

6.3 References

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APPENDIX

1

Review
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G TAN and others | *β-cell regeneration and differentiation* | **53**:3 | **R119**–R129

β-cell regeneration and differentiation: how close are we to the 'holy grail'?

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Journal of Molecular Endocrinology

Abstract

Diabetes can be managed by careful monitoring of blood glucose and timely delivery of exogenous insulin. However, even with fastidious compliance, people with diabetes can suffer from numerous complications including atherosclerosis, retinopathy, neuropathy, and kidney disease. This is because delivery of exogenous insulin coupled with glucose monitoring cannot provide the fine level of glucose control normally provided by endogenous β-cells in the context of intact islets. Moreover, a subset of people with diabetes lack awareness of hypoglycemic events; a status that can have grave consequences. Therefore, much effort has been focused on replacing lost or dysfunctional β-cells with cells derived from other sources. The advent of stem cell biology and cellular reprogramming strategies have provided impetus to this work and raised hopes that a β-cell replacement therapy is on the horizon. In this review, we look at two components that will be required for successful β-cell replacement therapy: a reliable and safe source of β-cells and a mechanism by which such cells can be delivered and protected from host immune destruction. Particular attention is paid to insulin-producing cells derived from pluripotent stem cells because this platform addresses the issue of scale, one of the more significant hurdles associated with potential cell-based therapies. We also review methods for encapsulating transplanted cells, a technique that allows grafts to evade immune attack and survive for a long term in the absence of ongoing immunosuppression. In surveying the literature, we conclude that there are still several substantial hurdles that need to be cleared before a stem cell-based β-cell replacement therapy for diabetes becomes a reality.

Key Words
- development
- diabetes (all)
- islet cells
- insulin

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Introduction

Diabetes mellitus encompasses a group of metabolic disorders that affect the ability to regulate blood glucose levels and can be classified into two main groups, type 1 and type 2. Previously known as juvenile-onset diabetes, Type 1 diabetes is thought to result from T-cell-mediated autoimmune destruction of insulin-producing β-cells and is believed to have a genetic component (reviewed in Concannon *et al.* (2009)), although, recently, both these contentions have been challenged (Skog *et al.* 2013). Type 2 diabetes is characterized by insulin resistance in

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peripheral tissues and is sometimes associated with β-cell dysfunction; both features resulting from prolonged exposure to elevated blood glucose levels (Tuomilehto *et al.* 2001).

Before the discovery of insulin, type 1 diabetes was almost always fatal. With the advent of insulin, diabetes was transformed into a chronic condition managed by careful monitoring of diet and blood glucose levels, in conjunction with insulin replacement therapy via s.c. injections or through an insulin pump (Tamborlane *et al.* 1979, Weissberg-Benchell *et al.* 2003, Renard *et al.* 2010). However, this system is imperfect as it fails to provide the fine control over blood glucose afforded by properly functioning islets. Moreover, inappropriate balance between insulin and glucose intake can cause severe hypoglycemia, a situation that can be exacerbated during acute illnesses (Krinsley *et al.* 2011). Critically, patients who are insulin dependent are sometimes unaware of hypoglycemic episodes, presenting the risk of loss of consciousness and the inability to be awakened from sleep (Cryer 2005).

At times where patients are at a high risk of severe hypoglycemia, replacement of insulin-producing β-cells through transplantation is considered. Originally, whole-organ pancreas transplantation was the only option. Successful pancreas transplants could restore normoglycemia and reduce, and sometimes reverse, secondary diabetic complications such as diabetic neuropathy (Bohman *et al.* 1985, Fioretto *et al.* 1998). Despite these benefits, similar to insulin therapy, pancreatic transplants are not without cost, with potential risks related to surgical complications, lifelong immunosuppression, and graft rejection. In order to avoid major invasive surgery, treatments involving the transplantation of cadaveric derived islets were developed. Successful transplantation improved glycemic control and often protected patients from hypoglycemia compared with pre-transplantation (Shapiro *et al.* 2000, 2006, Street *et al.* 2004). However, as in the case of pancreatic transplants, patients still require ongoing immunosuppression. Perhaps more importantly, in common with all transplantation-based therapies, the dearth of organ donors meant that this treatment option would only ever be available to a select few.

