UNIVERSIDADE DO ALGARVE

DISSERTAÇÃO EM CIÊNCIAS BIOMÉDICAS

TOWARDS DIRECT TRANSDIFFERENTIATION OF ADULT HUMAN CELLS TO THE PANCREATIC β-CELL FATE

TRABALHO EFECTUADO SOB A ORIENTAÇÃO DO PROFESSOR GUSTAVO TISCORNIA

FILIPA INÊS RODRIGUES DOS SANTOS

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Filipa Inês Rodrigues dos Santos

ACKNOWLEDGEMENTS

I would like to thank to Professor Gustavo Tiscornia.

ABSTRACT

Type 1 Diabetes mellitus (T1DM) is one of the most widespread metabolic disorders with epidemic dimension affecting almost 6% of the world's population. Autoimmune reaction causes selective destruction of the insulin-producing β-cells within the pancreatic islets, leading to both acute and long-term complications. Daily insulin injections treat but no dot cure diabetes. Any progress in obtaining large number of transplantable insulin producing cells would be a major advance towards a cure for the disease. In order to create an alternative source of β-cells we are developing a method to transdifferentiate adult human cells to the beta cell phenotype by direct reprogramming mediated by forced expression of an optimized set of pancreas specific transcription factors. The underlying experimental rationale is that sequential or combinatorial ectopic expression of transcription factors can induce recipient cells to establish a β-cell regulatory state. We cloned a set of transcription factors known to be involved in pancreatic development into viral vectors and used them to transdifferentiate adult human cell types in conditions known to favor β-cell differentiation.

RESUMO

A diabetes mellitus tipo 1 (DM1) é uma das alterações metabólicas mais comum a nível mundial, afectando quase 6% da população mundial. É uma doença auto-imune que tem como resultado a destruição das células β do pâncreas, produtoras de insulina. Caracteriza-se por hiperglicémia e insuficiência na produção de insulina, levando a complicações quer a curto quer a longo prazo. Não existe cura para a DM1. O único tratamento para os doentes com DM1 é a administração diária de insulina.

No entanto, a administração de insulina está frequentemente associada a episódios graves de hipoglicémia, para além de que não previne o aparecimento das complicações crónicas associadas à doença, tais como a retinopatia diabética e a doença renal terminal.

A obtenção em larga escala de células produtoras de insulina poderá ser um grande avanço para a cura da diabetes. Um dos métodos mais inovadores para a obtenção de células β é a transdiferenciação de células humanas adultas em células β produtoras de insulina, através da expressão ectópica de um conjunto de factores de transcrição que estão envolvidos no estabelecimento destas células.

O procedimento experimental baseou-se na expressão ectópica destes factores de transcrição para induzir células humanas adultas a estabelecer o estadio regulatório que define as células β. Para tal, foi clonada uma coleção de factores de transcrição envolvidos no desenvolvimento pancreático em vectores virais. Estes vectores foram posteriormente utilizados na transdiferenciação de células humanas adultas em condições que favorecem a diferenciação das células β.

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1. INTRODUCTION

Diabetes mellitus (DM) is one of the most widespread metabolic disorders with epidemic dimension affecting almost 6% of the world's population. In 2011, the International Diabetes Federation estimated that over 366 million people around the world had diabetes and is expected that in 2030, over 552 million people, or one adult in 10, will have diabetes mellitus. The global prevalence of diabetes is shifting significantly from the developed countries to the middle-income countries, which are acquiring a western lifestyle and dietary habits (Figure 1) (Kordowich et al. 2010; Nair & Aloysious 2011; Ashcroft & Rorsman 2012).

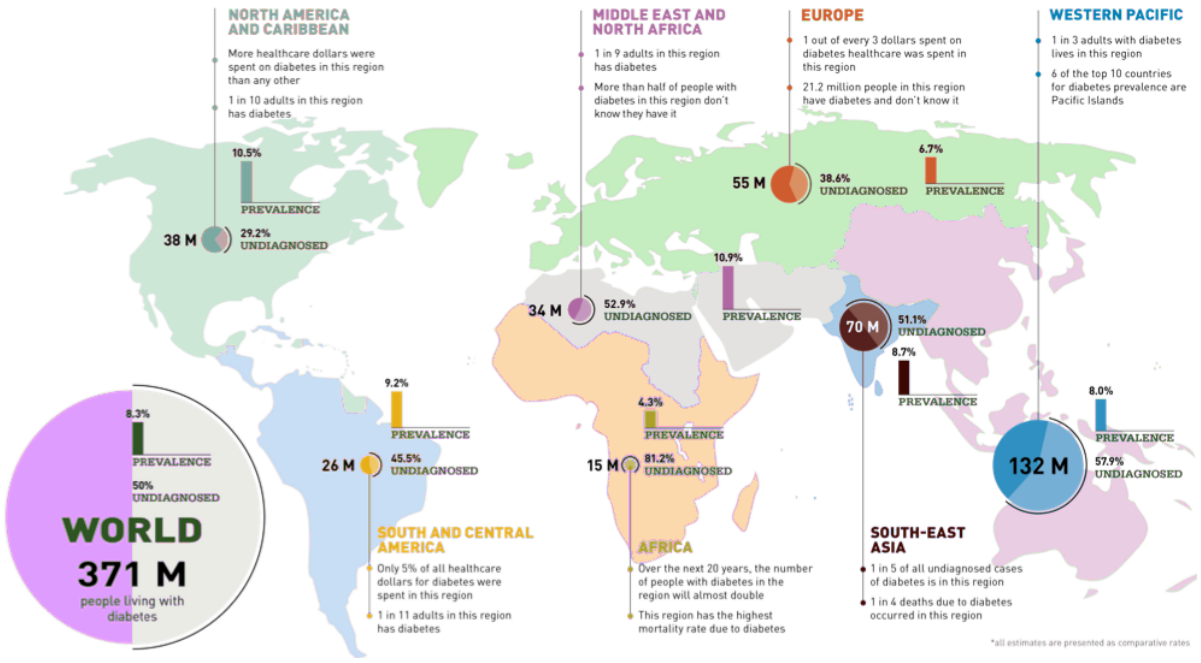

Figure 1: Worldwide diabetes prevalence in 2012. The number of people with diabetes is increasing in every country, with more than 371 million people worldwide being affected by the disease. Half of people with diabetes are undiagnosed. These estimates demonstrate the urgency for an effective prevention and treatment. All estimates are presented as comparative rates. *Adapted from* IDF Atlas fifth edition (In: http://www.idf.org/diabetesatlas).

DM is a chronic disorder that results from the targeted destruction of the insulin-producing beta(β)-cells in the pancreatic islets and it is characterized by high concentrations of blood glucose (hyperglycaemia). Insulin is produced and secreted by the β-cells. Cellular

uptake of glucose from the blood is regulated by the appropriate secretion of insulin by the pancreatic β-cells (Best et al. 2008; Domínguez-Bendala & Ricordi 2012a). Left untreated, this disease is fatal. However, even with correct medical treatment, DM patients may suffer from acute and long-term complications associated with the disease. The most common acute complication is diabetic ketoacidosis, a potentially life-threatening complication, which can lead to death if not treated over the period of 24 hours. Long-term complications include increased cardiovascular disease and accelerated atherosclerosis, hypercoagulability, amputation, retinopathy, nephropathy, neuropathy, dyslipidemia, microvascular damage to retina, with poor quality of life and death (Van Hoof et al. 2009).

Diabetes is manifested in two distinct forms: an absolute deficiency of insulin (type 1) and a relative deficiency of insulin (type 2) diabetes. Type 2 diabetes mellitus (T2DM) is caused by a combination of insulin resistance in varying degrees and β-cell failure, with a decrease by around 50% in the β-cell mass, together leading to inadequate insulin secretion which is not sufficient to maintain glycaemic control (Donath & Halban 2004; Pagliuca & Melton 2013).

Although most attention has focused on the increase in type 2 diabetes, a parallel rise in type 1 diabetes has occurred (Onkamo et al. 1999).Type 1 diabetes mellitus (T1DM) is a cell-specific autoimmune disorder in which the immune system of the affected individual attacks and destroys the pancreatic β-cells that secrete insulin in response to elevated blood sugar levels. It is usually (but not always) diagnosed during childhood and for that reason it is also referred as juvenile diabetes. T1DM affects genetically predisposed individuals.

There are susceptibility genes that are thought to be important regulators of the immune response. Susceptibility is largely inherited, residing predominantly in the human leukocyte antigen (HLA) class II DR and DQ, and to a lesser extent in a host of other genetic loci called insulin-dependent diabetes mellitus susceptibility genes (IDDM). The HLA locus has been known to confer 50% of the genetic susceptibility to the disease. There are other two candidate genes that, together, were confirmed to contribute about 15% of the risk, insulin-VNTR (IDDM2) and CTLA-4 (IDDM12) genes (Table 1). However, more elucidations about the mechanism by which they alter biology to predispose to disease remains to be elucidated. (Anjos & Polychronakos 2004; Devendra et al. 2004; Lambert et al. 2004; Concannon et al. 2009)

Table 1 - Important susceptibity loci for type 1 diabetes.

	Chromosome	Candidate genes/markers
IDDM1	6p21.31	HLA DR/DQ region Major role in presentation of peptides to T cells
IDDM2	11p15.5	Insulin-VNTR Protection associated with greater thymic insulin message
IDDM12	2q33	CTLA-4, CD28 Related to T-cell activation, thyroid autoimmunity

For many of those not listed (IDDM 3-11, 13-17), either the responsible gene remains unidentified or the gene function is uncertain in relation to type 1 diabetes.

Adapted from (Denis Daneman 2006).

T1DM can be also triggered by environmental factors that alter immune function, thereby initiating β-cell destruction. Putative triggers include viruses, such as congenital rubella or enteroviruses (but so far only congenital rubella syndrome has been associated with the disease), and environmental toxins, for example nitrosamines. Also, recent studies suggest that early exposure to cow's milk proteins, cereals or gluten may increase the risk of Type 1 diabetes. However, this needs to be confirmed (Devendra et al. 2004)

Approximately 10% of all diabetics suffer from Type 1 diabetes. In addition to racial and regional differences involving the genetic background and environmental triggers, other putative reasons for the increase in T1DM frequency are the rise of childhood obesity and increasing sedentary lifestyle, which leads to metabolic stress by development of insulin resistance and inflammatory injury of β-cells, causing their functional exhaustion and accelerating the onset and progression of the disease (The Diabetes Control and Complications Trial Research Group 1993; Yoon & Jun 2005; Denis Daneman 2006; Gangaram-Panday et al. 2007; Pozzilli et al. 2011).

The abnormal activation of the T-cell-mediated immune system in susceptible individuals leads to an inflammatory response within the islets (insulitis) as well as to a humoral response with production of antibodies to β-cell antigens (Devendra et al. 2004). This chronic process leads to selective destruction of the insulin-producing β-cells within the pancreatic islets. The resulting complete deficit of insulin, the main hormone regulating glucose as well as lipid and protein metabolism, causes hyperglycaemia (The Diabetes

Control and Complications Trial Research Group 1993; Zimmet et al. 2001; Steiner 2006; Van Hoof et al. 2009).

Because type 1 diabetic patients are unable to produce insulin, it is necessary to treat them with exogenous insulin in a daily basis, in order to maintain blood glucose levels within acceptable limits. Currently, insulin therapy is the treatment of choice for these patients, associated with specific diet and physical exercise programs (Zimmet et al. 2001; D Daneman 2006; Gangaram-Panday et al. 2007).

However, intensive insulin treatment has been associated with increasing severe hypoglycaemia, which is a frequent side effect of insulin therapy and a major obstacle to achieve normal glucose levels, leading to deterioration of glucose control and cardiovascular accidents. Up to 10-20% of long-standing T1DM patients have unstable metabolic control and hypoglycaemic episodes, which taken together can lead to progressive complications, as mentioned before. Trying to achieve glycaemic control in between narrow ranges is important, as the mortality rate in such individuals is high (McCrimmon 2008).

Whole pancreas transplant/Pancreas transplant alone (PTA) counteract many of the difficult aspects of type 1 DM, alleviating insulin-dependence, with improvement of glycaemic control, reduction of severe hypoglycaemic episodes, thus improving the quality of life of the patients and preventing long-term diabetic complications (The Diabetes Control and Complications Trial Research Group 1993; Ryan et al. 2006; Collombat et al. 2010). Nevertheless, the acute risks of pancreas transplant alone (PTA) include rejection, graft pancreatitis, intra-abdominal infection, venous or arterial thrombosis, and others (Gruessner & Sutherland 2005).There are also the long-term risks of immunosuppression with a possible increased risk of death. Thus, a PTA should be considered only if there are major concerns with glycaemic control not amenable to current therapy, since it has risks inherent to any major surgery (Humar et al. 2000; Becker & Odorico 2001; Ryan et al. 2006).

β-cell replacement therapy by allogeneic pancreatic islet transplantation might be a less invasive and safer option than pancreas transplantation, since the latter has a higher risk of perioperative morbidity and mortality. The islet transplant is a simpler procedure that involves percutaneous cannulation of the portal vein. Still, there is an associated risk of bleeding, puncture of the gallbladder, or abdominal pain (Ryan et al. 2002).

A final problem is the scarcity of pancreatic donors, which increases the imbalance between supply and demand. Thus, the protocol for islet transplantation remains far from ideal, as it requires a large supply of cadaveric material to treat a single patient (Ryan et al.

2006; Best et al. 2008; Ben-Othman et al. 2013a). Limited organ availability and the transplant associated immunosuppressive therapies prevent transplantation from becoming a widely available treatment option (Domínguez-Bendala & Ricordi 2012b).

For that, other alternatives must be found in order to efficiently treat the consequences of T1DM, but also type 2 diabetes, since both diseases will eventually result in a loss and/or insufficient number of β-cells (Kordowich et al. 2010; Collombat et al. 2010; Ben-Othman et al. 2013a).

In search for alternative treatments for T1DM, researchers are trying to find different sources of β-cells. An interesting option could be the generation of pancreatic β-cells from stem cells, progenitor or other cell subtypes, which represents one of the most promising research fields for β-cell replacement therapy. β-cells are an attractive case for cell replacement therapy because only a single cell type is missing and replacement can occur in non-endogenous sites, a surgical advantage since cells can be placed subcutaneously in minimal invasive surgeries.

However, it is still not known how to generate these fully functional β-cells. Therefore, to design rational protocols allowing the *in vitro* or *in vivo* generation of β-cells, it is important to gain further knowledge about the molecular mechanisms underlying the development of embryonic and adult β-cells, *in vivo* (Kordowich et al. 2010). A deeper understanding of the normal developmental processes that occur during pancreas formation, (transcription factors, extracellular signals, signaling pathways) is the key to understand cell fate decisions that determine whether or not progenitor cells will become islets and it is of greatest importance to the engineering of other cell types into β-cells (Van Hoof et al. 2009; Rieck et al. 2012; Ben-Othman et al. 2013a; Pagliuca & Melton 2013).

1.1 Pancreas Development

Most of our knowledge on pancreatic development comes from studies in model organisms, such as the mouse model. In fact, the most important molecular players are highly conserved between mouse and human.

Pancreas development can be divided in four main stages: 1) formation of definitive endoderm, 2) foregut differentiation, 3) endocrine specification, and 4) β-cell differentiation (Figure 2) (Domínguez-Bendala 2009; Guney & Gannon 2009; Van Hoof et al. 2009).

