Identification of proteins involved in pancreatic endocrine progenitor cell development

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A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

2017
School of Medical Sciences

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Abstract

University: The University of Manchester
Degree: PhD Stem Cell Research
Year: 2017
Name: Karolina Mosinska-Kodzik
Thesis title: **Identification of proteins involved in pancreatic endocrine progenitor cell development**

Pancreatic endocrine progenitor (PEP) cells can serve as an additional pool of cells for the Edmonton protocol (transportation of islets of Langerhans), a form of treatment for Type 1 Diabetes. This treatment is, however, limited by the shortage of donors. PEP cells can be efficiently generated *in vitro* from stem cells and following transplantation mature into functional β-cells. Additionally, unlike stem cells, PEP cells do not carry the risk of teratoma formation. However, currently available differentiation protocols still yield a mixed population of cells, therefore PEP cells have to be isolated based on their expression profile. PEP cells are currently identified based on expression of transcription factors (TFs). Although, TFs are nuclear proteins, therefore, isolation of intact PEP cells is not possible. In order to apply antibody-based cell sorting for PEP cells isolation cell membrane marker proteins specific to those cells need to be identified. Here a list of novel putative markers for PEP cells has been generated. This was achieved by transcriptomic and proteomic analysis of PEP cells generated from induced pluripotent stem cells (iPSCs). Additionally, data mining analysis applied here revealed signalling process previously not strongly associated with pancreatic development. Moreover, recently developed pancreatic mesenchymal stem cells (MSCs) were also tested for their potential to differentiate into PEP cells. Those cells were subjected to differentiation with a small compound Isx-9 and a step-wise protocol with conditioned media. However, this approach did not induce the expression of key TFs at the protein level.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Acknowledgement

First, I would also like to thank Prof Mark Dunne and Dr Karen Cosgrove for providing me with the opportunity to work on this project.

Secondly, I would like to thank my co-supervisor Dr Richard Unwin for all his help, guidance, time dedicated to this project and feedback.

Finally, thank you to my advisor Dr Liz Fitzgerald and to all past and present members of Dunne/Cosgrove group, especially to Dr Alexander Ryan, Dr Sophie Kellaway, Dr Aleksandra Toloczko and to my favourite research technician Ms Saba Khan.

Abbreviations

AI-pMSCs- adult islets-derived pancreatic mesenchymal stem cells

BMP- bone morphogenic protein

BSA - bovine serum albumin

Btc - betacellulin

cDNA- complementary DNA

CHI- Congenital Hyperinsulinism

CK19- Keratin 19

c-Kit- KIT Proto-Oncogene Receptor Tyrosine Kinase

DAPI - 4',6-diamidino-2-phenylindole

DDR1- Discoidin Domain Receptor Tyrosine Kinase 1

DISP1- Dispatched RND Transporter Family Member 1

DNER- Delta/Notch Like EGF Repeat Containing

ECM- extracellular matrix

EGF - epidermal growth factor

EMT - epithelial-mesenchymal transition

FGF- fibroblast growth factor

FoxA2- Forkhead Box A2

Gata4- GATA Binding Protein 4

Gata6- GATA Binding Protein 6

GLP1- Glucagon-like peptide-1

GSIS- Glucose-stimulated-insulin-secretion

hESCs- human embryonic stem cells

HGF- Hepatocyte growth factor

iPSCs- induced pluripotent stem cells

ISL1- ISL LIM Homeobox 1

Isx-9- Isoxazole 9

iTRAQ- Isobaric tag for relative and absolute quantitation

LC-MS- Liquid chromatography-mass spectrometry

LRP11- LDL Receptor Related Protein 11

MafA- MAF BZIP Transcription Factor A

MafB- MAF BZIP Transcription Factor B

mESCS- mouse embryonic stem cells

mRNA- messenger RNA

MS- mass spectrometry

MS/MS- tandem mass spectrometry

MSCs - mesenchymal stem cells

NANOG- Nanog Homeobox

NEUROD1- Neuronal Differentiation 1

NGS - normal goat serum

Nkx2.2- NK2 Homeobox 2

Nkx6.1- NK6 Homeobox 1

Oct4- POU Class 5 Homeobox 1

PBS - phosphate buffered saline

Pdx1- pancreatic and duodenal homeobox 1

PEP- Pancreatic Endocrine Progenitor Cells

PP- Pancreatic Progenitor Cells

PROM1- Prominin 1

SEZ6L2- Seizure Related 6 Homolog Like 2

Shh- Sonic Hedgehog

Sox17- SRY-Box 17

Sox2- SRY-Box 2

Sox9- SRY-Box 9

Ngn3- Neurogenin 3 (Neurog3)

T1D- Type 1 Diabetes

T2D- Type 2 Diabetes

TFs- Transcription factors

TMEM27- Transmembrane Protein 27

TPB - ((2S,5S)-(E,E)-8-(5-(4-(triuoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam

TROP2 (TACSTD2)- Tumor Associated Calcium Signal Transducer 2

TSPAN7- Tetraspanin 7

DCC- DCC Netrin 1 Receptor

ROBO2- Roundabout Guidance Receptor 2

FLRT3- Fibronectin Leucine Rich Transmembrane Protein 3

DSCAM- DS Cell Adhesion Molecule

UNC5C - Unc-5 Netrin Receptor C

MPZL2- Myelin Protein Zero Like 2

MPZL3- Myelin Protein Zero Like 3

NEO1- Neogenin 1

ITGB8- Integrin Subunit Beta 8

ITGA8- Integrin Subunit Alpha 8

ITGB6- Integrin Subunit Beta 6

ITGA1- Integrin Subunit Alpha 1

ALCAM- Activated Leukocyte Cell Adhesion Molecule

ATP1B1- ATPase Na+/K+ Transporting Subunit Beta 1

ATP2B1- ATPase Plasma Membrane Ca2+ Transporting 1

BCAM- Basal Cell Adhesion Molecule (Lutheran Blood Group)

CADM1- Cell Adhesion Molecule 1

Chapter 1

Introduction

The pancreas is an organ composed of several types of cells with complex development and physiology (Pandol, 2010; Pan and Wright, 2011). The majority of knowledge about pancreas function and development has been obtained from research on model organisms (e.g. Xenopus, Zebrafish and mouse), although, differences between species exist in the biology and development of this organ (Oliver-Krasinski *et al.*, 2009; Jennings *et al.*, 2015; Kim and Hebrok, 2001). Nevertheless, knowledge of mouse pancreatic development, with some success, has been applied to develop protocols for generation of β-like cells from human embryonic stem cells (D'Amour *et al.*, 2006; Pagliuca *et al.*, 2014; Rezania *et al.*, 2014). These protocols use a combination of factors important for pancreatic development, in order to mimic events occurring during embryogenesis. The efforts to generate functional, glucose-responsive β-like cells aim to improve currently available treatments for Type 1 Diabetes (T1D).

Type 1 Diabetes is a disease affecting pancreatic β-cells (Van Belle *et al.*, 2011). T1D can be partially reversed by islet transplantation (also known as the Edmonton protocol). However, this form of a treatment is limited by the shortage of donors. An additional pool of cells (e.g. pancreatic progenitor (PP) cells or pancreatic endocrine progenitor (PEP) cells) available for transplantation can be generated from stem cells. Currently, available differentiation protocols can generate PP and PEP cells with relatively high efficiency (approximately 90 % of

the cell population) (Rezania *et al.*, 2014). PP and PEP cells have the ability to mature into functional β-cells following transplantation and the risk associated with the formation of a teratoma is much lower compared to stem cells (Kroon *et al.*, 2008; Kelly *et al.*, 2011). One of the factors limiting the use of PP and PEP cells for transplantation-based treatment is the fact that current differentiation protocols still yield a mixed population of cells with a substantial number of undifferentiated stem cells present. PP and PEP cells can be identified based on their expression profile. Transcription factors (TFs) are currently the most commonly used markers for both progenitor cells types. However, TFs are nuclear fraction proteins and this limits their use as markers for the isolation of intact, live cells. Several cell membrane markers have been recently identified. Although, these markers lack specificity to PP and PEP cells and have not been broadly used (Fishman *et al.*, 2012; Kelly *et al.*, 2011; Hald *et al.*, 2012). Henceforth, this study will aim to determine novel, effective markers for PP and PEP cells.

1.1 Diabetes

Diabetes is a metabolic disease characterised by poor glycaemic control. This can be due to defects in insulin secretion by pancreatic β-cells, or loss of insulin sensitivity in skeletal muscle and adipose tissue (Guthrie and Guthrie, 2004). The prevalence of diabetes has increased markedly over past decades with 108 million of people living with diabetes in 1980 compared to 422 million in 2014 (World Health Organisation 2016). Based on the underlying molecular mechanisms and development of the disease, two main forms of diabetes (type 1 and type 2) have been defined (Najjar *et al.*, 2003).

1.1.1 Type 1 Diabetes

Type 1 diabetes accounts for 10 % of all cases of diabetes, however, this number might be an underestimate, as some cases of T1D are misdiagnosed as type

2 diabetes (T2D). (Atkinson, 2012). T1D is caused by autoimmune destruction of insulin-producing pancreatic β-cells (Van Belle *et al.*, 2011). The exact mechanisms causing the development of autoimmunity in T1D have not been yet elucidated (La Torre and Lernmark, 2010). The main factors to contribute to T1D include genetic predispositions and environmental factors. Almost 50 loci linked with the development of T1D have been identified so far, for example DR4-DQ8/DR3-DQ2 combination of human leukocyte antigen (HAL) genes (present in 90 % of children affected by T1D) and the IDDM2 locus (a region located upstream of the *insulin* promoter that contains variable number of tandem repeats) (Gillespie, 2006; Devendra and Eisenbarth, 2003; Pugliese *et al.*, 1997). Environmental factors associated with the T1D pathogenesis include virus infection, nutritional influences and vitamin D deficiency (Atkinson, 2012).

Complications associated with diabetes include several conditions caused by persistent high blood glucose leading to damage to small blood vessels. This includes: damage to blood vessels supplying nerves (neuropathy), damage to the blood vessels of the retina (retinopathy) that potentially can lead to blindness, and nephropathy caused by damage to the capillaries of the glomeruli. All of the above conditions can lead to increased rate of mortality (Harjutsalo *et al.*, 2011).

Currently, no cure for T1D is available and insulin injections are the most common form of treatment. This form of treatment has been greatly improved with the development of devices such as insulin pumps, which allow for more precise control of blood glucose levels and a lower frequency of hypoglycemic episodes compared to injection (Ghazanfar *et al.*, 2016). However, this solution still has certain disadvantages such as the delay between device glucose sensing and insulin delivery, skin infection at the site of infusion, a faster development of ketoacidosis compared to multiple injections methods and requires increased supervision by a specialist team (Cichocka *et al.*, 2016; Pickup, 2009). Therefore, a treatment that would restore functions of a healthy pancreas would eliminate disadvantages of a therapy based on exogenous insulin and by that improve patient

outcomes and reduce healthcare costs.

An alternative treatment, called the Edmonton protocol, involves transplantation of cadaveric islets into the hepatic portal vein of a patient. This treatment provides exogenous insulin independence with a reported success rate of 80 % at one year and 20 % at five years post-transplantation (Ryan *et al.*, 2005). In addition, this approaches have the benefit that transplanted β-cells can be placed in a non-endogenous site and within immunoprotective devices (Krishnan *et al.*, 2014; Jones *et al.*, 2008). This minimises the requirement for immunosuppressive treatments following transplantation and provides an opportunity for autologous transplants, which do not cause graft-vs-host disease and eliminate the requirement of a tissue/ organ donor (Krishnan *et al.*, 2014).

Currently, transplantation of islets depends on cells isolated from donors and is limited by the fact that for each transplantation, islet cells isolated from multiple (2-3) donors are required (McCall and James-Shapiro, 2012). Therefore, it is beneficial to develop methods to generate an additional pool of insulin-producing cells and this has become an attractive area of research. This includes research into regeneration of pancreatic β-cells or generation of new cells (Gillespie, 2006). Potential sources of an unlimited number of insulin-producing cells are pluripotent stem cell cultures, including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). However, generation of functional β-cells is still hampered by insufficient knowledge of the developmental process resulting in the formation of immature β-cells and difficulties in obtaining a pure population of fully differentiated cells (Pagliuca and Melton, 2013).

1.1.2 Type 2 Diabetes

Type 2 Diabetes accounts for 90 % of all diabetes cases worldwide (Organization, 2016). The pathogenesis of type 2 diabetes originates in dysregulation of the feedback loop between pancreatic beta cell and insulin sensitive tissue- muscle cells, adipocytes and liver. Insulin resistance of muscle and fat tissue leads to dimin-

ished glucose uptake, increased insulin synthesis and secretion as the pancreatic β-cells attempt to normalise blood glucose levels. This stress gradually leads to exhaustion of insulin-producing cells, reduction in their numbers and finally the inability to control glucose levels (Rahier *et al.*, 2008).

Dysfunction of beta cells, namely reduced glucose-stimulated secretion of insulin is yet another factor linked to the development of type 2 diabetes (Kahn *et al.*, 2014; Ashcroft and Rorsman, 2012). Therefore, in addition to reduced beta cell mass, remaining insulin-producing cells are not functioning properly. Treatment of type 2 diabetes is currently based on oral and injectable drugs designed to reduce and maintain blood glucose level close to the physiological range (Kahn *et al.*, 2014) and in later stages also includes insulin injections (Efrat, 2008).

1.2 Pancreatic Islets Biology

The pancreas is a gland composed of three main cell types: exocrine (comprising over 85% of the organ), ductal and endocrine. Cells forming exocrine and ductal structures are responsible for secretion (exocrine cells) and transport (ductal compartment) of digestive enzymes, water and ions into the intestine (Pandol, 2010). The endocrine compartment of the pancreas is composed of $\alpha, \beta, \delta, \epsilon$ and PP cells, which together form structures called islets of Langerhans. The main function of pancreatic islet cells is secretion of hormones: insulin (β-cells), glucagon (α-cells), somatostatin (δ- cells), ghrelin (ϵ- cells) and pancreatic peptide (PP- cells) (Cabrera *et al.*, 2006).

The vast majority of information regarding pancreas development, composition, physiology and ability to regenerate was obtained from studies of mouse model organisms (Jørgensen *et al.*, 2007; Pan and Wright, 2011). This was motivated by the limited access to human tissue and lack of cell line models of functional islet cells. However, several differences have been identified between human and rodent pancreatic islets, including islet cytoarchitecture, development and cell physiology (Dai *et al.*, 2012). Therefore data obtained from animal models not always can be directly translated into development and physiology of human pancreas. Albeit, limited but insightful observations regarding human pancreas development have been recently presented by Jennings et al. (2013). Where possible a review of human pancreas development will be presented here.

1.2.1 β-cells

The main function of pancreatic β-cells is biosynthesis and secretion of insulin, a hormone which lowers blood glucose levels.

Insulin secretion is stimulated by an increase in the concentration of food breakdown products such as monosaccharides, short peptides, amino acids and triacylglycerols, following a meal. Insulin triggers uptake of those metabolites by

muscles and fat tissue and simultaneously stops the release of similar molecules from body reserves (Wilcox, 2005).

Insulin release, following a meal, can be divided into two stages. The first, acute phase of insulin secretion starts with β-cells secreting the total of ready releasable pool, which is formed by the insulin contaminating granules docked at the cell membrane. Following this, cells start to release reserve pool of insulin, this is referred to as the chronic phase and lasts as long as the glucose stimulation (Rorsman and Renström, 2003).

In order to respond accurately to changing concentrations of blood glucose, islet cells are equipped with the glucose sensing mechanism. The first element of this mechanism in β-cells is a glucose-selective transporter. Three isoforms of the glucose transporter, GLUT1, 2 and 3, have been identified in rodent and human islets. In rodent β-cells, GLUT2 was identified as the main isoform, whereas in human insulin-producing cells the main isoforms are GLUT1 and GLUT3 (McCulloch *et al.*, 2011). Once within β-cells, glucose is phosphorylated by glucokinase; a hexokinase and the second element of the glucose sensing machinery. Expression of glucokinase is limited to only four types of tissue, including pancreatic β-cells, hepatic cells, glucose-sensitive neurones and enterocytes (Suckale and Solimena, 2008). Low affinity to glucose and inhibition by its own product (glucose-6-phosphate) make glucokinase the rate-limiting step in glucose metabolism (Fu et al., 2013). Following the generation of glucose-6-phosphate, phosphorylated glucose enters the glycolysis and tricarboxylic acid cycle to generate ATP. In this way, ATP links the increase in glucose concentration with activation of ATP-sensitive potassium channels in β-cell membrane. The increase in intracellular ATP/ADP ratio causes closure of K_{ATP} channels, which leads to cell membrane depolarization, followed by the activation and opening of L-type voltage-gated Ca^{2+} channels allowing for an influx of Ca^{2+}. The increase in the intracellular Ca^{2+} concentration stimulates exocytosis of granules containing insulin (Fu *et al.*, 2013) (Fig.1.1).

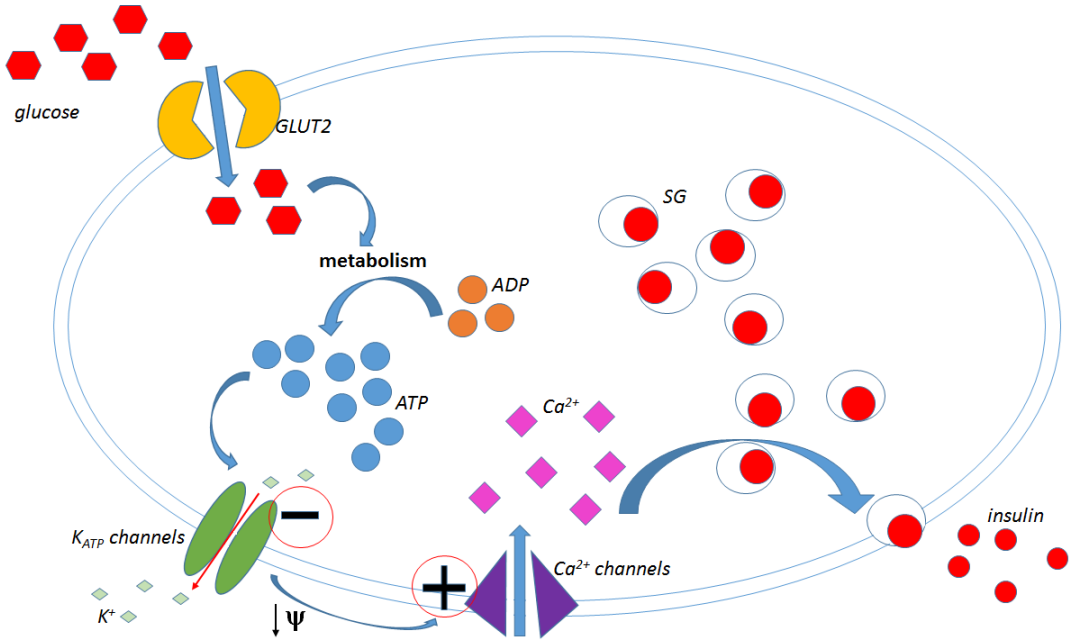

Figure 1.1: Glucose-stimulates insulin secretion. Glucose is transported in the β-cell by a GLUT transporter, is phosphorylated by glucokinase and enters the glycolysis and tricarboxylic acid cycle to generate ATP. The shift in the ATP/ADP ratio leads to the closure of K_{ATP} channels, cell membrane depolarisation, opening of L-type voltage-gated Ca^{2+} channels and Ca^{2+} influx. The increase in the intracellular Ca^{2+} concentration stimulates exocytosis of insulin-containing granules. GLUT2- glucose transporter; K_{ATP} channels- ATP-sensitive potassium channels, ψ- membrane potential, SG- secretory granules, adapted from (Rorsman and Renström, 2003).

Insulin secretion by β-cells is also additionally stimulated by the glucose metabolism products such as NADH, glutamate and malonyl-Co. These molecules further associate glucose metabolism with increased insulin secretion (Wollheim and Maechler, 1999). Moreover, gastric inhibitory polypeptide (GIP) and glucagon-like peptide (GLP-1) are hormones secreted from the gastrointestinal tract which can directly bind to their specific receptors on β-cell surface and moderate insulin secretion (MacDonald *et al.*, 2002; McIntosh *et al.*, 2009). Glucose stimulated insulin secretion (GSIS) is also influenced by free fatty acids (FFA), those metabolites interact with β-cells through the free fatty acid receptor (FFAR)-1 also known as GPR40 (Itoh *et al.*, 2003). The metabolism of FFA is a source of molecules such as diacylglycerol (DAG) that activate novel protein kinase C, previously implicated in insulin secretion (Fu *et al.*, 2013).

1.2.2 α-cells

The primary function of α-cells is the secretion of glucagon, a hormone exerting the opposite effect to insulin by stimulating the release of glucose from body energetic storage via activation of hepatic glycogenolysis and gluconeogenesis (Quesada *et al.*, 2008). The mechanism of glucagon secretions is still debatable, however some similarities to insulin secretion from β-cells have been observed. This includes the fact that both processes are regulated by glucose concentration and depend on the activation of cell membrane ion channels. Glucose is transported into α-cells through the GLUT1 transporter. When glucose concentration is low, α-cells K_{ATP} channels are closed causing cell membrane depolarisation, and activates voltage-dependent Na^+, Ca^{2+} and A-type K^+ channels. Ca^{2+} ions influx triggers exocytosis of glucagon- containing granules (Gaisano *et al.*, 2012; Sandoval and D'Alessio, 2015).

1.2.3 δ- cells, PP-cells and ϵ-cells

Pancreatic δ- cells produce and release somatostatin, which acts as an important regulator of both β and α-cells. Somatostatin has an inhibitory effect on glucagon and insulin secretion within pancreatic islets. However, the exact mechanism of somatostatin release and the pathways involved in its control still remain unclear (Adriaenssens *et al.*, 2016).

Pancreatic peptide- releasing cells comprise usually less than 1 % of all islet cells. PP cells in many species can also be found outside islet structures in the exocrine parenchyma, where they occur as single or clustered cells (Ekblad and Sundler, 2002). The function of PP cells are still not fully understood, however, their main functions are currently suggested to be linked with regulation of gastrointestinal motility (Batterham *et al.*, 2003) and energy expenditure (Liu *et al.*, 2008). In the context of pancreatic islet cell interactions, PP has been proposed to be involved in regulation of pancreatic exocrine cell secretion and

suppression of somatostatin secretion from δ- cells (Kim *et al.*, 2014).

The fifth, and most recently discovered type of pancreatic endocrine cells are ghrelin- secreting ϵ- cells (Wierup *et al.*, 2013). The function of ghrelin within pancreatic islets remains unknown but it has been hypothesised that ghrelin might act as a regulator of β-cells function and survival (Granata *et al.*, 2007). Additionally, based on the observation that ghrelin production is higher during pancreas development, its role as a promoter of cell growth and suppressor of apoptosis has been proposed (Andralojc *et al.*, 2009; Irako *et al.*, 2006).

1.3 Pancreatic Development

The majority of current knowledge regarding pancreatic development has been accumulated from observation of this process in model organisms e.g. the development of mouse pancreas.

The pancreas is a definitive endoderm derived organ (Pan and Wright, 2011). During embryonic development, in the gastrulation phase, three cell lineages arise. Gastrulation is initiated with the formation of a structure called the primitive streak. This structure introduces bilateral symmetry to the developing embryo and provides a landmark where definitive endoderm originates. Definitive endoderm is initially composed only of a thin sheet of cells that gradually develop into the primitive gut tube (McCracken and Wells, 2012).

The pancreatic region of gut endoderm is specified around Embryonic day 8.5 (E8.5) and is marked by expression of the Pancreatic and duodenal homeobox 1 (Pdx1) gene (Hebrok *et al.*, 1998). The first evidence of pancreas formation can be observed at E9.5 when two pancreatic buds are formed. According to their location, these are referred to as dorsal and ventral buds (Fig.1.2).

Based on observation of morphological changes, pancreas organogenesis in mice has been divided into two overlapping stages: primary (E9.5-E12.5) and secondary (starting at E13.5) transition. The primary transition is characterised by proliferation of pancreatic progenitor cells, which leads to transformation of the original cuboidal epithelium into a stratified structure. This is accompanied by formation and fusion of the microlumen, leading to the formation of initial branches and adding to the complexity of forming pancreatic buds (Oliver-Krasinski *et al.*, 2009). At E11.5, both ventral and dorsal buds are brought into close proximity as a consequence of gut tube movements that leads to the pancreatic bud fusion. Following pancreatic bud fusion, pancreatic progenitor cells continue to divide and form thickening epithelium. The size of the mature organ is determined at this stage by the number of generated progenitor cells (Stanger

et al., 2007).

At E12.5-13, significant morphological reorganisation and expansion of pancreatic epithelium occurs; this remodeling is referred to as the secondary transition. Simultaneously to this morphological reorganisation, progenitor cells differentiate into the acinar, ductal and endocrine lineage. Within extensively branching epithelium acinar cells are formed at the termini of ductal structures, whereas trunks become a domain containing proliferating and differentiating progenitors. Progenitor cells occupying this niche differentiate into pancreatic endocrine progenitors or ductal cells (Pan and Wright, 2011)(Fig.1.2).

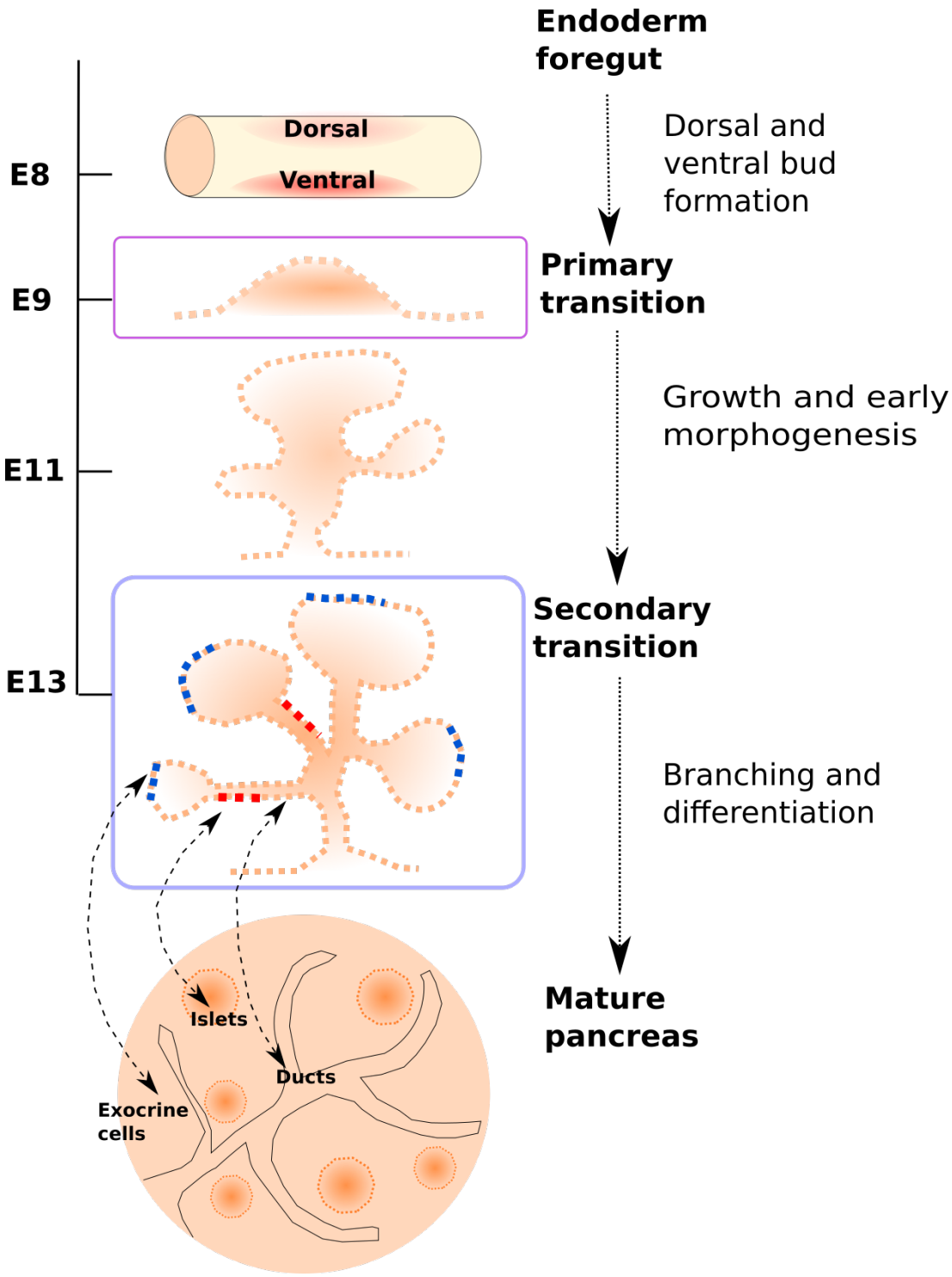

Figure 1.2: Mouse pancreatic organogenesis. Mouse pancreatic development can be divided into two phases: primary and secondary transition. During the primary transition, endoderm cells proliferate and cause initially thin cuboidal epithelium to expand into complex, stratified epithelium. During the secondary transition, epithelial cells continue to expand and remodel. This leads to the formation of an organ composed of acinar cells producing digestive enzymes, ductal cells forming a network connected to the duodenum and endocrine cells, which delaminates from ductal structures and form islets (blue dots- precursors of exocrine cells, red dots- pancreatic endocrine progenitor cells), adapted from (Pan and Wright, 2011).

1.3.1 Transcription Factors Crucial to Pancreatic Development

Various stages of pancreas development are defined by the transcription factors (TFs) that regulate essential molecular events, and lead to the acquisition of a specific cell identity. Those molecules are therefore often used as markers when the development of pancreatic cells is mimicked in the stem cell culture conditions (Fig.1.3).

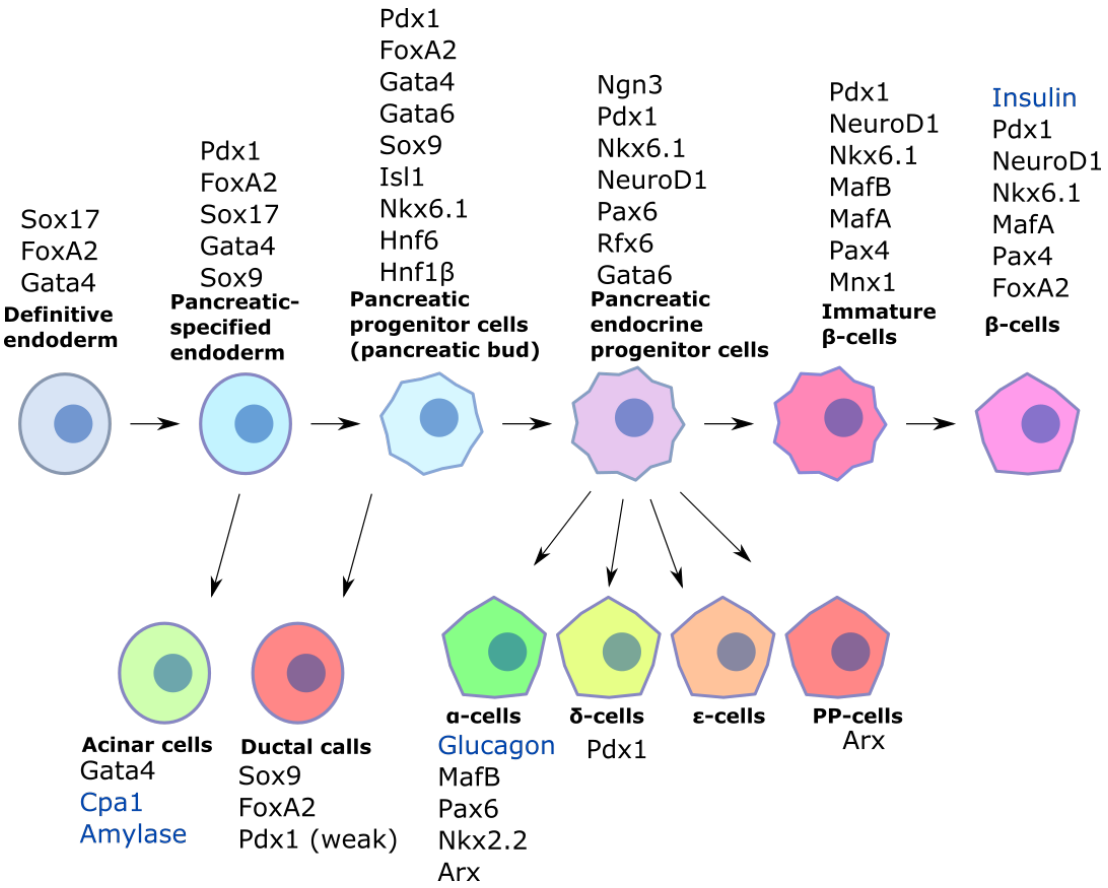

Figure 1.3: Transcription factors controlling development of pancreatic β-cells. Transcription markers and other factors (blue) use as markers to identified pancreatic progenitor cells at different stages of development; adapted from (Pan and Wright, 2011; Jennings *et al.*, 2015)

1.3.1.1 Definitive Endoderm Formation and Patterning

Definitive endoderm is generated from the inner germ layer of the embryo and gives rise to internal lining of the respiratory and digestive system and organs

such as thyroid, gallbladder, liver and pancreas (Zorn and Wells, 2009). Formation and segregation of endoderm and mesoderm are controlled by a TGFβ family growth factor- Nodal. Nodal interaction with type I and type II receptor, and Smad proteins leads to activation of its target genes: Sox17, FoxA2 and Gata4/6 (McCracken and Wells, 2012).

Following cell segregation between endodermal and mesodermal lineage, primitive gut tube undergoes patterning into regions that will form specific organs. This process is controlled by signalling molecules from neighbouring tissue such as notochord and cardiac mesoderm. Signalling events essential for specification of pancreatic endoderm include repression of Sonic Hedgehog (Shh) signalling by signals from adjacent notochord such as FGF (fibroblast growth factor) and activin ligands (dorsal bud); and inhibition of WNT and bone morphogenic protein (BMP) signalling (ventral bud) (Deutsch *et al.*, 2001; Rojas *et al.*, 2010).

FOXA2

FOXA2, a winged-helix TF during development marks definitive endoderm, however, its expression also persists in the mature pancreas (Spence *et al.*, 2009; Cano *et al.*, 2014; Lantz and Kaestner, 2005). In mice, FoxA2 has been shown to be involved in transcriptional regulation of genes specific to pancreatic β-cells such as Pdx1, and K_{ATP} channel subunits Sur1 and Kir6.2 (Gao *et al.*, 2008; Lantz and Kaestner, 2005; Lee *et al.*, 2002). In addition to being essential for pancreatic development, FoxA2 has also been linked to the maintenance of adult β-cells as its conditional deletion in those cells caused dysregulation of insulin secretion and hyperinsulinemic hypoglycemia (Sund *et al.*, 2001; Bastidas-Ponce *et al.*, 2017).

SOX17

SOX17 a member of HMG (high mobility group) is essential for definitive endoderm formation and with other TFs regulates expression of genes (e.g. Hnf1β)

involved in the development of multiple endoderm-derived organs (Kanai-Azuma *et al.*, 2002). Recently Sox17 was also established as a key regulator of cell segregation between pancreatic and hepatic lineage and loss of it expression caused atypical Pdx1 expression by liver bud and formation of pancreatic tissue in the common duct (Spence *et al.*, 2009).

GATA4 and GATA6

GATA4 and GATA6 are zinc finger type transcription factors and are involved in regulation of development of several organs (e.g. heart, liver and pancreas) (Zhao *et al.*, 2005; Holtzinger and Evans, 2005). Mice null for *Gata6* die before E7.5 due to defects in the development of extra-embryonic endoderm and mice null for *Gata4* die before E11 due to defective development of heart (Kuo *et al.*, 1997; Morrisey *et al.*, 1998). During pancreas development both Gata4 and Gata6 are co-expressed in pancreatic endoderm but successively their expression pattern becomes restricted to the exocrine compartment for *Gata4* and endocrine cells for *Gata6* (Ketola *et al.*, 2004). Moreover, recently GATA factors have also been shown to be essential for negative regulation of Shh expression in pancreatic endoderm as their conditional deletion caused conversion of pancreatic endoderm into stomach and intestinal fate (Xuan and Sussel, 2016).

1.3.2 Pancreatic Progenitors

PDX1

Pdx1, a homeobox transcription factor, is a key regulator implicated in early stages of pancreas development. Its expression marks the endoderm region that gives rise to pancreatic buds (Guz *et al.*, 1995; Ahlgren *et al.*, 1998). Its essential role in the pancreas development is confirmed by the observation that deletion of Pdx1 in mice causes pancreatic agenesis and a homozygous mutation in humans leads to congenital pancreatic agenesis, whereas heterozygous mutation causes the onset of maturity-onset diabetes of the young (MODY) and T2D (Offield *et al.*, 1996; Stoffers *et al.*, 1997). This TF can be used as a marker for pancreatic progenitor cells as Pdx1-expressing cells have been shown to give rise to ductal, exocrine and endocrine lineage (Hale *et al.*, 2005; Oliver-Krasinski *et al.*, 2009; Wescott *et al.*, 2009).

Pdx1 also plays an important function in the establishment and maintenance of β-cell identity. Deletion of Pdx1 in developing β-cells causes an increase in the Glucagon/Insulin- expressing cells ratio, this indicates that PDX1 favours β-cell over α-cell fate (Ahlgren *et al.*, 1998; Gannon *et al.*, 2008). Moreover, Pdx1 acting as a β-cell fate guarantor was recently linked with its potential function as a repressor of MafB, a TF characteristic for mature α-cells (Gao *et al.*, 2014). Therefore, PDX1 expression can be used to identify pancreatic progenitors at the early stages of development and β-cell progenitors at the later stages. Apart from its function during pancreas development PDX1 is also directly involved in the regulating of the *insulin* gene expression. A Pdx1 binding site was identified proximal to the insulin promoter (within 60 nucleotides upstream of the transcription start site) (Chakrabarti *et al.*, 2003; Barrow *et al.*, 2006). Therefore, PDX1 can be used as a marker of mature β-cells in addition to insulin.

ISLET1

Islet 1 (ISL1), a LIM-homeodomain transcription regulator, was first identified

as being involved in pancreatic endocrine cell functions in its role as an enhancer protein binding to the insulin promoter (Karlsson *et al.*, 1990). During pancreatic development, ISL1 is required for endocrine lineage specification and development of dorsal pancreatic mesenchyme (Ahlgren *et al.*, 1997). During mouse pancreas development expression of Isl1 is first noticeable at E9 and lack of its expression causes complete loss of endocrine islet cells and dorsal mesenchyme (Ahlgren *et al.*, 1997). ISL1 has also been reported to control proliferation and survival of pancreatic endocrine progenitors (PEP), therefore its expression can be utilised to identify a subpopulation of those cells (Du *et al.*, 2009; Guo *et al.*, 2011).

SOX9

SOX9 belongs to a high-mobility group (HMG) superfamily of TFs. This TF is first expressed in the mouse pancreas at E9.5 and marks pancreatic multipotent progenitor cells, its expression is gradually restricted to trunk cells at E12.5 and marks expression of bipotent cells that give rise to ductal and endocrine cells (Seymour *et al.*, 2007; Lioubinski *et al.*, 2003). The function of Sox9 as a regulator of progenitor cell numbers has been verified by studies of conditional knockout mouse models where both dorsal and ventral pancreatic sediments were small and formed by Sox9-expressing cells that escaped deletion (Seymour *et al.*, 2007). Sox9 is also essential for initiation of endocrine differentiation by activating expression of NGN3- a TF crucial for the development of pancreatic endocrine cells (Shih *et al.*, 2012). In the adult pancreas, the Sox9 expression is suppressed to ductal and centroacinar cells (Seymour *et al.*, 2007; Piper *et al.*, 2002).

1.3.3 Pancreatic Endocrine Progenitors

NEUROG3

The most important TF marking pancreatic endocrine progenitor cells in human is NEUROG3 and its homologue Neurogenin3 (Ngn3) in mice. This basic helix-loop-helix (bHLH) TF is expressed transiently during pancreatic development

and plays a key role in the specification of islet endocrine cell fate. Its expression is first detected in pancreatic epithelium at E9.5, increases until E15.5 and diminishes in the postnatal pancreas (Schwitzgebel *et al.*, 2000; Gradwohl *et al.*, 2000). Lineage tracing experiments has shown that all cells expressing Ngn3 are exclusively directed towards future endocrine differentiation (Gu *et al.*, 2002). The fact that Ngn3$^+$ cells are progenitors of islet cells has also been confirmed by the phenotype of the Ngn3 null mouse which lacks all endocrine cell types (Gradwohl *et al.*, 2000). Ngn3 is a direct regulator of several TFs essential for islet cell development, such as NeuroD1, Pax4, Pax6, Isl1 and it also suppresses its own expression (Gasa *et al.*, 2004; Gradwohl *et al.*, 2000).

NEUROD1

NeuroD1 (BETA2), a bHLH TF, is a direct downstream target of Ngn3 (Huang *et al.*, 2000). Mice deficient in NeuroD1 have poorly developed islets with a reduced number of β-cells and die of severe diabetes (Naya *et al.*, 1997).

In addition to its function in pancreas development, NeuroD1 is one of the TFs that binds to insulin and glucagon gene promoters and thus controls the expression of these two pancreatic islet hormones (Dumonteil *et al.*, 1998). The fact that NEUROD1 is a direct downstream target of NGN3 and its expression window is wider than that of NGN3 makes NEUROD1 a good endocrine cell marker during pancreatic development.

NKX2.2 and NKX6.1

Nkx2.2 in the mouse developing pancreas is first detected at E9.5 and its expression pattern overlaps with that of Pdx1 (Jørgensen *et al.*, 2007). Gradually its expression becomes restricted to Ngn3$^+$ cells and by E15.5, the Nkx2.2 expression is detected only in α, β and PP cells. Knockout Nkx2.2 mice lack β-cells and have a reduced number of α and PP cells (Sussel *et al.*, 1998; Arnes *et al.*, 2012).

Nkx6.1 expression is first detected in the pancreatic epithelium at E10 in mice

and around 30-33 days post conception (dpc) in humans (Jørgensen *et al.*, 2007; Jennings *et al.*, 2013). Nkx6.1 expression overlaps initially with Nkx2.2 expression but gradually becomes limited only to β-cells in the mature organ (Sander *et al.*, 2000; Schaffer *et al.*, 2013). Nkx6.1-deficient mice display reduced size of islets caused by a decreased number of β-cells (Sander *et al.*, 2000). Additionally, this TF has also been reported to suppress the expression of glucagon and regulate glucose-stimulated insulin secretion. Its inactivation in mature β-cells leads to defects in insulin production causing rapid onset of T2D (Schisler *et al.*, 2005; Taylor *et al.*, 2013).

MAFA and MAFB

MafA and MafB belong to the basic leucine- zipper (bZIP) family of TFs. MafB and MafA are involved in regulation of tissue- specific gene expression in several organs including brain, kidney, retina and pancreas (Tsuchiya *et al.*, 2015). In the developing mouse pancreas, MafB is expressed in progenitor cells from E12.5 and postnatally becomes restricted to α-cells (Hang and Stein, 2011). The expression of MafA, however, starts at E13.5 and is limited to maturing and mature β-cells (Matsuoka *et al.*, 2004). Mice deficient in MafB exhibit a reduced number of endocrine cells during islet embryonic development (Abdellatif *et al.*, 2015). The MafA knockout mice have no detectable defects in the developing pancreas but become glucose intolerant postnatally and in adult mice exhibit abnormal islets architecture (Zhang *et al.*, 2005).