Against this backdrop, research has focused on other potential sources of β-cells that could be used in place of donor-derived pancreatic tissue. Of particular interest are pluripotent stem cells, immortal stable cell lines that can be differentiated into any cell type found in the body, including insulin-producing β-cells. This review briefly discusses the use of this stem cell type as a source of β-cells, examines how such cells might be delivered, and clarifies what issues will need to be addressed before a stem cell-based therapy might become reality.

Human pluripotent stem cells

Pluripotent stem cells (PSCs) are immortal cells that can be differentiated into any cell type found in the body. Owing to this, much interest has been focused on the possibility of deriving insulin-producing β-cells from PSCs for the treatment of diabetes. There are two types of PSCs: Embryonic stem cells (ESCs) derived from blastocyst-stage human embryos and induced PSCs (iPSCs) (Fig. 1), derived by reprogramming somatic cells to an ESC-like state. Human ESCs were first cultured by Thomson *et al.* (1998) and paved the way for the subsequent reprogramming of somatic cells by Takahashi & Yamanaka (2006) and shortly thereafter by Thomson *et al.* (Yu *et al.* 2007), to generate iPSCs that grow under the same conditions as ESCs and display similar properties. Researchers using both mouse and human somatic cells found that the introduction of four ESC-associated factors, OCT3/4, SOX2, c-MYC, and KLF4 (Takahashi & Yamanaka 2006, Takahashi *et al.* 2007, Yu *et al.* 2009), or OCT4, SOX2, NANOG, and LIN28 (Yu *et al.* 2007), induced pluripotency. Subsequently, further research found that other combinations of factors, small molecules, and RNAs can be used to achieve an equivalent outcome. The differentiation potential, proliferative capacity, morphology, and gene expression profiles of iPSCs are highly similar to those of ESCs, but use of the former avoids the ethical complications of deriving ESCs from human blastocysts (Yu *et al.* 2007, Rao & Condic 2008).

In spite of their similarities, iPSCs hold slightly different promise than ESCs, namely the use of iPSCs for patient-specific therapy. Human iPSCs are a genetic match to the person from whom they were generated, theoretically circumventing the issue of immune rejection of iPSC derivatives. However, in the case of type 1 diabetes, it would not be unreasonable to suppose that iPSC-derived β-cells would be rejected by the same autoimmune mechanism that led to the disease in the first place. Therefore, at least for type 1 diabetes, it is questionable whether iPSCs would offer any advantages over ESCs as a source of new β-cells for transplantation therapies. Finally, the amount of work and cost required to derive, validate, and ensure safety for any given cell line means that individualized iPSCs may not be economically practical as a source of new β-cells. In this sense, a generalizable 'off-the-shelf' stem cell-derived product is likely to be more viable, particularly in the short term. This could either take the form of an ESC-derived product, or potentially

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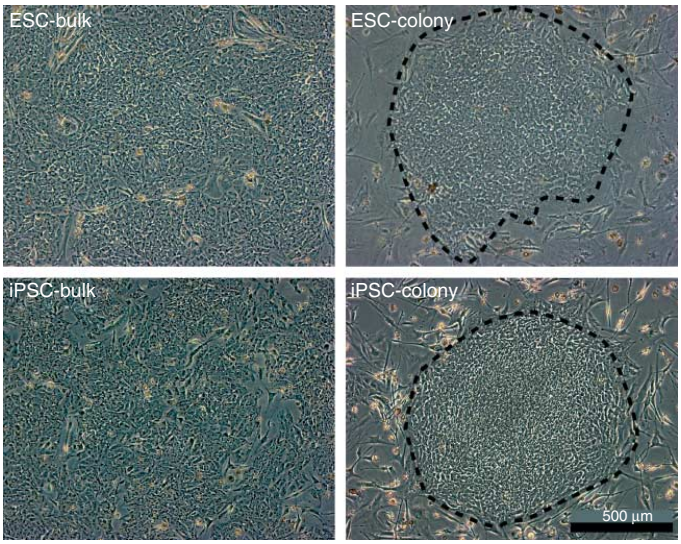

Figure 1
Morphology of human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) grown in monolayer cultures (left panels) and as single colonies (right panels) on mouse embryonic feeder cells (photographs taken using a ×5 objective lens).

cells differentiated from HLA-matched iPSCs sourced from pre-typed iPSC banks (Inoue *et al.* 2014). In either case, some form of immunoprotection for the graft or immunosuppression will be required.