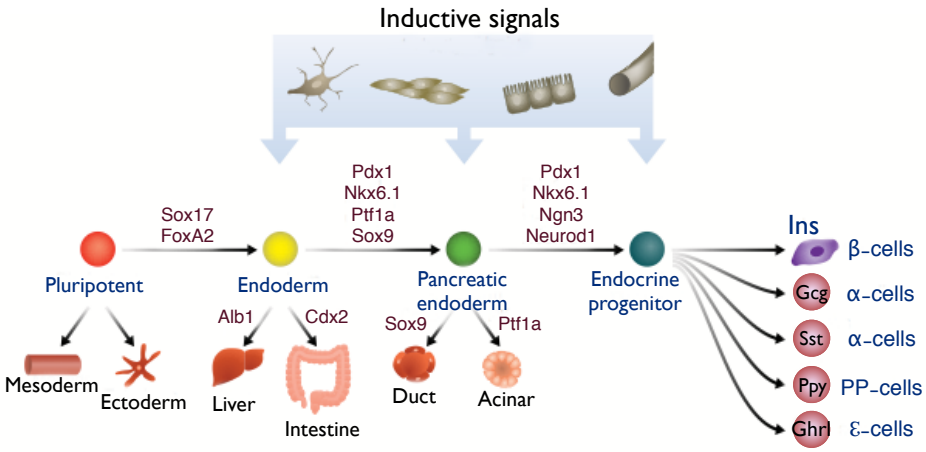

Figure 2: Critical developmental milestones for β-cell development. Pluripotent stem cells first acquire the identity of one of the three germ layers; pancreatic cells arise from the endodermal layer. A subset of endoderm is specified by Pdx1 expression to become pancreatic endoderm, which will further differentiate into acinar, ductal or endocrine fate. Endocrine progenitors express Ngn3 and consequently differentiate into the five hormone-expressing cell types within the islets, which will depend on which transcription factors are being expressed. Some of the most relevant transcription factors are listed. Ins, Insulin; Ccg, Glucagon; Sst, Somatostatin; Ppy, Pancreatic polypeptide; Ghrl, Ghrelin. *Adapted from* (Pagliuca & Melton 2013).

A variety of studies in the mouse model provided further insight on the morphological aspects of early pancreas formation.

Gastrulation defines the cellular organization in the three germ layers: ectoderm, mesoderm and endoderm. Closure of anterior endoderm will form the primitive gut tube, which will further develop into the foregut, the midgut and the hindgut. Several organs derive from endoderm, such as pancreas, liver, thyroid, lungs and intestine (Deutsch et al. 2001; Best et al. 2008). In the mouse, the gut tube becomes evident at embryonic day (e) e8.5-e9.0, after closure of anterior endoderm. The pancreas arises from two different embryonic growths of the dorsal and ventral regions of the foregut endoderm (Slack 1995). In contrast, the liver

develops from the ventral region, next to the ventral pancreas (Zaret 1996; Wells & Melton 1999; Zaret 2000).

The dorsal bud develops in proximity to the notochord (possibly mediated by a FGF or activin) and later to the dorsal aortic endothelial cells, that together provide inductive signals for early bud formation (Kim et al. 1997; Lammert et al. 2001). It is characterized by a robust expression of Pdx1 at e9.0, when the embryo turns and the endoderm is still closely associated with the notochord. The ventral bud develops from two lateral areas of the endoderm at the same level. However, the ventral and dorsal pancreas develop by means of different mechanisms due to different tissue formation context, i.e., opposing to the dorsal pancreas, the ventral pancreas will develop in close association with presumptive hepatic and bile duct endoderm. Despite that, communication between ventral pancreas and liver has not been reported, it is possible that interaction between these structures might be important for pancreas morphogenesis (Wells & Melton 1999; Jørgensen et al. 2007; Stanger & Hebrok 2013).

At embryonic day (e) 9.5 in the mouse (or gestational day 25 in humans), the dorsal and the ventral buds begin to evaginate: the pancreatic buds elongate and branch, sending finger-like epithelial protrusions into the surrounding mesenchyme with subsequent branching morphogenesis (Jørgensen et al. 2007; Guney & Gannon 2009). Lineage tracing studies have shown that early pancreatic buds are composed of multipotent pancreatic progenitor cells that give rise to exocrine, endocrine and duct cells (Zhou et al. 2007).

By e10.5, insulin and glucagon-positive cells can be detected in the primitive buds, but they do not express markers of mature β- and α- cells, respectively and it is thought that they are not the precursors of mature islets (Herrera 2000).

At e12.0, the dorsal and ventral buds start to rotate and give rise to one interconnected organ (Jørgensen et al. 2007; Guney & Gannon 2009).

At e13 (until e16 in the mouse), dramatic changes occur in the cellular architecture of the pancreas. A process named secondary transition initiates, during which a remarkable increase in the number of endocrine cells budding from the ductal epithelium is detected, particularly β-cells. Most of the hormone-expressing cells that will contribute to the formation of the mature islets begin to emerge at this stage, except for the early glucagon-positive endocrine cells, observed since e9.5 (Jørgensen et al. 2007; Oliver-Krasinski & Stoffers 2008). At the same time, rapid branching morphogenesis takes place, as well as acinar cell differentiation with increases in acinar enzyme gene expression (Gittes 2009).

To note that, despite most of the important molecular players are highly conserved between mouse and human, there are some differences in the overall morphology of the mature pancreas between humans and mice (Table 2). A brief example is the obvious difference in duration of pancreas formation. Also, in contrast to the organized mouse islets, that contain a large core of β-cells surrounded by a layer of α-cells and other endocrine cells, the human endocrine cells that compose islets have a more random pattern (Wieczorek et al. 1998).

Table 2 - Correspondence between human and mouse pancreatic development

Mouse developmental stage	Event	Human developmental stage
E8.5-9.5	Initiation of pancreatic development; evagination from the primitive foregut; Pdx1 expression	3-4 w.p.c
E9.5-10.5	Immunodetection of glucagon expression	8.5 w.p.c
E10.5-11.5	Immunodetection of insulin expression	7 w.p.c
E12.5	The two pancreatic buds fuse at the base	8 w.p.c
E13.5-14.5 onward	Formation of acini from ducts Initiation of secondary transition Immunodetection of somatostatin expression	8.5 w.p.c onward
E16.5-18.5	Immunodetection of PP expression	10 w.p.c
	Islet formation	12-13 w.p.c.

(E - embryonic day; w.p.c - weeks postconception)

Adapted from (Domínguez-Bendala 2009)

1.2 Anatomy of the Mature Pancreas

1.2.1 The β-cell

The mature pancreas is a solid glandular organ in the gastrointestinal tract and plays a crucial role in maintaining nutritional homeostasis through synthesis and secretion of hormones and enzymes (Kordowich et al. 2010). Three functionally distinct tissue types compose the mature pancreas: acinar, ductal and endocrine.

The exocrine pancreas consists of acinar cells that secrete digestive enzymes, such as amylases, lipases, proteases and nucleases into the pancreatic duct. Acinar cells also produce bicarbonate ions and electrolytes, which together with exocrine enzymes, are transported through the main duct into the duodenum, contributing to the food processing and digestion (Githens et al. 1994). Acinar and duct cells represent approximately 98% of the total organ mass. Endocrine cells represent less than 2% of the pancreatic tissue regulate nutrient metabolism and glucose homeostasis (Ben-Othman et al. 2013b).

The endocrine component of the pancreas is organized in small clusters of cells termed islets of Langerhans, first identified by the biologist Paul Langerhans in 1869. Each islet is a micro-organ containing hormone-producing cell types including alpha (α), beta (β), delta (δ), epsilon (ε) and PP cells, which produce and secrete glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively (Adrian 1978; Wierup et al. 2002; Heller et al. 2005; Collombat et al. 2006; Guney & Gannon 2009). Specifically, the hormones insulin (β-cells) and glucagon (α-cells) maintain glycaemic homeostasis (70-100mg/dl) by regulating the storage, metabolism and neogenesis of glucose.

Insulin is secreted in response to a variety of stimuli, including parasympathetic signals as acetylcholine (Gilon & Henquin 2001), the incretin peptides glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), high blood glucose levels and glucagon (Figure 3) (Baggio & Drucker 2007). Insulin activates the cellular uptake of glucose, by inducing its storage in the muscle, liver or adipose tissues, thereby reducing the blood glucose levels. In the liver, glucose is converted in glycogen, a reaction catalysed by the enzyme glycogen synthase. Liver glycogen serves as the main repository of glucose (Agius 2008).

On the other hand, when sugar levels are low, α-cells counteract the effects of insulin by secreting glucagon that will induce the catabolism of glycogen in the liver, in a process

called glycogenolysis. Glucagon also stimulates the conversion of noncarbohydrate substrates into glucose (gluconeogenesis) Taken together, theses processes will result in an increase of circulating glucose (Ben-Othman et al. 2013b).

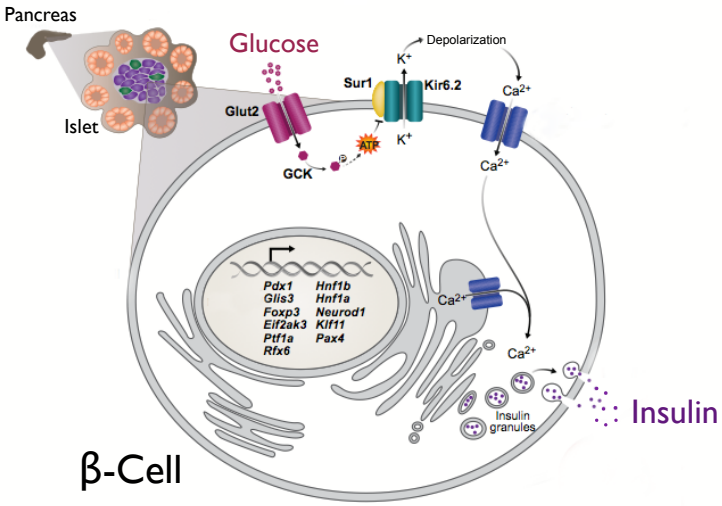

Figure 3: Functional β-cells secrete insulin in response to increasing glucose levels. In glucose-stimulated insulin secretion (GSIS), glucose is transported into the cell through glucose transporters (for example Glut1 or Glut2), where it is phosphorylated by glucokinase (GCK). The following metabolic reactions increase ATP levels. Rising ATP levels trigger the closure of potassium channels (Sur1 and Kir6.2 subunits), causing membrane depolarization and the opening of calcium channels. The resulting increase in intracellular calcium levels stimulates the exocytosis of insulin-containing granules and consequently leads to an increase in insulin levels in neighbouring blood vessels. Human genetic studies of maturity onset diabetes of the young (MODY) patients have identified a number of mutations that trigger diabetes, including those in genes encoding transcription factors (illustrated in the nucleus) and components of the GSIS pathway indicated in the figure. *Adapted from* (Pagliuca & Melton 2013).

1.2.2 Islet vasculature

The typical view of the islet cellular architecture has been formed based on early observations on the mouse, where β-cells cluster preferentially at the core of the structure and alpha, delta and PP cells are peripherally arranged. However, there are differences between species both in islet cell composition and cytoarchitecture: in human and nonhuman primates, alpha-cells are scattered throughout the islet rather than concentrated in the periphery (Wieczorek et al. 1998; Ballian 2007). There is evidence that these architectural differences have functional implications, suggesting that there is a possible correlation between the

pattern of distribution (clustered or scattered) and islet cell function. However, additional studies are needed to clarify this hypothesis (Ballian 2007).

Islets are very vascularised, to ensure the efficient secretion of endocrine hormones into the bloodstream. A complex network of capillary vessels and connective tissue, critical to their high metabolic activity, surrounds adult islets. Thus, although islets compose 1-2% of the total mass of the pancreas, they receive almost 15% of the overall blood flow to the organ, using 25% of the pancreatic oxygen supply. Islet blood flow is regulated by extracellular signals, such as hormones and nutrients that reach the islet vasculature from other tissues via the bloodstream. Also, islet perfusion determines communication between endocrine and exocrine cells and between different types of endocrine cells within islets (Konstantinova & Lammert 2004; Ballian 2007).

1.3 Pancreatic Specification

Pancreas formation is dependent on several successive prerequisites that need to be fulfilled. *Sonic hedgehog (Shh)* signaling mediates the gut endoderm patterning. Shh is highly expressed in the gut epithelium, but it is down-regulated in a *Ptf1α(p48)/Pdx1* region that will latter become the pancreas at e8.0. Both Shh repression and activation of Ptf1α and Pdx1 are required events for pancreatic specification. The areas defined by expression of Pdx1 and repression of Shh will start to branch out dorsally and ventrally (Domínguez-Bendala 2009).

1.3.1 Transcription factors involved in pancreatic differentiation

Pdx1

The pancreatic and duodenal homeobox 1 gene (*Pdx1*) is also known as insulin promoter factor 1 (*Ipf1*) or islet duodenum homeobox 1 (IDX1). It is expressed in islet β-cells, in the adult mouse, and its function is to bind and regulate the insulin promoter. During development, *Pdx1* expression is restricted to the dorsal and ventral walls of the pancreatic buds (Ohlsson et al. 1993). Mice homozygous for a targeted mutation in the *Pdx1* gene selectively lack the pancreas, demonstrating that *Pdx1* is needed for the formation of the pancreas. Also, direct cell lineage tracing in the mouse pancreas demonstrated that *Pdx1*-

expressing progenitors in the early embryo give rise to all pancreatic cells (Gu et al. 2003). Thus, *Pdx1* stimulates insulin gene transcription. Taken together, these data suggest that *Pdx1* is needed not only for early development of the primitive gut but also for the maturation of the β-cell (Jonsson & Ahlgren 1995).

Ptf1α (p48)

Ptf1α is the α-subunit of the pancreas-specific transcription factor 1 (*Ptf1*), a basic helix-loop-helix (bHLH) protein that was first described as a DNA-binding element that regulates the expression of α-amylase 2, elastase 2 and trypsin in the acinar pancreas (Cockell et al. 1989). This gene is key regulator of acinar tissue development, but also important for the initiation of pancreatic development (Krapp et al. 1998).

HNF-6 (OC-1)

HNF-6 (hepatocyte nuclear factor), also known as *Onecut-1* (OC-1), belong to the OC family of transcription factors (Lemaigre et al. 1996). During development, *HNF-6* is expressed in the epithelial cells that are precursors of the exocrine and endocrine pancreatic cells. *HNF-6* binds to and stimulates the *Ngn3* gene promoter (Jacquemin et al. 2000). Thus, *HNF-6* acts upstream of *Pdx1*, controlling its activity in the ventral and in the dorsal endoderm, highlighting its importance as a key regulator of pancreas development (Jacquemin et al. 2003).

Hlxb9

Human homeobox gene 9 (*Hlxb9*), also termed as *HB9*, its encoding protein (Harrison et al. 1994). *Hlxb9* regulates gene expression early in development (e8) in the notochord and in pancreatic endoderm (dorsal and ventral) (Li et al. 1999). It is also expressed in fully differentiated β-cells. *Hlxb9* expression precedes that of *Pdx1*, suggesting an active role in shaping the early events of pancreatic specification. *Hlxb9* knockouts show agenesis of the dorsal pancreas, with abnormal development of the ventral lobe (Harrison et al. 1999; Li et al. 1999).

