Reprogramming of Different Cell Types into Pancreatic Beta Cells by Using Transcription Factor Genes *Pdx1, Ngn3* and *MafA*

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Dedication

This thesis is dedicated to my father Nazmi Akinci.

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A. MAIN INTRODUCTION

1. The Pancreas

The pancreas is an endoderm derived glandular organ that is located in the abdominal cavity. It is made up of two different tissue types called exocrine and endocrine pancreas, both of which have different functions in the body. The exocrine part of the pancreas comprises the majority of the pancreatic mass (~90%) while endocrine part makes up only a small proportion of it (~2%) (Githens 1988). Three different cell types, termed acinar, centroacinar and pancreatic ductal cells, form the exocrine pancreas (Figure 1A). Acinar cells, the major cell population in the exocrine pancreas, secrete digestive enzymes (i.e proteases, amylase, lipase and nucleases) many of which are not active until they reach the digestive tract (Slack 1995) thereby protecting the pancreas from hydrolytic damage. Pancreatic ducts both transport these acinar cell digestive enzymes into the digestive tract and secrete bicarbonate ions which regulate the pH level in the duodenum. Centroacinar cells, which comprise the minor cell population in the exocrine pancreas, connect the acini (cluster of acinar cells) with pancreatic ducts. The endocrine part of the pancreas, on the other hand, is composed of five different cell types each of which can secrete different hormones directly into the blood stream (Figure 1B). These endocrine cells are insulin producing beta (β) cells, glucagon producing alpha (α) cells, somatostatin producing delta (δ) cells, pancreatic polypeptide producing (Pp) cells and ghrelin producing epsilon (ε) cells. Endocrine cell hormones are mainly involved in carbohydrate metabolism (Rovasio 2010). Insulin (hypoglycemic hormone), for instance, stimulates the muscle and adipose tissue cells to take up the glucose from the blood

stream. Glucagon (hyperglycemic hormone), on the other hand, has an opposite effect to that of insulin. It causes the conversion of glycogen stored in the liver into glucose which is then released into the blood stream. Somatostatin is synthesized and secreted by delta cells in pancreas, neuroendocrine cells in the central nervous system and cells in the gastrointestinal system (Reichlin 1983; Reichlin 1983). Consistent with its secretion from different cell types, somatostatin has a broad effect throughout the body mainly as an inhibitor of endocrine systems (Hauge-Evans, King et al. 2009) such as suppression of growth hormone secretion from the anterior pituitary (Spencer, Berry et al. 1991) and suppression of gastrointestinal peptide hormone release (Low 2004). In the pancreas somatostatin ensures the inhibition of insulin and glucagon release via a paracrine secretion (Rovasio 2010). Pancreatic polypeptide provides an inhibitory feedback mechanism for exocrine pancreatic secretion after meals (Lonovics, Devitt et al. 1981). Ghrelin hormone is mainly produced by stomach cells and epsilon cells in pancreas to a lesser extent. Beside its other roles such as stimulation of appetite, effects of ghrelin on beta cell development, function and survival have also been reviewed (Granata, Baragli et al. 2010).

The endocrine cells are organized as cell clusters which are called islets of Langerhans (Langerhans 1868) and these islets locate in close proximity to capillaries. Although the cytoarchitecture of islets depends on the animal species, they are scattered throughout the exocrine pancreas in the mammals (Gregoire and Bendayan 1986) more located in the tail than in the body and head of the human pancreas (Rovasio 2010). In rodents, beta cells tend to locate at the core of the islets and be surrounded by the other endocrine cell types. In humans, however, beta cells are distributed heterogeneously

throughout the islet. The number of beta cells in human islets is also proportionately less than those found in rodents (Cabrera, Berman et al. 2006). In a rodent islet, beta cells (~65-80%) are the main cell population, followed by alpha cells (~15-20%), delta cells (3-10%), Pp cells (3-5%) and epsilon cells (1%), respectively.

2. Pancreas Development

In mammals, after fertilization, the fertilized egg (ovum) undergoes a series of cell divisions with the result that a mass of cells is formed within the zona striata. The resulting structure is called a morula and the cells forming the morula are termed blastomeres. The cells of the morula are then arranged into an outer cell layer (trophectoderm) and an inner cell mass (ICM). This structure is called a blastocyst in which trophectoderm cells will develop to placenta and ICM will develop to embryo proper. The next developmental stage is termed gastrulation in which through the movement of cells, three embryonic layers (endoderm, ectoderm and mesoderm) are formed resulting in a structure called gastrula (Gray and Williams 1989). Before organogenesis, digestive track endoderm is regionalized into organ specific domains via anterior-posterior and dorsal-ventral patterning events which are driven by the inductive signals from adjacent mesodermal structures. During organogenesis, the pancreas develops as two separate protrusions from foregut endoderm that are surrounded by mesenchyme. These early pancreatic protrusions are called pancreatic ventral and dorsal buds, both of which fuse together to give rise to an intact pancreas (Figure 2). Formation of the pancreas starts with the condensation of mesenchyme that is lying on top of the dorsal foregut endoderm (Gittes 2009). This mesenchymal condensation is followed by

evagination of forget endoderm into the mesenchyme giving rise to the pancreatic dorsal bud (mouse 9.5 days post coitum (dpc)) (Gittes 2009). Inductive signals from the notochord, dorsal aorta and surrounding mesoderm are necessary for pancreatic dorsal bud formation (Neoptolemos 2010). Through these signals, activin-β_B and fibroblast growth factor 2 (FGF2) from the notochord suppress endodermal S*onic hedgehog* (*Shh*) expression thereby allowing pancreatic dorsal bud formation (Hebrok, Kim et al. 1998). Otherwise hedgehog signaling prevents pancreas development by repressing early expression of *Pancreatic and duodenal homeobox 1* (*Pdx1*) gene (Apelqvist, Ahlgren et al. 1997). Signals from endothelium of the dorsal aorta were also shown to be important for pancreatic dorsal bud development (Lammert, Cleaver et al. 2001). After the formation of dorsal bud, the pancreatic ventral bud arises by evagination of foregut endoderm underneath of hepatic bud (Gittes 2009). Following the bud formation these dorsal and ventral pancreatic buds keep forming new protrusions (mouse 10.5-11.5 dpc) resulting in highly branched structures under the control of proliferative signals from pancreatic mesenchyme (Slack 1995). The formation of dorsal and ventral pancreatic buds is morphologically similar but the signals which are controlling the bud formation are different. The absence of notochord, for instance, does not affect the ventral pancreas formation (Kim, Hebrok et al. 1997). Instead, lateral plate mesoderm (LPM) is necessary for pancreatic ventral bud formation (Kumar, Jordan et al. 2003). Signals from LPM are not identified yet but activin, bone morphogenetic protein 4 (BMP4) or retinoic acid (RA) are considered possible candidates (Kumar, Jordan et al. 2003). The pancreatic ventral bud arises from a region under the liver competent region (mouse 10 dpc). FGF signaling from cardiac mesoderm favors hepatic bud formation over pancreatic ventral bud

formation (Jung, Zheng et al. 1999). The septum transversum was also shown to have an effect in favor of liver formation over ventral pancreas formation via BMP signaling (Rossi, Dunn et al. 2001). On the other hand, a proper balance of BMP is necessary for pancreatic ventral bud formation since its complete absence was shown to prevent ventral bud formation (Kumar, Jordan et al. 2003). The next step is the coiling movement of the digestive track endoderm (mouse 11.5 dpc) which brings two pancreatic buds in close proximity to fuse and form an intact pancreas (mouse 12.5 dpc) (Pan and Wright 2011).

Pancreas development in rodents is divided into two stages. The first stage is called the primary transition (mouse 9.5-12.5 dpc, not seen in human) in which the first differentiated endocrine cells appear in the pancreatic dorsal bud. In this stage, pancreatic progenitor cells proliferate and form the multilayered epithelium. These cells are called primary pancreatic multi-potent progenitor cells (1° MPC) which can give rise to all pancreatic cell lineages. During the primary transition glucagon-producing alpha cells are the only endocrine cell line that originates from 1° MPCs. Initially all these endocrine cells are glucagon positive, then some of them become both glucagon and insulin positive and finally some of them turn into insulin positive only. Still a majority of them are only positive for glucagon (Cleaver and MacDonald 2010). These early endocrine cells are different from their mature counterparts and the functional importance of these cells is still unknown. Many microlumen structures are also formed during the primary transition. After the primary transition and fusion of buds, the pancreatic epithelium keeps expanding and is remodeled into a more complex plexus structure. Meanwhile the pancreatic epithelium is segregated into `tip` and `trunk` domains in which tip domains

include secondary pancreatic multipotent progenitor cells (2° MPC) and trunk domains include endocrine/duct bipotential progenitor cells.

The second stage is called the secondary transition (mouse 13.5-16.5 dpc) in which a massive wave of endocrine cell formation is seen. The endocrine cells formed at this stage are distinct from those formed in the primary transition (Cleaver and MacDonald 2010). In this stage formation of three main pancreatic lineages occurs: acinar cells, ductal cells and endocrine cells. Levels of insulin hormone and acinar enzymes are exponentially increased at this stage (Pictet R 1972). Acinar cells are differentiated from 2° MPCs at the tip domains (mouse 14.5 dpc) during the secondary transition and keep proliferating to increase the acinar cell mass after the secondary transition. During the secondary transition endocrine cells also differentiate from bipotent progenitor cells left behind in the trunk region as the tip region continues to grow (Zhou, Law et al. 2007). These endocrine cell precursors leave the trunk and aggregate into endocrine cell clusters, which are called islets of Langerhans, by birth (Piper, Brickwood et al. 2004). The exact mechanism of this cell escape from the trunk epithelium is not well understood and two different cellular processes have been proposed: a) epithelial-to-mesenchymal transition (EMT); and b) orthogonal cell division. In the EMT model, the mesenchymal state of escaped cells should be transient since they have to return back to an epithelial state before endocrine differentiation. The only support for this model is expression of S*nail homolog 2* (*Snail2*) in escaped cells after *Neurogenin 3* (*Ngn3*) expression (Rukstalis and Habener 2007). Snail2 is a close paralog of Snail1 which is the master regulator for EMT, working by repressing *E-cadherin* expression as well as other epithelial characteristics (del Barrio and Nieto 2002; Katoh and Katoh 2006). On the

other hand, according to the orthogonal cell division model, endocrine progenitor cells can change their cell division axis and start to divide parallel to the epithelial lumen (Pictet R 1972). In this model, asymmetric cell division retains one progenitor daughter cell in the epithelium (which may keep forming new endocrine progenitors or differentiate into ductal cells) and releases the other one out the epithelial tube. After birth, differentiated endocrine and exocrine cells start to proliferate, maintaining tissue growth in parallel with other embryonic organs. Postnatal replication of beta cells slows and stops until weaning. A distinguishable mature islet structure, in which a beta cell core is surrounded by other endocrine cells, is seen during the first few weeks after birth. Development of the early pancreas is summarized in Figure 3.

3. Gene Expression Patterns during Pancreas Development and Lineage Allocation

In mice at 8.5 dpc uncommitted prepancreatic foregut endoderm expresses *Pdx1*, *Motor neuron and pancreas homeobox* (*Mnx1*) (also known as *Hlxb9*), *Hepatocyte nuclear factor 1 beta* (*Hnf1b*) and *Forkhead box A2* (*FoxA2*) (also known as *Hnf3b*), *GATA binding protein 4* (*Gata4*), and *GATA binding protein 6* (*Gata6*). *Mnx1* is one of the earliest genes expressed during pancreas development and later then in the mature beta cells. Its expression is essential for pancreatic dorsal bud formation (Li, Arber et al. 1999). Unlike the dorsal bud, pancreatic ventral bud can develop in the absence of MNX1 but produces less beta cells than usual with an immature beta cell phenotype. *Pdx1* is another gene that is expressed during early pancreatic development and then in the mature beta cells. In the absence of PDX1, pancreas agenesis occurs (Jonsson, Carlsson et al. 1994) even though pancreatic buds form initially (mouse 9.5 dpc) without further

growth and branching. Neither a secondary transition nor acinar/endocrine cell formation is observed in the absence of PDX1 (Offield, Jetton et al. 1996). *Gata4* is another gene that is expressed throughout the early pancreatic epithelium and it was shown to control *Mnx1* and *Pdx1* expression in the pancreatic ventral bud (Watt, Zhao et al. 2007). In the absence of GATA4, ventral bud does not form but the dorsal bud does. After the secondary transition *Gata4* is expressed only in 2° MPCs at the tip of branching epithelium and then it is confined to acinar cells in the mature pancreas (Ketola, Otonkoski et al. 2004; Decker, Goldman et al. 2006). FOXA2 and HNF1B also control *Pdx1* expression by binding its promoter region during early pancreas development (Gerrish, Cissell et al. 2001). In the absence of HNF1B it was shown that the ventral pancreatic bud does not form without effecting formation of the pancreatic dorsal bud. However the progenitor cell population in that dorsal bud does not expand (Haumaitre, Barbacci et al. 2005). During the secondary transition *Hnf1b* is expressed in endocrine/duct bipotent progenitor cells located in the trunk epithelium and only in ductal cells in the adult pancreas (Solar, Cardalda et al. 2009). *Pancreas transcription factor 1a* (*Ptf1a*), which encodes a bHLH transcription factor, is expressed during early pancreas development (mouse 9.5 dpc) and then in the mature acinar cells only. Like PDX1, PTF1A is indispensable for pancreas organogenesis. PTF1A was also shown to be necessary for pancreatic ventral bud formation as well as proper development of pancreatic dorsal bud (Masui, Long et al. 2007). Beside its early role in pancreas development, *Ptf1a* expression is required for acinar cell differentiation (Krapp, Knöfler et al. 1998). In the absence of PTF1A, the exocrine pancreas fails to develop and the number of islet cells is reduced in mice (Krapp, Knöfler et al. 1998). Between 9.5-12 dpc

in mouse, *Ptf1a* and *Pdx1* are coexpressed in 1° MPCs (Kawaguchi, Cooper et al. 2002). Moreover, 1° MPCs also express *Mnx1, SRY-gene containing box 9* (*Sox9*), *Hnf1b, FoxA2, Hepatocyte nuclear factor 6* (*Hnf6*), *NK6 homeobox 1* (*Nkx6.1*), *Gata4* and *Gata6* (Pan and Wright 2011).

During the secondary transition, 2° MPCs at the tip epithelium express *Pdx1, Ptf1a, Carboxypeptidase A1* (*Cpa1), Sox9, Nkx6.1, Hnf1b, Gata4* and *Nuclear receptor subfamily 5 group A member 2* (*Nr5a2*). These cells have potential to give rise to all adult pancreatic lineages: acinar, ductal and endocrine cells. On the other hand, duct/endocrine bipotential progenitor cells at the trunk epithelium express *Pdx1, Sox9, Nkx6.1, Hnf1b, Hnf6, FoxA2* and *Gata4*. Endocrine progenitor cells arise from this duct/endocrine bipotent progenitor cell population by expressing intense amounts of *Ngn3* transiently (Schwitzgebel, Scheel et al. 2000; Gu, Dubauskaite et al. 2002; Jensen 2004). Beside activating other transcription factors (such as Myelin transcription factor 1 (MYT1), Islet1 (ISL1) and SNAIL2) which are necessary for endocrine progenitor cell formation, the primary role of NGN3 is activation of *Neurogenic differentiation 1* (*Neurod1*) by binding its promoter region (Huang, Liu et al. 2000). After *Ngn3* expression is turned off, NEUROD1 leads the endocrine differentiation program (Cleaver and MacDonald 2010). High levels of *Ngn3* expression differentiate these cells into endocrine precursor cells, which express *Mnx1, Myt1, Is1, Neurod1, Insulinoma associated 1* (*Insm1), Regulatory factor X 6* (*Rfx6*), *Paired box gene 6* (*Pax6*), *Nkx6.1, NK2 homeobox 2* (*Nkx2.2*), and *Snail2*. Endocrine precursor cells leave the trunk epithelium to form islets of Langerhans by assembling into endocrine cell clusters. By the time these cells leave the trunk epithelium, *Ngn3* expression is turned off (Smith, Gasa et al. 2003). Acinar cells,

however, differentiate from 2° MPCs at the tip epithelium at around 13.5 dpc in mice. These acinar cells express *Ptf1a, Gata4, Basic helix-loop-helix family member a15* (*Bhlha15*, also known as *Mist1), Nr5a2, Prospero-related homeobox 1* (*Prox1*) and keep proliferating after the secondary transition to increase the number of acinar cells. Meanwhile, duct/endocrine progenitor cell population also gives rise to ductal cells which express *Hnf6, Hnf1b, Sox9, Hes1* (*Hairy and enhancer of split 1*) and *FoxA2*.

4. Gene Expression Patterns during Endocrine Cell Development

Endocrine cell development is initiated by *Ngn3* expression and in the absence of NGN3 mice can not develop endocrine cells (Gradwohl, Dierich et al. 2000). Pan and Wright classified the transcription factors directing the endocrine cell fate into three main groups: a) general endocrine precursor differentiation factors; b) lineage allocation factors; and c) maturation factors (Pan and Wright 2011). *Neurod1*, *Insm1*, *Rfx6*, *Isl1*, all of which are direct targets for NGN3, and NKX6 family factors (*Nkx6.1* and *Nkx6.2*), which are not direct targets of NGN3, are considered general endocrine precursor differentiation factors. The main function of this class of transcription factors is facilitating the formation of the proper number of endocrine cells. The transcription factors which are on in endocrine precursor cells are MNX1, MYT1, ISL1, NEUROD1, INSM1, RFX6, PAX6, NKX6.1, NKX6.2 and SNAIL2. Lineage allocation class transcription factors control the formation of each adult endocrine cell from endocrine precursor cells. PDX1, NKX2.2, Aristaless related homeobox (ARX) and Paired box gene 4 (PAX4) are classified into this class of transcription factors. For instance, absence of NKX2.2 leads to increased numbers of epsilon cells and a decrease in the number of

other endocrine cells without affecting the total number of endocrine cells in mice (Sussel, Kalamaras et al. 1998). Absence of PAX4 also reduces the number of beta and delta cells while increasing the number of alpha and epsilon cells in mice (Prado, Pugh-Bernard et al. 2004). Moreover, ARX and PAX4 work antagonistically. *Arx* expressing cells can differentiate into alpha and Pp-cells (Collombat, Mansouri et al. 2003) while PAX4 has an opposite effect. Beside its pivotal role in early pancreas development, PDX1 is also essential for formation, proliferation and survival of adult beta and delta cells. During the secondary transition, deletion of an enhancer that is specifically required for *Pdx1* expression decreased the formation of beta cells but increased the number of other endocrine cells (Fujitani, Fujitani et al. 2006). After the decision of endocrine cell fate, maturation factors help the cells to go through terminal differentiation. MAFA, FOXA1, FOXA2 and NEUROD1, for instance, are considered maturation factors for beta cells.

5. Glucose Metabolism and Role of the Endocrine Pancreas

Glucose is an important source of energy for cells of the body. Even though most of the organs use free fatty acids as primary energy sources, glucose is used as the primary fuel in the brain (Siesjö 1988). Since the brain can not synthesize and/or store glucose, it requires a well regulated supply of glucose from the blood (Shrayyef and Gerich 2010). Most of the glucose in the blood circulation is used by brain, skeletal muscle, kidney, blood cells, splanchnic organs and adipose tissue, respectively (Gerich 1993). Even though there are some other players, the full glucose metabolism in large part is regulated by two endocrine hormones: insulin from beta cells and glucagon from

alpha cells. These hormones have converse effects during glucose metabolism. Release of insulin from beta cells depends mainly on the glucose level and to a lesser extent on mannose and fructose levels in the blood. Poorly metabolized sugars (galactose, xylose), however, do not stimulate insulin release (Grodsky, Batts et al. 1963). Free fatty acids and amino acids were also shown to have an influence on insulin secretion (Brass, Abelev et al. 2010). Glucose is transported into beta cells through a glucose transporter protein, SLC2A2 (also known as GLUT2) which locates at the beta cell membrane. Beside beta cells, SLC2A2 is also found in intestine, liver and kidney cell membranes (Bouché, Serdy et al. 2004). Glucose is then metabolized in the beta cells to produce the essential signals which are mandatory for exocytosis of insulin containing granules (Newgard and McGarry 1995). Briefly, after glucose is metabolized ATP is increased in the beta cells, and this results in the closure of ATP-dependent potassium channels on the cell membrane. Membrane is depolarized which causes the opening of calcium channels. The increased cytoplasmic calcium concentration activates protein kinases and interacts with the cell's secretory machinery which stimulates the exocytosis of insulin secretory vesicles.

Insulin has various roles in cell growth and differentiation as a signaling molecule but its major function is involved in glucose metabolism. When the glucose level increases in blood circulation after meals, insulin is pumped into the blood stream from beta cells. Once insulin reaches target cells in liver, kidney, muscle, and adipose tissue, it binds to its tyrosine kinase type receptor on the target cell surface which triggers a complex signaling pathway within the target cells. This signaling pathway involves a cascade of protein kinases and regulatory proteins. Insulin-receptor interaction has two

main consequences: a) glucose release into the plasma from liver and kidney is suppressed (Meyer, Dostou et al. 1998); and b) the glucose transporter protein SLC2A4 (also known as GLUT4), which normally locates intracellular via SLC2A4-containing vesicles, in muscle and adipose tissue cells is translocated to the cell membrane to increase the glucose uptake by these cells (Oster-Jorgensen, Pedersen et al. 1990; Chang, Chiang et al. 2004). Unlike in adipose tissue and muscle cells, insulin does not trigger the uptake of glucose in the liver and kidney (Saltiel and Kahn 2001). Instead, i) it promotes glycogenesis (glycogen synthesis from glucose) by stimulating glycogen synthase; ii) inhibits glycogenolysis (conversion of glycogen to glucose) by inhibiting glucose-6-phosphatase and phosphorylase both of which are glycogenolysis enzymes; and iii) inhibits gluconeogenesis (generation of glucose from non-carbohydrate carbon substrates) in the liver (Saltiel and Kahn 2001). Glucagon, on the other hand, increases the glucose level in the blood through glycogenolysis (Lecavalier, Bolli et al. 1989). It binds to its receptors on hepatocytes. Glucagon-receptor interaction increases intracellular cAMP which afterwards enhances glycogenolysis through phosphorylase stimulation (Magnusson, Rothman et al. 1995).

6. Diabetes Mellitus

Diabetes mellitus is a metabolic disease which is characterized by hyperglycemia, and complications (i.e. cardiovascular diseases, retinopathy, neuropathy, nephropathy and peripheral circulatory diseases) can be lethal unless treated (Bluestone, Herold et al. 2010). Diabetes was reported to be the sixth leading cause of death in the United States as of 2004 (Kung, Hoyert et al. 2008). It can result from defects during insulin secretion

and/or insulin action. There are two main types of diabetes which are called type 1 diabetes (insulin dependent diabetes) and type 2 diabetes (non-insulin dependent diabetes). In addition to type 1 and 2, there are also gestational diabetes, which is seen in pregnant women due to insufficient amount of insulin hormone, and MODY (Maturity onset diabetes of youth) type diabetes in which a single gene related to beta cell development or insulin action (i.e. *Hepatocyte nuclear factor 1 alpha* (*Hnf1α*), *Hnf1b, Hepatocyte nuclear factor 4 alpha* (*Hnf4α*), *Glucokinase* (*Gck*), *Neurod1*, *Pdx1*) is mutated. Type 1 diabetes, which accounts for 5-10% of all diagnosed cases of diabetes, results from almost complete loss of beta cells due to an autoimmune attack by T cells (Roep 2003). Loss of massive numbers of beta cells results in insulin shortage which must be compensated with external insulin hormone to control glucose homeostasis. Loss of the beta cells after the onset of disease is generally rapid and complete in younger children and patients already become dependent on exogenous insulin at the time of diagnosis. In older children and adults, however, some beta cells may survive after the onset of disease (Roep 2003). Even though the precise mechanism which triggers the autoimmune response in type 1 diabetes remains unclear, multiple genes and environmental factors (i.e. geographic location, seasonality, diet, viral exposure, chemicals, stress etc.) are known to be related to onset of the disease (Ali 2010). Family and twin studies have shown that there is a large genetic susceptibility for type 1 diabetes. However, a high percentage of recently diagnosed type 1 diabetic patients has no family history (Ali 2010). Although occurrence of type 1 diabetes due to a single gene mutation is rare, there are two different single gene defects causing type 1 diabetes. These are called IPEX syndrome, in which *Forkhead box P3* (*Foxp3*) gene is mutated, and APS-I

syndrome, in which the *Autoimmune regulator* (*Aire*) gene is mutated. Mutation of these genes causes loss of a majority of T lymphocytes which afterwards results in overwhelmed autoimmunity and development of diabetes. Instead of single gene mutations, genetic variations in several gene loci such as HLA, IDDM2, PTPN22, CD25 etc., were shown to influence type 1 diabetes (Ali 2010). Of these loci, major histocompatibility complex (referred as human leukocyte antigen in human) has the greatest contribution in type 1 diabetes occurrence. In type 2 diabetes, which accounts for 90-95% of all diagnosed cases of diabetes, the beta cells are not destroyed by T cells and keep providing insulin. However, the peripheral tissues (i.e. liver, muscle and adipose tissue) do not respond to insulin which is called insulin resistance. Existing beta cells try to overcome the insulin resistance by overproducing insulin. However, after a point glucose homeostasis is still impaired since beta cells are lost gradually by apoptosis. Up to 50% of total beta cell mass may be reduced in type 2 diabetes patients over time (Porte and Kahn 2001). Insulin resistance-triggered type 2 diabetes can occur due to defects at any point of insulin-receptor interaction, glucose uptake pathway and glucose phosphorylation after uptake into the target cells. In type 2 diabetic patients as well as obese people, the number of insulin receptors on the target cell membranes is reduced. However, even a low number of insulin receptors is enough for a moderate response to insulin. Based on this fact a reduced insulin response in patients should theoretically be due to post-binding defects (Fonseca and John-Kalarickal 2010). The main cause of type 2 diabetes is not certain yet. However, studies showed that it is linked with genetic factors and environmental factors (i.e. high fat and carbohydrate including diet, less physical activity, obesity, environmental toxins). In a study in which mouse insulin

receptor gene is disrupted ($IR^{+/-}$), it was shown that single gene disruption causes only a minor phenotypic effect. However, the combination of more than one minor defect like in insulin receptor and receptor activated intracellular proteins ($IR^{+/-}$ and IRS-$1^{+/-}$ double mutation) can mimic the insulin resistance in type 2 diabetes (Brüning, Winnay et al. 1997; Kadowaki 2000).

7. Current Treatments of Diabetes

The primary goal in diabetes therapy is restoring glucose homeostasis. Since the absence of insulin hormone in type 1 diabetes is the main reason for failure in glucose homeostasis, patients should be supplied with external insulin permanently or a new source supplying insulin should be created within the body. The current main therapy for type 1 diabetes is called insulin replacement in which patients take external insulin subcutaneously by daily shots. Beside multiple daily injections, there are insulin pumps that provide continuous insulin infusion for the patients (Lepore, Dodesini et al. 2003). External insulin was originally obtained from animal pancreases (i.e. bovine or porcine insulin), but its use is now discontinued due to immune reactions. Recombinant human insulin is now made in *Escherichia coli* and/or in *Saccharomyces cerevisiae* by recombinant DNA technology (Heller, Kozlovski et al. 2007). There are even commercially available human insulin analogues, the amino acid sequences of which are slightly modified to increase the absorbance efficiency of insulin after injection (Cohan and Peters 2010). Since the amount of insulin that the body needs depends on glucose levels of blood, which change over time, the frequent monitoring something required. The necessary amount of insulin in the body should be adjusted based on blood glucose

levels otherwise hypo- or hyperglycemia may occur. However, even if the blood glucose level is monitored frequently insulin replacement therapy can not completely mimic the normal insulin secretion from living beta cells. Another disadvantage is the fact that patients are dependent external insulin on for their entire life. Beside, other complications such as local allergic skin reactions at the site of insulin injection (Radermecker and Scheen 2007), systemic insulin allergy (Harmel, Mathur et al. 2004), loss of subcutaneous fat (lipoatrophy) at the site of insulin injection (Radermecker, Piérard et al. 2007), and accumulation of extra fat (lipohypertrophy) at the site of insulin injection (Vardar and Kızılcı 2007) have also been reported as side effects of the current insulin replacement therapy.

Transplantation of a cadaveric whole pancreas with or without kidney to type 1 patients is an alternative treatment which is first performed in 1966 in the University of Minnesota (Kelly, Lillehei et al. 1967) and improved over time. Recent advances in the field such as discovery of new immunosuppressive agents, improved matching between donor and recipient, and usage of bladder drainage, have positively improved the survival rate of grafts and reduced chance of graft rejection (Odorico, Becker et al. 2000). However, risks due to surgery and the use of immunosuppressive agents are still present which limits the application of this procedure to people who also need a kidney transplant and/or who cannot use insulin replacement therapy safely (Cohan and Peters 2010).

In addition to whole pancreas transplantation, successful infusion of isolated beta cells/islets from cadavers into type 1 diabetes patients was first reported in the University of Minnesota in 1977 (Najarian, Sutherland et al. 1977) and has been used recently as an experimental therapy. Most patients receiving islet transplantation maintained insulin

independence beyond 2 years (Farney, Hering et al. 1998) and the longest insulin independence was reported for 13 years (Robertson, Lanz et al. 2001). Unlike whole pancreas transplantation, advantages of beta cells/islets transplantation are less antigen loading, easier surgery and lower morbidity (Lakey, Mirbolooki et al. 2006). However, infusion of beta cells/islets into the liver through the portal vein was reported to cause some side effects including bleeding, portal vein thrombosis, and portal hypertension (Srinivasan, Huang et al. 2007). Like whole pancreas transplantations, cell infusion therapy also necessitates the suppression of the immune system lifelong. Moreover a shortage of cadaveric pancreas and the necessity for at least two cadaveric donors to isolate enough islet cells for one recipient are other obstacles for beta cells/islets transplantation treatments (Cohan and Peters 2010).

8. Possible Sources for Beta Cell Replacement

Problems such as the shortage of pancreas organ donors and necessity of intensive immunosuppression in current treatments for diabetes have caused researchers to seek for alternative sources of glucose-responsive insulin producing cells. Transplantation of beta cells from animals into humans (Xenotransplantation) is one of these possible ways to provide an alternative beta cell source. A high level of similarity between human and pig insulin protein (only one amino acid is different) (Lakey, Mirbolooki et al. 2006), lower sensitivity of porcine islets against autoimmunity than human islets (Koulmanda, Qipo et al. 2003) and successful genetic manipulations and breeding of pigs makes them the best candidate as beta cell donor for xenotransplantation studies. Transplantation of pig islets to non-human primates was performed in which diabetic animals controlled their glucose

metabolism more than 100 days after transplantation (Hering, Wijkstrom et al. 2006). However, many improvements are necessary for xenotransplantation studies before human clinical trials can take place, and immunosuppression is still essential for this type of treatment to prevent rejection of donor cells.

Another possible source for beta cell production is embryonic stem (ES) cells and/or induced pluripotent stem (IPS) cells. Soria et al. were first to show the potential of mouse ES cells to become insulin-positive cells which could rescue streptozotocin treated diabetic mice (Soria, Roche et al. 2000). There after, many other groups reported generation of insulin producing beta-like cells from mouse and human ES cells by using slightly different differentiation protocols. In these protocols, *in vivo* developmental principles of mouse pancreas were applied to *in vitro* systems (D'Amour, Bang et al. 2006; Kroon, Martinson et al. 2008; Zhang, Jiang et al. 2009). However, these differentiation protocols resulted in either a low number of insulin positive cells, teratoma formation, an immature beta cell phenotype (no c-peptide production and/or no glucose response), or improper endocrine cell specification (double positiveness for endocrine hormones) (Naujok, Burns et al. 2011).