Development of the pancreas

In order to differentiate PSCs *in vitro* toward a pancreatic cell fate, it is helpful to understand the process of pancreatic organogenesis. Extensive studies involving mouse models have led to a better understanding of the processes underlying early development of the embryo and the steps leading to the formation of complex tissues such as the pancreas. Using these processes as a roadmap, researchers have been able to guide PSCs through analogous stages of development toward the formation of desired cell types (Fig. 2). The pancreas is a derivative of definitive endoderm, one of the three primary germ layers generated during the process of gastrulation. Once generated, definitive endoderm folds into a primitive gut tube, which is then further regionalized into subdomains, a process that occurs under the influence of growth factors secreted by juxtaposed tissues. The pancreas arises from two patches of epithelium that evaginate dorsally and ventrally from the foregut endoderm, situated between the stomach and duodenum (Lammert *et al.* 2001, Field *et al.* 2003). The dorsal bud receives signals from the notochord and dorsal aorta and establishes a permissive environment for dorsal pancreatic specification within the gut endoderm (Hebrok *et al.* 1998). Conversely, the ventral bud is induced by a different set of signals originating from the adjacent cardiac mesenchyme and lateral plate mesoderm (Kumar *et al.* 2003). Following budding, the pancreatic primordia undergo considerable growth and branching, culminating in fusion of the two buds into a single organ (Villasenor *et al.* 2010). Over this period, expansion of the pancreatic epithelium is principally driven by mesenchymal cells, which secrete proliferative growth factors such as FGF10 (Bhushan *et al.* 2001). In mice, pancreatic development is accompanied by the branching of epithelial tubules; a stage sometimes referred to as the 'secondary transition' during which mono-hormonal insulin-expressing cells begin to emerge. During this time, acini form and begin to differentiate while mesenchymal derived growth factors continue to drive epithelial growth and new acinar formation (Landsman *et al.* 2011). Concomitant with this process, endocrine progenitors delaminate from the epithelium and aggregate to form islets (Bouwens & De Blay 1996). Maturation of endocrine cells within these islets generates glucagon, insulin, somatostatin, and pancreatic

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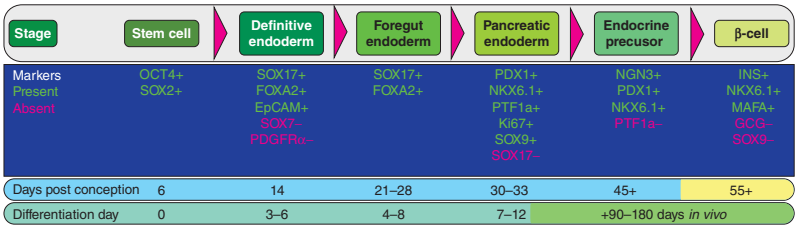

Figure 2
Timeline of pancreatic development *in vivo* and during PSC differentiation. Top line briefly summarizes the key cell types generated during pancreas development, while genes present or absent, which define these stages, are shown directly below. Days after conception indicate the number of days of embryonic/fetal development corresponding to each stage. Differentiation day gives an approximate estimate of the number of days required to reach these stages *in vitro*. Timelines have been based on studies by Piper *et al.* (2004), Riedel *et al.* (2012) and Jennings *et al.* (2013).

polypeptide-producing cells. Studies on mice suggest that endocrine precursors continue to differentiate throughout fetal development and for up to 3 weeks after birth. After this stage, endocrine tissue is maintained through a low frequency of replication (Dor *et al.* 2004, Brennand *et al.* 2007).

Although the process of human pancreatic development is thought to resemble that documented for mice, there are a number of points of difference that are pertinent to any discussion of PSC differentiation toward pancreatic lineages. First, it is arguable whether the developmental stages of the human pancreas can be precisely equated with those documented for mice (Sarkar *et al.* 2008). A key issue relating to the similarities of mouse and human pancreatic development centers on the appearance and function of cells expressing multiple hormones. Experiments conducted on mice suggest that insulin- and glucagon-expressing cells have distinct developmental origins (Herrera 2000). As such, it is unclear as to how and/or whether cells, which express multiple hormones during early mouse development (Teitelman *et al.* 1996), contribute to the adult mouse endocrine system. In human development, cells expressing multiple hormones range from 5 to 20% of hormone-positive cells, a fraction that remains relatively stable between weeks 10 and 20 of fetal life (Jeon *et al.* 2009). At this stage, it is still unclear whether such cells ever give rise to fully functional β-cells or whether they represent a fetal cell type that makes no contribution to the adult endocrine organ (Bocian-Sobkowska *et al.* 1999). Importantly, polyhormonal cells are a common feature of many PSCs to pancreatic differentiation protocols described to date (e.g., see D'Amour *et al.* (2006)) and, therefore, the potential of this cell type is an issue that needs to be urgently addressed.