1.3.2 Endocrine specification

Ngn3

Neurogenin 3 (*Ngn3*) encodes a class B bHLH factor. *Ngn3* induces the endocrine fate choice (Apelqvist et al. 1999), being a required factor for the development of all the endocrine cell lineages of the pancreas. Gain-of-function studies (Apelqvist et al. 1999), as well as lineage tracing experiments show that *Ngn3* is a cell-autonomous determinant and true marker of endocrine progenitor cells (Gu et al. 2002; Gu et al. 2003). The pattern of adoption of endocrine cell fates seems to have a specific timeline, suggesting that Ngn3-positive cells adapt their responses to the changing signaling in the bud environment. Early *Ngn3* expression in pancreatic progenitor cells (e8.5-e9) results in their differentiation into glucagon-producing cells (Domínguez-Bendala 2009). In contrast, during the secondary transition, there is a second wave of *Ngn3* expression, which activates the expression of other endocrine transcription factors, such as *Neurod1, Pax4* and *Nkx2.2* and the latter two will drive the differentiation into β-cells (Van Hoof et al. 2009).

Isl1

Isl1 is a LIM homeodomain protein, a family of proteins with a DNA binding homeodomain and two LIM domains, (the LIM domains are 50–60 amino acids in size and share two charac- teristic zinc finger domains, which are separated by two amino acids) (Zheng & Zhao 2007). During development, *Isl1* is required for the formation of the dorsal pancreatic mesenchyme. Also, *Isl1* expression in pancreatic epithelial cells drives differentiation of islet cells and it is expressed upon maturation of endocrine cells (e9 for glucagon-positive cells, e10.5-11 for insulin-positive cells) (Adolphs et al. 1997).

NeuroD/BETA2

BETA2, (beta-cell E-box transactivator 2, also known as *NeuroD*) is a cell-restricted bHLH, expressed in pancreatic β-cells, that was shown to be a component of the native insulin E-box-binding complex, which suggests that *BETA2* is an important regulator of the insulin gene (Naya et al. 1995). *BETA2*-deficient mice showed a dramatic reduction in the

number of β-cells and failed to develop well-organized, mature pancreatic islets, indicating that this transcription factor may be involved in the maintenance and proliferation of the islet cell types (Naya et al. 1997). Finally, *Ngn3* was shown to be involved in the upstream regulation of *BETA2* expression at an early stage of islet cell differentiation (Huang et al. 2000).

1.3.3 Islet cell sub-types: β-cell differentiation

While all the cells of the endocrine pancreas are thought to arise from a common endodermal precursor, little is known about the extracellular signals that direct beta cell differentiation from $Ngn3^+$ progenitors (Sussel et al. 1998). Here, a special emphasis will be done on the differentiation of endocrine β-cells, which are destroyed in T1DM, highlighting the role of the genes that act as master regulators in this transition.

Nkx2.2

Nkx2.2 is a member of the mammalian NK-homeobox transcription factor family and its expression is essential for normal beta cell development (Sussel et al. 1998). *Nkx2.2* is initially expressed at e9.5, upon formation of the dorsal pancreatic bud. However, during the secondary transition, when different exocrine and endocrine compartments can be identified, *Nkx2.2* expression becomes limited to most of the endocrine cell types, being further restricted to α, β and PP cells of the mature islet**.** In the adult, expression of *Nkx2.2* is preserved and is not detectable in the exocrine tissue (Sussel et al. 1998; Sander et al. 2000).

Nkx6.1

The transcription factor *Nkx6.1* is another member of the NK-homeodomain family. In the pancreas, *Nkx6.1* has an expression pattern similar to that of *Nkx2.2*, being first detected at e10.5 in the pancreatic epithelium. The major difference is that *Nkx6.1* expression becomes restricted to β-cells. Homozygotic mutation of the *Nkx6.1* gene in mice inhibits β-cell formation, which becomes evident during the secondary transition. *Nkx2.2* expression was not affected, suggesting that this gene acts upstream of *Nkx6.1*. Double *Nkx2.2/Nkx6.1* knockout mice experiments confirmed this hypothesis (Sander et al. 2000).

Pax4

Paired box-containing gene 4 (*Pax4*) is a member of the paired domain family of transcription factors, a family that shares a highly conserved motif with DNA-binding activity, named "paired box". Also, both *Pax4* and *Pax6* have a homeodomain (Walther et al. 1991; Dohrmann et al. 2000). $Pax4^{-/-}$ deficient mice do not generate neither insulin-producing β-cells nor δ-cells within the pancreas (Sosa-Pineda et al. 1997). There is also evidence that *Pax4* acts as a direct downstream target of *Ngn3* (Heremans et al. 2002; Smith et al. 2003).

Pax6

Paired box-containing gene 6 (*Pax6*) is also a member of the paired domain family of transcription factors (Walther et al. 1991). *Pax6* is known for being expressed in the CNS (Walther & Gruss 1991) and for its crucial role in eye development (Richardson et al. 1995). However, it is also expressed both in pancreas development and latter in alpha-, beta- or delta-cells in the adult pancreas (it is not expressed in the acinar tissue) (Sander et al. 1997; Dohrmann et al. 2000). *Pax6*-deficient mice show a dramatic reduction in all endocrine cells, with α-cells almost absent, suggesting that *Pax6* is required for the formation of α-cells (St-Onge et al. 1997).

MafA and MafB

MafA belongs to the *Maf* gene family of transcription factors containing a basic leucine zipper. This family has been associated with the regulation of cell differentiation in vertebrates (Ochi et al. 2004). *MafA* is expressed within the pancreas, preferentially in β-cells whereas *MafB* is expressed in α-cells. *MafA* has been identified as a transcription factor that binds to a promoter element of the insulin gene and is thought to regulate insulin transcription in response to serum glucose levels (Kataoka et al. 2002). *MafA* Knockout mice revealed glucose intolerance and developed diabetes, as well as diminished insulin transcription. Another observation was that *MafA*-deficient mice were unable to respond to glucose-stimulated insulin secretion (GSIS), suggesting that *MafA* is a key regulator of GSIS (Zhang et al. 2005).

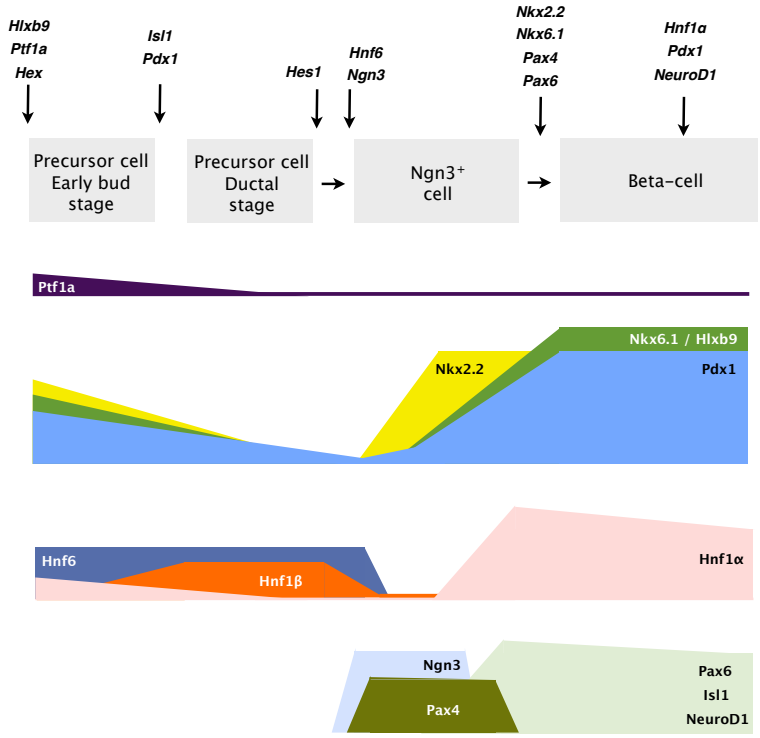

Figure 4: Essential expression patterns of transcription factors during beta-cell formation. At the top, arrows point to the developmental steps at which the indicated transcription factors have been shown to be indispensable in genetic knock-out studies of mice, irrespective of when these genes are expressed. At the bottom is a representation of the relative expression levels of different transcription factors throughout pancreatic development.

1.4 Possible strategies to generate new pancreatic β-cells

Insulin injections treat but do not cure T1DM (Best et al. 2008). Islet transplantation allows for insulin-independency in for a number of years. However, there are some obstacles underlying this approach since there are no enough donors to balance the demand, and it involves possible toxic imunossuppression (Halban 2004). To overcome this problem, the large scale in vitro production of β-cells for replacement therapy offers an aspiring solution.

The strategies for in vitro beta cell production are: 1) step-wise derivation of insulin-producing cells from embryonic stem cells, 2) In vitro β-cell expansion and redifferentiation, and 3) Reprogramming of a terminally differentiated cell type into insulin-producing cells.

1) Step-wise derivation of insulin-producing cells from embryonic stem cells

Embryonic stem cells (ESCs) have not been differentiated yet into phenotypically normal β-cells. Current protocols try to mimic the normal differentiation pathways that take place during normal pancreatic development. However, the major challenge is to discover strategies for designing protocols that follow the normal development. D'Amour et al. were the first to develop an efficient in vitro protocol for early induction of ESCs to a foregut-like phenotype by the addition of Activin-A, followed later by induction to a more specific pancreatic phenotype (D'Amour et al. 2005; D'Amour et al. 2006). Still, further improvements are necessary for generating functional β-like cells.

2) In vitro β-cell expansion and redifferentiation

Another strategy for producing β-cells is the induction of in vitro replication of existing β-cells to provide abundant source of human insulin-producing cells. However, the major obstacle is that the expansion of isolated human islets in tissue cultures is very difficult, since the islets show partial or complete loss of function (Beattie et al. 2002). In another hand, a recent cell-lineage report showed that islet insulin-expressing cells maintain a renewal capacity for islet regeneration after injury (Dor et al. 2004). Ouziel-Yahalom et al. described culture conditions that allowed replication of dedifferentiated human islets in culture and later favored their redifferentiation using a number of factors known to allow β-cell differentiation and proliferation. Nevertheless, these new differentiated cells were not glucose-responsive (Ouziel-Yahalom et al. 2006).

3) Reprogramming of a terminally differentiated cell type into insulin-producing cells

An alternative strategy to differentiating β-cells in a stepwise approach from embryonic stem cells is the reprogramming of terminally differentiated cell types into β-cells. This method for direct reprogramming allowed the reprogramming of fibroblasts to generate

iPSCs, cardiomyocytes, or neurons (Takahashi et al. 2007; Ieda et al. 2010; Vierbuchen et al. 2010). Due to this success, reprogramming other cell types into the β-cell phenotype could be of relevance in the regenerative medicine field.

One example of this reprogramming strategy was the demonstration of the direct reprogramming pancreatic exocrine tissue into the β-cell fate *in vivo* using a combination of three genes (*Pdx1*, *Ngn3* and *MafA*), delivered by adenoviral vectors (Zhou et al. 2008). The new insulin-positive cells were detected after injection of the adenoviral mix into the pancreas of $Rag1^{-/-}$ mice (a strain used to decrease the immune response induced when using a viral delivery method) (Wang et al. 2007). These cells expanded for up to three months after injection, after the adenoviruses had been cleared from the recipients. Importantly, these cells also expressed key markers of β-cell phenotype, such as *Nkx6.1*, *Glut2* and glucokinase and no longer expressed markers for the acinar function (Zhou et al. 2008). These induced cells showed to improve glycaemic control in diabetic mice, although diabetes was not completely reversed. One possible explanation for this observation could be that new insulin-positive cells did not cluster to form islets and β-cell communication is crucial for stimulation of glucose-mediated insulin secretion (Zhou et al. 2008).

Although acinar cells had been reprogrammed into β-cell in vivo, there is still not known how to drive differentiation of mouse or human cell types into a β-cell *in vitro.* This might be explained by the insufficient knowledge about the in vitro culture conditions that are necessary for the preservation of β-cell identity and function (Pagliuca & Melton 2013).

Another example of terminally differentiated cell reprogramming into β-cells was reported recently, this time using mouse endocrine α-cells. Mansouri et al. revealed that ectopic expression of *Pax4* was sufficient to transdifferentiate α-cells into β-cells *in vivo* (Collombat et al. 2009), which correlates with the common developmental pathway of these cell types. Also, current studies reported that α-cells have bivalent chromatin signatures at genes that are active in β-cells. This report suggests that α-cells may have the inherent capacity to be reprogrammed into β-cells, since this β-cell genes have already the capacity to be active (Bramswig & Everett 2013).

1.5 Aim of this work

Any progress in obtaining large number of transplantable insulin producing cells would be a major advance towards a cure for the disease.

The goal of this work was to set up the required tools to transdifferentiate adult human cells to the beta cell phenotype by direct reprogramming mediated by forced expression of an optimized set of pancreas specific transcription factors. The underlying experimental rationale is that sequential or combinatorial ectopic expression of transcription factors can induce recipient cells to establish a beta cell regulatory state.

1.6 Strategy

1.6.1 Pancreas Specific Transcription Factors

Differentiated adult somatic cells retain the capacity to be reprogrammed into other cell types (L. Baeyens et al 2009). This process presumably occurs because there is a change in the expression of key transcription factors - also called master switch genes - that alters the state of cell developmental commitment (Table 3).

Ectopic expression of particular combinations of transcription factors can result in direct conversion of one cell type into another by altering the underlying regulatory state of the initial cell (Papp & Plath 2011). Therefore, the main goal was to create a library of transcription factors known to play key roles in normal pancreatic development in doxycycline inducible lentiviral vectors and to attempt to find a combination of factors capable of transdifferentiating human fibroblasts to the β-cell state.

The strategy is to transduce an easily obtainable human cell type with the library and culture the cells in conditions known to favor beta cell phenotype maintenance.

