The evolving beta cell phenotype

Doctoral dissertation

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Abbreviations

AdDN-MafA: adenovirus dominant-negative MafA

AdGFP: adenovirus GFP

AdNeuroD1: adenovirus NeuroD1

AdPdx1: adenovirus Pdx1

ANOVA: analysis of variance

Arg: arginine

Arx: aristaless related homeobox

ATP: adenosine triphosphate

bHLH: basic helix-loop-helix

Cav1.2, Cav1.3: voltage-gated calcium channel alpha 1C, 1D subunit

cDNA: complimentary DNA

CDS: coding sequence

CMV: cytomegalovirus

Cox: cytochrome c oxidase

Cpt: carnitine-palmitoyl transferase

DAPI: 4'-6-Diamidino-2-phenylindole

DAVID: Database for Annotation, Visualization and Integrated Discovery

dChip: DNA-Chip analyser

dNTP: deoxyribonucleotide triphosphate

E47: E protein 47

EST: expressed sequence tag

FBS: fetal bovine serum

FC: fold change

Foxa2: forkhead box A2

GFP: green fluorescent protein

GK: Goto-Kakizaki rat

Gln: glutamine

Glp1r: glucagon-like peptide 1 receptor

Glut2: glucose transporter 2

Got: glutamine-oxalacetate transferase

Gpd: glycerol-3-phosphate dehydrogenase

GSIS: glucose-stimulated insulin secretion

Hes: hairy and enhancer of split

HSV: herpes simplex virus

INS-1: insulinoma 1

IPGTT: intraperitoneal glucose tolerance test

Isl1: islet 1

KCl: potassium chloride

Kir6.2: potassium inwardly-rectifying channel

KRBH: Krebs-Ringer bicarbonate buffer

LCB: lower confidence boundary

LCM: laser capture microdissection

Leu: leucine

LSM: laser scanning microscope

MafA: musculoaponeurotic fibrosarcoma oncogene homolog A

MafB: musculoaponeurotic fibrosarcoma oncogene homolog B

Mdh: malate dehydrogenase

Me: malic enzyme

MODY: mature onset diabetes in young

mRNA: messenger RNA

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

Nd: NADH dehydrogenase

NeuroD1: neurogenic differentiation 1

Ngn3: neurogenin 3

Nkx2.2, Nkx6.1: NK2 homeobox 2, NK6 homeobox 1

P: postnatal day

Pax4, Pax6: paired box 4, 6

PBS: phosphate buffered saline

Pc: pyruvate carboxylase

Pcsk1: proprotein convertase subtilisin/kexin type 1

Pdx1: pancreatic and duodenal homeobox 1

PK, PKM, PKLR: pyruvate kinase, muscle, liver and red blood cell isoform

PP: pancreatic polypeptide

Ptf1a: pancreas specific transcription factor 1a

PVDF: polyvinylidene fluoride

qPCR: quantitative real-time polymerase chain reaction

RBC: red blood cell

RPMI: Roswell Park Memorial Institute medium

RT-PCR: reverse transcription polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of mean

Slc: solute carrier

Snap: synaptosomal-associated protein

SNARE: soluble N-ethylmaleimide-sensitive factor activating protein receptor

Sox9: sex determining region Y box 9

Stx1a: syntaxin 1A

Stxbp: syntaxin binding protein

Suclg: succinate-CoA ligase

Sur1: sulfonylurea receptor 1

TBST: Tris buffered saline with Tween 20

UTR: untranslated region

Vamp: vesicle-associated membrane protein

WKY: Wistar-Kyoto rat

1. Introduction

The very essence of diabetes is the failure of the insulin-producing pancreatic beta cells, either due to autoimmune destruction in type 1 diabetes or as a consequence of permanent glucotoxicity combined with peripheral insulin resistance in type 2 diabetes. Diabetes mellitus in all its forms is a serious and costly disease and it has become a major health problem worldwide during the past few decades. People in many countries are becoming heavier, more sedentary and older [1, 2]; which increases the prevalence of type 2 diabetes [3] and could potentially result in a decline in life expectancy [4]. In addition, both type 1 and type 2 diabetes is complicated by severe end-organ damage in vital organs of the body including the retina, the kidney glomerulus and peripheral nerves, because of the long-term effects of chronic hyperglycaemia on the microvasculature [5]. People with diabetes are also at greater risk for developing artherosclerosis, affecting coronary, cerebrovascular and peripheral arterial circulation. The exact relationship between chronic hyperglycaemia and macrovascular complications has not been clearly established, however the evidence is accumulating, as early strict glycemic control seems to generate a positive legacy in terms of cardiovascular outcomes [6-8]. Taken together, data strongly imply that normalization of blood glucose levels is essential early in the timecourse of diabetes in order to prevent or delay micro- and macrovascular complications. There has been an impressive improvement in the treatment of diabetes with higher standards of care, advances in insulin therapy and new medications; however, patients still need to receive a life-long therapy and too many patients develop complications, and face reduced life quality and expectancy. Therefore, the quest for cure still remains.