Differentiation of pluripotent stem cells into pancreatic cells

Although numerous groups have published protocols for the differentiation of PSCs toward either pancreatic progenitors or endocrine cells, the most influential methods come from the biotechnology company, Viacyte, Inc. (http://viacyte.com). In a protocol developed by D'Amour *et al.*, combinations of growth factors involved in pancreatic development were used in a stage-specific manner to guide undifferentiated PSCs to insulin-expressing cells through a series of obligatory intermediate cell types identified through developmental studies (D'Amour *et al.* 2005, 2006, Kroon *et al.* 2008, Schulz *et al.* 2012). Since 2006, many other laboratories have published their own modifications of these pancreatic differentiation protocols (e.g., Jiang *et al.* 2007, Xu *et al.* 2011, Rezania *et al.* 2012).

Although there are a large number of elements that vary between pancreatic differentiation protocols, some common themes are evident. All methods are based on the ontogenetic framework described above that is used to rationalize the use of particular factors/treatments at specific stages of differentiation. To successfully differentiate PSCs to a pancreatic fate, it is recognized that multiple crucial developmental steps need to be accurately modeled (Fig. 2). These include the induction of definitive endoderm, the patterning and specification of endoderm to a pancreatic fate, and the generation of endocrine/exocrine cells (Biemar *et al.* 2001, Field *et al.* 2003, Nostro & Keller 2012). The first step invariably employs activin A to induce definitive endoderm. Activin A is a transforming growth factor beta (TGFβ) family member that mimics the action of Nodal, the ligand used by the embryo to drive development of the mesoderm and definitive endoderm (Osada & Wright 1999,

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Lowe *et al*. 2001, Kubo *et al*. 2004, Jiang *et al*. 2007, Kroon *et al*. 2008). Following this, definitive endoderm cells are treated with retinoic acid to induce foregut, from which pancreatic endoderm derives. This treatment was initially used in mouse ESCs for pancreatic differentiation (Micallef *et al*. 2005) and was rapidly adopted for human PSC systems (D'Amour *et al*. 2006). For both activin A (Nodal) and retinoic acid, key experiments defining the developmental role of these factors were conducted in model organisms such as xenopus, zebrafish, and mice (Conlon *et al*. 1994, Esni *et al*. 2001, Stafford & Prince 2002, Chen *et al*. 2004). Following the emergence of pancreatic endoderm, many methods include a treatment with the bone morphogenetic protein antagonist, noggin, or the small molecule analogs dorsomorphin or dorsomorphin homolog 1, because these factors promote the development of pancreatic progenitors defined by the expression of PDX1 (Jiang *et al*. 2007, Kroon *et al*. 2008, Bose *et al*. 2012). The final steps that are necessary to convert pancreatic endoderm to functional endocrine cells remain unclear. For this reason, many researchers transplant cells into immunocompromised mice at the pancreatic progenitor stage, allowing the final steps of differentiation and maturation to occur *in vivo* (Shim *et al*. 2007, Kroon *et al*. 2008, Rezania *et al*. 2012, Schulz *et al*. 2012, Rezania *et al*. 2013, Kirk *et al*. 2014). In instances where the final stages of differentiation were attempted *in vitro*, factors such as nicotinamide, insulin-like growth factor 1 (IGF1), and hepatocyte growth factor (HGF) were used to induce β-cell maturation, generating insulin- and glucagon-expressing cells (D'Amour *et al*. 2006, Jiang *et al*. 2007, Mfopou *et al*. 2010). However, unlike the cells that differentiate and mature *in vivo*, endocrine cells derived from a wholly *in vitro* differentiation approach frequently express more than one hormone and display an immature non-glucose-responsive phenotype (D'Amour *et al*. 2006, Basford *et al*. 2012, Micallef *et al*. 2012). Indeed, a direct comparison between insulin-expressing cells generated *in vitro* and those isolated from endogenous sources showed that, at a transcriptional level, PSC-derived β-cells most closely resembled fetal β-cells (Hrvatin *et al*. 2014).

In contrast to the success of generating pancreatic progenitors from PSCs, the overall yield of end-stage differentiated insulin-expressing β-cells remains low. For this reason, it is probable that if PSC-derived cells are used therapeutically, it is very likely that pancreatic progenitors – which retain both proliferative and differentiative capacities – will be the first choice for clinical trials.