Generation of beta cells by reprogramming of terminally differentiated other adult cells may be another possible new beta cell source. In differentiated cell types ectopic expression of transcription factors, which play a role in their normal developmental growth, and/or administration of extracellular soluble factors, can make reprogramming possible. In a pioneering study, for instance, Weintraub et al. activated the muscle cell phenotype in a variety of non-muscle tissue culture cells by overexpressing the myogenic factor MyoD (Weintraub, Tapscott et al. 1989). Some other examples of cell

reprogramming are, formation of hepatocyte-like cells from pancreatic exocrine cells (Shen, Slack et al. 2000), macrophages from B lymphocytes (Xie, Ye et al. 2004), neurons from fibroblasts (Vierbuchen, Ostermeier et al. 2010) and cardiomyocytes from fibroblasts (Ieda, Fu et al. 2010). Beta cell reprogramming from hepatocytes has been widely studied recently because of the developmental relatedness of liver and pancreas. Activation of pancreatic gene expression in hepatocyte was shown to be possible by ectopic expression of pancreatic master gene *Pdx1* in the mouse liver (Ferber, Halkin et al. 2000). Formation of insulin positive beta-like cells by ectopic expression of *Pdx1* in cultured human hepatocytes was also shown by Ferber`s group (Sapir, Shternhall et al. 2005). However, in all liver to pancreas reprogramming studies the number of insulin producing cells and amount of insulin produced/secreted by reprogrammed cells are low indicating that reprogramming is not yet effective (Meivar-Levy, Aviv et al. 2010). More recently, *in vivo* regeneration of pancreatic mouse endocrine cells from pancreatic exocrine cells was shown by overexpressing three important pancreatic transcription factor genes, *Pdx1*, *Ngn3* and *MafA* (Zhou, Brown et al. 2008).

Figure 1: Pancreatic cells

(**A**) Pancreatic exocrine cells. Adapted from "Developmental biology of the pancreas," by J. M. W. Slack, 1995, *Development*, *121*, p. 1569-1580 (**B**) Pancreatic endocrine cells. Adapted from "Pancreatic organogenesis- developmental mechanisms and implications for therapy," by H. Edlund, 2002, *Nature Review Genetics*, *3*, p. 524-532

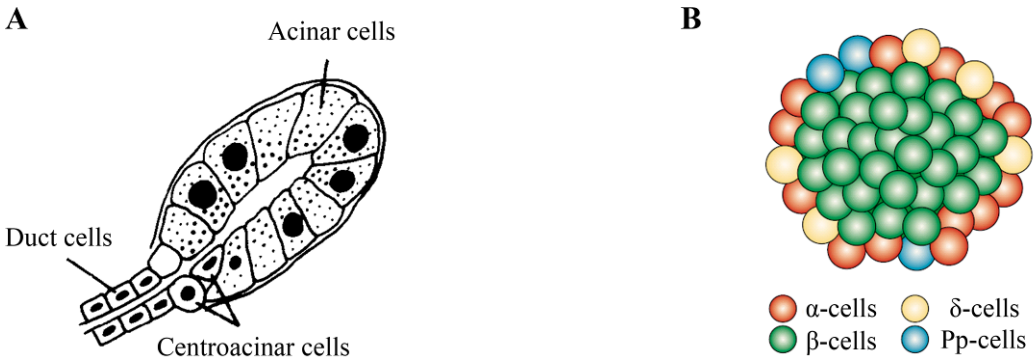

A
Acinar cells
Duct cells
Centroacinar cells
B
α-cells
δ-cells
β-cells
Pp-cells

Figure 2: Dorsal and ventral pancreatic buds in developing mouse embryo

Location of dorsal and ventral pancreatic buds in mouse embryo at 10 and 12 dpc respectively. Adapted from "Pancreatic organogenesis- developmental mechanisms and implications for therapy," by H. Edlund, 2002, *Nature Review Genetics*, *3,* p. 524-532

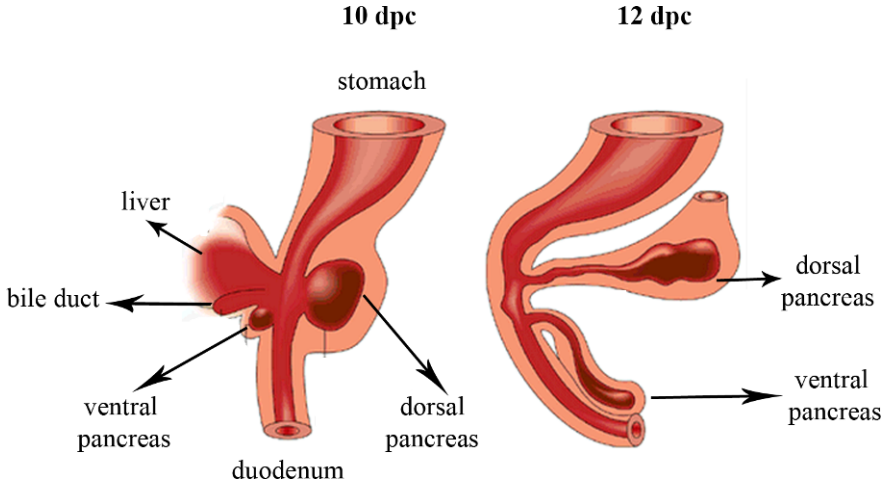
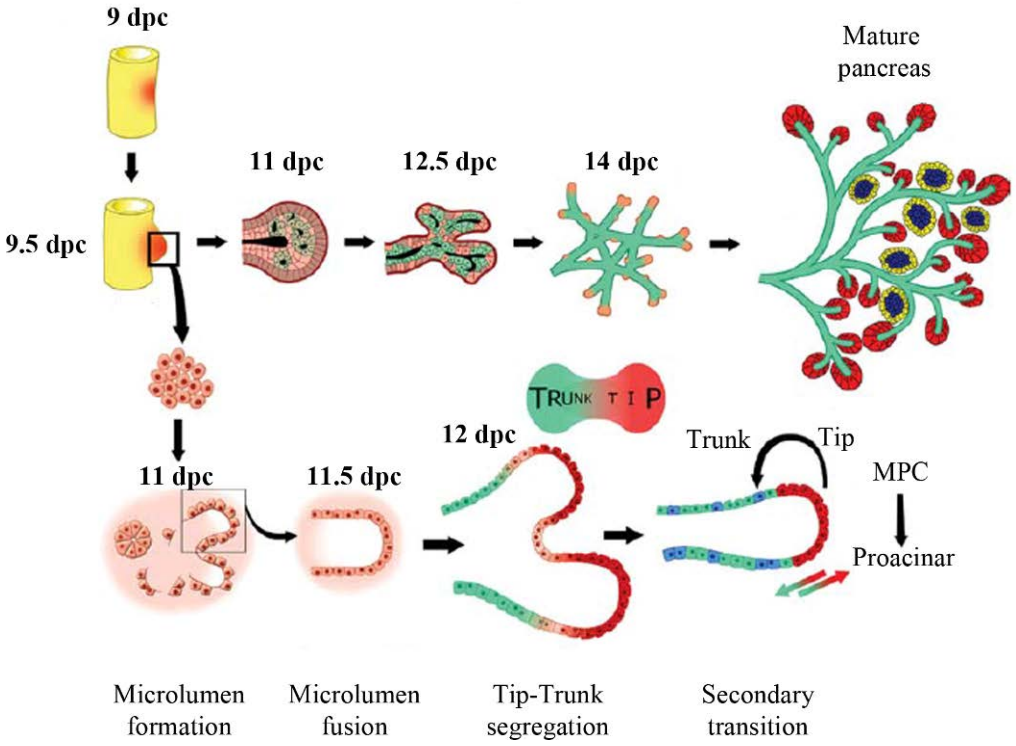

10 dpc
12 dpc
stomach
liver
bile duct
ventral pancreas
duodenum
dorsal pancreas
dorsal pancreas
ventral pancreas

Figure 3: Development of pancreas

Murine pancreas development from 9 dpc to adult. Adapted from “Pancreas organogenesis: from bud to plexus to gland,” by F. C. Pan and C. Wright, 2011, *Developmental Dynamics*, *240*, p. 530-565


9 dpc
Mature pancreas
11 dpc
12.5 dpc
14 dpc
9.5 dpc
TRUNK TIP
12 dpc
Trunk
Tip
MPC
11 dpc
11.5 dpc
Proacinar
Microlumen formation
Microlumen fusion
Tip-Trunk segregation
Secondary transition

B. EXPERIMENTAL OBJECTIVES

Diabetes is a life threatening disease the prevalence of which is increasing worldwide. According to American Diabetes Association (ADA) and World Health Organization (WHO) nearly 19 million people in United States are diagnosed with diabetes and more than 346 million people worldwide are diabetic. Despite common usage, therapy provided by external insulin shots can not imitate the normal insulin secretion pattern as efficiently as beta cells do. The shortage of cadaveric pancreases is the major limitation for beta cell/islet transplantation therapy in which immune suppression is also necessary. These limitations can potentially be overcome by reprogramming of terminally differentiated adult cells into insulin expressing, glucose sensitive beta-like cells. Any method providing beta cell neogenesis from a patient`s own adult cells by cell reprogramming will eliminate the necessity for immune suppression. Production of new beta cells from highly regenerative organs such as liver and/or from organs in which alteration of its cells does not affect the original function of the organ such as exocrine pancreas will also solve the shortage problem of starting cells. Based on this possibility many studies regarding beta cell reprogramming have been performed in liver cells both *in vivo* and *in vitro*. However, despite some promising results such as insulin expression, or amelioration of diabetic animals by newly produced insulin positive cells, none of these studies showed a true beta cell neogenesis. Overexpression of *Pdx1*, *Ngn3* and *MafA* in exocrine pancreas of mouse has been shown to produce insulin positive beta-like cells which were capable of rescuing streptozotocin-treated

NOD-SCID (non-obese diabetic severe combined immunodeficiency) diabetic mice (Zhou, Brown et al. 2008), although how beta-like these cells are is still not known.

In this thesis we used the same transcription factors; Pdx1, Ngn3 and MafA, but unlike Zhou et al., we cloned all three genes into a single adenoviral construct which we call Ad-PNM.

The first aim of this thesis was to investigate the reprogramming competency of different cell types from different species at different developmental stages into beta-like cells through Ad-PNM.

The second aim was to investigate the effect of Ad-PNM on the AR42J-B13 cell line, which gave the highest proportion of insulin positiveness after transduction with Ad-PNM, to see if the cell transformation we saw after Ad-PNM transduction is really a reprogramming event or not.

The third aim was to further characterize the effects of Ad-PNM on mouse hepatocyte-derived small cells, and to explore if small molecules and histone modifying agents can increase the effects.

Chapter 1 Investigation the Reprogramming Competency to Ad-PNM of Different Cell Types from Different Species at Different Developmental Stages

1.1. Introduction

In multi-cellular organisms during embryonic development, cells decide which developmental commitment pathways they will follow under the control of extracellular inducing factors, and eventually become one of about 200 different cell types of the vertebrate body (Slack 2006). Once a cell terminally differentiates it becomes relatively stable and persists long term with or without cell division. Recent studies showed that it is also possible to reprogram one differentiated cell type to another by overexpression of specific transcription factors which are responsible for the relevant commitment processes in normal development (Zhou, Brown et al. 2008). For instance some examples of reprogramming event that have been described are: formation of myogenic phenotype in a variety of non-muscle tissue culture cells by overexpression of *MyoD* (Weintraub, Tapscott et al. 1989), formation of hepatocytes from pancreatic exocrine cells (Shen, Slack et al. 2000), macrophages from B lymphocytes (Xie, Ye et al. 2004), neurons (Vierbuchen, Ostermeier et al. 2010) and cardiomyocytes (Ieda, Fu et al. 2010) from fibroblasts. In a recent study, generation of pancreatic beta cells from pancreatic exocrine cells in immunodeficient mice was shown by overexpression of *Pdx1*, *Ngn3* and *MafA* transcription factors *in vivo* (Zhou, Brown et al. 2008). Of these examples, formation of beta cells is of particular interest from the point of view of the necessity to find an alternative beta cell source because of the problems in current diabetes therapies.

In this study, we looked at the reprogramming competency of different cell types from different species at different developmental commitment stages into beta-like cells through overexpressing *Pdx1*, *Ngn3* and *MafA*. We used mouse hepatocyte-derived small cells (ASH), mouse primary hepatocytes, mouse embryonic fibroblasts (MEF) and mouse adult (tail tip) fibroblasts, rat primary hepatocytes, rat pancreatic exocrine cells (AR42J-B13), rat adult fibroblasts (CRL-1213) and rat multipotent adult progenitor cells (MAPC).

1.2. Experimental Design and Methods

1.2.1. Preparation of Recombinant Adenovirus

We constructed a single adenoviral vector coding three important pancreatic transcription factors; PDX1, NGN3 and MAFA from mouse and called it Ad-PNM (Figure 4). The translational termination sequences of the full length mouse *Pdx1* and *Ngn3* cDNAs were replaced with specific restriction sites by using polymerase chain replication (PCR). Amplified cDNAs were then cloned into pBluescript (KS+/-) as XbaI/BamHI fragments. *pBS-Pdx1-2A* and *pBS-Ngn3-2A* constructs were generated by ligation of *Pdx1* and *Ngn3* cDNAs to the coding region for the 2A peptide, which includes 18 amino acids, from the Foot and Mouth Disease Virus (FMDV). These two constructs were then joined together such that the coding region of the *Pdx1*-linked 2A was followed by the first amino acid codon of the *Ngn3* so that giving the construct *pBS-Pdx1-2A-Ngn3-2A*. This construct was then ligated to the *MafA* cDNA which is including its own the translational termination codon and cloned as BglII/EcoRV fragment in

pBluescript (KS+/-) to generate the polycistronic construct *pBS-Pdx1-2A-Ngn3-2A-MafA*. *Pdx1-2A-Ngn3-2A-MafA* construct was taken out as a single fragment by restriction digestion via SalI enzyme, the target sites of which locates internally upstream of *Pdx1* and downstream of *MafA,* and cloned into the shuttle vector, *pShuttleT2ALCAG5PL4-7*. Recombinant adenovirus containing *Pdx1-2A-Ngn3-2A-MafA* construct was produced by homologous recombination after electroporating the linearized shuttle vector into BJ5183 bacterial cells. This was done by using the pAdEasy adenoviral vector system (Agilent Technologies) according to the manufacturer's instructions.

1.2.2. *In Vitro* Culture of Cell Lines and Transfection with Ad-PNM

Mouse hepatocyte-derived small cells (ASH), which were derived from a primary mouse hepatocyte culture *in vitro,* were obtained from Dr. David Tosh (University of Bath, UK). ASH cells were maintained in low-glucose Dulbecco`s Modified Eagles medium (DMEM; Gibco) supplied with 10% (v/v) fetal bovine serum (FBS; Hyclone) and 1x antibiotic-antimycotic solution (anti-anti; Gibco).

Mouse embryonic fibroblasts (MEF) were isolated from prenatal mouse embryos. MEF cells were maintained in high-glucose DMEM supplied with 10% (v/v) FBS, 2mM L-glutamine (Gibco) and 1x anti-anti solution.

Mouse adult fibroblasts were obtained by culturing a piece of tail tip from an adult mouse *in vitro*. These tail tip-derived mouse adult fibroblasts were maintained in high-glucose DMEM supplied with 10% (v/v) FBS, 2mM L-glutamine and1x anti-anti solution.

Rat primary hepatocytes were isolated from adult male rats by two-step collagenase perfusion method described in (Seglen 1976). After the isolation rat primary hepatocytes were cultured in William`s E medium (Gibco) supplied with 10% (v/v) FBS, 2mM L-glutamine, 1x anti-anti and gentamycin solution (Gibco) overnight for attachment to plate. After cells attached to the plate they were maintained in low-glucose DMEM supplied with 10% (v/v) FBS, 2mM L-glutamine and 1x anti-anti solution.

Rat pancreatic exocrine-like cells (AR42J-B13), which were originally derived from a chemically induced pancreatic tumor (Longnecker, Lilja et al. 1979) were obtained from Dr. David Tosh.

Rat adult fibroblast cells (CRL-1213) were bought from ATCC and were maintained in high-glucose DMEM supplied with 10% (v/v) FBS, 2mM L-glutamine and 1x anti-anti solution.

Rat multipotent adult progenitor cells (MAPC), which were originally isolated from bone marrow and shown to have progenitor cell characters (Schwartz, Reyes et al. 2002), were obtained from Wei-Shou Hu`s Laboratory (University of Minnesota, MN). MAPCs were maintained in low-glucose DMEM and MCDB medium (Sigma) supplied with 2% (v/v) FBS, 1x anti-anti solution, 1x ITS (Insulin/Transferrin/Selenium; Sigma), 1mg/ml LA-BSA (Linoleic acid-bovine serum albumin; Sigma), 1x L-ascorbic acid, 0.5μM dexamethasone, 10ng/ml PDGF (Platelet-derived growth factor; Sigma), 10ng/ml EGF (Epidermal growth factor; Millipore), 0.01x LIF (Leukemia inhibitory factor; Millipore) and 0.1x β-mercaptoethanol (Gibco).

All the cell types were transduced with a dose of 25 multiplicity of infection (MOI) Ad-PNM. Next day the virus containing medium was replaced. Three days after

virus transduction, total RNA was isolated for reverse transcription–polymerase chain reaction (RT-PCR) and the cells were also fixed for immunostaining to see how they had responded.

1.2.3. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA from the cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's manual. RNA samples were then treated with DNase enzyme (Promega) to remove possible genomic DNA contamination. A PCR was performed with the RNA template at 60°C for 35 cycles and resulting products were run on 1% agarose gel to confirm the absence of genomic DNA contamination. cDNAs were then synthesized by reverse transcription from 2μg total RNA using SuperScript III Reverse Transcriptase, oligo$(dT)_{20}$ and dNTP (10mM) (Invitrogen). The gene expression pattern between the cells with and without Ad-PNM was then compared by performing PCR. PCR conditions were: 94°C 3 min initial denaturation, 94°C 30 s denaturation, 60°C 30 s annealing, 72°C 1 min extension, 72°C 5 min final extension and overall 30-35 cycles were performed. Primers used for RT-PCR are listed in Table 1. PCR products were then run on 1% (w/v) agarose gel (Invitrogen), in TAE (Tris-Acetic Acid-EDTA) buffer (Bio-Rad Laboratories) including ethidium bromide, by electrophoresis at 100V. Gels were visualized with a Bio-Rad Gel Documentation System model 2000.

1.2.4. Immunostaining

The cells were fixed with 4% (w/v) paraformaldehyde (Sigma) in PBS (Phosphate buffered saline; Sigma) for 20 min. After fixation cells were washed three times with

0.1% (v/v) Tween 20 (Bio-Rad) in PBS (PBS-T) for 5 min in each wash. Cells were then permeabilized with 0.2% (v/v) Triton X-100 (Sigma) in PBS for 15 min. After 1 hour blocking with PBS-T including 1% (w/v) BSA (Bovine serum albumin; Sigma), cells were incubated overnight with primary antibodies at 4°C and then 1 hour with secondary antibodies at room temperature. Primary antibodies used were as follows: guinea pig anti-insulin (1:200 dilution, Sigma); rabbit anti-Pdx1 (1:2000 dilution, Upstate); rabbit anti-C-peptide (1:100 dilution, Cell Signaling), rabbit anti-Ngn3 (1:100 dilution, Santa Cruz Biotechnology); and rabbit anti-MafA (1:100 dilution, Santa Cruz Biotechnology). Secondary antibodies used are as follows: alexa fluor 480 goat anti-rabbit IgG (1:500 dilution, Invitrogen) and alexa fluor 594 goat anti-guinea pig IgG (1:500 dilution, Invitrogen). Images were taken using a Leica DMI6000 B inverted microscope.

1.3. Results

When cultured cells are transduced with three adenoviruses carrying different genes, individual cells may receive one, two or three genes randomly. To remove this source of variation we have designed an adenoviral vector carrying all three genes in order (Figure 4). The coding regions of *Pdx1*, *Ngn3* and *MafA* are joined by 2A sequences, which are responsible from autocleavage of the extending polypeptide during protein synthesis (de Felipe 2004). The whole construct is under control of the highly active *cags* (cytomegalovirus-chicken *β*-actin) promoter (Chung, Andersson et al. 2002). This vector (Ad-PNM) was shown to infect almost all of the cells efficiently and expression of all three exogenous proteins was detected by immunostaining (Figure 10).

Three days after Ad-PNM induction, in all eight cell types used in this study *Insulin* genes (there are two different insulin genes in rodents: *Ins1* and *Ins2*) expression was detected in the RT-PCR but at varying intensities. In addition to *Insulin*, the most responsive four cell types (rat primary hepatocytes, rat MAPCs, AR42J-B13 cells and ASH cells) also activated some other important beta cell markers with different expression patterns (Figure 5). After Ad-PNM induction, rat primary hepatocytes started to express alpha cell hormone gene *glucagon* (*Gcg*), beta cell transcription factor genes *Iapp* (*Islet amyloid polypeptide*), *Pax4*, *Isl1*, *Mnx1*, beta cell membrane protein genes *Slc2a2*, *Kcnj11* (P*otassium inwardly rectifying channel subfamily J member 11*; also known as *Kir6.2*), and *Abcc8* (*ATP-binding cassette sub-family C CFTR/MRP member 8*; also known as *Sur1*), endogenous counterparts of *Pdx1* and *MafA* as well as *Cpe* (*Carboxypeptidase E*) (Figure 5A). Rat MAPCs expressed beta cell transcription factor genes *Iapp*, *Pax4*, *Neurod1*, *Nkx2.2*, *Isl1*, *Mnx1*, beta cell membrane protein genes *Slc2a2*, *Kcnj11* and *Abcc8* (Figure 5B). AR42J-B13 cells expressed beta cell transcription factor genes *Iapp*, *Pax4*, *Neurod1*, *Nkx2.2*, beta cell membrane protein gene *Abcc8* as well as *Cpe* and *Pcsk2* (Figure 5C). ASH cells began to express Pp-cell hormone gene *Pancreatic polypeptide* (*Ppy*), beta cell transcription factor genes *Iapp*, *Pax4*, *Mnx1*, beta cell membrane protein genes *Slc2a2*, *Kcnj11* and *Abcc8* as well as *Cpe* and *Pcsk2 (Proprotein convertase subtilisi/kexin type 2*; also known as *PC2*) (Figure 5D). However none of these cell types activated the entire group of beta cell genes likely to be necessary for beta cell function. In addition to activating some of the beta cell genes, these cells also downregulated the expression of some of their original genes like *Pou5f1* (*POU domain class 5 transcription factor 1*; also known as *Oct4*) in Rat MAPCs, *Pancreatic amylase*

(*Amy2*) in AR42J-B13 cells, *Albumin* (*Alb*) in ASH cells. The other four cell types which are rat adult fibroblasts (CRL-1213), mouse embryonic fibroblasts, mouse primary hepatocytes and mouse adult (tail tip) fibroblasts showed little or no response in a majority of the beta cell genes apart from *Insulin* (Figure 5E, 5F, 5G, 5H).

Although *Insulin* genes were detected for all the cell types in RT-PCR, insulin immunostaining was only detected in five cell types which are rat primary hepatocytes (Figure 6A-A`), ASH cells (Figure 6B-B`), AR42J-B13 cells (Figure 6C-C`), rat MAPCs (Figure 6D) and CRL-1213 (Figure 6E). In addition to insulin, expression of C-peptide, which is a side product formed during proinsulin to insulin transition, and its co-localization with insulin in the same cells, were also shown for rat primary hepatocytes (Figure 6A`-A``), ASH cells (Figure 6B`-B``) and AR42J-B13 cells (Figure 6C`-C``).

Based on these results we concluded that the transformation competency of the cells into insulin expressing beta cell-like state is higher for a) rat cell lines compared to mouse cell lines; b) progenitor-like cell lines compared to the terminally differentiated cell lines; and c) cells which are developmentally related to beta cells such as hepatocytes and acinar cells compared to the developmentally unrelated cell lines such as fibroblasts.

1.4. Discussion

Three days after transduction with Ad-PNM, all cell types used in this study showed *Insulin* genes activation in RT-PCR at various intensities. In addition to the *Insulin* genes, some other beta cell genes were also activated in four cell types: rat primary hepatocytes, ASH cells, rat MAPCs and AR42J-B13 cells. However none of these cell types activated all the important beta cell genes. After transduction with Ad-

PNM, rat primary hepatocytes lacked *Nkx2.2* and *Neurod1* expression; ASH cells lacked *Neurod1*, *Nkx2.2*, *Isl1*, endogenous *Pdx1* and endogenous *MafA* expression; rat MAPCs lacked endogenous *Pdx1* and endogenous *MafA* expression; AR42J-B13 cells lacked *Isl1*, *Mnx1*, endogenous *Pdx1*, endogenous *MafA*, *Slc2a2* and *Kcnj11* expression.

Activation of *Insulin* genes together with some other beta cell markers shows the ability of the *Pdx1*, *Ngn3* and *MafA* combination to lead the transformation of different cell types into beta cell-like state. Downregulation of the major product genes in the recipient cells also supports this theory. Immunocytological detection of the co-existence of C-peptide together with insulin protein in the same cells also demonstrated that detectable insulin expression was coming from the transformed cells rather then being tissue culture medium insulin which can be absorbed by dead and/or dying cells. On the other hand, the absence of essential beta cell genes such as *Kcnj11*, *Abcc8* and *Slc2a2*, as well as endogenous counterparts of input genes, showed that the transformation into a beta cell-like state was not complete. In real beta cells, the insulin amount to be released into the blood stream is adjusted according to glucose amount in the blood. This glucose dependent insulin release function of beta cells is controlled by KCNJ11, ABCC8 as well as SLC2A2 membrane proteins. This is why these proteins are indispensable for a true beta cell phenotype. Moreover, in adult beta cells *Pdx1* and *MafA* are master genes driving *Insulin* gene expression as well as other beta cell transcription factors. To obtain a true beta cell phenotype endogenous *Pdx1* and *MafA* should be activated and stay on after the removal of their exogenous counterparts. In our experiments none of the cell types were transformed into true beta cells because they did not fulfil two requirements

mentioned above: a) all of the genes of the cell type of interest should be activated; and b) the endogenous counterparts of input genes (if any used) should be activated.

In this study we used eight different cell lines from two different species: rat and mouse. Based on the response of these cell types against *Pdx1*, *Ngn3* and *MafA* combination, our overall observation was that rat cell types no matter what their developmental stage are transformed better than their mouse counterparts. In mouse primary hepatoytes, for instance, only *Ins1* and *Gcg* genes were activated (Figure 5G). However, rat primary hepatocytes activated more beta cell genes after transduction with Ad-PNM (Figure 5A). Comparison of rat and mouse fibroblasts gave a similar result. While mouse adult (tail tip) fibroblasts were activating *Ins2* and *Iapp* genes only (Figure 5H), rat adult fibroblasts activated *Ins1*, *Ins2*, endogenous *Pdx1* and *Kcnj11* genes (Figure 5E). Immunocytologic detection of insulin protein was also achieved in rat fibroblast cells unlike mouse tail tip fibroblast cells. Of the mouse cell lines we used in this study, only mouse hepatocyte-derived small cells (ASH) showed a significant response to Ad-PNM (Figure 5D). We conclude that this was due to the progenitor cell-like phenotype of these ASH cells. Similar to the ASH cells response, rat MAPCs, a progenitor cell type, activated more beta cell genes after transduction with Ad-PNM than rat fibroblast cells did (Figure 5B and 5E). On the other hand, even though rat primary hepatocytes and rat pancreatic exocrine cell line AR42J-B13 cells are not progenitor-like cells, they showed a significant response to Ad-PNM by activating many beta cell genes (Figure 5A and 5C). Our results suggest that the competency of these two cell types to give rise to a beta cell-like state is due to their developmental relatedness to beta cells. During embryogenesis of the liver, the principal functional cell type of which is the

hepatocyte, and the pancreas, arise from neighboring regions of the same embryonic tissue, the definitive endoderm. Different signals from surrounding tissues like FGFs from the cardiac mesoderm (Jung, Zheng et al. 1999) and BMP from the septum transversum (Rossi, Dunn et al. 2001), and activin-β_B and FGF2 from the notochord (Kim, Hebrok et al. 1997) directs the formation of liver and pancreatic buds respectively from definitive endoderm. For that reason hepatocytes and beta cells should share a similar epigenetic memory to a large extent. Similarly, pancreatic exocrine and endocrine cells arise from a same progenitor cell population during pancreas development (Percival and Slack 1999) and they should share a similar epigenetic memory which makes them more competent to be transformed into each other than any other unrelated cell types. Even though rat primary hepatocytes and the rat pancreatic exocrine cell line AR42J-B13 did not activate all important beta cell genes after transduction with Ad-PNM, their higher response supports the transformation competency theory of developmentally related cells mentioned above.

Figure 4: Structure of adenovirus carrying *Pdx1, Ngn3* and *MafA* (Ad-PNM).

Pdx1, Ngn3 and *MafA* are linked to each other respectively through 2A sequence which provides autocleavage of extending polypeptide during protein synthesis. The whole construct is under the control of *cags* promoter.

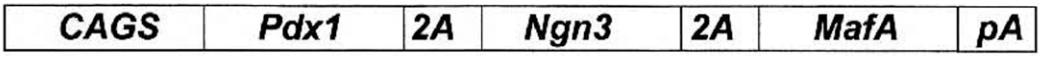
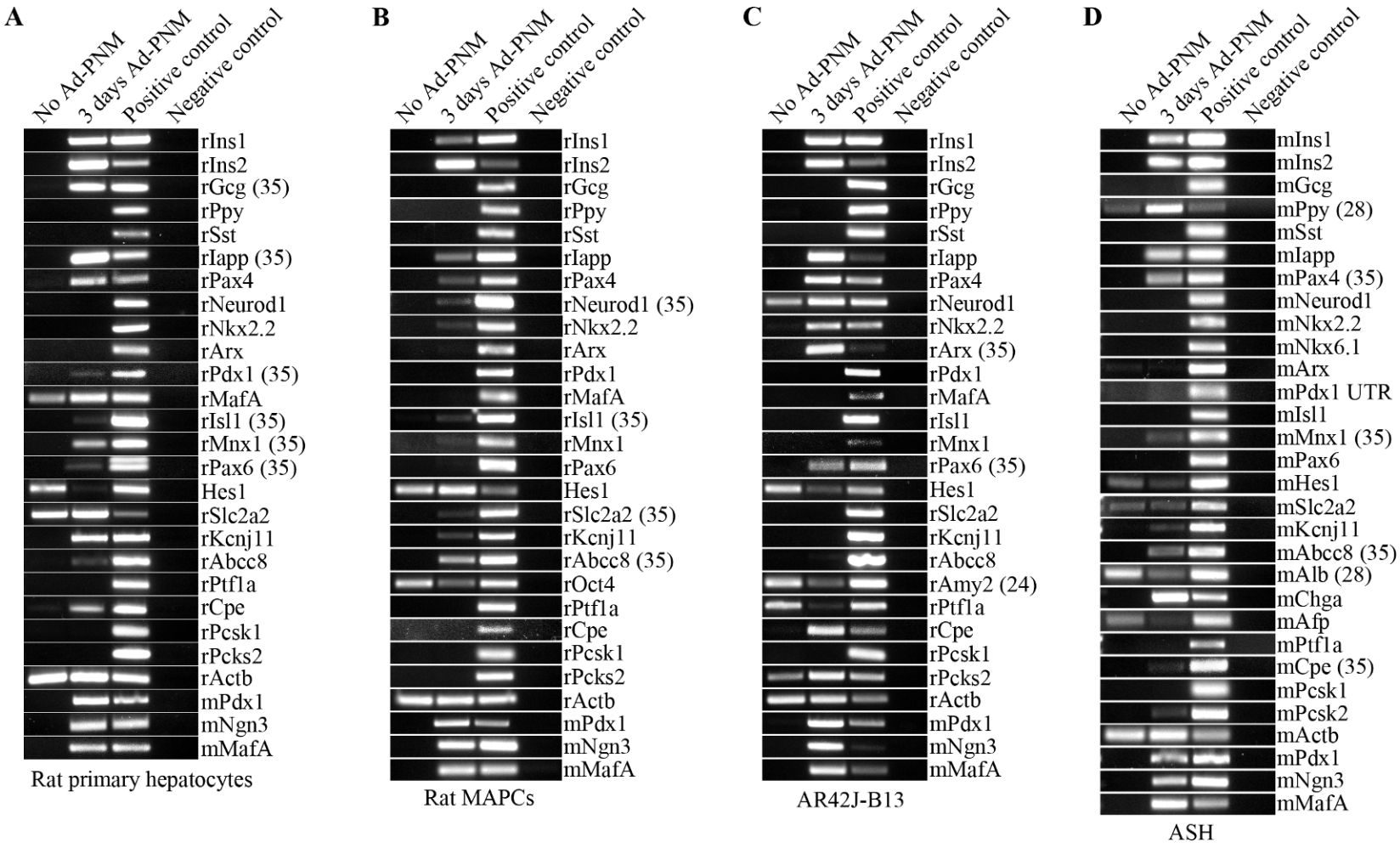

CAGS
Pdx1
2A
Ngn3
2A
MafA
pA

Figure 5: Gene expression profile of different cell types after Ad-PNM (RT-PCR)

Different cell types from mouse and rat were transduced with Ad-PNM. Gene expression profile of most responding cell types: (**A**) Rat primary hepatocytes, (**B**) Rat MAPCs, (**C**) AR42J-B13 cells, (**D**) ASH cells and cells showing little or no response: (**E**) Rat adult fibroblasts, (**F**) Mouse embryonic fibroblasts, (**G**) Mouse primary hepatocytes, (**H**) Mouse adult fibroblasts were shown by RT-PCR. 30 cycles of PCR were performed for each primer pairs unless it is stated next to gene name.