Both transcription factors are reported to have a regulatory function in the expression of key pancreatic endocrine cell-specific genes. MafB was reported to occupy promoter regions of genes such as Glut2, Pdx1 and Nkx6.1 (Vanhoose *et al.*, 2008; Artner *et al.*, 2007). MafA was found to regulate expression of Pdx1, NeuroD1, Glut2 and insulin (Samaras *et al.*, 2003; Vanhoose *et al.*, 2008; Zhang *et al.*, 2005).

TFs are currently the most reliable tool for identification of pancreatic progenitor cells within developing pancreas or an *in vitro* cell culture. Despite the fact that their spatiotemporal expression pattern often characterises more than one stage of development, the combination of several TF still allows for the distinction between multipotent progenitor cell and endocrine precursor cell. The main limitation of this approach is, however, nuclear localisation of TF as this requires cell membrane destruction in order to identify potential progenitor cells and does not allow for isolation of intact, functional cells.

1.3.4 Signalling Pathways Involved in Pancreatic Organogenesis

Pancreas development from gut tube endoderm is controlled by patterning events and crosstalk between several signalling pathways. The key pathways include TGFβ, Wnt, Notch, PI3K, Sonic Hedgehog and retinoic acid signalling.

1.3.4.1 The Transforming Growth Factor β Signalling Pathway

During pancreas development, TGFβ signals originate in notochord and are crucial for the formation of definitive endoderm and mesoderm (Hebrok *et al.*, 1998).

TGFβ receptor ligands are categorised into two subfamilies: TGFβ/ Activin/ Nodal subfamily and BMP/growth and differentiation factor (GDF)/ Muellerian inhibiting substance (MIS) superfamily. The TGFβ signalling cascade starts with a ligand binding to type II receptor. This recruits and activates, through phosphorylation, the type I receptor. Activated type I receptors phosphorylate Smad proteins. To date eight distinct Smad proteins have been identified, Smad proteins can be divided into three functional groups: receptor-regulated (R-Smads: 1, 2, 3, 5 and 8), co-mediator (co-Smads: Smad4) and inhibitory (I-Smads: 6 and 7). Following activation, R-Smad proteins form homotrimers or a heteromeric complex with the co-mediator, Smad4. The R-SMAD/co-SMAD complex is translocated to the nucleus where it can activate its downstream target genes. Inhibitory Smads compete for binding with R-Smad and in that way negatively regulate TGFβ signalling (Shi *et al.*, 2003) (Fig.1.4).

In pancreatic development TGFβ signalling is required at several stages: Nodal signalling is necessary for the induction of endoderm and mesoderm development, BMP and Activin have been reported to induce expression of Pdx1 in both ventral and dorsal pancreatic primordium (Kumar *et al.*, 2003; Hebrok *et al.*, 1998; McCracken and Wells, 2012). TGFβ signalling ligands are also used in the design of the protocols to simulate pancreatic β-cell differentiation *in vitro*.

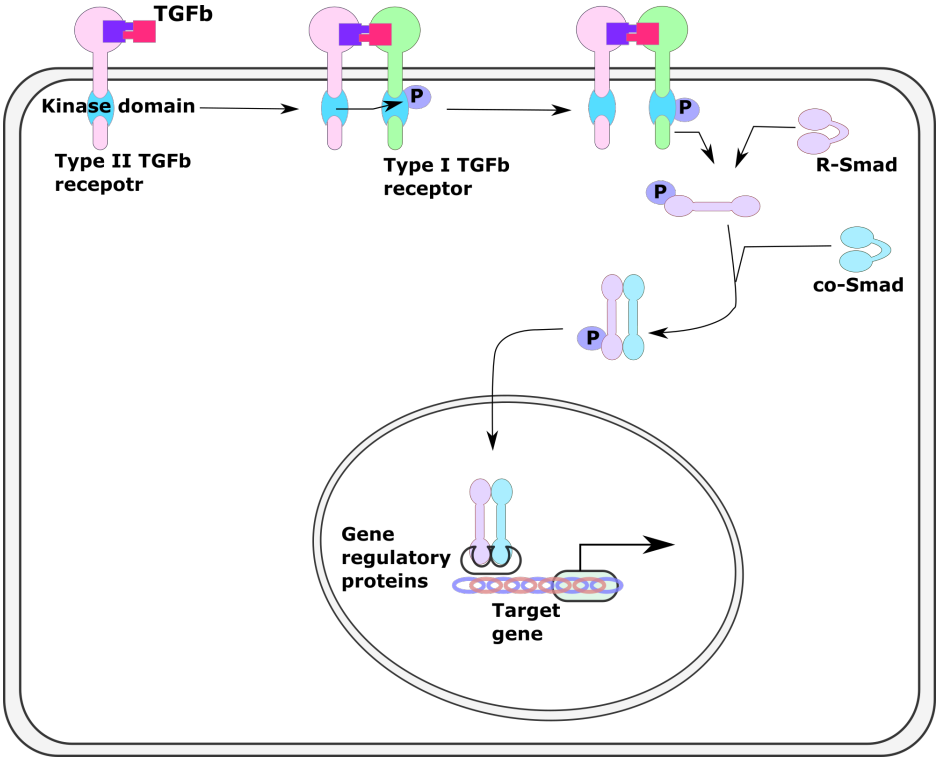

Figure 1.4: TGFβ signalling pathway. Binding of TGFβ ligand to type II receptor promotes receptor dimerisation with type I receptor and leads to its transphosphorylation. Activated type I receptor phosphorylates and activates R-SMADs, which interact with co-SMAD (i.e. Smad4). This complex is translocated to the cell nucleus where activates transcription of target genes; adapted from (Massague, 2012).

Activin A, a recombinant protein with receptor binding ability similar to Nodal is applied to induce stem cells (e.g. hESCs and iPSCs) to differentiate into definitive endoderm (Pagliuca and Melton, 2013). Additionally, BMP receptor antagonists are also used at a later stage to induce the expression of PDX1 and NKX6.1 (Nostro *et al.*, 2011).

1.3.4.2 The Wnt Signalling Pathway

Wnt signalling is a form of short- range signal transduction, in which the Wnt ligand, the protein with a covalently attached palmitoleate group, conveys stimulation to other cell within short range (Takada *et al.*, 2006).

Downstream signalling cascades triggered by Wnt cytokines were divided into Wnt/β-catenin (canonical)- exerting an effect on cell fate specification and non-canonical- involved in the establishment of cell polarity (Loh *et al.*, 2016).

The main component of Wnt/β-catenin signalling is cytoplasmic protein- β-catenin. In the absence of the Wnt ligand β-catenin is phosphorylated by the destruction complex, becomes ubiquitinated and degrades by a proteasome. The destruction complex is composed of Axin (the scaffolding protein), adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3β). The elimination of β-catenin from the cytoplasm by this constitutively active complex prevents β-catenin translocation to nucleus and expression of its target genes.

When Wnt ligand forms a complex with the Frizzled receptor and its coreceptor low-density lipoprotein receptor related protein 5 or 6 (LRP5/6), a scaffolding protein Dishevelled (Dvl) is required to this complex and this results in phosphorylation of the LRP5/6. Activation of LRP5/6 leads to relocation of the destruction complex components to the receptors and disturbs β-catenin degradation by this complex. As a result, β-catenin accumulates in the cell cytoplasm and translocates to the nucleus (MacDonald *et al.*, 2009; Loh *et al.*, 2016) (Fig. 1.5).

During pancreas development, following definitive endoderm specification, the formation of the pro-pancreatic domain in the foregut requires inhibition of Wnt signalling (Murtaugh, 2008). This has been reflected by the application of Wnt receptor agonist (e.g. Wnt3a) or GSK3β inhibitor (e.g. CHIR98014) at initial stages of the *in vitro* differentiation protocols (D'Amour *et al.*, 2006; Kroon *et al.*, 2008; Rezania *et al.*, 2014; Pagliuca *et al.*, 2014).

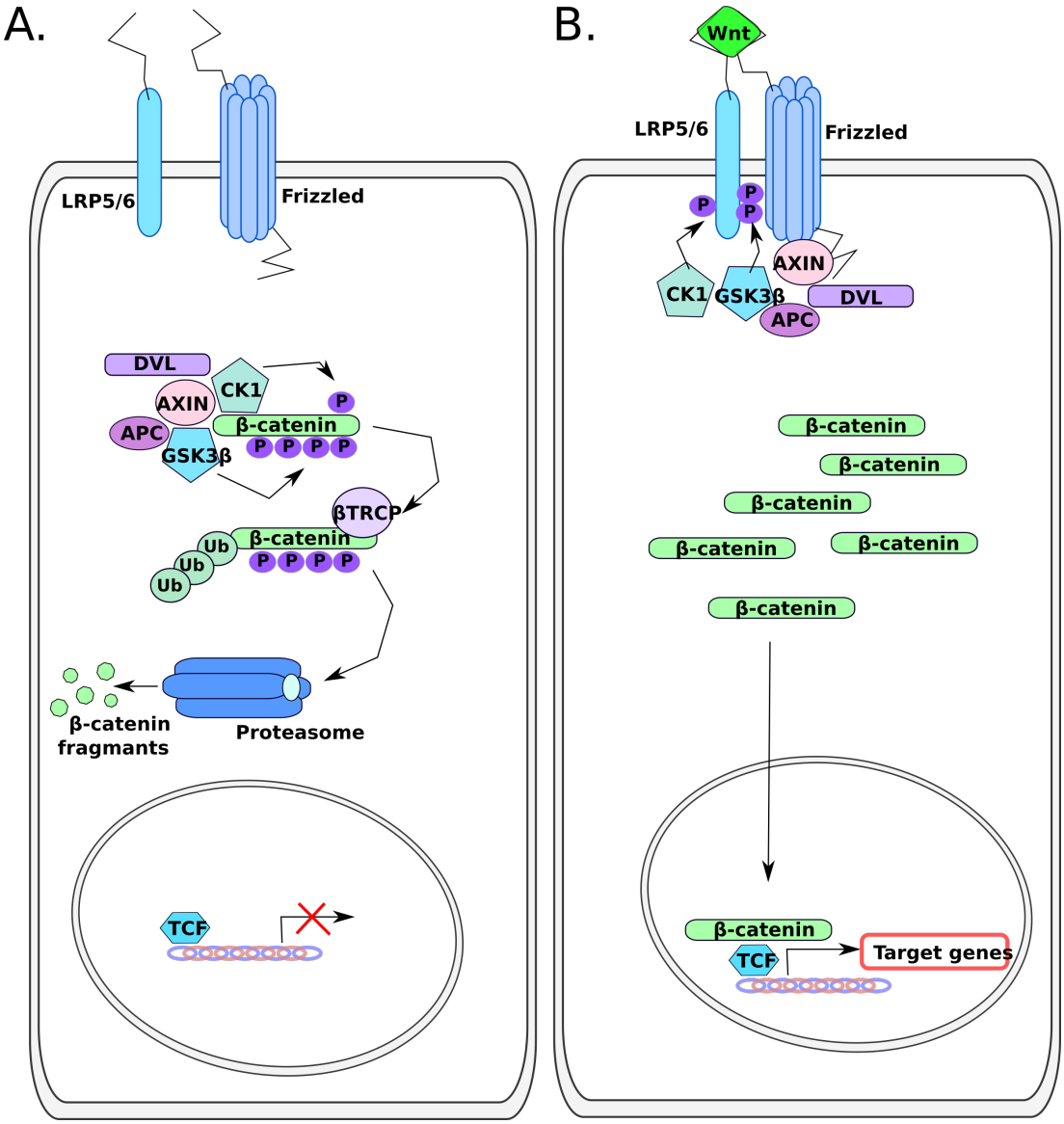

Figure 1.5: Canonical Wnt Signalling. When Wnt ligand is absent (A.) the destruction complex binds to cytoplasmic β-catenin molecules and targets them for degradation. In the presence of the Wnt protein (B.), its interaction with Frizzled and LRP5/6 co-receptors leads to activation of Dishevelled (Dvl). This causes disruption of the destruction complex by recruiting its compounds to the Dvl-receptor complex (Murtaugh, 2008) (DVL-Dishevelled, CK1- Casein kinase 1, APC- Adenomatous polyposis coli gene product, GSK3β- Glycogen synthase kinase 3 beta, βTRCP- Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase, Ub- Ubiquitin, TCF- T-cell factor/lymphoid enhancer factor).

1.3.4.3 The Notch Signalling Pathway

Notch signalling is an evolutionarily conserved, short-range, cell-cell signal transduction system. The four Notch receptors (Notch 1-4) and five Delta-Serrate-Lad (DSL) type ligands (Jag 1, Jag2, Dll1, Dll3 and Dll4) have been identified in mammals (Andersson *et al.*, 2011). The Notch signalling cascade starts with binding of a ligand, that is present on the surface of the ligand-expressing cell to the Notch extracellular domain (NECD) of the Notch receptor on adjacent cells. This triggers activation of membrane-bound proteases, which cut non-NECD domain into the Notch transmembrane fragment (NTN) and Notch intracellular domain (NICD). Two families of proteases are involved in this process: ADAM (a disintegrin and metalloprotease) and γ-secretase complex. Following proteolytic cleavage, the NICD is translocated to the nucleus where it interacts with a DNA binding protein RBFJ-κ (also known as CSL and CBF1). NICD/RBFJ-κ complex recruits co-activator Mastermind-like (Maml) protein to activates expression of target genes, such as a transcription factor- HES1 (Li *et al.*, 2015) (Fig. 1.6).

HES1 plays a crucial role in the determining the fate of multipotent pancreatic progenitors, control of the pool of pancreatic progenitors and is a transcriptional repressor of NGN3 (a transcription factor essential for the establishment of pancreatic endocrine progenitors) (Lee *et al.*, 2001; Gu *et al.*, 2002). NGN3$^+$ cells are progenitors for all islet endocrine cell types, therefore induction of this TF expression is essential for the generation of functional β-cells. This link between Notch signalling, regulation of Hes1 and Ngn3 expression, has been explored in *in vitro* differentiation of stem cells towards pancreatic β-cells. To stimulate expression of NGN3 several differentiation protocols implemented use of inhibitors of γ-secretase following generation of PDX1$^+$ cells (e.g. γ-Secretase inhibitor XX) (Rezania *et al.*, 2014).

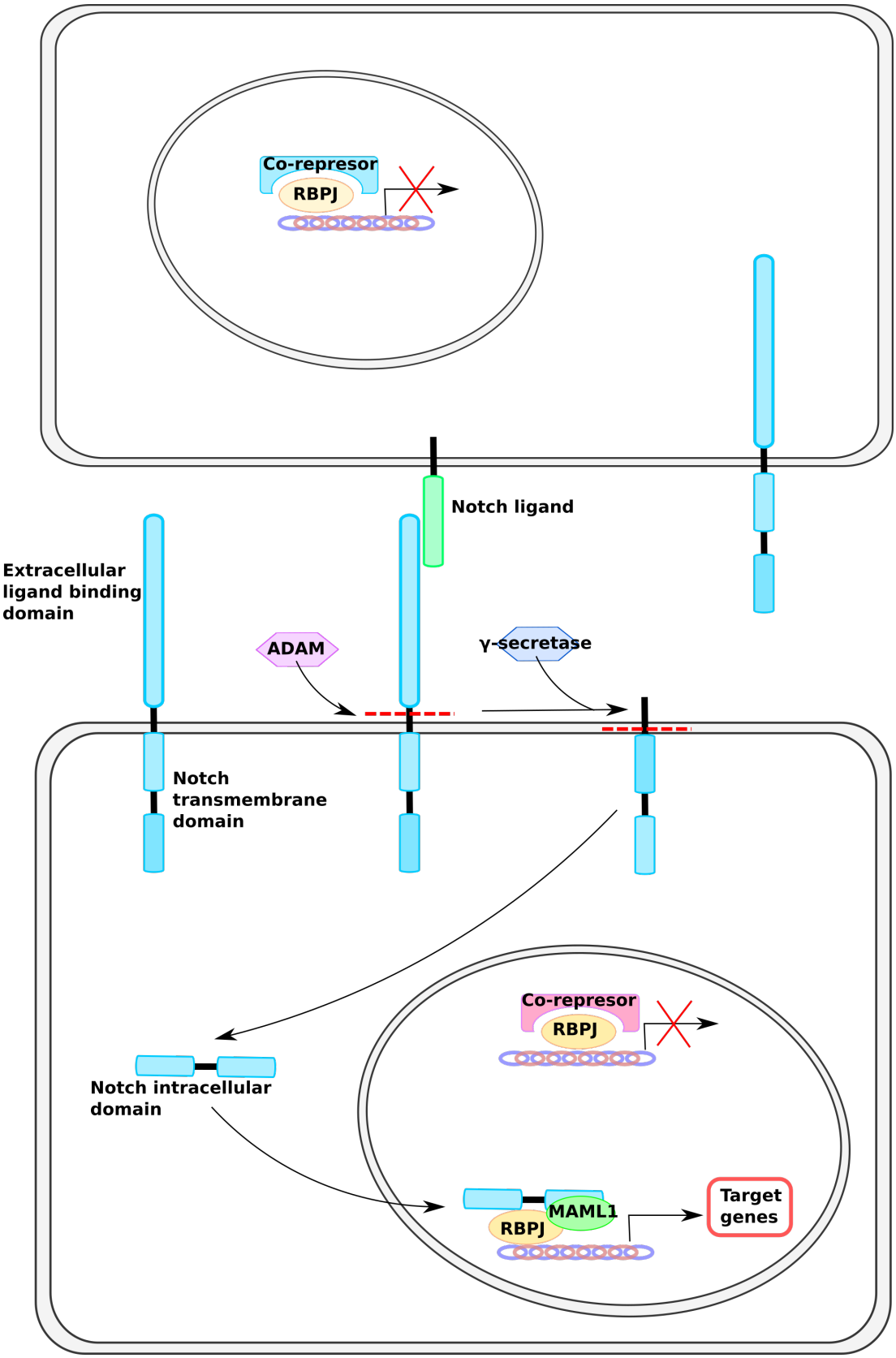

Figure 1.6: Notch Signalling. Binding between the Notch ligand and Notch receptor leads to the subsequent receptor proteolytic cleavage by ADAM and by γ-secretase complex. Formed in this process Notch intracellular domain (NICD)is translocated to the cell nucleus where it interacts with the DNA-binding protein RBPJ-κ and the co-activator Mastermind (MAM) promoting expression of target genes; adapted from (Bray, 2016).

1.3.4.4 Phosphoinositide 3-kinases Signalling

Phosphoinositide 3-kinases (PI3Ks) are a group of kinases involved in regulation of multiple cellular processes, including proliferation, cytoskeletal rearrangements, migration and apoptosis (Champeris Tsaniras and Jones, 2010). These signalling pathways start with activation of receptor tyrosine kinases, cytokine receptor, G-protein coupled receptor and other cellular events able to activate PI3Ks and lead to the production of phosphatidylinositol (3,4,5) trisphosphate (PIP3). PIP3 interacts with cell cytoplasm proteins such as Akt (Vanhaesebroeck *et al.*, 2012). PI3K signalling has been shown to be essential for the regulation of stem cells renewal and negatively regulate differentiation of these cells (Paling *et al.*, 2004). Therefore suppression of this signalling (e.g. with wortmannin) has been incorporated in step-wise protocols to induce definitive endoderm formation (Zhang *et al.*, 2009).

More recently, the activation of PI3K signalling has been shown to stimulate expression of PDX1 in ductal cells during pancreas regeneration (Watanabe *et al.*, 2008). This suggests that this signalling might also enhance PDX1 expression when implemented during *in vitro* generation of β-cells.

1.3.4.5 Trans-Retinoic Acid Signalling

Trans-retinoic acid is an important factor during early endoderm patterning and outgrowth of dorsal pancreatic bud. Mouse embryos deficient for an enzyme involved in the generation of endogenous retinoic acid (Raldh2), lack Pdx1 expression in the dorsal endoderm and have agenesis of dorsal pancreas (Molotkov *et al.*, 2005; Martín *et al.*, 2005). Retinoic acid has also been utilised to induce PDX1 expression in several differentiation protocols (Rezania *et al.*, 2014; D'Amour *et al.*, 2006; Pagliuca *et al.*, 2014).

1.4 Pancreatic Progenitor Cells

During pancreas development, cells marked by expression of Pdx1 transcription factors are generally considered to be pancreatic progenitors (PPs) that have the potential to differentiate into all types of cells found in the mature pancreas (Jonsson *et al.*, 1994). Ngn3-expressing cells are considered to be precursors of all types of pancreatic endocrine cells, whereas MafA-positive cells are a fraction of β-cell precursors (Rukstalis and Habener, 2009; Gu *et al.*, 2002).

Limited regeneration potential of the adult pancreas followed injury, mainly observed in mouse models, has led to the proposal that a dormant population of progenitor cells can be present in this gland (Wang *et al.*, 1995; Inada *et al.*, 2008; Bonner-Weir *et al.*, 2004; Teta *et al.*, 2007). As a source of progenitor cells in pancreas, ductal structures, exocrine compartment as well as islet cells have been proposed. Ductal cells have been shown to give rise to the precursors of islet cell during embryogenesis (Kopp *et al.*, 2011), however, the potential of ductal cells of the adult pancreas to differentiate into endocrine cells is still controversial. For example, transdifferentiation of ductal cells into islet-cells has been observed in the adult rats following injury caused by duct ligation (Wang *et al.*, 1995), however, lineage tracing of $Sox9^+$ cells in the adult mice following pancreatic duct ligation failed to confirm this observation (Kopp *et al.*, 2011). Moreover, lineage tracing studies of postnatal β-cells in mice after pancreatectomy have indicated that these terminally differentiated cells have proliferation capacity and are a source of regenerative potential of pancreas (Dor *et al.*, 2004). Also, exocrine cells have been shown to be able to generate β-like cells *in vitro* when exposed to epidermal growth factor and nicotinamide (Baeyens *et al.*, 2009). More recently, it has also been proposed that very small embryonic-like stem cells (VSELs) which reside in the pancreas can be source of pancreatic progenitor cells in the adult pancreas (Dor *et al.*, 2004; Bhartiya and Patel, 2015). In conclusion, the origin of pancreatic progenitor cells in mature pancreatic tissue is still controversial.

1.4.1 Markers for Pancreatic Progenitor Cells

Recent progress made in the field of stem cell based generation of pancreatic β-cells highlighted the need for efficient verification of the identity of those cells. The ultimate goal of research attempting to create *in vitro* substitutes of pancreatic endocrine cells is to utilise these cells for the treatment of diabetes. Pancreatic progenitor cells can potentially serve as an additional source of cells for transplantation along with cadaveric β-cells. This would help with shortage of donors' tissue since currently for each transplantation islets isolated from 2-3 donors are required (McCall and James-Shapiro, 2012).

The main limiting factors currently associated with the use of β-cells generated *in vitro* are the immature state and heterogeneous character of these cells. The fact that among induced β-cells or progenitor cells might also be undifferentiated pluripotent cells links this approach with a risk of teratoma formation. To address this issue an efforts have been made by several research groups to generate a panel of pancreatic progenitor cell markers that could facilitate isolation of pancreatic progenitor cells (Table 1.1 and 1.2).

Table 1.1: Putative markers for isolation of pancreatic progenitor cells.

Marker	Stage	Comments	Ref.
CD24	$PDX1^+$-cells	• source of cells: differentiated hESCs • strong expression in undifferentiated hESCs (co-expressed with NANOG and OCT4) • weak expression in $SOX17^+$ and $FOXA2^+$ (definitive endoderm cells) • multiple $CD24^+$ cells present prior to the onset of $PDX1^+$ cell formation	(Jiang *et al.*, 2011)
CD142 (F3)	$PDX1^+/NKX6.1^+$-cells	• source of cells: differentiated hESCs • $CD142^+$ fraction also contained $NKX6.1^-$-cells	(Kelly *et al.*, 2011)
CD200 CD318	Endocrine cells	• CD200 and CD318- enriched fractions contained also a small fraction of non-endocrine cells (chromogranin$^-$ cells)	
c-Kit	$Pdx1^+$, $Ngn3^+$ and $insulin^+$- cells	• source of cells: differentiated mESCs • c-Kit is also a marker for hematopoietic stem cells (Shin *et al.*, 2014) -this limits its used for isolation of progenitor cells from tissue • c-Kit positive fraction also contained cells that were not pancreatic progenitor cells	(Ma *et al.*, 2012)
CD133 (PROM1)	Developing and adult mouse pancreas	• a transmembrane glycoprotein • is also a marker for hematopoietic stem cells (Miraglia *et al.*, 1997; Yin *et al.*, 1997) • isolated $CD133^+$-cells were forming clonal colonies *in vitro* and could be differentiated into pancreatic endocrine and acinar cells	(Suzuki *et al.*, 2004)

Table 1.2: Putative markers for isolation of pancreatic progenitor cells. Continued.

Marker	Stage	Comments	Ref.
DDR1	Ductal/endocrine progenitor cells	• transcriptomic set of data obtained from Nng3- knockout and wild-type mice, data-mining approach combined with in situ hybridization and immunohistochemistry • Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase, which is activated by interactions with ECM compounds such as fibrillar collagen (Vogel, 1999)	(Hald *et al.*, 2012)
DISP1, SEZ6L2, LRP11, TSPAN7, TMEM27	Endocrine cells	• partially overlaps with the expression of Ngn3 and Nkx6.1 (mouse) • in human and mouse pancreas these markers stain pancreatic islets	(Altirriba *et al.*, 2010); (Hald *et al.*, 2012); (Lindskog *et al.*, 2010)
DNER	$Ngn3^+$	• a Notch signalling ligand • its expression has also been detected in adult mouse pancreas and overlaps with that of with glucagon	(Hald *et al.*, 2012)
GRP50, TROP2	$PDX1^+$-cells	• source of cells: differentiated hESCs • identified based on RT-qPCR and microarray data, however, the expression profile of GPR50 and TROP2 were not validated at the protein level • GPR50 was linked with a potential role in the regulation of energy metabolism (Ivanova *et al.*, 2007) • TROP2 was reported to have a function in the growth of epithelial cell-originating cancers (Fong *et al.*, 2008)	(Fishman *et al.*, 2012)

Several cell membrane proteins have been proposed to be putative markers of pancreatic progenitor (PP) cells, however, their usability for the isolation of PPs from mixed cell population has yet to be verified. For example CD24 has been proposed to be a marker for PDX1$^+$ progenitor cells by Jiang et al., although subsequent validation of this marker by Kelly et al. revealed its expression pattern to be widespread and not limited to PDX1$^+$-cells (Jiang *et al.*, 2011; Kelly *et al.*, 2011). Other markers such as c-Kit and CD133 are also expressed in hematopoietic stem cells and therefore of limited use for detection of pancreatic progenitor cells within tissue. Whereas, markers such as GRP50 and TROP2 were only analysed at the mRNA level and their expression at the protein level in PP cells has yet to be verified (Fishman *et al.*, 2012). Expression of DDR1 and DNER correlated with expression of NGN3 and has been validated in mouse and human tissue, however, the utility of those markers for isolation of progenitor cells from differentiated culture of hESCs has yet not been tested. Therefore validation of existing and identification of novel putative markers for PP cells would be beneficial for the transplantation-based treatment for T1D and basic biology research aimed to elucidate the processes controlling development of pancreas.

1.5 Project Aims

This project aimed to identify transmembrane proteins that could serve as markers for pancreatic progenitor cells. In order to achieve that:

- a population of PDX1$^+$/NGN3$^+$/NEUROD1$^+$ cells had to be generated from stem cells;
- generated progenitor cells had to be characterised and expression of the key TFs (PDX1,NGN3 and NEUROD1) by these cells needed to be verified;
- omic (transcriptomic/proteomic) profile of these cells had to be produced and analysed to elucidate novel cell membrane markers for pancreatic progenitor cells.

Chapter 2

Methods

2.1 CHI pMSC and AI pMSC Cell Lines

2.1.1 CHI pMSC Cells

CHI pMSC cells were developed from pancreatic tissue obtained from Congenital hyperinsulinism patients. Tissue, obtained with permission and consent of the patients' legal guardians, following removal was washed in KRH (Krebs-Ringer-Hepes) buffer with 0.1 % (w/v) BSA and 5.6 mM glucose. Following this, tissue was digested with 0.75 mg/ml Liberase (Roche, Germany) prepared in KRH buffer supplemented with 1.5 mg/ml egg white trypsin inhibitors (Sigma-Aldrich, UK) and 1.5 mg/ml soybean trypsin inhibitor (Gibco, UK). Tissue digestion was carried out for approximately 3 minutes at 37 °C with moderate agitation, followed by vigorous shaking for 1 minute. This procedure was repeated three times. Tissue digestion was stopped by addition of ice cold KRH buffer and mixture was centrifuged for 1 minute at 150 x g. This step was repeated further three times. Majority of islet was removed from the digested tissue mixture under a dissection microscope and the remaining exocrine- enriched fraction was transferred to RPMI 1640 5.5 mM glucose (0 mM glucose RPMI 1640 mixed 1:1 with 11 mM glucose RPMI 1640) supplemented with 10 % (v/v) FBS (Gibco, UK) and 1x Penicillin-Streptomycin (x100 solution, Gibco, UK). Initial cell culture

was performed in 6- well tissue culture plates with approximately 20 mg of wet tissue per well. Following 48 hour-long incubation, non-adhered tissue/ cells were removed and fresh media added to the remaining/ attached cells. Cells were cultured unlit 70 % confluence with media changed every 48 h.

2.1.2 AI pMSC Cell Lines

Adult islets pMSC cells were developed from islets obtained from Oxford Centre for Islet Transplantation. Islets were washed with PBS (Sigma- Aldrich, UK) and transferred to 6- well tissue culture plates with 5.5 mM glucose RPMI supplemented with 10 % (v/v) FBS (Gibco, UK) and 1x Penicillin-Streptomycin (x100 solution, Gibco, UK). Approximately, 20 - 50 islets were seeded per well. Following 48 hour-long incubation, non-adhered tissue/ cells were removed and fresh media added to the remaining/ attached cells. Cells were cultured unlit 70 % confluence with media changed every 48 h.

2.2 CHI pMSCs Differentiation

2.2.1 Differentiation into Adipocytes, Chondrocytes and Osteocytes

CHI pMSC were differentiated into adipocytes, chondrocytes and osteocytes with StemPro Adipogenesis, StemPro Chondrogenesis or StemPro Osteogenesis Differentiation Kit (Gibco, UK), respectively, following manufacturer instruction. Briefly, cells expanded in MesenPRO (Gibco, UK) or DMEM with 10 % (v/v) FBS (Gibco, UK) up to 70 % confluence prior to differentiation. Then cell monolayer was rinsed with DPBS without Calcium and Magnesium (Sigma, UK) and expansion medium was replaced with differentiation medium. Cells differentiation was carried out for 14 days and differentiation medium was replaced with fresh every four days. For differentiation into chondrocytes cells were cultured as a cell

micromass. Cell differentiated into osteocytes were exposed to the differentiation media for additional seven days. Successful differentiation into adipocytes was verified by Oil Red O staining, differentiation into chondrocytes was confirmed with Alcian Blue staining and differentiation into osteocytes was detected with Alizarin Red staining.

2.2.2 Differentiation into Pancreatic Progenitor Cells

CHI pMSC cells were expanded to 70-80 % confluence in MesenPRO (Gibco, UK) or DMEM with 10 % (v/v) FBS (Gibco, UK). Following that cells were maintained in serum free DMEM/F12 supplemented with 1 % (v/v) ITS (Gibco, UK), 100 μg/ml ascorbic acid (Sigma, UK) and 2mM L-glutamine (Gibco, UK)for one day. Next cells were exposed to differentiation medium DMEM/F12 supplemented with 1 % (v/v) ITS (Gibco, UK), 100 μg/ml ascorbic acid (Sigma, UK) and 2 mM L-glutamine (Gibco, UK), 10 μg/ml Exendin-4 (Sigma, UK), 20 ng/ml Betacellulin (PeproTech, USA), 20 ng/ml EGF (Calbiochem, UK) and 10 mM nicotinamide for seven days as a monolayer and then for 14 days as spheroids in ultra-low adherence plates (Corning, UK). The differentiation medium was additionally supplemented as follows: day 1: 100 ng/ml Activin A (PeproTech, USA), 100 ng/ml Dkk (PeproTech, USA) and 10 μM retinoic acid (Sigma, UK); day 2-3: 100 ng/ml Activin A (PeproTech, USA), 100 ng/ml Dkk (PeproTech, USA). Following differentiation day 3, medium was changed every four days.

2.3 iPS Cells

iPS cells were routinely maintained in Essential 8 media (Gibco, UK). Cells were cultured in 6 well plates (Corning, UK) coated with Vitronectin (ThermoFisher, UK) and split at 1:6 or 1:8 ratio. Prior to splitting cells were rinsed with DPBS without Calcium and Magnesium (Sigma, UK) and exposed to Versene solution (Gibco, UK). Following that cells were squirted with pre-warmed complete Es-

sential 8 media collected in a 15 ml tube and, depending on split ratio, required volumes were transferred to a fresh plate. Cell culture media was replenished daily.

2.4 iPS Cell Differentiation

Cells for differentiation were plated on matrigel (BD Biosciences, UK) and maintained in E9 medium for initial 48 h. iPS cells were differentiated into $Pdx1^+$ and $Pdx1^+$/$NeuroD1^+$ with a recently published protocol (Rezania *et al.* 2014). Briefly, cells were maintained in MCDB 131 (Gibco, UK) supplemented with 10 mM glucose, 1.5 g/l sodium bicarbonate, 2 mM L-glutamine and 0.5 % (w/v) BSA (Roche, fatty acid free) for days 1 to 5 (stage 1). Stage 1 (day 1 to 3) differentiation media was further supplemented as follows, day 1: 100 ng/ml Activin A (PeproTech, USA) and 3 μM CHIR99021 (Sigma, UK), day 2: 100 ng/ml Activin A and 0.3 μM CHIR99021, day 3: 100 ng/ml Activin A. Stage 2 medium for day 4 and 5 was supplemented with 0.25 mM ascorbic acid (Sigma, UK) and 50 ng/ml KGF (PeproTech, USA). Stage 3 medium for differentiation days 6 to 10 was supplemented with 10 mM glucose, 2.5 g/l sodium bicarbonate, 2 mM L-glutamine, 2 % (w/v) BSA and 0.25 mM ascorbic acid. Additionally, day 6 and 7 medium was supplemented with 50 ng/ml KGF, 0.25 μM SANT-1 (Sigma, UK), 1 μM retinoic acid (Sigma, UK), 200 nM LDN193189 (Generon, UK), 0.5 % (v/v) ITS-x (Life Technologies, UK) and 100 nM TPB (Sigma, UK). Day 8 to 10 (stage 4) medium was supplemented with 2 ng/ml KGF, 0.25 μM SANT-1, 0.1 μM retinoic acid, 200 nM LDN193189, 0.5 % (v/v) ITS-x, 100 nM TPB. On the last day of the stage 4 differentiation media was additionally supplemented with 10 μM Y-27632 (Sigma, UK) and cell were incubated with it for 4 hours before transforming cells growing as a monolayer into spheroids growing in transwell permeable supports 24 mm inserts with 0.4 μm polyester membranes (Costar, USA). Cells growing on inserts were exposed to medium supplemented with 20 mM glucose, 1.5 g/l sodium bicarbonate, 2 mM l-glutamine, 2 % (w/v) BSA, 0.25

μM SANT-1, 0.05 μM retinoic acid, 100 nM LDN193189, 0.5 % (v/v) ITS-X, 1 muM T3 (Sigma, UK), 10 muM ALK5 Inhibitor II (Cayman Chemical, UK), 10 μM zinc sulfate and 10 μg/ml heparin (Sigma, UK). Cells were harvested for gene expression, immunoblotting and mass spectrometry analysis at S0, S4 and S5.

2.5 RNA Extraction

On average 3 to 5 x 10^6 cells were used for RNA extraction. RNA extraction from cell lines was performed using ISOLATE II RNA Mini Kit (BioLine, UK) following manufacturer's instructions. In brief, cell cultured as a monolayer were washed with PBS (Sigma, UK) and lysed with 350 to 700 μl of RLY buffer containing 0.1 % (v/v) of β-mercaptoethanol. Next, the lysate was loaded on the shredder column and spun at 11 000 x g for 1 min. The binding properties were adjusted by adding equal volume of 70 % (v/v) ethanol to the homogenised lysate and mixed by pipetting. The lysate was loaded onto the spin column placed in a collection tube, spun at 11 000 x g for 30 sec and column was transferred to fresh collection tube. The silica column was washed with 350 μl of Membrane Desalting Buffer in preparation for DNase I treatment. The sample was treated with 95 μl of DNase I mixture (10 μl of DNase I and 90 μl of RDN buffer) for 15 min at room temperature. Following that the membrane was washed with 200 μl of Wash Buffer RW1, centrifuged at 11 000 x g for 30 sec and placed in a new collection tube. Wash Buffer RW2 (600 μl) was added onto a silica column and spun at 11 000 x g for 30 sec. That step was repeated with 200 μl of RW2 buffer with centrifugation time extended to 2 min in order to ensure complete removal of ethanol containing RW2 buffer. The column was placed in a new collection tube and RNA was eluted with 30 to 60 μl of RNase- free water by centrifugation at 11 000 x g for 1 min. Isolated RNA was stored at -80.

An additional DNase I treatment was performed with RQ1 DNase kit (Promega, USA). Per each DNase reaction between 8 to 1 μg of nucleic acid in a volume of 37 μl was used. The treatment was performed in a total volume of 50 μl, with

5 μl of 10x buffer and 8 μl of the enzyme (at 1u/μl). The mixture of nucleic acid, 10x buffer and enzyme was incubated at 37 °C for 30 min. Following that 5μl of Stop Solution was added to each sample and the mix was incubated for additional 10 min at 65 °C.

Before cDNA synthesis, RNA was purified by ethanol precipitation. To each sample 2.2 volume of ice cold 100 % molecular grade ethanol (Sigma, UK) and 0.1 volume of 3 M sodium acetate were added. Next, the samples were stored at -20 °C for minimum 20 min. This incubation was followed by 20 min centrifugation at 12 000 rpm at 4 ° and ethanol was decanted. RNA was washed with 70 % (v/v) ethanol, centrifuged for 10 min at 12 000 rpm at 4 ° and air dried. Finally, the sample was re-suspended in RNase-free water.

2.6 cDNA Synthesis

Between 0.25 to 1 μg were used for cDNA synthesis. SensiFAST cDNA Synthesis Kit (BioLine, UK) was utilised. Following manufacturer's instructions, 4 μl of 5 x buffer and 1μl of reverse transcriptase were combined with 15 μl of RNA. The reaction was mixed gently by pipetting and incubated at 25 °C for 10 min (primer annealing), next for 42 °C for 15 min (reverse transcription) and finally at 85 °C for 5 min (inactivation). cDNA was stored at -20 °C.

2.7 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The RT-PCR was performed using the Taq DNA RT-PCR kit (Invitrogen, UK). Possible variation between assays was minimised by preparing a master-mix for each set of reactions. The master-mix composition is detailed in the Table 2.2. The final volume of each PCR reaction was 25 μl. Prime sequence is shown in Table 2.1. Amplification was performed with following steps: initial denaturation 2 min at 94 °C, denaturation at 45 sec at 94 °C, annealing 30 sec at temperature specific for each pair of primers (Table 2.1), extension 30 sec at 72 °C and final extension 2 min at 72 °C . On average 30 to 35 cycles of amplification were performed for each reaction. Each test was match with a no-RT control and when possible with a positive control. PCR products were separated by agarose gel electrophoresis.

Table 2.1: List of primers used for RT-PCR (T_A- annealing temperature, bp-base pairs.

Gene	Accession Number	Primers 5'-3'	T_A (C°)	Product size
FOXA2	NM021784.4	F: GCACTCGGCTTCCAGTATGC R: TGCTCACGGAGGAGTAGCC	60	104 bp
PDX1	NM000209.3	F: GGAGCCGGAGGAGAACAAG R: CTCGGTCAAGTTCAACATGACAG	60	138 bp
SOX17	NM022454.3	F: GCATGACTCCGGTGTGAATCT R: TCACACGTCAGGATAGTTGCAGT	60	103 bp
SOX9	NM000346.3	F: AGCGAAATCAACGAGAAACT R: ATCCCCTCAAAATGGTAATG	55	222 bp
ISL1	Obtained from Sigma	F: CTAATATCCAGGGGATGACAG R: CTGGTAACTTTGTACTTCCAC	60	101 bp
GAPDH	M17851.1	F: ATTGCCCTCAACGACCAC R: GGTCCACCACCCTGTTGC	60	79 bp
PPIA	from Xu et al. (2009)	F: CCCACCGTGTTCTTCGACAT R: CCAGTGCTCAGAGCACGAAA	60	116 bp

Table 2.2: RT-PCR Master Mix.

Master Mix Component	Volume (μl)	Final Concentration
PCR Buffer	2.5	x1
dNTPs	0.5	200 μM
$MgCl_2$	0.75	1.5 mM
Forward Primer	0.25	100 nM
Reverse Primer	0.25	100 nM
DNase/RNase-Free Water	16.55	-
Taq Polymerase	0.25	1U
Template	4	10-30 ng/reaction

2.8 Agarose Gel Electrophoresis

PCR products were separated on 2 % (w/v) agarose gel. The gel was prepared by dissolving agarose (BioLine, UK) in 1xTAE (Tris base- acetic acid- EDTA) buffer and pouring into casting tray. PCR products were visualised with Gel Red (BioLien, UK) of which 10 μl were added to 50 ml of agarose/TAE buffer mixture. Gels were electrophoresed for approximately 40 to 60 min at 100 eV. Electrophoresis results were visualised with a Bio-RAD UV transilluminator.

2.9 Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Changes in the genes expression in differentiated cells were analysed with qRT-PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, UK) and Taqman probes (Table 2.3). Each reaction contained: 10 μl of Master Mix, 5 μl of DNase/RNase-free waster, 1 μl of Taqman probe and 4 μl of template (2.5-10 ng/reaction). Data were collected using StepOnePlus Real-Time PCR System (Applied Biosystems) and normalised to undifferentiated iPS cells (S0) using the $\Delta\Delta$ Ct method (StepOnePlus settings Table 2.4). Statistical analysis was performed with GraphPad Prism software using one-sample t-test.

Table 2.3: List of primers used for qRT-PCR.

Gene	Taqman Probe	Source
NGN3	HS01875204_s1	Applied Biosystems
PDX1	HS00236830_m1	Applied Biosystems
B2M	4332653	Applied Biosystems
PPIA	4332647	Applied Biosystems

Table 2.4: qRT-PCR Settings.