Table 3 - Summary of transcriptional regulators involved in the formation of the early gut tube through to the specification of islet cell type

Cell type	Transcription factor	Function	Reference
Gut tube	GATA4	Foregut morphogenesis; differentiation of both exocrine and endocrine lineage; transactivates glucagon gene	Bossard and Zaret, 2000; Kertola et al., 2004; and Ritz-Laser et al., 2005
	FOXA2	Homozygous knockout lethal in mice due to lack of endoderm and notochord; required for alpha-cell lineage	Monaghan et al., 1993; and Lee et al.,2005
Pancreas specification	PDX1	Essential for pancreatic development; differentiation of alpha- and beta-cells; transactivator of insulin gene	Jonsson et al., 1994; Offield et al., 1996; and Hui and Perfetti, 2002
	SOX9	Expressed in all pancreatic progenitors cells; islet organization; restricted to duct cells later in development	Piper et al., 2002; Akiyama Ddagger et al., 2005; and Seymour et al., 2007
	SOX4	Broadly expressed in pancreatic buds with subsequent restriction to islets	Wilson et al., 2005
	HLXB9	Expressed in all pancreatic progenitors with restriction to beta-cells during differentiation	Harrison et al., 1999; and Li et al., 1999
	PBX1	Expressed in pre-pancreatic epithelium; required for both exocrine and endocrine differentiation	Dutta et al., 2001; and Kim et al., 2002
	PTF1a	Expressed in all pancreatic progenitors; essential for exocrine development	Krapp et al., 1996; and Kawaguchi et al., 2002
	HNF1β	Expressed in early endoderm; regulator of HNF6 expression	Barbacci et al., 1999; and Poll et al., 2006
	HNF6	Pancreatic precursor cell specification; regulates PDX1 and NGN3 expression; islet and duct cell formation	Jacquemin et al., 2000; Jacquemin et al., 2003; and Poll et al., 2006
Endocrine specification	NGN3	Differentiation of endocrine lineage	Apelqvist et al., 1999; Jensen et al., 2000; Schwitzgebel et al., 2000; and Sugiyama et al., 2007
	ISL1	Differentiation of all islet cell types	Apelqvist et al., 1997;
	NEUROD1	Differentiation of all islet cell types; islet organization; transactivates insulin gene	Naya et al., 1997;
Islet cell subtypes	HNF4α	Transactivation of HNF1α and insulin gene	Gragnoli et al., 1997; and Bartoov-Shifman et al., 2002
	HNF1α	Transactivation of PDX1 and insulin gene	Emens et al., 1992; and Guerrish et al., 2001
	PAX6	Differentiation of all islet cell types; transactivates glucogon gene	Sander et al., 1997; and St-Onge et al., 1997
	PAX4	Formation of alpha- and beta-cells	Sosa-Pineda et al., 1997; and Smith et al., 1999
	NKX2.2	Transactivation of NKX6.1 and insulin genes in beta-cells progenitors	Sussel et al., 1998
	NKX6.1	Differentiation of beta-cells	Sander et al., 2000
	MAFA	Transactivates insulin gene	Kataoka et al., 2002
	GATA6	Differentiation of endocrine lineage; expressed in beta cells	Ketola et al., 2004

Adapted from (Best et al. 2008).

1.6.2 Lentiviral Delivery

Conversion of somatic cells into a beta cell phenotype will be done by ectopic expression of the lineage-specific transcription factors using a Tet-ON Lentiviral Vector. The Tet-ON system allows the tight control of the gene expression. In this system, the gene expression is turned on when Tetracycline (Tc) or Doxycicline (Dox) is present in the culture media. In contrast, expression is turned off in the Tet-ON System by removing Dox from the media. The genes of interest are expressed under control of Tetracycline Responsive Element (TRE). In the presence of Dox, the reverse tetracycicline transactivator (rtTA) binds the TRE element and thereby activates transcription of the genes (Figure 5).

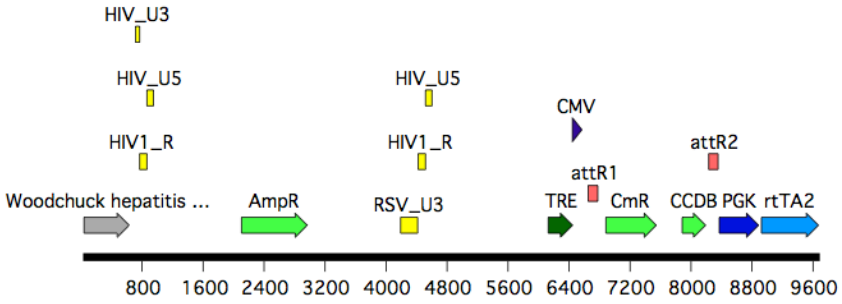

Figure 5: Representation of the Tet-ON Lentiviral Vector (9.6Kb). In grey, Woodchuck hepatitis responsive element; AmpR, ampicillin resistance gene; TRE, tetracycline responsive element; CmR, chloramphenicol resistance gene; rtTA2, reverse tetracycline transactivator.

1.6.3 The reporter vector

Cell identity will be identified using a lentiviral reporter vector expressing GFP and RFP under the control of the Insulin and Pdx1 promoters respectively (Figure 6). Cells turning on the reporter could be isolated by FACS and analyzed by PCR to identify the identity of the transcription factors inserted in their genome. Stability of the phenotypic transformation could be assessed by testing reporter activity in conditions that repress the expression of the pancreatic transcription from the lentiviral vectors (Figure 7).

Figure 6: Schematic representation of the lentiviral dual-reporter system containing the Pdx1 and insulin promoters driving expression of a monomeric red fluorescent protein and enhanced green fluorescent protein (Szabat et al. 2009). *Pdx1* is a master regulator of pancreatic development, islet survival, and beta cell function. Insulin is the main functional product of the cell type we wish to obtain by transdifferentiation.

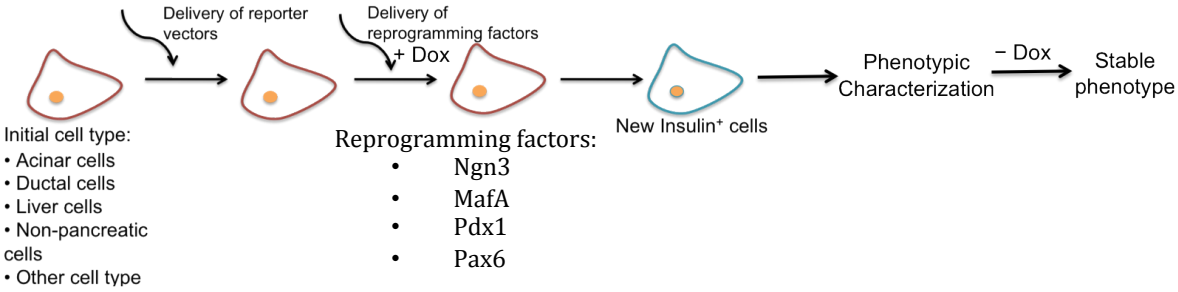

Figure 7: Schematic representation of the strategy used for direct reprogramming into beta cell phenotype mediated by expression of pancreatic transcription factors. Ectopic co-expression of the reporter vector and the reprogramming factors in an initial cell type

2. METHODS AND RESULTS

The first and basic task of this thesis was to clone a library of pancreas specific transcription factors into a doxycicline inducible lentiviral vector. Cloning the transcription factors (TF) into such an inducible lentiviral vector was considered crucial, as induction of expression of the TF would be needed for triggering transdifferentiation. Nevertheless, repression of expression would be important to determine if the phenotypic conversion would be stable in the absence of exogenous TF expression. Therefore we attempted several different cloning strategies.

2.1 The Gateway Cloning System

The Gateway System is a cloning method that provides an efficient way to clone a gene of interest into a vector of choice, based on the site-specific recombination properties of the bacteriophage lambda. The system is based on recombination between DNA sequences present in bacterial genomes (*att*B), and in the phage genome (*att*P). When a phage infects a bacterium, there is a recombination reaction between the *att*B and the *att*P sequences, which will result in integration of the phage DNA into the bacterial genome (Hartley 2000).

The product of recombination between an attB site and an attP (ie, a BP recombination reaction) site produces a new recombination site called attL. In turn, an attL sequence can recombine with another sequence named attR (ie, a LR recombination reaction) to restore an attB site.

These recombination reactions are the basis of the Gateway Cloning System, a commercially available system designed to facilitate the cloning of genes of interest and their subsequent transfer to more complex vectors for a number of purposes. In a first step, the gene of interest is amplified by PCR with primers containing the attB1 and attB2 sites in the forward and reverse gene specific primers respectively. This PCR fragment is introduced into pDonor, a plasmid containing a ccdB toxic gene (flanked by attP1 and attP2) in a recombination reaction catalyzed by the enzyme BP clonase, resulting in the exchange of the gene of interest for the ccdB cassette, generating a new plasmid called pEntry (Figure 8).

The reaction mixture is transformed into ccdB sensitive bacteria, ensuring that only recombinant clones (clones with a pDonor backbone carrying the gene of interest flanked by

attL1 and attL2 sites) are obtained. These can be analyzed by restriction digest analysis and sequencing to confirm their identity and integrity.

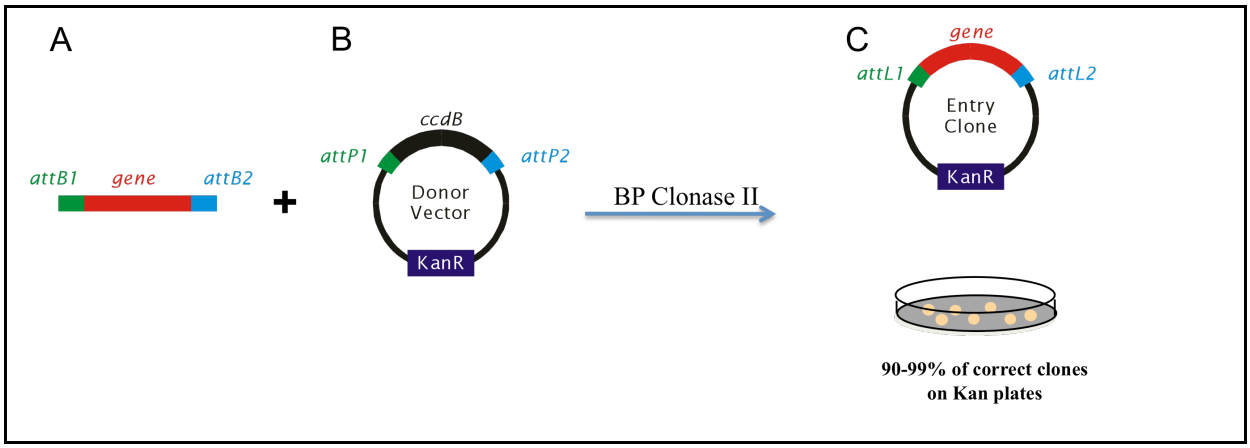

Figure 8: Schematic representation of the BP recombination reaction in order to generate the desired Entry Clone. A) PCR amplification using primers that have the attB1 and attB2 sites flanking the gene of interest. B) The Donor Vector contains a ccdB toxic gene flanked by attP1 and attP2, and a kanamycin resistance gene for positive selection. C) After the BP recombination reaction of the gene of interest into the Donor vector, the ligation reaction is transformed into ccdB sensitive bacteria and selected with kanamycin. The resulting clones will have the pEntry clone, which now contains the gene of interest flanked by attL1 and attL2 sites. Adapted from Invitrogen.

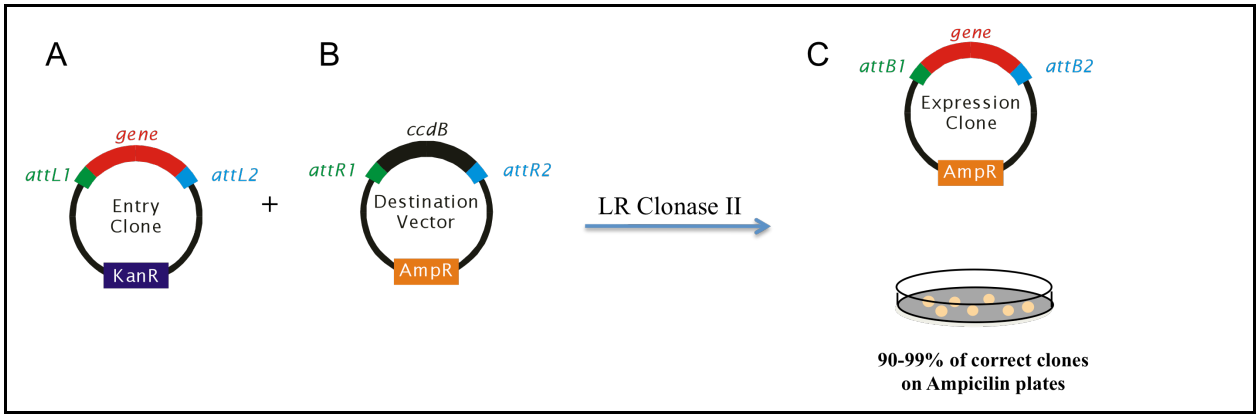

Figure 9: Schematic representation of the LR recombination reaction in order to clone Entry Clone into Destination Vector. A) Entry clone with the gene of interest flanked by attL1 and attL2 sites. B) Destination vector containing a ccdB gene and chloramphenicol resistance gene flanked by attR1 and attR2 sites. C) After LR recombination reaction of these two vectors, the ligation reaction is transformed into ccdB sensitive bacteria and selected for ampicillin resistance. The final plasmid (named expression clone) will now have attB1 and attB2 sites that result from recombination of attL and attR sites. Adapted from Invitrogen.

Once the Entry clones are obtained, the DNA of interest can be cloned into a destination vector in a recombination reaction catalyzed by the enzyme LR Clonase II, resulting in the formation of sticky ends in the attL sequences that will consequently match with the attR sticky ends in the destination vector. This will result in the exchange of the gene of interest for the ccdB gene producing a new plasmid, termed Expression clone (Figure 9). The expression clone now contains the gene of interest and a resultant recombinant att sequence, named attB site and is now ready to transfect into E.Coli following selection for the recombinant clones.

This site-specific recombination cloning system allows the efficient transfer of gene-coding sequences from one vector (Entry clone) to any expression clone modified with the necessary recombination sites flanking the insertion site for the gene-coding sequences (Destination vector).

2.2 Construction of the Gateway Destination Vector

We obtained a number of pancreas specific transcription factors from a library of transcription factors that had been cloned into pEntry vectors. Of note, this library had been designed for the creation of fusion proteins and therefore the open reading frames lack the stop codons. We chose to clone these factors into an appropriate destination vector and planned to add stop codons at a later stage.

Our goal was to clone a collection of pancreatic specific transcription factors into a doxycycline regulatable lentiviral vector. Therefore, we required the appropriate gateway destination vector.

In order to generate the final destination vector, the lentiviral vector Lenti-Tet-ON-ires-GFP was used (Figure 10).

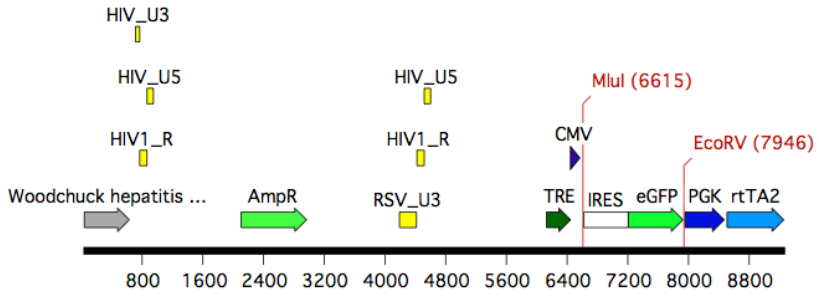

Figure 10: Representation of the original vector Lenti-Tet-ON-IRES-GFP.

The original plasmid was digested with restriction enzymes (MluI and EcoRV) and its backbone was gel purified using QIAquick gel extration Kit (Qiagen). Because MluI cut leaves a 5´-overhang, the original backbone was incubated with dNTPs and Klenow fragment for 15 minutes at 65C, following incubations at 25ºC for 15 minutes and 75ºC for 20 minutes. This procedure resulted in the fill-in of the 5'-overhang and the generation of blunt DNA ends. The vector backbone was then treated with alkaline phosphatase to remove the 5' phosphates in order to minimize re-ligation of the vector. The plasmid containing the destination cassette was digested with EcoRV and gel purified using QIAquick gel extration Kit (Qiagen). After that, the destination cassette (contained the attR1 and attR2 sites and both a ccdB gene and a chloramphenicol resistance marker) was ligated into the vector using a T4 DNA ligase. The ligation reaction was transformed into electrocompetent bacteria resistant to ccdB and selected for Ampicilin.