Delivery of stem cell-derived products

Before any cellular based therapy can be used for the treatment of diabetes, it is necessary to consider how such cells would be delivered and what potential pitfalls may be encountered. In the case of type 2 diabetes, it would be theoretically possible to use patient-specific iPSC-derived pancreatic progenitors or β-cells without the need for immunosuppression. However, for type 1 diabetes, the impact of immune-mediated destruction of the transplanted islet cells will still need to be addressed, even if patient iPSCs are the source. Moreover, in either case, the question of safety of the transplanted cells also needs to be considered. In particular, as undifferentiated PSCs have the potential to form teratomas in xenotransplants, there is an ongoing concern that PSC-based cellular therapies may pose a safety risk (Hentze *et al*. 2009).

As most differentiation protocols enrich rather than purify cell types of interest, the presence of other cell types could represent a safety hazard, particularly if these off-target cells retained substantial proliferative potential. Furthermore, if differentiation is not 100% efficient, undifferentiated human PSCs with teratoma-forming potential may persist. Indeed, a number of studies reported that when differentiated cultures were transplanted into animal models, teratoma formation was sometimes observed (Sipione *et al*. 2004, Fujikawa *et al*. 2005, Kroon *et al*. 2008, Stadtfeld *et al*. 2008). These tumors are similar to spontaneous human teratomas that contain derivatives of each germ layer, endoderm, mesoderm, and ectoderm.

There are a number of ways this safety issue could be addressed. First, improvements in differentiation efficiency could reduce the frequency of unwanted cell types to negligible levels (Hentze *et al*. 2009). Secondly, procedures for purifying the desired cell types or for selecting against unwanted cell types could be developed. Having said this, any positive selection strategy that uses physical purification methods, such as cell selection using antibodies, is likely to be too expensive to apply on a large scale. Therefore, if purification methods are to be employed, it is likely that such methods would use drug-based selection against specific unwanted contaminant cell types. Lastly, as an additional safety precaution, cells could be encapsulated in a device that restricted their dispersion and facilitated their retrieval in the event of unwanted growth or differentiation. This approach also has the advantage that such a device could also serve to shield the cells from the immune system.

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Appendix 1 Published review

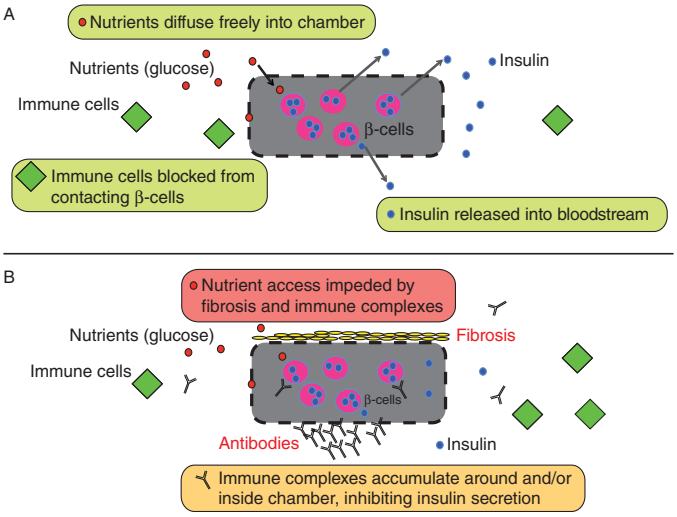

Figure 3

Promises and pitfalls of macroencapsulation devices for the delivery and protection of endogenously produced β-cells. (A) Idealized scenario in which the device allows free diffusion of glucose, nutrients, and insulin but excludes harmful immune cells. (B) Possible problems that may occur with macroencapsulation devices, including fibrosis covering the device and the entry of harmful anti-β-cell antibodies or immunoinflammatory molecules or cytokines.

Journal of Molecular Endocrinology

overgrowth and the subsequent necrosis of the transplanted islets (de Vos & Marchetti 2002).