StepOnePlus	Settings
Experiment Type	Quantification- Comparative Ct ($\Delta\Delta$ CT)
Reagent	Taqman Reagents
Ramp Speed	Fast
Template	cDNA
Reporter	FAM
Passive Reference	ROX

2.10 Immunocytochemistry

For immunostaining experiments cells were cultured on a glass coverslips. All following steps were performed at room temperature. Prior to staining cells were washed with 1xPBS (Sigma, UK) three times to remove any residual culture media and fixed with 4 % (w/v) PFA (Sigma, UK) in PBS pH 7.4 for 15 min. Following that cells were washed with PBS three times and permeabilized with PBS containing 0.1 % (v/v) of Triton-X100 for 10 min. Next cells were incubated for 1 h with blocking solution of 10 % (v/v) normal goat serum (NGS) in PBS. Cells were washed once with PBS and incubated with primary antibody diluted in 3 % (v/v) NGS in PBS for 1 h. Cells were rinsed three times with PBS with 0.1 % (v/v) Tween20 (PBST) and incubated with secondary antibody diluted in 3 % (v/v) NGS in PBS for 1 h in dark. The secondary antibody was decanted and cells were washed with PBS three times. Coverslips were mounted with ProLong Antifade Reagent (Invitrogen, UK) and left in dark over night to dry. Staining was observed using a snapshot widefield upright microscope (Olympus BX51). As a negative control cell incubated with blocking solution without primary antibody were used. A list of antibodies used is presented in Table 2.5.

Table 2.5: Antibodies used for immunocytochemistry (Ms- mouse, Rb- rabbit, Gt-goat).

Antigen	Source	Catalog Number	Dilution
αSMA	Abcam	ab5694	1:400
VIMENTIN	Abcam	ab8978	1:200
NESTIN	EMD Millipore	MAB5326	1:200
PDX1	Santa Cruz	sc-14662	1:50
NEUROD1	PTG	12081-1-AP	1:200
SOX2	Abcam	ab97959	1:500
OCT4	Abcam	ab19857	1:200
NANOG	Abcam	ab21624	1:50
SSEA4	Abcam	ab16287	1:70
TRA-1-60	Abcam	ab16288	1:500
FOXA2	R&D Systems	AF2400	1:50
SOX17	Cell Signalling	81778S	1:3000
AF488 anti-Rb	Thermo Fisher	A-11008	1:200
AF488 anti-Ms	Thermo Fisher	A-11001	1:200
Cy5 anti-Rb	Abcam	ab6564	1:200
Cy3 anti-Rb	Thermo Fisher	A10520	1:200
AF488 anti-Gt	Thermo Fisher	A11055	1:200

2.11 Immunocytochemistry on Cryosections

Stage 5 differentiated iPS cells were snap- frozen in liquid nitrogen and stored in -80 ∘C until further use. Cryopreserved spheroids were cut into 5 μm sections and allowed to adjust to room temperature prior to staining for 10 min. Sections were fixed with 3 % (w/v) PFA for 15 min at room temperature, washed with PBS three times (each was 5 min) and permeabilised with 0.2 % (v/v) Triton X-100 (Sigma, UK) for 20 min and washed three times with PBS. Following that sections were blocked with 2 % (v/v) donkey serum, 2 % (w/v) BSA (Sigma, UK) and 50 mM glycine (Sigma, UK) in PBS for 1 hour. Next section were washed three times with PBS and incubated with the primary antibody diluted in the working buffer (blocking buffer diluted in PBS 1:10 with 0.1 % (v/v) Triton X-100) overnight at 4°C. Sections were washed three times with working buffer for 10 min each wash with moderate agitation. Next, sections were incubated with the secondary antibody diluted in the working buffer for 1 hour at room temperature in dark. Following incubation with the secondary antibody, sections

were washed with PBS three times, each time for 10 min with moderate agitation. Coverslips were mounted with ProLong Antifade Reagent (Invitrogen) and left in dark over night to dry. Staining was observed using a snapshot widefields upright microscope (Olympus BX51). A list of antibodies used is presented in Table 2.6.

Table 2.6: Antibodies used for immunocytochemistry on cryosections (Ms- mouse, Rb- rabbit).

Antigen	Source	Catalog Number	Dilution
NKX6.1	DSHB	F55A12-S	1:20
PDX1	Abcam	ab47267	1:800
NEUROD1	PTG	12081-1-AP	1:100
Ax488 anti-Ms	Thermo Fisher	A-11001	1:250
Cy5 anti-Rb	Abcam	ab6564	1:100

2.12 Protein Extraction

Protein for Western Blot experiments were extracted using RIPA buffer with 1x protease inhibitor cocktail (Promega, USA) or 1M TEAB buffer with 0.01 % (w/v) SDS. Cell monolayer was lysed with RIPA buffer on ice and removed from flask with cell scraper, transferred to 1.5 ml tube and incubated on ice for 15 min. The lysate was then centrifuged at 12000 xg at 4 °C for 5 min. Supernatant was collected and stored at -80 °C.

Proteins for iTRAQ MS/MS where isolated with 1M triethylammonium bicarbonate (TEAB) buffer with 0.1% w/v SDS. Cell monolayer was lysed, removed from flask using cell scraper, transferred to 1.5 ml tube and stored at -80 °C.

2.13 SDS-PAGE

On average 40-60 μg of protein extracts/ well were loaded on gel. Proteins were separated using 12 or 4-20 % precast stain- free gels (Biorad, UK). Following electrophoresis proteins were visualised using stain-free technology on Gel Doc XR System (Biorad, UK).

2.14 Western Blotting

Proteins were transferred using the PVDF Mini transfer packs and Trans-Blot Turbo device (Biorad, UK). Membranes were then placed in 5 % (w/v) milk-TBST blocking solution for 1 h. Membranes were then probed primary antibody and horseradish peroxidase conjugated secondary antibody (1:10000 dilution). Membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Merckmillipore, UK). Proteins were visualised with Gel Doc XR System (Biorad, UK).

Membranes were stripped with low pH solution (25mM glycine-HCl, 1 % (w/v) SDS, pH 2) for 30 min at 37 °C. Following that membranes were washed twice with PBS for 10 min, blocked with blocking solution and re-probed with primary antibody. List of antibodies used was presented in Table 2.7.

Table 2.7: Antibodies used for immunblotting.

Antigen	Source	Catalog Number	Dilution	Predicted Size (kDa)
PDX1	Santa Cruz	sc-14662	1:200	46
NEUROD1	Proteintech Group	12081-1-AP	1:500	50
SOX2	Abcam	ab97959	1:1000	34
LAMIN B1	Abcam	ab16048	1:1000	68
NKX6.1	DSHB	F55A12-s	1:200	46
SOX9	Cell Signaling Technology	82630	1:1000	70
SOX17	Cell Signaling Technology	81778S	1:1000	55
VIMENTIN	Abcam	ab8978	1:1000	57
ISL1	Proteintech Group	15661-1-AP	1:2000	39-45
PAX6	Abcam	ab5790	1:1000	47
GAPDH	Cell Signalling	2118S	1:1000	37

2.15 Flow Cytometry

Cells were harvested with trypsin and re-suspended at $1x10^6$ cell/ml in ice cold PBS with 10 % (v/v) normal goat serum (NGS) and 1 % (w/v) sodium azide and incubated for 15 min on ice. For each staining 100 μl of cell suspension was transferred to fresh centrifuge tube and incubated with the primary antibody diluted in 3 % (v/v) NGS in PBS for 45 min at room temperature. Next, cells were

washed with ice cold PBS, spun at 1500 rpm for 5 min at 4 °C and re-suspended in ice cold PBS-this was repeated further 2 times. Following this, cells were the secondary antibody diluted in 3 % (v/v) NGS in PBS and incubated for 30 min at room temperature in dark. Cell were washed three times and re-suspended in 100 μl of ice cold PBS. Cell counting was performed with Beckman Coulter Cyan ADP with 635 nm excitation. Post-acquisition fluorescence compensation and gating were performed with Summit V4.3 software. As a negative control cells incubated with isotype control antibodies were used. List of antibodies/isotype controls was presented in the Table 2.8.

Table 2.8: Antibodies and isotype controls used for flow cytometer experiments (Ms- mouse, Rb- rabbit).

Antigen	Source	Catalog Number	Concentration/Dilution	Isotype Control
CD90	Abcam	ab23894	1 μg/ml	Ms IgG1
CD29	Abcam	ab52971	1 mg/ml	Ms IgG1
CD44	Abcam	ab6124	1 μg/ml	Ms IgG2a
CD73	Miltenyi Biotec	130-095-185	10 μg/ml	Ms IgG1
CD45	Abcam	ab10559	0.2 μg/ml	Rb IgG
Rb IgG	AbD Serotech	STAR159	variable	-
Ms IgG1	Cell Signalling	5415	variable	-
Ms IgG2a	Thermo Fisher	MG2A00	variable	-
Ax488 anti-Rb	Thermo Fisher	A-11008	1:200	-
Ax488 anti-Ms	Thermo Fisher	A-11001	1:200	-
Cy5 anti-Rb	Abcam	ab6564	1:200	-

2.16 cDNA Arrays

The cDNA arrays were performed by the Genomic Technologies and Bioinformatics Core Facilities in the Faculty of Biology, Medicine and Health, The University of Manchester. The RNA samples obtained from three independent biological replicates were processed according to Affymetrix GeneChip protocol (Affymetrix,USA). Briefly, total of 100 ng of RNA was used to generate labelled targets using the GeneChip WT PLUS Reagent Kit and hybridized to GeneChip Human Transcriptome Array 2.0. Microarrays were then washed and stained with the Fluidics FS450 script. Images were acquired with a GCS 3000HR scanner.

Table 2.9: List of mRNA samples obtained from differentiated iPS cells that have been used for cDNA arrays analysis

No	passage no	stage 0	stage 4	stage 5
1	p34	+	+	+
2	p39	+	+	+
3	p50	+	+	+

2.17 iTRAQ-MS/MS

2.17.1 Sample Preparation

Prior to iTRAQ-MS/MS, samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue in order to ensure protein integrity. Protein concentration in samples collected from untreated iPS cells (stage 0) and cells differentiated towards $Pdx1^+$ (stage 4), and $Ngn3^+$ (stage 5) was assessed with Bradford assay (Sigma, UK). Volume containing 100 μg of protein was transferred to a fresh tube and adjusted to 40 μl with 1M TEAB (Sigma, UK). Next cysteine disulphide bonds were reduced with 0.1 volume of 50 mM DTT (Sigma, UK), mixed, spun and incubated at 60 °C for 45 min. To prevent re-formation of disulphide bonds, cysteine residues were blocked by addition of 0.1 volume of 150 mM iodoacetic acid (IAA) (Sigma, UK), mixed, centrifuged and incubated for further 10 min at room temperature. Following that, samples were treated with 10 μg of trypsin (Promega, USA) in 1M TEAB buffer, mixed, centrifuged and incubated overnight at 37 °C.

2.17.2 Peptide Labelling

The volume of digested samples was reduced in a SpeedVac (Eppendorf, UK) to approximately 20 μl and adjusted 30 μl with 1M TEAB buffer prior to labelling. The 8-plex iTRAQ reagents (AB Sciex, UK) were reconstituted in 70 μl of isopropanol, mixed and centrifuged. Labelling was performed by transferring selected reconstituted iTRAQ reagent to a corresponding sample. Samples

with iTRAQ labels were mixed, pulse spun and incubated for 2 h at room temperature. Following labelling all 8 protein samples were pooled together, mixed, divided into two and transferred to a SpeedVac in order to reduce samples volume. Samples were stored in -20 °C. Technical replicates were prepared for iPS cell stage 0 (x2), stage 4 (x3) and stage 5 (x3).

2.17.3 Liquid Chromatography

iTRAQ-labelled samples were fractionated off-line with high-pH reverse-phase chromatography (3 μm Extend-C18 column; 4.6 x 100 mm; Agilent,USA). Prior to iTRAQ-protein samples fractionation, the column was used for three quality control runs, each with a mixture of known peptides. Samples were re-suspended in the mixture of 97 % (v/v) buffer A (water with 0.1% v/v ammonium hydroxide) and 3 % (v/v) buffer B (acetonitrile with 0.1 % v/v ammonium hydroxide). The samples were mixed, centrifuged and transferred to a glass sample vail for automated sample loading onto the chromatography column. The sample was fractionated by high pH reversed phase chromatography run on Agilent 1200 series HPLC system at 45 °C. Peptides were eluted using a gradient from 3 to 40 % (v/v) buffer B over 30 min at 0.75 ml/min, 85 fractions were collected on a 96-well plate. Collected fractions were dried in a SpeedVac and stored at -20 °C.

2.17.4 Tandem Mass Spectrometry

Fractionated samples were re-suspended in 180 μl of 3% v/v acetonitrile with 0.1 % (v/v) trifluoroacetic acid and 60 μl was transferred to an autosampler for loading onto a trapping column for clean-up/desalting (Symmetry C18 Trap; 5 μl, 180 μl x 20 μl, Waters). Peptides were eluted over an analytical column (nanoACQUITY UPLC BEH C18 Column; 1.7 μm, 75 μm x 250 mm, Waters) at 300 nl/min using a solvent gradient starting from 3 % (v/v) acentonitrile, 0.1 (v/v) formic acid to 40 % (v/v) acetonitrile , 0.1 % (v/v) formic acid.

The low-pH reverse-phase chromatography was run on-line to a QSTAR Elite

MS (AB Sciex; Analyst software QS 2.0). The mass spectrometer was set up to acquire a time-of-flight spectrometry scan for 1 s, followed with two MS/MS scans, each 1.5 s (as previously described, (Unwin *et al.*, 2010).

Data files obtained from tandem MS were analysed with Protein Pilot 4.0 (AB Sciex) using setting as specified in Table 2.10 .

Table 2.10: Protein identification and relative quantification were obtained with The Protein Pilot software (version 4.0) using the Paragon algorithm for search.

Option	Selected
Paragon Method	Human Peptide iTRAQ-8-Plex
Sample Type	iTRAQ 8plex (peptide Labelled)
Cys Alkylation	Iodoacetamide
Digestion	Trypsin
Instrument	QSTAR ESI
Species	Homo sapiens
Processing	Quantitate/ Bias Correction/ Biological modification
Database	Uniport (updated 08 March 2011)
Search Effort	Thorough ID
Results Quality	Detected Protein Threshold: 0.05; with False Discovery Rate Analysis

Table 2.11: Protein samples used for iTRAQ-MS/MS. S0- stage 0, S4- stage 4, S5- stage 5, 113-121-iTRAQ labels.

No	passage no	113	114	115	116	117	118	119	121
1	p34	S0	S0	S4	S4	S4	S5	S5	S5
2	p39	S0	S0	S4	S4	S4	S5	S5	S5

2.17.5 Data Analysis

Ratio values obtained from iTRAQ-MS/MS experiments were averaged and transformed into a $\log_2$ values. For statistical analysis of these data sets two-tailed t-student test assuming unequal variances was utilised.

The probe intensity level data generated by GeneChip Command Console software were analysed by the Genomic Technologies and Bioinformatics Core Facilities in the Faculty of Biology, Medicine and Health. The initial bio-informatics analysis consisted of: technical quality control using dChip software, normalisation and expression analysis using RMA (RMA normalisation on exons), assessment of experimental performance using Principal Component Analysis with

Partek Software (Partek Inc, USA) and statistical analysis with limma and QVALUE software. Post-analysis was performed with Ingenuity Pathway Analysis (IPA, Qiagen, UK) and Panther Gene Ontology (http://pantherdb.org/).

The IPA analysis was performed against Affymetrix GeneChip Human Transcriptome Array (HTA) for cDNA arrays and all identified proteins for iTRAQ-MS/MS data. Cut-offs were set for p-value ≤ 0.05 and log fold change cut-off from (-1) to 1 for cDNA arrays, and p-value cut-off ≤ 0.05 and log fold change cut-off from (-0.5) to 0.5 for iTRAQ-MS/MS.

Data analysis was also performed with PANTHER (Protein Annotation Through Evolutionary Relationship) classification system (http://www.pantherdb.org/). For this analysis input lists were generated by applying p-value cut-off ≤ 0.05 and log fold change cut-off from (-1) to 1 for cDNA arrays, and p-value cut-off ≤ 0.05 and log fold change cut-off from (-0.5) to 0.5 for iTRAQ-MS/MS. The overrepresentation test was performed against REACTOME database.

Chapter 3

Primary Cell Lines Derived from Pancreatic Tissue

3.1 Introduction

A panel of cell lines developed form pancreatic tissue was recently established in Dunne/Cosgrove laboratory. Three cell lines were developed from pancreatic tissue obtained from patients suffering from Congenital Hyperinsulinism (CHI) and has been previously characterised and described elsewhere (Kellaway, 2016). Those cell lines showed features of mesenchymal stem cells and based on previous research were proposed to have a potential to be successfully differentiated into pancreatic progenitor cells.

Congenital Hyperinsulinism (CHI) is a genetic disorder affecting newborns and infants. Physiologically it is manifested by dysregulated insulin secretion leading to abnormally low blood sugar levels (James *et al.*, 2009). CHI has complex genetic background with several known mutations linked to this disease. The most common genetic abnormalities casing CHI affect *ABCC8* and *KCNJ11*, genes encoding subunits of K_{ATP} channel. Dysfunction of K_{ATP} channels within pancreatic β-cells leads to constitutive cell depolarisation and insulin secretion (Rahman *et al.*, 2015). Based on morphology and genetic background CHI has

been divided into three subtypes: FCHI (affected β-cells are clustered together forming a lesion), DCHI (all islets cells in the pancreas are affected) and atypical (a morphological mosaics in a part of the organ) (Rahman *et al.*, 2015). CHI tissue- derived cell lines were previously observed to show increased proliferation rate than cell lines derived from adult tissue and this was also shown here (Kellaway, 2016). Although, whether this effect is due to young age of individuals from whom tissue was obtained or an effect of CHI related mutations has not yet been clearly established.

Multiple research groups have previously developed primary cell lines from both the endocrine and the exocrine fraction of pancreatic tissue. In most cases, cell lines of pancreatic origin were reported to have a similar phenotype and resembled mesenchymal stem cells (Zulewski *et al.*, 2001; Ouziel-Yahalom *et al.*, 2006; Eberhardt *et al.*, 2006; Fanjul *et al.*, 2010).

Mesenchymal stem cells (MSCs) are multipotent cells with a crucial role in tissue repair and regeneration (Dimarino *et al.*, 2013; Murphy *et al.*, 2013). MSCs were originally isolated from bone marrow (Friedenstein *et al.*, 1970; Pittenger *et al.*, 1999) and subsequently from other types of tissue, such as adipose tissue (Zuk *et al.*, 2002), umbilical cord (Can and Balci, 2011), placenta (Fukuchi *et al.*, 2004) and pancreas (Eberhardt *et al.*, 2006; Gallo *et al.*, 2007; Zha *et al.*, 2016). Those multipotent cells can be differentiated into other mesodermal cell types such as adipocytes, chondrocytes and osteoblasts (Pittenger *et al.*, 1999). More recently MSCs were also reported to be successfully differentiated into non-mesodermal lineage cell types, including hepatocytes (Aurich *et al.*, 2009; Xu *et al.*, 2015), neurones (Black and Woodbury, 2001) and pancreatic endocrine cells (Zanini *et al.*, 2011; Marappagounder *et al.*, 2013; Li *et al.*, 2013; Kao *et al.*, 2015).

Mesenchymal stem cells are characterised as spindle-like shape, plastic-adherent cells with the ability to form colonies (Pittenger *et al.*, 1999). MSCs were also reported to express class VI intermediate filament protein- Nestin (Xie *et al.*,

2015) and two other cytoskeleton proteins, Vimentin and alpha-smooth muscle actin (Eberhardt *et al.*, 2006; Kinner *et al.*, 2002). Additionally, minimum criteria for defining MSCs proposed by the International Society for Cellular Therapy include expression of specific cell surface markers such as CD90, CD73, CD105 (more than 95 % of the population) and lack of the expression of CD45 (less than 2 % of the population). MSCs must also demonstrate potential to differentiate into mesodermal lineage (Dominici *et al.*, 2006) (Table 3.1).

Table 3.1: Criteria for mesenchymal stem cells (MSC) identification.

Criteria	Cells Phenotype	Ref.
Cell culture	Cell adhere to the plastic in normal culture condition	(Dominici *et al.*, 2006)
Specific cell surface antigen expression	Positive ($>$ 95 %): CD105 (Endoglin, SH2), CD73 (Ecto-5 nucleotidase, SH3, SH4) and CD90 (Thy-1); Negative ($<$ 2 %): CD45, CD34, CD14 or CD11b, CD79a or CD19, HLA-DR	(Dominici *et al.*, 2006)
In vitro differentiation	osteoblasts, adipocytes, chondroblasts	(Dominici *et al.*, 2006)
Other molecules expressed by MSCs	CD13, CD29, CD44, and CD10	(Buhring *et al.*, 2007)

The experiments presented here aimed to:

- verify identity of three previously developed mesenchymal stem cell lines (CHI-pMSCs; cell lines established from CHI patents' tissue (Kellaway, 2016));
- verify identity of one more CHI-derived cell line (CH-pMSC4);
- characterise cell line developed from adult pancreatic islets (AI-pMSCs);

Both CH-pMSCs and AI-pMSCs were also tested for their potential to differentiate into pancreatic progenitor cells (Chapter 4).

3.2 AI-pMSC and CH-pMSC Cells are Mesenchymal Stem Cells

Adult human pancreatic islets obtain from the Oxford Centre for Islets Transplantation and exocrine pancreatic tissue obtained from the Central Manchester University Hospital were used to establish AI-pMSC and CHI-pMSC cell lines, respectively (Tab.3.2 and 3.3).

Table 3.2: CHI-pMSCs were developed from tissue obtained from CHI patients following partial pancreatectomy.

Cell Line	Age	Disease Type
CHI-pMSC1	4 months	FCHI
CHI-pMSC2	17 months	Atypical
CHI-pMSC3	11 weeks	DCHI
CHI-pMSC4	3 months	DCHI

Table 3.3: AI-pMSCs were developed from pancreatic islets obtained from the Oxford Centre for Islets Transplantation.

Cell Line	Age	BMI	Purity	Viability
AI-pMSC	41	34	60 %	75 %

The majority of human pancreatic islets that were obtained from adult donors, attached within first 48 h forming growth areas when placed in the cell culture dish. Following this short adaptation period, outgrowth and migration of cells from the original adherence spot was observed. Cells outgrowing from the original sphere had elongated, fibroblast-like morphology. After a second passage, cells with fibroblast-like morphology were the dominant type of cells in the culture (Fig.3.1, A, B and C). AI-pMSC cells were passaged up to p8 when the first signs of senescence were observed, such as an increase in granulation and formation of cell debris.

Similarly, when islet-excised fractions of pancreatic tissue obtained from CHI patients were placed in the culture, a growth area of attached cells was formed within first 24 h. Originally, those cells had a polygonal shape. However, an

outgrowth of elongated, spindle-shaped cells was observed on the edges of primary adherence zones. Following sequential passaging, the major population of cells had elongated, fibroblast-like morphology (Fig.3.1 D, E and F). CHI-pMSCs were maintained in culture up to passage 20.

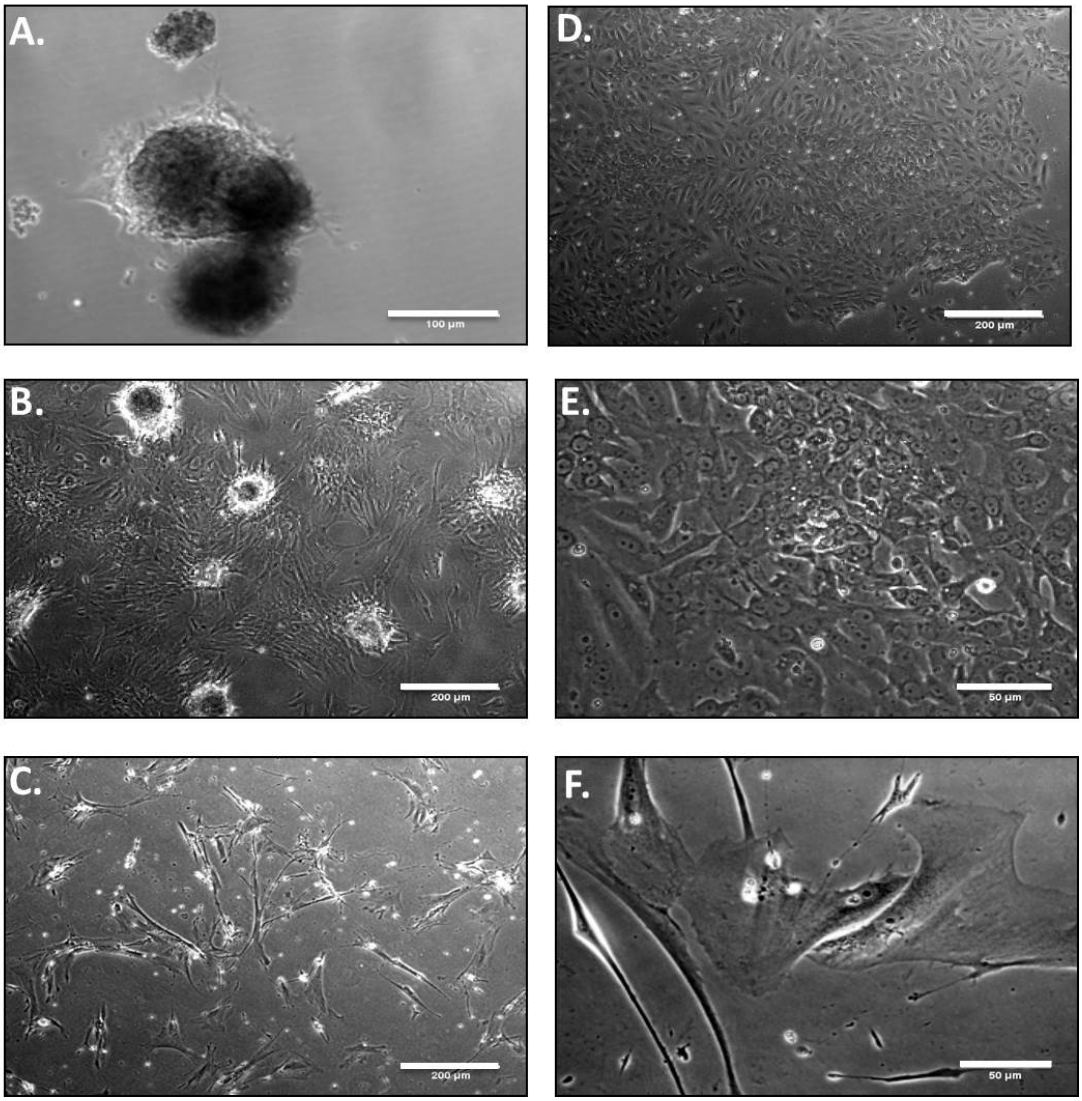

Figure 3.1: AI-pMSC and CH-pMSC cells morphology. After several days in expansion medium outgrowth of fibroblast like cells from the original adhesion spot was observed; After passage 1 fibroblast like cells were dominant type of cells observed; **A:** AI-pMSC cells passage 0 48 h in culture (scale bar = 100 μm); **B:** AI-pMSC cells passage 0 14 days in culture (scale bar = 200 μm); **C:** AI-pMSC cells passage 10 (scale bar = 200 μm); **D:** CH-pMSC4 cells passage 0 four days in culture (scale bar = 200 μm); **E:** CH-pMSC4 cells passage 0 four days in culture (scale bar = 50 μm); **F:** CH-pMSC4 cells passage 8 (scale bar = 50 μm).

AI-pMSC ad CHI-pMSC4 cells were also tested for the expression of cell cytoskeleton proteins that were previously established to be mesenchymal markers such as: a type III intermediate filament protein- Vimentin, a type VI intermediate filament protein- Nestin and α-smooth muscle actin (α-SMA). Both cell lines expressed those markers at the protein level, suggesting their potential mesenchymal stem cell properties (Fig.3.2).

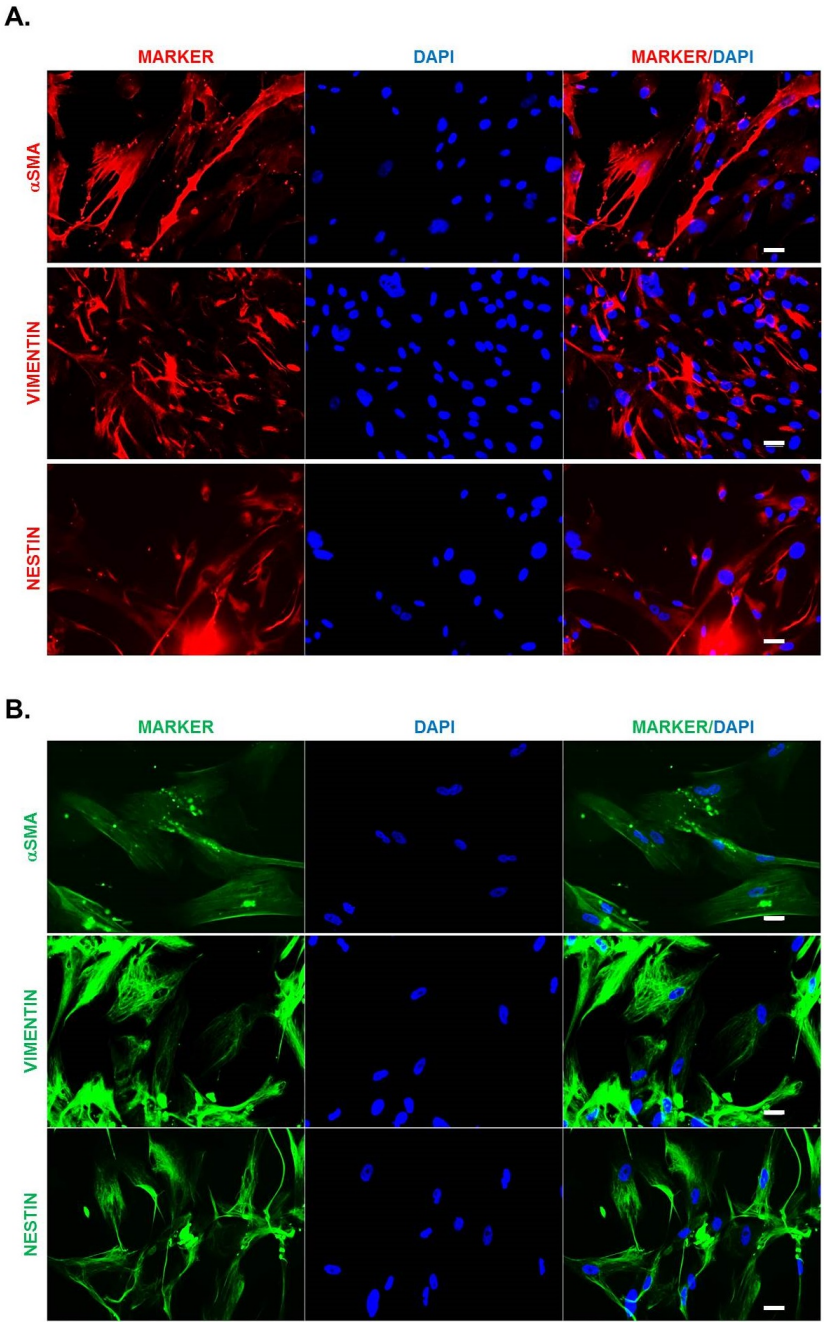

Figure 3.2: CHI-pMSC4 and AI-MSC cells express VIMENTIN, NESTIN and α-SMA. CHI-pMSC4 and AI-MSC cells tested positive for common mesenchymal stem cell cytoskeleton proteins: VIMENTIN, NESTIN and α-SMA; Panel **A**: CHI-pMSCs; Panel **B:** AI-pMSCs; (scale bar= 50 μm).

In addition to distinctive morphology and expression of characteristic cytoskeleton proteins CHI-MSCs also tested positive for cell surface markers specific to mesenchymal stem cell such as CD44, CD90, CD29, CD73 and did not expressed CD45- a marker for hematopoietic stem cells. This experiment was performed on an early (p5) and late (p13) passage numbers for CHI-pMSC4. According to the minimum criteria for MSCs minimum of 95 % of the cell population has to test positive for those markers to allow for that cell line to be classified as a mesenchymal stem cells. The low passage cultures of CH-pMSC4 cells were in minimum 95 % positive for three out of four selected markers, with only cells stained with CD90 being slightly below this cutoff, testing positive in 93 %. However, for cells above passage 10, observed expression of CD markers was noticeable below this cutoff (Fig.3.3).

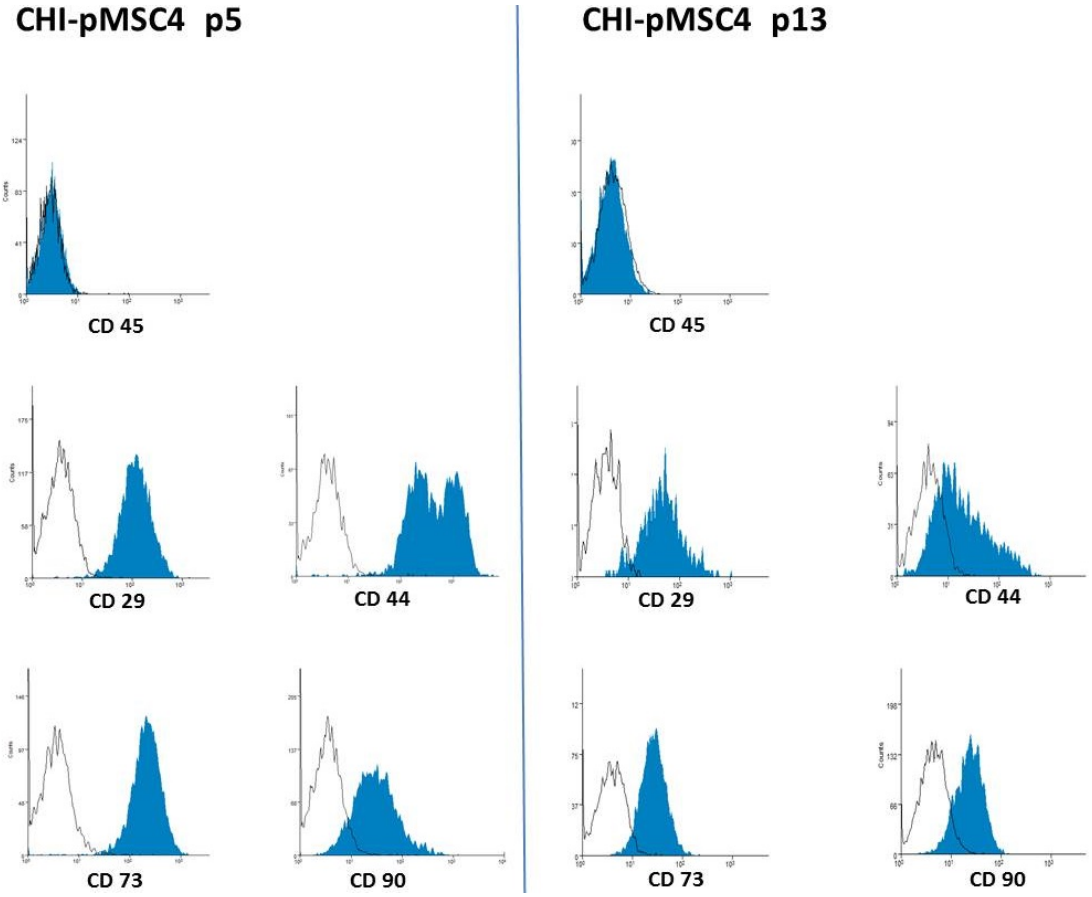

Figure 3.3: CH-pMSC4 cell surface antigen analysis by flow cytometry. Cell expanded from the exocrine- enriched fraction of pancreatic tissue tested positive for CD29 (95 %), CD44 (96 %), CD73 (95 %) and CD90 (93 %) when tested at early passage number (p5). Above passage 10, a trend indicating a decrease in the number of cells expressing those markers was observed; CD29 (75 %), CD44 (31 %), CD73 (70 %), CD90 (66 %). Early and late passage CHI-pMSC4 tested negative for CD45. Plots show isotype control staining (white histogram) versus specific antibody staining (blue histogram).

3.3 Differentiation of CHI-pMSC2 and CHI-pMSC4 Cells into Adipocytes, Chondrocytes and Osteocytes

CHI-pMSC2 and CHI-pMSC4 cells were tested for their ability to differentiate into the mesenchymal lineage. Cells between passage four and 10 were cultured in conditions that stimulate differentiation into adipocytes for 14 days. Following culture in adipocyte-selective conditions, lipid droplets were stained with Oil O

Red (Fig 3.4 panel: B and E). Undifferentiated controls generally did not develop lipid inclusions, however, on two occasions lipid droplets were also observed in one out of three technical controls for CHI-pMSC2 and CHI-pMSC4 (data not shown).

Chondrogenic differentiation was also attempted on cells between four and 10 passages. CHI-pMSC2 and CHI-pMSC4 cells maintained as a micromass culture were exposed to chondrocyte-selective medium for 14 days. Negative control cells were also set as a micromass, however, flattening and atrophy of that structure was observed when maintained in non-differentiating media. Differentiation into cartilage was confirmed by selectively staining cartilage extracellular matrix with alcian blue. Cells in control conditions shown no signs of aggrecan staining (Fig.3.4 panel: A and D).

Differentiation into osteoblasts was also successful when performed on cells between passage four and 10. After 21 days in osteo-selective medium, CHI-pMSC2 and CHI-pMSC4 cell lines were fixed and presence of extracellular calcium deposits was indicated by alizarin red staining. Cells in control conditions showed no evidence of mineralization (Fig.3.4 panel C and F).

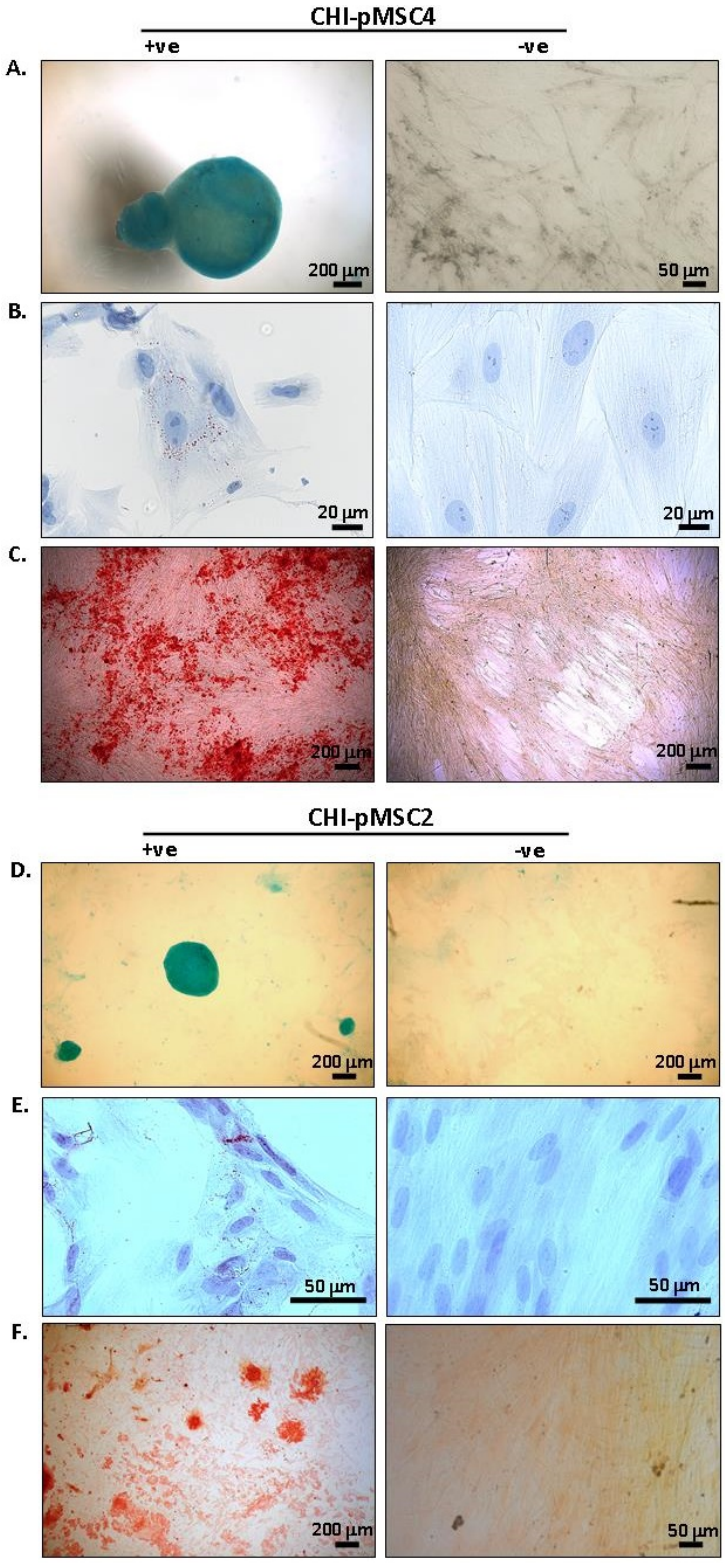

Figure 3.4: Differentiation of CHI-pMSC cells into mesodermal lineage cells: chondrocytes, adipocytes and osteoblasts. Representative image of CHI-pMSC2 and CHI-pMSC4 cell differentiated into chondrocites. Following 14 days culture in chondrogenic conditions, cell micromass was stained with alcian blue: **(A, D)**. For adipogeninc differentiation Nes cells were maintained in the adipo-selective medium for 14 days. Accumulating lipid droplets stained red with oil O red **(B, E)**. To induce osteogenesis Nes cells were cultured for 21 days in the osteo-selective medium. Accumulation of calcium deposits was detected by alizarin red staining **(C, F)**.

3.4 Growth and expression profile of AI-pMSC and CHI-pMSC cells

The majority of results presented here and in the next chapter were obtained from experiments performed on CHI-pMSC2 cells as this cell line continue to grow up to passage 20 and could be passaged in a 1:4 ratio. CHI-pMSC3 and CHI-pMSC4 persisted in the culture up to passage 15 and were passaged 1:3, whereas CH-pMSC1 cells were passaged 1:2 and stopped proliferating at passage 12. AI-pMSC cells were cultured up to passage 10 and passaged in 1:2 ratio. As expected, proliferation of CHI-pMSC2 cells was significantly higher when compared to the proliferation of AI-pMSC cells. Also, doubling time for CHI-pMSC2 cells was only 45.44 h in contrast to 99.89 h for AI-pMSC cells (Fig.3.5).