The resultant plasmid (from now on termed destination vector – also see Figure 5) was confirmed by restriction and sequencing (Figure 11).

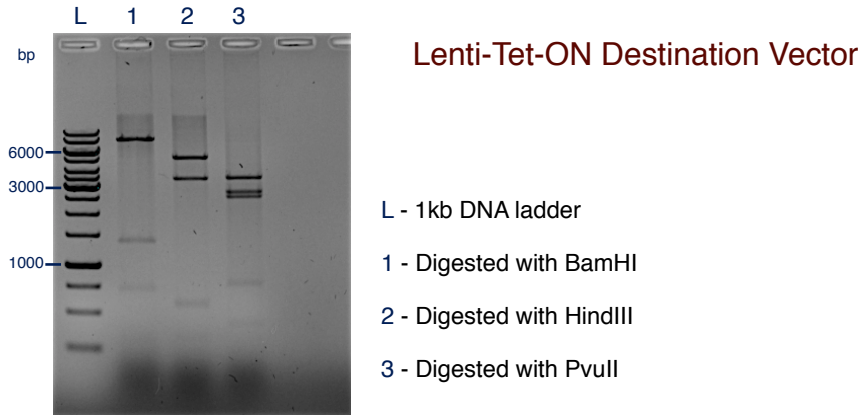

Figure 11: Restriction pattern of Lenti-Tet-ON Destination Vector analysed by agarose electrophoresis. The expected bands are: in lane 1) 7.4kb, 1.34kb, o.7kb and 0.25kb; in lane 2) 4.9kb, 3.34kb, 0.58kb, 0.55 kb and 0.3 kb; in lane 3) 3.39kb, 2.65kb, 2.52kb and 0.77kb.

The genes of interest were cloned into the destination vector using Gateway technology, as described before. The genes were obtained from a commercial library already cloned into pEntry vectors and lacking stop codons as explained above. The transcription

factors were transferred into the destination vectors by a LR recombination reaction between the entry clone and the converted gateway destination vector.

The first library of genes cloned into gateway destination vector (Library 1) was already cloned in pEntry vector by the time in which the cloning has started.

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Table 4: Library 1 - Genes cloned in pEntry vector	
pE-ISL1	pE-UBA2
pE-HADHB	pE-ES1
pE-NeuroD	pE-IL1
pE-MafA	pE-COX2
pE-CKD4	pE-M4T1
pE-PAX6	

The identity of clones in pEntry was confirmed by restriction and sequencing. All the clones showed the right restriction pattern (data not shown). For the clones which identity was confirmed by sequencing, a LR recombination reaction was performed in order to clone the genes of interest into the destination vector (Figure 12), followed by new restriction and sequencing to confirm the final clones that were obtained (Figure 13).

Note that the sequences of transcription factors belonging to Library 1 did not contained the stop codon, since these plasmids were first generated for the construction of recombinant proteins. Nevertheless, since the goal of this work was to test the effect of ectopic expression of several transcription factors in order to reprogram somatic cells into the beta cell phenotype, the stop codons would need to be added be added to the sequences at a later step (see Site-directed mutagenesis protocol below).

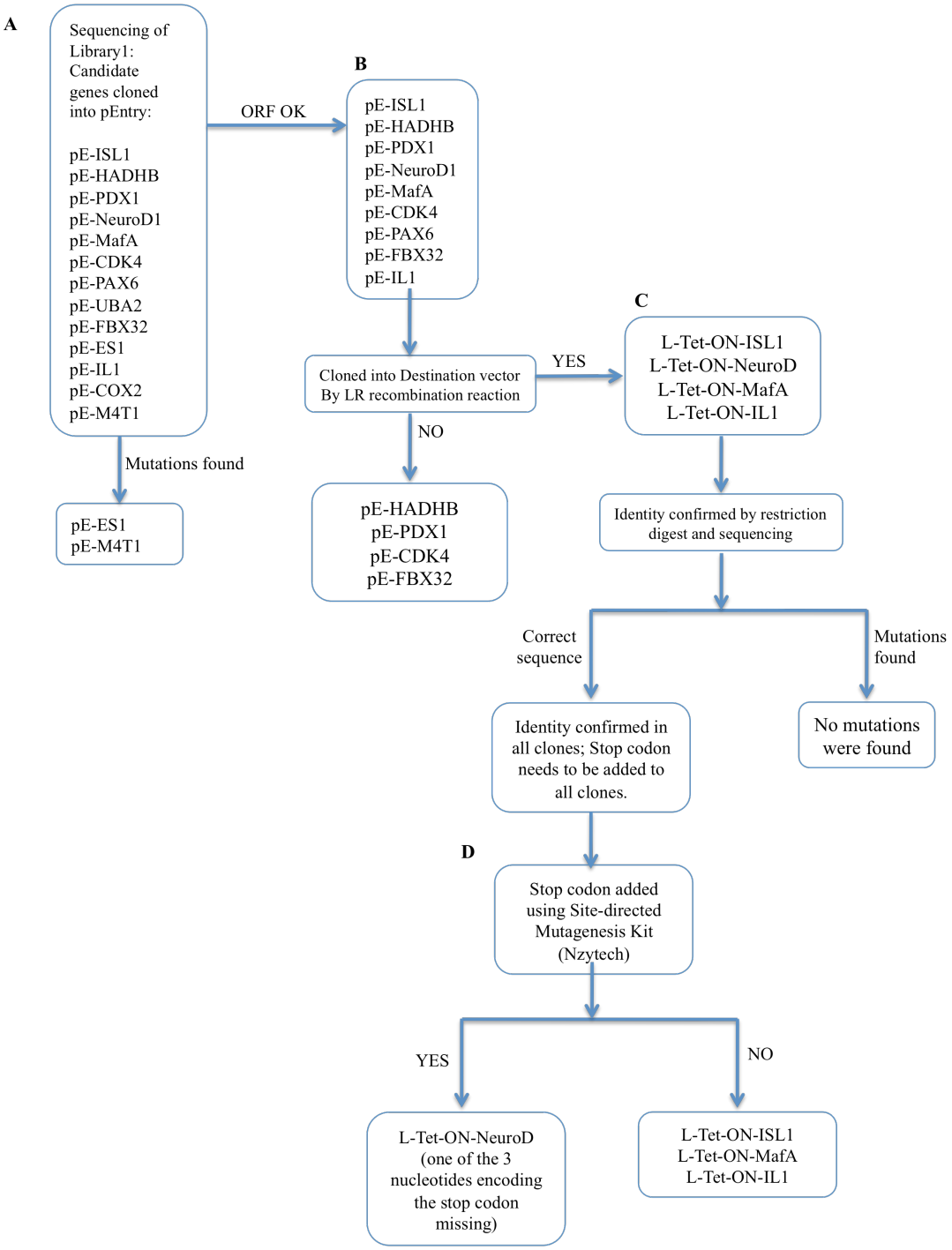

Figure 12: Schematic representation of the generation of the final expression clones using the Gateway System for clones in Library1. A) Library1 containing several transcription factors cloned into pEntry vector. B) Clones which identity was confirmed by sequencing; LR recombination reaction into destination vector. C) Clones successfully cloned into destination vector were confirmed by restriction enzyme digestion and sequencing. D) Stop codon was added into the sequences of genes cloned in C) using Site-Directed Mutagenesis Kit (Nzytech). The success and failure of the different strategies is here represented as “YES” or “NO”, respectively.

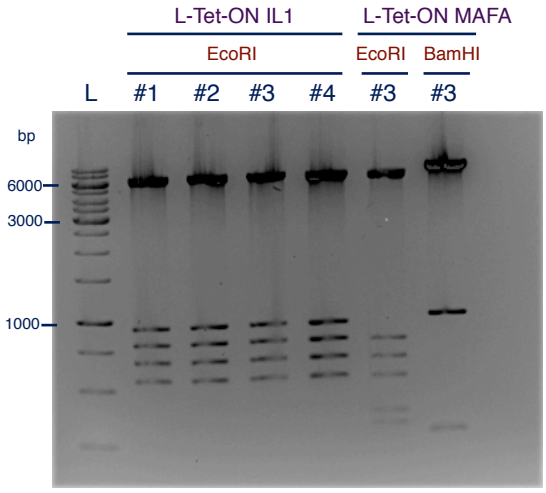

Figure 13: Restriction pattern of Lenti-Tet-ON-IL1 (lanes 2 to 5) and Lenti-Tet-ON-MAFA (lanes 6 and 7) analysed by agarose electrophoresis. L, 1kb letter; #1, #2, #3 and #4 refer to different clones that were subjected to restriction. The expected bands are: For Lenti-Tet-ON-IL1: 6kb, 0.8kb, 0.76kb, 0.68kb and 0.6kb; Lenti-Tet-ON-MAFA: Cut with EcoRI: 6kb, 0.76kb, 0.68kb, 0.6kb, 0.35kb and 0.3kb; Cut with BamHI: 7kb, 1.1kb and 0.27kb.

Despite multiple attempts, we were unable to clone some of our candidate transcription factors into our destination vector by LR recombination. This situation is not unheard of when using the gateway system to clone inserts into complex constructs such as the LentiTETON destination vectors. Apparently, the system is dependent on the nature and sequence of the inserts being cloned and to obtain spuriously recombined and incorrect clones is sometimes known to occur.

In order to reconstruct the missing stop codons in the final expression clones we subjected them to Site-directed Mutagenesis Kit (Nzytech).

The Site-directed Mutagenesis Kit is a simple procedure designed to make modifications in a vector of choice, such as point mutations, and insertions or deletions of single or multiple amino acids (Figure 14). In this specific situation, the system was used for addition of the stop codons missing in the gene sequences of Library 1. Therefore, two synthetic oligonucleotide primers containing the mutation (in this case the stop codon) were designed, each one annealing to the same sequence, on opposite strands of the vector. PCR

amplification of the plasmid using these primers will generate a mutated plasmid containing a nick at the end of each circular strand.

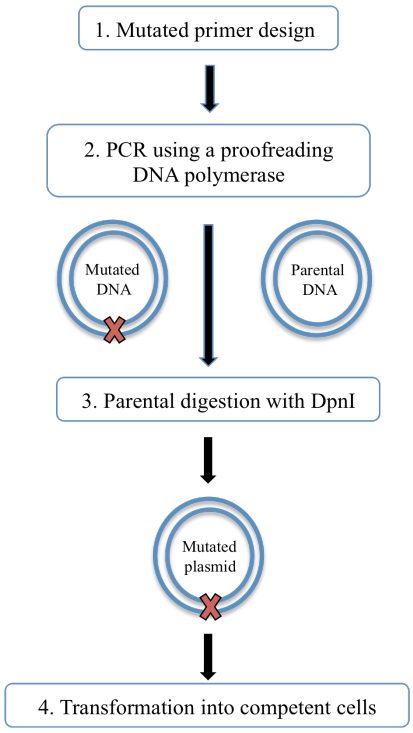

Figure 14: Schematic representation of the Site-directed mutagenesis method. *Adapted from* http://www.stratagene.com/manuals/200516.pdf.

After that, the PCR product is digested with DpnI, which is an endonuclease specific for methylated DNA. DpnI is used to digest the parental DNA template and select for plasmids containing the desired mutation (the synthetic DNA is not methylated and therefore resists to DpnI digestion; DNA isolated from almost *E.coli* strains is dam methylated and consequently vulnerable to digestion with DpnI).

The plasmid is finally transformed into competent cells and the latter will repair the nicks in the mutated vector.

At the completion of this thesis, the Lenti-Tet-ON-NeuroD1 construct has been partially modified to reconstruct the stop codon (one base of the stop codon is still missing

and will require another round of mutagenesis) and the other three constructs (ISL1, MAFA and IL1) are awaiting repair.

In summary, this cloning approach was not as efficient as hoped for.

2.3 Cloning With Vaccinia Virus DNA polymerase using In-Fusion Kit (Clonetech)

In order to clone the genes of interest into the lentiviral vector L-TET-ON, another strategy was adopted, which consisted in cloning the DNA of interest into the vector of choice by means of a recombineering technology based in recombination of virus *in vivo*.

This technology uses Vaccinia Virus DNA polymerase (VVpol), a polymerase that encodes a 3'- to 5'-proofreading exonuclease that is able to degrade the ends of the duplex DNA and therefore expose single-stranded DNA tails. As a result, complementary single-stranded DNA tails can recombine giving rise to stable recombinant molecules. For this reason, VVpol can be used for directional cloning of PCR products (Figure 15).

The target vector is linearized using restriction enzymes and the DNA of interest can be amplified by PCR using forward and reverse primers that contain a sequence of 16-18bp that is homologous to the two ends of the linearized vector. These products are then coincubated with VVpol, which will degrade the ends of the DNAs (through its 3'- to 5' exonuclease activity) and expose the areas of complementary sequence. The single-stranded DNAs can anneal spontaneously since they share complementary sequences (this reaction is enhanced by adding vaccinia virus single-strand DNA binding protein – or I3 protein) and will form non-covalently linked joint molecules. After transformation into *E. coli*, the E. coli DNA repair systems convert these molecules into stable recombinants. Because this cloning method can be used with any combination of homologous ends, it is a very useful system for directional cloning of PCR products into a target vector.

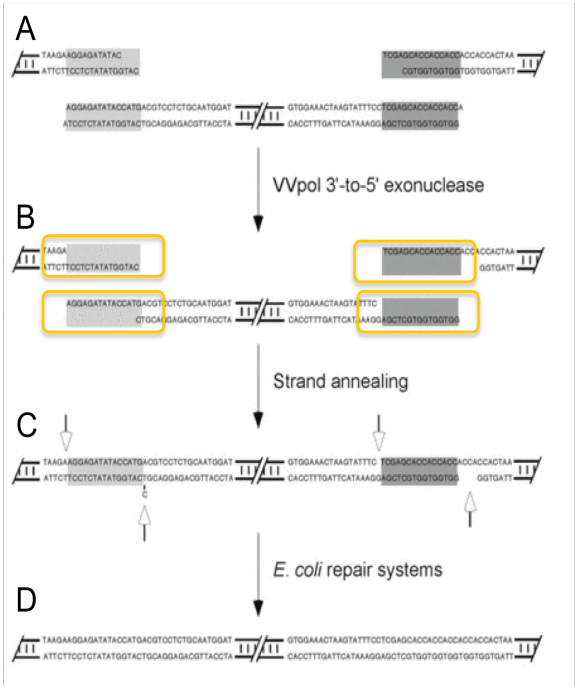

Figure 15: Principles of the cloning method using Vaccinia Virus DNA polymerase (VVpol). A) Digestion of the target vector with restriction enzymes and amplification of the gene of interest using primers that contain the sequences homologous to the vector ends. B) Co-incubation of both the linearized vector and the PCR product with VVpol. The 3'- to 5' exonuclease activity of VVpol degrades and exposes the complementary sequences of the DNAs (see yellow boxes). C) Single-strand annealing of the complementary ends (this reaction is catalysed by I3 proein) and generation of joint molecules, that can contain a mix of gaps, nicks or extra nucleotides, as a result of the variation in the coverage of exonuclease attack (white arrows). D) These joint molecules can then be transfected into *E. coli* and its repairing system will convert the joint molecules into stable recombinants. *Adapted from* (Irwin et al. 2012).