The potential drawbacks of macroencapsulation devices have been partly addressed in the construction of the Theracyte device. This device ranges in length from ~2 to 4 cm, with a rectangular planar shape. The device contains a loading dock and is made of a biocompatible bi-layered polytetrafluoroethylene membrane. Studies have demonstrated that when the device containing neonatal pig cells positive for insulin and glucagon was transplanted into monkeys, the cells remained viable for up to 8 weeks with no inflammatory reaction (Elliott *et al.* 2005). Since these initial studies, a number of groups have shown that such a device can support the growth and differentiation of fetal or PSC-derived pancreatic progenitors and that this composite bioartificial pancreas can restore glucose control in mouse models of experimentally induced diabetes (Lee *et al.* 2009, Ludwig *et al.* 2012, Bruin *et al.* 2013, Rezania *et al.* 2013, Kirk *et al.* 2014). Currently, the US biotechnology company, ViaCyte, Inc., is applying to conduct clinical trials for a combination product that incorporates PSC-derived pancreatic progenitors and a two-dimensional planar device (Encaptra), which has shared the characteristics of the Theracyte device.

A potential issue with devices of this nature concerns their carrying capacity in relation to size. Kirk *et al.* (2014) have recently reported poor results when PSC-derived pancreatic progenitors were incorporated into a 5 μl device compared with the 20 μl device. The larger device, ~2× 1 cm, demonstrated a sufficient capacity to control glycemia after alloxan treatment of severe combined immunodeficiency/beige mice. Owing to the planar nature of the device, increasing the capacity requires a linear increase in the total area, that is, for a 1 cm wide device, increasing the volume from 20 to 40 μl requires a doubling in length. If a proportionately larger device is required for humans, then an average human weighing 70 kg might require a device 46 m long! Clearly, further innovations in the structure of these devices may be required in order for them to be ready for clinical application.

An alternative approach would be to devise a treatment in which β-cells were replenished from endogenous sources – either through the activation of endogenous pancreatic stem cells or by the *in vivo* reprogramming of non-endocrine cells toward a β-cell phenotype (Zhou *et al.* 2008, Smukler *et al.* 2011). However, in the case of type 1 diabetes, any regenerative treatment may still require the issue of the immune system

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to be addressed – and, currently, it is unclear how activated endogenous pancreatic stem cells would fare if the ongoing immune assault could not be attenuated or eliminated. Similarly, neo-β-cells brought into existence through reprogramming approaches or growth factor treatment would still face a hostile immune system that would need to be addressed.

One potential solution to immune-mediated destruction of a cellular therapeutic product would be to engineer glucose-responsive non-β-cells that could avoid immune surveillance. However, this might not be possible if the proteins required for regulated insulin release are the target of autoimmunity. In particular, a recent work demonstrating that pro-insulin itself could be a key autoantigen indicates the potential difficulties in avoiding ongoing immunological assault (Pathiraja *et al.* 2014).

Similarly, in type 2 diabetes, it seems difficult to envisage a scenario in which newly derived β-cells would not fall victim to the same adverse conditions that precipitated the decline in β-cell function often associated with this disease (Potter *et al.* 2014). As such, for both types of diabetes, it might transpire that exogenously produced β-cells will represent the best hope of achieving normoglycemia for patients with either type 1 or type 2 diabetes.

The quest to find a new treatment for diabetes has been punctuated with obstacles that reflect the various complexities of the disease. The need to find a new source of insulin-producing β-cells has driven research into the potential use of stem cells, which, in turn, has benefited from decades of assiduous developmental studies. In the case of type 1 diabetes, the need to deal with sustained attack from the immune system has prompted the development of encapsulation strategies that have relied heavily on materials science and transplantation biology. If a workable solution is to be achieved, it will probably require the coming together of several streams of research that span different fields of scientific endeavor. Optimistically, this quest, based on scientific insight, will prove to be more fruitful than that of the mythical quest for the holy grail.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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APPENDIX

2

Appendix 2 Statistics

Table 3.1, appendix 2. Percentage of EPCAM+CXCR4+ cell population at differentiation day 3 as described in chapter 3. FACS data points taken from 3 independent experiments.			
	Experiment 1	**Experiment 2**	**Experiment 3**
No Activin A condition	2.3	2.54	3.28
Low Activin A condition	56.18	57.71	58.24
Loh et al., 2014 condition	78.48	80.34	238.97
Current protocol condition	61.37	64.21	63.22

Table 3.2, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 3. Data was derived from 3 independent experiments and relates to a comparison of No Activin A Condition verses Loh et al. Condition and the expression of CXCR4+EPCAM+ cell population. The table contains the results of a unpaired t-test performed using PRISM6.	
No Activin A vs Loh et al. Condition	
Unpaired t test	
P value	< 0.0001
P value Summary	****
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 116.5 df= 4
Mean ± SEM of No Activin A condition	2.707 ± 0.2949, n=3
Mean ± SEM of Loh et al. condition	79.66 ± 0.5909, n=3
Difference between means	76.95 ± 0.6604
95% Confidence Interval	75.12 to 78.78
R squared	0.9997