A
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
rIns1
rIns2
rGcg (35)
rPpy
rSst
rIapp (35)
rPax4
rNeurod1
rNkx2.2
rArx
rPdx1 (35)
rMafA
rIsl1 (35)
rMnx1 (35)
rPax6 (35)
Hes1
rSlc2a2
rKcnj11
rAbcc8
rPtf1a
rCpe
rPcsk1
rPcks2
rActb
mPdx1
mNgn3
mMafA
Rat primary hepatocytes
B
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
rIns1
rIns2
rGcg
rPpy
rSst
rIapp
rPax4
rNeurod1 (35)
rNkx2.2
rArx
rPdx1
rMafA
rIsl1 (35)
rMnx1
rPax6
Hes1
rSlc2a2 (35)
rKcnj11
rAbcc8 (35)
rOct4
rPtf1a
rCpe
rPcsk1
rPcks2
rActb
mPdx1
mNgn3
mMafA
Rat MAPCs
C
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
rIns1
rIns2
rGcg
rPpy
rSst
rIapp
rPax4
rNeurod1
rNkx2.2
rArx (35)
rPdx1
rMafA
rIsl1
rMnx1
rPax6 (35)
Hes1
rSlc2a2
rKcnj11
rAbcc8
rAmy2 (24)
rPtf1a
rCpe
rPcsk1
rPcks2
rActb
mPdx1
mNgn3
mMafA
AR42J-B13
D
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
mIns1
mIns2
mGcg
mPpy (28)
mSst
mIapp
mPax4 (35)
mNeurod1
mNkx2.2
mNkx6.1
mArx
mPdx1 UTR
mIsl1
mMnx1 (35)
mPax6
mHes1
mSlc2a2
mKcnj11
mAbcc8 (35)
mAlb (28)
mChga
mAfp
mPtf1a
mCpe (35)
mPcsk1
mPcsk2
mActb
mPdx1
mNgn3
mMafA
ASH

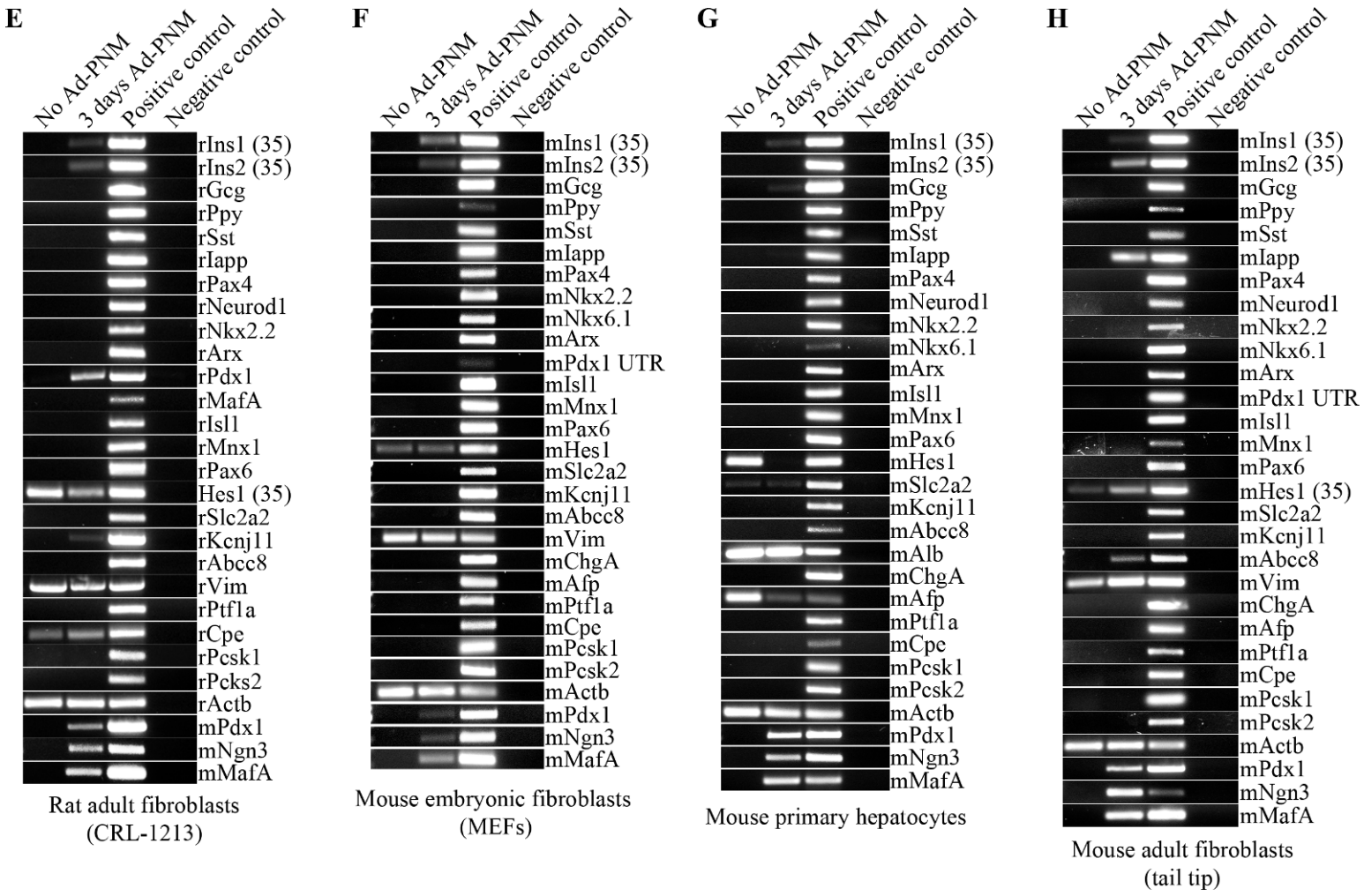

E
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
rIns1 (35)
rIns2 (35)
rGcg
rPpy
rSst
rIapp
rPax4
rNeurod1
rNkx2.2
rArx
rPdx1
rMafA
rIsl1
rMnx1
rPax6
Hes1 (35)
rSlc2a2
rKcnj11
rAbcc8
rVim
rPtf1a
rCpe
rPcsk1
rPcks2
rActb
mPdx1
mNgn3
mMafA
Rat adult fibroblasts
(CRL-1213)
F
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
mIns1 (35)
mIns2 (35)
mGcg
mPpy
mSst
mIapp
mPax4
mNkx2.2
mNkx6.1
mArx
mPdx1 UTR
mIsl1
mMnx1
mPax6
mHes1
mSlc2a2
mKcnj11
mAbcc8
mVim
mChgA
mAfp
mPtf1a
mCpe
mPcsk1
mPcsk2
mActb
mPdx1
mNgn3
mMafA
Mouse embryonic fibroblasts
(MEFs)
G
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
mIns1 (35)
mIns2 (35)
mGcg
mPpy
mSst
mIapp
mPax4
mNeurod1
mNkx2.2
mNkx6.1
mArx
mIsl1
mMnx1
mPax6
mHes1
mSlc2a2
mKcnj11
mAbcc8
mAlb
mChgA
mAfp
mPtf1a
mCpe
mPcsk1
mPcsk2
mActb
mPdx1
mNgn3
mMafA
Mouse primary hepatocytes
H
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
mIns1 (35)
mIns2 (35)
mGcg
mPpy
mSst
mIapp
mPax4
mNeurod1
mNkx2.2
mNkx6.1
mArx
mPdx1 UTR
mIsl1
mMnx1
mPax6
mHes1 (35)
mSlc2a2
mKcnj11
mAbcc8
mVim
mChgA
mAfp
mPtf1a
mCpe
mPcsk1
mPcsk2
mActb
mPdx1
mNgn3
mMafA
Mouse adult fibroblasts
(tail tip)

Figure 6: Insulin and C-peptide immunostaining in different cell types after Ad-PNM

Different cell types from mouse and rat were transduced with Ad-PNM. 3 days after Ad-PNM transduction expression of insulin protein (red) in (**A**-**A`**) Primary rat hepatocytes, (**B**-**B`**) ASH cells, (**C**-**C`**) AR42J-B13 cells, (**D**) Rat MAPCs and (**E**) Rat fibroblasts were demonstrated through immunostaining. Expression of C-peptide (green) in (**A``**) Primary rat hepatocytes, (**B``**) ASH cells and (**C``**) AR42J-B13 cells was also demonstrated. Insulin expression was detected only in viral PDX1 (green) expressing cells (**A**, **B**, **C**, **D**, **E**).

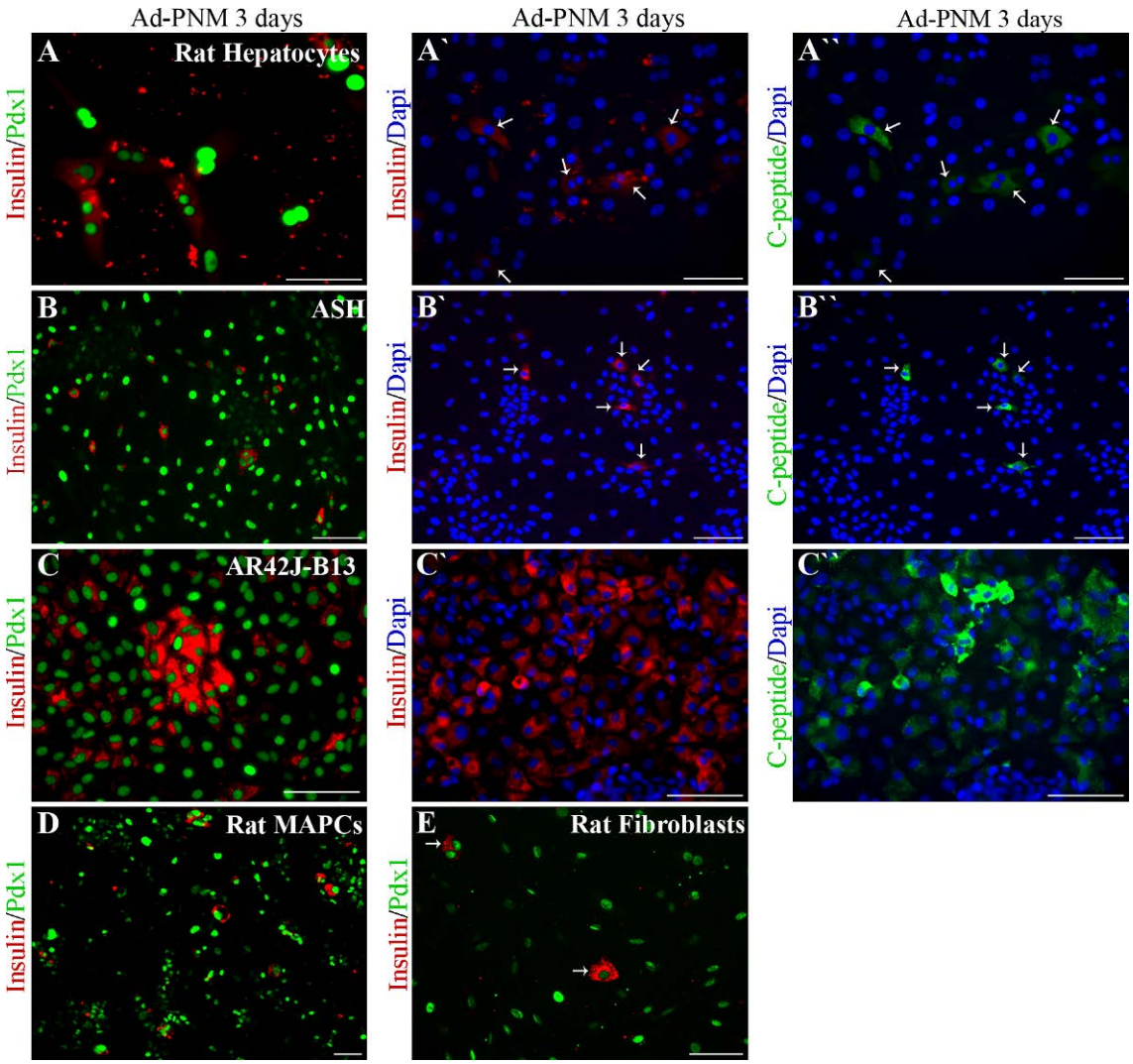

Ad-PNM 3 days
Ad-PNM 3 days
Ad-PNM 3 days
A
Rat Hepatocytes
A`
A``
B
ASH
B`
B``
C
AR42J-B13
C`
C``
D
Rat MAPCs
E
Rat Fibroblasts
Insulin/Pdx1
Insulin/Dapi
C-peptide/Dapi

Table 1: Rat and mouse primer pairs used in RT-PCR

Rat Primers used in RT-PCR		
Primer name	**Forward Sequence 5`-3`**	**Reverse Sequence 5`-3`**
Ins1	CTACAATCATAGACCATCAGCA	CAGTTGGTAGAGGGAGCAGAT
Ins2	CCCTGTGGATCCGCTTCCTGC	GTGCCAAGGTCTGAAGGTCA
Gcg	GCTGGCAGCATGCCCCTCAA	CCTTTGCTGCCTGGCCCTCC
Ppy	CTATCCACTTGGGTGGCTCT	ATCAAACCCACCAGAAGGC
Sst	CCCAGACTCCGTCAGTTTCT	GAAGTTCTTGCAGCCAGCTT
Iapp	GGCTGTAGTTCCTGAAGCTT	AAGGTTGTTGCTGGAGCGAA
Pax4	TGGAGAAAGAGTTCCAGCGT	CTTGGGATGTGGTGTCACTG
Neurod1	TTTCAAACACGAACCATCCA	CCTGTTTCTTCCAAAGGCAG
Nkx2.2	CACGCGCTCAAAGCCCAGGA	GGAGAGAGCGGTGGGGCAGA
Arx	AGGACGCCGAGGGCAAGGAT	TCTCCCGCTTGCGCCACTTG
Pdx1	GTAGTAGCGGGACAACGAGC	CAGTTGGGAGCCTGATTCTC
Ngn3	AAACTTCGAAGCGAGCAGAG	CATCCTGAGGTTGGGAAAAA
MafA	TCCAGGCTGGTGCGCGAAAG	GCAAGCCCACTCAGGAGCCG
Isl1	TGATGCGCGCCCGCTCTAAG	TGACTCGGGGACTGAGGCCG
Mnx1	CCTGTCGCGACCCAAGCGTT	CCCAGCAGCTCCTCCTCCGT
Pax6	GGCCGTGCGACATTTCCCGA	GCCGTCTGCGCCCATCTGTT
Hes1	GGCCACCTGGCCAACTGCAT	GCTGGAAGGCGACACTGCG
Slc2a2	GCCATCTTCCTCTTCGTCAG	ACCTGGTTCCCTTCTGGTCT
Kcnj11	CTGGAAGGAAGCCAGTCTTG	CAGTGTCCCCCAGACAAAGT
Abcc8	AGCTGCGCTTCTGCCTCACG	GGCACCCTGCTGGCTCTGTG
Amy2a	GCCTACTGACAGAGCCCTTG	TGGTCCAATCCAGTCATTCA
Ptf1a	CCTCTCCAAGGTAGACACGC	CTTGGGATGTGGTGTCACTG
Cpe	GACCGGCGGTACAGCTCGCG	CTGCGCCCCACCGTGTAGAT
Pcsk2	ACACAGCTCCGCACATTCGCA	TGAGATCCACAACCGCCCTCCA
Prss1	GTGTATCCTCCAACGATCTTGT	CACTTCTGATCCTAGCCCTTG
Cpa1	CCAGAAGTCCAACTGCAAGT	CAGTCTGTGGCAATGAGAACT
Ctrb1	GAATAGCATCCTCTCCGTTGAC	GTCCTGCTTTGCCCTTGT
Rbpjl	CATCTCCGAACCACACCTTG	CTCCAGTGCCTCATATCAGC
Actb	TCCGTAAAGACCTCTATGCC	GGAGGGGCCGGACTCATCGT

Mouse Primers used in RT-PCR		
Primer name	**Forward Sequence 5`-3`**	**Reverse Sequence 5`-3`**
Ins1	TAGTGACCAGCTATAATCAGAG	ACGCCAAGGTCTGAAGGTCC
Ins2	CCCTGCTGGCCCTGCTCTT	AGGTCTGAAGGTCACCTGCT
Gcg	TGTCTACACCTGTTCGCAGC	TCCTCATGCGCTTCTGTCTG
Ppy	GGCCCAACACTCACTAGCTC	CTGCTCAGGTGTCGCATAGT
Sst	GGACCTGCGACTAGACTGAC	GGCTCCAGGGCATCATTCTC
Iapp	CGTTCCAGCAACAACCTTGG	CCCTATTTGGATCCCCTGCC
Pax4	CCACCTCTCTGCCTGAAGAC	CCCACAGCATAGCTGACAGA
Neurod1	GCCTTTACCATGCACTACCC	CCCGGGAATAGTGAAACTGA
Nkx2.2	AGCGACAACCCCTACACTCGC	GCCTGAGTCAGTCGCCCGAC
Arx	TCTCTTCCTCCGGATACCCC	GATTCGCATTCGCTTCCCTG
Ngn3	CCGGATGACGCCAAACTTACA	ACACCAGTGCTCCCGGGAG
Pdx1	CCGGACATCTCCCCATACGAAGT	CGCACAATCTTGCTCCGGCTCT
MafA	ATCATCACTCTGCCCACCAT	AGTCGGATGACCTCCTCCTT
Isl1	CACTATTTGCCACCTAGCCAC	AAATACTGATTACACTCCGCAC
Mnx1	CTCATCTGAGGACGACTCGC	CCAGGTAGCCATCTTTCGCA
Pax6	CGTTGACATTTAAACTCTGGGGC	CAGCACCTGGACTTTTGCAT
Hes1	GAGGCTGCCAAGGTTTTTGG	ACTTTACGGGTAGCAGTGGC
Slc2a2	CGACCAGGAGGAACCACGCG	TGAGCCTTGCTGACACCAGCC
Kcnj11	CAAGATGCACTTCAGGCAAAA	ACGATGTCTGGTGGATGTCA
Abcc8	CCTGCAGCCAGACATAGACA	GCTCTTGGTCTTGCCTGTTC
Afp	ACTCACCCCAACCTTCCTGTC	CAGCAGTGGCTGATACCAGA
Alb	CAGATGACAGGGCGGAACTT	AGGTGCTTTCTGGGTGTAGC
Chga	AGCCAGACTACAGACCCACT	TGACTTCCAGGACGCACTTC
Ptf1a	TCAGCGAGCTGGTGCAAGCC	AAGACGCGGCCAACCCGATG
Pcsk1	CACAGACCAGCGAATAACAA	AGCAAAGATACCAGCAGCCA
Pcsk2	AAGAAGACGCAGCCTACACC	TTGTATGCTACGCCGACTCC
Cpe	TTTTCCAAAGCTTGGCTCGC	TGTGATTGCCAGGTAGCCAG
Actb	GCAGCTCCTTCGTTGCCGGT	TACAGCCCGGGGAGCATCGT

Chapter 2 Investigation the Effect of Ad-PNM on Mouse Embryonic Hepatoblasts (E14-16)

2.1. Introduction

Developmental relatedness of liver and pancreas makes liver cells one of the best candidates for beta cell formation studies. It is likely that the competence of relevant genes to be upregulated depends on their chromatin configuration and this will be more similar the closer the developmental lineage (Slack 2007). *In vitro* formation of insulin-positive cells with expression of other beta cell markers from Ad-PNM transduced rat primary hepatocytes shown in Chapter B1 also supports this idea. In addition, we also showed (Chapter B1) that progenitor-like cells are more susceptible to be reprogrammed into beta-like cells than differentiated cells. Based on these two facts, we investigated how efficiently mouse embryonic (E14-16) hepatoblast cells can be reprogrammed into insulin expressing beta-like cells through Ad-PNM transduction. For this purpose we used CD1 mice and *Pdx1-GFP* transgenic mice in which GFP protein is under control of the *Pdx1* promoter.

2.2. Experimental Design and Methods

2.2.1. Isolation and Ad-PNM Transduction of E14-16 Mouse Hepatoblasts

Mouse embryos from *Pdx1-GFP* transgenic mice and/or CD1 mice were harvested at 14-16 dpc. Livers were excised from embryos and minced into small pieces in 0.5mg/ml collagenase (Worthington) enzyme. After 30 min incubation at 37°C liver

tissues were dissociated into single cells by pipeting and passed through a 100μm membrane. Dissociated embryonic hepatoblasts were plated on collagen (Invitrogen) coated plates and maintained in high glucose DMEM supplemented with 10% FBS and 1x anti-anti solution. The next day cells were transduced with 25moi Ad-PNM and the virus was removed the day after transduction by changing the medium.

2.2.2. Immunostaining

Five days after the Ad-PNM transduction cells were fixed and stained as described in Chapter B1. Primary antibodies used were as follows: guinea pig anti-insulin (1:200 dilution, Sigma); rabbit anti-Pdx1 (1:2000 dilution, Upstate); rabbit anti-C-peptide (1:100 dilution, Cell Signaling); and mouse anti-E-cadherin (1:250 dilution, BD Bioscience). Secondary antibodies used were as follows: alexa fluor 480 goat anti-rabbit IgG (1:500 dilution, Invitrogen), alexa fluor 594 goat anti-guinea pig IgG (1:500 dilution, Invitrogen) and alexa fluor 488 anti-mouse IgG (1:500 dilution, Invitrogen). Images were taken using a Leica DMI6000 B inverted microscope.

2.2.3. Fluorescence Activated Cell Sorting (FACS) of GFP Positive Cells

Five days after the Ad-PNM transduction, cells from *Pdx1-GFP* transgenic mouse were dissociated from tissue culture plates with TryplE and were collected into FACS tubes. 7-Aminoactinomycin D (7-ADD) was also added into the cell suspension to label and thereby exclude dead cells during cell sorting. For gating, cells with no Ad-PNM were used as negative control. GFP positive cells were then sorted with a FACS Aria.

2.2.4. qRT-PCR

Total RNA isolation and cDNA synthesis from GFP-positive sorted cells (5 days in culture) control cells (no Ad-PNM, 5 days in culture) and E14-16 hepatoblasts (after isolation, not cultured *in vitro*) were performed as described in Chapter B1. The gene expression pattern between GFP-positive cells, 0 and 5 day control was then compared by performing qRT-PCR for mouse *Alb, Alpha fetoprotein* (*Afp), Transferrin* (*Trf*), *Hnf4a, Ins1, Ins2, Gcg, Somatostatin* (*Sst*), *Ppy,* endogenous *Pdx1, Pdx1, Ngn3, MafA, Kcnj11, Abcc8, Insm1, Slc2a2, Neurod1, Pax4, Rfx6, Isl1, Gck, Pcsk1, Pcsk2, Cpe, Nkx2.2, Nkx6.1, Mnx1, Solute carrier family 30 (zinc transporter) member 2* (*Slc30a2*), *Arx, Amy2,* and *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*). After an initial denaturation at 95°C for 30 s, reaction mixtures were subjected to 40 cycles of amplification using the following conditions: 95°C for 5 s denaturation, and 60°C for 10 s annealing and extension. The primer pairs used for the qRT-PCR are listed in Table 2.

2.2.5. ELISA for Glucose-Stimulated Insulin Release

After sorting the Ad-PNM treated GFP-positive E14-16 hepatoblasts of *Pdx1-GFP* transgenic mouse, the cells (~10^5) were incubated in KRB (Krebs–Ringer buffer) supplemented with 2.8mM (low glucose environment) and then in 20mM (high glucose environment) glucose for 1 h at 37°C in each, respectively. For the control cells, E14-16 hepatoblasts cultured *in vitro* without Ad-PNM were used. The KRB was then removed and kept at −80°C. The amount of insulin released into the medium was measured using an Ultrasensitive Mouse Insulin ELISA kit (Mercodia), according to the manufacturer's instructions.

2.3. Results

2.3.1. CD1 Mouse

Experiments performed with CD1 mouse E14 embryos showed that after the isolation some hepatoblasts were completely dissociated into single cells whereas some of them remained as cell clusters (Figure 7A). After plating on collagen coated plates these hepatoblast clusters layered immediately and grew as distinct colonies among the mesenchymal cells (Figure 7B). Immunostaining for insulin protein showed that some cells within these colonies start to express insulin 5 days after Ad-PNM transduction (Figure 7C and D). C-peptide was also shown to colocalize with insulin (Figure 7D and D`). *E-cadherin* is a hepatoblast marker (Nitou, Sugiyama et al. 2002) and immunostaining demonstrated that cells expressing insulin were no longer E-cadherin positive (Figure 7E). However insulin negative cells were still positive for E-cadherin.

2.3.2. Pdx1-GFP Mouse

Transduction of *Pdx1-GFP* transgenic mouse E14-16 hepatoblasts with Ad-PNM resulted in the expression of GFP in some cells 5 days after Ad-PNM transduction (Figure 8A). Even though the fraction of GFP-positive cells (1%) was low, we sorted nearly 10^5 cells out of 10^7 cells. From these sorted cells we performed qRT-PCR to identify the gene expression profile and ELISA to examine glucose stimulated insulin release of the cells. We also performed immunostaining on a sample of these cells before we sorted them.

Immunostaining of these cells showed that not all but some of these GFP-positive cells were also insulin positive (Figure 8B and C). Also there were some cells which were positive for insulin but not GFP.

The qRT-PCR of GPF-positive cells showed that these cells upregulated many pancreatic endocrine cell markers including the islet hormone genes *Ins1, Ins2, Gcg, Sst, Ppy,* glucose stimulated insulin release-related cell membrane protein genes *Kcnj11, Abcc8, Slc2a2,* insulin maturation-related protein genes *Pcsk1, Pcsk2, Cpe,* and beta cell transcription factors such as endogenous *Pdx1*, *Neurod1*, *Pax4*, *Rfx6, Isl1*, *Insm1* (Figure 9). Moreover GFP-positive cells downregulated the expression of liver markers such as *Alb, Afp, Trf* and *Hnf4a* to some extent although not completely (Figure 9). However GFP-positive cells are still missing some typical beta cell transcription factors such as *Nkx2.2*, *Nkx6.1* and *Mnx1*.

ELISA results showed that Ad-PNM transduced GFP-positive cells can secrete insulin and that the release of insulin was to some degree controlled by the glucose level (Figure 10). Hepatoblast cells cultured for 5 days with no Ad-PNM transduction had trace amounts of insulin released into the medium. Ad-PNM transduced GFP-positive cells, however, increased the amount of released insulin in low glucose and even more in high glucose environments (Figure 10).

2.4. Discussion

The qRT-PCR results showed that the GFP-positive cells have indeed upregulated the endogenous *Pdx1* gene, and were not simply showing a false-positive upregulation of GFP (Figure 9). This is the first time that we have seen the upregulation of endogenous

Pdx1 in mouse cells. The activation of endogenous counterparts of the input genes is essential for real reprogramming events. Otherwise even if target genes are upregulated, the cells remain dependent on input genes to maintain their new phenotype. Beside endogenous *Pdx1*, the GFP-positive cells upregulated also other important beta cells markers such as beta cell hormone genes (*Ins1* and *Ins2*), insulin processing protein genes (*Pcks1*, *Pcsk2* and *Cpe*), beta cell membrane protein genes responsible for glucose stimulated insulin release (*Kcnj11* and *Abcc8*) and many beta cell transcription factors (*Neurod1*, *Pax4*, *Isl1*, *Isnm1*, *Rfx6*). Moreover liver cell markers such as *Alb*, *Afp*, *Trf* and *Hnf4a* were downregulated in these cells. However even though the effects of Ad-PNM on these cells were dramatic, these cells were not fully reprogrammed into genuine beta cells. Beside *Insulin* genes, the expression of *Gcg*, *Ppy* and *Sst* encoding other pancreatic hormones, indicates that this was not a true beta cell phenotype. Moreover, even though the liver markers were downregulated significantly, they were not completely turned off.

A modest degree of glucose stimulated insulin release was shown by the GFP-positive cells. This glucose dependent insulin release of GFP-positive cells was consistent with the observed *Kcnj11* and *Abcc8* upregulation. Even though the results are encouraging, the amount of released insulin was still lower than insulin amount released by genuine beta cells.

The results indicate that the embryonic hepatoblasts are more susceptible to the effects of Ad-PNM than adult hepatocytes. Although the transformation to a beta cell phenotype was not complete, it should be noted that the GFP-positive population contained many cells which were not insulin positive and may represent other types of pancreatic precursors. It is therefore possible that the minority of insulin-positive, GFP-

positive cells do have a genuine beta cell phenotype, although to establish this for sure would require considerable additional work.

Figure 7: Insulin, C-peptide and E-cadherin immunostaining in CD1 mouse embryonic hepatoblasts after Ad-PNM

(**A**) Hepatoblast cell clusters immediately after isolation from E14-16 mouse embryo, (**B**) Expanding hepatoblasts in culture 1 day after isolation, (**C**) Insulin (red) and viral PDX1 (green) positive hepatoblasts 5 days after Ad-PNM transduction, (**D**) Insulin (red) and (**D`**) C-peptide (green) colocalization in the same cells, (**E**) Expression of Insulin (red) and E-cadherin (green) in different cells. DAPI (blue). Scale bars =100μm.

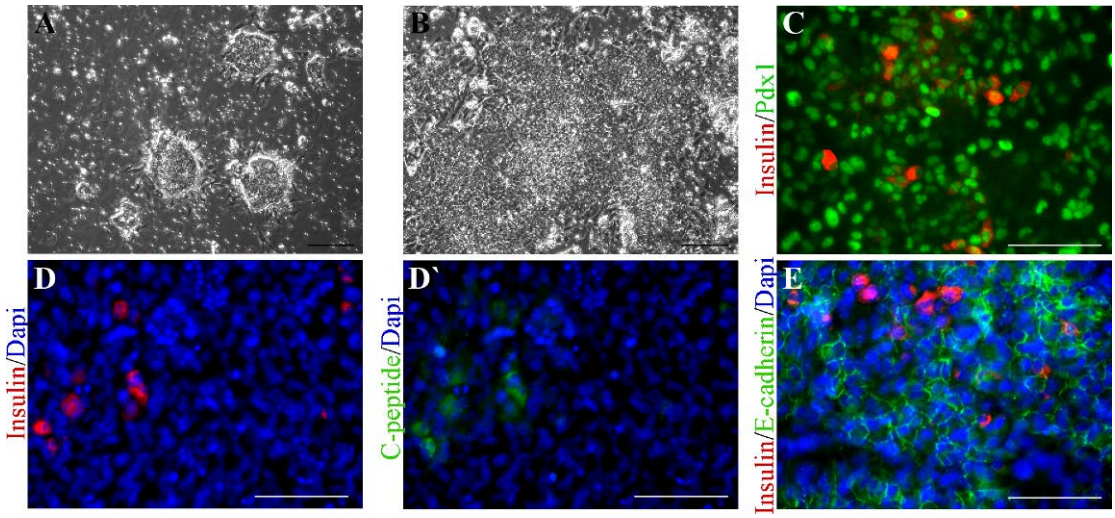

A
B
C
Insulin/Pdx1
D
Insulin/Dapi
D`
C-peptide/Dapi
E
Insulin/E-cadherin/Dapi

Figure 8: GFP expression and insulin immunostaining in *Pdx1-GFP* mouse embryonic hepatoblasts after Ad-PNM

GFP expression (**A**) and Insulin (red) staining (**B**, **C**) in mouse embryonic hepatoblasts (E14-16) 5 days after Ad-PNM transduction. Scale bars =100μm.