A number of commercially purchased clones from our candidate list, as well as genes from library 1 that were not successfully cloned by gateway technology were amplified by PCR, cloned into pBluntZero using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and confirmed by sequencing and subjected to In-fusion cloning. Of note, in amplifying the transcription factors, MluI and EcoRV restriction sites were created upstream and downstream of the transcription factors open reading frames. Our rationale was that amplifying the transcription factors (TFs) open reading frames would further allow the cloning of the TFs into the lentiviral vector Lenti-Tet-ON using two alternative strategies: a) In-phusion cloning and b) Directional cloning using MluI and EcoRV restriction sites.

This commercially available technique (Zero Blunt TOPO PCR Cloning Kit) is a rapid and simple cloning strategy used for the direct insertion of blunt-end PCR products into a

plasmid vector. It is a cloning technique based in the Topoisomerase I from Vaccinia virus, a virus-encoded eukaryotic type I topoisomerase that has the ability to cleave and rejoin DNA strands with high specificity (Shuman 1991). The Topoisomerase I binds to duplex DNA in specific sequences (5'-CCCTT) cleaving the phosphodiester backbone after 5'-CCCTT in one strand. In the cleavage reaction, the energy is conserved by the formation of a covalent bond between the 3' phosphate of the cleaved strand and the tyrosyl residue of topoisomerase (Tyr-274). The enzyme has the ability to religate to a heterologous acceptor DNA, creating a recombinant molecule (Shuman 1994).All the resulting vectors cloned using this strategy were restriction digested with enzymes and sequenced to confirm the identity of the pBluntZero plasmids containing the target genes.

The library of genes successfully clone in pBluntZero were then used for two different cloning strategies: 1) T4 DNA ligase cloning strategy and 2) In-fusion Cloning.

In the T4 DNA ligase cloning strategy, both the initial vector (L-Tet-ON-IRES-GFP) and the genes cloned into pBluntZero were digested with MluI and EcoRV, and gel purified using QIAquick gel extration Kit (Qiagen).

Aftter gel purification, each gene of interest was introduced into the L-Tet-ON vector in a ligation reaction with T4 DNA Ligase. The ligation reactions were then transformed into DH5α electrocompetent bacteria and the resulting plasmids were digested with restriction enzymes and sequenced to confirm their identity.

In the In-Fusion cloning approach, the initial vector was digested with MluI and EcoRV, to generate the target linearized backbone following gel purification (QIAquick gel extration Kit, Qiagen). The genes of interest (that were cloned into pBluntZero) were amplified using primers containing the sequence homologous to the two ends of the linearized vector and gel purified (QIAquick gel extration Kit, Qiagen). Co-incubation with VVpol and transfection into *E.coli* was performed as described before. The identity of the resulting clones was confirmed by restriction enzyme digestion followed by sequencing (Figure 16).

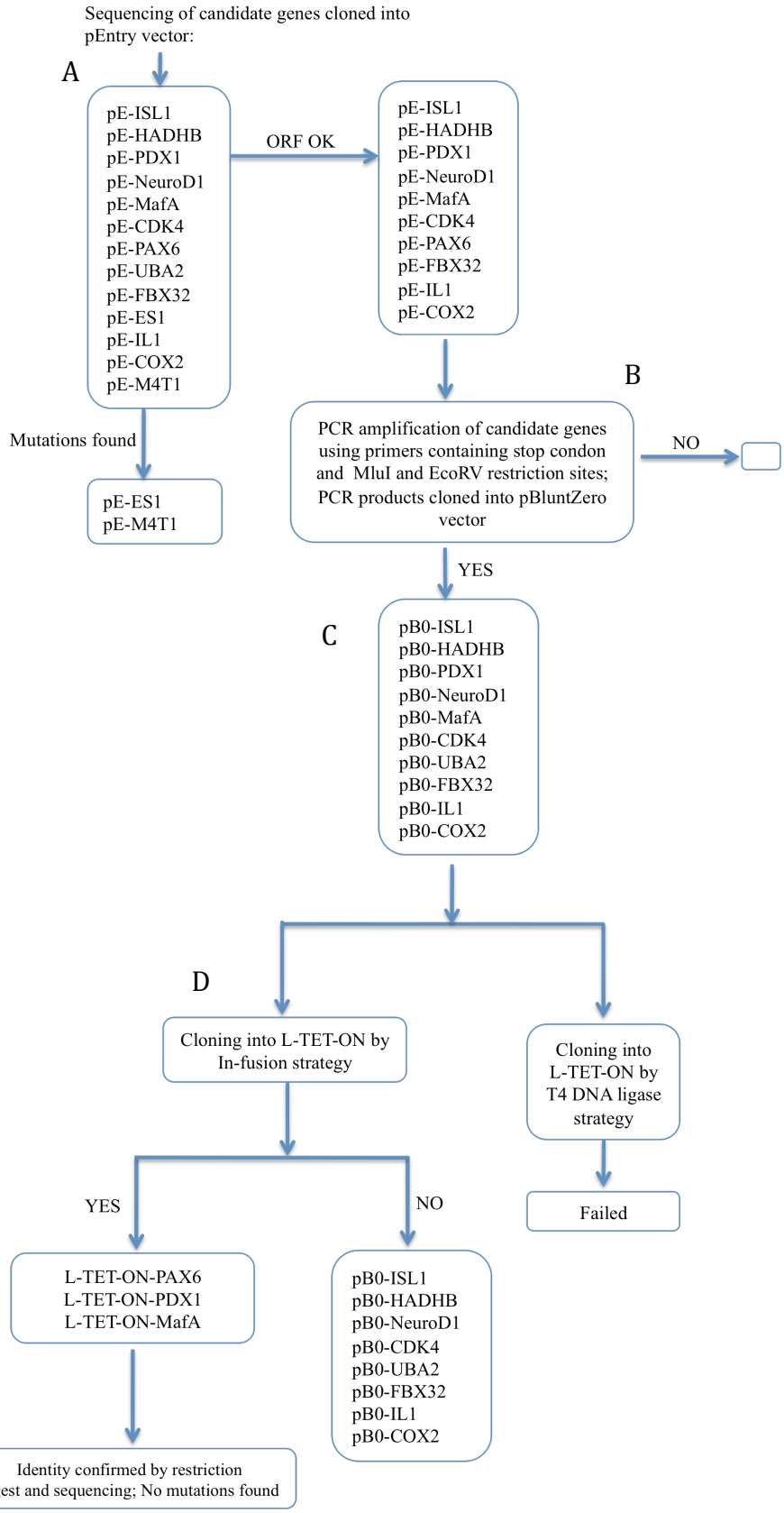

Figure 16: Schematic representation of the method used for generation of the final expression clones using the In-fusion technology or the T4 DNA Ligase strategy. A) Library1 containing several transcription factors cloned

into pEntry vector. B) Clones which identity was confirmed by sequencing; Amplification by PCR of the genes in Library 1 using primers containing each stop codon and MluI and EcoRV restriction sites. C) Clones successfully cloned into pBluntZero were confirmed by restriction enzyme digestion and sequencing. D) Two different strategies were used to clone the candidate genes into L-Tet-ON vector: the In-Fusion technology and the T4 DNA ligase. The resulting clones were restriction digested and sequenced to confirm their identities. The success and failure of the different strategies is here represented as "YES" or "NO", respectively.

Other genes showed to code for proteins that drive pancreas formation. For this reason, a second library of candidate genes was created (named Library 2). This library contains sequence-validated full-length protein-coding (FL-CDS) human cDNAs acquired from the Mammalian Gene Collection (Invitrogen), each one containing a start and a stop codon.

Table 5: Library 2 - cDNAs from the Mammalian Gene Collection

GATA4	MSI2
HHEX	NKX6.1
HNF1B	PAX4
FOXA2	PROX1
HNF4A	SOX9
HNF6	TFCP2L1
HOPX	

The cDNAs clones were isolated from *E.coli* and analyzed by restriction followed by PCR amplification with flanking primers containing MluI and EcoRV restriction sites. The resulting PCR products were cloned into pBluntZero plasmid, as explained before. The identity of the clones was confirmed by restriction and sequencing. The cDNAs were introduced into the L-Tet-ON vector by the In-Fusion Cloning Kit (Clonetech), as mentioned before (Figure 17).

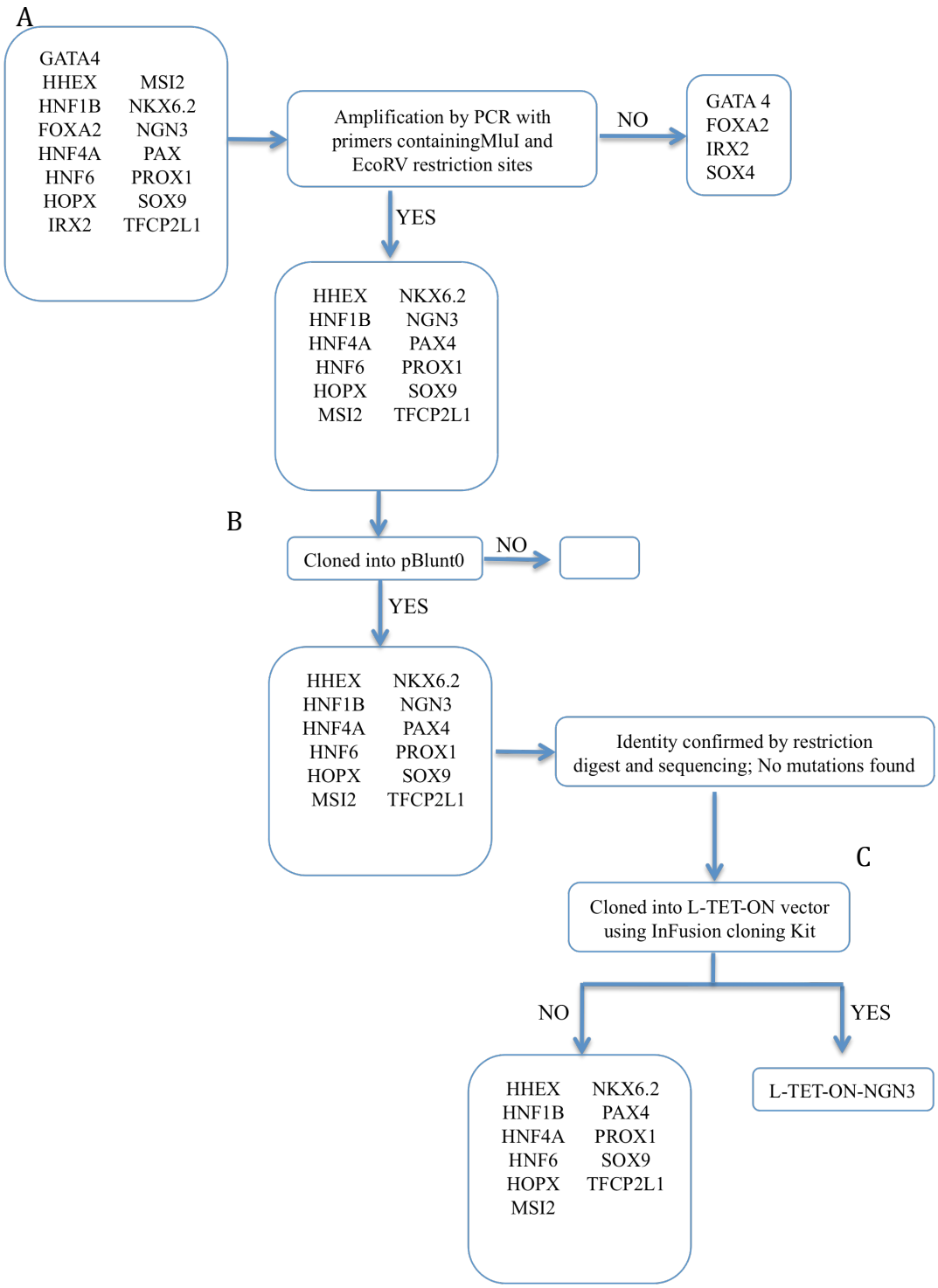

Figure 17: schematic representation showing the construction of the expression clones using the In-Fusion system. A) Library 2: cDNAs acquired from the Mammalian Gene Collection (Invitrogen) amplified by PCR using a forward and reverse primers containing an MluI and EcoRV restriction sites, respectively. B) PCR products were successfully cloned into pBluntZero vector and their identity and integrity was confirmed by restriction and sequencing. C) cDNAs were posteriorly cloned into L-Tet-ON vector by In-fusion strategy. The resulting clones were restriction digested and sequenced to confirm their identities. The success and failure of the different strategies is here represented as “YES” or “NO”, respectively.

A third strategy was used in order to isolate transcription factors thought to play a role in adult human islets. Since we had access to total mRNA from adult human islets, total mRNA was amplified by RT-PCR with attB flanked primers for a list of several candidate cDNAs that codify for these transcription factors (Table 6).

The candidate cDNAs that were amplified with success were cloned into pDonor using the Gateway cloning system, as explained before. pDonor is a plasmid containing a ccdB toxic gene (flanked by attP1 and attP2). The recombination reaction was catalyzed by the enzyme BP clonase, resulting in the exchange of the gene of interest for the ccdB cassette, generating a new plasmid called pEntry.

The resultant pEntry vectors were digested with restriction enzymes and sequenced to confirm their identity and integrity followed by cloning into the destination vector, also using gateway cloning strategy, as mentioned above (Figure 18).