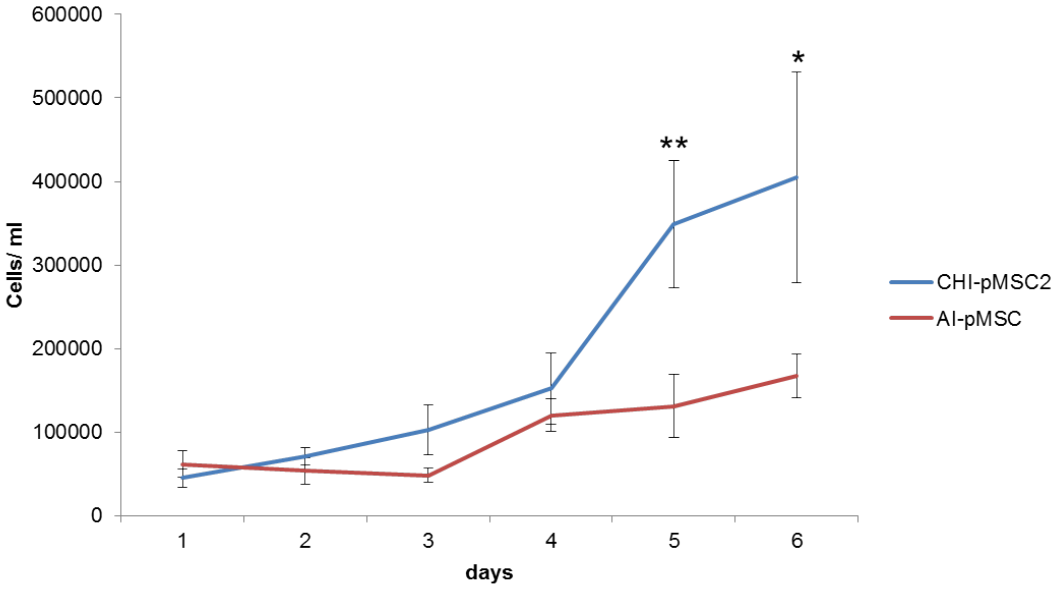

Figure 3.5: Growth curves for AI-pMSC and CHI-pMSC2 cell lines ((**) $p \leq 0.01$; (*) $p < 0.05$).

Expression pattern of key transcription factors (TFs) involved in pancreatic islet development was also tested for CHI-pMSC and AI-pMSC cells. The key TF involved in the development of pancreatic endocrine cells- *PDX1* was not expressed by AI-pMSC or CHI-pMSC cells at the mRNA level. However, both cell lines expressed *ISL1*, a TF linked with proliferation, survival and maturation of pancreatic cells. The expression of ISL1 was also detected at the protein level in all four CHI-pMSC cell lines. Both, CHI-pMSC and AI-pMSC cell lines, also tested positive for the expression of SOX9 at the mRNA and in the case of CHI-pMSC cells also at the proteins level. Additionally, CHI-pMSC cells were also found to express transcription factors characteristic for early stages of pancreatic cell development, such as *SOX17*, *FOXA2* and *PAX6* (Fig.3.6).

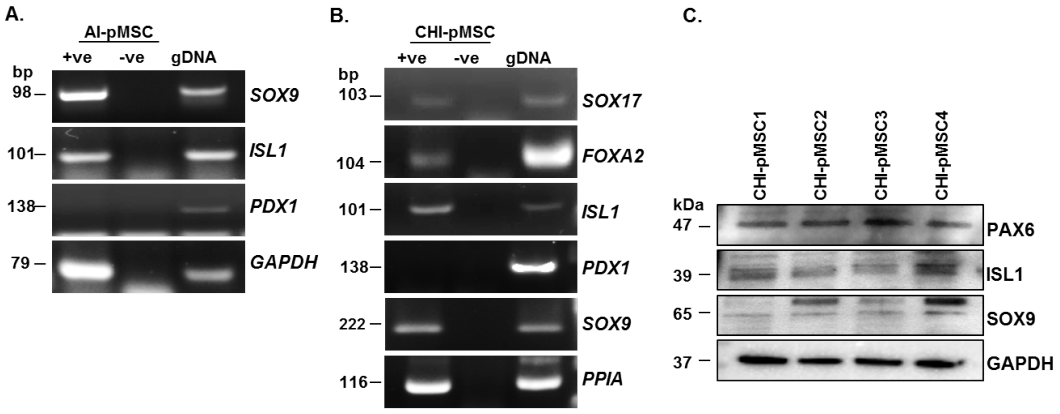

Figure 3.6: Characterisation of CHI-pMSC and AI-pMSC cell lines. Expression profile of key TFs involves in pancreatic endocrine cell development for AI-pMSC cells (**A**) and CHI-pMSC cells (**B**); Expression of PAX6, SOX9 and ISL1 was also confirmed at the protein level with immunoblotting (**C**).

3.5 Discussion

It was previously reported that pancreatic cells in culture generate a population of mesenchymal stem cells (Eberhardt *et al.*, 2006; Gallo *et al.*, 2007). This phenomenon was first linked with a possibility that pancreatic cells in *in vitro* conditions undergo the process of epithelial mesenchymal transition (EMT)(Gershengorn *et al.*, 2004; Fanjul *et al.*, 2010; Lima *et al.*, 2013). However, this observation was not supported by the result of genetic lineage tracing experiments in rodents (Atouf *et al.*, 2007). Based on that observation it was postulated that cells with mesenchymal morphology, rather than from de-differentiated endocrine cells, originated from a population of resident MSCs that eventually have over-grown epithelial pancreatic cells (Kayali *et al.*, 2007; Gallo *et al.*, 2007). The exact nature of changes cells of pancreatic origin undergo in *in vitro* culture remains controversial, nevertheless, MSC populations obtained from cells of pancreatic origin are an attractive source of cells for research into β-cells generation and treatment of Type 1 Diabetes. Here, as it was previously reported, human pancreatic islet and exocrine cells placed in *in vitro* culture conditions resulted in a population of fibroblast-like, plastic adherent cells. The primary focus of this chapter was on verifying if those cells, AI-pMSC and CHI-pMSC, were mesenchymal stem cells and on subsequent characterization of those cell lines.

Both types of cell lines developed from pancreatic tissue, AI-pMSC and CHI-pMSC, following passage 4 were composed purely of cells resembling MSCs. Additionally, CHI-pMSC and AI-pMSC cell lines expressed Vimentin, α smooth muscle actin and Nestin, cytoskeleton proteins normally found in mesenchymal stem cells (Xie *et al.*, 2015; Eberhardt *et al.*, 2006; Kinner *et al.*, 2002). CHI-pMSC cells, additionally, tested positive for CD29, CD44, CD73 and CD90 markers, with a minimum of 93-95 % of the population expressing these proteins; and were negative for CD45, a hematopoietic cell marker. However, a decrease in the percentage of cells positive for CD markers was observed for CHI-pMSC cells

above passage 10. This could possibly indicate gradual loss of stem- like phenotype of cells cultured for a prolonged time.

The ability to differentiate into another type of cells is also characteristic of all stem cells. However, in the case of MSCs, this key feature was reported to be lost upon long-term *in vitro* cultivation (Bonab *et al.*, 2006). For those reasons all experiments aiming to differentiated CHI-pMSC cells into adipocytes, chondrocytes and osteoblasts were performed on cells between passage 4 and 10. CHI-pMSC2 and CHI-pMSC4 cells successfully differentiated into all three mesodermal lineage cell types.

Following expansion, AI-pMSC and CHI-pMSC cell lines maintained expression of TFs involved in the development of the pancreas and pancreatic endocrine cells. Both AI-pMSC and CHI-pMSC cells expressed SOX9, a master regulator involved in maintaining a pool of pancreatic progenitor cells (Seymour *et al.*, 2007) and ISL1, a TFs involved in pancreatic endocrine cells proliferation, maturation and survival (Du *et al.*, 2009). Expression of ISL1 and SOX9 by pancreatic MSC was previously reported (Eberhardt *et al.*, 2006; Baertschiger *et al.*, 2008). CHI-pMSC cells were also found to express *SOX17* and *FOXA2*, transcription regulators associated with the development of endoderm. Expression of endoderm-specific TFs was also previously observed in MSCs established from Wharton's Jelly (Nekanti *et al.*, 2010). However, no expression of PDX1, a transcription regulator essential for pancreas development, was detected for CHI-pMSC or AI-pMSC cell line.

Differentiation of MSCs into pancreatic progenitors, presented in next chapter, requires a high number of cells. Therefore, the growth of CHI-pMSC and AI-pMSC cells were compared. CHI-pMSC cells had shorter doubling time and remain proliferative at late passage numbers. The greater proliferative potential of CHI-pMSC cells comparing to AI-pMSC could be potentially contributed to the very young age of patients from whom the pancreatic tissue was obtained. As it was previously observed that MSCs obtained from Wharton's Jelly had

greater proliferative potential that bone marrow derived MSCs obtained from adult donors (Nekanti *et al.*, 2010). The greater potential of CHI-pMSC cells could also be linked with Congenital Hyperinsulinism, as its pathological phenotype was observed to include increased proliferation of ductal and exocrine compartments (Salisbury *et al.*, 2015). However, the nature of this observation has not yet been elucidated.

In summary, cell lines established from exocrine and endocrine pancreatic tissue had characteristics of mesenchymal stem cells. CHI-pMSC cells had greater proliferation potential and therefore were selected as a model for differentiation experiments presented in the next chapter.

Chapter 4

Differentiation of Mesenchymal Stem Cells towards Pancreatic Progenitor Cells

4.1 Introduction

Isolation of mesenchymal stem cells (MSCs) from pancreatic tissue has been previously described (Zulewski *et al.*, 2001; Zanini *et al.*, 2011; Ramiya *et al.*, 2000; Seeberger *et al.*, 2006). The exact function of MSCs in pancreas remains undefined, although it was hypothesised that those cells are a population of dormant stem cells that possess tissue regeneration potential (Eberhardt *et al.*, 2006). Additionally, it has also been previously demonstrated that MSCs developed from pancreatic islets can be re-differentiated into insulin producing cells (Eberhardt *et al.*, 2006). Protocols aiming to induce differentiation of β-like cells from MSCs involve combinations of serum free conditions, non-adherent plasticware and media supplementation with various growth and differentiation factors. Extrinsic factors utilised for this purpose include: activin A, nicotinamide, retinoic acid, betacellulin, exendin 4 and HGF and EGF (Table 4.1) (Zulewski *et al.*, 2001; Gallo *et al.*, 2007). Those compounds were shown to stimulate β-cell prolifera-

tion, improve cells survival and enhance insulin expression.

Table 4.1: Biologically active compounds used to stimulate MSCs differentiation into β-like cells.

Compound	**Function**	**Reference**
Betacellulin	stimulates proliferation of pancreatic progenitor cells, sustains expression of PDX1 and enhances differentiation stem cells towards β-cells	(Demeterco *et al.*, 2000; Cho *et al.*, 2008)
Exendin-4 & GLP1	has anti-apoptotic effect on pancreatic islets and stimulates β-cell neogenesis	(Xu *et al.*, 1999; Farilla *et al.*, 2003)
Activin A, Retinoic acid	induces differentiation towards β-cells	(Demeterco *et al.*, 2000; Shi *et al.*, 2005)
Nicotinamide	induces differentiation and maturation of fetal pancreatic cells, has been extensively used as a maturation factor in differentiation of mESCs and hESCs towards pancreatic β-cells	(Otonkoski *et al.*, 1993; Champeris Tsaniras and Jones, 2010)
HGF	stimulates proliferation and differentiation of insulin- expressing cells	(Otonkoski *et al.*, 1994; Movassat *et al.*, 2003)
EGF	important in pancreatic development, survival and in β-cell proliferation	(Miettinen *et al.*, 2006)

In addition to extrinsic factors, other differentiation enhancers such as extracellular matrix and induction of 3D multicellular aggregates, have also been utilised for the induction of MSC differentiation (Cesarz and Tamama, 2016). 3D culture techniques are favourable in terms of closely imitating the conditions of a stem cell niche within the tissue. Such spheroid cultures of stem cells are regarded to be more physiologically accurate by reflecting the *in vivo* cell-cell and cell-matrix interactions, the gradient of nutrients and oxygen as well as enhancing cell polarity establishment (Labusca, 2015). MSCs cultured under such conditions were previously reported to significantly improve their differentiation potential (Baraniak and McDevitt, 2012). For example, cells cultured as spheroids were observed to demonstrate increased potential towards differentiation into osteocytes and adipocytes (Wang *et al.*, 2009; Yamaguchi *et al.*, 2014).

Changes between 3D cell cultures and conventional 2D culture were also observed in cell cytoskeleton organisation, tolerance to ischemic conditions, migration ability, gene expression profile and anti-inflammatory compounds secreted by MSCs (Tsai *et al.*, 2015; Cesarz and Tamama, 2016).

In this chapter potential of pancreatic MSC lines derived from CHI patients' tissue (CHI-pMSC) to differentiate into pancreatic progenitors was tested with two protocols: a step-wise approach with conditioned media (Zanini *et al.*, 2011) and an Isx-9 based protocol (Dioum *et al.*, 2011).

The protocol developed by Zanini et al. (2011) used as a starting point a population of MSCs developed from human adult pancreatic islets, this resembled the human pancreatic tissue origin of CHI-pMSC. This differentiation has led to the generation of β-like cells that shown expression of PDX1 and insulin at the protein level. The main steps of this protocol included treatment of 3D cell cultures with a mixture of extrinsic compounds such as activin A, betacellulin, nicotinamide, exendin-4, and EGF.

In addition to the stepwise protocol, a differentiation with a small compound, called Isx-9 was tested on CHI-pMSC. Isx-9 is a small molecule compound, containing an isoxozol ring, which was reported to enhance expression of neuronal and β-cell specific genes. Isx-9 was first described as a cardiogenic factor, which stimulates expression of Nkx2.5 in mouse pluripotent cells (Sadek *et al.*, 2008). In subsequent research, this compound was also found to induce expression of neurogenic genes, mainly NEUROD1 (Schneider *et al.*, 2008) and TFs involved in specification and maturation of pancreatic progenitors and β-cells. The proposed mechanism of gene expression enhancement by Isx-9 in pancreatic cells involves activation of histone acetyltransferase (HAT) p300, a transcriptional co-activator able to interact with a vast array of transcription factors. By this epigenetic modification, Isx-9 can activate transcription of genes such as *PDX1*, *NGN3*, *NEUROD1* and *MAFA* (Dioum *et al.*, 2011).

The overall aim of this project was to identify cell surface proteins that could

be used as markers for isolation of pancreatic progenitor (PP) cells. Currently, PP cells are identified based on expression of specific transcription factors. However, this approach does not allow for isolation of intact PP cells as selection based on TFs requires disintegration of the cell membrane. The aim of the experiments presented in this chapter was to:

- test the ability of CH-pMSCs to differentiate into PP cells using step-wise differentiation protocol;
- test the ability of CH-pMSCs to differentiate into PP cells when treated with Isx-9;

If successfully generated PP cells could be further analysed using a proteomic approach in order to identify novel pancreatic progenitor cell makers.

4.2 Differentiation of CHI-pMSC Cells with Conditioned Media Protocol

A protocol used here for differentiation of CHI-pMSC was based on research reported by Zanini et al. (2011) with modifications developed by Dr Kellaway (Zanini *et al.*, 2011; Kellaway, 2016). This protocol included supplementation of growth media with factors enhancing β-cells maturation and survival, such as activin A, betacellulin, nicotinamide, exendin-4 and EGF (Fig.4.1, panel A). The expression of key transcription factors involved in β-cell differentiation, PDX1- a TF marking pancreatic progenitor cells and NEUROD1- a TF downstream of NGN3, which is a key regulator of pancreatic endocrine progenitor development, was tested at the end stage 2, stage 3 and stage 4 by immunoblotting. Expression of PDX1 and NEUROD1 was not detected at the protein level for any of differentiation stages. Additionally, expression of type III intermediate filament protein specific to mesenchymal cells- Vimentin was tested. This aimed to test if this protocol in addition to expected induction of pancreas specific genes also stimulates the reduction in the expression of proteins specific to the mesenchymal phenotype. Although expression of PDX1 and NEUROD1 was not detected at the protein level following differentiation, the expression of Vimentin was noticeably reduced for cells cultured as spheroids. Interestingly, the reduction in the expression of Vimentin was observed not only for cell spheroids treated with the conditioned media but also for it time match control. The time match control cells were maintained in serum free media as cell spheroids, however, those cells were not exposed to any of the pancreas differentiation enhancing factors.

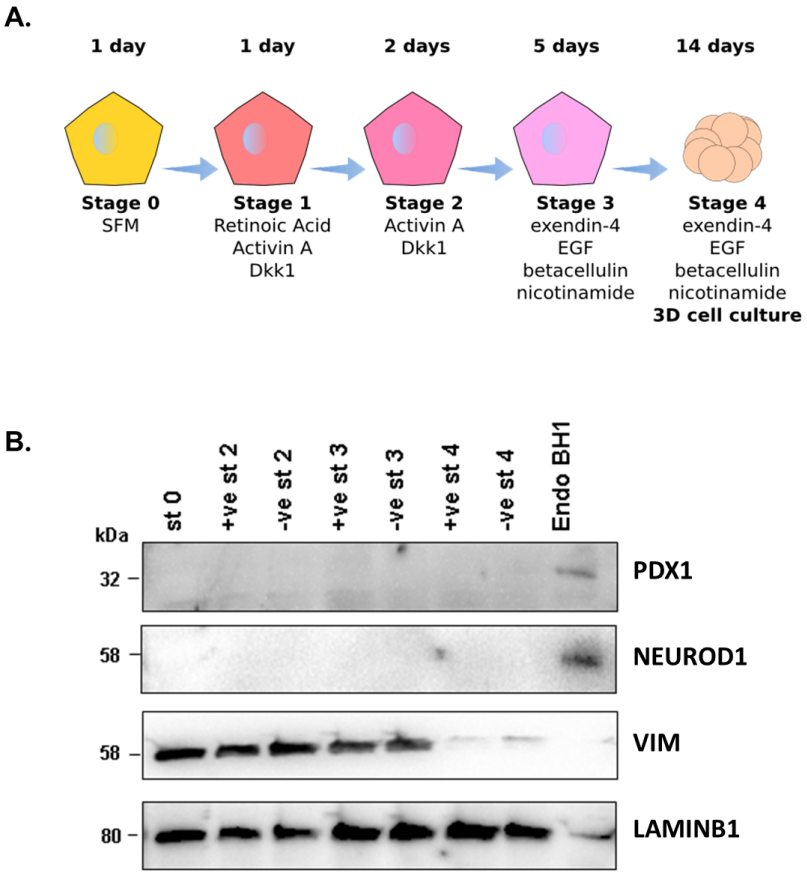

Figure 4.1: Step-wise differentiation of CHI-pMSCs. **Panel A**: an overview on the differentiation protocol- cells growing in serum free media (SFM) for 1 day were then exposed to SFM supplemented with retinoic acid, activin A and Dkk1 for 1 day. Next, cells were exposed to SFM with activin A and Dkk1 for a further 2 days. Following that, cells were maintained in SFM with exendin-4, EGF, betacellulin and nicotinamide for 5 days. Finally, cells were transferred to ultra-low adherence plates and maintained as spheroids in media supplemented as per stage 3. **Panel B**: analysis of PDX1, NEUROD1 and VIMENTIN expression levels in treated CHI-pMSC cells. (EndoBH1 cells were used as a positive control for PDX1 and NEUROD1 expression).

4.3 CHI-pMSC cells differentiation with Isx-9

The protocol based on research by Zanini et al. (2011) failed to induce expression of PDX1 and NEUROD1 at the protein level in CHI-pMSCs, therefore an alternative protocol based on Isx-9 has also been tested.

Previous studies with Isx-9 show that cells exposed to this compound have enhanced/ sustained expression of key transcription factors regulating development of pancreatic endocrine progenitors. The list of TFs reported to be induced by treatment with Isx9 includes PDX1, NGN3, MAFA and NEUROD1. Here, expression of *PDX1* and *NEUROD1* at the mRNA level was detected for CHI-pMSCs treated with Isx-9 for 48 and 96 h. Whereas cells growing in control conditions (control: serum free media (SFM) and vehicle control: SFM with DMSO) did not express *PDX1* or *NEUROD1* at levels detectable by RT-PCR (Fig.4.2, panel A).

However, expression of PDX1 was not detected in CHI-pMSC cells exposed to Isx-9 for 48 and 96 h, when tested at the protein level with immunoblotting. This result was confirmed by testing CHI-pMSCs exposed to Isx-9 for 96h with a different anybody against PDX1 by immunocytochemistry. Also, expression of NEUROD1 in CHI-pMSCs treated with Isx-9 for 96h was not detected when tested with immunocytochemistry (Fig.4.2, panel B and C).

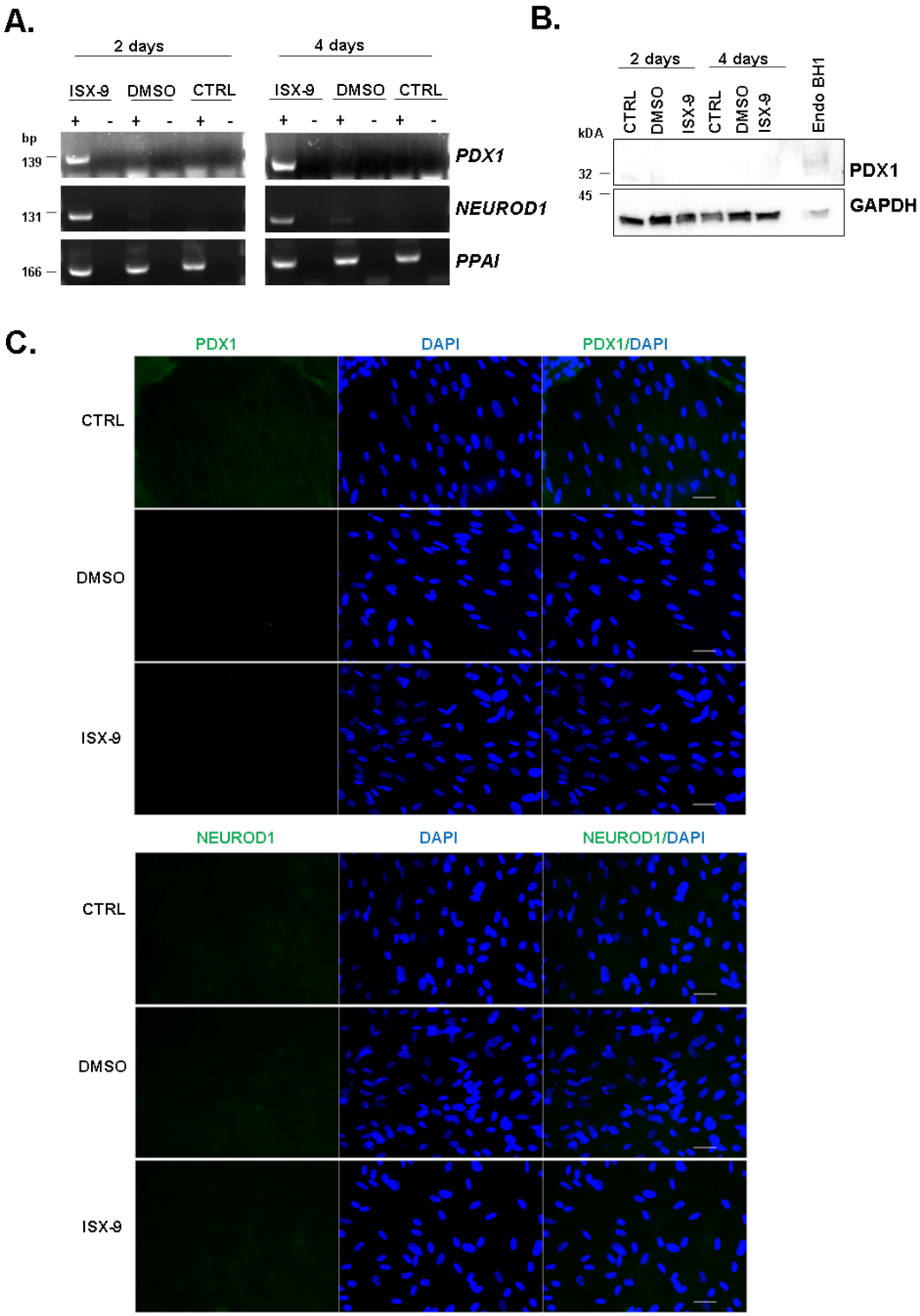

Figure 4.2: Isx-9 induces expression of *PDX1* and *NEUROD1* only at the mRNA level. **Panel A**: CHI-pMSCs were exposed to Isx-9 for 48 h and 96 h. Following this treatment expression of *PDX1* and *NEUROD1* at the mRNA level was detected. **Panel B**: Expression of PDX1 by Isx-9 treated cells was tested by Western blotting. No expression was detected at the protein level. **Panel C**: CHI-pMSCs treated with Isx-9 for 96 h were tested with immunocytochemistry for expression of PDX1 and NEUROD1. No expression was detected (scale bar = 50 μm).

4.4 Discussion

Mesenchymal stem cells are considered to be a valuable source of cells for regenerative medicine. The most advanced research involving MSCs for clinical applications targeted degenerative diseases affecting cartilage and osseous tissue (Murphy *et al.*, 2013). MSCs were also proposed to be a source of cells for treatment of Type 1 Diabetes and their potential to differentiate into pancreatic β-like cells has been demonstrated previously (Zanini *et al.*, 2011; Zulewski *et al.*, 2001; Ramiya *et al.*, 2000). In this chapter, such ability of MSCs developed from the exocrine-enriched fraction of the pancreas was explored.

Two differentiation protocols were tested: a step-wise differentiation utilising several compounds inducing β-cell maturation and survival, and differentiation with Isx-9. Additionally, an effect of 3D culture on MSCs differentiation has also been observed.

Here a protocol developed by Zanini et al. (2011) was adapted for differentiation of CHI-pMSC cells. However, expression of key pancreatic progenitor TFs was not detected at the protein level. This result is contrary to the outcome achieved by Zanini et al. (2011). Although, only a weak expression of PDX1 was observed in the original research and this was obtained only for MSCs derived from pancreatic islet cells. Also, tested in the same research, MSC line derived from bone marrow did not show expression of PDX1 following treatment (Zanini *et al.*, 2011). This lead to the conclusion that currently available protocols for generation of pancreatic β-cells from MSCs require further optimisation and the origin of the MSCs might be an important factor that needs to be considered in the differentiation process.

Culturing cells in 3D conditions was previously reported to enhance the differentiation potential of MSCs (Wang *et al.*, 2009). This type of cell culture was also reported to induce MSCs differentiation towards β-like cell and was a part of the step-wise protocol published by Zanini et al. (2011). However, changes in the

expression of Vimentin were observed not only in cells exposed to the conditioned media but also in the time match control. Those control cells were also maintained as spheroids but in a serum free media. Therefore the observed reduction in the level of Vimentin expression was rather due to the cell growth as spheroids than to their exposure to the differentiation factors.

Vimentin is a major intermediate filament protein in MSCs and was reported to act as an integrator of proteins involved in cell adhesion, migration and signalling (Ivaska *et al.*, 2007). Downregulation of Vimentin was observed during differentiation of myogenic cell towards myotubes and in myogenesis following injury (Wu *et al.*, 2007; Vaittinen *et al.*, 2001). Therefore, noticeable reduction in the expression of this protein might indicate that spheroid culture of CHI-pMSC to some degree altered the phenotype of those cells towards less mesenchymal one.

MSC cells were also treated with a small molecule inducer previously reported to stimulate expression of key TFs involved in β-cell differentiation (Dioum *et al.*, 2011). Dioum et al. showed that exposure of MIN6 cells or human pancreatic islets cultured for several months to Isx-9 for 24 and 48 h resulted in the induction of genes such as PDX1, NEUROD1 and NGN3 at the protein level. Data presented in this chapter indeed showed that this treatment induced expression of *PDX1* and *NEUROD1* in MSCs after treatment with Isx-9 for 48 and 96 h in conventional 2D cell culture conditions. However, expression of PDX1 or NEUROD1 at the protein level was not detected in Isx-9 treated cells. Isx-9 has been reported to induce genes expression by epigenetic modulation of chromatin. The proposed mechanism of Isx-9 induction of pancreas related TFs expression involved activation of ERK1/2 and increase in histone acetylation (Dioum *et al.*, 2011). However, based on the data presented in this chapter induction of *PDX1* and *NEUROD1* transcription has not resulted in translation and generation of functional proteins that would control the expression of their downstream effectors.

In summary, MSCs might be a source of β-cells for regenerative medicine application, however, currently available differentiation protocol are characterised by low success rates. Here, a direct differentiation with a small molecule inducer and a step wise protocol were utilised in order to generate a population of pancreatic progenitors from MSC. Only induction of CHI-pMSCs with Isx-9 resulted in expression of *PDX1* and *NEUROD1* at the mRNA level. Since presented in this chapter attempts to use CHI-pMSCs to generated pancreatic progenitor cells that express key TFs at the protein level failed, iPS cells were used as an *in vitro* model for further differentiation (Chapter 5).

Chapter 5

Differentiation of iPS Cells towards Pancreatic Progenitors

5.1 Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPS cells) are an important model for basic biological research, regenerative medicine, disease modeling and drug development. However, the use of human embryonic stem cells in research is controversial (ethical dilemma specific to the origin of these cells) and often of limited access (Lo and Parham, 2009). Therefore, iPS cells that can be induced from any adult somatic cell type are an attractive alternative. Moreover, it was also reported that iPS cells might, to some extent, retain an epigenetic memory of the somatic tissue they have been developed from. This retained pattern of chromatin modifications can influence properties of iPS cells and enhance their capacity to differentiate towards cells of initial type (Bar-Nur *et al.*, 2011; Vaskova *et al.*, 2013; Liang and Zhang, 2013).

Several recently developed step-wise differentiation protocols aimed at the generation of mature and functional pancreatic β-cells from hES and iPS cell lines (Rezania *et al.*, 2012; Pagliuca *et al.*, 2014; Rezania *et al.*, 2014). These protocols attempt to imitate *in vivo* development of pancreatic β-cells under defined

cell culture conditions. However, pancreas development is a complex process orchestrated by multiple extrinsic signals in addition to the extensive network of transcription factors (TFs) of which the plethora is still being explored (Conrad *et al.*, 2014). Therefore, even the most successful differentiation protocols are so far characterised by limited efficiency, generating a low percentage of mature, functional β-cells (Hrvatin *et al.*, 2014). Additionally, the vast majority of knowledge applied to generate these protocols is based on rodent studies. Although it was observed that the spatiotemporal gene expression of key TFs regulating pancreas development seems to be similar between mouse and human (Jennings *et al.*, 2013), it remains undetermined how well knowledge of rodent pancreas development reflects events occurring during human pancreas development (Pan and Brissova, 2014).

The first step in *in vitro* differentiation of stem cells into pancreatic endocrine cells usually involves induction of definitive endoderm. This was first effectively achieved by activating TGFβ and Wnt signalling with Activin A and Wnt3a, respectively (D'Amour *et al.*, 2005, 2006). Cells at this stage can be characterised by expression of TFs such as SOX17 and FOXA2 (D'Amour *et al.*, 2005). Definitive endoderm cells can then be induced towards pancreatic endoderm with a combination of factors such as retinoic acid and FGF7. The key TF expressed by cells at this stage of the differentiation process is PDX1 (Wilson *et al.*, 2003; Pagliuca *et al.*, 2014; Rezania *et al.*, 2014). Following on from this, differentiation protocols aim to mimic *in vivo* determination of bipotent progenitors that give rise to the population of ductal and endocrine progenitor cells. This is accomplished with a cocktail of factors such as KGF, retinoic acid, sonic hedgehog pathway inhibitor, BMP signalling inhibitor and PKC activator (Rezania *et al.*, 2014). The key TFs marking bipotent stage cells are SOX9, PDX1 and NKX6.1 (Jennings *et al.*, 2015). The final stage of stem cell differentiation into endocrine progenitors involves induction of NGN3 and NEUROD1 positive cells (Lyttle *et al.*, 2008). This is achieved with a similar cocktail of factors as above, with the

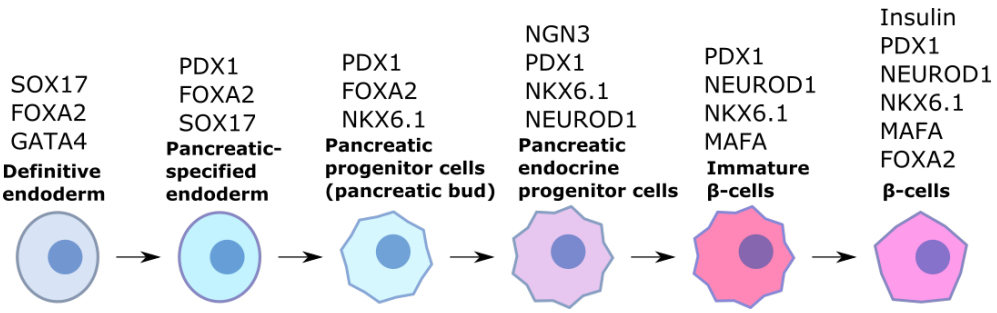

Figure 5.1: Key TFs controlling human pancreas development, which has been used in this project to identify cells at specific developmental stages (Jennings *et al.*, 2015).

addition of TGFβ receptor inhibitor, as well as factors supporting cell survival and maturation of β-cells. Additionally, to establish basal-apical cell polarity, 2D cell culture conditions can be changed to the air-liquid interface (Fig.5.1) (Rezania *et al.*, 2014).

iPSCs utilised in the following experiments were established from the pancreatic mesenchymal stem cell line- CH-pMSC through retrovirus-mediated gene transduction, specifically using Sendai reprogramming virus (SeV) and this was conducted by Dr Sophie Kellaway (Kellaway, 2016). Cells were transduced with Klf4, Oct4, Sox2 and c-Myc. Successful reprogramming was then initially confirmed by staining with anti-TRA 1-81 antibody for initial colony selection. Clearance of the SeV was verified with qRT-PCR. Finally, the pluripotent character of generated cells was confirmed by staining with anti- OCT4, NANOG and SOX2, flow cytometer for SSEAA, TRA 1-81 and TRA 1-60, and three germ layer random differentiation (Kellaway, 2016).

It was hypothesised that the pancreatic origin of the mesenchymal stem cells used for iPSCs generation could possibly enhance pancreatic endocrine differentiation. The aim of experiments presented here was to:

- differentiate iPS cells into pancreatic and endocrine progenitor cells (this was achieved with the use of protocol developed by Rezania et al. (2014);
- confirm progenitor characteristic of these cells by testing for the expression of stage specific TFs;

Pancreatic and endocrine progenitor cells generated in this way were later used for transcriptomics and proteomics analysis (Chapter 6).

5.2 iPS Cells Generated from Pancreatic Mesenchymal Stem Cells

Prior to differentiation iPS cells were tested for pluripotency markers. A panel of five commonly used markers of pluripotent stem cells was utilised. The expression of stem cell associated TFs involved in the maintenance of pluripotency, OCT4, NANOG and SOX2, and two cell surface antigens, SSEA4 and TRA 1-60 was analysed by immunocytochemistry (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Nakagawa *et al.*, 2007; Zhao *et al.*, 2012). Cells tested positive for all three pluripotent cell specific TFs and the staining also showed their nuclear localisation, as it would be expected for the active transcription regulators. Moreover, cells also showed expression of the cell surface antigens specific for pluripotent cells; SSEA4 (a glycolipid carbohydrate) and TRA 1-60 (a high molecular weight glycoprotein antigens). Stable expression of these markers was tested at different passage numbers between p38 to p50, and expression of all five markers was observed for all tested passage numbers. A representative outcome of this test is shown in Fig.5.2. This indicates that iPS cells generated from pCH-MSC line had pluripotent identity and that it was sustained up to passage 50; the highest passage used for transcriptomics and proteomic analysis (see Chapter 6).

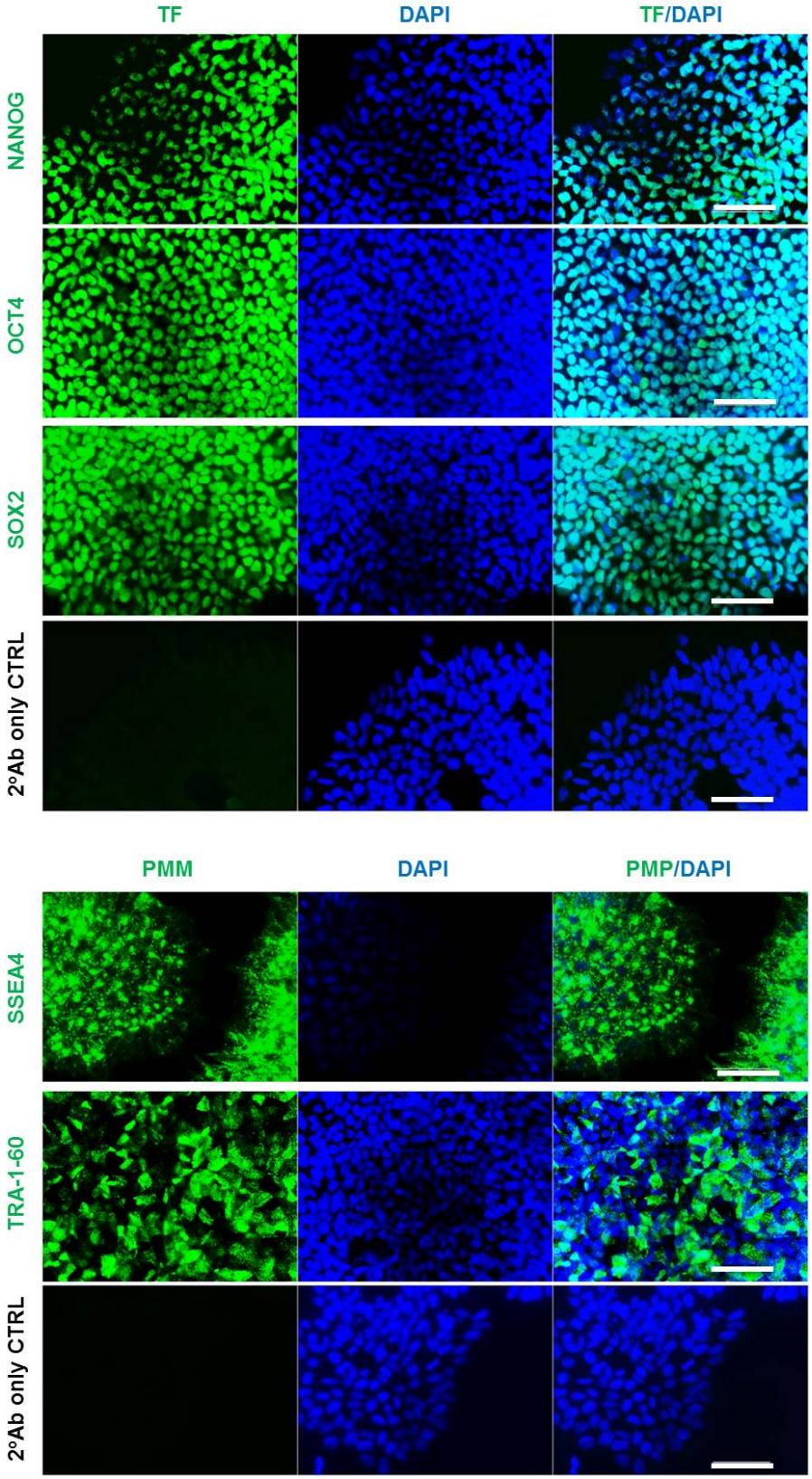

Figure 5.2: iPS cell generated from pancreatic MSC express markers for undifferentiated pluripotent human stem cells. Representative outcome from three independent experiments. iPS cells were tested at passage 38, 42 and 50; this panel shows data obtained for p50 (scale bar= 50 μm), PMM- plasma membrane marker, TFs- transcription factors.

5.3 Pancreatic Progenitors Generated From iPS Cells

iPS cells were differentiated using a protocol recently published by Rezania et al. (2014), with small modifications (see Methods section for more details, briefly iPS cells were exposed to ActivinA and CHIR 99021 for S1; KGF and ascorbic acid for S2; SANT-1, LDN193189, TPB, KGF and retinoic acid for S3-S4; SANT-1, LDN193189, TPB, ALK5iII and T3 for S5). Expression of key TFs characteristic for each stage of differentiation, such as OCT4 (a pluripotency regulator for S0), FOXA2 (one of TFs crucial for the development of definitive endoderm), PDX1 (key determinant of pancreatic endoderm for S4 and S5), NEUROD1 (a TF downstream of Ngn3 for S5) and NKX6.1 (a TF involved in the establishment of islet cell identity for S5) were analysed first by immunoblotting and confirmed with immunocytochemistry. Undifferentiated iPS cells (S0) were found to express OCT4, and a trend indicating decrease in OCT4 expression from S0 to S5 was observed, although this TF remained detectable at the final stage S5 (Fig.5.2 and Fig.5.3 panel C). Expression of definitive endoderm marker- FOXA2, was first observed at S2 of the protocol and persisted until S5. Pancreatic progenitor and endocrine progenitor cells, which respectively correspond to S4 and S5 of the protocol, were defined by expression of PDX1, first detected at S4, followed by expression of NKX6.1, and NEUROD1 detected only at S5 (Fig.5.3 panel C).

Expression of NGN3 could only be analysed at the mRNA level due to the lack of a specific antibody. Ngn3 is transiently expressed during pancreatic development and marks the endocrine progenitor population. An increase in expression of this TF was observed at S5 when compared to undifferentiated iPS cells (Fig.5.3 panel B). Similarly, a rise in the expression of Pdx1 at the mRNA level was also observed in S5 cells. Data obtained by qRT-PCR for the expression of Pdx1 correlates with the observed induction of expression of this transcription factor at the protein level (Fig.5.3 panel B and C).

In summary, successful induction of iPS cells towards both: pancreatic progenitors and endocrine progenitors was obtained with this directed differentiation. The outcome of this induction was confirmed by Western blotting for key markers (PDX1, NKX6.1 and NEUROD1) at the end of S4 and S5, and by qRT-PCR for *PDX1* and *NGN3* at the end of S5.