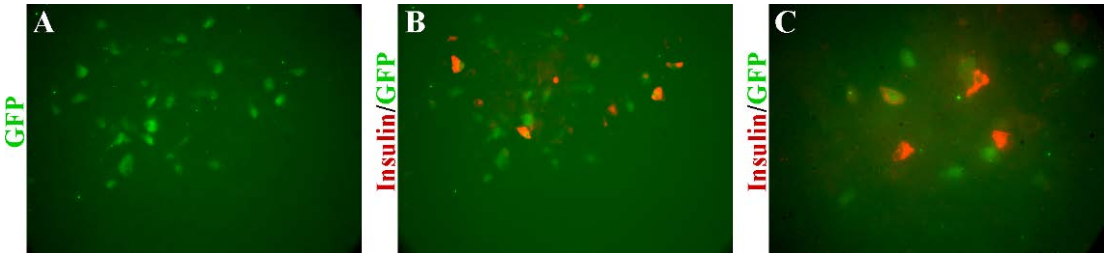

A
GFP
B
Insulin/GFP
C
Insulin/GFP

Figure 9: Gene expression profile of E14-16 mouse embryonic hepatoblasts after Ad-PNM (qRT-PCR)

Gene expression profile between day 0 E14-16 mouse hepatoblasts (immediately after isolation, No Ad-PNM) (white bars), day 5 E14-16 mouse hepatoblasts (cultured 5 days in vitro, No Ad-PNM) (gray bars) and GFP-positive FACS sorted E14-16 mouse hepatoblasts (cultured 5 days in vitro, transduced with Ad-PNM) (black bars) were compared with qRT-PCR. Top panel is showing the expression pattern of pancreatic markers. Bottom panel is showing the expression pattern of liver markers. Data are means ± S.E. (n = 3).

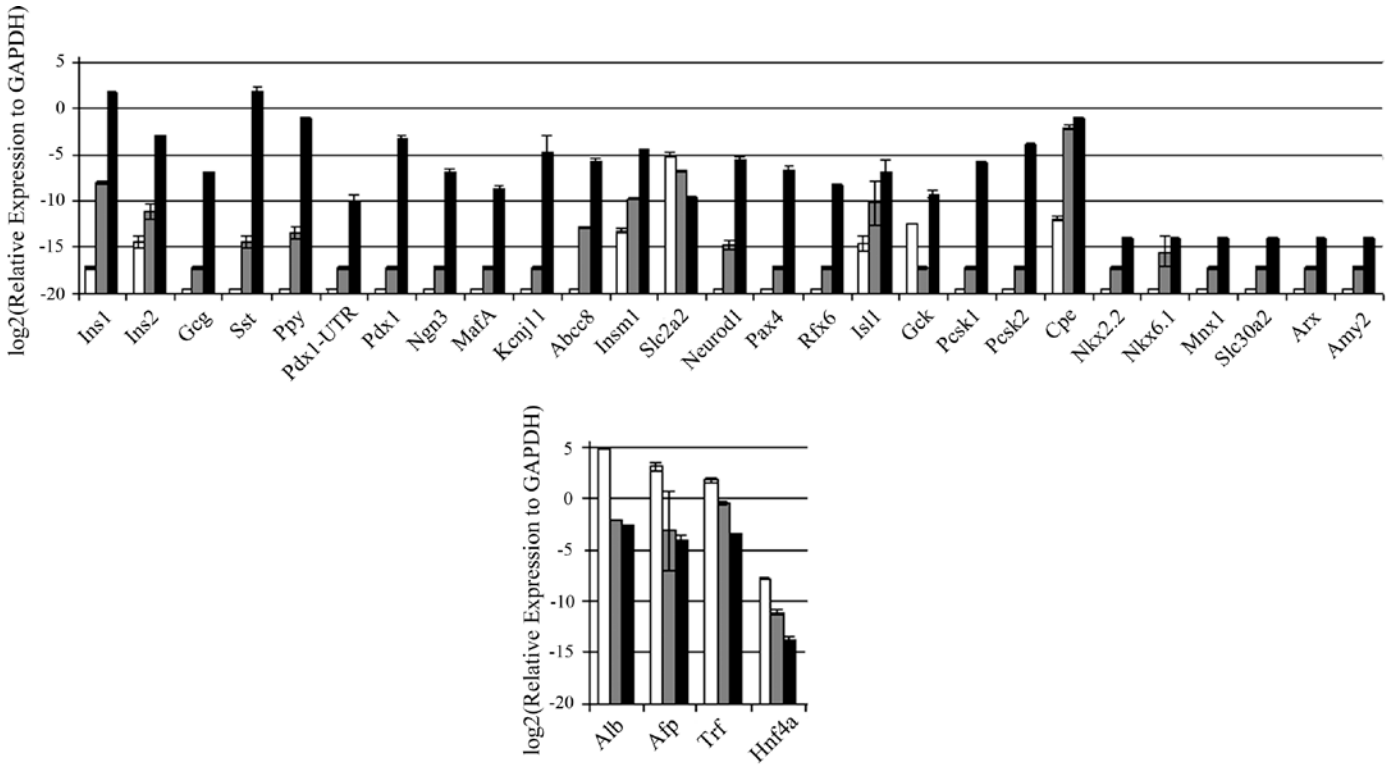

log2(Relative Expression to GAPDH)
5
0
-5
-10
-15
-20
Ins1
Ins2
Geg
Sst
Ppy
Pdx1-UTR
Pdx1
Ngn3
MafA
Kcnj11
Abcc8
Insm1
Slc2a2
Neurod1
Pax4
Rfx6
Isl1
Gck
Pcsk1
Pcsk2
Cpe
Nkx2.2
Nkx6.1
Mnx1
Slc30a2
Arx
Amy2
log2(Relative Expression to GAPDH)
5
0
-5
-10
-15
-20
Alb
Afp
Trf
Hnf4a
Day 0 E14-16 mouse hepatoblasts (immediately after isolation, No Ad-PNM)
Day 5 E14-16 mouse hepatoblasts (5 days in culture, No Ad-PNM)
GFP-positive FACS sorted E14-16 mouse hepatoblasts (5 days in culture, transduced with Ad-PNM)

Figure 10: Secreted insulin from E14-16 mouse embryonic hepatoblasts after Ad-PNM

The amount of insulin in the medium released from E14-16 mouse hepatoblasts (cultured 5 days in vitro, No Ad-PNM) and GFP-positive FACS sorted E14-16 mouse hepatoblasts (cultured 5 days in vitro, transduced with Ad-PNM) was measured by ELISA. GFP-positive cells were stimulated either with low-glucose or high-glucose. Data are means ± S.E. (n = 2).

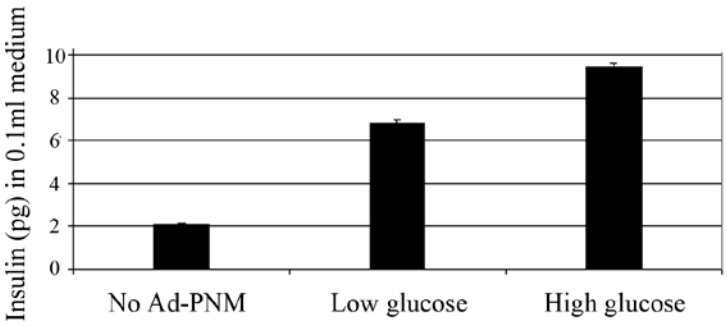

Insulin (pg) in 0.1ml medium
10
8
6
4
2
0
No Ad-PNM
Low glucose
High glucose

Table 2: Mouse primers used in qRT-PCR

Mouse Primers used in qRT-PCR		
Primer name	**Forward Sequence 5`-3`**	**Reverse Sequence 5`-3`**
Alb	CAGTCAGCCAGTTCACCATAG	TGTGTTGCCGATGAGTCTG
Ins1	GCCATGTTGAAACAATGACCTG	GCCAAACAGCAAAGTCCAG
Ins2	CATGGGTGTGTAGAAGAAGCC	TTTGTCAAGCAGCACCTTTG
Gcg	CAGCATGCCTCTCAAATTCATC	ACATTCACCAGCGACTACAG
Sst	GGCATCATTCTCTGTCTGGTT	AGACTCCGTCAGTTTCTGC
Ppy	GCTGGACCTGTACTCTCCTA	TTGGCTTGATTCCCTGCTC
Amy2	TCTCTGTGTTGGAAAATGAAATCT	CCTGGAGACATAAAGGCAGTT
Pdx1	GCAGTACGGGTCCTCTTGT	GATGAAATCCACCAAAGCTCAC
MafA	GCCAACTTCTCGTATTTCTCCT	CACATTCTGGAGAGCGAGAAG
Pax4	AGAAGCTGAAATGGGAAGCA	GGGGACTGTGCAGAGATGAT
Nkx2.2	CTTATCCAATCGCTCCACCTT	TCCAGAACCATCGCTACAAG
Nkx6.1	TCCGAGTCCTGCTTCTTCT	CACGCTTGGCCTATTCTCTG
Rfx6	CAATAAATGCCTCCACTGTAGC	TCTTACCATATCCAACAGCACA
Isl1	GCCTGTAAACCACCATCATGT	GTGCAAGGACAAGAAACGC
Mnx1	GCTGCGTTTCCATTTCATTCG	CAGTTCAAGCTCAACAAGTACC
Neurod1	ACACTCATCTGTCCAGCTTG	AGATCGTCACTATTCAGAACCTTT
Arx	TGTGGGCTGTCTCAGGA	GGTCTGAGCACTTTTCTAGGAG
Abcc8	GAATGATGACAGCTGCTCCA	TCTTCTTATGCCCAAACCTCTG
Slc2a2	CTCTTCCAACGTGGTCCCTA	GAGCCCCTCGTAGGTTTTTC
Kcnj11	TTGGAAGGCGTGGTACAAAC	CCCCATAGAATCTCGTCAGC
Gck	ATAAGCCAGTGGTGGAGTGG	CCAACACAGGTGTGAAGTGG
Gapdh	GTGGAGTCATACTGGAACATGTAG	AATGGTGAAGGTCGGTGTG

Chapter 3 Investigation the Effect of Ad-PNM on Rat Pancreatic Exocrine Cell Line AR42J-B13

3.1. Introduction

PDX1 is a major pancreatic transcription factor. It belongs to the ParaHox gene family. Expression of *Pdx1* is necessary both for formation of the pancreatic buds in the embryo and subsequently for beta cell formation and function (Jonsson, Carlsson et al. 1994; Guz, Montminy et al. 1995). NGN3 is a member of basic helix-loop-helix transcription factor family. Expression of *Ngn3* is essential for the formation of endocrine progenitor cells in the trunk epithelium of developing pancreas (Gradwohl, Dierich et al. 2000). MAFA is a member of the basic leucine zipper transcription factor family. Expression of *MafA* is essential for maturation of beta cells (Kataoka, Han et al. 2002). Expression of *Pdx1* and *MafA* genes is not only indispensable during pancreas organogenesis but they also positively regulate the expression of *Insulin* genes in beta cells.

In the study of Zhou et al, the authors transformed pancreatic exocrine cells into insulin expressing beta-like cells *in vivo* by overexpressing the ectopic *Pdx1*, *Ngn3* and *MafA* genes. They also reported that these insulin-positive cells restored the blood glucose level in streptozotocin-treated immunodeficient diabetic mice (Zhou, Brown et al. 2008). However, it is difficult to investigate the molecular mechanisms of cell transformation *in vivo*. For that reason we felt it important to establish an *in vitro* model for the process which is more amenable to study. For this purpose we selected the AR42J-B13 cell line which was shown as described above to give an extreme response to

Ad-PNM by transforming more than 70% of the cells into an insulin-positive state. AR42J-B13 is a rat cell line, which was originally derived from a chemically induced pancreatic tumor (Longnecker, Lilja et al. 1979), with a pancreatic exocrine phenotype. The cells are easy to grow in tissue culture and they express high levels of typical exocrine cell products like amylase, trypsin and carboxypeptidase A1. Unlike primary cultures of exocrine pancreas cells, AR42J-B13 cells have a stable phenotype during *in vitro* culture for a long period. Although there are reports in the literature of insulin-positive cells arising from this line following culture on Matrigel and treatment with various growth factors (Mashima, Shibata et al. 1996; Aldibbiat, Marriott et al. 2008), we have not used such conditions in this study and saw no spontaneous endocrine differentiation of the AR42J-B13 cells under the conditions used.

In this study, we investigated the effect of *Pdx1*, *Ngn3* and *MafA* combination on rat exocrine cell line AR42J-B13 in a more detailed way to see the changes in their gene and protein expression profiles. Moreover we asked if the cells can gain glucose-controlled insulin releasing mechanism and restore blood glucose levels in streptozotocin-treated immunodeficient diabetic mice.

3.2. Experimental Design and Methods

3.2.1. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

AR42J-B13 cells were cultured and transduced with Ad-PNM as described in Chapter B1. Total RNA isolation, DNase treatment and cDNA synthesis from AR42J-B13 cells were performed as described in Chapter B1. The gene expression pattern

between AR42J-B13 cells with and without Ad-PNM was then compared by performing PCR for rat *Ins1, Ins2, Gcg, Ppy, Sst, Iapp, Pax4, Neurod1, Nkx2.2, Arx, Pdx1, Ngn3, MafA, Nkx6.1, Isl1 , Mnx1, Pax6, Hes1, Slc2a2, Kcnj11, Abcc8, Cpe, Pcsk2, Amy2* (24 cycles), *Prss1* (*Trypsin*), *Cpa1* (27 cycles), *Ctrb1* (*Chymotrypsinogen B1*; 25 cycles), *Rbpjl* (*Recombination signal binding protein for immunoglobulin kappa J region-like*; 26 cycles), *Ptf1a* and *Actb* (*Beta actin*), in addition to mouse *Pdx1, Ngn3* and *MafA*. Rat whole pancreas cDNA was used as a positive control for *Gcg*, *Sst* and *Ppy*. cDNA from Ad-PNM-transduced HEK (human embryonic kidney)-293 cells was used as positive control for the input genes mouse *Pdx1, Ngn3* and *MafA*. cDNA from RIN-m5F cells was used as positive control for the rest of the genes. After an initial denaturation at 94°C for 3 min, the reaction mixtures were subjected to 30 cycles (unless otherwise indicated) of amplification using the following conditions: 94°C 30 s denaturation, 60°C 30 s annealing, 72°C 1 min extension. This was followed by a final extension step at 72°C for 5 min. Primer pairs used for RT-PCR are listed in Table 1. PCR products were then run on 1% (w/v) agarose gel (Invitrogen) including ethidium bromide, in TAE buffer (Bio-Rad Laboratories), by electrophoresis at 100V. Agarose gels were visualized with a Bio-Rad Gel Documentation System model 2000.

3.2.2. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

After transduction with Ad-PNM, AR42J-B13 cells were maintained in low-glucose DMEM medium supplemented with 200μM of the anti-mitotic agent araC (cytosine arabinofuranoside; Sigma) to suppress the continued proliferation of non-transformed AR42J-B13 cells. Total RNAs were isolated at different time points (0-12

days post-infection) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's manual. DNase treatment and cDNA synthesis from these cells were performed as described in Chapter B1. The gene expression pattern between AR42J-B13 cells with and without Ad-PNM was then compared by performing qRT-PCR for rat *Ins1*, *Ins2*, *Iapp*, *Pax4*, *Neurod1*, *Nkx2.2*, *Arx*, endogenous *Pdx1*, endogenous *Ngn3*, endogenous *MafA*, *Isl1*, *Mnx1*, *Hes1*, *Slc2a2*, *Kcnj11*, *Abcc8*, *Amy2*, *Ptf1a*, *Cpe*, *Pcsk2*, *Pcsk1*, *Prss1*, *Cpa*, *Ctrb1*, *Rbpj1* and *Gapdh*. After an initial denaturation at 95°C for 30 s, reaction mixtures were subjected to 40 cycles of amplification using the following conditions: 95°C for 5 s denaturation, and 60°C for 10 s annealing and extension. The primer pairs used for the qRT-PCR are listed in Table 3.

3.2.3. Immunostaining

Immunostaining experiments were performed at different time points (0-12 days post-infection) as described in Chapter B1. Number of insulin-positive cells was also counted out of Ad-PNM bearing cells at different time points to see if there is any change in number of insulin-positive cells over time.

3.2.4. EdU (5`-ethynl-2`-deoxyuridine) Labeling and Staining

5×10^5 AR42J-B13 cells were used in EdU (5`-ethynl-2`-deoxyuridine) labeling experiments. EdU (10μM) from the Click-iT EdU 594 Imaging Kit (Invitrogen) was used for labeling. After the incubation (overnight and/or 1 hr), cells were fixed and stained for EdU, according to the manufacturer’s instructions to detect the number of cells in cycle

during the EdU treatment. Images were taken using a Leica DMI6000 B inverted microscope.

3.2.5. ELISA for Total Insulin Content and Glucose-Stimulated Insulin Release

AR42J-B13 cells (5×10^5) were plated in each well of a six-well plate. For control groups, cells in three wells were cultured without transduction. For experimental group, cells in the remaining three wells were transduced with Ad-PNM for overnight. After overnight incubation medium was changed and virus was removed. At 3 days after Ad-PNM administration, DMEM medium was removed and cells were incubated either with 2.8mM (low glucose environment) or 20mM (high glucose environment) glucose in KRB for 1 h at 37°C. In some experiments 30mM KCl was included to effect depolarization of the cells. The KRB was then removed and kept at −20°C for measurement of insulin release into the medium. After the removal of KRB, the cells were scraped into 0.18 M HCl/35% ethanol. The cells were homogenized by sonication with a Branson Sonifier 450 (6×10 s on ice with 1 min waiting on ice between pulses) and were rotated overnight at 4°C. The next day, the cells in acid/ethanol were subjected to centrifugation (10000 g for 30 min at 4°C). The supernatant was collected and kept at −20°C to measure the total amount of insulin in the cells. The remaining cell pellets after centrifugation were resuspended in 0.1M NaOH to measure the total amount of protein in the cells. The amount of the insulin released into the medium and the total insulin in the cells were measured using an Ultrasensitive Rat Insulin ELISA kit (Mercodia), according to the manufacturer's instructions. The total protein amount in the cells was measured using the

BCA (bicinchoninic acid) protein assay kit (Thermo Scientific), according to manufacturer's instructions.

3.2.6. Mouse Experiments

For the cell transplantation experiments**,** adult male NOD-SCID mice weighing 22–27g (up to 42 days old) were injected intraperitoneally with streptozotocin (Sigma) at a dose of 120 mg/kg of body mass. The mice were considered diabetic when their blood glucose level reached a stable value over 400 mg/dl. The diabetic mice were then transplanted with Ad-PNM-transduced AR42J-B13 cells or with Ad-GFP-transduced AR42J-B13 cells as control. An aliquot of 2×10^6 cells were transplanted under the kidney membrane of each mouse. Blood was drawn from the tail vein and blood glucose levels were measured with a glucose meter every two days after the transplantation to see the change in the blood glucose level. At 10 days after cell transplantation, the kidneys with transplanted cells were removed and blood glucose levels of mice were monitored for two more days. The blood glucose changes were quantified by measuring the area under the curve, and comparisons were made using a Student's *t* test. The excised kidneys were fixed in 10% (v/v) formalin (Fisher) and were processed for tissue sectioning. After antigen retrieval, tissue sections were immunostained for insulin, E-cadherin and DAPI (4`,6-diamidino-2-phenylindole) as described above. The mouse anti-E-cadherin (1:500 dilution, BD Bioscience Pharmingen) primary antibody and Alexa Fluor® 488 goat anti-mouse IgG (1:500 dilution, Invitrogen) secondary antibody were used to stain E-cadherin protein.

For expression of exogenous *Pdx1*, *Ngn3* and *MafA* in the exocrine pancreas, adult CD1 mice were anaesthetized with avertin. A portion of skin was shaved and wiped with betadine. A small incision was then made and the pancreas everted from the wound. Ad-GFP or Ad-PNM (100µl; 10^{11} plaque-forming units/ml) were injected into the splenic lobe of the pancreas using an insulin needle. This caused formation of a visible bubble of fluid within the pancreas. The pancreas was reinternalized and the wound repaired in layers. The mice were killed 7 days later. The pancreases were removed, fixed in 10% (v/v) formalin (Fisher) and processed for tissue sectioning (frozen for GFP and paraffin for immunostaining). The tissue sections were stained for insulin and Ngn3 as described above. The animal experiments were carried out under IACUC (Institutional Animal Care and Use Committee) protocol 1007A85640 of the University of Minnesota.

3.3. Results

3.3.1. Effects of *Pdx1, Ngn3* and *MafA* on AR42J-B13 Cells

Administration of different adenoviral doses from 1 to 100 MOI indicated that for AR42J-B13 cells the optimum MOI was 25 and this dose was used in the experiments to be described.

One day after Ad-PNM administration, cells changed their shape immediately from an oval structure to a more flattened fibroblast-like shape (Figure 11A and 11B). In the control group, in which cells were transduced with an adenovirus encoding GFP protein (Ad-GFP), cells did not have this phenotype (Figure11C) which showed that the change in cell shape was not a consequence of adenovirus infection itself.

In addition to cell shape, Ad-PNM also altered the gene expression pattern. RT-PCR results are shown at 3 days after Ad-PNM administration (Figure 12) and qRT-PCR over a time course of 12 days (Figure 13). AraC, which kills dividing cells, was added into the cell culture medium for the 12 day studies to remove untransduced cells that continue to divide and would otherwise overgrow the cultures. qRT-PCR results therefore reflected just the Ad-PNM-transduced cells. After transduction with Ad-PNM, AR42J-B13 cells started to express *Ins1* and *Ins2* genes. *Iapp* and *Pax4* genes, which are not normally expressed in AR42J-B13 cells, were also activated. Moreover, expression level of genes encoding the beta cell transcription factors such as NEUROD1, NKX2.2 and the pro-insulin processing enzymes CPE and PCSK2, which were all expressed to some extent in the untreated cells, were increased in Ad-PNM treated AR42J-B13 cells. *Abcc8*, one of the genes for the ATP-sensitive potassium channel, was up-regulated, but *Kcnj11,* the gene for another component of this channel, was not. While a majority of the beta cell markers were up-regulated, the expression of all the exocrine acinar cell markers (*Amy2, Prss1, Cpa1, Ctrb1, Rbpj1 and Ptf1a)* was down-regulated, as was the centroacinar marker *Hes1* in Ad-PNM-treated cells. There was approximately 100-fold fall in *Amy2*, *Prss1* and *Cpa1* transcripts, whereas the fall was approximately 10-fold in *Ctrb1, Rbpj1 and Ptf1a*. Moreover, the genes encoding the hormones produced by the non-beta endocrine cell types found in the pancreas such as *Gcg*, *Ppy*, *Sst* and *Ghrl* (ghrelin) were not up-regulated.

Based on RT- and qRT-PCR results, the Ad-PNM-treated AR42J-B13 cells transformed their phenotype into something more resembling that of pancreatic beta cells than that of pancreatic exocrine cells. On the other hand, the indispensable genes for a

beta cell phenotype, such as membrane channel proteins encoding *Slc2a2* and *Kcnj11*, were not significantly up-regulated (Figures 12 and 13). One of the unique characters of beta cells is to secrete insulin in response to a rise in blood glucose concentration. The essential components of this mechanism are not all induced in our cells. These results indicate that the reprogramming of AR42J-B13 cells into pancreatic beta cells is incomplete. In addition, even though the reduction of exocrine cell gene expression, is striking, it is not complete. Moreover, expression is not induced from the endogenous counterparts of the three input genes. When designing the Ad-PNM, only the coding regions of *Pdx1, Ngn3 and MafA* were cloned into the vector. In other words, there was no 5` or 3` untranslated region (UTR) of input genes so that their mRNA could be distinguished from those of endogenous *Pdx1, Ngn3* and *MafA* using suitable primers in PCR. Although the expression of *Ngn3* might be expected to be confined to the period of formation of endocrine precursors, we would have expected to see *Pdx1* and *MafA* expressed long-term in genuine beta cells, and it was not.

We also showed the existence of insulin protein, its side product C-peptide, the major exocrine protein amylase, and the three input proteins PDX1, NGN3 and MAFA by immunostaining. Immunostained insulin protein was first observed at day 3 in the majority of Ad-PNM-transduced cells (Figure 14). These insulin-positive cells could persist in cell culture for at least 4 weeks (results are not shown). Some of the cells did not become insulin positive even though they are transduced with Ad-PNM (especially highly Pdx1, Ngn3, MafA positive cells were not insulin positive) but approximately 70% of Ad-PNM bearing cells did so and this proportion remained similar over at least 12 days (Figure 14D). The cells were shown to have C-peptide which was co-localized

with insulin. C-peptide is normally excised during the maturation of pro-insulin to the mature two chain structure and its presence in insulin-positive cells indicates that the pro-insulin protein is properly processed after expression (Figure 14E). In addition, the presence of C-peptide also indicated that immunostained insulin was produced by cells instead of taken from the culture medium by dead and/or dying cells. Moreover, co-immunostaining for amylase and insulin proteins showed that the insulin positive cells completely lost amylase protein which is one of the major products of pancreatic exocrine cells (Figure 14F). In these immunostaining experiments araC was not used so there were still untransduced AR42J-B13 cells present which were growing rapidly. This provides the internal positive control for amylase staining in Figure 14F.

Another change we observed was that Ad-PNM transduced cells were slowing and/or stopping their cell cycle whereas untransduced cells kept proliferating rapidly. To verify this observation we looked at the proliferation ability of AR42J-B13 cells before and after Ad-PNM transduction by EdU administration and staining (Figure 15). A 4 h pulse of EdU labeled approximately 47% of the untreated AR42J-B13 cells as expected for a rapidly dividing population (Figures 15A and 15D). When we added Ad-PNM after such a 4 h pulse and examined the insulin-positive cells 3 days after Ad-PNM administration, a high proportion of the cells were labeled (~27%), although somewhat less than 47%, indicating that the transformation may occur either in or out of S-phase (Figures 15B and 15D). In other words, the stage of the cell cycle was not important for the cells to turn into an insulin-positive cell. However, if the cells were transduced with Ad-PNM first, left for 3 days and then were given an overnight label with EdU, the proportion of insulin-positive cells that were also EdU-labeled was extremely small

(~1%) (Figure 15C and 15D). This showed that cell division ceases almost entirely following the transformation. When we suppress the overgrowth by the untransformed AR42J-B13 cells by using araC, the fibroblast-shaped insulin-positive cells persisted for an extended period. We have maintained Ad-PNM-transduced cells in cultures with araC for 4 weeks, although there is some cell death of the insulin-positive cells over this period.

3.3.2. Insulin Content and Secretion in Transformed Cells

To quantify and compare the amount of the insulin produced, we measured total levels cells as well as secreted insulin in the medium by ELISA in AR42J-B13 cells with or without Ad-PNM. ELISA results showed a substantial insulin increase in Ad-PNM-transduced cells (Figure 16A). However, the insulin level in Ad-PNM transduced cells did not reach the insulin level of mature beta cells which can contain as much as 5% of insulin as a proportion of total cell protein. Since glucose-stimulated insulin release is the key physiological property of genuine beta cells we examined this property by treating the cells with 2.8mM (low) or 20mM (high) glucose in KRB for 1 h and then analyzing the released insulin content of the medium by ELISA (Figure 16B). In addition to low and high glucose environment, we also tested the effect of KCl which produces complete depolarization of beta cells and causes them to emit a maximal amount of insulin. Untreated AR42J-B13 cells did not secrete any insulin as expected. The Ad-PNM-transduced cells, on the other hand did secrete insulin, but in a completely unregulated manner, showing no effect of glucose or KCl. Based on these results, we concluded that the transformed cells did secrete insulin in a constitutive, but unregulated, manner.

3.3.3. Insulin-Expressing AR42J-B13 Cells Rescue Diabetic Mice

Insulin-expressing cell transplantation to ameliorate diabetes in experimental animals is one of the standard assays for a beta cell phenotype in diabetes studies. To fulfill this assay, we transplanted insulin-expressing AR42J-B13 cells to an immune-deficient (NOD-SCID) mice line which will tolerate a graft of rat cells. Despite their name (non-obese diabetic), these mice are not diabetic, and diabetes is induced by an injection of the toxin streptozotocin, a chemical which preferentially destroys beta cells in pancreas to generate a model of Type 1 diabetes.

Once the animals become diabetic (blood glucose level>400mg/dl), we transplanted Ad-PNM- transduced insulin-positive AR42J-B13 cells under the kidney capsule (only one kidney per animal was grafted) and then monitored the mice every two days. The fall in blood glucose indicates a relief of the diabetes. Following this, we removed the grafted kidneys for analysis. The rise of blood glucose after removal of the grafted kidney indicated that the relief of diabetes was in fact due to the graft and not to a non-specific effect of surgery or to consequent effects on feeding behaviour. Because of the risk of tumor formation from residual non-transformed AR42J-B13 cells, these experiments were terminated at 20 days after cell transplantation. Three out of ten mice were successfully engrafted with Ad-PNM-treated cells (■) (Figure 17A). Immunostaining of kidney sections from the excised kidneys demonstrated the persistent presence of insulin-positive rat cells under the kidney capsule (Figure 17B). The controls consisted of one mouse receiving Ad-GFP-treated cells (Δ) and three receiving Ad-PNM-treated cells, in which the graft did not take (●). The results were quantified by measuring

the area under the curve for each mouse from days 9–17 inclusive. This gave mean values of 1285 (S.D.=426) for the active grafts, 2531 (S.D.=385) for the grafts that did not take, and 2780 for the diabetic control. Comparison of the active grafts with those that did not take showed a *P* value of 0.020. Comparison of the active grafts with diabetic control gave a *P* value of 0.026. By contrast, comparison of the grafts that did not take with the diabetic control showed no difference (*P*=0.379). This result indicates that the Ad-PNM-treated AR42J-B13 cells are capable of relieving diabetes in an animal model, even though they do not exhibit glucose-sensitive insulin secretion.

3.3.4. Transduction of Exocrine Pancreas *in vivo*

In addition to transformation we have seen in pancreatic exocrine cell lines *in vitro*, we wanted to see if Ad-PNM can transform pancreatic exocrine cells *in vivo* (Zhou, Brown et al. 2008). For this purpose, Ad-GFP or Ad-PNM were injected directly into the mouse pancreas as described previously (Zhou, Brown et al. 2008) and the results are shown in Figure 18. Injection of Ad-GFP showed a very high efficiency of transduction in exocrine tissue, with most cells expressing GFP protein. A small number of beta cells in the islets, visualized by immunostaining for insulin, were also transduced with Ad-GFP (Figure 18A). When we gave a similar dose of Ad-PNM, once again a high level of transduction was detected by expression of the vector-encoded proteins. Figure 18B shows the expression of Ngn3, which is not normally found in mature exocrine or endocrine cells. The sections were stained for insulin and it was evident that a proportion of the scattered cells expressing NGN3 were also expressing insulin. The insulin expression was not necessarily observed in those cells expressing the highest levels of

NGN3, rather it was more likely to be observed in those with intermediate levels. This experiment confirms that the basic observation of Zhou et al. (Zhou, Brown et al. 2008) was correct. On the other hand it was difficult to establish for certain the phenotype of scattered cells *in vivo*. Consistent with the study of the AR42J-B13 cells, the insulin-positive cells *in vivo* superficially resembled exocrine cells making insulin, rather than true beta cells.