Table 6: Library 3 - Candidate genes thought to play a role in human islets that were amplified by PCR

CyclinD2	PBX1
GATA4	PTF1a
HNF3B	SOX4
HNF6	GATA6
HOPX	HDAC9
NKX2.2	PROX1
NKX6.1	SOX9
TERT	

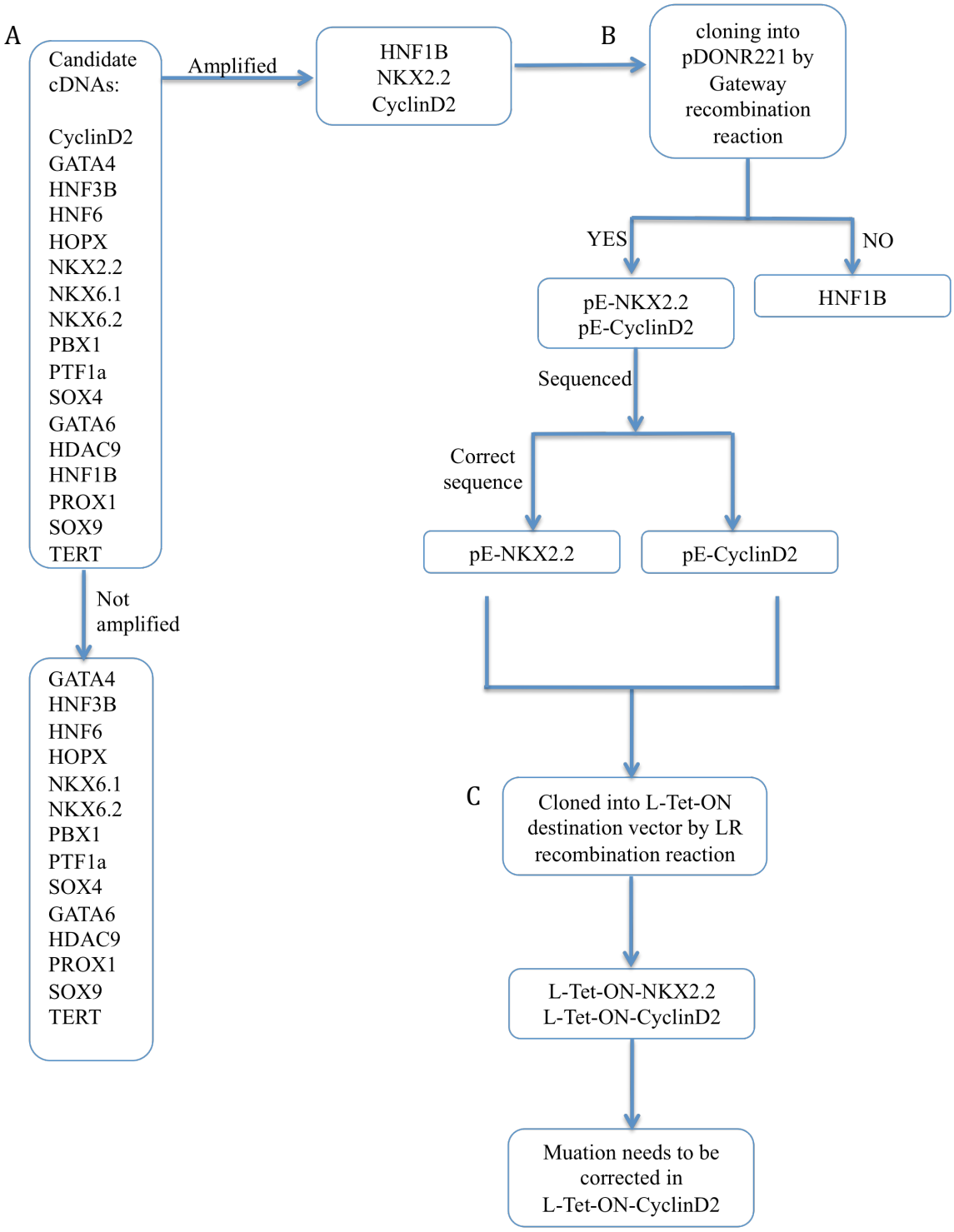

Figure 18: schematic representation showing the construction of expression clones by the gateway system. A) Candidate cDNAs from total mRNA from adult human islets were amplified by RT-PCR with attB flanked primers for a list these several candidate cDNAs. B) PCR products that were successfully cloned into pDONR221 vector and which identity and integrity was confirmed by restriction and sequencing. C) cDNAs were posteriorly cloned into L-Tet-ON vector by gateway cloning system. The resulting clones were sequenced to confirm their identities. The success and failure of the different strategies is here represented as "YES" or "NO", respectively.

Finally, because only three cDNAs were amplified using human islets total mRNA, the last strategy used was to isolate putative transcription factors thought to play a role in human islets by amplification of more cDNAs from 293T total mRNA. To achieve this, total mRNA was amplified by RT-PCR with attB flanked primers for the same list of several candidate cDNAs (Table 6). PBX1 was successfully amplified by RT-PCR, and further cloned into pDonor vector using the Gateway cloning system, creating a pEntry vector. Although PBX1 had been cloned into pDonor, the sequencing results revealed that the actual sequence belonged to PBX2. Therefore, the cloning of this cDNA into the destination vector was not continued (Figure 19).

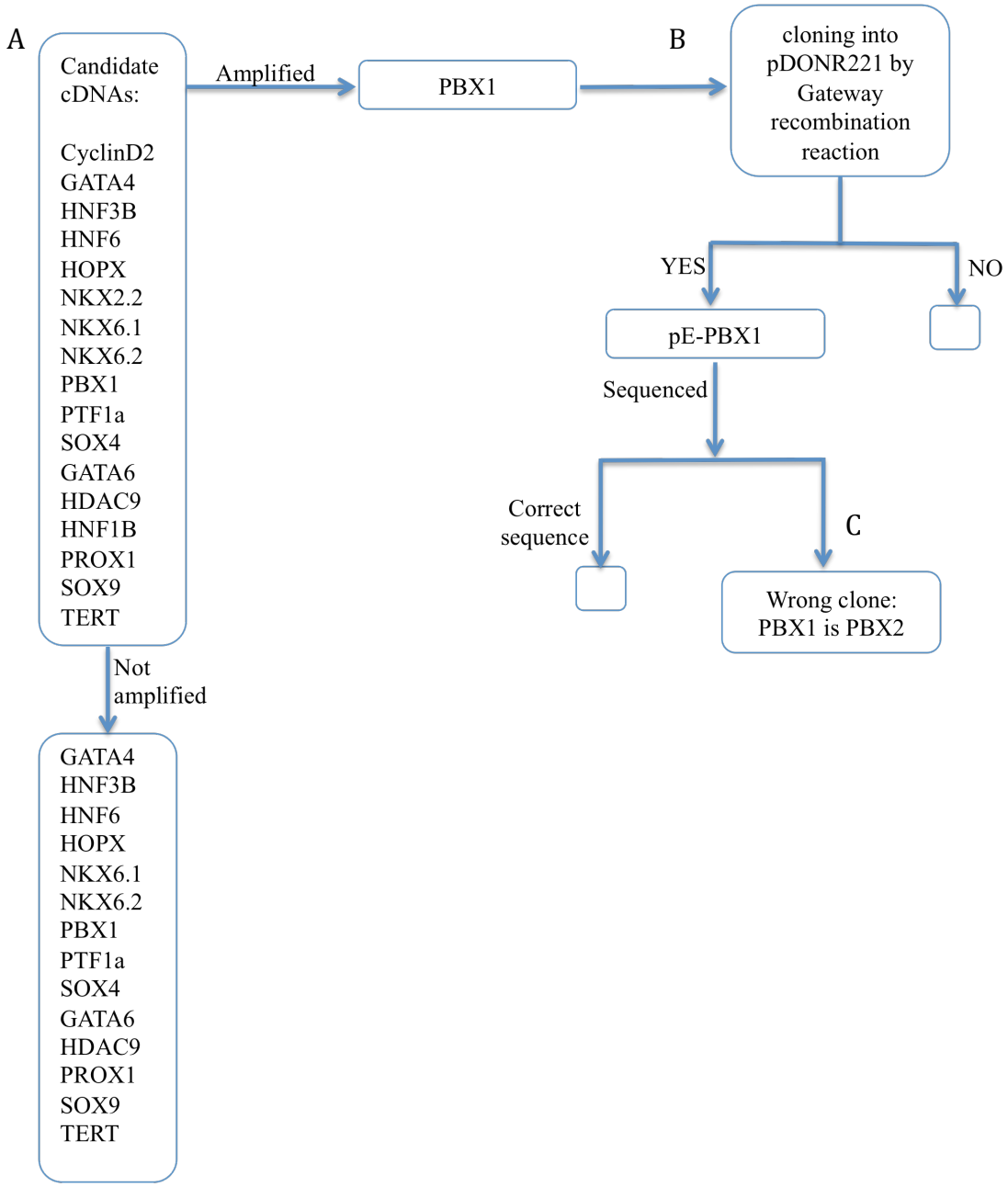

Figure 19: Strategy for the production of expression clones using the gateway system. A) Candidate cDNAs from 293T total mRNA were amplified by RT-PCR with attB flanked primers for a list these several candidate cDNAs. B) PCR products that were successfully cloned into pDONR221 vector. C) The sequencin results showed that pE-PBX1 was actually the wrong clone, and therefore this method was discontinued. The success and failure of the different strategies is here represented as “YES” or “NO”, respectively.

2.4 Virus Production Protocol

The following protocol was used for the production of Lenti-tet-ON-Ngn3, Lenti-tet-ON-Pax6, Lenti-Tet-ON-Pdx1, Lenti-Tet-ON-MafA. A lentiviral vector hPGK-GFP was used as a positive control.

- Seed $4x10^6$ 293T cells in a 10cm plate; when cells reach 90% confluency proceed to transfection;

Transfection procedure:

-	10 cm plate
Vector plasmid	10.0 µg
PMDL	6.5 µg
Rev	2.5 µg
VSV-G	3.5 µg
NaCl 150mM	890 µL
PEI (polyethylenimine)	90 µL
Total volume of transfection mix	1 mL

- For DNA/PEI complex formation, the reaction must be carried out in an isotonic solution of NaCl (150mM);
- The final volume of the reaction must be 1/10 of the volume in which cells were grown (otherwise the complex will precipitate);
- Mix DNA into appropriate volume of NaCl 150mM;
- Add PEI in a 4:1 ratio (4 µg of PEI per 1 µg of DNA);
- Mix well (vortex immediately);
- Leave at room temperature for 5 to 10 minutes;
- Add homogeneously the solution (dropwise) that contains the DNA/PEI complex into the plate and swirl;
- Incubate at 37ºC for six hours; Change the medium 6 hours after transfection for 7.5 mL of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% PenStrep (Invitrogen) and 1% Glutamax (Invitrogen);
- Incubate cells at 32ºC;
- Collect the medium at 48 hours after transfection; Add 7.5mL of fresh medium to the plate and incubate cells at 32ºC;
- Filter the medium through a 0.45 µm filter;

- Aliquot in 1.5mL tubes and proceed to storage at -20ºC for short term use or at -80ºC for long term storage;
- Collect the medium 24 hours later; Add 7.5mL of fresh medium and incubate cells at 32ºC;
- Filter the medium as mentioned before, aliquot in 1.5mL tubes and proceed to storage at -20ºC for short term use or at -80ºC for long term storage;
- Collect the medium 24 hours later;
- Filter the medium as mentioned before, aliquot in 1.5mL tubes and proceed to storage at -20ºC for short term use or at -80ºC for long term storage.

For the production of pTiger-Pdx1mRFP-Ins1eGFP, the following protocol was used. The lentivirus pTigerCMVeGFP and pTigerCMVmRFP were used as positive controls.

- Seed 293T cells ($4x10^6$ cells per plate) in a 10cm plate; when cells reach 90% confluency proceed to transfection;

Transfection procedure:

-	10 cm plate
Vector plasmid	9.0 µg
pCPREnv	15.0 µg
pCI-VSVG	18.0 µg
NaCl 150mM	890 µL
PEI (polyethylenimine)	90 µL
Total volume of transfection mix	1 mL

- For DNA/PEI complex formation, the reaction must be carried out in an isotonic solution of NaCl (150mM);
- The final volume of the reaction should be 1/10 of the volume in which cells were grown (otherwise the complex would precipitate);
- Mix the DNA into an appropriate volume of NaCl 150mM;
- Add PEI in a 4:1 ratio (4 µg of PEI per 1 µg of DNA) and vortex;
- Leave at room temperature for 5 to 10 minutes;
- Add homogeneously the solution (dropwise) that contains the DNA/PEI complex into the plate and swirl;

- Incubate at 37ºC for sixteen hours; Change the medium sixteen hours after transfection for 7.5 mL of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% PenStrep (Invitrogen) and 1% Glutamax (Invitrogen);
- Incubate cells at 32ºC;
- Collect the medium at 48 hours after transfection; Add 7.5mL of fresh medium to the plate and incubate cells at 32ºC;
- Filter the medium through a 0.45 µm filtre;
- Aliquot in 1.5mL tubes and proceed to storage at -20ºC for short term use or at -80ºC for long term storage;
- Collect the medium 24 hours later; Add 7.5mL of fresh medium and incubate cells at 32ºC;
- Filter the medium as mentioned before, aliquot in 1.5mL tubes and proceed to storage at -20ºC for short term use or at -80ºC for long term storage;
- Collect the medium 24 hours later;
- Filter the medium as mentioned before, aliquot in 1.5mL tubes and proceed to storage at -20ºC for short term use or at -80ºC for long term storage.

2.5 **Biological Titre**

1) For the biological titre of the lentivirus Lenti-Tet-ON (MafA, Pdx1, Pax6 and Ngn3), the lentivirus Lenti-hPGK-GFP was used.
2) For the biological titre of the reporter pTiger-Pdx1mRFP-Ins1eGFP, the lentivirus pTigerCMVeGFP was used.

Titration procedure:

- Seed 293T cells ($1x10^5$ per well) in a 24 well plate
- Make ten-fold serial dilutions of the viral particles in PBS;
- The best dilution should have 10-30% GFP positive cells.

Serial dilutions:

1) Undiluted (100 µL of viral particles)
2) 1:10 dilution (10 µL of viral particles in 90 µL of PBS)
3) 1:100 dilution (10 µL of 1:10 dilution in 90 µL of PBS)
4) 1:1000 dilution (10 µL of 1:100 dilution in 90 µL of PBS)
   - Use 10 µL of each dilution to transduce 293T cells in 24 wells in 500 µL final volume.
   - Incubate cells at 37ºC and 48 hours later analyse by flow cytometry;
   - Calculate the transducing units per microliter (TU/µL) according to:

TU/ µL = (P x N / 100 x V) x D,

Where:
P = % GFP positive cells
N = number of cells seeded per well
V = volume of supernatant
D = dilution: undiluted =1
1:10 dilution =10
1:100 = 100
1:1000 = 1000

After flow cytometry analysis, the titration results were:

- TU/ µL (Lenti-Tet-ON vectors) = 3000 units/µL
- TU/ µL (pTiger-Pdx1mRFP-Ins1eGFP) = 190.6 units/ µL

2.6 Transdifferentiation Experiment

For the transdifferentiation experiment, the following protocol was followed.

- Seed $5x10^4$ human Dermal Fibroblasts (Invitrogen) per well, in two 6-well cluster
- Culture cells in DMEM (Invitrogen) medium supplemented with 10% FBS (Invitrogen), 1% PenStrep (Invitrogen) and 1% glutamine (Invitrogen);
- Add doxycycline to the medium in just one of the clusters;
- Calculate the multiplicity of infection (MOI) for both the Lenti-Tet-ON vectors and the pTiger-Pdx1mRFP-Ins1eGFP.

Transduction procedure:

1) All wells without Doxycycline

Well#1: $5x10^4$ cells Negative control (no virus)	**Well#2:** $5x10^4$ cells pTiger-Pdx1mRFP-Ins1eGFP MOI of 2	**Well#3:** $5x10^4$ cells Positive control pTigerCMVeGFP MOI of 2
Well#4: $5x10^4$ cells pTiger-Pdx1mRFP-Ins1eGFP L-Tet-ON-MafA L-Tet-ON-Pdx1 L-Tet-ON-Pax6 L-Tet-ON-Ngn3 MOI of 2	**Well#5:** $5x10^4$ cells pTiger-Pdx1mRFP-Ins1eGFP L-Tet-ON-MafA L-Tet-ON-Pdx1 L-Tet-ON-Pax6 L-Tet-ON-Ngn3 MOI of 10	----------

2) Add doxycycline to all wells (1μg/mL)

Well#1: $5x10^4$ cells Negative control (no virus)	**Well#2:** $5x10^4$ cells pTiger-Pdx1mRFP-Ins1eGFP MOI of 2	**Well#3:** $5x10^4$ cells Positive control pTigerCMVeGFP MOI of 2
Well#4: $5x10^4$ cells pTiger-Pdx1mRFP-Ins1eGFP L-Tet-ON-MafA L-Tet-ON-Pdx1 L-Tet-ON-Pax6 L-Tet-ON-Ngn3 MOI of 2	**Well#5:** $5x10^4$ cells pTiger-Pdx1mRFP-Ins1eGFP L-Tet-ON-MafA L-Tet-ON-Pdx1 L-Tet-ON-Pax6 L-Tet-ON-Ngn3 MOI of 10	----------

- Add each solution containing the viral particles into each well;
- Incubate cells at 37ºC and 16 hours later replace medium for pancreatic induction medium: cells were cultured in N2B27 medium supplemented with 0.1 mM β-mercaptoethanol (Invitrogen), 2 mM glutamine (Invitrogen), 1 mM MEM non-essential aminoacids (Gibco), 0.5%BSA(Sigma), 1% B27 (Invitrogen), 20 ng/mL basic fibroblast growth factor (R&DSystem), and 1% insulin-transferrin-selenium (Invitrogen) to further encourage transdifferentiation;
- Refresh medium every 48 hours.