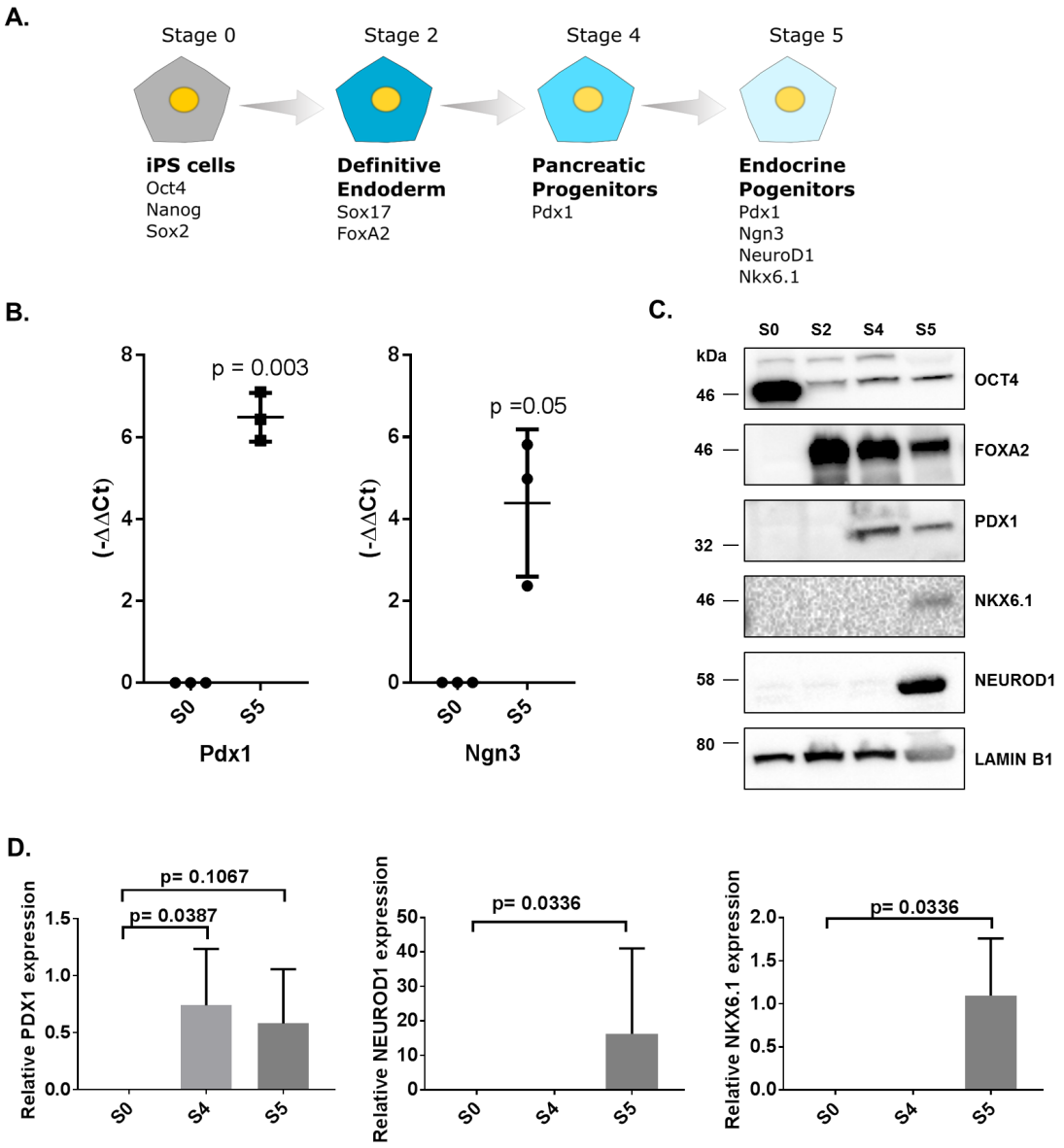

Figure 5.3: The differentiation of iPS cells towards pancreatic progenitor cells. **Panel A**: an overview of key differentiation stages. Starting from stage 0: undifferentiated iPS cells were characterised by OCT4/NANOG/SOX2 expression, stage two: definitive endoderm stage (SOX17 and FOXA2 positive cells), stage 4: pancreatic progenitor stage (PDX1-positive cells) and stage 5: endocrine progenitors (PDX1/NGN3/NKX6.1/NEUROD1-positive cells). **Panel B**: endocrine progenitor cells (S5) were tested for expression of key TFs: *PDX1* and *NGN3* by qRT-PCR. An increase in *PDX1* and *NGN3* expression is presented as a $\log_2$(fold change) comparing to expression at S0 (mean $\pm$ SD, n= 3: passage 34, 50 and 54); data were analysed with one-sample t-test. **Panel C**: expression of TFs specific to each differentiation stage was analysed at the protein level by immunoblotting (n= 3, passage 34, 39, and 50). **Panel D**: Quantification of PDX1, NEUROD1 and NKX6.1 expression (data were analysed with KruskalWallis test followed by Dunn's multiple comparisons test).

5.4 Differentiation Protocol Efficiency

Following generation of pancreatic progenitors, the efficiency of the utilised differentiation protocol was investigated. Cells were tested with immunocytochemistry at stage two, four and five for expression of TFs indicative of each stage. This allowed for percentage analysis of successfully induced cells and verification of nuclear localisation of crucial transcription inducers.

Differentiation of iPS cells into definitive endoderm was achieved by exposing cells to Activin A and an inhibitor of GSK-3 (CHIR 99021) at S1 followed by treatment with KGF and ascorbic acid (S2) (as it has been reported previously by Pagliuca et al. (2014) and Rezania et al. (2014)). iPS cells differentiated into definitive endoderm were analysed for expression of FOXA2 and SOX17. Positive staining was observed for both TFs, with efficiency of 91.7 % ± 4.9 of cell stained for FOXA2 and 90.7 % ± 1.3 stained for SOX17 (mean ± SD). A nuclear localisation was observed for both TFs (Fig.5.4).

In order to generate pancreatic progenitors S2 cells were treated with a cocktail of the sonic hedgehog signalling inhibitor (SANT-1), BMP signalling inhibitor (LDN193189), PKC activator (TPB), KGF and retinoic acid for S3 and S4. At the end of S4 cells were tested for the expression of PDX1. Cells expressing this TF comprised 81.8 %± 2.7 of total cell population, additionally a nuclear localisation of PDX1 was observed (Fig.5.5).

A similar cocktail of SANT-1, LDN193189 and TPB, but supplemented with TGF-β receptor antagonist (ALK5iII) and thyroid hormone-T3 (3,3,5-Triiodo-L-thyronine sodium salt) was utilized for promoting activation of endocrine program in S4 cells. Cells at the end of S5 were then tested for the expression of PDX1, NKX6.1 and NEUROD1. Nearly 82 % of these cells tested positive for expression of NeuroD1 (81.7 %± 2.6). PDX1 expressing cells constituted more than a half of cell population at this stage (51.4 %± 18.2) and only 6.5 % of cells were positive for NKX6.1 (6.5 %± 1).

In summary, the expression of key, stage-specific TFs was verified with immunocytochemistry, confirming nuclear localisation of tested proteins. The efficiency of this directed protocol decreased with each stage of differentiation. However, an acceptable outcome was achieved with over 50 % of the cell population expressing key TFs at each stage. The only exception was observed for S5 NKX6.1-positive cells, that constituted less than 10 % of cell population. This differentiation protocol allowed for generation a population of pancreatic progenitor and pancreatic endocrine progenitor cells that could be analysed using transcriptomics and proteomics approach. In this way cell surface proteins differentially expressed between stem and progenitor cells could be elucidated. Such cell membrane proteins could be used as a putative marker for pancreatic progenitor cells.

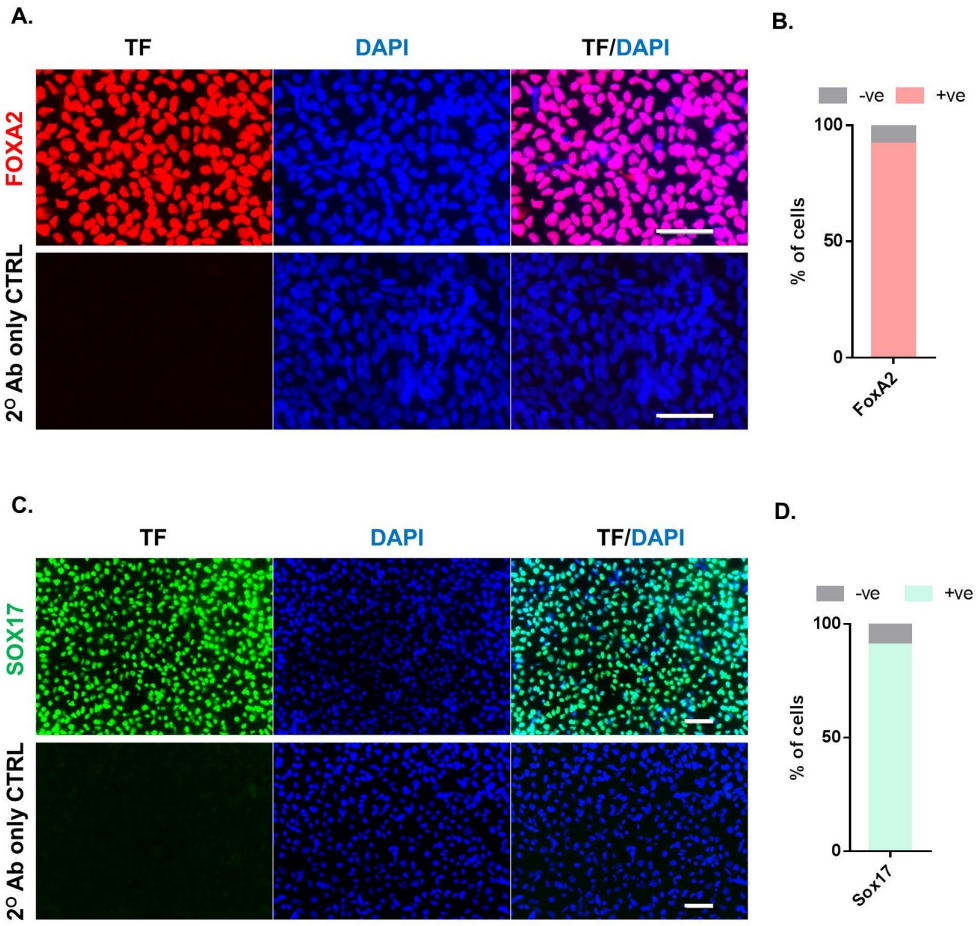

Figure 5.4: iPS cells differentiated into definitive endoderm ($SOX17^+$ and $FOXA2^+$- cells)- differentiation efficiency. iPS cells were tested at passage 30, 34 and 44 for FOXA2, and passage 34, 44 and 50 for SOX17. Positive staining for FOXA2 with nuclear localisation (**panel A**) was observed for 91.7 % ± 4.9 of cells (**panel B**). S2 cells also tested positive for SOX17 (**panel C**) with 90.7 % ± 1.3 of cells showing nuclear staining (**panel D**) (scale bar= 50 μm; mean ± SD, n= 3, n= 3).

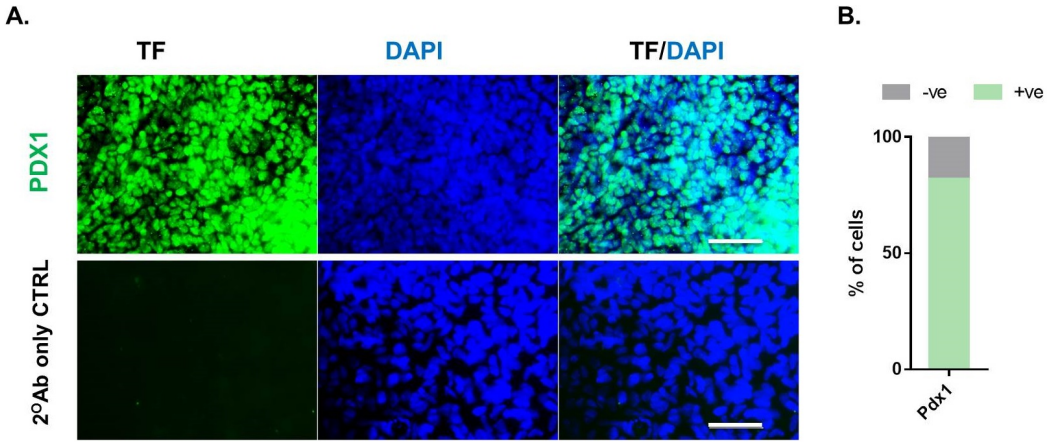

Figure 5.5: iPS cells differentiated into pancreatic progenitor cells ($PDX1^{+}$- cells)-differentiation efficiency. **Panel A**: expression and nuclear localisation of PDX1 were tested by immunocytochemistry. iPS cells were tested at passage 36, 38 and 44. **Panel B**: the efficiency of differentiation at this stage was 81.8 %± 2.7 (scale bar= 50 μm; mean ± SD, n= 3).

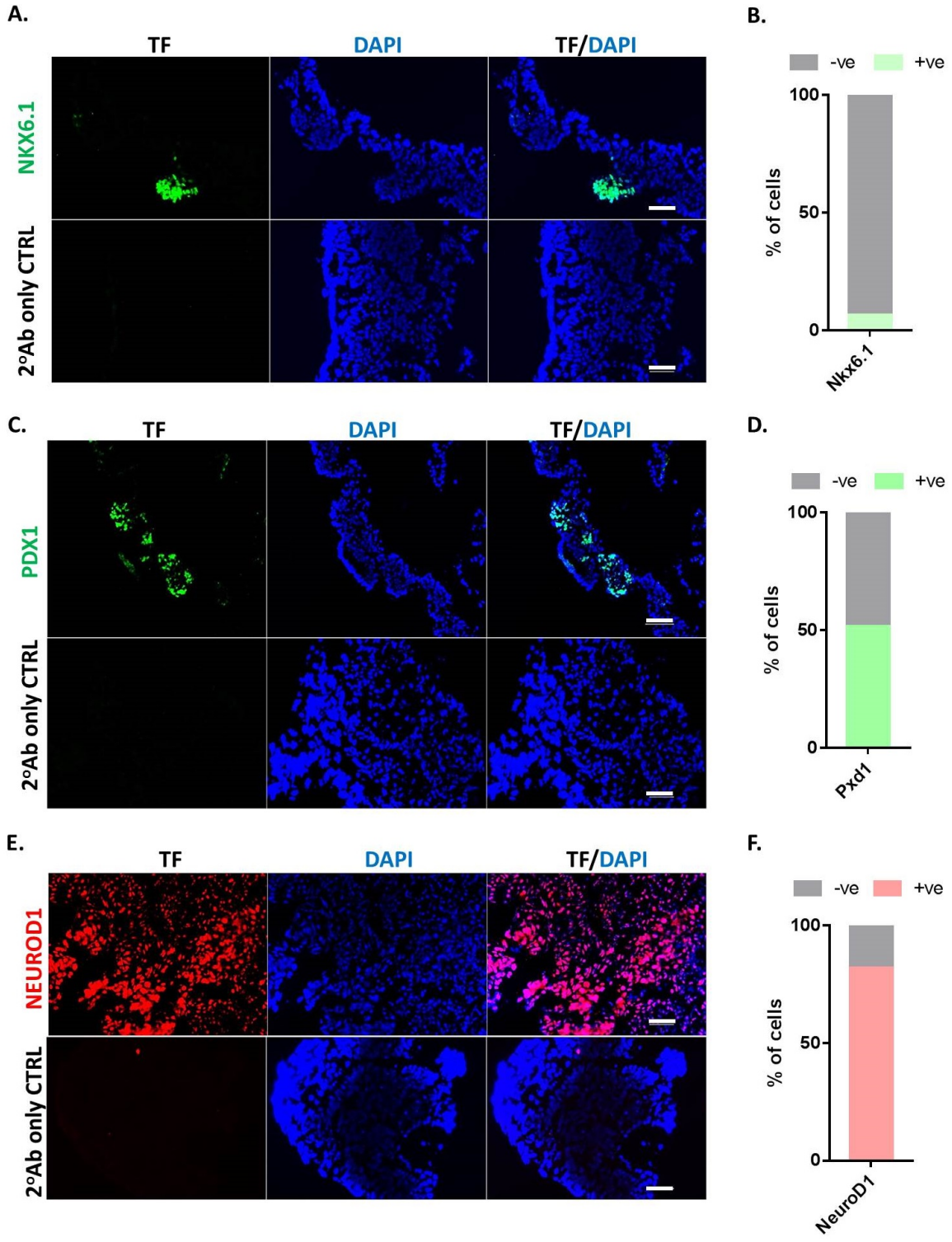

Figure 5.6: iPS cells differentiated into pancreatic endocrine progenitor cells (PDX1$^+$, NEUROD1$^+$ and NKX6.1$^+$- cells)- differentiation efficiency. S5 cells were induced towards pancreatic endocrine progenitor cell fate and analysed for expression of key TFs at the end of S5. Those cells were found to express NKX6.1 (6.5 %± 1 of cells) **(panel A and B)**, PDX1 (51.4 %± 18.2) (**panel C and D**) and NEUROD1 (81.7 %± 2.6) (**panel E and F**) (scale bar= 50 μm; mean ± SD, n= 3, passage 36, 38, and 44).

5.5 Expression of NESTIN and CK19 in S5 Cells

Results obtained with S5 cells immunostaining for NKX6.1 PDX1 and NEUROD1 suggested that cells at this stage of differentiation might be in fact a mixed population. To test this hypothesis and gain an inside into the nature of that cell, cryosections of S5 spheroids were stained for CK19 (a ductal cell marker) and NESTIN (a marker for neuronal stem cells and immature β-cells).

The majority of S5 cells stained positive for CK19 with a small fraction of NESTIN positive cells. The weak overlap between CK19 and NESTIN stain was also observed for several cells, mostly localised to the border between two cell populations (Fig.5.7).

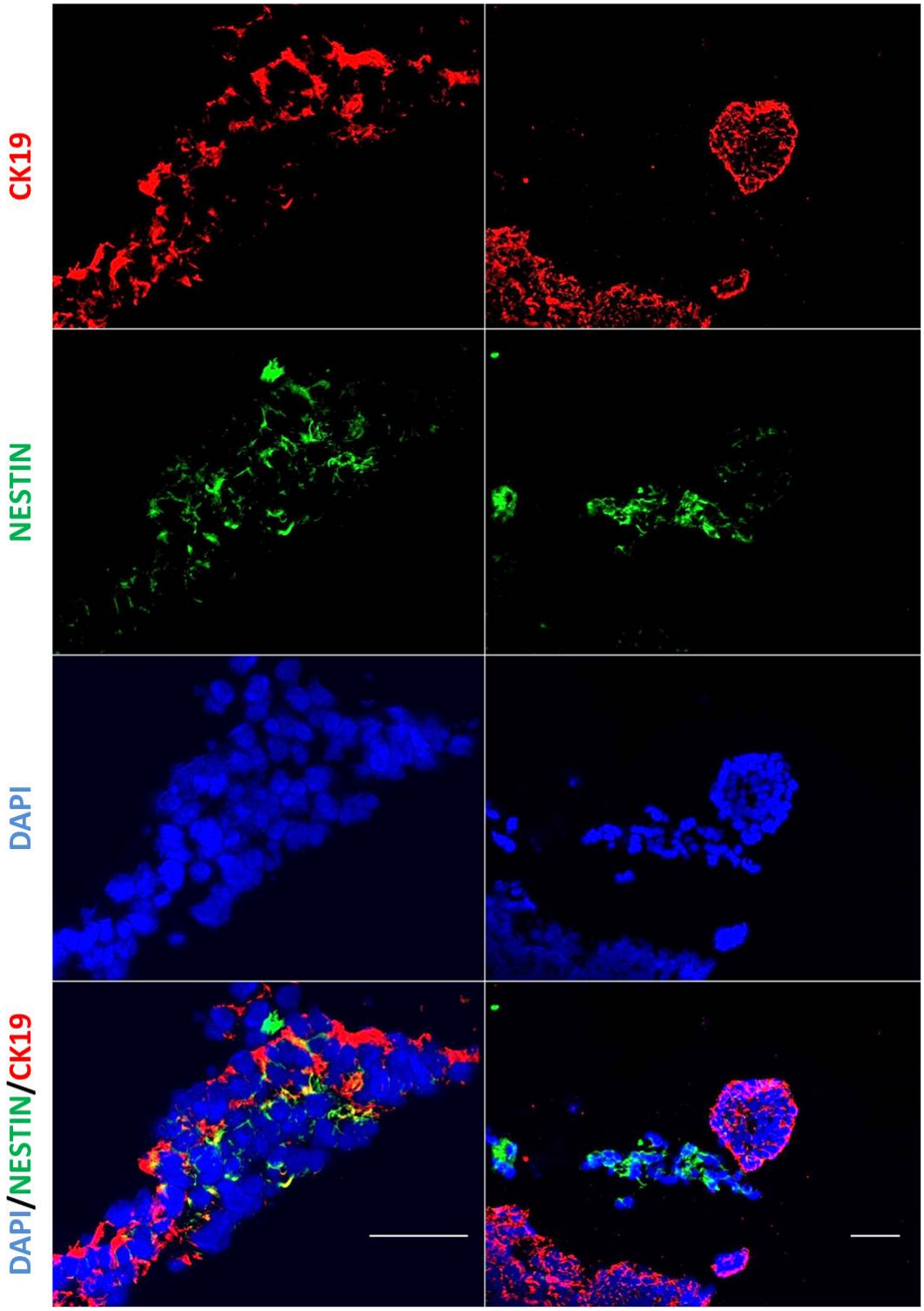

Figure 5.7: S5 cells express NESTIN and CK19. S5 cells can be divided into two distinctive populations: NESTIN^{+} and CK19^{+} (scale bar= 50 μ, n= 3, passage 36, 38, and 44).

5.6 Discussion

The ability of stem cells to become virtually any type of cell found in the human body makes them a perfect candidate for regenerative medicine. The current state of knowledge, however, does not permit their safe application for the regenerative treatment (Steinbeck and Studer, 2015). The major limiting factor preventing the therapeutic use of these cells is, ironically, their differentiation potential that if not restrained could lead to teratoma formation. Over past decades a variety of differentiation protocols aiming to mimic the development of pancreatic β-cells have been generated. This effort, combined with basic developmental biology research, lead to a significant increase in our understanding of molecular mechanisms involved in the formation of β-cells, islets and the entire pancreas. This knowledge can be used to improve currently available treatment for diseases such as T1D. Currently, ES and iPS cells are an irreplaceable model for further research into β-cells development and biology.

The aim of experiments presented here was to generate a population of pancreatic endocrine progenitor cells that could be further analysed by transcriptomic and proteomic methods in order to gain an insight into key molecular processes involved in pancreas development. The iPS cell line, utilised here, was established by reprogramming pancreatic mesenchymal stem cells by ectopic expression of TFs innate to pluripotent stem cells, such as Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006; Schmidt and Plath, 2012). iPS cells obtained in this process were stably expressing pluripotency markers, including TFs such as Oct4, Sox2, Nanog, and membrane proteins characteristic for this state: SSEA4 and TRA-1-60. Those iPS cells were subjected to a directed differentiation protocol developed by Rezania et al. (2014) and generated a population of pancreatic and endocrine progenitors. The transformation from undifferentiated, pluripotent stage 0 cells (S0) to S4 (pancreatic progenitors) and S5 (endocrine progenitors) were verified by qPCR, immunoblotting for key TFs, and immunohistochemistry.

Stage 5 cells were not expected to function as insulin secreting cells therefore functional assessment (e.g. measurement of glucose induced insulin secretion) has not been performed.

As expected, immunoblotting revealed that undifferentiated iPS cells (S0) were positive for stage-specific TF- OCT4. Expression of OCT4 showed a trend to diminish during differentiation, however, it was still detectable at the S5, indicating that within S5 cells some cells were still displaying pluripotency features. A subpopulation of undifferentiated, pluripotent cells co-existing within differentiated cells was previously reported and remains one of the major obstacle limiting the use of stem cells for transplantation-based therapy (Herberts *et al.*, 2011). This could be addressed by the antibody-based sorting of differentiated cells, however, stage specific markers localised within cell membrane are required to facilitate this approach. Additionally, research investigating de-differentiated β-cells in diabetic mice observed that expression of Ngn3 can be accompanied by Oct4 (Talchai *et al.*, 2012). De-differentiated β-cells did not contribute to the insulin production and displayed endocrine progenitor-like phenotype (Talchai *et al.*, 2012). Therefore it is a possibility that Oct4 expression is preserved till progenitor cell stage during pancreas organogenesis. This, however, would have to be further tested with in the developing pancreatic cells (e.g. with OCT4/NGN3 immunofluorescence dual staining).

FOXA2, as well as a marker for definitive endoderm cells, is also a master regulator involved in pancreatic endocrine cell development and in the regulation of insulin homeostasis. For example, FOXA2 was reported to stimulate Pdx1 expression by binding to its cis-regulatory element in pancreatic β-cells (Ben-Shushan *et al.*, 2001). It is also directly linked with the expression of genes coding K-ATP channel subunits SUR1 and KIR6.2 (Lantz *et al.*, 2004). Therefore, expression of FOXA2 was expected to remain detectable in S5 cells. Stage 2 cells showed expression of FOXA2 and this TF persisted until stage 5.

Expression of pancreatic progenitors specific TF- PDX1 was initially observed

in S4 cells and continued to S5. Final stage (S5) cells were also marked by expression of NEUROD1, which correlates with the outcome previously reported by (Rezania *et al.*, 2014). Moreover, expression of *NGN3*, a bHLH transcription factor that plays a key role in endocrine progenitor cells formation was evident at the end of S5, and this was also observed by (Rezania *et al.*, 2014). In contrast to results obtained by Rezania et al. (2014) where expression of NKX6.1 was first detected at S4, here expression of this TF was not observed till S5 cells. The discrepancy in the delay of NKX6.1 appearance could be attributed to multiple factors, such as the cell line used in these experiments or modifications of S1 and S2 media supplementation that were introduced here in order to enhance differentiation into definitive endodermal cells. Verifying the exact cause of this discrepancy would require further investigation into optimal conditions for the induction of S4 cells to Ngn3-positive endocrine progenitors. In the course of this project expression of Ngn3 could only be evaluated at the mRNA level, as no suitable antibody was available. Expression of both Pdx1 and Nng3 at the mRNA was therefore assessed by qRT-PCR using $\Delta\Delta$Ct method. S5 cells showed increased expression of *PDX1* and *NGN3* with changes in the range between 60-137 and 2-56-fold upregulation, respectively. Analysis of corrected ΔCt values with the t-test statistics has indicated difference in the expression levels between S0 and S5 cells with p-values 0.003 for *PDX1* and 0.05 for *NGN3*. However, low number of biological replicates did not allow assuring normal distribution of the ΔCt values. Therefore analysis with Wilcoxon signed-rank test was performed and generated p-values above the 0.05 cut-off. Nevertheless, NGN3 expression and activity was indirectly demonstrated by expression of NEUROD1- another bHLH TF induced by NGN3 during endocrine progenitor formation and detected in differentiated cells at the end of S5.

It has been proposed that iPS cells retain some epigenetic memory of their tissue of origin (e.g. by harbouring some of the DNA methylation signature specific to their tissue of origin) (Liang and Zhang, 2013). Therefore, differentia-

tion of iPS cells generated from a pancreatic origin might be more efficient when compared to differentiation of hES cells, in which epigenetic identity must be established from the very beginning. Yet epigenetic memory of iPS cells was observed to diminish gradually during cell culture (Liang and Zhang, 2013). Here, efficiency of differentiation protocol was estimated based on immunocytochemistry staining for crucial, stage-specific TFs. Cells at the end of S2 showed a high differentiation rate with a minimum of 90 % of cells testing positive for SOX17 and FOXA2. Within S4 cells over 80 % of cells tested positive for the pancreatic progenitors' marker- PDX1; with this rate decreasing to 51 % at S5. This yield was lower than the one reported by Rezania et al. (2014), where Pdx1 positive cells were consisting of 99.5 % of all cells at the end of S4 and 99 % at the end of S5. However, this to some degree might be due to the fact that data reported by Rezania et al. (2014) was obtained by flow cytometry, a more accurate approach. Percentage of S5 cells positive for NKX6.1 was also notably lower when compared to previously reported data, specifically 6.5 % for iPS cells, compared to over 60 % for hES cells reported by Rezania et al. (2014). However, expression of NEUROD1 for S5 progenitors generated from iPS cells was close to 82 %; compared to approximately 75 % observed for the hES cells utilised in the original protocol.

The efficiency of PDX1$^+$ cell generation was also lower when compared with protocol recently developed for hESCs by Russ et al. (2015) where expression of PDX1 was observed in $>$ 88 % of the total cell number at the pre-NGN3$^+$ stage. Similarly, expression of NEUROD1 was reported to reach 95 % of the C-peptide$^+$ β-like cells, which would correspond to S6-S7 of protocol used here (Russ *et al.*, 2015). However, the percentage of NEUROD1$^+$ cells was not reported for a stage corresponding to S5 in this study, therefore direct comparison between protocol used here to differentiate iPS cells and that developed by Russ et al. (2015) is not possible.

Unlike protocols aiming to generate functional β-cells, the goal of this research was to obtain a population of NGN3$^+$ cells that could be used for tran-

scriptome/proteome analysis in order to elucidate putative cell surface markers for endocrine progenitor cell isolation. Therefore, the differentiation protocol here was only conducted up to stage previously described as a starting point for endocrine cells maturation that is NGN3$^+$/NEUROD1$^+$/NKX6.1$^+$ cells (Gu *et al.*, 2002). Additionally, progenitor cells generated *in vitro* has been previously shown to differentiate into functional, mature β-cells once transplanted into diabetic mice (Bruin *et al.*, 2013; Rezania *et al.*, 2012). Therefore if progenitor cells can be efficiently generated *in vitro* and removal of undifferentiated cells can be achieved, these cells can serve as an additional pool of cells for transplantation-based treatment of T1D.

Interestingly, the expression pattern obtained with immunocytochemistry for NKX6.1 suggested that S5 cell might contain a subpopulation of cells with a distinct characteristic. NKX6.1$^+$ cells were clustered and localised to the edge of spheroids. Similar pattern was observed for cells stained with NESTIN, an intermediate filament protein that has been proposed to be a marker for neuronal stem cells (Cattaneo and McKay, 1990; Dahlstrand *et al.*, 1995), was linked with processes specific to differentiating cells including migration and mitosis (Li-Jing *et al.*, 2015; Yan *et al.*, 2016), and was observed to be expressed in developing pancreas (Hunziker and Stein, 2000; Street *et al.*, 2004). Unfortunately, due to this project time constraints more detailed analysis of NESTIN$^+$ cells and possible overlap between NESTIN and NKX6.1 expression in PP cells was not performed.

In summary, iPS cells generated from pancreatic mesenchymal stem cells were successfully differentiated into pancreatic and endocrine progenitors. Cells obtained at the end point were expressing key stage-specific TFs, such as FOXA2, PDX1, NKX6.1 and NEUROD1 at the protein level. Additionally, those TFs showed nuclear localisation pattern, suggesting that they are interacting with their downstream targets. However, a satisfactory outcome was achieved with the differentiation protocol, the main limiting factor constituted generation of a mixed cell population that is in approximately 20 % composed of cells of un-

known phenotype. This presented a restriction of this experimental approach which, however, can be justified by current lack of ultimately better and available research model.

Chapter 6

Molecular Changes during Endocrine Progenitor Cells Development

6.1 Introduction

As a model of pancreatic endocrine cell development, iPS cells generated from pancreatic mesenchymal stem cells (CHI-pMSC) were differentiated using a recently published protocol (Rezania *et al.*, 2014), yielding a population of cells expressing known markers of pancreatic progenitor cells (S4 cells) and islet endocrine progenitors (S5 cells). Those cells were subjected to transcriptome and proteome analysis with cDNA arrays and iTRAQ-mass spectrometry.

6.1.1 cDNA arrays

Gene expression microarrays allow analysis of the transcription profile of thousands of genes in one experiment. This technology is based on hybridization between a cell-derived cDNA mixture and a library of sequences attached to the surface of a chip (Hoheisel, 2006). Expression profiling with this technology allows for detection and relative quantification of mRNA molecules in the sample

of interest. Here, expression profiles were compared between undifferentiated iPS cells (S0), cells differentiated towards pancreatic progenitors (S4) and islet endocrine progenitor cells (S5) using Gene Chip Human Transcriptome Array 2.0 (Affymetrix). This type of array provides high resolution that covers over 40 000 protein coding genes. The main aim of this experiment was to identify differentially expressed genes between S0 and S5 cells, with the emphasis on genes encoding for cell membrane proteins. Data generated from transcriptome profiling were combined with data obtained from mass spectrometry-based analysis of matching control/differentiated cell populations. This allowed pinpointing several molecular events and pathways previously not associated closely with pancreatic islet cell development.

6.2 Transcriptomics versus Proteomics

Proteins are functional units encoded by the cell genome. Cell nature, functions, morphology and its close proximity environment (such as ECM) are directly linked to proteins the cell produces. Synthesis of those molecules is therefore strictly controlled at multiple levels (Alberts *et al.*, 2002).

Several mechanisms are involved in the control of gene expression and are implemented at the stage of mRNA as well as at the stage of protein production. This includes processes such as changes in chromatin structure, initiation of transcription, stability/ decay of mRNA, destabilisation by non-coding RNAs, regulation of translation as well as protein degradation (Belasco, 2010).

With the development of techniques such as microarrays, it has become possible to determine the expression level of thousands of genes in a single experiment (Brown and Botstein, 1999). However, the utility of direct translation of changes measured at the mRNA level into changes taking place at the level of a cell was put into question (Vogel and Marcotte, 2012). Several recent studies aiming at the direct comparison of mRNA and protein abundance revealed discrepancy between expression levels observed for those molecules (Griffin *et al.*, 2002; Schmidt *et al.*,

2007; Tian *et al.*, 2004; Lu *et al.*, 2009). Tian et al. (2004) correlated expression level of mRNA and proteins in hematopoietic cell lines undergoing myeloid differentiation, and reported that the expression of genes as measured at the mRNA level reflected only 40% of changes in the expression at the protein level. A higher correlation was observed when quantification of transcriptome-proteome obtained from *S. pombe* was performed. In this case, relative quantification was performed between all proteins expressed by *S. pombe* cells in the vegetative state and this was shown to correlate in 61 % with the corresponding transcriptome profile (r_s=0.61). Additionally, the functional pathway-protein analysis revealed that certain proteins display higher/lower correlation than observed at the level of a cell. The highest correlation was observed for kinases (80 %), proteins involved in cell cycle regulations (67 %) and amino acids biosynthesis (63 %), whereas the lowest correlation was noticed for transporters (21 %) and UPR pathway (12 %) (Schmidt *et al.*, 2007).

6.2.1 Mass Spectrometry

Mass spectrometry (MS) is a highly sensitive technique that allows for the analysis of complex samples to the level where its singular components can be identified and quantified (Glish and Vachet, 2003). Here, this method was applied for the characterisation of the cell proteome in order to determine differentially expressed proteins that could be utilised as markers of pancreatic progenitor cells. The key question of this project addressed changes at the protein level of expression between stem cell and pancreatic progenitor cells, therefore relative quantification mass spectrometry was utilised. Specifically, an iTRAQ (isobaric tag for relative and absolute quantitation) tandem mass spectrometry based approach was applied. The general outline of the experimental approach applied here included: protein enzymatic digestion, peptide labelling, liquid chromatography fractionation and tandem mass spectrometry. Proteins, prior to MS, were subjected to the enzymatic digestion which generated a mixture of peptides that could be ionised

and identified with MS based on their mass to charge ratio (Glish and Vachet, 2003). This approach is referred to as "bottom-up" and allows for the detection of a large number of proteins due to its high resolution and comprehensive coverage (Wu *et al.*, 2009). This was also coupled with the use of tandem mass spectrometry (MS/MS) mode, which is favoured for the analysis of complex samples such as total cell proteome and applies the use of two consequential stages of MS (Aebersold and Mann, 2003). During the initial stage of MS/MS ions are formed in the ion source and separated by mass-to- charge ratio. Following this, ions of the specific mass-to-charge ratio (precursor ions) are selected and undergo dissociation in the collision cell. Formed in this way product ions are separated and detected in the second round of MS/MS (El-Aneed *et al.*, 2009). Prior to tandem MS, samples were also fractionated by liquid chromatography (LC) in order to reduce their complexity.

iTRAQ (isobaric tag for relative and absolute quantitation) system allows for reliable detection of changes in protein abundance between comparative samples. This system also permits analysis of up to eight samples simultaneously minimising variance accumulated due to separate LC-MS runs (Ross *et al.*, 2004; Unwin *et al.*, 2010). As shown in Fig. 6.1, prior to fractionation peptides from each sample are labelled with isobaric tags. Each tag is composed of three groups: reactive, balance and reporter group. The reactive group of the tag binds to free amino groups of peptides (N-terminal and on lysine side chain) allowing for the labelling of virtually all peptides in the sample. The balance group compensates for the mass variance between reporter groups, whereas the reporter group provides the quantitative information. All tags are composed of the same elements, however, their reporter groups differ in the proportion of heavy and light atoms. Different masses of reporter groups are balanced out within the tag by the ratio of heavy/light atoms in the corresponding balance group, which reduces the effect of labelling on chromatographic separation of samples (Ross *et al.*, 2004). During the second round of MS, when product ions are generated, reporter groups are

detached and appear as eight distinct masses (113-119 and 121 kDa). This allows quantifying peptides/ proteins of interest from multiple samples in a single experiment (Fig. 6.1).

In this chapter data obtained from transcriptomic (cDNA arrays) and proteomic (iTRAQ-MS/MS) screening were analysed using IPA (Ingenuity Pathway Analysis, Qiagene), Panther ontology software (http://pantherdb.org) and String: functional protein association networks (http://string-db.org; version 10.0). Data analysis using this software revealed novel pathways relating to the development of pancreatic endocrine cells and allowed to identify several cell membrane proteins that can served as markers for PP and PEP cells.

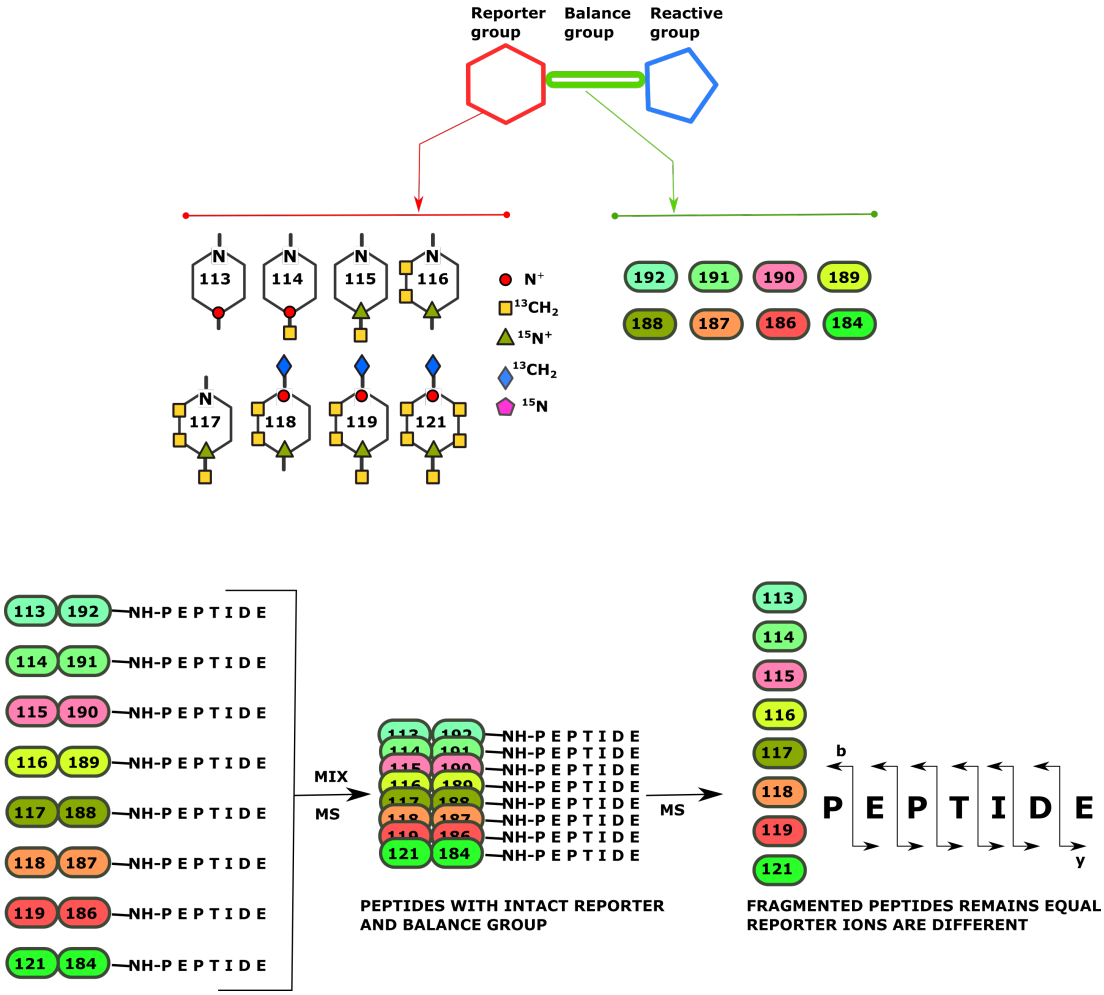

Figure 6.1: Isobaric tags for relative and absolute quantification (iTRAQ)-MS/MS. iTRAQ based tandem mass spectrometry involves the use of chemical tags. Those tags are chemically identical and are composed of the same mixture of heavy and light atoms but at different ratios. Each tag is composed of a reporter, balance and reactive group. The reactive group of the tag binds to the N-terminus of peptides within the sample. The combined masses of the balance and reporter groups are identical for each tag, however, their individual masses differ. During, MS/MS analysis tags are fragmented and the reporter groups are detached from their balance groups, this allows for simultaneous quantification of peptides from eight independent samples; adapted from (Ross *et al.*, 2004; Unwin *et al.*, 2010)).

6.3 Results

6.3.1 TFs Factors Regulating Endocrine Progenitors Differentiation

Prior to the microarray and iTRAQ-MS/MS experiments, the increased expression of Pdx1 and Ngn3 in S5 cells was verified with the qPCR based approach (Chapter 5). Similarly, those TFs were later confirmed to be upregulated with cDNA array analysis. Using this method expression of Pdx1 was 4.22-fold upregulated for S4 and 2.86-fold upregulated for S5, and Ngn3 expression 2.63- fold upregulated for S5, $p \leq 0.05$ (Fig.6.2, panel A).

Additionally, expression of genes marking stem cells, such as *OCT4*, *SOX2* and *NANOG* was significantly decreased in S4 and S5 cells. This also correlates with data presented in Chapter 5, where expression of *OCT4* in S4 and S5 cells was shown to be reduced at the protein level.

cDNA arrays also revealed a significant increase in the expression of several other transcription factors previously associated and utilised as markers of pancreatic endocrine cells development. This included key regulators involved in as early stages as primitive gut tube and prospective pancreatic endoderm formation, and as advance as pancreatic progenitors and endocrine progenitor cell establishment (Fig.6.2, panel A).

The highest fold-increase among key TFs involved in the pro-endocrine cell development was observed for *RFX6*; 35.8-fold for S4 and 49.1 fold-change for S5 ($p \leq 0.05$). Upregulated expression was also detected for several TFs with no previous strong association to pancreas development. This included two *RFX* winged-helix transcription factors: *RFX3*, *RFX2*; two members of the Sox family of transcription factors: *SOX5* and *SOX6* and *c-MAF* (Fig.6.2, panel B).