3.4. Discussion

Generation of a particular adult cell type by direct reprogramming from another terminally differentiated adult cell type is of interest for many reasons. First of all, direct reprogramming studies have an important intellectual issue because it raises the question of whether the 200 or so visible cell types are the only possible stable states of vertebrate cells or whether numerous other states are potentially stable but are not accessed during normal development. This question can be experimentally probed by experiments in which developmental transcription factors are misexpressed in order to drive cells into a new developmental state. Because of the dense web of regulatory connections between developmental transcription factors, overexpression of a small number of factors can sometimes push the cell into a new state which involves a change in activity of thousands of genes. In case of medical practice, direct cell reprogramming can potentially solve many current problems such as shortage of a cell population to be transplanted, usage of immunosuppression therapy in cell transplanted-patients, or bypassing the need for ES cells to create a particular adult cell type. In the case of generation of the beta cell phenotype, direct reprogramming is also important from a practical point of view.

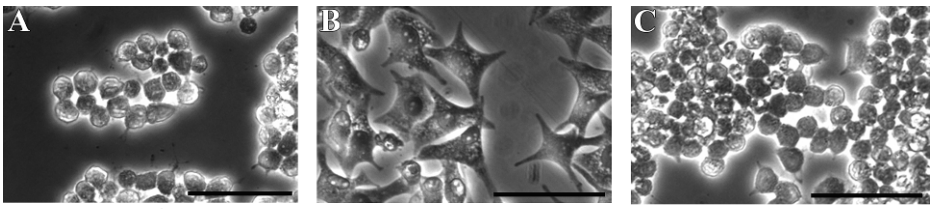
Following the publication by Zhou et al. (Zhou, Brown et al. 2008) we were motivated to perform the present study. In that study, formation of insulin-positive cells in the exocrine pancreas of mice after direct injection of three different adenoviruses encoding the three genes *Pdx1*, *Ngn3* and *MafA* was shown by Zhou et al. Our results (Figure 18) indicated that the procedure can certainly induce ectopic insulin-positive cells, but it is not easy to prove that they are genuine beta cells with the full range of properties expected of this cell type. In order to facilitate mechanistic studies we carried out our experiments in *in vitro* by using a well known pancreatic exocrine cell line AR42J-B13. Our results showed that there is a remarkable phenotypic change in these cells after the ectopic expression the three genes. The cells change morphology to a flat fibroblast-like shape, stop dividing, down-regulate the expression of a range of exocrine markers, express insulin from both the endogenous genes and display a number of characteristic transcription factors normally found in beta cells. However, the reprogramming is not complete, since the endogenous counterparts of the introduced genes were not up-regulated, and some components of the all-important glucose-sensing mechanism of beta cells were not induced. We showed this both from the lack of expression of the *Slc2a2* and *Kcnj11* genes and from the lack of glucose-induced insulin secretion by these cells. In a previous study (Aldibbiat, Marriott et al. 2008), which involved formation of insulin-positive cells from AR42J-B13 cells cultured on Matrigel and treated with growth factors, it was also reported that insulin secretion from these cells was not glucose-regulated. On the other hand, recently Lima et al. showed that ectopic expression of *Pax4* together with *Pdx1*, *Ngn3* and *MafA* can stimulate glucose-controlled insulin secretion in AR42J-B13 cells (Lima, Docherty et al. 2012). Even though

endogenous *Pax4* was activated significantly in our cells after transduction with Ad-PNM, we still did not observe glucose-regulated insulin secretion in the current study.

If cellular reprogramming to beta cells does eventually become a method for clinical use, it is likely to take the form of *in vitro* cell transformation followed by a graft similar to the current islet transplantation procedure. This is because direct introduction of genes into the pancreas is likely to pose risks of oncogenic transformations as seen in other types of gene therapy (Flotte 2007). Thus it is necessary to show that the transformed cells can indeed cure diabetes following transformation. In our case we have shown that the Ad-PNM-treated AR42J-B13 cells can cure streptozotocin induced diabetes. However, we have good evidence that the cells are not glucose-responsive, so their curative ability must derive from their constitutive insulin secretion. This is a reminder that the ability to rescue experimental diabetes in rodents is not a very discriminating characteristic. For treatment of human diabetes, cells must have the exact glucose responsiveness of real beta cells or they will not be significantly better than the present regimes of insulin injection. Whether beta cells are obtained by direct reprogramming or from other sources, such as directed differentiation of pluripotent stem cells (Murry and Keller 2008), it will be necessary to carefully characterize the phenotype of the cells before proceeding.

Figure 11: Effect of Ad-PNM on cell shape

Shape of the AR42J-B13 cells changed from an oval structure to a more flattened fibroblast-like shape after Ad-PNM transduction. AR42J-B13 cells (**A**) with no treatment, (**B**) with Ad-PNM, (**C**) with Ad-GFP. Scale bars =100μm.



No virus Ad-PNM Ad-GFP

Figure 12: Gene expression profile of AR42J-B13 cells after Ad-PNM (RT-PCR)

AR42J-B13 cells were transduced with Ad-PNM. 3 days after Ad-PNM transduction gene expression profile between Ad-PNM transduced and nontransduced cells were compared through RT-PCR.

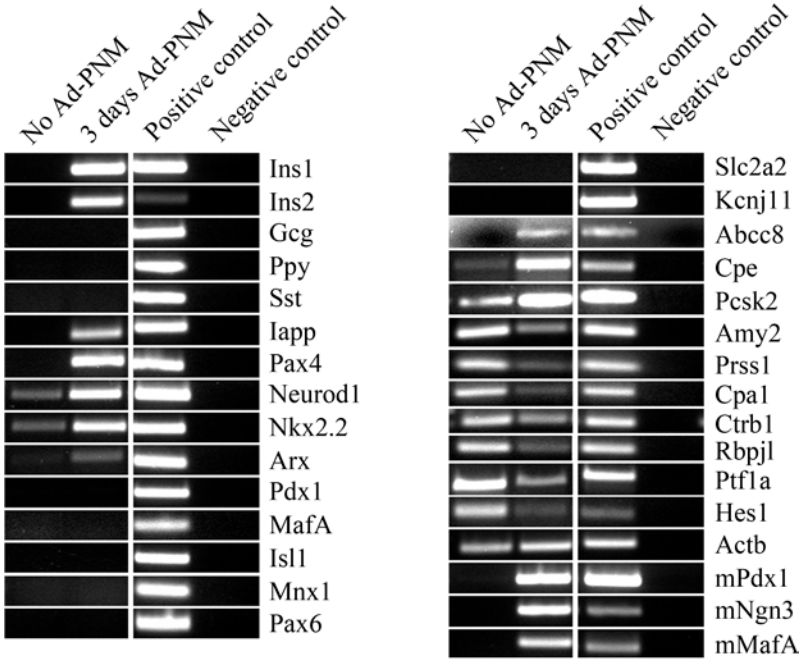
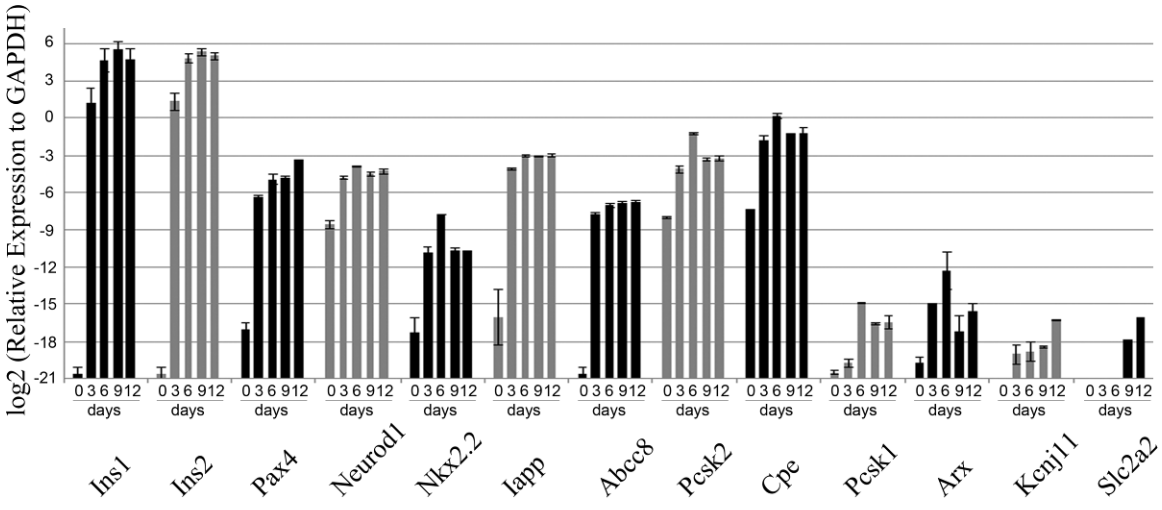

No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
Ins1
Ins2
Gcg
Ppy
Sst
Iapp
Pax4
Neurod1
Nkx2.2
Arx
Pdx1
MafA
Isl1
Mnx1
Pax6
No Ad-PNM
3 days Ad-PNM
Positive control
Negative control
Slc2a2
Kcnj11
Abcc8
Cpe
Pcsk2
Amy2
Prss1
Cpa1
Ctrb1
Rbpjl
Ptf1a
Hes1
Actb
mPdx1
mNgn3
mMafA

Figure 13: Gene expression profile of AR42J-B13 cells after Ad-PNM (qRT-PCR)

AR42J-B13 cells were transduced with Ad-PNM. Gene expression profile between Ad-PNM transduced and nontransduced cells were compared through qRT-PCR at different time points (i.e 0, 3, 6, 9 and 12 days after Ad-PNM). Top panel is showing the expression pattern of endocrine cell markers. Bottom panel is showing the expression pattern of exocrine cell markers.


log2 (Relative Expression to GAPDH)
6
3
0
-3
-6
-9
-12
-15
-18
-21
0 3 6 9 12
days
Ins1
Ins2
Pax4
Neurod1
Nkx2.2
Iapp
Abcc8
Pcsk2
Cpe
Pcsk1
Arx
Kcnj11
Slc2a2

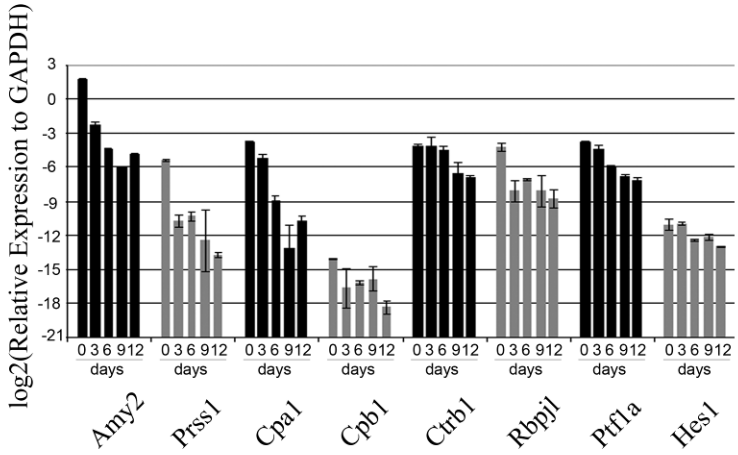

log2(Relative Expression to GAPDH)
3
0
-3
-6
-9
-12
-15
-18
-21
0 3 6 9 12
days
Amy2
Prss1
Cpa1
Cpb1
Ctrb1
Rbpjl
Ptf1a
Hes1

Figure 14: Expression of insulin and exogenous Pdx1, Ngn3 and MafA in AR42JB13 cells

AR42J-B13 cells were fixed and immunostained 3, 6, 9, 12 days after Ad-PNM transduction for (**A**) insulin (red) and Pdx1 (green), (**B**) insulin (red) and Ngn3 (green), (**C**) insulin (red) and MafA (green). (**D**) Percentage of insulin-positive cells out of the population of Ad-PNM bearing cells over time. Data are means ± S.E. (n =3). (**E**) C-peptide (green), insulin (red) and DAPI (blue) at day 12. (**F**) Amylase (green), insulin (red) and DAPI (blue) at day 6. Scale bars =100μm.

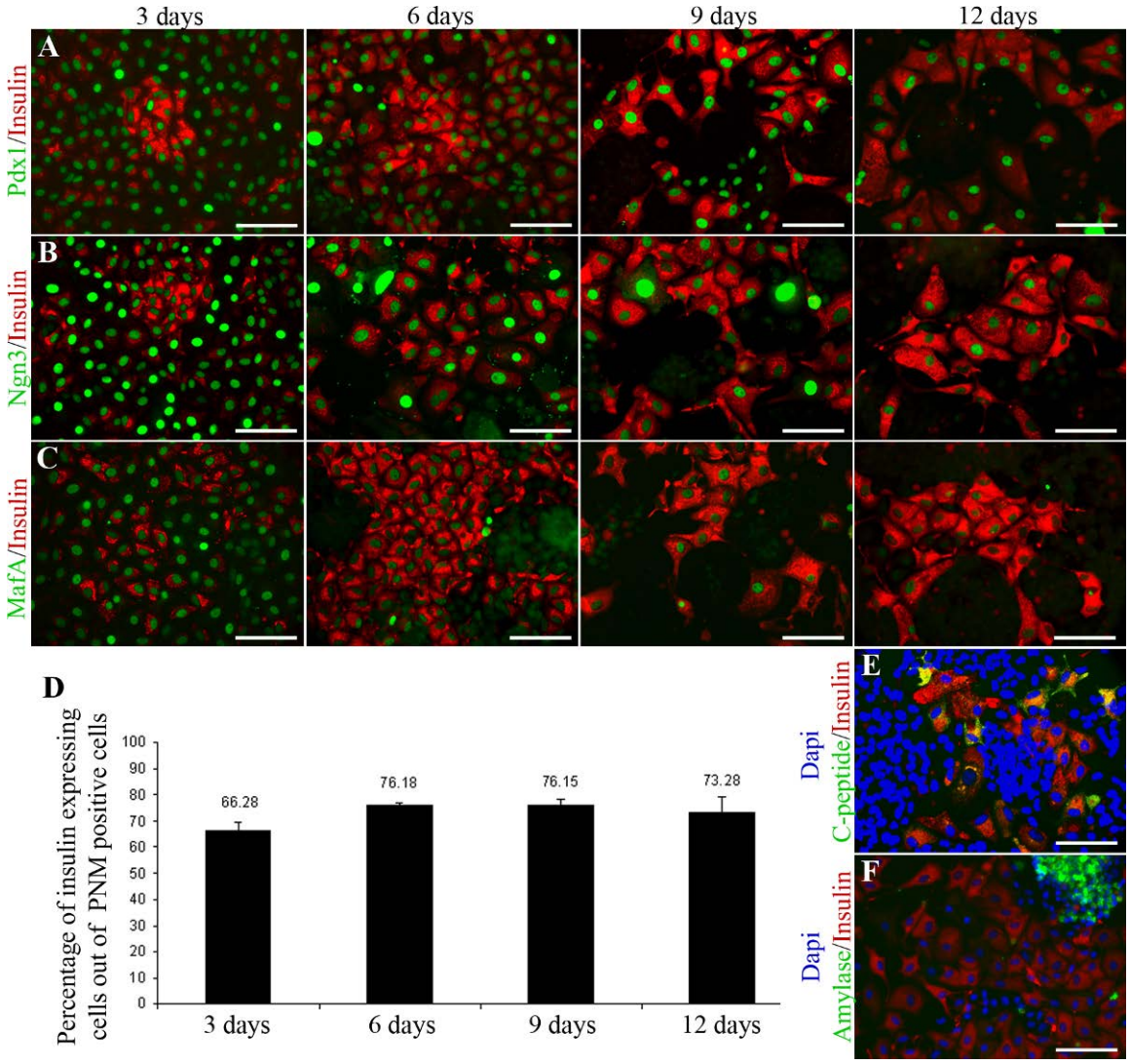
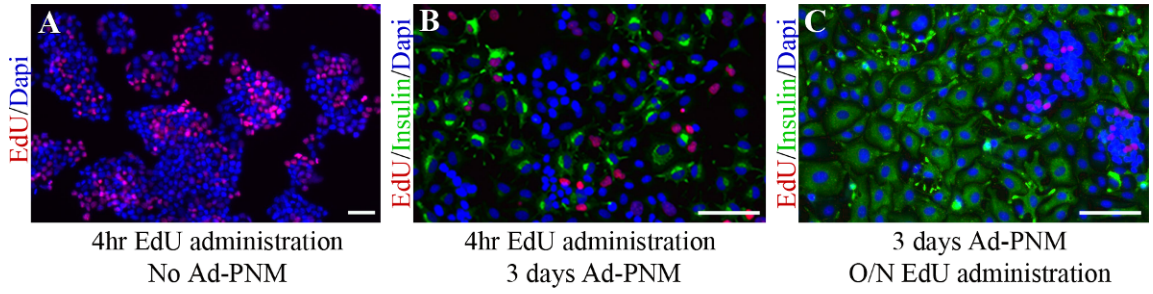

3 days
6 days
9 days
12 days
A
Pdx1/Insulin
B
Ngn3/Insulin
C
MafA/Insulin
D
Percentage of insulin expressing cells out of PNM positive cells
100
90
80
70
60
50
40
30
20
10
0
66.28
76.18
76.15
73.28
3 days
6 days
9 days
12 days
E
Dapi
C-peptide/Insulin
F
Dapi
Amylase/Insulin

Figure 15: AR42J-B13 cell proliferation assay with EdU

(**A**) AR42J-B13 cells were given EdU for 4 h. Then they were fixed and stained for EdU (red) and DAPI (blue). (**B**) AR42J-B13 cells were given EduU for 4 h. Then the cells were transduced with Ad-PNM. 3 days after, cells were fixed and stained for EdU (red), insulin (green) and DAPI (blue). (**C**). AR42J-B13 cells were transduced with Ad-PNM. At 3 days these cells were given EdU overnight. Next day they were fixed and stained for EdU (red), insulin (green) and DAPI (blue). Scale bars =100μm. The red colour is slightly enhanced in (**B** and **C**) to be consistent with (**A**). (**D**) Left-hand bar: percentage of proliferating cells out of total population. Middle and right-hand bars: percentage of proliferating insulin-positive cells out of total insulin-positive cells. Data are means ± S.E. (n =3).


A
EdU/Dapi
B
EdU/Insulin/Dapi
C
EdU/Insulin/Dapi
4hr EdU administration
No Ad-PNM
4hr EdU administration
3 days Ad-PNM
3 days Ad-PNM
O/N EdU administration

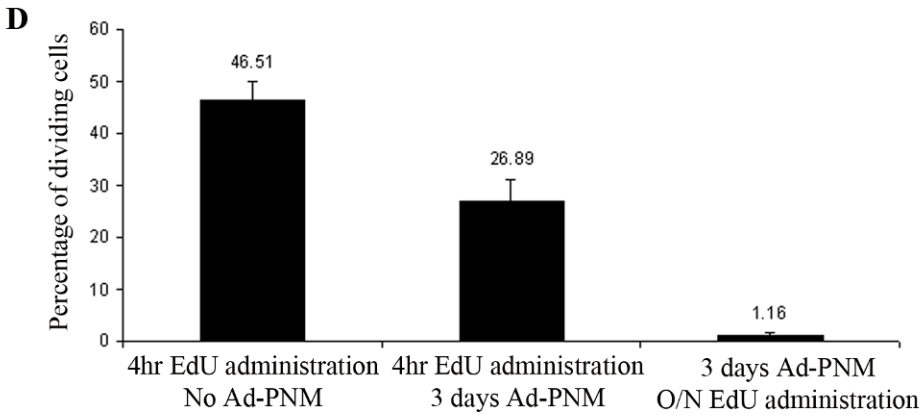

D
Percentage of dividing cells
60
50
40
30
20
10
0
46.51
26.89
1.16
4hr EdU administration
No Ad-PNM
4hr EdU administration
3 days Ad-PNM
3 days Ad-PNM
O/N EdU administration

Figure 16: Total and secreted insulin amount of AR42J-B13 cells by ELISA

(**A**) The total insulin amount in the AR42J-B13 cells with and without Ad-PNM was measured by ELISA. (**B**) The amount of insulin in the medium released from AR42J-B13 cells with and without Ad–PNM was measured by ELISA. Cells were stimulated either with low-glucose (open bars) or high-glucose (closed bars) and KCl. Experiments with Ad-PNM-treated cells were performed 3 days after transduction. Data are means ± S.E. (n =3).

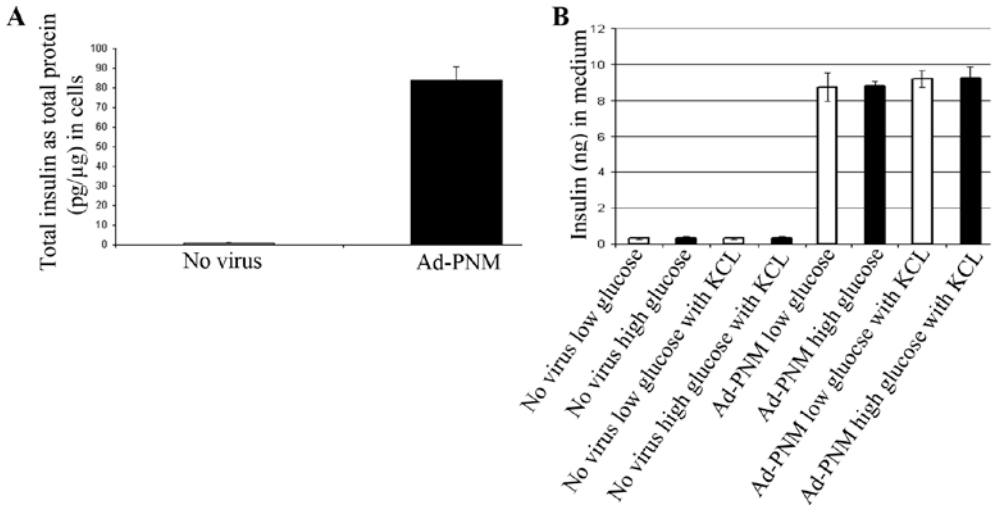

A
Total insulin as total protein (pg/μg) in cells
100
90
80
70
60
50
40
30
20
10
0
No virus
Ad-PNM
B
Insulin (ng) in medium
12
10
8
6
4
2
0
No virus low glucose
No virus high glucose
No virus low glucose with KCL
No virus high glucose with KCL
Ad-PNM low glucose
Ad-PNM high glucose
Ad-PNM low gluocse with KCL
Ad-PNM high glucose with KCL

Figure 17: Amelioration of diabetes by Ad-PNM-transduced AR42J-B13 cells

(**A**) Mice were given STZ to induce diabetes at day 0. Cells were transplanted under the kidney capsule at day 7 (grey arrow). Blood glucose levels were then measured every 2 days. At day 17 the transplanted kidneys were removed and analyzed for the presence of graft cells (black arrow). Δ, Ad-GFP-treated cells (control); ■, Ad-PNM-treated cells, n =3 and one mouse died after kidney removal; ●, Ad-PNM-treated but without successful engraftment, n =3. Results are means ± S.E. (**B**) Explanted kidneys were stained for insulin (red), E-cadherin (green) and DAPI (blue). Scale bar =100μm.

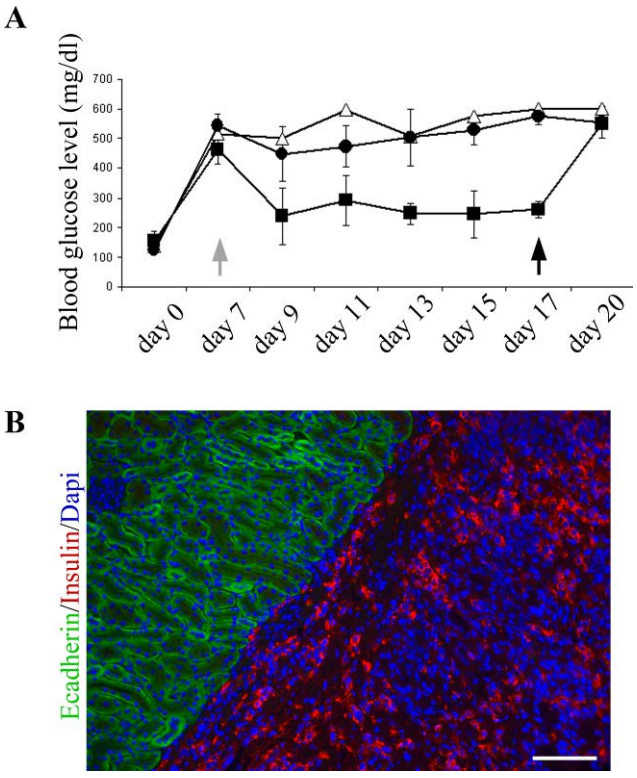

A
Blood glucose level (mg/dl)
700
600
500
400
300
200
100
0
day 0
day 7
day 9
day 11
day 13
day 15
day 17
day 20
B
Ecadherin/Insulin/Dapi

Figure 18: Transduction of the exocrine pancreas *in vivo*

(**A**) Injection of pancreas with Ad-GFP, visualized by GFP fluorescence, showing a high level of transduction in exocrine tissue. (**B**) Transduction with Ad-PNM, immunostained for Ngn3 (green) and insulin (red). Scale bars =100μm.

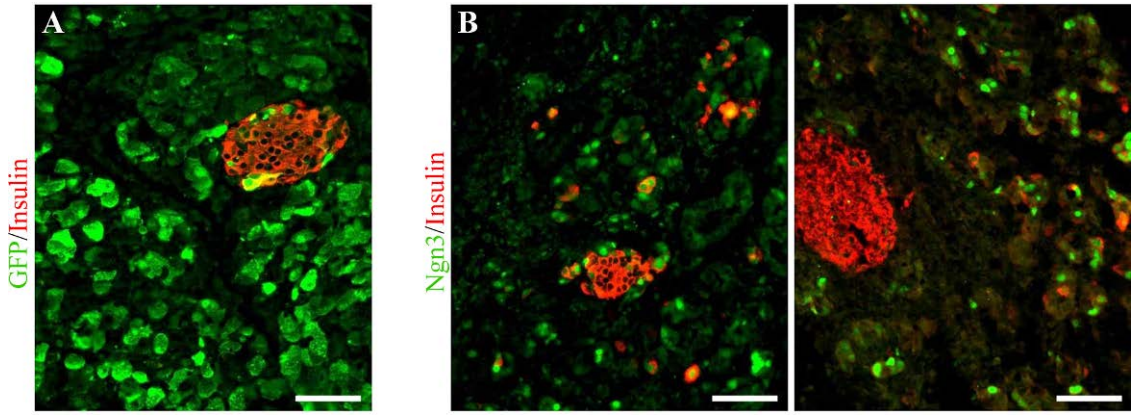

A
GFP/Insulin
B
Ngn3/Insulin

Table 3: Rat primers used in qRT-PCR

Rat Primers used in qRT-PCR		
Primer Name	**Forward Sequence 5`-3`**	**Reverse Sequence 5`-3`**
Ins1	CAATCATAGACCATCAGCAAGC	AGAAACCACGTTCCCCAC
Ins2	CCCAGGCTTTTGTCAAACAG	GTGCCAAGGTCTGAAGGTC
Iapp	CCACTGAAAGGGATCTTGAGAC	TTCCGTTTGTCCACCTGAG
Pax4	GGGCAGTATCCAGATTCAGTTG	GGCATCTGTGTTTCCCATTTC
Neurod1	ATGTCTTCCACGTCAAGCC	GAGAAGTTGCCATTGATGCTG
Nkx2.2	GGTCAAGATCTGGTTCCAAAAC	GTCACCTCCATACCTTTCTCAG
Arx	AATCTAACCCATCCCCAACAC	CTCTTCCTGGTACTGATTGCTC
Endogenous Pdx1	CCCGAGCTTCTGAAAACTTTG	CTTTTCATTGTCCTCAGTTGGG
Endogenous Ngn3	TCCAGACGCAATTTACTCCAG	CTAGTTCTCCGGGCTCAAAAG
Endogenous MafA	TCTTTCTGTGAGCGCGG	TCAGAGTCCGAACCGAGG
Isl1	TTGTTAGGGACGGGAAAACC	CTACACAGCGGAAACATTCG
Mnx1	GCTTTCCTACTCGTATCCTCAG	TTCCCCAAGAGGTTCGATTG
Hes1	AGAAAAATTCCTCGTCCCCG	TTTCATTTATTCTTGCCCGGC
Slc2a2	CACATCCTACTTGGCCTATCTG	TCAGTGCCCCTTAGTCTTTTC
Kcnj11	TCAGTAAGCAATGAGCAGGG	CAACCTCTGGACTGATATGCC
Abcc8	AGAAGCTCCTAGAGTACACCG	TGTAGGGAGTTGGAGATGGAG
Amy2	GCAGACCTTTCATTTTCCAAGAG	ACAAAACCCCAACCTTCTCC
Ptf1a	AGGACCCCAGAAAACTCAAC	CAATATGCACAAAGACACAGCC
Cpe	ATGGTAATGAGGCGGTTGG	AGGGCATGATATGGATTCGTG
Pcsk1	CTCAGCCCTTCCTACTTGTG	CATTGACAAACTGCCTCTTCG
Pcsk2	GCATAAAGACGGAGAGGAAGAG	TGGTAAAAGTGGTACAGGCC
Prss1	GTGTATCCTCCAACGATCTTGT	CACTTCTGATCCTAGCCCTTG
Cpa1	CCAGAAGTCCAACTGCAAGT	CAGTCTGTGGCAATGAGAACT
Cpb1	CACGTTGCTTATCAGTACCTCA	GCCTCTCACTACAGTTGACTT
Ctrb1	GAATAGCATCCTCTCCGTTGAC	GTCCTGCTTTGCCCTTGT
Rbpjl	CATCTCCGAACCACACCTTG	CTCCAGTGCCTCATATCAGC
Gapdh	TCCAGTATGACTCTACCCACG	CACGACATACTCAGCACCAG

Chapter 4 Comparision of Epigenetic Patterns of Developmentally Related Cells and Effect of Ad-PNM on Epigenetic Modifications

4.1. Introduction

During formation of a specific cell lineage, configuration of active genes encoding specific differentiation products is important. After formation of a cell lineage configuration of regulatory genes that maintain the state in a stable manner is important as well. For both it is clear that an essential feature of the differentiated cell state is the chromatin state of key genes. Two types of epigenetic modification; histone tail modifications and DNA methylation have been shown to be important in this context.

Acetylation of the lysine residues on the histone tails is one of the modifications which regulates the expression of genes, generally in favor of gene activation (Hebbes, Thorne et al. 1988). This reaction is catalyzed by histone acetyltransferase (HAT) enzymes by which acetyl groups are transferred from acetyl-coenzyme A to lysine residues. Acetylation of the lysine residues removes the positive charge of the epsilon amino groups which reduces the interaction between histone tails and the negatively charged phosphate groups of DNA, thus relaxing the chromatin structure at the acetylated regions. In the relaxed chromatin area, transcription factors and the general transcription machinery members can easily reach their target genes and can initiate transcription. On the other hand, deacetylation of the lysine residues by histone deacetylase (HDAC) enzymes reverses this effect, condensing the chromatin structure in the deacetylated regions. A condensed chromatin area is not easily accessible by transcription factors or the general transcription machinery so that genes in that area are not transcribed.

Methylation of lysine and arginine residues of the histones tails is another histone tail modification regulating gene expression (Chen, Ma et al. 1999). This reaction is catalyzed either by lysine specific (SET domain containing or non-SET domain containing) or arginine specific histone methyltransferase (HMT) enzymes by which S-adenosyl methionine is used as methyl donor. While methylation of lysine 4 on H3 (H3K4) and the arginine residues on H3 (H3R) as well as H4 (H4R) can stimulate active gene expression, dimethylation of lysine 9 on H3 ($H3K9Me_2$) and trimethylation of lysine 27 on H3 ($H3K27Me_3$) are signatures of transcriptional repression (Jenuwein and Allis 2001).