After 21 days of *in vitro* cell culture, we looked for GFP and RFP positive cells, which are indicators of the reporter pTiger-Pdx1mRFP-Ins1eGFP activity. The positive control lentiviral vector pTigerCMVeGFP expressed GFP in both conditions (with doxycycline and without), as expected. However, there was neither GFP nor RFP activity in the reporter pTiger-Pdx1mRFP-Ins1eGFP, indicating that there was no activation of the both insulin and Pdx1 promoters. Although a week GFP signal was observed in Figures 20 and 21, we postulated that this could be due to a contamination, since the signal was detected both in the minus and plus Doxycycline conditions. This contamination might arose from the positive viral control pTigerCMVeGFP at the time of transduction.

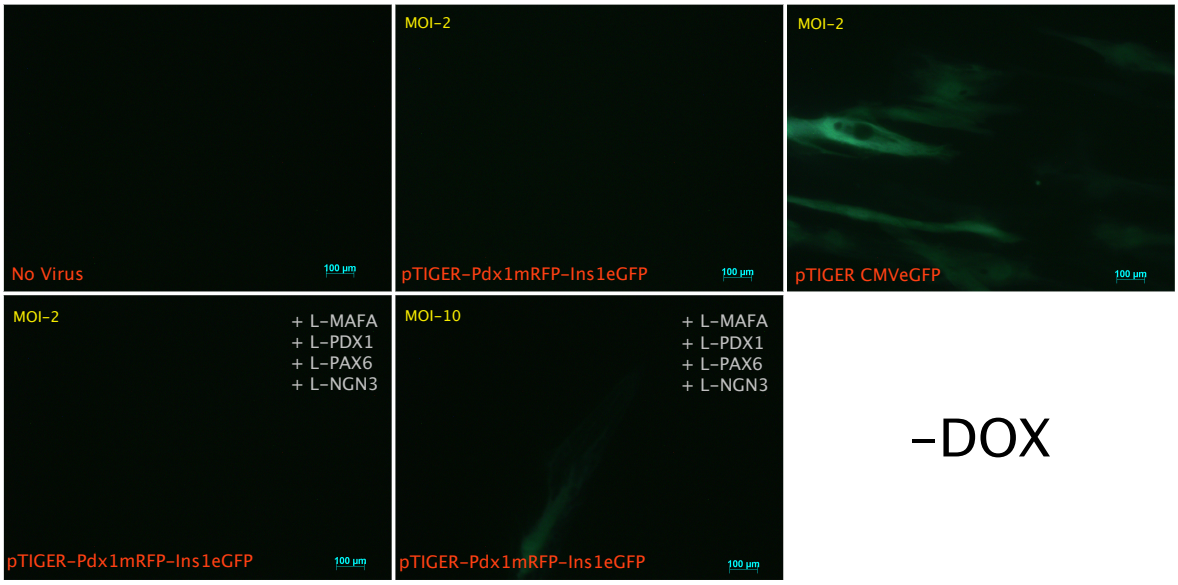

Figure 20: Effect of the ectopic expression of MafA, Pdx1, Pax6 and Ngn3 in the transdifferentiation of human dermal fibroblasts into the β-cell phenotype, after 21 days in culture without the addition of doxycycline in the medium. GFP expression indicates the reporter pTiger-Pdx1mRFP-Ins1eGFP activity. The top three panels are the negative and positive controls. The negative controls (No virus; pTiger-Pdx1mRFP-Ins1eGFP only) show no GFP activity, as expected. In the positive control (pTigerCMVeGFP) there is GFP expression. In the bottom two panels are the experimental conditions. A very few amount of cells expressed GFP.

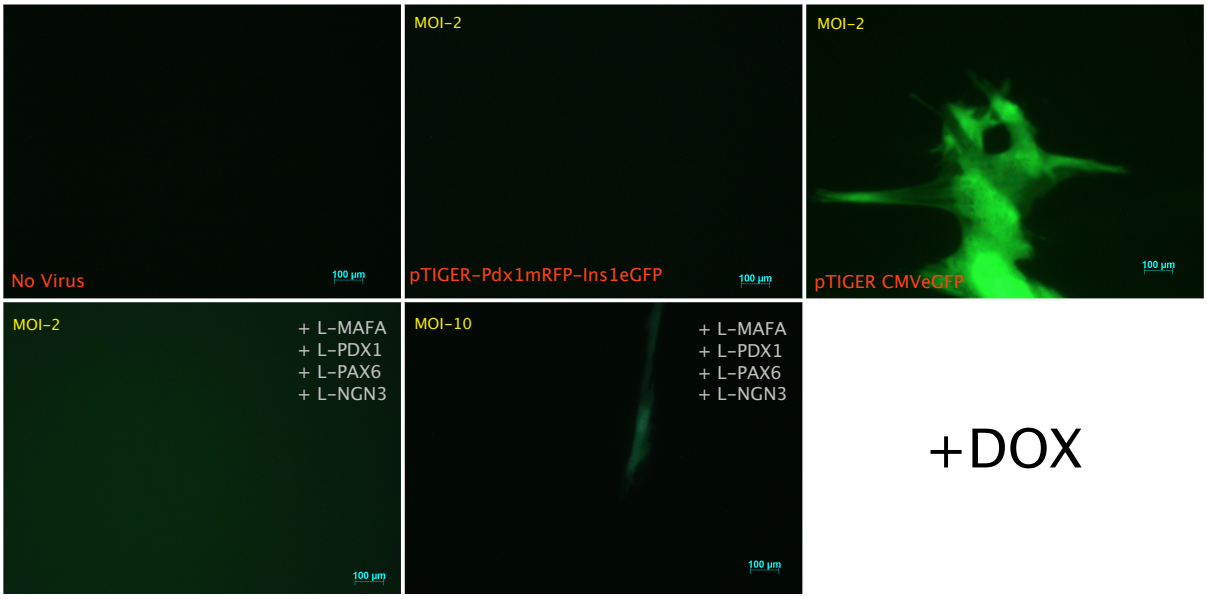

Figure 21: Effect of the ectopic expression of MafA, Pdx1, Pax6 and Ngn3 in the transdifferentiation of human dermal fibroblasts into the β-cell phenotype, after 21 days in culture with the addition of doxycycline (1µg/mL) into the medium. GFP expression indicates the reporter pTiger-Pdx1mRFP-Ins1eGFP activity. The top three panels are the negative and positive controls. The negative controls (No virus; pTiger-Pdx1mRFP-Ins1eGFP only) show no GFP activity, as expected. In the positive control (pTigerCMVeGFP) there is GFP expression. In the bottom two panels are the experimental conditions. A very few amount of cells expressed GFP.

3. DISCUSSION AND CONCLUSION

This thesis is part of a larger project ongoing in the Molecular and Regenerative Medicine Laboratory at University of Algarve. The goal of the overall project is to develop a method to transdifferentiate adult human cells to pancreatic β-cells. The strategy chosen to achieve this is by forced over-expression of pancreas specific transcription factors.

Direct transdifferentiation is the process by which a given cell type is driven to replace the underlying regulatory state that establishes and maintains its cellular phenotype for an alternative regulatory state that will establish a different cellular phenotype. The first report that such a process could be driven by transcription factors that act as 'master regulators' came in 1989 with the work of Weintraub et al. who demonstrated that over-expression of the muscle specific transcription factor MyoD was capable of activating the expression of muscle specific genes in a number of different cell types (Weintraub & Tapscott 1989). This report was eventually followed by others, in which transdifferentiation was demonstrated for a number of cellular transitions, which have been discussed in the introduction of this work.

In particular, efforts have been made by multiple groups worldwide to develop transdifferentiation methods to create pancreatic β-cells for the treatment of diabetes. Most of these efforts have tested the use of one or a few pancreas specific transcription factors starting from a range of initial cell types and have been partially successful, as a number of groups have demonstrated the feasibility of obtaining insulin-producing cells (Zhou et al. 2008). However, the phenotype obtained still falls short of a fully complete beta cell phenotype, in particular regarding the ability of the transdifferentiated cells to sense glucose concentrations and to appropriately secrete insulin in response. To date, there has been no report of a systematic search for an optimized set of transcription factors capable of driving transdifferentiation to the beta cell fate.

Cloning a collection of transcription factors

Such a project must start with the development of a collection of pancreas specific transcription factors clones into an appropriate vector. A review of the literature led to the identification of 22 transcription factors known to be involved in pancreatic development or function of pancreatic β-cells. We obtained these transcription factors from three sources:

a) A commercial library of genes that are already available in pEntry vectors of the Gateway system. Of note, the availability of these clones invited the use of a strategy based on Gateway recombination technology. A second point to keep in mind is that all these clones lacked stop codons, as the library had been designed to create fusion protein expression vectors.

b) An attempt was made to obtain beta cell specific genes directly from total RNA from human islet samples by RT-PCR

c) A set of commercially available cDNA clones (complete with stop codons) in mammalian expression vectors.

Choice of vector

An important aspect of the projects design relates to the choice of delivery vector for the transcription factors. Such a delivery vector would need to be practical and efficient in terms of delivery, so a lentiviral vector was chosen. Lentiviral vectors have a carrying capacity of up to 8 Kb, which was more than sufficient for all our chosen transcription factors. In addition, they are capable of efficient transduction of both dividing and non-dividing cells of various cell types.

In addition, inducible expression was a desirable feature due to the necessity of ascertaining the phenotypic stability of any transdifferentiation we could obtain. By using an inducible expression vector, it would be possible to deliver the transcription factors to the initial cell type, turn on the expression to induce transdifferentiation, and eventually turn

expression off in order to determine if the new regulatory state could be sustained in the absence of expression of exogenous factors.

All these characteristics are present in our lentiviral vector of choice, which contains all the elements required for doxycicline-regulated induction of expression. This lentiviral vector contains a TET-ON system, in which the transcription factor to be expressed is under the control of a TET-ON promoter. This promoter is only functional if a transactivator is expressed in the cell and if doxycicline is present in the cell culture medium. The lentiviral vector contains a constitutive promoter expressing the required transactivator.

However, this lentiviral vector, being a complex construct, has multiple restriction enzyme sites, making cloning by traditional restriction enzyme cutting and T4 DNA ligase techniques challenging due to the scarcity of unique restriction sites in the appropriate positions.

Cloning by Gateway recombination

The availability of a number of transcription factors already cloned in pEntry vectors led us to attempt cloning by use of the Gateway recombination system. This required us to transform our lentiviral vector of choice into a Gateway destination vector by introducing a Gateway destination cassette at the appropriate position in the lentiviral vector. This was achieved by classic cloning in which the destination cassette was amplified incorporating MluI and EcoRV restiction sites into the forward and reverse primers. The fragment was cloned into a cloning vector and sequenced. In a second step, the MluI-EcoRV destination cassette was cloned into the vector similarly cut with MluI and EcoRV.

The collection of clones in pEntry vectors (without stop codons) was sequenced to ensure they contained no mutations, and subjected to an LR recombination reaction for transfer into the destination vector. This strategy, while straightforward, was partially successful. Out of 9 clones we attempted to clone using this strategy we obtained 4 (*IL-1, ISL-1, NeuroD1* and *MafA*).

Despite repeated attempts, we were unable to obtain correct clones for the remaining 5 clopnes (*Pax6, HADHB, PDX1, CDK4* and *FBX32*). The reason for this remains unclear, but is not unheard of when using Gateway technology.

In essence, the reaction employed is one of recombination and sensitive to the nature of the sequences involved in the reaction; we hypothesize that the unsuccessful clones underwent unwanted recombination reactions that resulted in clones with incorrect structure.

In order to correct the sequence of the clones we had obtained in terms of reconstructing the missing stop codon, we utilized a commercially available strategy (Quick Change Mutagenesis Kit-Stratagene). This system consists in performing a PCR reaction using primers containing the mutations that one wants to introduce into the plasmid on an intact plasmid molecule. Successive rounds of PCR result in the amplification of a molecule with modified sequence. We partially corrected one of the clones (Lenti-TET-ON-NeuroD1) using this strategy. This clone awaits complete correction, as do the other three clones we obtained (*IL1*, *ISL-1* and *MafA*).

Cloning using the In-Phusion strategy

We then attempted a different strategy: In-Phusion cloning. This approach involves mixing two fragments: a) the linearized vector and b) the insert that is to be cloned. Of note, both fragments need to have a sequence overlap of 15 bp at their respective ends, as explained in the results section. The Vvpol enzyme (derived from vaccinia virus) has a 3´to 5´exonuclease activity and exposes variable length 5´overhangs at the ends of both fragments. Since the fragments posses 15 base overlaps of identical sequence, complementary overhangs are created, allowing the binding through base complementary of the fragments. These are transformed into bacteria and repaired in vivo by DNA nick repairing enzymes.

Using this system, we successfully cloned four transcription factors (*Ngn3, Pax6, Pdx1* and *MafA)* out of 11 attempted. Despite repeated attempt, the remaining factors could not be cloned. The reasons for this failure are unclear, but we suspect that the particular lentiviral backbone we have chosen to clone our transcription factors into is rather unstable.

A first attempt at transdifferentiation

As the first four transcription factors (*Ngn3, Pdx1, MafA* and *Pax6*) became available we decide to attempt a transdifferentiation experiment. To do so, lentiviral preparations were made of the four lentiTET-ON TFs. The cells of choice were adult human fibroblasts obtained from a commercial vendor. The design involved co- transduction of the cells along with a reporter lentiviral vector carrying RFP under the control of a pdx1 promoter and GFP under the control of an insulin promoter. The rationale is that a transdifferentiation event would cause upregulation of fluorescence.

However, no reporter activity was evident after three weeks of culture post-transduction. Lack of transduction was not the cause, as a control lentiviral vector expressing GFP was capable of transducing cells. We conclude that this particular combination of transcription factors was incapable of transdifferentiation of human adult fibroblasts under the condition used.

Future directions

Despite intense work and repeated attempts, a number of transcription factors remain to be cloned. Success in this cloning effort is obviously crucial to the development of the project. We intend to obtain a different lentiviral vector backbone with the same characteristics (TET-ON system) which has a better set of available restriction sites for cloning and which will hopefully be more stable in cloning.

Once a more complete set of transcription factors is obtained, attempts will be made to transduce several different cell types with a large collection of TFs in order to test for transdifferentiation.

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