Appendix 2 Statistics

Table 3.3, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 3. Data was derived from 3 independent experiments and relates to a comparison of Low Activin A Condition verses Loh et al. Condition. The table contains the results of a unpaired t-test performed using PRISM6.	
Low Levels Activin A vs Loh et al. Condition	
Unpaired t test	
P value	< 0.0001
P value Summary	****
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 26.07 df= 4
Mean ± SEM of Low Levels Activin A condition	57.38 ± 0.6176, n=3
Mean ± SEM of Loh et al. condition	79.66 ± 0.5909, n=3
Difference between means	22.28 ± 0.8547
95% Confidence Interval	19.91 to 24.65
R squared	0.9941

Table 3.4, appendix 2. Relative gene expression of CXCR4 in TetO PDX1 Line at differentiation day 4 and day 7 in the presence and absence of Doxycycline as described in chapter 4. Data points taken from 3 independent experiments.			
	Experiment 1	**Experiment 2**	**Experiment 3**
Condition 1 (day 4)	135.84	105.84	196.14
Condition 2 (day 4)	0.41	8.5	11.2
Condition 3 (day 7)	102.95	110.33	108.06
Condition 4 (day 7)	141.61	145.59	135.84
Condition 5 (day 7)	151.77	139.66	142.56
Condition 6 (day 7)	2.09	7.34	5.75
Condition 7 (day 7)	2.093	2.306	3.377
Condition 8 (day 7)	51.12	31.25	25.033
Condition 9 (day 7)	4.944	4.876	6.75
Condition 10 (day 7)	0.676	6.479	0.936

Appendix 2 Statistics

Table 3.5, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 4, figure 4.9. Data was derived from 3 independent experiments and relates to a comparison of Condition 1 verses Condition 2. The table contains the results of a unpaired t-test performed using PRISM6.	
Condition 1 vs Condition 2 (day 4)	
Unpaired t test	
P value	0.0065
P value Summary	**
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 5.205 df= 4
Mean ± SEM of Condition 1	145.9 ± 26.55, n=3
Mean ± SEM of Condition 2	6.703± 3.242, n=3
Difference between means	-139.2 ± 26.75
95% Confidence Interval	-213.5 to -64.97
R squared	0.8714

Table 3.6, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 4, figure 4.10. Data was derived from 3 independent experiments and relates to a comparison of Condition 3 verses Condition 4. The table contains the results of a unpaired t-test performed using PRISM6.	
Condition 3 vs Condition 4 (day 7)	
Unpaired t test	
P value	0.0007
P value Summary	***
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 9.485 df= 4
Mean ± SEM of Condition 3	107.1 ± 2.182, n=3
Mean ± SEM of Condition 4	141.0± 2.830, n=3
Difference between means	33.90 ± 3.574
95% Confidence Interval	23.98 to 43.82
R squared	0.9574

Appendix 2 Statistics

Table 3.7, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 4, figure 4.11. Data was derived from 3 independent experiments and relates to a comparison of Condition 5 verses Condition 6. The table contains the results of a unpaired t-test performed using PRISM6.	
Condition 5 vs Condition 6 (day 7)	
Unpaired t test	
P value	<0.0001
P value Summary	****
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 35.18 df= 4
Mean ± SEM of Condition 5	144.7 ± 3.651, n=3
Mean ± SEM of Condition 6	5.060 ± 1.554, n=3
Difference between means	-139.6 ± 3.968
95% Confidence Interval	-150.6 to -128.6
R squared	0.9968

Table 3.8, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 4, figure 4.12. Data was derived from 3 independent experiments and relates to a comparison of Condition 7 verses Condition 8. The table contains the results of a unpaired t-test performed using PRISM6.	
Condition 7 vs Condition 8 (day 7)	
Unpaired t test	
P value	0.0135
P value Summary	*
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 4.216 df= 4
Mean ± SEM of Condition 7	2.592 ± 0.3973, n=3
Mean ± SEM of Condition 8	35.08 ± 7.877, n=3
Difference between means	33.21 ± 7.877
95% Confidence Interval	11.34 to 55.08
R squared	0.8163