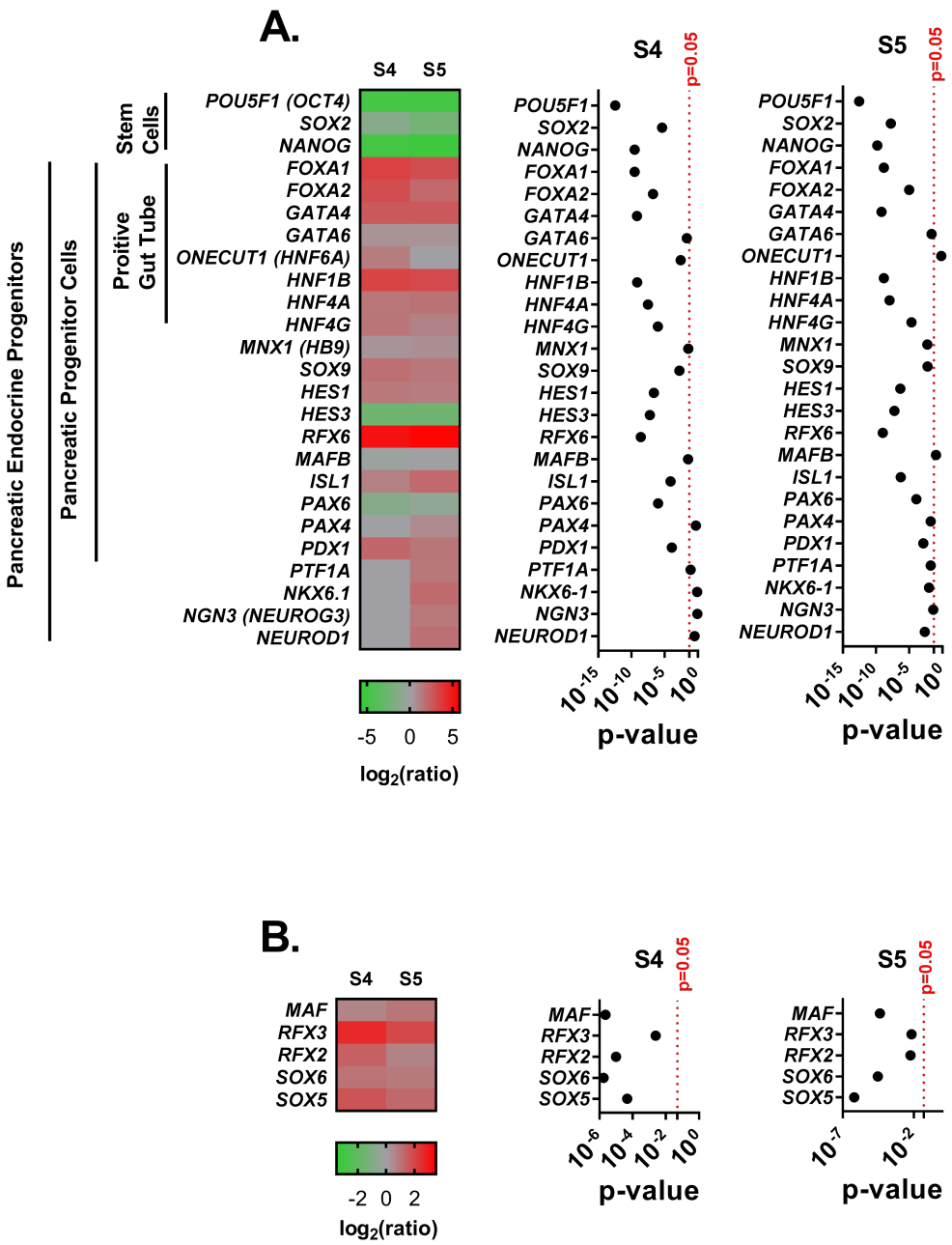

Figure 6.2: Expression factors involved in the development of pancreatic endocrine precursors development. Panel **A**: heat map illustrating the expression of key transcription factors regulating development of pancreatic endocrine progenitors at the mRNA level. Panel **B**: expression of transcription factors potentially involved in regulation of pancreas development- mRNA level. (Heat maps generated using fold change values in relation to S0 expression on a logarithmic scale; only differential expression values with $p \leq 0.05$ were presented in the heat map.)

6.3.2 Current Markers for Pancreatic Progenitor Cells

The expression profile of several membrane proteins previously reported to be putative markers for pancreatic progenitor cells was altered at the mRNA level at S4 and S5. From the sixteen previously reported genes encoding prospective progenitor cell surface markers (discussed in Chapter 1), this study found 13 significantly altered ($p \leq 0.05$). Out of the thirteen, seven showed moderate upregulation at S5 in comparison to stage 0. This included genes encoding markers previously associated with $PDX1^+$ cells, such as TROP2 (TACSTD2) and PROM1, here observed to be upregulated at S4 ($PDX1^+$ cells) and also S5 ($PDX1^+/NGN3^+$ cells). CD142 (encoded by F3 gene) a marker for $PDX1^+$ cells here was upregulated only in S5 cells. Genes encoding for $PDX1^+/NGN3^+$ cell markers such as DNER and c-KIT were here indeed observed to be upregulated at the mRNA level in S5 cells only. However, other putative cell surface markers associated with $PDX1^+$/ $NGN3^+$, such as GPR50, TMEM27, SEZ6L2, DDR1, CD318 and CD200 were downregulated in comparison to S0. With GPR50 showing the greatest decrease in the expression at the mRNA level: -20.32 and -37-fold, for S4 and S5 respectively. Additionally, a cell surface proteins proposed to be a putative marker for mature β-cells, TSPAN7 was here upregulated 2-fold and SLC30A8 (1.2-fold change) at S5 compared to S0(Fig. 6.3).

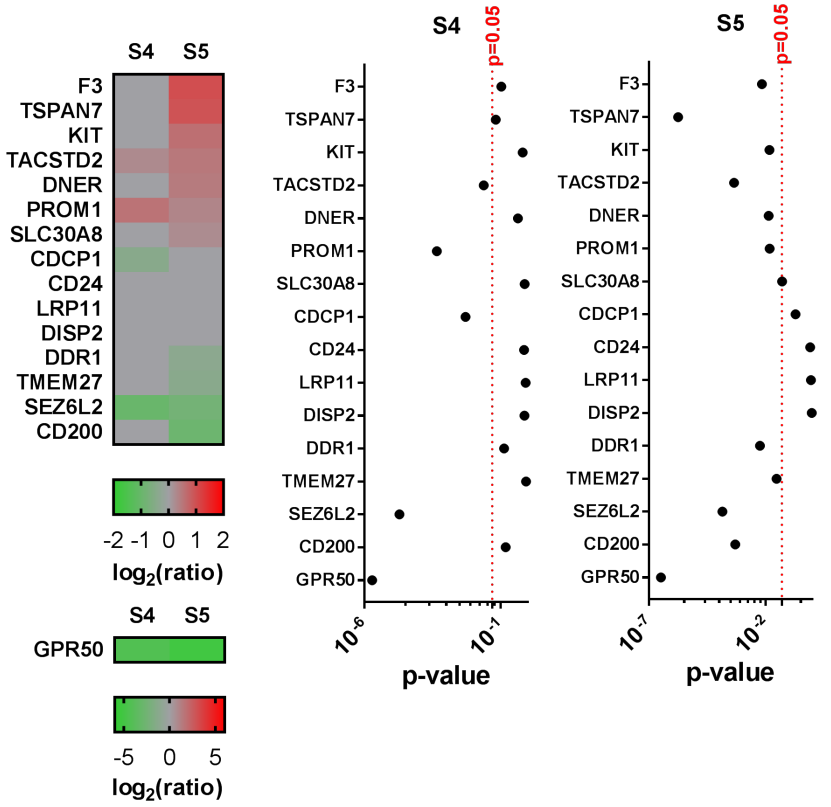

Figure 6.3: Current markers for pancreatic progenitor cells. Expression of several membrane proteins previously reported to mark pancreatic progenitor cells was also detected at the mRNA level for S4 and S5 cells. (Heat maps generated using fold change values in relation to S0 expression on a logarithmic scale; only differential expression values with $p \leq 0.05$ were presented in the heat map.)

6.3.3 Correlation between cDNA Arrays and iTRAQ-MS/MS Data

Genes differentially expressed at the mRNA level consisted of 30.0 % of those encoding cytoplasmic proteins, 17.0 % encoding nuclear proteins, 13.5 % encoding extracellular proteins and 32.5 % encoded for proteins linked to other cellular compartments. Out of the differentially expressed proteins identified by mass spectrometry more than half (51.5 %) constituted cytoplasmic fraction, 30.7 % nuclear fraction, 9.0 % plasma membrane proteins, 3.9 % extracellular proteins and 4.9 % proteins localised to other cellular compartments (Fig. 6.4) .

A total of 1616 proteins were identified and quantified with iTRAQ-MS/MS for S5 cells. From these, expression of 1591 proteins was also quantified with cDNA arrays. A moderate positive correlation (Spearman's Rank r_s= 0.5; p < 0.0001) between these two sets of data was observed (Fig. 6.4).

Additionally, multiple genes differentially expressed between S5 and S0 cells were also identified with cDNA arrays. However, the expression level of these genes was not obtained with mass spectrometry. This is potentially due to limitations of currently available mass spectrometry techniques (Angel *et al.*, 2012).

Disproportional enrichment of proteins from different cell compartments between mass spectrometry and cDNA array data sets indicated the necessity to utilise specific background for statistical overrepresentation test when possible. Therefore, to reduce the possibility of a false outcome, expression data were analysed with Ingenuity Pathway Analysis (IPA) software against a specific background. The analysis was performed against Affymetrix Gene Chip Human Transcriptome Array (HTA) for cDNA arrays and against all identified proteins for iTRAQ-MS/MS data. Cut-offs were set for p-value $\leq$ 0.05 and log fold change cut-off from (-1) to 1 for cDNA arrays, and p-value cut-off $\leq$ 0.05 and log fold change cut-off from (-0.5) to 0.5 for iTRAQ-MS/MS. Canonical pathway analysis identified 127 and 53 significantly (p $\leq$ 0.05) altered pathways for cDNA

arrays and iTRAQ- MS/MS data, respectively. From these, 13 pathways were altered in both sets of data (Fig. 6.5 and 6.6). These pathways represented cellular processes such as metabolism (FXR/RXR Activation, LXR/RXR Activation), modification of cell cytoskeleton (ILK Signalling) or re-arrangement of ECM (Atherosclerosis Signalling, Hepatic Fibrosis / Hepatic Stellate Cell Activation). From the list of overlapping, altered pathways, those linked to cell cytoskeleton alteration and ECM components remodelling were selected for further data mining analysis (Fig. 6.5 and 6.6).

Similar results were obtained when expression data were analysed with PANTHER (Protein Annotation Through Evolutionary Relationship) classification system (http:// www.pantherdb.org/). For this analysis input lists were generated by applying p-value cut-off ≤ 0.05 and log fold change cut-off from (-1) to 1 for cDNA arrays, and p-value cut-off ≤ 0.05 and log fold change cut-off from (-0.5) to 0.5 for iTRAQ-MS/MS. The overrepresentation test was performed against REACTOME database. The analysis revealed 28 pathways being significantly ($p \leq 0.05$) overrepresented for cDNA arrays data set and 66 pathways significantly ($p \leq 0.05$) overrepresented for MS data set (Appendix A). Five pathways out of both data sets overlapped, these included processes/pathways such as Developmental Biology, Metabolism, Extracellular Matrix Organisation and Axon Guidance (Appendix A). In addition to ECM re-arrangement, also identified by IPA based analysis, axon guidance was selected for further data mining analysis.

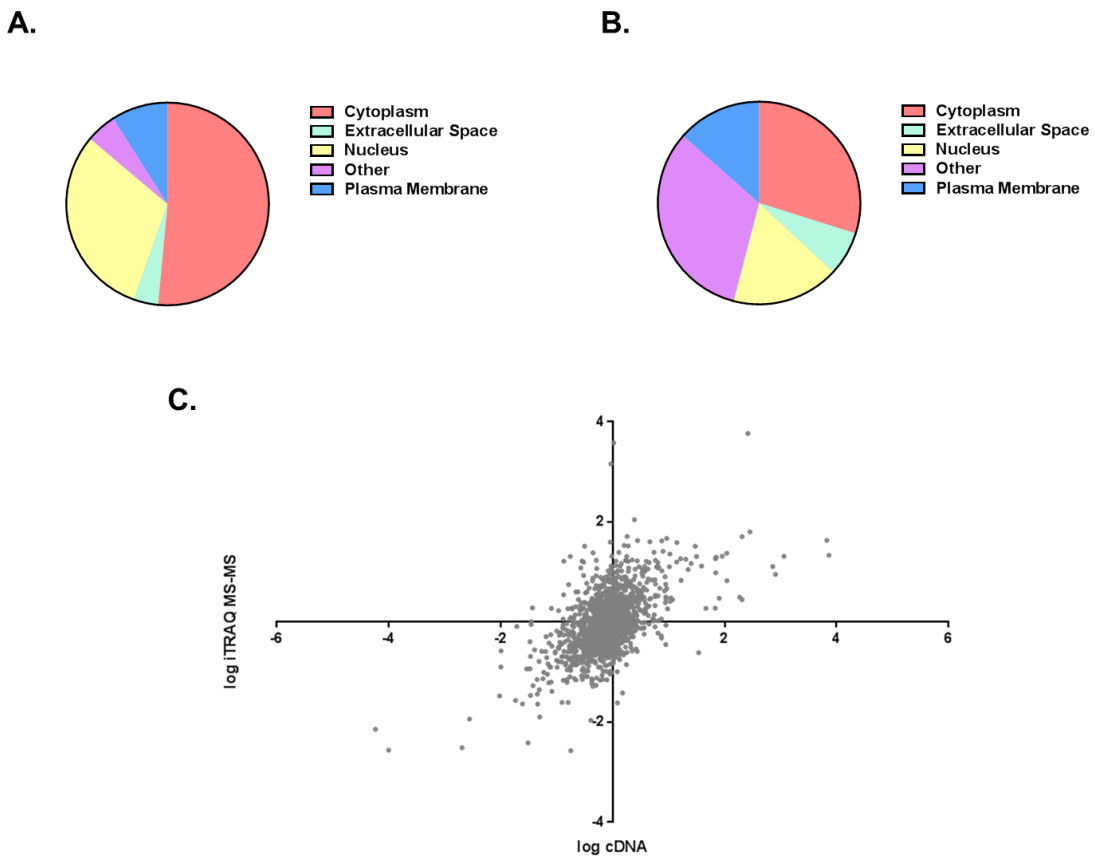

Figure 6.4: Correlation between cDNA array data and MS/MS data set. Panel **A:**percentage of proteins from various cell compartments identified by iTRAQ-MS/MS. Panel **B:** percentage of proteins from various cell compartments identified by cDNA arrays. Panel **C:** Scattered plot of mRNA (cDNA) versus related protein expression ratio. Genes/proteins that were identified and quantified at the protein and mRNA level showed moderate positive correlation with r_s= 0.5.

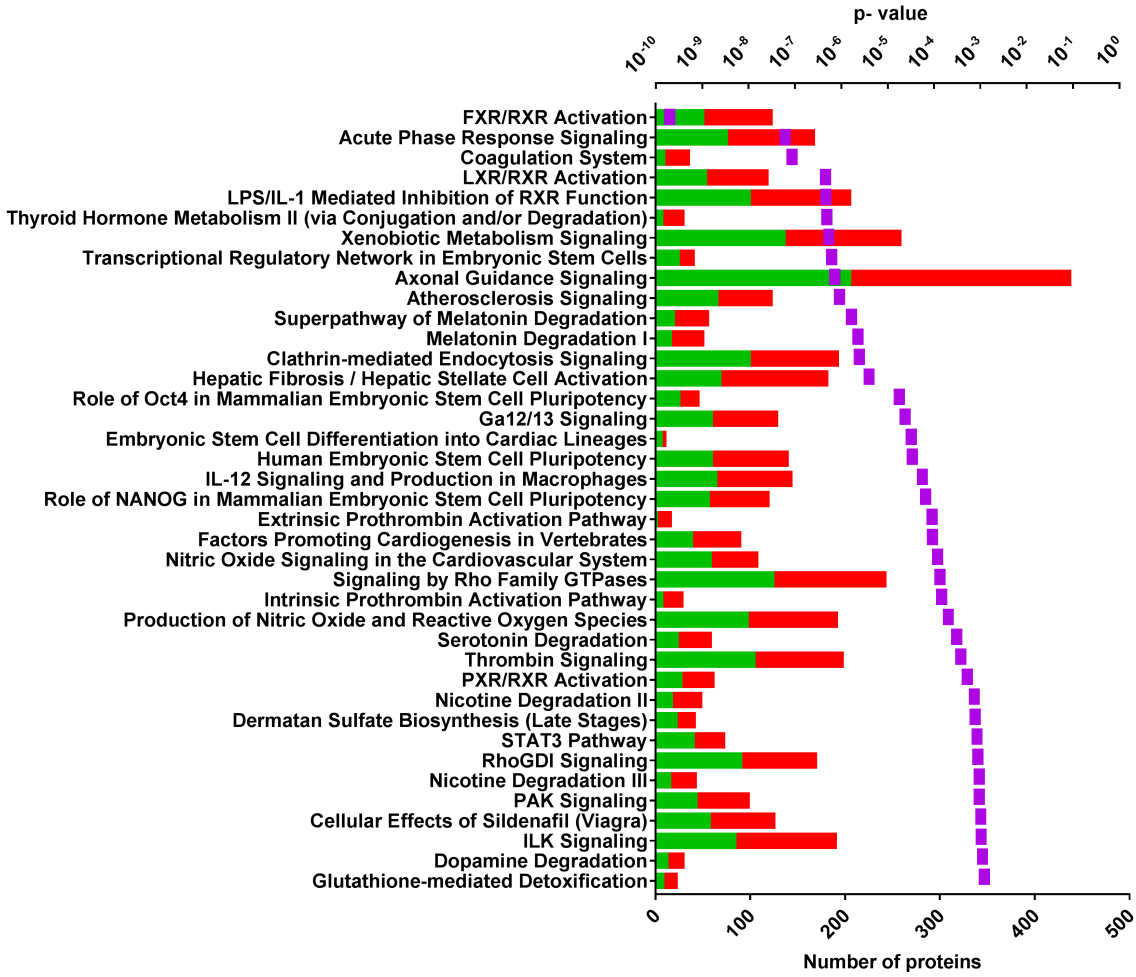

Figure 6.5: Pathways altered at the mRNA (cDNA) level between undifferentiated iPS cells (S0) and pancreatic endocrine progenitors (S5). Data analysed with IPA. Only pathways with $p \leq 0.001$ are presented here; the full list of pathways with $p \leq 0.05$ can be found in Appendix B. Green-downregulated, red-upregulated, purple- p-value.

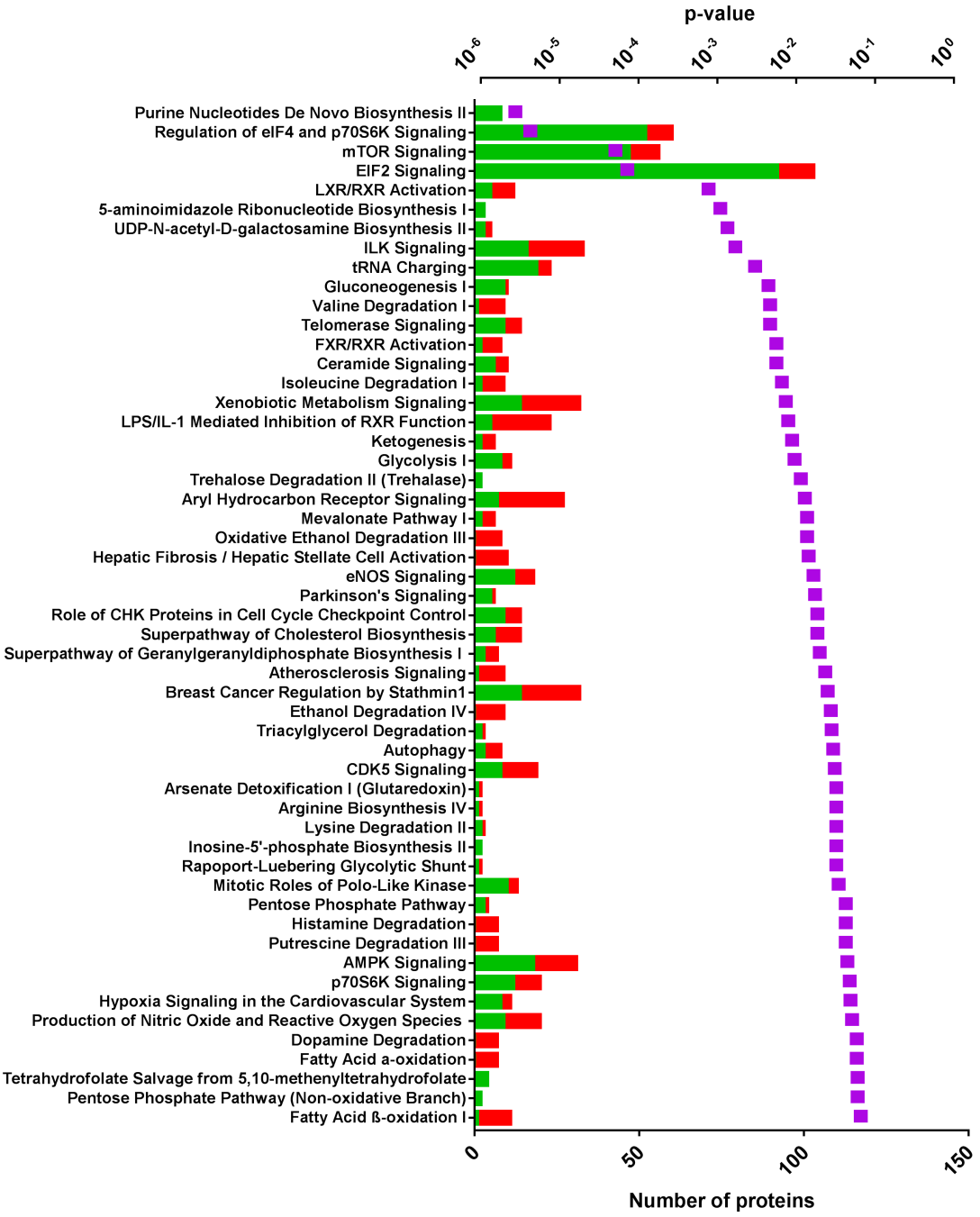

Figure 6.6: Pathway altered at the protein level between undifferentiated iPS cells (S0) and pancreatic endocrine progenitors (S5). Data analysed with IPA. Only pathways with $p \leq 0.05$ are presented here; green-downregulated, red- up-regulated, purple- p-value.

6.3.4 Membrane Proteins

Expression data at the mRNA and protein level were obtained for 1591 genes. From these, 111 genes were encoding plasma membrane/ membrane-associated proteins. Out of those 111 genes, 55 were altered at the mRNA level and 49 were altered at the protein level in S5 cells compared to S0 ($p \leq 0.05$). Moreover, 29 genes had altered expression profile at both the mRNA and protein level and showed strong positive correlation with $r_s = 0.7$ for S5 and $r_s = 0.8$ for S4 (Fig.6.7, panel A and B).

From membrane proteins with altered expression, several also had transmembrane/ extracellular domain which advocated their selection for potential pancreatic progenitor cell biomarkers, this included: ALCAM, ATP1B1, ATP2B1, BCAM and CADM1 (Fig.6.7 and Table6.1).

Table 6.1: Cell membrane proteins with upregulated expression in S4 and S5 cells comparing to S0. Expression presented as a fold change if significant; $p \leq 0.05$.

	S4		S5	
Gene	mRNA	protein	mRNA	protein
ALCAM	2.6	3.4	2.5	2.4
ATP1B1	1.2	$p > 0.05$	1.7	2.3
ATP2B1	2.1	1.9	2.6	2.4
BCAM	2.7	1.7	1.9	1.4
CADM1	2.6	2.9	2.3	2.4

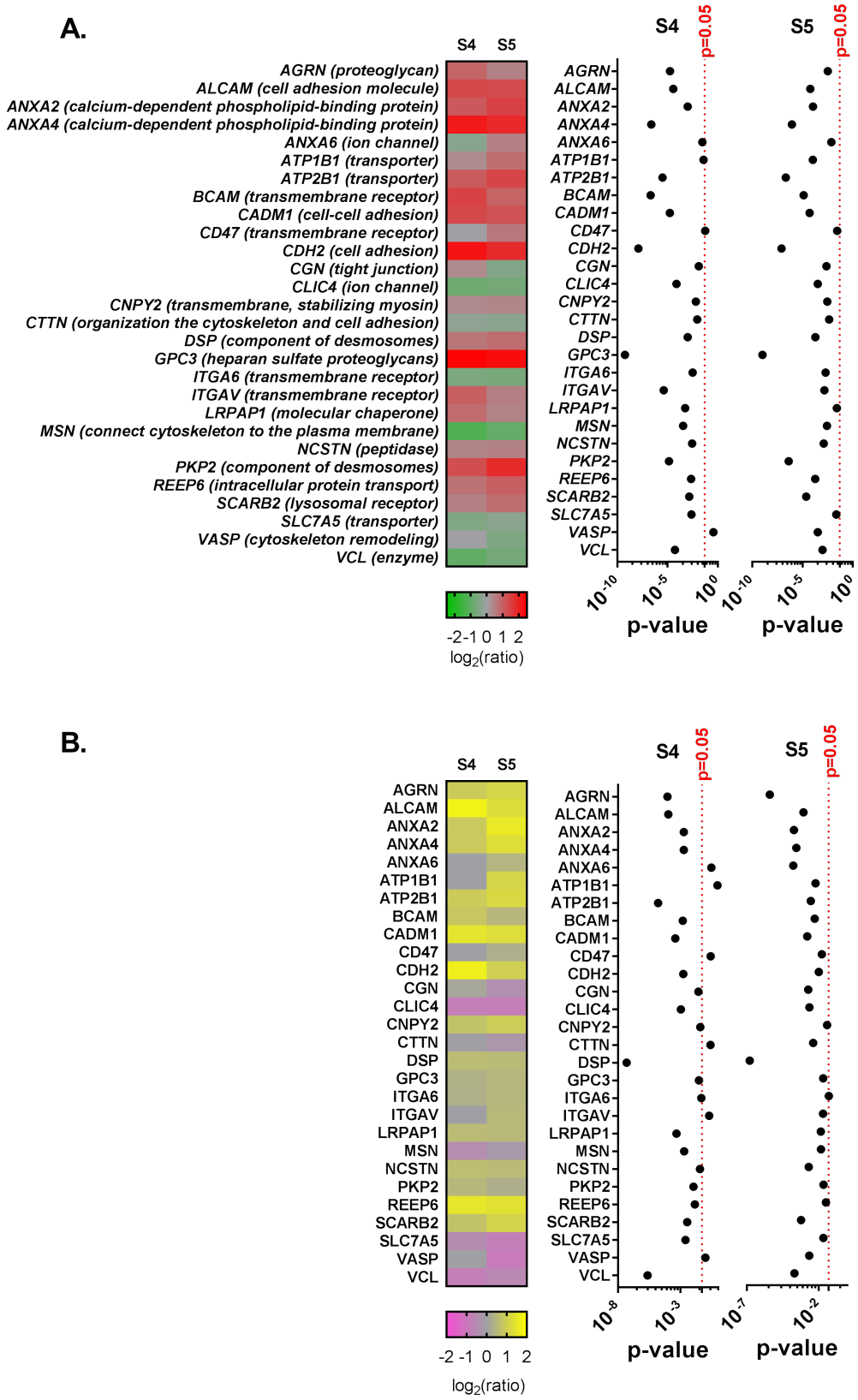

Figure 6.7: Plasma membrane and membrane associated proteins with altered expression profile at S5 (pancreatic endocrine progenitors);genes encoding for proteins with expression profile changed at both, the mRNA (A) and protein level (B).

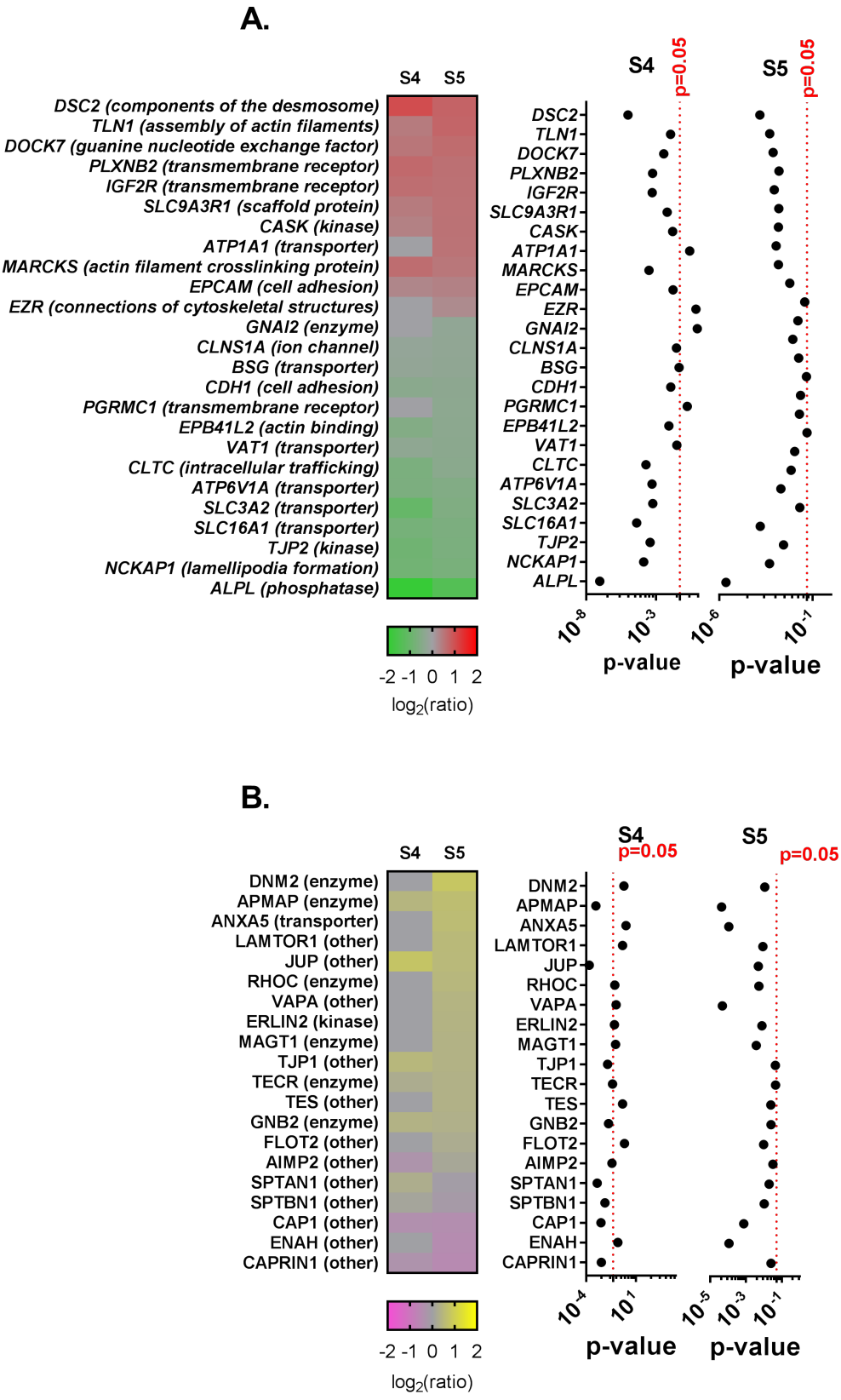

Figure 6.8: Plasma membrane/ membrane associated proteins with expression profile changed only at the mRNA level (panel **A**) and only at the protein level (panel **B**).

6.3.5 Role of ECM Proteins During Pancreatic Progenitors Differentiation

ECM provides support for cells and is involved in the regulation of essential cell biology processes such as attachment, migration, differentiation, proliferation and survival. ECM compounds are secreted by cells and compose their external environment. ECM also shapes cells and is involved in signal transduction from outside the cell to its cytoskeleton. Expression of genes encoding for various ECM proteins such as collagens, laminins, Vitronectin, Reelin, Fibronectin, thrombospodins and integrins was altered is S4/S5 cells compared to S0.

Fifteen genes encoding several forms of collagen were significantly upregulated ($p \leq 0.05$) in S5 cells and seven genes out of these were also upregulated in S4 cells compared to S0. The highest expression was observed for *COL3A1* in S5 cells (6.2-fold increase). More than 2-fold increase in expression was also observed in both S4 and S5 cell populations for genes encoding *COL2A1* and *COL12A1*. Also, expression of *COL4A5*, *COL4A2* and *COL4A1* was upregulated at the mRNA level in S4 and S5 cells. Interestingly, a slightly different collagen expression pattern was observed when analysed at the protein level. The strongest upregulation with over 4-fold increase in expression was detected for COL4A1, over 3-fold increase for COL18A1 for both S5 and S4 cells. The expression of COL4A2 was also nearly 2-fold higher for S4 and S5 cells, compared to S0 cells. A moderate increase in the expression level of COL26A1 and COL2A1 was also detected for both S4 and S5 cells (Fig.6.9, panel A and B, changes in expression are presented in a logarithmic scale).

The expression of genes coding laminins was also upregulated in S4 and S5 cells compared to S0 cells. The highest increase in expression, at the mRNA level, was observed for the gene encoding *LAMA4* (3- fold increase),*LAMA1* and *LAMB1* (2-fold for both). At the protein level most upregulated expression was observed for LAMA1, nearly 2-fold increase in S4 cells and nearly 2.5-fold increase

in S5 cells. Expression of two other detected laminins, LAMB1 and LAMC1, were also significantly upregulated in S4 and S5 cells compared to S0 cells.

In addition, the expression of the genes encoding for tenascin C, Thrombospodin 1 and 3 was also significantly increased in S5 cells compared to S0 cells. In contrast, expression levels of two other proteins from thrombospodin family, THBS2 and THBS4 were significantly downregulated. For THBS2 the expression level for S4 and S5 cells was over 4-fold decreased compared to S0 cells. Expression of tenascin and thrombospodins was only measured only at the mRNA level.

Vitronectin (VTN), Reelin (RELN) and Fibronectin (FN1), next to COL3A1, were ECM proteins with the greatest fold change increase in the expression at the mRNA level in S4 and S5 cells when compared to S0 cells. Expression of VTN was 14.42-fold higher for S4 cells and 7.46-fold higher for S5 cells comparing to S0 cells. Expression of RELN increased by nearly 7-fold for S5 cells and over 2.5-fold for S4 cells. Whereas, expression of FN1 was increased by nearly 6-fold for S4 and 5.5-fold for S5 cells.

An increase in the expression of FN1 and VTN was also detected at the protein level, with over 4-fold increase at S4 and over 3-fold increase at S5 for FN1 and over 1.5-fold increase at S4 and S5 for VTN, compared to S0 cells (Fig.6.9, panel A and B). No data regarding the expression RELN at the protein level was obtained by mass spectrometry.

This research was concentrated on protein quantification from cell lysates and the fraction of ECM, which is composed of secreted proteins, was not collected and analysed. Therefore to observe noticeable changes at the protein level between S4/S5 cells compared to S0 in ECM proteins was unexpected. The limitation of the experimental design to the cell lysates also does not allow to fully addressing changes in the ECM protein expression.

Integrins, a large family of cell surface proteins connecting cell cytoskeleton with ECM, also showed altered expression profile. Expression of integrin subunit

β8 was increased by more than 6-fold for S4 and S5 cells and over 2-fold increase at the mRNA level was detected for Integrin subunits α8 and β6, compared to S0 cells. At the protein level only the expression of Integrin α6, αv and β1 subunits was moderately upregulated in S5 cells and α6 and β1 in S4 cells, compared to S0 population (Fig.6.9, panel A and B).

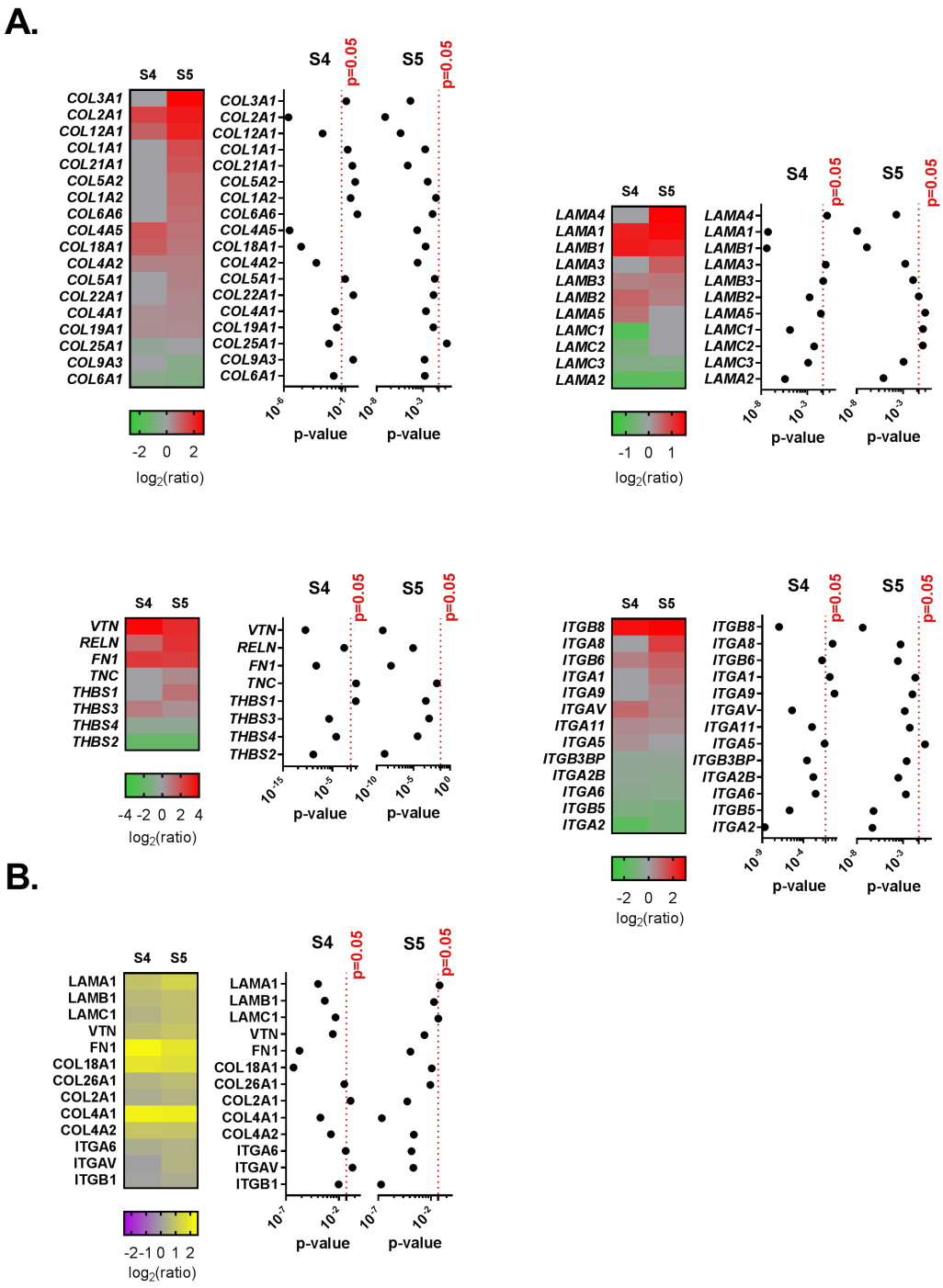

Figure 6.9: ECM components differentially expressed by S4 and S5 progenitor cells. Panel **A**: relative expression of ECM compounds at the mRNA level. Panel **B**: relative expression of ECM compounds at the protein level. (Heat maps were generated using fold change values in relation to S0 expression in a logarithmic scale; only differential expression values with p$\leq$ 0.05 were presented in the heat map.)

6.3.6 Endocrine Progenitor Cells Delamination

To form functional islets, pro-endocrine cells have to delaminate from ductal structures, migrate and cluster together in a highly organised manner. This process has recently been shown to be controlled by NGN3, a transcription factor also known to be indispensable for islet endocrine cells development (Gouzi *et al.*, 2011). Here, expression of *NGN3* was 2.63- fold increased for S5 cells, comparing to S0 cell. No significant changes in expression of *NGN3* between S0 and S4 cell populations were observed at the mRNA level and NGN3 was not detected by mass spectrometry (Fig.6.2, panel A).

In addition to *NGN3*, several well-established markers of EMT were upregulated in S5 and S4 cells. This included: intermediate filament protein- Vimentin (mRNA: S4: 3.6/ S5: 5; protein: S4: 2.72/S5: 3.3-fold), cell-cell junctions component- N-cadherin (CDH2) (mRNA: S4: 4.6/S5: 3.6; protein: S4: 3.2/S5: 2.1) and a transcription factor Snail2 (S5: 2.14- fold change, detected only at the mRNA level). Expression of two other Snail family transcription factor members was downregulated: Snail3 (S5: (-1.2)-fold change, not detected at the protein level) and Snail1 (S4: (-1.9)- fold, not detected at the protein level). This was accompanied by, also EMT-related, significant downregulation of E-cadherin (CDH1) gene expression (S4: (-1.33)/ S5: (-1.3)- fold; however, not significantly changed at the protein level), and altered expression levels of the genes encoding proteins involved in cell cytoskeleton remodeling such as RhoB (S4: 1.43/ S5: 1.6-fold change, at the mRNA level; RHOB was not detected by mass spectrometry), RhoC (upregulated only at the proteins level in S5 cells; 1.4-fold change), and RhoA expression at the protein level significantly downregulated in S5 cells; ((-1.5)- fold change) (Fig.6.10, panel A and B).

Also, expression levels of Cell Division Cycle 42 (Cdc42), a small GTPase that belongs to the Rho-subfamily (S4: 1.19, S5: 1.22- fold change; however, not significantly changed at the protein level) and its downstream target, N-Wasp (S4:(-1.23), not detected by mass spectrometry), were altered at the mRNA level.

Additionally, expression of genes encoding Doublecortin (Dcx), a protein involved in regulation of neuronal migration by modulating stability and organisation of microtubules, raised significantly at the mRNA level between S4 (1.91-fold increase), S5 (3.42-increase) and S0 cells (Fig.6.10, panel A and B); however its expression was not detected by mass spectrometry.

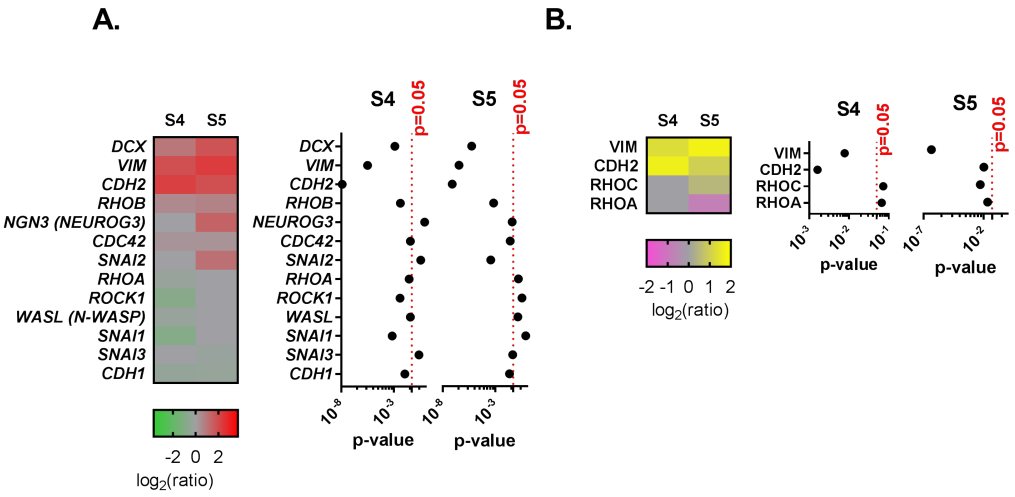

Figure 6.10: Genes associated with EMT, cell migration and pancreatic progenitor cell delamination showed altered expression pattern for S4 and S5 cells. Panel **A**: microarray data; panel **B**: protein relative quantification with iTRAQ-MS/MS. (Heat maps generated using fold change values in relation to S0 expression on a logarithmic scale; only differential expression values with $p \leq 0.05$ were presented in the heat map.)