In addition to histone tail modifications, DNA base methylation in CpG rich areas can also regulate gene expression. This reaction is catalyzed by DNA methyl transferase (DNMT) enzymes by which a methyl group is transferred from S-adenosyl methionine to 5 position of the cytosine pyrimidine ring in CpG dinucleotide (Hergersberg 1991). Active genes are usually associated with a relative undermethylation of CpGs in their regulatory regions ("CpG islands") (Bird 2002). Repressed genes, on the other hand, are associated with a high level of methylation in their regulatory regions. Methylated DNA may repress the active transcription via two possible mechanisms: a) preventing the binding of the general transcription machinery physically as well as other transcription factors to methylated promoter regions; and b) Accumulation of specific transcriptional repressor proteins (methyl-CpG-binding domain proteins) which recognize the methylated CpGs (Kass, Pruss et al. 1997) and prevent the binding of the general transcription machinery as well as other transcription factors which favor gene transcription.

Acinar cells of exocrine pancreas are considered to be developmentally close to beta cells of endocrine pancreas since both exocrine and endocrine regions of the pancreas arise from the same endodermal cell population during pancreas development (Percival and Slack 1999; Gu, Dubauskaite et al. 2002). In other words, before endocrine and exocrine lineage commitment these cells are believed to share the same epigenetic pattern. The common impression is that different adult cells which are developmentally related can be reprogrammed into each other by overexpression of selected transcription factors, typically ones that are involved in the normal embryonic development of the cell type in question like *Pdx1*, *Ngn3* and *MafA* for beta cells (Gu, Dubauskaite et al. 2002; Zhou and Melton 2008)

The notion of high reprogramming competency of developmentally related cell types into each other is supported by our previous results where we showed that primary rat hepatocytes and rat pancreatic exocrine cell line AR42J-B13 can activate not all but many beta cell genes after transduction with Ad-PNM. Compared to AR42J-B13 cells, primary rat hepatocytes activated more beta cell genes based on RT-PCR results (Figure 5). However, by immunostaining the number of insulin positive cells after Ad-PNM transduction was higher in AR42J-B13 cells than rat primary hepatocytes (Figure 6).

In this study we compared the epigenetic state (histone tail modifications and DNA methylation) of developmentally related (RIN-m5F and AR42J-B13) and unrelated (IRPT and AR42J-B13) cells for three important beta cell genes: *Ins1*, *Ins2* and *Pdx1*. We also examined the epigenetic modifications of the same three genes for AR42J-B13 cells after transduction with Ad-PNM.

Some of the important cis-regulatory elements and CpGs at the proximal promoters and first exons of *Ins1*, *Ins2* and *Pdx1* genes are shown in Figure 19, 20 and 21, respectively. *Ins1* and *Ins2* basal promoters include a TATA box (*TATAA*; ~25-30bp upstream from start of transcription) which binds transcription factor IID (TFIID) through TATA box binding protein (TBP). Binding of TFIID to TATA box will then recruit the other members of the general transcription machinery for active gene transcription (Patikoglou, Kim et al. 1999). CAAT box (*CCAAT*; ~75-80bp upstream from start of transcription), which normally binds CAAT binding protein (CBP) and CAAT/enhancer binding protein (C/EBP), is combined with a CRE box both in *Ins1* and *Ins2* genes of rodents. CRE/CAAT combined site was shown to bind nuclear transcription factor-Y (NF-Y) (Eggers, Siemann et al. 1998). However, neither CRE/CAAT combined box nor CAAT box alone are present in other mammals. This fact raises the idea that CAAT box may not have a role in *Insulin* gene expression (Hay and Docherty 2006). Homeodomain protein-binding, *TAAT* motif-including (except for A2) A boxes are present in *Ins1* (A1, A2, A3 and A4) and *Ins2* (A1, A2 and A3) proximal promoters of rat (Melloul, Marshak et al. 2002). A1 and A3 boxes are the most conserved and were shown to control *Insulin* genes expression by binding the PDX1 transcription factor (Melloul, Marshak et al. 2002). C1 box is another conserved regulatory element in *Ins1* and *Ins2* promoters and it was shown to control I*nsulin* genes expression by binding MAFA transcription factor (Olbrot, Rud et al. 2002). E boxes (*CANNTG*), which binds basic-helix-loop- helix (bHLH) class of transcription factors, are also present in *Ins1* (E1 and E2 boxes) and *Ins2* (E1 box) promoters of rat (Karlsson, Edlund et al. 1987). Unlike *Ins1* and *Ins2* promoters, *Pdx1* promoter does not contain a TATA box. On the other

hand CAAT and E boxes are present at the proximal promoter of *Pdx1* gene (Campbell and Macfarlane 2002).

4.2. Experimental Design and Methods

4.2.1. ChIP (Chromatin Immunoprecipitation) for Histone Tail Modifications and qPCR (Quantitative Polymerase Chain Reaction)

AR42J-B13 cells (10^7) with no Ad-PNM, AR42J-B13 cells 3 days after administration of Ad-PNM, RIN-m5F cells or IRPT cells were plated on 20 cm tissue culture dishes. Formaldehyde (36.5%; Sigma) was added to the dishes to a final concentration of 1% in each dish and cells were incubated for 10 min at room temperature. The cross-linking reaction was quenched by addition of 2ml of 1.25M glycine (Sigma). The cells were then scraped into 50ml conical tubes and were subjected to centrifugation (10000 g for 15 min at 4°C). The supernatant was discarded after centrifugation. Cell and nucleus lysis buffers (Magna ChIP™ G Kit, Millipore) were added to the remaining cell pellets. The cells/DNAs were sheared by sonication using a Branson Sonifier 450 (15×10 s on ice with 1 min waiting on ice between pulses). Then ChIP (chromatin immunoprecipitation) was performed for $H3K4Me_3$ (trimethylated Lys4 of histone 3), $H3K9Me_2$ (dimethylated Lys9 of histone 3), $H3K27Me_3$ (trimethylated Lys27 of histone 3) and H3Ac (acetylated histone 3) by using Magna ChIP™ G Kit (Millipore), according to the manufacturer's instructions. Antibodies used for ChIP were: rabbit anti-$H3K4Me_3$ mAb (monoclonal antibody; Cell Signaling Technology), mouse anti-$H3K9Me_2$ mAb (Abcam), mouse anti-$H3K27Me_3$ mAb (Abcam) and rabbit anti-

H3Ac polyclonal antibody (Millipore). For each immunoprecipitation reaction 5μg of antibody was used. After immunoprecipitation of DNA fragments bearing the histone modifications mentioned above, qPCR was performed by using specific primer pairs (Table 4) which span approximately −200bp (base pair) to +50bp of the *Ins1*, *Ins2* and *Pdx1* genes. These regions include a number of important regulatory sites as shown in Figure 19, 20 and 21 (German, Ashcroft et al. 1995; Gannon, Gamer et al. 2001; Campbell and Macfarlane 2002; Iype, Francis et al. 2005; Miyatsuka, Matsuoka et al. 2007). In qPCR, reaction mixtures were incubated at 50°C for 2 min and then at 95°C for 10 min for initial denaturation. Reaction mixtures were then subjected to 40 cycles of amplification using the following conditions: 95°C for 15 s denaturation, 60°C for 30 s annealing and 60°C for 30 s extension. An Eppendorf Realplex^4 mastercycler was used for these qPCRs. To normalize the ChIP data, the fold enrichment method (also known as signal over background) was used in which ChIP signals were divided by non-specific antibody signals.

4.2.2. DNA Methylation Assay with Bisulfite Sequencing

Isolation of genomic DNA from AR42J-B13 cells with no Ad-PNM, from AR42J-B13 cells with 3 days after Ad-PNM, RIN-m5F cells or IRPT cells was performed with QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. Bisulfite conversion of isolated genomic DNA was performed with the EZ DNA methylation-gold kit (Zymo Research), according to the manufacturer's instructions. Bisulfite-treated DNA-specific primer pairs (Table 3) which are spanning the proximal promoters and first exons of *Ins1*, *Ins2* and *Pdx1* genes were designed by

using MethPrimer software (Li and Dahiya 2002). To amplify the bisulfite-treated genomic DNA, a PCR was performed. In this PCR, samples were heated at 95 °C for 10 min as initial denaturation. Reaction mixtures were then subjected to 40 cycles of amplification using the following conditions: 95°C for 30 s denaturation, 50°C for 40 s annealing, 72°C for 1 min extension. This was followed by a final extension step at 72°C for 7 min. Hot Taq DNA Polymerase (Zymo Research) was used in these PCR reactions. Amplified PCR products were then run on 1% (w/v) agarose gel in 1x TAE buffer by electrophoresis at 100 V. DNA fragments of interest were cut out from the gel and purified with Wizard SV Gel and PCR Clean-Up System (Promega), according to the manufacturer's instructions. Purified PCR products were cloned into the pCR4-TOPO vector with TOPO TA Cloning Kit (Invitrogen) for subsequent sequencing. Plasmids including the DNA fragments of interest were transformed into the chemically competent One Shot TOP10 cells provided by the kit, according to the manufacturer's instructions. Cells were grown on LB (Luria–Bertani) agar plates containing kanamycin. From each plate ten colonies were picked up randomly and grown in kanamaycin including LB broth overnight at 37°C in a shaking incubator. The next day plasmid DNAs were isolated by using Wizard Plus SV Minipreps DNA Purification System (Promega), according to the manufacturer's instructions. Each collected clone was restriction-digested with EcoRI (Promega) to verify that they contain the right DNA fragments of interest. Then ten different clones for each DNA fragment were sequenced by the DNA Sequencing and Analysis Facility, Biomedical Genomics Center, University of Minnesota, MN, U.S.A.

4.3 Results

4.3.1. Effects on Histone Tail Modifications at *Pdx1* and *Insulin* Genes

Methylation and acetylation of histone tails are functionally important either for up-regulation or repression of a gene (Jenuwein and Allis 2001; Eberharter and Becker 2002). Modifications such as H3K4Me$_3$ and H3Ac for instance, are two of the indicators for active transcription when they are found in histones at the promoter region of a gene. Modifications such as H3K9Me$_2$ and H3K27Me$_3$ are two indicators of transcriptional repression.

During a cell reprogramming event, regulatory genes controlling the cell phenotype as well as the genes encoding the differentiation products for the cell in question are expected to be activated. For this reason we examined four histone tail modifications (H3K4Me$_3$, H3Ac, H3K9Me$_2$ and H3K27Me$_3$) at the promoter of the endogenous *Pdx1*, *Ins1* and *Ins2* genes (German, Ashcroft et al. 1995; Gannon, Gamer et al. 2001; Campbell and Macfarlane 2002; Iype, Francis et al. 2005; Miyatsuka, Matsuoka et al. 2007) (Figure 22). The housekeeping gene *Gapdh* (which is active in all cell types) was used as positive control (Figure 22A). We compared RIN-m5F cells (a rat insulinoma cell line which is *Ins1*+, *Ins2*+ and *Pdx1*+), AR42J-B13 cells without Ad-PNM (which are *Ins1*−, *Ins2*− and *Pdx1*−), the AR42J-B13 cells with Ad-PNM (which are *Ins1*+, *Ins2*+ and *Pdx1*−) and IRPT cells (a rat kidney cell line which is *Ins1*−, *Ins2*− and *Pdx1*−). We first performed chromatin immunoprecipitation (ChIP) for H3K4Me$_3$, H3K9Me$_2$, H3K27Me$_3$ and H3Ac modifications and then ran qRT–PCR for the promoter regions under study. For the Ad-PNM-transduced cells, ChIP was conducted 3 days after Ad-PNM transduction.

At the *Pdx1* promoter of the AR42J-B13 cells, Ad-PNM increased the proportion of H3K4Me$_3$ to a modest extent and it did not change the level of H3K9Me$_2$, H3K27Me$_3$ or H3Ac (Figure 22B). This result was consistent with the failure to activate endogenous *Pdx1* gene as shown by the RT–PCR studies. At both the *Ins1* and *Ins2* promoters of AR42J-B13 cells, Ad-PNM reduced the inhibitory H3K9Me$_2$ and H3K27Me$_3$ proportions to a degree (not statistically significant for *Ins2* H3K27Me$_3$) (Figures 22C and 22D). This result was consistent with the up-regulation of *Ins1* and *Ins2* genes as shown by RT–PCR studies. Before the ChIP experiments, we had hypothesized that the chromatin for regulatory genes such as *Pdx1* might be 'open' in pancreatic exocrine cells and 'closed' in non-pancreatic cells such as the kidney. As shown in Figure 22B this is the case in relation to the active expression indicator H3K4Me$_3$ and the two inhibitory indicators H3K9Me$_2$ and H3K27Me$_3$. However since we see no significant up-regulation of endogenous *Pdx1* with Ad- PNM it may be that the chromatin is not really accessible and is rendered inactive by other modifications not examined in the present study.

4.3.2. Effects on Promoter Methylation at *Pdx1* and *Insulin* Genes

Together with histone tail modifications such as methylation and acetylation, the methylation of the DNA base cytosine at promoter regions of the genes is another important epigenetic factor affecting gene expression. The methylation of cytosines at CpG islands is usually associated with repression of gene activity (Bird 2002). To examine the effects of Ad-PNM on the DNA methylation pattern in treated cells, we compared the same four cell populations used for the ChIP analysis and performed bisulfite sequencing for the same promoter regions of *Pdx1, Ins1* and *Ins2* genes. Our

results showed that the expression of the *Pdx1* gene is not under the control of DNA methylation at the sites examined, as there is no CpG methylation at either proximal promoter or exons in any of the cell types (Figure 23C). Unlike *Pdx1*, the expression of the *Ins1* gene correlated with DNA methylation at the proximal promoter (Figure 23A). In RIN-m5F cells (which are *Ins1*+), the CpG methylation ratio was only 2% at the proximal promoter of *Ins1* as compared with 63% for the whole gene. In IRPT cells (which are *Ins1*−), the CpG methylation ratio was 90% at the proximal promoter of *Ins1* compared with 47% for the whole gene. This result suggests that demethylation of the proximal promoter of the *Ins1* gene was necessary for the gene expression. Like IRPT cells, the proximal promoter of the *Ins1* gene of AR42J-B13 cells (which are *Ins1*−) is highly methylated at 90%. After transduction with Ad-PNM (such that the cells become *Ins1*+), CpG methylation at the proximal promoter was reduced to 28%, with little change of methylation in the *Ins1* exon. Unlike *Ins1*, the expression of the *Ins2* gene may not be under the control of DNA methylation at the sites examined as there was no correlation with expression state (Figure 23B). In the RIN-m5F cells (*Ins2*+), the methylation ratio was 88% at the proximal promoter of *Ins2* and 89% throughout the whole gene. In IRPT cells (*Ins2*−), the methylation ratio was 42% at the proximal promoter of *Ins2* and 40% throughout the whole gene. After transduction with Ad-PNM the methylation ratio in AR42J-B13 cells fell slightly from 40% to 35% at the proximal promoter of *Ins2* and from 54% to 49% throughout the whole gene.

In summary, our results from the DNA base methylation study indicated that there was no pre-existing competence of these genes for upregulation in the pancreatic cells as

compared with the kidney cells, and that of the genes examined *Ins1* showed promoter demethylation following Ad-PNM treatment, whereas *Ins2* does not.

4.4. Discussion

Both exocrine and endocrine cell types arise from the same endodermal cell population of the pancreatic bud during pancreas development (Percival and Slack 1999; Gu, Dubauskaite et al. 2002). As a result of their developmental relatedness, endocrine and exocrine cells might share some aspects of chromatin state, and this may facilitate interconversion by overexpression of selected transcription factors. For instance, in pancreatic exocrine cells perhaps the genes characteristic of pancreatic endocrine cells, while not being expressed, are still accessible to transcription factors rather than being sequestered in inactive regions of chromatin. For this reason we performed histone tail modification and DNA base methylation studies for *Pdx1*, *Ins1* and *Ins2* genes. In the case of *Pdx1*, which is a key regulatory gene in pancreatic development, the histone tail modification studies did suggest an open configuration in the pancreatic, but not the non-pancreatic, cell types. Although our line of AR42J-B13 cells does not express *Pdx1*, other lines of the original cells in use elsewhere have been reported to do so in their normal state (Aldibbiat, Marriott et al. 2008; Ogihara, Fujitani et al. 2008). With regard to the *Insulin* genes, the results of the histone tail modification and DNA base methylation studies indicate that the introduction of the *Pdx1*, *Ngn3* and *MafA* combination itself modifies the chromatin to promote expression.

Figure 19: Rat *Ins1* gene

Location of primer pairs used for ChIP, and CpGs sequenced for methylation analysis, in the genomic sequence of *Ins1*. Black = proximal promoter; Blue =5` UTR; Red =First exon. The sequences in brackets indicate known regulatory elements. Primers for ChIP-qPCR are highlighted in yellow. CGs shown here in large type were sequenced in the DNA methylation assay.

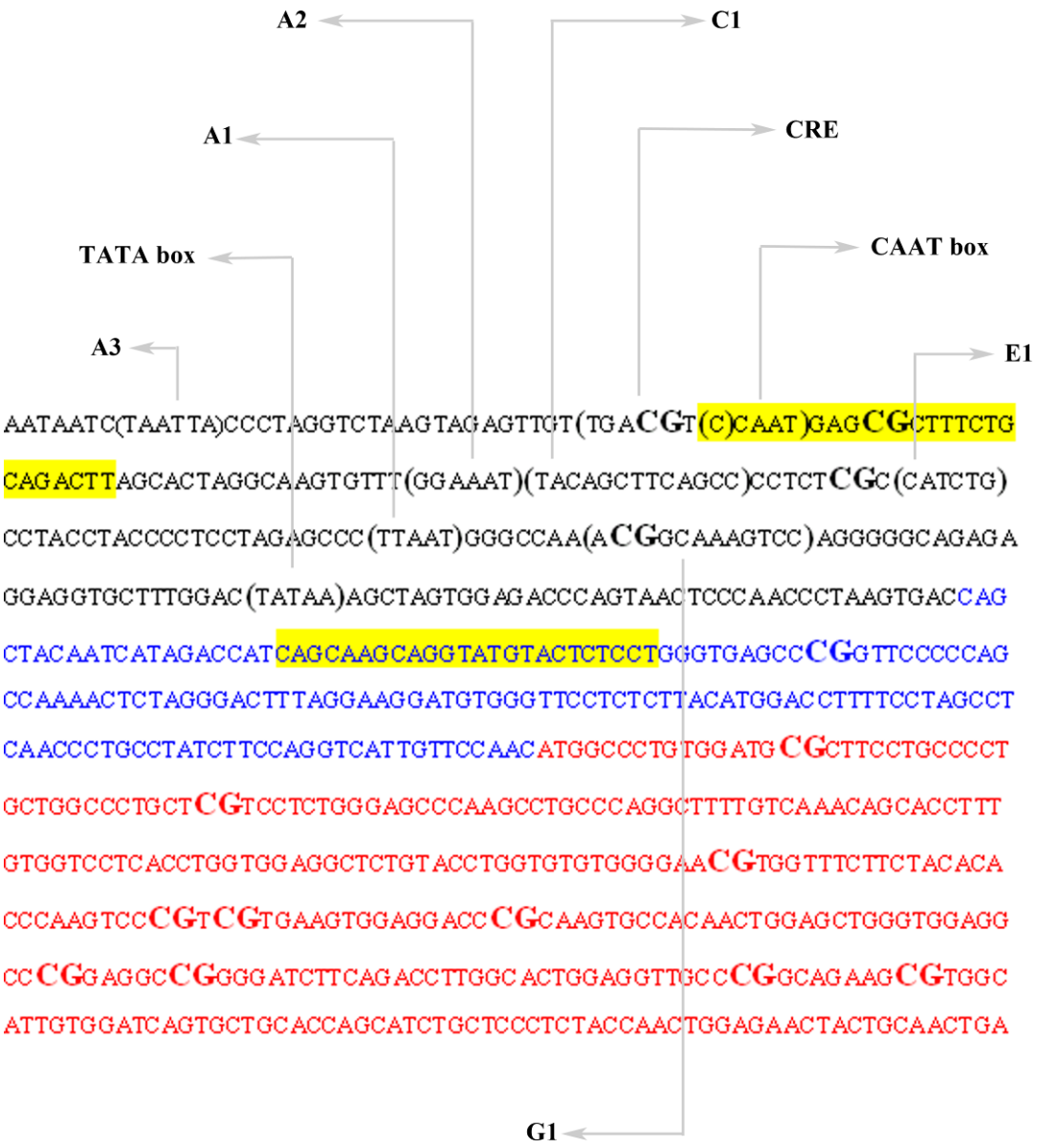

A2
C1
A1
CRE
TATA box
CAAT box
A3
E1
AATAATC(TAATTA)CCCTAGGTCTAAGTAGAGTTGT(TGACGT(C)CAAT)GAGCGCTTTCTG
CAGACTTAGCACTAGGCAAGTGTTT(GGAAAT)(TACAGCTTCAGCC)CCTCTCGC(CATCTG)
CCTACCTACCCCTCCTAGAGCCC(TTAAT)GGGCCAA(ACGGCAAAGTCC)AGGGGGCAGAGA
GGAGGTGCTTTGGAC(TATAA)AGCTAGTGGAGACCCAGTAACTCCCAACCCTAAGTGACCAG
CTACAATCATAGACCATCAGCAAGCAGGTATGTACTCTCCTGGGTGAGCCCGGTTCCCCCAG
CCAAAACTCTAGGGACTTTAGGAAGGATGTGGGTTCCTCTCTTACATGGACCTTTTCCTAGCCT
CAACCCTGCCTATCTTCCAGGTCATTGTTCCAACATGGCCCTGTGGATGCGCTTCCTGCCCCT
GCTGGCCCTGCTCGTCCTCTGGGAGCCCAAGCCTGCCCAGGCTTTTGTCAAACAGCACCTTT
GTGGTCCTCACCTGGTGGAGGCTCTGTACCTGGTGTGTGGGGAACGTGGTTTCTTCTACACA
CCCAAGTCCCGTCGTGAAGTGGAGGACCCGCAAGTGCCACAACTGGAGCTGGGTGGAGG
CCCGGAGGCCGGGGATCTTCAGACCTTGGCACTGGAGGTTGCCCGGCAGAAGCGTGGC
ATTGTGGATCAGTGCTGCACCAGCATCTGCTCCCTCTACCAACTGGAGAACTACTGCAACTGA
G1

Figure 20: Rat *Ins2* gene

Location of primer pairs used for ChIP, and CpGs sequenced for methylation analysis, in the genomic sequence of *Ins2*. Black = proximal promoter; Red =First exon. The sequences in brackets indicate known regulatory elements. Primers for ChIP-qPCR are highlighted in yellow. CGs shown here in large type were sequenced in the DNA methylation assay.

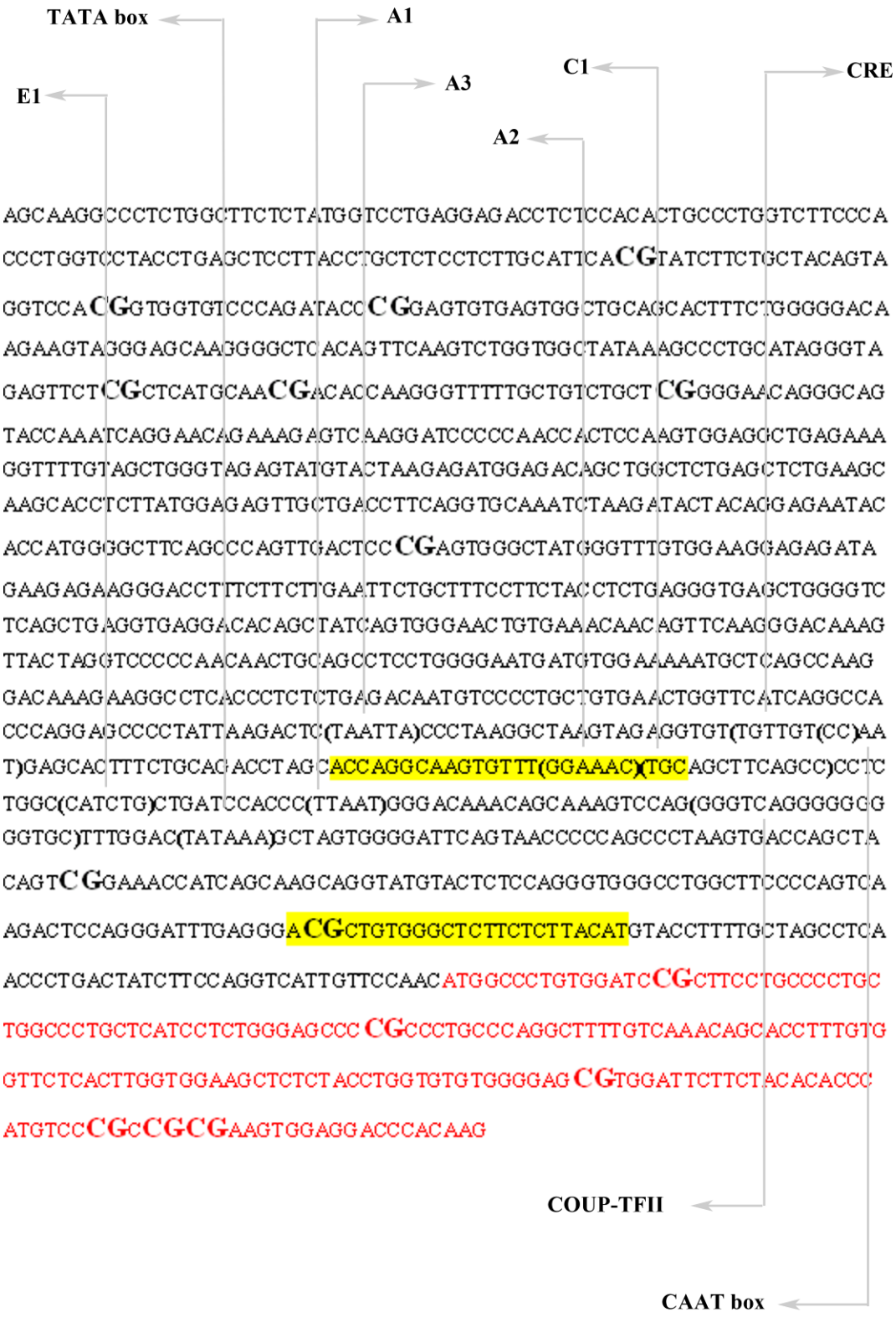

TATA box
A1
E1
A3
C1
CRE
A2
AGCAAGGCCCTCTGGCTTCTCTATGGTCCTGAGGAGACCTCTCCACACTGCCCTGGTCTTCCCA
CCCTGGTCCTACCTGAGCTCCTTACCTGCTCTCCTCTTGCATTCACGTATCTTCTGCTACAGTA
GGTCCACGGTGGTGTCCCAGATACCCGGAGTGTGAGTGGCTGCAGCACTTTCTGGGGGACA
AGAAGTAGGGAGCAAGGGGCTCACAGTTCAAGTCTGGTGGCTATAAAGCCCTGCATAGGGTA
GAGTTCTCGCTCATGCAACGACACCAAGGGTTTTTGCTGTCTGCTCGGGAACAGGGCAG
TACCAAATCAGGAACAGAAAGAGTCAAGGATCCCCCAACCACTCCAAGTGGAGGCTGAGAAA
GGTTTTGTAGCTGGGTAGAGTATGTACTAAGAGATGGAGACAGCTGGCTCTGAGCTCTGAAGC
AAGCACCTCTTATGGAGAGTTGCTGACCTTCAGGTGCAAATCTAAGATACTACAGGAGAATAC
ACCATGGGGCTTCAGCCCAGTTGACTCCCGAGTGGGCTATGGGTTTGTGGAAGGAGAGATA
GAAGAGAAGGGACCTTTCTTCTTGAATTCTGCTTTCCTTCTACCTCTGAGGGTGAGCTGGGGTC
TCAGCTGAGGTGAGGACACAGCTATCAGTGGGAACTGTGAAACAACAGTTCAAGGGACAAAG
TTACTAGGTCCCCCAACAACTGCAGCCTCCTGGGGAATGATGTGGAAAAATGCTCAGCCAAG
GACAAAGAAGGCCTCACCCTCTCTGAGACAATGTCCCCTGCTGTGAACTGGTTCATCAGGCCA
CCCAGGAGCCCCTATTAAGACTC(TAATTA)CCCTAAGGCTAAGTAGAGGTGT(TGTTGT(CC)AA
T)GAGCACTTTCTGCAGACCTAGCACCAGGCAAGTGTTT(GGAAAC)(TGCAGCTTCAGCC)CCTC
TGGC(CATCTG)CTGATCCACCC(TTAAT)GGGACAAACAGCAAAGTCCAG(GGGTCAGGGGGGG
GGTGC)TTTGGAC(TATAAA)GCTAGTGGGGATTCAGTAACCCCCAGCCCTAAGTGACCAGCTA
CAGTCGGAAACCATCAGCAAGCAGGTATGTACTCTCCAGGGTGGGCCTGGCTTCCCCAGTCA
AGACTCCAGGGATTTGAGGGACGCTGTGGGCTCTTCTCTTACATGTACCTTTTGCTAGCCTCA
ACCCTGACTATCTTCCAGGTCATTGTTCCAACATGGCCCTGTGGATCCGCTTCCTGCCCCTGC
TGGCCCTGCTCATCCTCTGGGAGCCCCGCCCTGCCCAGGCTTTTGTCAAACAGCACCTTTGTG
GTTCTCACTTGGTGGAAGCTCTCTACCTGGTGTGTGGGGAGCGTGGATTCTTCTACACACCC
ATGTCCCGCCGCGAAGTGGAGGACCCACAAG
COUP-TFII
CAAT box

Figure 21: Rat *Pdx1* gene

Location of primer pairs used for ChIP, and CpGs sequenced for methylation analysis, in the genomic sequence of *Pdx1*. Black = proximal promoter; Blue =5` UTR; Red =First exon. The sequences in brackets indicate known regulatory elements. Primers for ChIP-qPCR are highlighted in yellow. CGs shown here in large type were sequenced in the DNA methylation assay.