Appendix 2 Statistics

Table 3.9, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 4, figure 4.13. Data was derived from 3 independent experiments and relates to a comparison of Condition 9 verses Condition 10. The table contains the results of a unpaired t-test performed using PRISM6.	
Condition 9 vs Condition 10 (day 7)	
Unpaired t test	
P value	0.2285
P value Summary	ns
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 1.421 df= 4
Mean ± SEM of Condition 9	5.523 ± 0.6136, n=3
Mean ± SEM of Condition 10	2.697 ± 1.892, n=3
Difference between means	-2.826 ± 1.989
95% Confidence Interval	-8.350 to 2.697
R squared	0.3353

Table 3.10, appendix 2. Relative gene expression of NKX6.1 in Parent and PDX1 ER Line at differentiation day 6 and day 7 in the presence and absence of 4OHT as described in chapter 5. Data points taken from 3 independent experiments.

	Experiment 1	**Experiment 2**	**Experiment 3**
day 6 Parent +4OHT	26.18	26.92	27.03
day 6 PDX1 ER	33.25	32.36	33.3
day 6 PDX1 ER +4OHT	33.03	32.82	32.46
day 7 Parent +4OHT	27.78	26.48	27.73
day 7 PDX1 ER	33.49	33.43	33.4
day 7 PDX1 ER +4OHT	32.52	33.33	32.87

Appendix 2 Statistics

Table 3.11, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 5, figure 5.10. Data was derived from 3 independent experiments and relates to a comparison of Day 6 Parent +4OHT verses Day 6 PDX1 ER Line. The table contains the results of a unpaired t-test performed using PRISM6.	
Day 6 Parent +4OHT vs PDX1 ER Line	
Unpaired t test	
P value	0.0001
P value Summary	***
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 15.44 df= 4
Mean ± SEM of Parent +4OHT (day 6)	26.71 ± 0.2669, n=3
Mean ± SEM of PDX1 ER Line (day 6)	32.97 ± 0.3053, n=3
Difference between means	6.260 ± 0.4055
95% Confidence Interval	5.134 to 7.386
R squared	0.9835

Table 3.12, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 5, figure 5.10. Data was derived from 3 independent experiments and relates to a comparison of Day 6 Parent +4OHT verses Day 6 PDX1 ER Line +4OHT. The table contains the results of a unpaired t-test performed using PRISM6.	
Day 6 Parent +4OHT vs PDX1 ER Line +4OHT	
Unpaired t test	
P value	< 0.0001
P value Summary	****
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 19.27 df= 4
Mean ± SEM of Parent +4OHT (day 6)	26.71 ± 0.2669, n=3
Mean ± SEM of PDX1 ER Line +4OHT (day 6)	32.77 ± 0.1664, n=3
Difference between means	0.060 ± 0.3145
95% Confidence Interval	5.187 to 6.933
R squared	0.9893

Appendix 2 Statistics

Table 3.13, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 5, figure 5.11. Data was derived from 3 independent experiments and relates to a comparison of Day 7 Parent +4OHT verses Day 7 PDX1 ER Line. The table contains the results of a unpaired t-test performed using PRISM6.	
Day 7 Parent +4OHT vs PDX1 ER Line	
Unpaired t test	
P value	0.0001
P value Summary	***
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 14.34 df= 4
Mean ± SEM of Parent +4OHT (day 7)	27.33 ± 0.4252, n=3
Mean ± SEM of PDX1 ER Line (day 7)	33.44 ± 0.02646, n=3
Difference between means	6.110 ± 0.4261
95% Confidence Interval	-4.927 to 7.293
R squared	0.9809

Table 3.14, Appendix 2. This table contains calculations relating to the analysis of experiments described in chapter 5, figure 5.11. Data was derived from 3 independent experiments and relates to a comparison of Day 7 Parent +4OHT verses PDX1 ER Line +4OHT. The table contains the results of a unpaired t-test performed using PRISM6.	
Day 7 Parent +4OHT vs PDX1 ER Line +4OHT	
Unpaired t test	
P value	0.0003
P value Summary	***
Significantly different? (P<0.05)	Yes
One or two tailed P value	Two-Tailed
t, df	t= 11.48 df= 4
Mean ± SEM of Parent +4OHT (day 7)	27.33 ± 0.4252, n=3
Mean ± SEM of PDX1 ER Line +4OHT (day 7)	32.91 ± 0.2345, n=3
Difference between means	-5.577 ± 0.4856
95% Confidence Interval	4.228 to 6.925
R squared	0.9706