6.3.7 T-box Transcription Factors

Members of the T-box transcription factor family have been previously implicated in the control of cell migration and segregation in the CNS and heart (Song *et al.*, 2006; Hatcher *et al.*, 2004). This large family of proteins acts during multiple developmental stages, is involved in major signalling pathways, has multiple downstream targets and can exert different effects depending on their spatiotemporal expression patterns and tissue context (Begum and Papaioannou, 2011). Here, expression of several members of the T-box gene family was significantly altered during the development of pancreatic endocrine progenitor cells. Expression of *TBX3*, a transcription factor previously linked with liver development, has been upregulated by over 17-fold for S4 and over 23-fold for S5 cells. Other members of T-box transcription regulators, with increased expression in pancreatic endocrine progenitor cells (S5), included: *TBX20* (3.0- fold change), *TBX5* (2.3- fold change) and *TBX2* (3.5- fold change). Also, expression of *TBX1* was significantly upregulated (1.6- fold change), however, this upregulation was only observed for pancreatic progenitor (S4) cells.

Analysis of protein- protein interactions performed with STRING v10 (Szklarczyk *et al.*, 2015) revealed several other molecules possibly interacting with *TBX3*. Among 10 network components linked to *TBX3*, seven proteins were detected to have upregulated expression profile, with *GATA3* (2.4- upregulation) and *IHH* (2.2- upregulation) showing the highest increase for S4 and S5 cells (Fig.6.11, panel A and B). However, changes in the expression of TBX factors and associated proteins were only detected at the mRNA level.

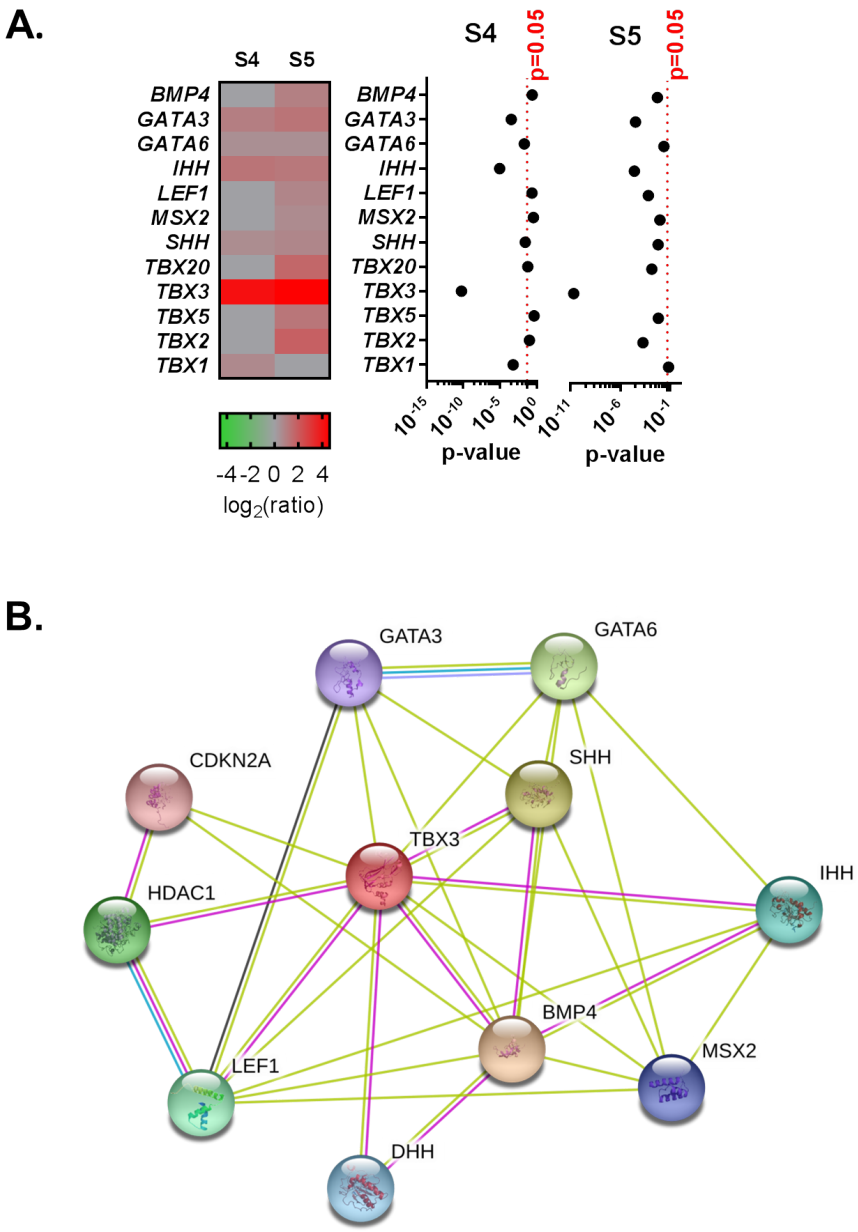

Figure 6.11: T-box transcription factor network during pancreatic progenitor cells development. Panel **A.**: heat map representing relative expression levels of the T-box family factors and Tbx3 network compounds. Panel **B.**: results of STRING protein-protein interaction for Tbx3 protein. Different colour edges represent proteins association resulted from text mining- yellow, known interactions from curated databases- blue, known interactions experimentally determined- pink, co-expression- black and protein homology- violet. (The heat map visualises fold change values in relation to S0 in a logarithmic scale; only differential expression values with $p \leq 0.05$ were presented in the heat map).

6.3.8 Robo/Slit and Netrin/Dcc Signalling

Signalling through Robo (Roundabout) and Dcc receptors is one of the major molecular mechanisms regulating axon guidance (Chilton, 2006). Both receptors were also found to be expressed outside the CNS and were implicated in the regulation of cell migration (Araújo and Tear, 2003). Analysis of cDNA array data reviled that both receptors and other compounds of their signalling pathways showed altered expression profile. Among genes encoding for Robo receptors, the greatest upregulation was observed for the *ROBO2* receptor for S4 cells (4.7-fold change), S5 cell also expressed *ROBO2* at a significantly higher levels than S0 cells, albeit, lower than S4 cells (1.8- fold change). *ROBO1* and *ROBO3* expression were downregulated compared to S0 cells with the fold change of (-1.4) for *ROBO1* and (-1.2) for *ROBO3* for S4 cells and (-1.7) for *ROBO1* and (-1.3) for *ROBO3* for S5 cells. Moreover, expression was upregulated for Slit family members (Robo receptor ligands), both *SLIT2*(S4: 1.82/S5:2.5) and *SLIT3* (S4: 1.80/S5: 2.5) expression was upregulated, whereas expression of SLIT1 was not significantly changed compared to S0 cells.

Robo receptors require GTPase-activating proteins (GAPs) for their signal transduction, such as Slit-RoboGAPs (srGAPs) (Ghose and Van Vactor, 2002). Here, expression of four genes encoding for srGAPs was observed to be altered comparing to S0 cells. Two genes, encoding for *srGAP1* and *srGAP3* were detected to be significantly upregulated in S4 and S5 cells with a fold change of S4: 1.72 and for *srGAP1* and S4: 1.24 and S5: 1.3 for srGAP3. Whereas, *srGAP2* and *srGAP2b* were shown to be downregulated in S4 and S5 cells when comparing to S0 cells (*srGAP2* fold change for S4: (-2.1), S5: (-1.7); *srGAP2b* fold change for S4: (-1.82) and S5: (-1.83))at the mRNA level (Fig.6.12, panel A).

Additionally, Slit can also act as a ligand for the Eva-1 receptor. The mammalian homologue of this receptor is MPZL protein family and an increased in the expression of genes encoding for MPZL proteins was observed at the mRNA

level for both, S4 and S5 cells. The most upregulated *MPZL2* gene showed a 9-fold increase in S4 cells and 7.32 for S5 cells. Two other members of MPZL gene family, *MPZL1* and *MPZL3* were upregulated by 1.3 and 1.85 in S4 cells, and 1.36 and 1.55 for S5 cells, respectively (Fig. 6.12, panel A and B).

While Slit acts as a repulsive ligand, members of another extracellular axon guidance molecule family, the Netrins, can function as a both a chemorepellent and a chemoattractant. The effect of Netrins on a cell is determined by the type of receptors expressed on the cell surface. Netrins mediate an attractive effect through Dcc and Neogenin (NEO1) receptor, whereas the repulsive effect can be produced through Dcc and UNC-5 receptors. Here, expression of *DCC* and *UNC-5c* has been upregulated over S4: 9.4/S5: 11.1 and S4: 7.9/S5: 29.7- fold at the mRNA level, respectively. However, expression of three other members of UNC-5 receptor family, UNC-5a, b and d was downregulated. Also, expression of NEO1 and Dscam, a protein recently identified as yet another receptor of Netrin1 (NTN1), was moderately upregulated for S4 cells (fold change: 1.7 and 4, respectively) and for S5 cells (fold change: 1.6 and 2.05) compared to S0 cells. Expression of Netrin 1, in contrast, was downregulated for S4 by fold change of (-1.35) and (-1.2) for S5 cells (Fig.6.12, panel C).

String analysis revealed additional components previously shown to interact with Netrin/Dcc/UNC-5 signalling and significantly altered at the mRNA level during differentiation towards pancreatic and endocrine progenitors. This included proteins such as, a Fibronectin Leucine Rich Transmembrane Protein 3 (FLRT3) (S4: 23.8 and S5: 25.6- fold upregulated), a transcription repressor PRDM1 (PR/SET Domain 1) (S4: 4/S5: 4.34-fold upregulated) and a DNA-binding protein involved in regulation of transcription and chromatin remodelling, SATB Homeobox2 (SATB2) (S4:(-1.82)/S5: (-1.6)-fold change) compared to S0 (Fig.6.12, panel C and D).

Unfortunately, changes in the expression of genes involved in axon guidance were only observed at the mRNA level and none of these proteins was detected

by mass spectrometry.

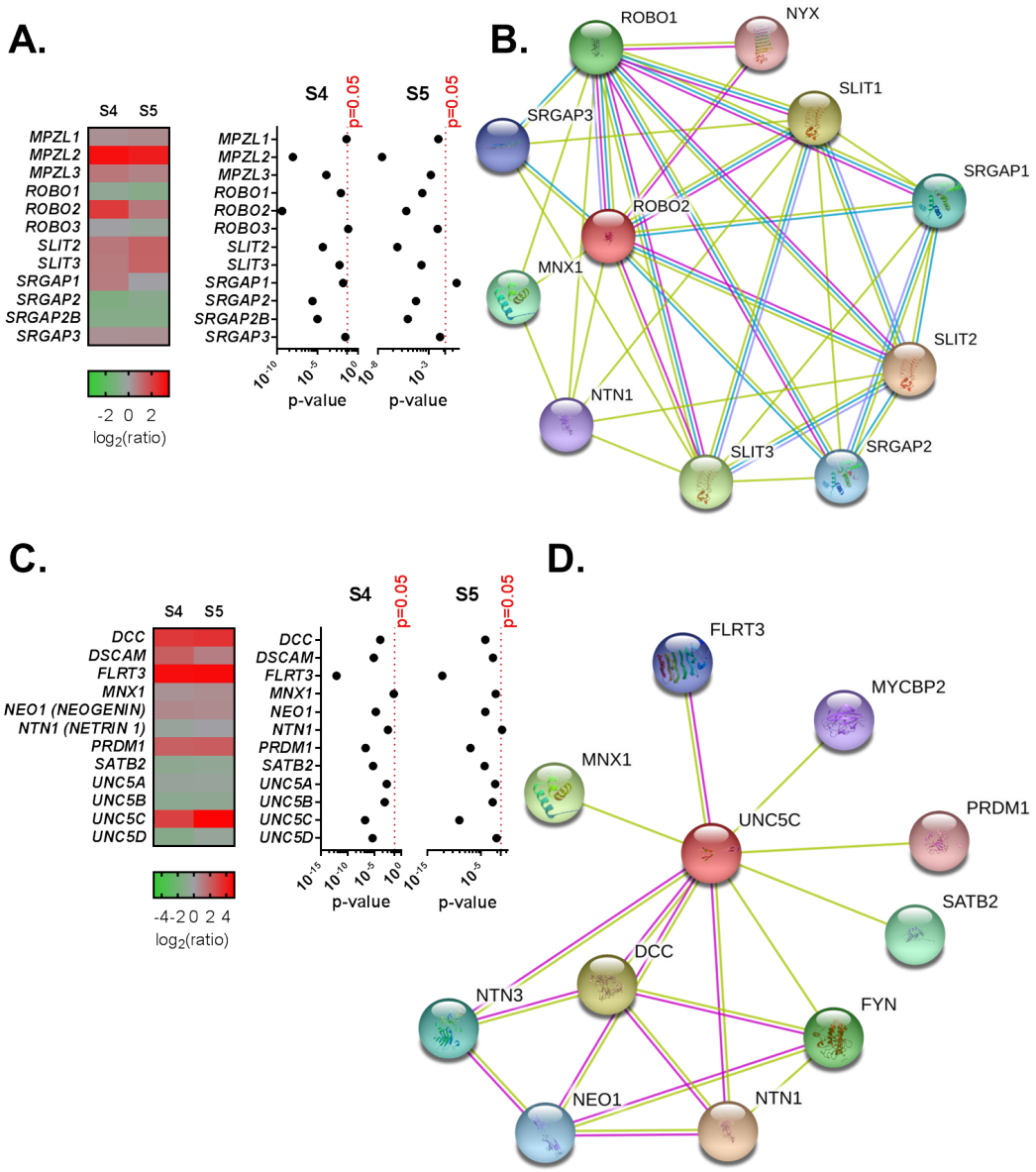

Figure 6.12: Genes associated with neuronal cells development showed altered expression pattern in pancreatic progenitors and pro-endocrine cells. Panel **A** and **C**: heat maps illustrating changes at the mRNA level. Panel **B** and **D**: String: functional protein association networks. Different colour edges represent proteins association resulted from text mining- yellow, known interactions from curated databases- blue, known interactions experimentally determined- pink, co-expression- black and protein homology- violet. (Heat maps were generated using fold change values in relation to S0 expression on a logarithmic scale; only differential expression values with p$\leq$ 0.05 were presented in the heat map.)

6.4 Discussion

6.4.1 Transcription Factors and Pancreatic Progenitor Cell Markers

Expression of several previously established pancreatic progenitor (PP) markers was assessed at the mRNA level. The upregulated expression was observed only for seven out of 13 genes. The strongest upregulation was observed for genes encoding for CD142 (a PDX1$^+$-cell marker) and TSPAN7 (a pancreatic endocrine cell marker) (Hald *et al.*, 2012; Kelly *et al.*, 2011). Although the expression of PP markers only moderately correlated with the anticipated characteristics of S4 and S5 cells, a good correlation was observed for key TFs. Stage 4 and 5 cells were marked by a strong expression of *PDX1*, *NKX6.1*, *NEUROD1* and *NGN3*. The previous research aiming to identify PP markers utilised a model organism or stem cell cultures (Jiang *et al.*, 2011; Kelly *et al.*, 2011; Ma *et al.*, 2012; Hald *et al.*, 2012; Fishman *et al.*, 2012). Both approaches present certain limitations, such as developmental differences between species or low efficiency of differentiation protocol. Here, one of the most recent, multi-step protocols for differentiation of stem cells into mature β-cells was utilised (Rezania *et al.*, 2014). Several others recently developed protocols applied similar differentiation conditions and induce progenitor cell population with a comparable efficiency (Pagliuca *et al.*, 2014; Russ *et al.*, 2015; Zhu *et al.*, 2016). The protocol developed by Rezania et al. (2014) reported improved efficiency with 50 % of final stage cells expressing insulin and key, β-cell specific transcription factors, including PDX1 and NKX6.1. The protocol efficiency for the earlier stages has also been reported to be as high as 99 % for PDX1$^+$ cells (S4 and S5 cells) (Rezania *et al.*, 2014). This potentially would allow for more accurate identification of stage specific cell membrane proteins that could be utilised for identification of PP cells.

In addition to the well-established, key TFs orchestrating pancreas develop-

ment (PDX1, NGN3 and NKX6.1), cDNA array analysis revealed several other transcription regulators with yet undefined function in pancreatic progenitors. This included two members of the Sox transcription factor family, *SOX6* and *SOX5*. Both TFs showed upregulated expression in S4 and S5 cells compared to S0 cells. Those TFs belong to the SOXD group (one of the several groups within SOX TFs family) and were previously linked with the regulation of chondrogenesis and segregation of progenitor cells during differentiation of cortical neurones (Lefebvre, 2010; Azim *et al.*, 2009). SOX6 was also found to be involved in the regulation of pancreatic β-cell proliferation, by controlling the expression of cyclin D (Iguchi *et al.*, 2007).

Moreover, *RFX3* and *RFX2*, two members of the RFX transcription factor family also showed significant upregulation at the mRNA level in S4 and S5 cells. RFX3 has been previously found to be an important fate determinant during pancreatic β-cell development. Pancreatic islets lacking Rfx3 expression were characterised by a reduced number of insulin-producing cells and incompletely developed β-cell progenitors with a reduced Glut-2, Gck and insulin expression (Ait-Lounis *et al.*, 2010). Rfx2 is mainly linked with the process of ciliogenesis in vertebrate (Chung *et al.*, 2012), however, its putative function during pancreas development has not yet been demonstrated.

MAF (c-MAF) is a transcription factor belonging to the large Maf family. Two other members of this family- MafA and MafB play an important function in pancreas development (Abdellatif *et al.*, 2015).c-Maf has been previously shown to be involved in the regulation of lens, liver and kidneys development. Moreover, c-Maf has been found to be expressed in developing pancreatic α- and β-cells and in areas around the branching ducts (Tsuchiya *et al.*, 2006). However, the exact function of c-Maf in pancreas organogenesis has not yet been elucidated (Zhang and Guo, 2015).

6.4.2 The Upregulation of Membrane Proteins in Pancreatic Progenitor Cells

Several membrane spanning proteins with an upregulated expression at the mRNA and protein level in S4/S5 cells were identified: ALCAM and BCAM, AGRN, ATP1B1 ATP2B1 and CADM1. Those proteins could serve as putative cell surface markers for isolation of pancreatic progenitor cells.

ALCAM (Activated Leukocyte Cell Adhesion Molecule, CD166) and BCAM (Lutheran/ Basal Cell Adhesion Molecule) are transmembrane proteins that belong to the immunoglobulin superfamily (Bowen *et al.*, 1997; Kikkawa *et al.*, 2013). ALCAM was reported to be expressed in a wide spectrum of cell types including fibroblasts, neurons and epithelial cells, although its expression has been observed to be restricted to a subset of cells characterised by dynamic growth and/or migration (e.g. neuronal cell during development) (Swart, 2002). Within the cell membrane, ALCAM is localised at the cell adhesive complex. This cellular localisation suggests a possible involvement of ALCAM in the control and maintenance of tissue architecture (Ofori-Acquah and King, 2008). Additionally, ALCAM was proposed to be a prospective marker for cancer stem cells and its expression was shown to be altered in several types of cancer, including melanoma, breast, colorectal, prostate and pancreatic cancer (Ofori-Acquah and King, 2008; Zhang *et al.*, 2016). Moreover, ALCAM was revealed to play a role in processes such as immune response, haematopoiesis and neurogenesis (von Bauer *et al.*, 2013; Wade *et al.*, 2012; Chitteti *et al.*, 2014). Its function in the development of the CNS was associated with axonogenesis, axon guidance and muscle innervation (Swart, 2002). Involvement and function of ALCAM in pancreatic progenitor cell development has not been previously investigated, however, its well-documented association with progenitor cell populations (e.g. neural stem/precursor cells) potentially establishes this protein as a promising biomarker candidate (Ofori-Acquah and King, 2008; Sundberg *et al.*, 2009).

BCAM interacts with Laminin $\alpha 5$, its only known ligand. This interaction was reported to modulate cell adhesion/migration potential. BCAM and integrin $\alpha 3\beta 1$, both recognise LG domain of laminin and bind to it in a competitive manner. Binding of laminin 511 (a heterotrimer composed of $\alpha 5\beta 1\gamma 1$ chains) by integrin $\alpha 3\beta 1$ promotes cell adhesion, whereas the binding of BCAM results in a suppression of cell adhesion and induction of a migratory phenotype in which cells display elongated pseudopodia (Kikkawa *et al.*, 2013). Involvement of BCAM in pancreatic progenitor cell development and migration has not been previously investigated. Furthermore, here changes in the expression of genes encoding several laminin chains (including $\alpha 5$, $\beta 1$ and $\gamma 1$) has been observed in S4 and S5 cells compared to S0 (Fig.6.9). However, no significant changes in the expression of genes encoding for integrin subunit $\alpha 3$ has been detected here in S4 or S5 cells. Therefore elucidation of BCAM functions and its possible implication in the acquisition of a migratory phenotype by pancreatic progenitor during development requires further investigation.

Agrin (AGRN) is a macromolecule that belongs to the group of glycoproteins called heparan sulphate proteoglycans. Agrin expression has been reported in the CNS and in non-neuronal tissue, including lung and kidney (Groffen *et al.*, 1998; Halfter *et al.*, 1997). It was also shown to play an important role in the development of both, peripheral and CNS, particularly in the formation of neuromuscular synapses and axon/ dendrites branching (Kim *et al.*, 2006; Banks *et al.*, 2003; Mantych and Ferreira, 2001). A transmembrane form of Agrin has also been shown to influence cytoskeleton organisation and induce the formation of filopodia when clustered with the membrane of axon/ dendrites (Annies *et al.*, 2006). Additionally, Agrin has been shown to bind to sodium/potassium-ATPases and by acting as a competitive antagonist it has been revealed to block the activity of those ion pumps, through modulation of the membrane potential of neurones (Hilgenberg *et al.*, 2006; Tidow *et al.*, 2010). The function of Agrin in pancreas development has not yet been identified.

CADM1 (Cell Adhesion Molecule, also known as SynCAM) a transmembrane protein that has been previously described to play a role in cancer progression and in synapse formation (Murakami, 2005; Biederer, 2002). CADM1 has been observed to be expressed in several organs during development, however, its function has not yet been fully elucidated. Among others, it was reported to be expressed in epithelial and neuroepithelial cells forming developing lung, liver, gut and several regions of the brain (Fujita *et al.*, 2005; Pietri *et al.*, 2008). Expression of CADM1 was also observed in developing mouse pancreas starting around E12.5. This observation was proposed to support CADM1 role in the maturation of pancreatic islets and in the establishment of islet-neuronal crest derivatives interaction (Shimada *et al.*, 2012; Suckow *et al.*, 2008).

ATP1B1 encodes a subunit of sodium/potassium–ATPase, a member of P-type primary ion transport ATPases. ATP2B1 encodes a subunit of plasma membrane calcium ATPase (PMCA) that belongs to the same family of ATP-dependent transporters. Both transporters are ubiquitously expressed and are involved in the maintenance of cellular homeostasis (Dunbar and Caplan, 2001). To summarise, the majority of those proteins have been previously reported to be involved in the development and cell organisation within a tissue, however, their function during pancreas organogenesis requires further investigation.

6.4.3 ECM Components in Pancreatic Progenitor Development

Pathway analysis of cDNA array and mass spectrometry data revealed a set of cellular processes altered in S5 cells as compared to S0 cells. One of these altered processes that were identified with Panther overrepresentation test was Extracellular Matrix Organisation. Similarly, analysis with IPA identified an alteration in pathways such as Atherosclerosis Signalling and Hepatic Stellate Cell Activation that might also indicate re-arrangement of ECM (Fig.6.5 and 6.6). ECM components play an important role in development, morphogenesis and

organs formation. ECM is a prime component of the cellular microenvironment and is involved in the regulation of cell proliferation, death and differentiation (Lu *et al.*, 2011).

Integrins are transmembrane proteins which provide a structural link between ECM and cell cytoskeleton. Their function, in addition to cell adhesion, involves a transduction of signal and activation of pathways linking cell response to its environment (including ECM) (Bökel *et al.*, 2002). Expression of integrins, $\alpha v\beta 3$, $\alpha v\beta 5$ and $\beta 1$ (all, but $\beta 3$, had an altered expression profile in S4/S5 cells) has been observed in developing human islets and was described to play a role in adhesion and migration of endocrine progenitors (Cirulli *et al.*, 2000). Additionally, integrin $\beta 1$ was also established to regulate the branching morphogenesis during pancreas development (Shih *et al.*, 2016). Correspondingly, ITGB1 (integrin $\beta 1$) was observed to be significantly upregulated at the protein level for S4 and S5 cells as reported in this study. Moreover, the expression of ITGAV was increased at the mRNA and protein level for S4 and S5 cells. However, mRNA expression profile of *ITGB5* was significantly downregulated for S4 and S5 cells and expression of *ITGB3* was not significantly changed. Unfortunately, data revealing expression profile of those integrins at the protein level were not obtained in the iTRAQ-MS/MS analysis. In addition, *ITGB8* and *ITGB6* were shown to be strongly upregulated (Fig.6.9). These two genes encode for integrin subunits and have not been previously reported to be involved in pancreas development. ITGB8 and ITGB6 in combination with ITGAV could potentially form $\alpha v\beta 8$ and $\alpha v\beta 6$, the two out of eight integrins that recognise RGD (Arg-Gly-Asp) sequence and activate the cytokine TGFβ (Aluwihare *et al.*, 2009). TGFβ proprotein forms a noncovalent complex with latency associated peptide-LAP and requires alternation prior to its interaction with a receptor (Khalil, 1999; Robertson *et al.*, 2015). Integrins such as $\alpha v\beta 8$ and $\alpha v\beta 6$ disrupt the latent complex contributing to the formation of an active TGFβ signalling molecule (Khalil, 1999). In addition, activation of TGFβ cytokine can be facilitated by Thrombospondin 1 (THBS1), a

matricellular protein, which expression was also significantly upregulated at the mRNA level for S5 cells compared to S0 cells.

THBS1 and yet another extracellular matrix glycoprotein- Reelin (RELN), were both implicated in the control of radial neurones migration during neocortex development (Trommsdorff *et al.*, 1999; Hashimoto-Torii *et al.*, 2008). RELN encoding gene, similarly to THBS1 gene, was upregulated in S5 cells as compared to S0 cells. THBS1 and RELN proteins can interact with Apolipoprotein E receptor 2 (ApoER2), Very low density lipoprotein receptor (VLDLR), and with Dab1 (Massalini *et al.*, 2009). RELN and THBS1 share some of their downstream targets, however, molecular events downstream of these molecules differ between RELN and THBS1- initiated signalling. For instance, RELN and THBS1 were both observed to execute an effect on migrating neuronal progenitors although of a divergent nature. Reelin promotes destabilisation of chains of migrating progenitor cells, whereas THBS1 stabilises progenitor cell migration as chains (Massalini *et al.*, 2009).

In addition to a role in the CNS, THBS1 and Reelin were reported to be implicated in processes such as cell motility and cell adhesion. Nonetheless, no previous reports describing a potential function of these proteins in pancreatic development were reported (Yuan *et al.*, 2012; Lawler, 2002). Although an increase in the RELN and THBS1 expression was observed for S5 cells, the initial common components of the THBS1/Reelin signalling (ApoER2 and VLDLR) were downregulated for S4/S5 cells compared to S0 cells (ApoER2: S4:(-3.10)/S5:-1.94) and VLDLR: S4:(-3.89)/S5: not significantly changed). Therefore, further investigation into an exact nature and physiological effects of THBS1 and Reelin during pancreas development are required.

Collagens are structural proteins of ECM. Several forms of collagen were previously reported to be involved in the formation of the pancreatic basement membrane, including COL-I, COL-III, COL-V (assemble into fibrillar structures), COL-VI (beaded filaments) and COL-IV (forms a planar hexagonal network)

(Stendahl *et al.*, 2009). The proteomic analysis of S4 and S5 cells revealed increased expression of collagen type IV when compared to S0 cells. Other pancreas associated forms of collagen were undetectable at the protein level. Collagen type I, III, V and VI showed upregulated expression pattern at the mRNA level for pancreatic progenitor cell population.

Other groups of structural ECM proteins that were detected to be upregulated in S4 and S5 cells compared to S0 cells included laminins. Only limited data are available regarding laminin types that form the ECM of pancreatic progenitor cells. Laminin 1 was previously reported to be the dominant isoform detected in developing mouse pancreas (Jiang *et al.*, 1999). This isoform was also upregulated in S4 and S5 cells at the mRNA and protein level. Other isoforms of detected laminins with an increased expression included Laminin 3 and 2, however, this observation was not verified at the protein level.

6.4.4 Pancreatic Endocrine Progenitor Cells Delamination

In addition to ECM organisation, axon guidance and several other pathways linked to the modification of cell cytoskeleton such as ILK and RhoGDI signalling were also identified by pathway analysis. This, combined with the recently reported observation that Ngn3, a transcription factor critical for pancreatic endocrine progenitors is a regulator of islet precursor cells migration prompted data mining analysis into a potential upregulation of proteins essential for cell migration (Gouzi *et al.*, 2011).

The molecular control of axon guidance, neuronal progenitor cell migration and development of pancreatic endocrine compartment has several common elements (Nobrega-Pereira and Marin, 2009; Gu *et al.*, 2004). Both, development of neuronal cells and pancreatic endocrine cells, require an active form of NGN, a bHLH transcription factor. NGN1 and NGN2 are implicated in the control of cortical neurogenesis, where they function as regulators of neuronal fate ac-

quisition as well as inducers of cell migration, whereas NGN3 is indispensable for pancreas development (Ge *et al.*, 2006). NGN3 was reported to have a dual function in the establishment of pancreatic endocrine cells, by acting as a key upstream regulator of several transcription factors controlling the fate of endocrine progenitor cells, such as NEUROD1, NKX2.2, PAX4 and orchestrating delamination of further islet cells from the trunk (Gouzi *et al.*, 2011). The pancreatic progenitor's delamination is a process resembling epithelial-mesenchymal transition (EMT) and leads to subsequent cells delamination, migration and clustering into islets of Langerhans. This was first observed to occur in the endocrine progenitors characterised by the expression of Ngn3 (Gouzi *et al.*, 2011). Other EMT marker proteins, such as Snail2, Vimentin and N-cadherin, were also reported to play a role in delamination and be co-express with NGN3 in pancreatic endocrine precursors (Rukstalis and Habener, 2009).

Transcriptome analysis of S4 and S5 cells, which correspond respectively to pancreatic progenitors and endocrine progenitors, revealed increased expression of NGN3 and SNAIL2 in S5 cells when compared to S0 undifferentiated cells. This was accompanied by upregulated expression of Vimentin and N-Cadherin (CDH2) observed at the mRNA and protein level. Upregulation of Vimentin and N-cadherin together with decreased levels of E-cadherin (CDH1) expression (also observed at the mRNA level for S4 and S5 cells) is characteristic to EMT, a process of transforming epithelial cells into more migratory cells with the mesenchymal phenotype (Thiery *et al.*, 2009).

Changes in cell polarity are considered as another hallmark of cellular acquisition of a mesenchymal/migratory phenotype. This process requires the reorganisation of the cell cytoskeleton, cell-cell and cell-ECM interactions. Key modulators of this process are Rho, Rac and Cdc42 GTPases (Etienne-Manneville, 2008). Recently, it was shown that the inactivation of Cdc42 and its downstream effector N-WASP is crucial for normal delamination and maturation of precursor endocrine cells (Kesavan *et al.*, 2014). Here, the expression of Cdc42 was observed

to be moderately upregulated for S4 and S5 cells at the mRNA level compared to S0 cells. However, expression of N-WASP was downregulated at S4 and remains unchanged for S5 cells. This might indicate that activities of Cdc42 and N-WASP during cell delamination are not regulated at the level of transcription. Another small GTPase involved in cytoskeleton organisation and potentially regulated by neurogenic bHLH transcription factor is RhoA (Ge *et al.*, 2006). As reported in this study, the mRNA expression of RhoA was moderately downregulated for S4 cells when compared to S0 cells and this corresponded with its significantly downregulated expression at the protein level in S5 cells. This observation is in agreement with the data presented by Ge et al. (2006) for migrating progenitor neuronal cells, showing that NGN1/2 negatively controls levels of RHOA. In addition to a downregulation of RHOA, Ngn1 and Ngn2 were observed to upregulate Doublecortin (DCX) and p35 during neurogenesis (Ge *et al.*, 2006). Here, expression of DCX, a protein regulating microtubule organisation, was observed to be increased at the mRNA level for both S4 and S5 cells, indicating a possible role for DCX in cytoskeleton regulation during pancreatic progenitor cells delamination.

In summary, increased expression of *NGN3* in S5 cells was accompanied by upregulated expression of Vimentin, N-Cadherin, Snail2 and Dcx, all functionally linked to EMT. This potentially establishes pancreatic progenitors as migratory cells with mesenchymal- like characteristics, however, this has yet to be verified by further research.

6.4.5 Factors Regulating Axon Guidance in Pancreatic Progenitors Development

In this study, genes encoding proteins associated with the development of neuronal cells (specifically with the process of axon migration towards its specific target) were identified as upregulated in S4/S5 cells when compared to S0 cells. These included genes encoding for members of T-box transcription regulators,

Robo/Slit signalling and Netrins signalling (Fig. 6.11 and 6.12).

T-box family transcription factors have been previously shown to regulate migration of progenitor cells (e.g. regulation of motor neurones migration during hindbrain development and cardiac cell migration in developing heart), although expression and function of those TFs in pancreas development has not yet been well characterised (Song *et al.*, 2006; Hatcher *et al.*, 2004). Here, four Tbx TFs had an altered expression profile: *TBX3* expression was increased in S4 and S5 cell and the expression of *TBX2*, *TBX5* and *TBX20* was elevated in S5 cells.

TBX3 had the most upregulation expression among detected T-box family transcription factors. Tbx3 expression, together with Tbx2, has been previously observed during mouse pancreas development (Begum and Papaioannou, 2011). Tbx3 was expressed in pancreatic mesenchyme during early stages of development (E9.5) and in exocrine cells in the postnatal and adult pancreas, Tbx2 was expressed during pancreas development in the pancreatic vasculature and in the fetal and adult pancreas in endocrine and ductal cells (Zhou *et al.*, 2007; Begum and Papaioannou, 2011). Additionally, Tbx3 was previously reported to be implicated in the hepatic development (a process controlled by a set of TFs similar to the one involved in the pancreatic development) as a key factor regulating proliferation and segregation of hepatic progenitors (Suzuki *et al.*, 2008). However, no downstream targets of Tbx3 in pancreas development are currently known and TBX3 function during human pancreas development also requires further clarification. Tbx20 was observed to be expressed by motor neurones within the hindbrain and was revealed to be essential for the process of neuronal progenitor cell migration (Song *et al.*, 2006). Moreover, Tbx20 expression was detected during cardiac development, where it plays an essential role during expansion of cardiac precursor cells and their segregation into the chamber and non-chamber myocardium (Chakraborty and Yutzey, 2012; Singh *et al.*, 2005). Tbx20 was reported to be an upstream negative regulator of Tbx2 myocardial cells; however, the exact nature of the interaction between those two TFs has not yet been fully

determined as an opposite effect: Tbx20 stimulating Tbx2 expression was found in neuronal cells (Song *et al.*, 2006). The interaction between Tbx TFs during development might be even more complex since, in addition to its function as a negative regulator of Tbx2 expression in myocardial cells, Tbx20 has been shown to synergistically function as an activator of Tbx5 expression in these cells (Singh *et al.*, 2005; Stennard *et al.*, 2003). Here, expression of *TBX20*, *TBX2* and *TBX5* was enhanced in S5 cells at the mRNA level, however, further research is required in order to verify this expression pattern at the protein level and elucidate the interaction between those factors in the developing pancreas. Interestingly, Tbx2 was also found to be implicated in the development of pancreatic β-cells in *Xenopus* and was hypothesised to be a downstream target of Ngn3 (Oropeza and Horb, 2012). This, however, has yet to be verified in the developing mammalian pancreas.

Another network of proteins essential for neuronal cells development and axon guidance that was altered during the transition from undifferentiated iPS cells towards pancreatic/ endocrine progenitors included proteins involved in the Robo/Slit signalling. Roundabout molecule (Robo) is a transmembrane receptor involved in the control of commissural neurones crossing midline during the CNS development. Its ligand, Slit, is a midline neurone repellent molecule (Araújo and Tear, 2003). Both, Robo and Slit family member proteins were also found to be involved in regulation of cell migration outside of the CNS (Yuasa-Kawada *et al.*, 2009).

In regards to pancreatic progenitor cells development, an increase in the expression of Robo2 was observed at the mRNA level for both S4 and S5 cells compared to S0 cells (Fig.6.12). In contrast, expression of another member of Robo family- Robo1 was decreased for S4 and S5 cells. The expression of genes encoding members of Slit family, Slit2 and Slit3, was increased for S4 and S5 cells at the mRNA level when compared to S0 cells. In addition to the role as a ligand for the Robo family of receptors, Slit molecules also bind to EVA-1, yet another

transmembrane receptor (Fujisawa *et al.*, 2007). A mammalian homologue of *C. elegans* EVA-1, MPZL2 was also observed to be upregulated in both S4 and S5 cells when compared to S0 cells.

Netrins are a class of proteins involved in the regulation of axon guidance (Dickson, 2002). In S4/S5 cells expression of Netrin 1 (NTN1) was downregulated in S4 cells, however, no changes in its expression were observed for S5 cells as compared to S0 cells. Netrins are ligands for several receptors including DCC (Deleted in Colorectal Cancer), Neogenin, DSCAM (Down's syndrome cell adhesion molecule) and UNC5 homolog family, expression of these genes was upregulated in S4 and S5 cells. These receptors are transmembrane proteins involved in the control of axon guidance as well as other cell migration related processes (Rajasekharan and Kennedy, 2009; Ypsilanti *et al.*, 2010). Netrin1 can act as a chemoattractant or a chemorepellent depending on the composition of receptors available on the surface of a cell. Expression of *DCC*, *DSCAM*, *NEO1* and *UNC5c* was elevated in S4 and S5, however, expression of *DCC* and *UNC5c* showed increasing trend between S4 and S5, whereas expression of *DSCAM* showed decreasing trend between S4 and S5 cells. Interestingly, *DCC* and *UNC5c* receptors have been previously reported to mediate the repulsive effect of Netrin 1.

Both, Robo/Slit and Netrins signalling are key proteins involved in cytoskeletal dynamics. Robo receptor exerts its action through GTPase-activating proteins (GAPs), such as Slit-RoboGAPs (srGAPs) (Wong *et al.*, 2001). SrGAPs interact with WASP/WAVE proteins, that are implicated in actin remodelling by regulating the activity of its downstream target small GTPases such as Cdc42, RhoA and Rac1 (Wong *et al.*, 2001). Netrins effect on the cell cytoskeleton can be executed through several pathways, including signalling through small GTPases as well as various kinases, including FAK and PI3K (Barallobre *et al.*, 2005).

To summarise, in addition to increased expression of *NGN3* and several EMT markers in pancreatic/endocrine progenitors, expression of proteins previously described as regulators of precursor cell migration was also observed to be altered.

This includes signalling molecules such as Netrin1 and Slit and their relevant receptors, including Robo2, Dcc, Neogenin, Dscam and Unc5. Moreover, expression of transcription factors linked to the regulation of cell migration during organogenesis, such as members of Tbx family showed upregulated profile for S4/S5 cells. This supports previous observation by Gouzi et al. (2011). that pancreatic progenitor cells activate signalling cascade that allows for cell morphology changes and provides means necessary for migrating cells to navigate and form mature islets structures.

6.4.6 Summary

Similarly to previously reported observations that expression at the mRNA level reflects changes occurring at the proteins level only partially, here a moderate positive correlation (r_s= 0.5) between mRNA and protein expression was observed (Griffin *et al.*, 2002; Schmidt *et al.*, 2007; Tian *et al.*, 2004; Lu *et al.*, 2009). This discrepancy between mRNA and protein levels was previously related in a first instance to post-transcriptional regulation of the gene expression (Tian *et al.*, 2004).

In this study, data obtained from transcriptomics and proteomics were combined in order to elucidate changes in the expression of cell surface proteins between stem cells and pancreatic endocrine progenitor cells. This allowed for comparison between 1591 genes identified between both sets of data as well as for supplementing mass spectrometry data with transcriptome analysis. The main limiting factor of this study was incomplete coverage of differentially expressed genes in both sets of data. Multiple changes were observed only at the level of mRNA and expression of those changes will have yet to be verified at the protein level. Additionally, a correlation between both data sets reached only 50%, therefore not all changes observed at the mRNA level can confidently be translated to the proteome. However, expression at the mRNA level of genes encoding for cell membrane proteins such as *ALCAM*, *ATP1B1*, *ATP2B1*, *BCAM* and

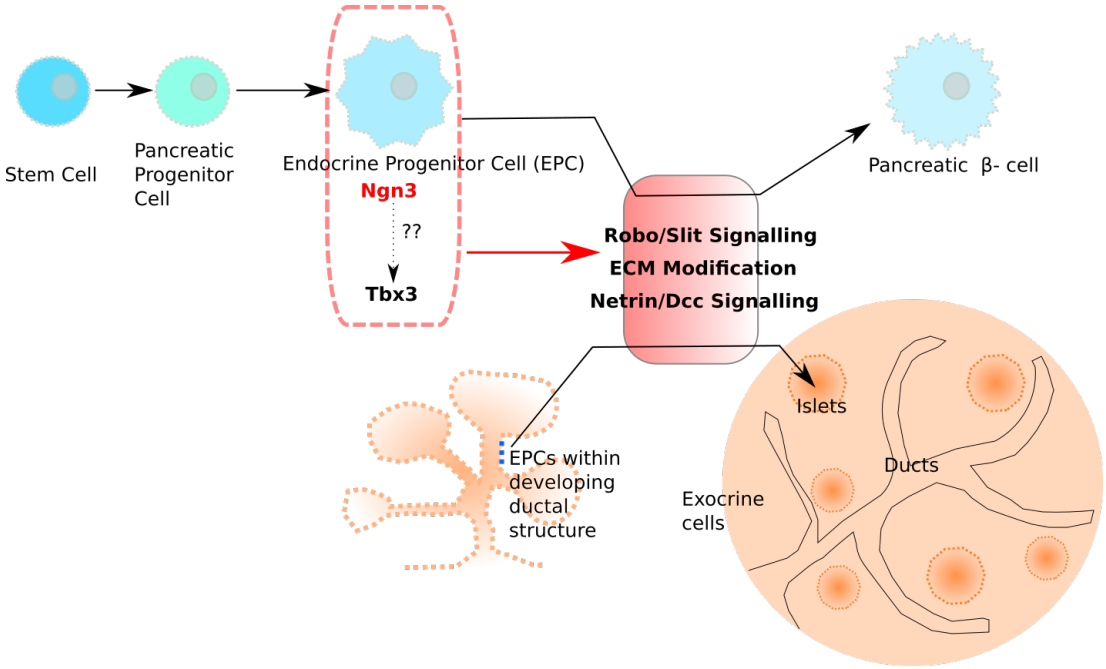

Figure 6.13: Upregulated expression of Robo/Slit, Netrin/Dcc signalling and modification of ECM are characteristic for pancreatic endocrine progenitor cells (EPCs). Analysis of the differential expression data between EPCs and stem cells revealed that $Ngn3^{+}$ cells also show upregulated expression of genes associated with cell motility and segregation, such as Tbx3 transcription factor, and components of Robo/Slit and Netrin/Dcc in addition to ECM modification.

CADM1 appears to correspond well with their expression at the protein level. However, changes in the expression of NGN3, several EMT markers, expression of proteins involved in cell motility, including Netrin/Dcc and Robo/Slit signalling, still requires validation at the protein level. Nevertheless, differential expression of multiple components involved in ECM modification and enhancement of cell migratory abilities exposes this area as a potential source of insight into the nature of elusive pancreatic endocrine progenitors (Fig. 6.13).