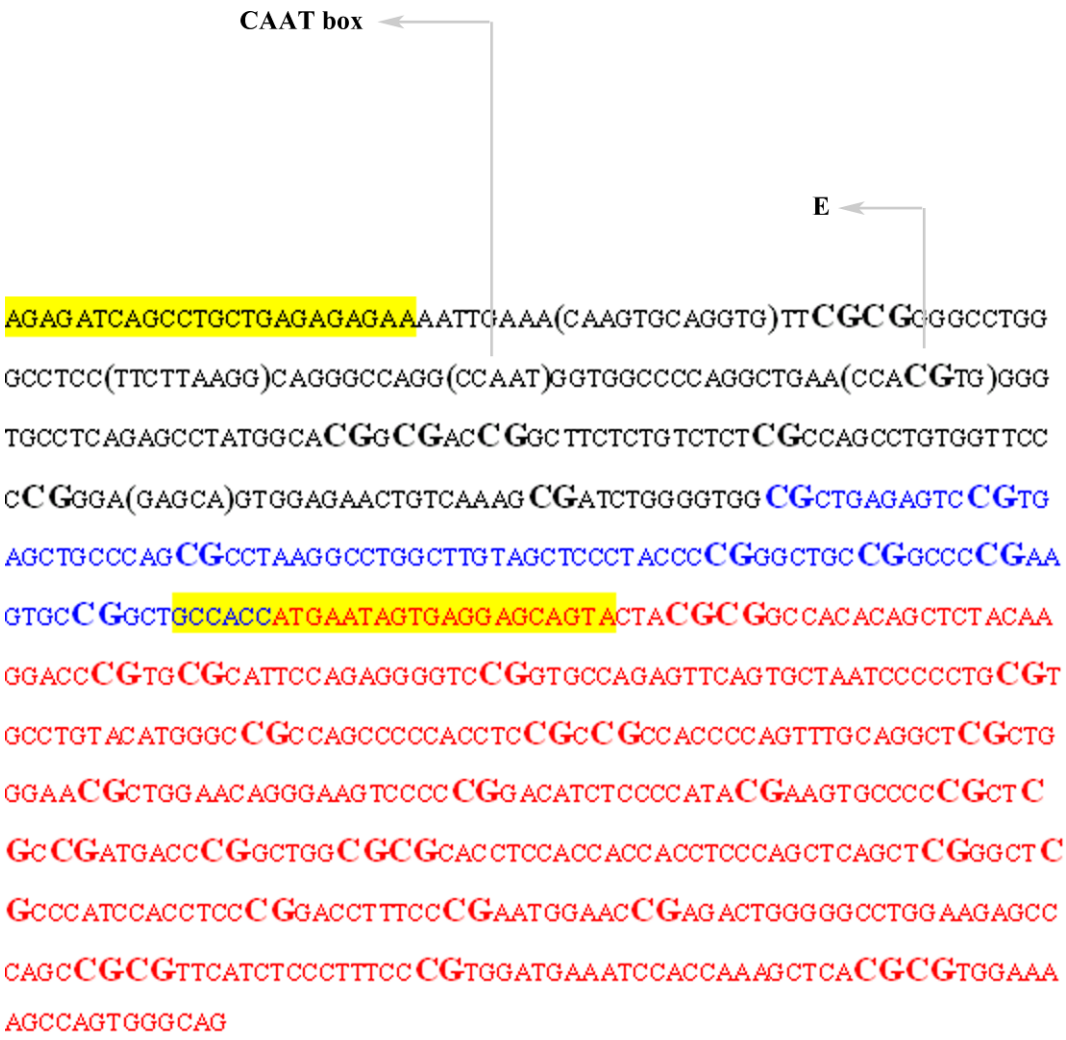

CAAT box
E
AGAGATCAGCCTGCTGAGAGAGAAAATTGAAA(CAAGTGCAGGTG)TTCGCGCGGGCCTGG
GCCTCC(TTCTTAAGG)CAGGGCCAGG(CCAAT)GGTGGCCCCAGGCTGAA(CCACGTG)GGG
TGCCTCAGAGCCTATGGCACGGCGACCGGCTTCTCTGTCTCTCGCCAGCCTGTGGTTCC
CCGGGA(GAGCA)GTGGAGAACTGTCAAAGCGATCTGGGGTGGCGCTGAGAGTCCGTG
AGCTGCCCAGCGCCTAAGGCCTGGCTTGTAGCTCCCTACCCCGGGCTGCCGGCCCCGAA
GTGCCGGCTGCCACCATGAATAGTGAGGAGCAGTACTACGCGGCCACACAGCTCTACAA
GGACCCGTGCGCATTCCAGAGGGGTCCGGTGCCAGAGTTCAGTGCTAATCCCCCTGCGT
GCCTGTACATGGGCCGCCAGCCCCCACCTCCGCCGCCACCCCAGTTTGCAGGCTCGCTG
GGAACGCTGGAACAGGGAAGTCCCCCGGACATCTCCCCATACGAAGTGCCCCCGCTC
GCCGATGACCCGGCTGGCGCGCACCTCCACCACCACCTCCCAGCTCAGCTCGGGCTC
GCCCATCCACCTCCCGGACCTTTCCCGAATGGAACCGAGACTGGGGGCCTGGAAGAGCC
CAGCCGCGTTCATCTCCCTTTCCCGTGGATGAAATCCACCAAAGCTCACGCGTGGAAA
AGCCAGTGGGCAG

Figure 22: Changes of the histone tail modifications at the promoter regions of the *Pdx1*, *Ins1* and *Ins2* genes after Ad-PNM

ChIP assays were carried out to compare the proportion of the histone tail modifications ($H3K4Me_3$, $H3K9Me_2$, $H3K27Me_3$ and H3Ac) at the promoters of the *Gapdh* (**A**), *Pdx1* (**B**), *Ins1* (**C**) and *Ins2* (**D**) genes. The cell types analyzed are RIN-m5F cells (stippled bars), AR42J-B13 cells without Ad-PNM (open bars), AR42J-B13 cells with Ad-PNM (closed bars) and IRPT cells (diagonal hatched bars). The y-axis shows fold change of samples over negative control rat IgG. Data are means ± S.E. (n =3).

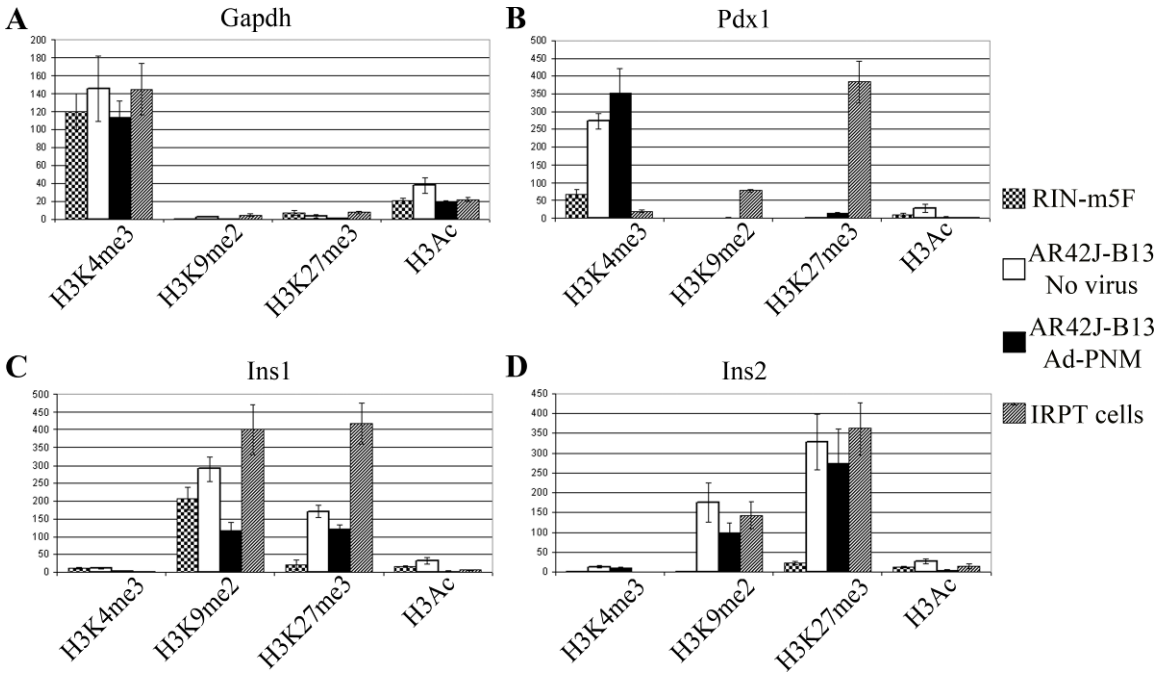

A
Gapdh
B
Pdx1
C
Ins1
D
Ins2
H3K4me3
H3K9me2
H3K27me3
H3Ac
RIN-m5F
AR42J-B13 No virus
AR42J-B13 Ad-PNM
IRPT cells

Figure 23: Changes of the DNA methylation pattern of the *Pdx1*, *Ins1* and *Ins2* genes after Ad-PNM

Comparison of the CpG methylation pattern of the *Ins1* (**A**), *Ins2* (**B**) and *Pdx1* (**C**) genes in RIN-m5F cells, AR42J-B13 cells with and without Ad-PNM, and IRPT cells. Each circle, divided into ten wedges, represents ten different clones for the same CpG. Open wedges represent unmethylated CpGs and closed wedges represent methylated CpGs. Intact open circles represent CpGs that were not covered by the bisulfite sequencing. UTR, untranslated region.

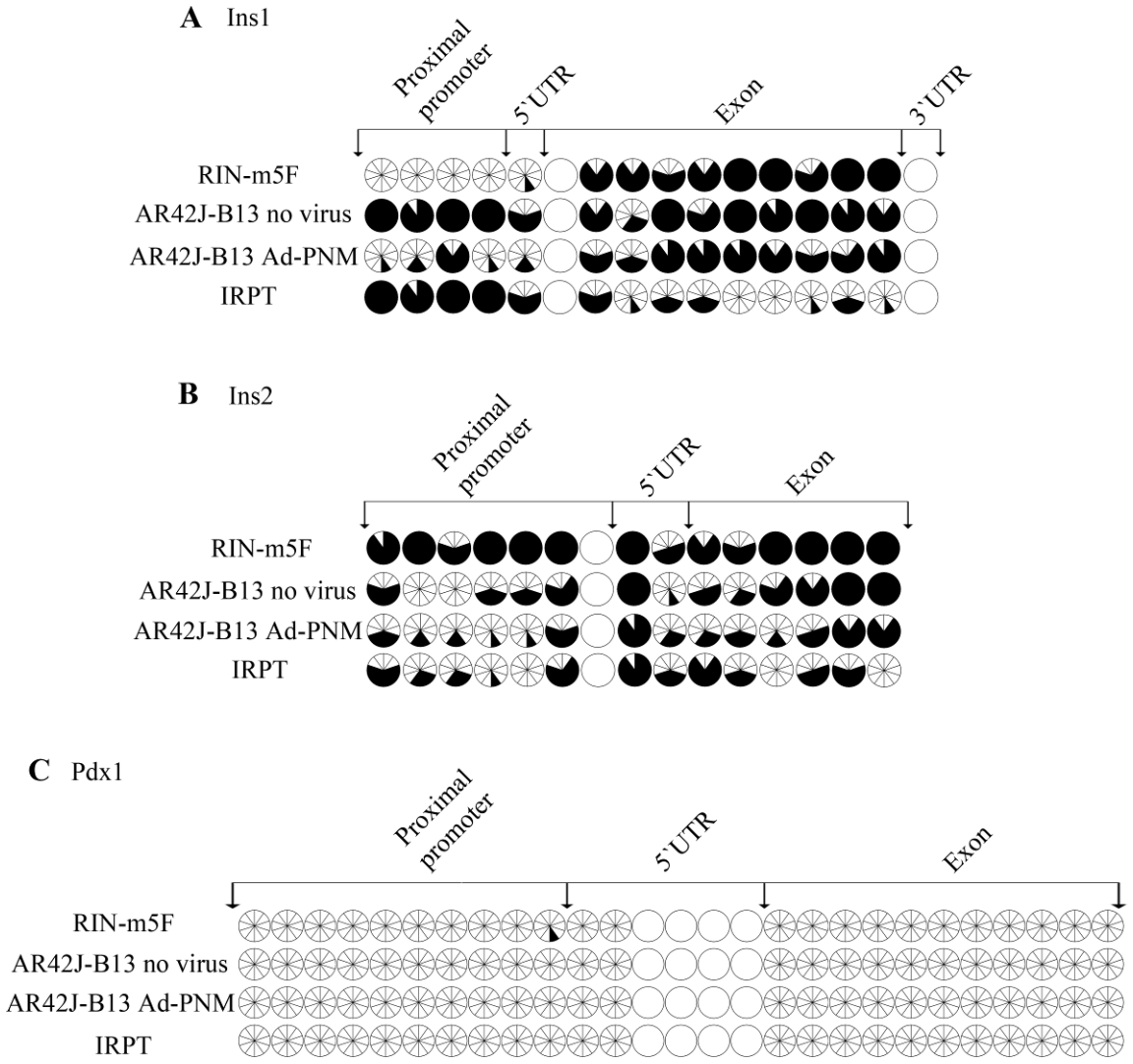

A Ins1
Proximal promoter
5`UTR
Exon
3`UTR
RIN-m5F
AR42J-B13 no virus
AR42J-B13 Ad-PNM
IRPT
B Ins2
Proximal promoter
5`UTR
Exon
RIN-m5F
AR42J-B13 no virus
AR42J-B13 Ad-PNM
IRPT
C Pdx1
Proximal promoter
5`UTR
Exon
RIN-m5F
AR42J-B13 no virus
AR42J-B13 Ad-PNM
IRPT

Table 4: Rat primers used in ChIP qPCR

Rat Primers used in ChIP qPCR		
Primer Name	**Forward Sequence 5`-3`**	**Reverse Sequence 5`-3`**
Ins1	CCAATGAGCGCTTTCTGCAGACTT	AGGAGAGTACATACCTGCTTGCTG
Ins2	ACCAGGCAAGTGTTTGGAAACTGC	ATGTAAGAGAAGAGCCCACAGCGT
Pdx1	AGAGATCAGCCTGCTGAGAGAGAA	TACTGCTCCTCACTATTCATGGTGGC
Gapdh	CTTTACGGGTGCACGTAGCTCA	TTTCACCTGGCACTGCACAAGAAG
Rat Primers used in DNA methylation assay		
Ins1 set1	TTTTAGGTTTAAGTAGAGTTGTTGA	CCCTAAAATTTTAACTAAAAAAACC
Ins1 set2	GTTTTTTGTTTTTGTTGGTTTTGTT	CACCCAACTCCAATTATAACACTTAC
Ins1 set3	TTTTTGGGAGTTTAAGTTTGTTTAG	ATACAACACTAATCCACAATACCAC
Ins2 set1	TTTGAGTTTTTTATTTGTTTTTTTT	CTAATTTAATACTACCCTATTCCCC
Ins2 set2	GATTATAAAGTTAGTGGGGATTTAGTAAT	TATTTAACAAAAACCTAAACAAAAC
Ins2 set3	GTATTTTTGTGGTTTTTATTTGGTG	ACTTACCTTATAAATCCTCCACTTC
Pdx1 set1	AGGAGAGATTAGTTTGTTGAGAGAGAA	AAAAACTACAAACCAAACCTTAAAC
Pdx1 set2	ATTATGAATAGTGAGGAGTAGTATTA	AAAAACTTCCCTATTCCAAC
Pdx1 set3	GTTGGAATAGGGAAGTTTTT	ACTTACCTACCCACTAACTTTTCCAC

Chapter 5 Effects of Small Molecules Together with Ad-PNM on Mouse Hepatocyte-Derived Small Cells

5.1 Introduction

The mouse hepatocyte-derived small cells, which are called ASH cells here, were derived from primary mouse hepatocytes maintained in tissue culture over a month by Dr. David Tosh`s group in University of Bath, UK. They express many of the hepatocyte genes such as *Alb*, *Trf*. It should be noted that expression of *Alb* and *Trf* in ASH cells is lower than the primary hepatocytes. ASH cells are smaller than primary hepatocytes. Unlike primary hepatocytes ASH cells are also highly proliferative in tissue culture and so far they have been maintained over thirty passages in tissue culture without losing their phenotype.

In this study we decided to use ASH cells to see if small molecules can increase the reprogramming efficiency of Ad-PNM for two reasons. Firstly, in Figure 6B by using immunostaining technique for insulin protein we showed that nearly 2% of the ASH cells become insulin-positive after transduction with Ad-PNM. Stimulation of the expression of beta cell genes was also demonstrated in Figure 5D. So any significant increase in the number of insulin-positive cells via small molecules can be easily detected in ASH cells. Secondly, unlike primary hepatocytes ASH cells are highly proliferative, phenotypically stable and form a homogenous population in tissue culture.

Small molecules which target genes, enzymes and/or signaling proteins have recently been commonly used in reprogramming studies. So far many different small molecules have been identified and used to alter cell fate and function (Shi, Tae Do et al.

2008; Xu, Shi et al. 2008). Maintenance and differentiation of stem cells as well as reprogramming of different cell types into each other are some examples in which the right small molecules favor the processes. Based on this fact to see if any small molecules can increase the reprogramming efficiency of Ad-PNM, we tested some of them together with Ad-PNM on ASH cells. We chose thirteen different small molecules which either were shown or thought to favor beta cell formation, replication and/or survival in other systems. We classified these small molecules into three groups which are: a) chromatin modification-related agents such as trichostatin A (TSA), valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA), a diazepin-quinazolin-amine derivative (BIX-01294) and 5-azacytosine (5-AzaC); b) signaling/metabolism agents such as pirinixic acid (WY-14643) (an agonist of PPARα and γ), *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) (an inhibitor of Notch), mycophenolic acid (MPA) (an inhibitor of IMP dehydrogenase which is used as immunosuppressant), 5'-*N*-ethylcarboxamidoadenosine (NECA) (an agonist of adenosine) and nicotinamide; and c) retinoic acid (RA) and inhibitors of its synthesis such as disulfiram (tetraethylthiuram disulphide) and diethylaminobenzaldehyde (DEAB).

5.1.1 Chromatin Modification-Related Agents

In mammals, histone deacetylase (HDAC) enzymes are classified into four classes; I to IV. Of the HDAC enzyme inhibitors, VPA inhibits only class I HDAC enzymes (Gottlicher, Minucci et al. 2001), while TSA and SAHA inhibit both class I and II HDAC enzymes (Yoshida, Kijima et al. 1990; Richon, Emiliani et al. 1998; Zhou, Marks et al. 2001).

Inhibition of histone methyltransferase (HMT) enzymes can impair the methylation of histone tails hence altering the gene expression profile. For instance, inhibition of G9a HMT enzyme by BIX-01294 was shown to lower $H3K9Me_2$ levels, allowing the active transcription of *Mage-a2* gene which is otherwise silent in ES cells (Kubicek, O'Sullivan et al. 2007).

DNA base methylation was shown to be removed when the cells are cultured with 5-AzaC (Taylor and Jones 1982) which is a cytosine analogue and can incorporate into DNA. 5-AzaC can form an irreversible covalent bond with DNMT enzyme thus depleting activity of the enzyme (Creusot, Acs et al. 1982).

5.1.2. Signaling/Metabolism Agents

WY-14643 is an agonist of peroxisome proliferator activated receptor-α and -γ (PPARα&γ) which can act as transcription factors to increase the expression of many genes reviewed in (Mandard, Müller et al. 2004). When rodents are fed for long periods of time with WY-14643, it was shown to cause hepatocellular tumors in mice and rats (Marsman and Popp 1994; Gonzalez, Peters et al. 1998) via a nongenotoxic mechanism (Peters, Cattley et al. 1997). The carcinogenic role of WY-14643 was then shown to be due to its hypomethylation effect on genomic DNA as well as on the promoter region of *C-myc* gene which induces cell proliferation (Ge, Wang et al. 2001).

Nicotinamide is the amide derivative of nicotinic acid. It was shown to have a protective effect on beta cell survival as well as function in the animal studies (Knip, Douek et al. 2000). In many studies describing protocols for insulin producing cell formation from human and mouse ES cells, nicotinamide was used together with other

molecules (Soria, Roche et al. 2000; Hori, Rulifson et al. 2002; Ku, Zhang et al. 2004; Miyazaki, Yamato et al. 2004; Segev, Fishman et al. 2004; Bai, Meredith et al. 2005; Shi, Hou et al. 2005). Effects of nicotinamide alone on the beta cell development from ES cells have also been reported (Vaca, Berná et al. 2003; Vaca, Berná et al. 2008).

Notch signaling can mediate many important cellular functions such as differentiation, proliferation and apoptosis in numerous tissue types. Notch proteins are cell surface transmembrane-spanning receptors and their interaction with proposed ligands initiates a signaling cascade within the cells. Activation of notch signaling upon ligand binding is accompanied by proteolytic cleavage of the intracellular domain of notch protein (NICD) by the gamma-secretase protease complex (Andersson, Sandberg et al. 2011). Upon release, the NICD translocates into the nucleus and associates with DNA-binding proteins (such as recombination signal binding protein for immunoglobulin kappa J region (RBPJ-$_K$) in mammals) to form a transcriptional activator, which activates the expression of a set of target genes, including *Hes1* (Apelqvist, Li et al. 1999). During pancreas development, HES1 represses the expression of *Ngn3* which is necessary for the formation of endocrine cell precursors (Jensen, Pedersen et al. 2000; Lee, Smith et al. 2001). Notch signaling was also shown to inhibit acinar and ductal cell development (Esni, Ghosh et al. 2004; Fujikura, Hosoda et al. 2006). DAPT was shown to functionally inhibit gamma-secretase activity (Dovey, John et al. 2001) hence inhibiting notch signaling as well as *Hes1* gene activation. Thus, DAPT administration to ASH cells together with Ad-PNM may stimulate endogenous *Ngn3* expression which can be expected to contribute to beta cell neogenesis.

MPA, the metabolite of the prodrug mycophenolate mofetil, reduces the guanine nucleotide level in the cells by inhibiting GMP synthesis through inosine monophosphate dehydrogenase (IMPDH) inhibition (Ransom 1995). Due to its inhibitory role on guanine synthesis, it has an antiproliferative effect on cells by preventing DNA replication (Allison and Eugui 2000). MPA is also widely used as an immunosuppressive drug as well as an antineoplastic agent. In a recent study carried out with zebrafish larvae, MPA was shown to induce precocious formation of secondary islets in zebrafish pancreas with a significant increase in the number of formed islets when MPA is given during larval development (Rovira, Huang et al. 2011). The authors also demonstrated that the precocious formation and increased number of beta cells through MPA was due to the inhibition of IMPDH with resulting depletion of intracellular guanosine level (Rovira, Huang et al. 2011). However, how intracellular guanosine levels are involved in beta cell differentiation is still unknown.

Recently it was also shown that MPA can cause the translocation from nucleolus to nucleoplasm of nucleolar proteins such as nucleolin, nucleophosmin and nucleostemin, hence changing the nucleolar architecture (Huang, Ji et al. 2008). These proteins are preferentially expressed in stem cells and in cancer cells (Tsai and McKay 2002). Translocation of nucleolar proteins was shown to be due to the inhibition by MPA of rRNA synthesis (Huang, Ji et al. 2008). Related to this, MPA-induced nucleolar stress activates p53 through the inhibition of MDM2 by ribosomal proteins L5 and L11 (Sun, Dai et al. 2008).

NECA is a nonspecific agonist of adenosine (Fredholm, IJzerman et al. 2001) which can activate adenosine G protein-coupled receptor signaling. Recently, the

adenosine signaling has been shown to be important in beta cell regeneration (Andersson, Adams et al. 2012). In this paper, the authors showed that some adenosine signaling-related small molecules particularly NECA can enhance the regeneration of the beta cells by contributing to their proliferation in zebrafish larvae in which beta cells had been depleted chemically (Andersson, Adams et al. 2012).

5.1.3. Retinoic Acid and Its Modulators

RA is an active derivative of the endogenous vitamin A which is the only source for RA since it can not be synthesized *de novo*. RA is a lipophilic small molecule and can diffuse through the cell membrane. In chordate embryos and adults, RA regulates several important cellular functions including cellular proliferation and differentiation (Escriva, Holland et al. 2002). The correct balance of RA is also necessary for patterning of the anteroposterior axis of the body in vertebrates by controlling the expression of *Hox* genes (Durston, Timmermans et al. 1989; Hogan, Thaller et al. 1992). In many other studies, RA was also shown to function in developing the facial region and forebrain (Schneider, Hu et al. 2001), eye (Matt, Dupé et al. 2005), inner ear (Romand, Dollé et al. 2006), lung (Wang, Dollé et al. 2006), kidney (Batourina, Gim et al. 2001) and pancreas (Rochette-Egly and Germain 2009). RA functions by binding to nuclear retinoic acid receptor (RAR) family members which are RARα, RARβ and RARγ (Ross, McCaffery et al. 2000). After ligand binding RARs form heterodimers with retinoid-X receptors (RXRs), including RXRα, RXRβ and RXRγ. RAR/RXR dimers will then bind to DNA sequences which are called RA-response elements (RARE) and activate their target genes. Retinol (vitamin A) is first transformed into retinaldehyde which is catalyzed by two enzyme

families: cytosolic alcohol dehydrogenases (ADHs) and microsomal retinol dehydrogenases (RDHs). Retinaldehyde is then oxidized to RA which is carried out by three retinaldehyde dehydrogenase enzymes (RALDH1, RALDH2, RALDH3).

RA appears to have critical role in development of the pancreas (Martín, Gallego-Llamas et al. 2005). In this study, the expression of *Raldh2* in mesoderm which surrounds the budding pancreatic epithelium was shown to be essential for *Pdx1* expression in the pancreatic dorsal bud, since in its absence the pancreatic dorsal bud did not express *Pdx1* (Martín, Gallego-Llamas et al. 2005). In a recent study, *Raldh1* expression was demonstrated in pancreatic epithelium of mouse and human at a period coinciding with the massive beta cell formation (Öström, Loffler et al. 2008). The authors also showed that transgenic mice expressing a dominant negative form of RARα under the control of the *Pdx1* promoter (*Pdx1* is expressed both in pancreatic progenitor cells and beta cells) did not develop dorsal or ventral pancreas and died after birth. These results prove the necessity of RA signaling in early pancreatic progenitor cells of both dorsal and ventral pancreas. In the same study, by using an *ex vivo* explant system, in which the authors cultured the mouse 10.5 dpc dorsal pancreatic buds in serum-free defined medium with or without RA, they showed that 25nM RA provides ~2.5 fold increase in the number of insulin-positive cells compared to control. However higher concentrations of RA such as 50nM and 100nM inhibited endocrine cell formation (Öström, Loffler et al. 2008). These results demonstrated the stimulatory effect of RA on beta cell neogenesis as well as its inhibitory effect on beta cell formation at high concentrations. For that reason in addition to RA we also tested the effect of RA inhibitors such as disulfiram and DEAB both of

which inhibit the aldehyde dehydrogenase enzyme activity thereby impairing RA signaling.

5.2. Experimental Design and Methods

5.2.1. Killing Curve for Small Molecules

To determine the final concentrations of the small molecules to be used in this study, we performed a killing curve assays for each small molecule. For this purpose 10^5 mouse hepatocyte-derived small (ASH) cells were plated into each well of a 6-well plate and were maintained in low-glucose DMEM medium which was supplied with 10% (v/v) FBS and 1x anti-anti solution. One day after we plated the cells, incremental concentrations of small molecules were included in the culture. The cells were incubated with these small molecules for two days and then the small molecules were removed by refreshing the medium. The highest concentration which causes no more cell death than of the control cells was chosen as the final concentration for the given small molecule. These final concentrations were as follows: 1mM for TSA (diluted in dH_2O), 1mM for VPA (diluted in dH_2O, Sigma), 5μM for SAHA (diluted in DMSO; Sigma), 2μM for BIX-01294 (diluted in dH_2O; Tocris), 5μM for 5-AzaC (diluted in DMSO), 75μM for WY-14643 (diluted in DMSO; Cayman), 10μM for DAPT (diluted in DMSO; Sigma), 1μM for MPA (diluted in DMSO; Sigma), 10μM for NECA (diluted in DMSO, Tocris), 5mM for Nicotinamide (diluted in dH_2O; Sigma), 100nm for Disulfiram (diluted in DMSO; Sigma), 10μM for DEAB (diluted in DMSO; Aldrich), 10μM for RA (diluted in DMSO).

5.2.2. Administration of Small Molecules and Ad-PNM to the Cells

After the determination of the final concentrations for each small molecule, the same number of ASH cells (10^5) was plated into each well of 6-well plates (Day 0). The cells were allowed to attach to the plates overnight. The next day, each small molecule was individually given to the cells in different wells at the final concentrations mentioned above (Day 1). After two days incubation with small molecules, the number of the cells per well was calculated and 15 MOI Ad-PNM was given without removing the small molecules from the medium (Day 3). The next day, both Ad-PNM and the small molecules containing medium was removed (Day 4). The cells were maintained in the culture for two more days before analysis.

To see if the small molecules increased the reprogramming efficiency of Ad-PNM, we decided to count the number of insulin expressing cells as the read out. For this purpose, ASH cells were fixed and immunostained for insulin and PDX1 proteins (Day 6) as described previously. The number of insulin positive cells as a fraction of viral Pdx1 bearing cells was calculated by counting the insulin-positive cells from ten randomly chosen different areas in each well. Three different wells were counted for each experimental group and two different experiments were set up at different times (n=2x3).

RNA isolation, cDNA synthesis and qRT-PCR experiments for ASH cells, ASH cells with DAPT, ASH cells with Ad-PNM, ASH cells with DAPT and Ad-PNM were performed as described in Chapters B1 and B3. ELISA for glucose stimulated insulin release for ASH cells, ASH cells with DAPT, ASH cells with Ad-PNM, ASH cells with DAPT and Ad-PNM were performed as described in Chapter B3.

5.3 Results

Contribution of the different small molecules to the reprogramming efficiency of Ad-PNM on ASH cells was shown in the Figure 24. Of the thirteen small molecules used in this study, DAPT, NECA and BIX-01294 yielded ~6% (3 fold increase), ~4% (2 fold increase) and ~4% (2 fold increase) respectively in the number of insulin-positive cells as a fraction of the Ad-PNM bearing cells, where the efficiency of Ad-PNM alone was 2%. Unlike DAPT and NECA, other signaling/metabolism agents such as WY-14643 (~3%), MPA (~2.5%) and nicotinamide (~2.5%) showed a slight or no increase in the number of insulin-positive cells compared to Ad-PNM bearing cells only. Among chromatin modifying agents, BIX-01294 (~4%) was the only one making a significant contribution to the efficiency of reprogramming into an insulin-positive state through Ad-PNM. The rest of them either did not increase the number of insulin-positive cells (VPA and SAHA; ~2%) or made it worse than Ad-PNM alone (TSA and 5-AzaC; less than ~1%). RA (~2.5%) and its negative modulator DEAB (~3%) slightly increased the efficiency of Ad-PNM whereas another RA modulator disulfiram (~1.5%) made it worse. Moreover administration of most responding three small molecules DAPT, NECA and BIX-01294 at the same time resulted in significant increase (~12%) in number of insulin positive cells (Figure 24).

Gene expression profile comparison between small molecules only, Ad-PNM only and small molecules + Ad-PNM including cells with qRT-PCR revealed that when used together with Ad-PNM, small molecules (DAPT+NECA+BIX) also increased the

expression level of many beta cell genes such as *Ins1*, *Ins2*, *Pax4*, *Neurod1*, *Insm1*, *Isl1*, *Pcsk1*, *Pcsk2*, *Cpe*, and *Mnx1* (Figure 25).

Like the increase in expression of beta cell markers, ELISA results showed that when used together with Ad-PNM, small molecules (DAPT+NECA+BIX) yielded a slight increase in the amount of insulin released into the medium (Figure 26).

5.4. Discussion

Of thirteen small molecules used in this study, the notch signal blocking agent DAPT gave the best result with a three fold increase in the number of insulin-positive ASH cells compared to those that were induced with Ad-PNM only. This favorable effect of DAPT was probably due to its ability to deplete the notch signal driven-expression of *Hes1* which otherwise suppresses the pancreatic endocrine cell formation. Through the inhibition of gamma-secretase protein complex, DAPT prevents the expression of *Hes1* gene which allows the expression on endocrine cell differentiation factor *Ngn3*. Our results for DAPT is consistent with the studies performed with developing zebrafish larvae (Parsons, Pisharath et al. 2009; Rovira, Huang et al. 2011) which demonstrated the favorable effect of DAPT on beta cell formation.

Among chromatin modifying agents tested in our hands, the G9a HMT enzyme inhibitor BIX-01294 was the only one which significantly increased the number of insulin-positive ASH cells by almost two fold. This was probably due to its effect on lowering $H3K9Me_2$ level thereby allowing the active transcription of many genes (Kubicek, O'Sullivan et al. 2007). The HDAC enzyme inhibitors such as TSA, VPA and SAHA, however, did not increase the number of insulin-positive ASH cells unlike a

report in which HDAC enzyme inhibitors were shown to favor *Ngn3* expression thereby increasing the number of endocrine cells in rat pancreatic bud cultured *in vitro* (Haumaitre, Lenoir et al. 2008). Like most of the histone modification-related small molecules used here, the DNA base methylation-related small molecule 5-AzaC, which depletes the activity of DNMT enzyme also did not increase the number of insulin-positive ASH cells.