Chapter 7

Summary and Further Work

This project tested two cell models; pancreatic mesenchyme stem cells and iPS cells in their capability to differentiate into pancreatic progenitor cells (PP cells). Following iPSC differentiation into $PDX1^+$ and $PDX1^+/NGN3^+/NEUROD1^+$ cells, these cells were analysed with cDNA arrays and iTRAQ-MS/MS to identify putative cell surface markers . The aim of this study was to improve methods for isolation of intact pancreatic progenitor cells for the treatment of Type 1 Diabetes. Current techniques for the identification and isolation of progenitor cells are based on expression profiles of specific transcription factors. However, this requires partial disintegration of the cell membrane as specific TFs are localised within the nuclear compartment of the cell. Therefore, identification of a panel of cell surface markers for pancreatic progenitor cells would remarkably improve the isolation of these cells and enhance the drive towards their use for therapeutic treatment for diabetes.

7.1 Pancreatic Mesenchymal Stem Cells

The first step of this study involved generation of pancreatic progenitor cells that expressed key, stage specific TFs, including PDX1, NGN3, NEUROD1, and NKX6.1. Mesenchymal stem cells derived from human pancreatic tissue CH-pMSC were the first *in vitro* model tested for its ability to generate a population

of PP cells. Mesenchymal stem cells are a promising source of cells for regenerative medical applications. MSCs have features of stem cells, including the ability to self-renew and capacity to differentiate into functional cells, mainly of mesodermal lineage (Kim and Cho, 2015). MSCs can be identified based on expression of cell cytoskeleton proteins such as Nestin, Vimentin and α-SMA, cell surface antigens such as CD29, CD44, CD73 and CD90, lack of expression of CD49 and ability to differentiate into chondrocytes, osteoblasts, and adipocytes when exposed to selective media (Dominici *et al.*, 2006).

Cell lines derived from the exocrine fraction of human pancreatic tissue (CHI-pMSCs) and described in Chapter 3 had morphological characteristics of mesenchymal stem cells, expressed Nestin, Vimentin and αSMA when tested by immunocytochemistry. These cells also expressed the cell surface markers CD29, CD44, CD73 and CD90 when analysed by flow cytometry. Additionally, expression of CD45 was not detected when tested by flow cytometry. CHI-pMSCs were also successfully differentiated into chondrocytes, osteoblasts and adipocytes.

Following confirmation of the "stem-cell like" nature of CHI-pMSCs this cell line were tested for its capacity to differentiate into pancreatic progenitor cells. Mesenchymal stem cells (CH-pMSC) were treated with Isx-9 compound and expression of PP cell- specific TFs was then tested at the mRNA and protein level. Isx-9 was previously shown to induce expression of islet-specific TFs in human islets maintained over prolonged time in cell culture (Dioum *et al.*, 2011), induce differentiation of fibroblasts to myofibroblasts (Velasquez *et al.*, 2013), neuroepithelial stem cells to sensory neurons (Ali *et al.*, 2016) and, in combination with other small-molecules, mouse fibroblast to neurons (Li *et al.*, 2015). This broad differentiation capacity of Isx-9 was linked with its ability to moderate activity of p300/CBP (Dioum *et al.*, 2011). CREB-binding protein (CBP) and p300 are involved in activation of hundreds of transcription factors by binding to the activation domain of a TF and due to its histone acetyltransferase capacity modulating chromatin activity (Vo and Goodman, 2001; Chan and La Thangue, 2001).

Presented here, results indicate that CHI-pMSCs treated with Isx-9 expressed TFs such as PDX1 and NEUROD1 at the mRNA level only; however the expression of those TFs was undetectable at the protein level when analysed by immunoblotting and immunocytochemistry. This lead to the conclusion that the effect of Isx-9 was limited to the transcriptional level only. Alternatively, it might also suggest the pro-endocrine effect of Isx-9 depends on the origin of the treated cells and is restricted to cells derived from islets.

A Step-wise differentiation protocol with several pro-endocrine compounds was also tested in addition to the Isx-9 based protocol. This protocol was adapted from results presented by Zanini et al. (2011) and involved a change from planar 2D cell growth conditions to spheroid cell culture. Zanini et al. reported that MSCs derived from human pancreatic islets expressed PDX1 at the protein level following treatment with conditioned media. However, the $PDX1^{+}$ cells comprised of only $\sim$ 15-20 % of the total treated cell population. Additionally, the protocol failed to induce PDX1 expression in MSCs derived from bone marrow. Here, the induction of key TFs expression in cells exposed to the conditioned media was not detected when tested with immunoblotting, suggesting a lack of translation at the protein level. This might indicate that further optimisation of this differentiation protocol is required.

7.2 Differentiation of iPS Cells towards Pancreatic Progenitor Cells

Several attempts to differentiate MSCs into PP cells failed to induce expression of the key TFs (PDX1 and NEUROD1) at the protein level. Therefore, a protocol recently developed by Razania et al. (2014) was used for the differentiation of iPS cell. Those iPS cells were derived from CH-pMSCs and showed expression of markers characteristic for the ES/iPScellssuch as OCT4, SOX2, NANOG, SSEA4 and TRA-1-60 .

Following iPS cell differentiation to PPs, the expression of TFs characteristic for those cells was verified by qRT-PCR, immunoblotting and immunohistochemistry. S4 cells expressed PDX1 at the protein level and based on that were assumed to be a population of pancreatic progenitors. S5 cells were $PDX1^+/NEUROD1^+/NKX6.1^+$ characteristic of pancreatic endocrine progenitors. Immunohistochemistry staining was also used to confirm nuclear localisation of PDX1, NEUROD1 and NKX6.1.

In addition, efficiency of this differentiation protocol was also estimated and for S2 and arose to $\sim$ 90 % $FOXA2^+/SOX17^+$ cells, for S4 $\sim$ 80 % PDX^+ and for S5 above 50 % for $PDX1^+$ and $NEUROD1^+$ but only 6.5 % for $NKX6.1^+$.

Moreover, an interesting pattern of CK19 and NESTIN expression was observed with $NESTIN^+$ cells forming a cluster adjacent to the majority of $CK19^+$ cells. Only a small number of cells showed co-expression of both CK19 and NESTIN. This pattern was also very similar to that observed for NKX6.1 cells, although further research is required to determine the exact nature of these $NESTIN^+$ cells.

7.3 Molecular Changes during Endocrine Progenitor Cell Development

Following differentiation of iPS cells towards pancreatic progenitor (S4) and endocrine progenitor cells (S5), total mRNA and proteins were extracted from S0 (undifferentiated iPS cells), S4 and S5 and used for cDNA array and mass spectrometry analysis. Transcriptomics and proteomics analysis of those cells revealed several processes specific to S4 and S5 cells, such as:

- **Changes in the expression of ECM components.** The most upregulated genes encoding ECM components were Collagen Type III α 1 Chain (COL3A1), Collagen Type II α 1 Chain (COL2A1), Laminin Subunit α 4, Laminin Subunit α 1, Vitronectin (VTN), Reelin (RELN) and Fibronectin

1 (FN1). Among the most upregulated ECM proteins were Collagen Type IV α 1 Chain (COL4A1) and Collagen Type XVIII α 1 Chain (COL18A1), Laminin α1 (LAMA1) and Fibronectin 1 (FN1). However, this study was designed to analyse proteins extracted from the membrane, cytoplasmic and nuclear cell fractions, whereas components of ECM are secreted proteins and this fraction was not collected for analysis in this study. Therefore, a more ECM protein-orientated study would be needed to verify this observation. This would address any discrepancy observed between gene expression at the mRNA and protein expression.

- **Changes in the expression of integrins.** In this study upregulation of integrin subunits α6, αv and β1 was detected at the protein level in S5 cells. Those subunits were previously linked with the development of the pancreas, however, their function in pancreatic progenitor cells requires further research. Additionally, several other genes encoding for integrin subunits were also upregulated at the mRNA level but not detected by mass spectrometry, this included; *ITGB8*, *ITGA8*, *ITGB6* and *ITGA1*. Expression of these integrins at the protein level also needs further verification.

- **Changes in the expression of EMT-related genes.** In addition to the upregulation of *NGN3* expression, which has been previously linked with the control of islet progenitor cell migration, genes encoding for *DCX*, *SNAIL2*, *RHOB*, *VIMENTIN* and *N-CADHERIN* were also upregulated at the mRNA level. Additionally, expression of VIMENTIN and N-CADHERIN were also increased at the protein level. The mechanism of pancreatic progenitor cell delamination, migration, and formation of islets is still unknown. Presented here protein expression profile was limited only to VIMENTIN and N-CADHERIN as its associated components, therefore further research is needed to elucidate other proteins involved in the formation of islet structures.

- **Changes in the expression of T-box transcription factors.** The expression of a transcription factor linked to cell migration, *TBX3*, was observed to be over 20-fold increased in S5 cells. Additionally, three other T-box TFs (*TBX20*, *TBX2* and *TBX5*) were also upregulated in S5 cell as well as several genes encoding for proteins linked to those TFs. The function of TBX2 during pancreas development was previously reported in *Xenopus* and mouse (Begum and Papaioannou, 2011). However, the function these TFs in the human pancreas and the downstream targets are still unknown. Moreover, changes in the expression of these TFs were only observed at the mRNA level in this study and have yet to be verified at the protein level.

- **Changes in the expression of *ROBO*, *NETRIN* and *DCC* signalling components.** Receptors involved in the control of axon guidance were shown here to have an altered expression pattern in S4 and S5 cells. Genes encoding for Myelin Protein Zero Like 2 (MPZL2), DCC Netrin 1 Receptor (DCC) and Unc-5 Netrin Receptor C (UNC5C) were upregulated over 7-fold in S4 and S5 cells and Roundabout Guidance Receptor 2 (ROBO2) expression in S4 cells was increased 4-fold compared to S0. Several other components linked to these receptors also had an altered expression pattern. It is possible that a similar mechanism to the one previously observed in axon guidance is involved in the pancreatic progenitor cell migration. However, the expression profile presented here is limited to mRNA expression and the expression of ROBO2, MPZL2, DCC and UNC5C is yet to be evaluated at a protein level.

In addition, several membrane proteins that could potentially serve as markers for pancreatic progenitor cells were also identified (Table 7.1). Those cell membrane proteins included receptors involved in cell migration, integrins, transporters and cell adhesion molecules. However, not all of the proteins listed in table 7.1 were detected by mass spectrometry, therefore verification of their expression at the protein level is still needed.

Table 7.1: List of putative pancreatic progenitor cell markers (ND- not detected).

Gene Name	Function	FC S4 mRNA	FC S5 mRNA	p val S4 mRNA	p val S5 mRNA	FC S4 protein	FC S5 protein	p val S4	p val S5
DCC	cell migration	9.38	11.07	1.1E-04	6.0E-05	ND	ND	*	*
ROBO2	cell migration	4.68	1.82	4.0E-10	6.4E-06	ND	ND	*	*
FLRT3	unknown	23.83	25.62	8.0E-13	6.2E-13	ND	ND	*	*
DSCAM	cell migration	4.04	2.05	6.7E-06	1.7E-03	ND	ND	*	*
UNC5C	cell migration	7.90	29.69	1.6E-07	9.4E-10	ND	ND	*	*
MPZL2	cell migration	9.03	7.32	8.8E-09	2.5E-08	ND	ND	*	*
MPZL3	cell migration	1.85	1.55	1.3E-04	1.7E-03	ND	ND	*	*
NEO1	cell migration	1.68	1.56	1.6E-05	6.0E-05	ND	ND	*	*
ITGB8	ECM-cytoskeleton signalling	6.60	7.91	1.1E-07	4.4E-08	ND	ND	*	*
ITGA8	ECM-cytoskeleton signalling	p$\geq$ 0.05	3.61	p$\geq$ 0.05	5.3E-04	ND	ND	*	*
ITGB6	ECM-cytoskeleton signalling	1.53	2.27	1.9E-02	2.7E-04	ND	ND	*	*
ITGA1	ECM-cytoskeleton signalling	p$\geq$ 0.05	1.81	p$\geq$ 0.05	2.1E-02	ND	ND	*	*
ALCAM	cell adhesion	2.59	2.47	3.8E-05	5.8E-05	1.72	2.42	1.05E-04	8.88E-04
ATP1B1	transporter	1.24	1.72	3.9E-02	1.0E-04	p$\geq$ 0.05	2.29	p $\geq$ 0.05	6.12E-03
ATP2B1	transporter	2.09	2.64	3.2E-06	2.1E-07	-1.12	2.41	1.60E-05	2.86E-03
BCAM	cell adhesion	2.74	1.93	2.1E-07	1.3E-05	-1.26	1.40	1.47E-03	5.35E-03
CADM1	cell adhesion	2.58	2.32	1.7E-05	5.0E-05	1.43	2.42	3.82E-04	1.63E-03

7.4 Limitations of this Study

The main limitations of this study are:

- *In vitro* model used for identification of markers. iPS cells differentiated towards $PDX1^+/NGN3^+/NEUROD1^+$ cells were analysed in comparison to undifferentiated cells. However, it remains to be established how well this mimics human pancreatic cell development.

- The efficiency of the differentiation protocol was estimated to ~ 50 %; thus suggesting a heterogeneous S5 cell population.

- Only a limited data was obtained by mass spectrometry. There was a discrepancy observed between changes detected at the mRNA level and protein level. Numerous genes showing over 5-fold upregulation in S4 and S5 cells at the mRNA level were not detected by this technique.

7.5 Clinical Application

Cell surface markers for pancreatic progenitor cells would allow for isolation cells from a tissue or a heterogeneous cell population without the need to disintegrate the cell membrane. Isolation of intact PPs would benefit the research into the generation of pancreatic β-cells and the treatment of diabetes. Such a solution for T1D treatment based on an implant containing immature cells derived from hESCs has recently been reported to reach the clinic in the STEP ONE Trial (Safety, Tolerability, and Efficacy of PEC-Encap Combination Product in Type One Diabetes) (http://viacyte.com/products/product-overview-pipeline/). However, only limited information is available regarding the state of the differentiated cells used for this implant, however, markers for PPs could be utilised to enhance this therapeutic approach.

PP cell surface markers could also allow for isolation of these cells from donor pancreatic tissue in addition to human pancreatic islets. Currently, islets collected

from 2-3 donors are required for each transplantation (McCall and James-Shapiro, 2012). However, if progenitor cells are present in the adult pancreas, they could be used to increase the pool of cells available for the transplantation.

7.6 Future Work

Future work would involve:

- Testing the expression of prospective cell surface markers listed in the table 7.1 at the protein level; first by immunoblotting to verify the expression profile between S0, S2, S4 and S5, and then by immunostaining to test their co-expression with transcription factors such as PDX1, NEUROD1 and NKX6.1.

- Testing expression of prospective markers by immunostaining on developing human pancreatic tissue sections.

- Testing new markers for isolation of pancreatic progenitor cells by flow cytometry or Dynabeads/MagniSort technology.

- An interesting expression pattern was observed for CK19 and NESTIN in S5 cells. Immunostaining to test NESTIN and NKX6.1, PDX1 and NEUROD1 would provide more information about the nature of those cells.

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Appendices

Appendix A

Reactome pathways	FE iTRAQ-MS/MS	PV iTRAQ-MS/MS	FE microarrays	PV microarrays
Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S (R-HSA-72662)	18.51	1.91E-32	N/A	N/A
Amino acid synthesis and interconversion (transamination) (R-HSA-70614)	8.52	0.0426	N/A	N/A
Apoptosis (R-HSA-109581)	4.05	0.0000561	N/A	N/A
Apoptotic execution phase (R-HSA-75153)	6.72	0.000709	N/A	N/A
Assembly of the pre-replicative complex (R-HSA-68867)	5.35	0.00289	N/A	N/A
Attenuation phase (R-HSA-3371568)	12	0.0247	N/A	N/A
AUF1 (hnRNP D0) binds and destabilizes mRNA (R-HSA-450408)	6.22	0.00157	N/A	N/A
Axon guidance (R-HSA-422475)	**2.77**	**8.17E-08**	**2.04**	**0.00000329**
Beta oxidation of decanoyl-CoA to octanoyl-CoA-CoA (R-HSA-77346)	28	0.00229	N/A	N/A
Beta oxidation of octanoyl-CoA to hexanoyl-CoA (R-HSA-77348)	28	0.00229	N/A	N/A
Cap-dependent Translation Initiation (R-HSA-72737)	16.94	3.35E-59	N/A	N/A
Cell Cycle (R-HSA-1640170)	2.63	0.000000512	N/A	N/A
Cell Cycle Checkpoints (R-HSA-69620)	3.21	0.00822	N/A	N/A
Cell Cycle, Mitotic (R-HSA-69278)	2.79	0.00000115	N/A	N/A
Cellular response to heat stress (R-HSA-3371556)	5.79	0.00000936	N/A	N/A
Cellular responses to stress (R-HSA-2262752)	2.43	0.0154	N/A	N/A
Cooperation of PDCL (PhLP1) and TRiC/CCT in G-protein beta folding (R-HSA-6814122)	7.33	0.000927	N/A	N/A
Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding (R-HSA-389958)	7.87	0.00564	N/A	N/A
Cytosolic tRNA aminoacylation (R-HSA-379716)	18.67	2.78E-12	N/A	N/A
Degradation of beta-catenin by the destruction complex (R-HSA-195253)	4.78	0.00426	N/A	N/A
Degradation of the extracellular matrix (R-HSA-1474228)	3.47	0.0245	N/A	N/A
Developmental Biology (R-HSA-1266738)	**2.12**	**0.000101**	**2.09**	**3.84E-11**
Disease (R-HSA-1643685)	3.61	3E-29	N/A	N/A
DNA Replication (R-HSA-69306)	4.67	0.00023	N/A	N/A
DNA Replication Pre-Initiation (R-HSA-69002)	4.61	0.0064	N/A	N/A
DNA strand elongation (R-HSA-69190)	8.75	0.00063	N/A	N/A
ECM proteoglycans (R-HSA-3000178)	**4.48**	**0.0412**	**3.18**	**0.0464**
Eukaryotic Translation Elongation (R-HSA-156842)	17.87	3.24E-50	N/A	N/A
Eukaryotic Translation Initiation (R-HSA-72613)	16.94	3.35E-59	N/A	N/A
Eukaryotic Translation Termination (R-HSA-72764)	17.76	3.45E-49	N/A	N/A
Extracellular matrix organization (R-HSA-1474244)	**2.97**	**0.000261**	**2.45**	**0.00000843**

Folding of actin by CCT/TriC (R-HSA-390450)	22.4	0.0000082	N/A	N/A
Formation of a pool of free 40S subunits (R-HSA-72689)	17.74	1.54E-53	N/A	N/A
Formation of Fibrin Clot (Clotting Cascade) (R-HSA-140877)	N/A	N/A	4.42	0.0229
Formation of the ternary complex, and subsequently, the 43S complex (R-HSA-72695)	19.76	8.9E-31	N/A	N/A
Formation of tubulin folding intermediates by CCT/TriC (R-HSA-389960)	10.08	0.000762	N/A	N/A
G1/S Transition (R-HSA-69206)	4.63	0.000119	N/A	N/A
G2/M Checkpoints (R-HSA-69481)	3.89	0.000416	N/A	N/A
G2/M Transition (R-HSA-69275)	3.33	0.0028	N/A	N/A
Gene Expression (R-HSA-74160)	3.37	2.98E-51	N/A	N/A
Gluconeogenesis (R-HSA-70263)	7	0.0454	N/A	N/A
Glucose metabolism (R-HSA-70326)	5.89	0.0000518	N/A	N/A
Glycolysis (R-HSA-70171)	10.27	0.0000336	N/A	N/A
GPCR downstream signaling (R-HSA-388396)	0.23	0.0000393	N/A	N/A
GTP hydrolysis and joining of the 60S ribosomal subunit (R-HSA-72706)	17.5	2.06E-58	N/A	N/A
Hemostasis (R-HSA-109582)	N/A	N/A	2.13	2.66E-08
HIV Infection (R-HSA-162906)	3.42	0.000111	N/A	N/A
Host Interactions of HIV factors (R-HSA-162909)	4.52	0.0000792	N/A	N/A
Infectious disease (R-HSA-5663205)	7.27	6.91E-44	N/A	N/A
Influenza Infection (R-HSA-168254)	12.4	1.17E-45	N/A	N/A
Influenza Life Cycle (R-HSA-168255)	13.19	1.54E-46	N/A	N/A
Influenza Viral RNA Transcription and Replication (R-HSA-168273)	13.47	1.47E-45	N/A	N/A
Integration of provirus (R-HSA-162592)	15.56	0.0384	N/A	N/A
Integrin cell surface interactions (R-HSA-216083)	N/A	N/A	3.43	0.0019
L13a-mediated translational silencing of Ceruloplasmin expression (R-HSA-156827)	17.41	2.19E-57	N/A	N/A
L1CAM interactions (R-HSA-373760)	3.9	0.011	N/A	N/A
Laminin interactions (R-HSA-3000157)	9.33	0.000352	N/A	N/A
M Phase (R-HSA-68886)	2.99	0.000368	N/A	N/A
M/G1 Transition (R-HSA-68874)	4.61	0.0064	N/A	N/A
Major pathway of rRNA processing in the nucleolus and cytosol (R-HSA-6791226)	9.61	6.19E-34	N/A	N/A
Metabolism (R-HSA-1430728)	**3.31**	**1.47E-58**	**1.53**	**3.25E-07**
Metabolism of amino acids and derivatives (R-HSA-71291)	7.89	4.21E-50	N/A	N/A
Metabolism of carbohydrates (R-HSA-71387)	3.51	0.000000647	N/A	N/A
Metabolism of fat-soluble vitamins (R-HSA-6806667)	N/A	N/A	4.08	0.00659
Metabolism of nucleotides (R-HSA-15869)	5.29	0.0000901	N/A	N/A
Metabolism of polyamines (R-HSA-351202)	4.33	0.0269	N/A	N/A
Metabolism of proteins (R-HSA-392499)	3.37	9.05E-39	N/A	N/A
Metabolism of vitamins and cofactors (R-HSA-196854)	N/A	N/A	2.72	0.00278
Metallothioneins bind metals (R-HSA-5661231)	N/A	N/A	10.84	0.000428
Mitochondrial Fatty Acid Beta-Oxidation (R-HSA-77289)	13.18	0.00046	N/A	N/A
mitochondrial fatty acid beta-oxidation of saturated fatty acids (R-HSA-77286)	21	0.00103	N/A	N/A
Mitotic G1-G1/S phases (R-HSA-453279)	4.03	0.000475	N/A	N/A
Mitotic G2-G2/M phases (R-HSA-453274)	3.29	0.00332	N/A	N/A
Mitotic Prophase (R-HSA-68875)	3.84	0.0474	N/A	N/A
mRNA Splicing - Major Pathway (R-HSA-72163)	5.63	1.41E-12	N/A	N/A
mRNA Splicing - Minor Pathway (R-HSA-72165)	5.38	0.0429	N/A	N/A
mRNA Splicing (R-HSA-72172)	5.38	5.2E-12	N/A	N/A
Muscle contraction (R-HSA-397014)	N/A	N/A	2.62	0.000144
Non-integrin membrane-ECM interactions (R-HSA-3000171)	6.17	0.000611	4.27	0.000432
Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC) (R-HSA-975957)	15.86	1.38E-50	N/A	N/A
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC) (R-HSA-975956)	17.68	5.93E-50	N/A	N/A
Nonsense-Mediated Decay (NMD) (R-HSA-927802)	15.86	1.38E-50	N/A	N/A
Nuclear import of Rev protein (R-HSA-180746)	7.47	0.0289	N/A	N/A
Olfactory Signaling Pathway (R-HSA-381753)	N/A	N/A	¡ 0.2	0.000000127
Orc1 removal from chromatin (R-HSA-68949)	5.13	0.0046	N/A	N/A
Peptide chain elongation (R-HSA-156902)	18.25	5.88E-49	N/A	N/A
Phase II conjugation (R-HSA-156580)	N/A	N/A	2.94	0.0203
Platelet activation, signaling and aggregation (R-HSA-76002)	N/A	N/A	2.41	0.0000519
Platelet degranulation (R-HSA-114608)	N/A	N/A	2.94	0.00141

Post-translational protein modification (R-HSA-597592)	1.86	0.0196	N/A	N/A
POU5F1 (OCT4), SOX2, NANOG activate genes related to proliferation (R-HSA-2892247)	N/A	N/A	12.14	0.00000635
Prefoldin mediated transfer of substrate to CCT/TriC (R-HSA-389957)	9.33	0.00143	N/A	N/A
Processing of Capped Intron-Containing Pre-mRNA (R-HSA-72203)	5.01	2.36E-13	N/A	N/A
Programmed Cell Death (R-HSA-5357801)	3.98	0.0000781	N/A	N/A
Purine metabolism (R-HSA-73847)	7.41	0.00914	N/A	N/A
Purine ribonucleoside monophosphate biosynthesis (R-HSA-73817)	16.33	0.000628	N/A	N/A
Regulation of beta-cell development (R-HSA-186712)	N/A	N/A	5.94	0.000967
Regulation of DNA replication (R-HSA-69304)	4.79	0.00949	N/A	N/A
Regulation of HSF1-mediated heat shock response (R-HSA-3371453)	5.01	0.0138	N/A	N/A
Regulation of mRNA stability by proteins that bind AU-rich elements (R-HSA-450531)	4.23	0.0343	N/A	N/A
Removal of licensing factors from origins (R-HSA-69300)	4.99	0.00619	N/A	N/A
Response to elevated platelet cytosolic Ca2+ (R-HSA-76005)	N/A	N/A	3.03	0.000312
Response to metal ions (R-HSA-5660526)	N/A	N/A	10.84	0.000428
Retinoid metabolism and transport (R-HSA-975634)	N/A	N/A	4.93	0.000588
RHO GTPase Effectors (R-HSA-195258)	2.81	0.00459	N/A	N/A
Ribosomal scanning and start codon recognition (R-HSA-72702)	18.83	1.01E-32	N/A	N/A
rRNA processing (R-HSA-72312)	8.99	3.22E-34	N/A	N/A
rRNA processing in the nucleus and cytosol (R-HSA-8868773)	9.33	1.68E-34	N/A	N/A
S Phase (R-HSA-69242)	4.16	0.000593	N/A	N/A
Selenoamino acid metabolism (R-HSA-2408522)	16.07	1.32E-52	N/A	N/A
Selenocysteine synthesis (R-HSA-2408557)	17.04	1.3E-45	N/A	N/A
Semaphorin interactions (R-HSA-373755)	5.01	0.0138	N/A	N/A
SeMet incorporation into proteins (R-HSA-2408517)	20.36	0.000017	N/A	N/A
Signaling by EGFR (R-HSA-177929)	N/A	N/A	1.93	0.03
Signaling by FGFR1 (R-HSA-5654736)	N/A	N/A	1.93	0.0465
Signaling by PDGF (R-HSA-186797)	N/A	N/A	2.02	0.00323
SRP-dependent cotranslational protein targeting to membrane (R-HSA-1799339)	15	6.65E-46	N/A	N/A
Switching of origins to a post-replicative state (R-HSA-69052)	5.13	0.0046	N/A	N/A
Syndecan interactions (R-HSA-3000170)	N/A	N/A	5.4	0.0171
Synthesis of DNA (R-HSA-69239)	5.04	0.0000749	N/A	N/A
The citric acid (TCA) cycle and respiratory electron transport (R-HSA-1428517)	3.26	0.0186	N/A	N/A
The role of GTSE1 in G2/M progression after G2 checkpoint (R-HSA-8852276)	4.73	0.0109	N/A	N/A
Transcriptional regulation of pluripotent stem cells (R-HSA-452723)	N/A	N/A	5.46	0.000974
Translation (R-HSA-72766)	14.8	4E-64	N/A	N/A
Translation initiation complex formation (R-HSA-72649)	18.83	1.01E-32	N/A	N/A
Transmission across Chemical Synapses (R-HSA-112315)	N/A	N/A	2.43	0.00182
tRNA Aminoacylation (R-HSA-379724)	12	8.34E-11	N/A	N/A
Viral mRNA Translation (R-HSA-192823)	17.62	2.24E-46	N/A	N/A

Appendix B

Table B.1: Pathway identified with IPA; cDNA arrays.

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
FXR/RXR Activation	1.62E-10	50	122	41%	72	122	59%
Acute Phase Response Signaling	5.01E-08	75	167	45%	92	167	55%
Coagulation System	7.08E-08	9	35	26%	26	35	74%
LXR/RXR Activation	3.72E-07	53	118	45%	65	118	55%
LPS/IL-1 Mediated Inhibition of RXR Function	3.8E-07	99	206	48%	106	206	51%
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	3.98E-07	7	29	24%	22	29	76%
Xenobiotic Metabolism Signaling	4.37E-07	136	259	53%	122	259	47%
Transcriptional Regulatory Network in Embryonic Stem Cells	5.01E-07	24	40	60%	16	40	40%
Axonal Guidance Signaling	5.89E-07	205	439	47%	232	439	53%
Atherosclerosis Signaling	7.41E-07	65	122	53%	57	122	47%
Superpathway of Melatonin Degradation	1.35E-06	19	55	35%	36	55	65%
Melatonin Degradation I	1.86E-06	16	50	32%	34	50	68%
Clathrin-mediated Endocytosis Signaling	2E-06	99	194	51%	93	194	48%
Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.24E-06	68	181	38%	113	181	62%
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	1.45E-05	25	45	56%	20	45	44%

Table B.1: Pathway identified with IPA; cDNA arrays (continued).

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
G?12/13 Signaling	1.95E-05	59	128	46%	69	128	54%
Embryonic Stem Cell Differentiation into Cardiac Lineages	2.63E-05	6	10	60%	4	10	40%
Human Embryonic Stem Cell Pluripotency	2.75E-05	59	139	42%	80	139	58%
IL-12 Signaling and Production in Macrophages	4.57E-05	64	143	45%	79	143	55%
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	5.37E-05	56	119	47%	63	119	53%
Extrinsic Prothrombin Activation Pathway	7.41E-05	1	16	6%	15	16	94%
Factors Promoting Cardiogenesis in Vertebrates	7.59E-05	38	89	43%	51	89	57%
Nitric Oxide Signaling in the Cardiovascular System	9.77E-05	58	107	54%	49	107	46%
Signaling by Rho Family GTPases	0.00011	124	243	51%	118	243	49%
Intrinsic Prothrombin Activation Pathway	0.00012	7	28	25%	21	28	75%
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.000166	97	191	51%	94	191	49%
Serotonin Degradation	0.000251	23	58	40%	35	58	60%
Thrombin Signaling	0.000309	104	198	53%	93	198	47%
PXR/RXR Activation	0.000427	27	61	44%	34	61	56%
Nicotine Degradation II	0.000603	17	48	35%	31	48	65%
Dermatan Sulfate Biosynthesis (Late Stages)	0.000631	22	41	54%	19	41	46%
STAT3 Pathway	0.000692	40	72	56%	32	72	44%
RhoGDI Signaling	0.000724	90	170	53%	79	170	46%
Nicotine Degradation III	0.000776	15	42	36%	27	42	64%
PAK Signaling	0.000776	43	98	44%	55	98	56%
Cellular Effects of Sildenafil (Viagra)	0.000832	57	125	46%	68	125	54%
ILK Signaling	0.000851	84	191	44%	106	191	55%
Dopamine Degradation	0.000912	12	29	41%	17	29	59%
Glutathione-mediated Detoxification	0.001	8	23	35%	14	23	61%
Chondroitin Sulfate Biosynthesis (Late Stages)	0.001148	22	44	50%	22	44	50%
Wnt/?-catenin Signaling	0.001175	82	166	49%	83	166	50%
VEGF Family Ligand-Receptor Interactions	0.001318	45	85	53%	40	85	47%

Table B.1: Pathway identified with IPA; cDNA arrays (continued).

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
NRF2-mediated Oxidative Stress Response	0.001349	91	187	49%	95	187	51%
Ephrin Receptor Signaling	0.001622	79	170	46%	90	170	53%
Dermatan Sulfate Biosynthesis	0.001698	29	54	54%	25	54	46%
Maturity Onset Diabetes of Young (MODY) Signaling	0.001905	5	19	26%	14	19	74%
Heparan Sulfate Biosynthesis (Late Stages)	0.002344	31	56	55%	25	56	45%
Glioma Signaling	0.002344	52	108	48%	55	108	51%
Role of Tissue Factor in Cancer	0.002512	58	118	49%	59	118	50%
Synaptic Long Term Depression	0.002818	92	138	67%	46	138	33%
p53 Signaling	0.002884	59	110	54%	50	110	45%
Tec Kinase Signaling	0.003236	92	159	58%	66	159	42%
Calcium Signaling	0.003236	84	169	50%	83	169	49%
VDR/RXR Activation	0.003548	40	76	53%	36	76	47%
Mouse Embryonic Stem Cell Pluripotency	0.00389	52	104	50%	52	104	50%
ERK/MAPK Signaling	0.003981	109	192	57%	83	192	43%
Chondroitin Sulfate Biosynthesis	0.004266	27	52	52%	25	52	48%
G Beta Gamma Signaling	0.004467	51	87	59%	35	87	40%
Sperm Motility	0.005012	69	116	59%	46	116	40%
Growth Hormone Signaling	0.005623	43	80	54%	37	80	46%
eNOS Signaling	0.005754	87	147	59%	59	147	40%
Heparan Sulfate Biosynthesis	0.006026	36	63	57%	27	63	43%
Phospholipase C Signaling	0.006761	129	221	58%	91	221	41%
CXCR4 Signaling	0.007762	85	161	53%	75	161	47%
Aryl Hydrocarbon Receptor Signaling	0.008318	69	132	52%	62	132	47%
G-Protein Coupled Receptor Signaling	0.00871	144	268	54%	124	268	46%
IL-8 Signaling	0.008913	103	194	53%	89	194	46%
Circadian Rhythm Signaling	0.00912	19	33	58%	14	33	42%
EGF Signaling	0.00955	32	67	48%	35	67	52%

Table B.1: Pathway identified with IPA; cDNA arrays (continued).

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
Role of NFAT in Cardiac Hypertrophy	0.010233	101	186	54%	84	186	45%
Cardiomyocyte Differentiation via BMP Receptors	0.010471	6	19	32%	13	19	68%
Oncostatin M Signaling	0.010965	21	34	62%	13	34	38%
cAMP-mediated signaling	0.01122	120	219	55%	99	219	45%
TR/RXR Activation	0.011749	47	97	48%	50	97	52%
Relaxin Signaling	0.012023	73	147	50%	73	147	50%
Renin-Angiotensin Signaling	0.012303	61	117	52%	56	117	48%
Macropinocytosis Signaling	0.012882	38	79	48%	41	79	52%
PTEN Signaling	0.013183	63	118	53%	54	118	46%
Endothelin-1 Signaling	0.01349	101	180	56%	79	180	44%
p70S6K Signaling	0.014125	75	129	58%	54	129	42%
Thyronamine and Iodothyronamine Metabolism	0.015488	1	3	33%	2	3	67%
Thyroid Hormone Metabolism I (via Deiodination)	0.015488	1	3	33%	2	3	67%
Reelin Signaling in Neurons	0.015849	48	91	53%	43	91	47%
Ephrin B Signaling	0.016218	38	72	53%	33	72	46%
GM-CSF Signaling	0.016218	41	72	57%	31	72	43%
Agranulocyte Adhesion and Diapedesis	0.016596	81	173	47%	92	173	53%
IL-3 Signaling	0.016982	47	82	57%	35	82	43%
Neuropathic Pain Signaling In Dorsal Horn Neurons	0.017783	56	112	50%	56	112	50%
Thrombopoietin Signaling	0.018197	34	64	53%	30	64	47%
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.019498	137	296	46%	159	296	54%
IGF-1 Signaling	0.020893	53	104	51%	51	104	49%
Corticotropin Releasing Hormone Signaling	0.023988	57	106	54%	49	106	46%
FAK Signaling	0.023988	40	96	42%	55	96	57%
Dopamine-DARPP32 Feedback in cAMP Signaling	0.023988	97	158	61%	61	158	39%
CREB Signaling in Neurons	0.025119	100	180	56%	79	180	44%
CNTF Signaling	0.025704	28	58	48%	30	58	52%

Table B.1: Pathway identified with IPA; cDNA arrays (continued).

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
HER-2 Signaling in Breast Cancer	0.025704	45	87	52%	42	87	48%
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.026915	97	225	43%	128	225	57%
Role of JAK2 in Hormone-like Cytokine Signaling	0.027542	23	32	72%	9	32	28%
Paxillin Signaling	0.027542	45	108	42%	63	108	58%
Ephrin A Signaling	0.028184	26	59	44%	33	59	56%
Bupropion Degradation	0.028184	10	24	42%	14	24	58%
Uracil Degradation II (Reductive)	0.029512	1	4	25%	3	4	75%
Amyotrophic Lateral Sclerosis Signaling	0.029512	48	109	44%	61	109	56%
VEGF Signaling	0.0302	50	99	51%	49	99	49%
Regulation of the Epithelial-Mesenchymal Transition Pathway	0.030903	82	184	45%	102	184	55%
Semaphorin Signaling in Neurons	0.032359	29	51	57%	22	51	43%
Ubiquinol-10 Biosynthesis (Eukaryotic)	0.032359	5	17	29%	12	17	71%
Actin Cytoskeleton Signaling	0.033884	109	219	50%	110	219	50%
FLT3 Signaling in Hematopoietic Progenitor Cells	0.035481	41	81	51%	40	81	49%
RAR Activation	0.036308	97	187	52%	89	187	48%
Prolactin Signaling	0.038019	44	82	54%	38	82	46%
HGF Signaling	0.038905	55	113	49%	58	113	51%
Acetone Degradation I (to Methylglyoxal)	0.038905	11	26	42%	15	26	58%
Protein Kinase A Signaling	0.042658	186	374	50%	186	374	50%
UVB-Induced MAPK Signaling	0.044668	31	64	48%	33	64	52%
NF-?B Signaling	0.045709	87	170	51%	83	170	49%
ErbB Signaling	0.046774	42	95	44%	53	95	56%
Citrulline-Nitric Oxide Cycle	0.046774	3	5	60%	2	5	40%
Putrescine Degradation III	0.046774	10	19	53%	9	19	47%
Leukocyte Extravasation Signaling	0.047863	108	204	53%	96	204	47%
Calcium-induced T Lymphocyte Apoptosis	0.047863	39	55	71%	16	55	29%
Neuregulin Signaling	0.047863	42	85	49%	42	85	49%

Table B.1: Pathway identified with IPA; cDNA arrays (continued).

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
NF-?B Activation by Viruses	0.047863	45	85	53%	40	85	47%
GDNF Family Ligand-Receptor Interactions	0.048978	35	75	47%	40	75	53%
Synaptic Long Term Potentiation	0.050119	66	117	56%	51	117	44%

Table B.2: Pathways identified with IPA; iTRAQ-MS/MS arrays.

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
Purine Nucleotides De Novo Biosynthesis II	2.23872E-06	8	9	89%	0	9	0%
Regulation of eIF4 and p70S6K Signaling	3.46737E-06	52	86	60%	8	86	9%
mTOR Signaling	4.16869E-05	47	86	55%	9	86	10%
EIF2 Signaling	5.88844E-05	92	135	68%	11	135	8%
LXR/RXR Activation	0.000630957	5	27	19%	7	27	26%
5-aminoimidazole Ribonucleotide Biosynthesis I	0.000891251	3	3	100%	0	3	0%
UDP-N-acetyl-D-galactosamine Biosynthesis II	0.001096478	3	6	50%	2	6	33%
ILK Signaling	0.001380384	16	67	24%	17	67	25%
tRNA Charging	0.002454709	19	32	59%	4	32	13%
Gluconeogenesis I	0.003630781	9	12	75%	1	12	8%
Valine Degradation I	0.003801894	1	17	6%	8	17	47%
Telomerase Signaling	0.003801894	9	28	32%	5	28	18%
FXR/RXR Activation	0.004570882	2	23	9%	6	23	26%
Ceramide Signaling	0.004570882	6	23	26%	4	23	17%
Isoleucine Degradation I	0.005370318	2	13	15%	7	13	54%
Xenobiotic Metabolism Signaling	0.006025596	14	63	22%	18	63	29%
LPS/IL-1 Mediated Inhibition of RXR Function	0.006456542	5	43	12%	18	43	42%
Ketogenesis	0.00724436	2	9	22%	4	9	44%
Glycolysis I	0.007762471	8	14	57%	3	14	21%
Trehalose Degradation II (Trehalase)	0.009332543	2	2	100%	0	2	0%
Aryl Hydrocarbon Receptor Signaling	0.010471285	7	46	15%	20	46	43%
Mevalonate Pathway I	0.011220185	2	10	20%	4	10	40%
Oxidative Ethanol Degradation III	0.011220185	0	10	0%	8	10	80%
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.011748976	0	27	0%	10	27	37%
eNOS Signaling	0.013489629	12	34	35%	6	34	18%
Parkinson's Signaling	0.014125375	5	6	83%	1	6	17%
Role of CHK Proteins in Cell Cycle Checkpoint Control	0.015135612	9	22	41%	5	22	23%

Table B.2: Pathways identified with IPA; iTRAQ-MS/MS arrays (continued).

Pathway name	p-val	Downreg.	Total	% Downreg.	Upreg.	Total	% Upreg.
Superpathway of Cholesterol Biosynthesis	0.015135612	6	22	27%	8	22	36%
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	0.016218101	3	11	27%	4	11	36%
Atherosclerosis Signaling	0.019054607	1	17	6%	8	17	47%
Breast Cancer Regulation by Stathmin1	0.020417379	14	73	19%	18	73	25%
Ethanol Degradation IV	0.022387211	0	12	0%	9	12	75%
Triacylglycerol Degradation	0.022908677	2	7	29%	1	7	14%
autophagy	0.023988329	3	18	17%	5	18	28%
CDK5 Signaling	0.025118864	8	31	26%	11	31	35%
Arsenate Detoxification I (Glutaredoxin)	0.02630268	1	3	33%	1	3	33%
Arginine Biosynthesis IV	0.02630268	1	3	33%	1	3	33%
Lysine Degradation II	0.02630268	2	3	67%	1	3	33%
Inosine-5'-phosphate Biosynthesis II	0.02630268	2	3	67%	0	3	0%
Rapoport-Luebering Glycolytic Shunt	0.02630268	1	3	33%	1	3	33%
Mitotic Roles of Polo-Like Kinase	0.028183829	10	25	40%	3	25	12%
Pentose Phosphate Pathway	0.034673685	3	8	38%	1	8	13%
Histamine Degradation	0.034673685	0	8	0%	7	8	88%
Putrescine Degradation III	0.034673685	0	8	0%	7	8	88%
AMPK Signaling	0.036307805	18	63	29%	13	63	21%
p70S6K Signaling	0.038904514	12	41	29%	8	41	20%
Hypoxia Signaling in the Cardiovascular System	0.039810717	8	27	30%	3	27	11%
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.041686938	9	49	18%	11	49	22%
Dopamine Degradation	0.047863009	0	9	0%	7	9	78%
Fatty Acid α-oxidation	0.047863009	0	9	0%	7	9	78%
Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate	0.048977882	4	4	100%	0	4	0%
Pentose Phosphate Pathway (Non-oxidative Branch)	0.048977882	2	4	50%	0	4	0%
Fatty Acid β-oxidation I	0.05370318	1	22	5%	10	22	45%