The adenosine agonist NECA is another small molecule that resulted in an almost two fold increase in the number of insulin-positive ASH cells. It has recently been showed that NECA acts through the adenosine receptor A2aa and increases the proliferation of specifically beta cells during beta cell regeneration (Andersson, Adams et al. 2012). Beside NECA, the authors described three more adenosine signaling-related small molecules enhancing the beta cell regeneration during the development of zebrafish larvae in which beta cells were depleted previously. Even though we have not tested the proliferation ability of NECA-treated insulin-positive ASH cells, it should be noted that these cells were already highly proliferative without NECA. Unlike DAPT and NECA, other signaling/metabolism agents such as WY-14643, MPA or nicotinamide did not make any significant contribution into the formation of insulin-positive cells. Also, neither RA nor its negative modulators improved the results.

When we used these three most effective small molecules (DAPT, BIX-01294 and NECA) together on the ASH cells, they increased the number of insulin positive cells almost 6 fold (~12%). This was an expected result since these three small molecules have different mode of actions and targets in the cells.

qRT-PCR and ELISA results demonstrated that there is also a slight increase in the expression level of many beta cells genes as well as in the amount of the insulin released into the medium from the cells when DAPT+NECA+BIX combination used together with Ad-PNM. However, increase in the transcript level as well as secreted insulin is probably due to increase in the number of the responding cells. We concluded that when used together with Ad-PNM, DAPT+NECA+BIX can increase the quantity of transformed cells but not the quality of transformation since a) there in no activation of a different beta cell marker in DAPT+NECA+BIX & Ad-PNM including samples as distinct from Ad-PNM only including cells; and b) there is no increase in the ratio of released insulin in low and high glucose environment.

Figure 24: Effect of small molecules on cell reprogramming

Thirteen small molecules were given to ASH cells together with Ad-PNM. Their effect in reprogramming efficiency was quantified by comparing the fraction of insulin-positive cells after insulin immunostaining. ASH cells were treated with small molecules for 2 days, then were given Ad-PNM overnight and insulin immunostaining was performed 3 days after Ad-PNM transduction. Data are means ± S.E. (n =3).

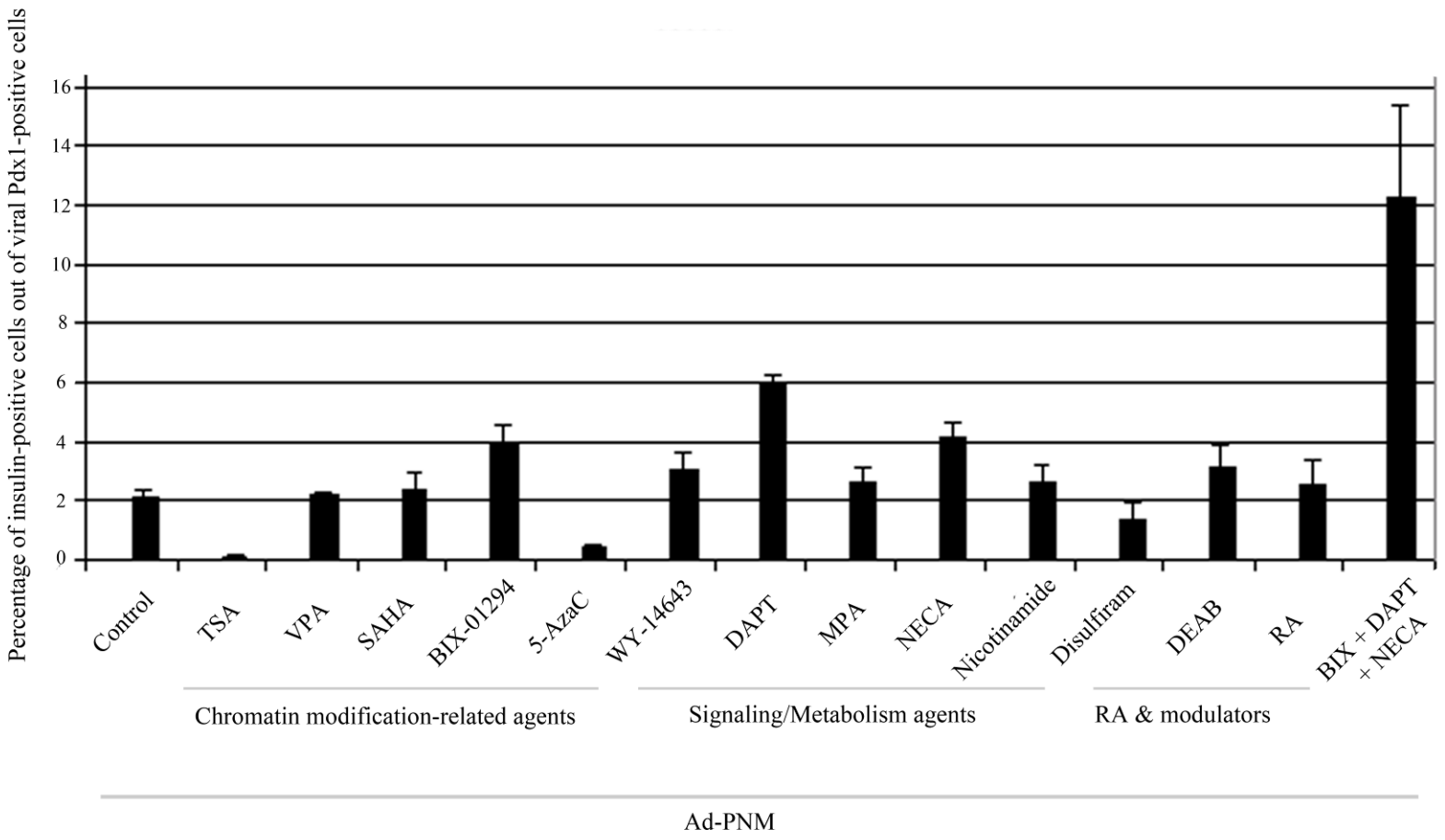

Percentage of insulin-positive cells out of viral Pdx1-positive cells
16
14
12
10
8
6
4
2
0
Control
TSA
VPA
SAHA
BIX-01294
5-AzaC
WY-14643
DAPT
MPA
NECA
Nicotinamide
Disulfiram
DEAB
RA
BIX + DAPT + NECA
Chromatin modification-related agents
Signaling/Metabolism agents
RA & modulators
Ad-PNM

Figure 25: Gene expression profile of ASH cells after small molecules and Ad-PNM

Gene expression profile of ASH cells with no treatment (white bars), with DAPT only (light grey bars), with DAPT+NECA+BIX (dark grey bars), Ad-PNM only (black bars), with DAPT & Ad-PNM (grey dotted bars) and with DAPT+NECA+BIX & Ad-PNM (grey dotted bars) were quantified by qRT-PCR. ASH cells only group was maintained in culture for three days. ASH cells with small molecule(s) group were maintained in medium supplemented with small molecule(s) for three days. ASH cells with Ad-PNM were maintained in culture for three days in which Ad-PNM were given the cells at day 0. ASH cells with small molecule(s) and Ad-PNM were maintained in medium supplemented with small molecule(s) for five days in which Ad-PNM were given the cells at day 2. Data are means ± S.E. (n =3).

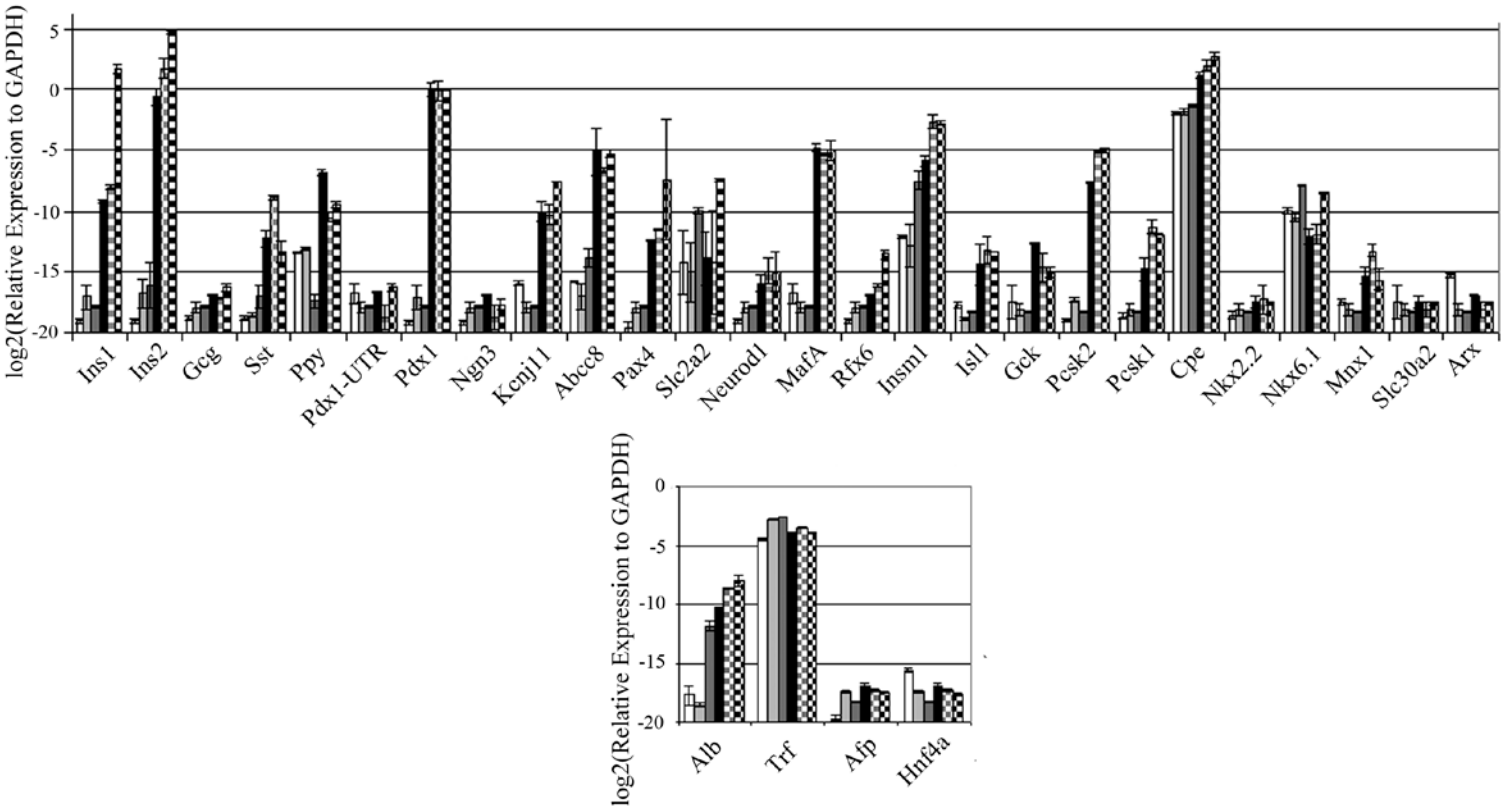
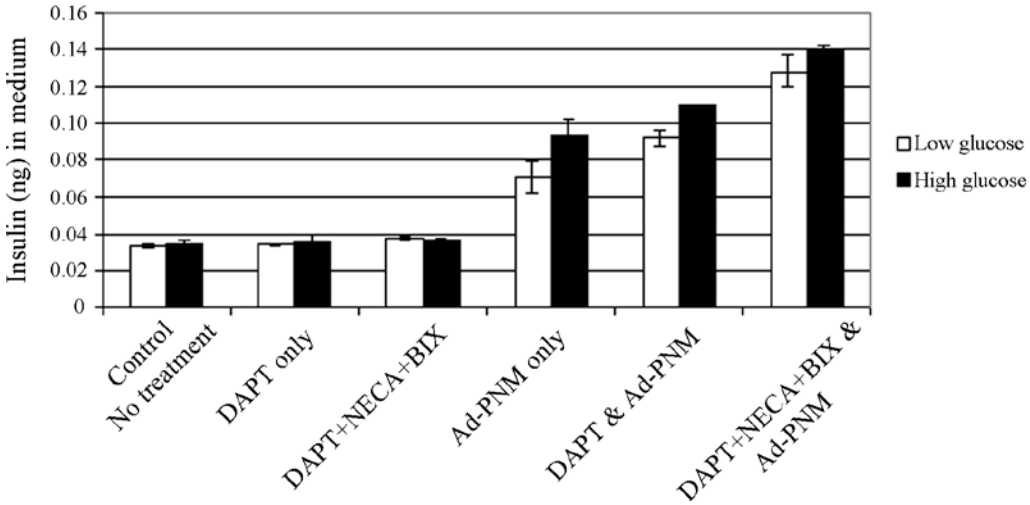

log2(Relative Expression to GAPDH)
5
0
-5
-10
-15
-20
Ins1
Ins2
Gcg
Sst
Ppy
Pdx1-UTR
Pdx1
Ngn3
Kcnj11
Abcc8
Pax4
Slc2a2
Neurod1
MafA
Rfx6
Insm1
Isl1
Gck
Pcsk2
Pcsk1
Cpe
Nkx2.2
Nkx6.1
Mnx1
Slc30a2
Arx
log2(Relative Expression to GAPDH)
0
-5
-10
-15
-20
Alb
Trf
Afp
Hnf4a
Control, No treatment
DAPT only
DAPT+NECA+BIX
Ad-PNM only
DAPT & Ad-PNM
DAPT+NECA+BIX & Ad-PNM

Figure 26: Secreted insulin amount of ASH cells after small molecules and Ad-PNM by ELISA

The amount of insulin in the medium released from ASH cells with no treatment, with DAPT only, with DAPT+NECA+BIX, with Ad–PNM only, with DAPT and Ad-PNM, with DAPT+NECA+BIX and Ad-PNM was measured by ELISA. Cells were stimulated either with low-glucose (white bars) or high-glucose (black bars). Data are means ± S.E. (n =3).


Insulin (ng) in medium
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Control
No treatment
DAPT only
DAPT+NECA+BIX
Ad-PNM only
DAPT & Ad-PNM
DAPT+NECA+BIX &
Ad-PNM
Low glucose
High glucose

Chapter 6 Attempts to Sort/Enrich Insulin-Positive Cells after Ad-PNM Transduction

6.1. Introduction

In Chapter B1 we showed the expression of *Insulin* genes by RT-PCR in eight different cell types at varying intensities upon Ad-PNM transduction. Some of these cell types activated more beta cell markers than others in addition to *Insulin* genes. Even though the expression of *Insulin* genes was shown for all the cell types in RT-PCR, insulin immunostaining was only detected in five cell types, including primary rat hepatocytes (Figure 6A-A`), ASH cells (Figure 6B-B`), AR42J-B13 cells (Figure 6C-C`), rat MAPCs (Figure 6D) and CRL-1213 (Figure 6E). On the other hand, for four of these cell types (except AR42J-B13 cells) the number of insulin positive cells as a fraction of Ad-PNM bearing cells was low; at about 2%. The low concentration of the insulin-positive cells made it difficult to further analyze these cells to see how close to a genuine beta cell phenotype they are. The ideal way to get trustworthy results from these cells was to increase the ability to sort and/or enrich the fraction of these insulin-positive cells. To accomplish this, we focused on two methods: a) Newport green staining and fluorescence-activated cell sorting (FACS), and b) Insulin immunostaining and FACS or laser capture microdissection (LCM).

Newport green is a fluorescent indicator which has a specific affinity for zinc (Zn^{2+}) molecule (Gee, Zhou et al. 2002). Intracellular Zn^{2+} rich cells can be stained and visualized with Newport green without any need for fixation and/or permeabilization steps hence keeping the cells alive during and after staining. In addition to its other

critical functions in biological systems (Beyersmann and Haase 2001), Zn^{2+} is also indispensable for beta cell biology. Insulin proteins are packed and stored in secretory vesicles of beta cells as solid hexamers and two Zn^{2+} ions are necessary for this packaging (Chausmer 1998). For that reason, intracellular Zn^{2+} ions are exceptionally abundant in beta cells and can be stained with Newport green staining.

Newport green was shown to stain insulin expressing, intracellular zinc-rich beta cells (Lukowiak, Vandewalle et al. 2001), as well as insulin-positive cells derived from human ES cells (Liew, Shah et al. 2008) without fixing and permeabilizing the cells thereby keeping them alive. However, in our experiments in which we stained the Ad-PNM-transduced ASH cells with Newport green dye we did not see any difference between insulin-positive and -negative cells. Incubation of experimental and control groups with extra Zn^{2+} resulted in Newport green positivness both for Ad-PNM transduced and nontransduced cells. Since there was no true Newport green staining we could not sort insulin positive cells through FACS.

6.2. Experimental Design and Methods

6.2.1 Newport Green Staining

10^5 ASH cells were plated into each well of a 6-well plate and were maintained in low-glucose DMEM which was supplied with 10% (v/v) FBS and 1x anti-anti solution (Day 0). The cells were allowed to attach to the plates overnight. The next day, 15 MOI Ad-PNM was given to the cells (Day 1). The day after virus administration, Ad-PNM containing medium was removed (Day 2). Three days after Ad-PNM transduction (Day

5) the cells were stained with 1μM Newport green (Invitrogen). To do this ASH cells were washed twice with PBS. The cells were then incubated in 1ml PBS supplied with 1μM newport green and 1μl pyronic acid (Invitrogen) for 1 h at 37°C.

6.2.2 FACS and LCM Sorting Following Insulin Immunostaining

ASH cells were plated into either T-175 tissue culture flasks (for FACS) or PEN-membrane coated tissue slides (Leica) (for LCM) and were maintained in low-glucose DMEM which was supplied with 10% (v/v) FBS and 1x anti-anti solution (Day 0). For FACS, a total of $5x10^7$ ASH cells were maintained in ten T-175 tissue culture flasks. For LCM $5x10^5$ ASH cells were plated into each slide. The cells were allowed to attach the plates and slides overnight. The next day, 15 MOI Ad-PNM was given to the cells (Day 1). The next day Ad-PNM containing medium was removed (Day 2). Three days after Ad-PNM transduction (Day 5) the cells were fixed and stained for insulin protein.

For FACS, ASH cells were dissociated from tissue culture flasks with TrypLE (Gibco) and were collected into RNase-free 1.5ml centrifuge tubes. Formalin fixation, permeabilization and all remaining steps for insulin staining were carried out with ASH cell suspension in the centrifuge tubes. All the solutions used during staining except formalin were supplied with 0.1% (v/v) active diethylpyrocarbonate (DEPC, Sigma) to reduce RNA degradation. Guinea pig anti-insulin (1:200 dilution, Sigma) antibody was used for primary antibody staining. R-Phycoerythrin conjugated donkey anti-guinea pig (1:500 dilution, Jackson ImmunoResearch) antibody was used for secondary antibody staining. After the cells were stained for 1 h with each antibody, insulin-positive cells were sorted with FACS machine. Approximately 10^5 insulin-positive ASH cells (1%)

were separated from $5x10^7$ ASH cells. Total RNA from the sorted cells was isolated using the FFPE RNA isolation kit (Qiagen), which is designed for RNA isolation from formalin fixed paraffin embedded tissues, according to the manufacturer's manual.

DNase treatment and cDNA synthesis were performed as described in Chapter B1. One difference was that RNA samples were incubated at 70°C for 15 min before cDNA synthesis to reduce the formaldehyde induced cross linking. Random primers (Invitrogen) instead of oligo$(dT)_{20}$ were also used during cDNA synthesis. The gene expression pattern between insulin-positive and negative ASH cells was then compared by performing qRT–PCR as described in Chapter B3. The primer pairs used in this qRT–PCR were designed for small amplicons such as less than 100bp and were listed in Table 5.

6.3. Results

LCM sorting of formalin-fixed, insulin-immunostained ASH cells posed another challenge as we could not isolate enough cells for applications such as qRT-PCR. The problems we encountered during LCM were as follows: a) The laser which dissects the cells out is not fine enough. In order not to burn the insulin-positive cell of interest with the laser a wide circle should be drawn which then burns the neighboring insulin-positive cells. b) The slide and the cells on it start to dry during laser dissection which causes a background signal which makes it difficult to identify the true insulin-positive cells. c) Laser dissection of a single cell takes at least 10 sec and considering at least 10^5 cells is necessary for RNA isolation from formalin fixed cells, it takes roughly 250 h to isolate enough number of cells with LCM.

FACS of formalin-fixed, insulin-immunostained ASH cells gave better results than LCM. Approximately 10^5 insulin-positive ASH cells (1%) were sorted from $5x10^7$ ASH cells with FACS. The whole procedure from fixation/staining to sorting took 7-8 h during which cells were preserved in active DEPC-containing solutions. 4μg total RNA was isolated from the sorted cells. This amount was lower compared to the total RNA yield from control cells but high enough to prepare cDNA for qRT-PCR. The gene expression difference between Ad-PNM-transduced, insulin-immunostained, FACS-sorted ASH cells and no virus-treated, secondary antibody-immunostained, FACS-sorted ASH cells was shown in Figure 27.

6.4. Discussion

The fraction of insulin-positive cells for Ad-PNM-transduced AR42J-B13 cells was approximately 70%. This ratio was high enough to get accurate and reliable results from a heterogenous cell population in which Ad-PNM bearing insulin-positive cells, Ad-PNM bearing insulin-negative cells, and cells with no Ad-PNM are present. However the low fraction of insulin-positive cells in other cell types such as ASH (~2%) after Ad-PNM transduction made it difficult to further analyze the insulin-positive cells. For that reason, in this section we were motivated to find out a way to sort/enrich the fraction of insulin-positive cells from remaining unresponsive cells.

Unlike cells which have a unique cell surface protein, until now there are no known cell surface proteins for beta cells. A2B5 antibody, which primarily binds to gangliosides of various neural tissue cells, was shown to bind to plasma membrane of islet cells (Eisenbarth, Shimizu et al. 1982). However, none of the insulin-positive cell

types we obtained after Ad-PNM transduction were stained with this antibody (results are not shown).

The impossibility of a live beta cell sorting via FACS due to the absence of a unique cell surface protein in beta cells was overcome by the use of Newport green staining which stains the intracellular Zn^{2+} ions highly abundant in beta cells (Lukowiak, Vandewalle et al. 2001; Liew, Shah et al. 2008). However, like A2B5, Newport green staining did not label our insulin-positive cells. As proven in many of the experiments performed in this dissertation, even though the effects of Ad-PNM are dramatic on the cell types we used, the reprogramming event is not complete in any of them and eventual insulin-positive cells are not true beta cells. Because of this incomplete reprogramming, our insulin positive cells may not have developed proper targets for A2B5 antibody hence remaining irresponsive. For the same reason, intracellular insulin content may not have packed into cellular granules properly thereby eliminating the necessity of high intracellular Zn^{2+} content. Alternatively the failure of Newport green staining may simply have been due to the low amount of the insulin produced by our cells.

After these unsuccessful attempts to sort insulin-positive cells alive, we decided to sort them via insulin immunostaining. Since we have to fix and permeabilize the cells for immunostaining of cytoplasmic insulin protein, the cells will not be alive after sorting which highly limits the downstream applications (i.e. glucose stimulated insulin response, cell transplantation) for these cells. However, the gene expression profile of these enriched insulin-positive cells can be quantified by qRT-PCR if a sufficiently high quality and quantity of total RNA can be isolated. Isolation of total RNA from formalin fixed cells/tissues is challenging because a) RNA bases show covalent modifications

(Evers, Fowler et al. 2011), and b) RNA strands are fragmented into smaller pieces (Hewitt, Lewis et al. 2008) after formalin fixation. These effects of formalin on RNA fragments make RNA isolation and cDNA synthesis from isolated RNA difficult. To overcome these problems we first reduced the duration of cell fixation (15 min) and primary/secondary antibody staining (30 min for each). The solutions used during staining and sorting were supplemented with active DEPC to prevent further strand fragmentation. The LCM technique was not practical to collect enough cells because of the reasons mentioned above. However, we succeeded to sort 10^5 insulin-positive cells out of more than $5x10^7$ cells in a reasonable time (4-5 h) with FACS. Total RNA from these cells was isolated using a FFPE RNA isolation kit (Qiagen) which was designed for RNA isolation from formalin treated samples. Briefly kit uses proteinase K enzyme to degrade the proteins cross-linked to RNA to make RNA isolation more efficient. Moreover during the isolation cell extracts are also heated up to 80°C to reverse the modifications on the RNA bases. Still RNA is fragmented into small pieces and we used random primers instead of oligo$(dT)_{20}$ when making cDNAs from these fragmented RNAs. We also designed primers to be used in qRT-PCR for small amplicons such as less than 100 bp. All these small modifications performed during experiments improved the qRT-PCR results to a large extent.

All in all, even though it does not allow us to perform more downstream applications after the sorting, FACS isolation of the formalin fixed, insulin-immunostained ASH cells gave us an opportunity to quantify the gene expression profile of these enriched insulin positive cells in a more accurate and reliable way than those that not sorted, 2% insulin-positive cell including, heterogenous ASH cell population.

Figure 27: Gene expression profile of insulin immunostained FACS sorted ASH cells

Gene expression profiles of FACS sorted insulin-positive ASH cells after Ad-PNM transduction were quantified by qRT-PCR. ASH cells were transduced with Ad-PNM. Three days after transduction, the cells were fixed with formalin and immunostained for insulin protein. Insulin-positive ASH cells were sorted using FACS. qRT-PCR was performed for the insulin-positive (grey bars) and -negative cells (white bars) for some important beta cell markers. Data are means ± S.E. (n =2).

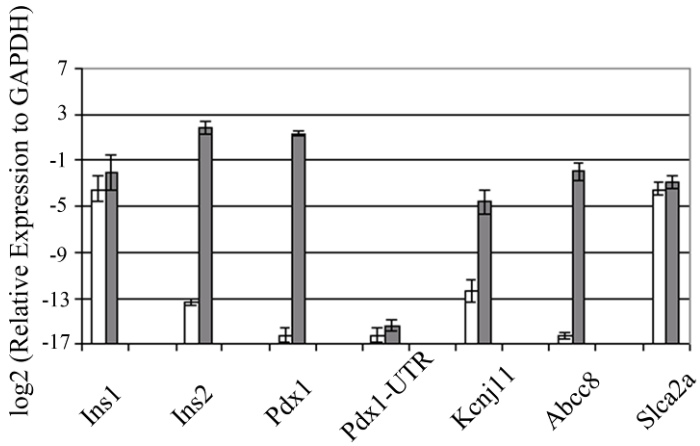

log2 (Relative Expression to GAPDH)
7
3
-1
-5
-9
-13
-17
Ins1
Ins2
Pdx1
Pdx1-UTR
Kcnj11
Abcc8
Slca2a

Table 5: Mouse primers used for small amplicons

Mouse Primers used for small amplicons		
Primer name	**Forward Sequence 5`-3`**	**Reverse Sequence 5`-3`**
Ins1	TGCTCCCTCTACCAGCTG	CATTGCAGAGGGGTGGG
Ins2	GGCTTCTTCTACACACCCATG	CACCCAGCTCCAGTTGTG
Pdx1	GATGAAATCCACCAAAGCTCAC	TCTTGTTTTCCTCGGGTTCC
Pdx1 UTR	CACAGCCCTCCAGCATC	CCGCTCACCCTCAGACT
Slc2a2	AATTACCGACAGCCCATCC	TCCCATTGATTCCTGAGAACTG
Kcnj11	CGGAGAGGGCACCAATG	AGGAAGGCAGATGAAAAGGAG
Abcc8	CATGGAGACCAGACTCAGATTG	ACTGATTCGCTGACGCTG

Chapter 7 Conclusion and Future Directions

The loss of beta cells at the onset of type 1 and at the late stage of type 2 diabetes necessitates a new source of insulin hormone for proper control of the glucose metabolism. Current therapies such as insulin replacement (i.e. insulin shots, insulin pumps) and islets and/or whole pancreas transplantation can treat the disease to some extent but not cure it. The production of insulin-producing, glucose-responsive beta-like cells from patients` own cells can solve many of the problems encountered in the present treatments. Due to this possibility, many studies related to beta-like cell reprogramming have been performed recently. In one of these studies Zhou et al. showed the ability of the combination of *Pdx1*, *Ngn3* and *MafA* genes to reprogram the mouse exocrine acinar cells into insulin producing beta-like cells.

In this dissertation we studied the effect of the same transcription factors used by Zhou et al., but cloned all three genes into single adenoviral construct. First, we investigated the reprogramming competency to *Pdx1*, *Ngn3* and *MafA* of different cell types at different developmental stages from mouse and rat. Second, we looked at the effect of these three genes on a pancreatic rat exocrine cell line AR42J-B13 to see if this gene combination provides a true beta cell reprogramming event. Next, we investigated the effect of some small molecules to see if they increased the reprogramming efficiency of this gene combination. Finally we tried to sort the small fraction of insulin-positive cells formed after *Pdx1*, *Ngn3*, *MafA* transduction to enrich the insulin-positive cell population hence getting more accurate data from them.

Transduction of different cell types with Ad-PNM showed that the *Pdx1, Ngn3* and *MafA* gene combination is enough to stimulate the expression of *Insulin* genes with varying intensities depending on cell type. Rat cell lines responded to Ad-PNM in a better way than mouse cell lines by activating more beta cell genes. The number of responding cells to Ad-PNM was also higher for the rat cell lines than the mouse cell lines. Moreover progenitor-like cells as well as the cells developmentally related to beta cells are prone to be reprogrammed into beta-like state. Supporting this final statement, embryonic mouse heptoblasts from *Pdx1-GFP* transgenic mice gave the most promising result by activating the endogenous *Pdx1* as well as many other important beta cell genes upon transduction with Ad-PNM.

Further characterization of the effect of Ad-PNM on the most resposive cell type AR42J-B13 cells showed that after Ad-PNM the cells became post-mitotic, began to express *Insulin* genes together with many other beta cell genes, produced mature insulin hormone, changed the epigenetic state of their chromatin, and rescued diabetic mice if transplanted into the kidney capture. However, some important beta cell genes were not activated thereby making these cells unresponsive to glucose which is an indispensable beta cell quality. It is obvious that even though the changes are dramatic, the combination of *Pdx1, Ngn3* and *MafA* did not provide a true beta cell reprogramming *in vitro* at least for this cell system.

Among some of the small molecules which had been reported to favor beta cell formation, regeneration and/or survival, three of them including DAPT, BIX-01294 and NECA increased the effect of Ad-PNM on mouse hepatocyte-derived small cells.

Moreover when all three were used together they showed a better efficiency. Even though there was a significant increase in the number of insulin positive cells, the fraction of the Ad-PNM-responding cells was still low after the small molecules. For that reason the effect of small molecules on reprogramming efficiency of Ad-PNM was compared by counting the fraction of insulin-positive cells out of Ad-PNM-bearing cells between control and experimental cell groups instead of performing qRT-PCR.

Of the attempts that we performed to increase the low fraction of insulin-positive cells via different sorting techniques to get more accurate and reliable results from Ad-PNM responding cells, only fluorescence-activated cell sorting of insulin-immunostained cells worked. Even though these cells were enriched through the FACS, because the cells were nonviable not alive we could not perform any downstream application that necessitate living cells. This system allows us to do qRT-PCR only.

To form insulin-expressing beta–like cells from other cell types, the combination of *Pdx1*, *Ngn3* and *MafA* is one of the best candidates because of the roles of these factors during beta cell development. Dramatic effects of this combination on the cells tested also support this idea. Incomplete reprogramming of the tested cell systems, however, is still an important issue which needs to be solved in the future studies. We verified that three small molecules reported previously by others increase the number of insulin-positive cells after Ad-PNM. For that reason simultaneous administration of a true combination of small molecules and transcription factors can provide genuine beta cell formation *in vitro*.

Our preliminary results (not shown here) demonstrated that low and high levels of Ad-PNM prevent the insulin-positive cell formation. For our experiments 25 MOI Ad-PNM was the optimum dose and gave the highest number of insulin-positive cells. This result underscores the importance of amount of the transcription factors for reprogramming events. For that reason instead of simultaneous changes in the amount of *Pdx1*, *Ngn3* and *MafA* genes, their different combinations should be tested in future experiments.

The main purpose behind these experiments was to identify an alternative beta cell source for diabetic patients. For this purpose, all these experiments should also be upgraded to human cell studies to see if they can be reprogrammed into true beta cells.

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