Much effort has been made to provide a curative therapy for both type 1 and type 2 diabetes by reconstituting endogenous insulin production with the replenishment of the insulin-producing pancreatic beta cells. Currently, the only forms of clinically available beta cell replacement are whole-organ pancreas transplantation and pancreatic islet transplantation. Whole-organ pancreas transplantation has three specific categories: the most commonly performed simultaneous pancreas-kidney transplantation (SPK), pancreas after kidney transplantation (PAK), and pancreas transplant alone (PTA). The

progress in the past years has been steady and the outcomes with pancreas transplantation are excellent [9] with satisfying results in terms of glycemic control. Still, the procedure has many drawbacks. The surgery has about 4-5% mortality and patients have to receive a life-long immunosuppressive therapy [9]. In certain cases, however, whole pancreas transplantation could be a reasonable choice of treatment, for instance in patients with end-stage renal disease, or in patients with extreme lability of diabetes [10, 11].

Islet transplantation has been in the focus of attention since the late 1970's. It is a minimally invasive procedure, complications are rare, and in recent years rapid advances have been made in transplant technology. The goal of the transplantation is to infuse enough beta cells in form of islets to control blood glucose levels without insulin injections. The islets are isolated from cadaver pancreases by enzymatic digestion and mechanical stimuli. Isolated islets are then injected into the portal vein system to the liver with a catheter through a small surgical incision on the abdomen. Transplanted islets release insulin and ensure an almost physiologic glycemic control, however, long-term follow-up on islet recipients showed that graft function decreases over time. Moreover, patients have to face a life-long immunosuppressive therapy. Beta cell failure after islet transplant is not fully understood, rejection as well as the deleterious effects of immunosuppressants, and suboptimal location of islet grafts may play role in the process. The landmark study in the islet transplantation field, the so-called Edmonton trial described a new protocol that uses a much larger amount of islet cells and a different type of immunosuppressive therapy. Scientists from Edmonton, Canada reported that seven out of seven patients who received islet cell transplants no longer needed to take insulin, and their blood glucose concentrations were normal a year after surgery [12]. The five year follow-up study on sixty-five patients has been published, and the results are still promising concerning to the control of blood glucose levels, but only 10% of the patients remained totally insulin-independent at 5 years [13]. These findings point to the need for further progress in improving islet engraftment, preserving islet function, and reducing toxic immunosuppression.

The application of this treatment is restricted by the very limited availability of donor tissue. Nowadays, due to the limited supply of cadaveric pancreases, the main

indication for islet transplantation is problematic hypoglycemia. Islet cells are obtained from cadavers and at least two cadavers are needed per transplant. Furthermore, as with any transplant, the islet cells must be immunologically compatible and freshly obtained. These requirements are difficult to meet, and the list of patients in wait for transplants far exceeds the available donor tissue.

The shortage of donor organs spurs the research into alternative means of generating beta cells. Essentially, a limitless supply of competent insulin-producing beta cells would be desirable, and cell-based therapies for both type 1 and type 2 diabetes are heavily investigated. Differentiated beta cells are difficult to culture and expand, whereas, stem or progenitor cells hold the capacity to multiply in culture and they are multipotent, in a sense that they are capable of forming cells from more than one germ layer. Presently, it is not clear whether it will be desirable to produce only beta cells for diabetes therapy or whether other types of pancreatic islet cells are also necessary. Theoretically, insulin-secreting beta cells that are derived *in vitro* from stem or precursor cells under experimental conditions could be transplanted into patients. Obviously, researchers have encountered many obstacles during the procedure of stem cell differentiation and the road to stem cell therapy will be long. However, this field of research holds the promise for a future curative therapy for diabetes.

Clearly, the understanding of beta cell development, and beta cell biology with detailed mechanisms of insulin transcription, translation and secretion is needed in order to provide better treatment and hopefully, one day, a cure for patients with diabetes. Over the past few decades, beta cells have been studied extensively. Most research has focused on the development of the pancreatic beta cells, and on the regulation and molecular biology of insulin synthesis and release. Consequently, many features of beta cell biology are now better understood, and a detailed description of the current knowledge is provided below. However, there are many aspects of beta cell biology yet to be described, and the current thesis is aimed at investigating the natural course of beta cell maturation and phenotypic changes and impairment in diabetic conditions.

1.1 Beta cell generation

A beta cell is defined by its ability to store large amounts of insulin and to secrete it in a regulated manner in response to a demand, such as glucose stimulation [14]. There are different approaches to obtain functional, insulin-producing cells from various types of progenitor cells. The possible tissue sources for generating beta cells include (1) fetal tissue {(a) human embryonic stem cells [15, 16], and (b) umbilical cord blood for mesenchymal stromal cells [17, 18]}, and (2) adult tissue {(c) induced pluripotent stem cells from human somatic cells [18], (d) bone marrow-derived mesenchymal stromal cells [19], (e) organ specific stem or progenitor cells such as oval cells [20], (f) duct epithelial cells [21], (g) acinar cells [22] and (h) liver cells [23, 24]}. Early attempts to generate beta cells directly from embryonic stem cells or other cell sources were successful in producing insulin containing cells [25-27], however, these cells were not true beta cells as they failed to secrete insulin in a regulated manner and they did not contain proinsulin-derived C-peptide [28, 29]. Later, it has become evident, that most effective protocols for the derivation of cells that express insulin recapitulate normal embryonic development, and such protocols using human embryonic stem cells yielded a cell population with more beta cell like characteristics [15]. Of note, these *in vitro* derived cells still fail to function like normal beta cells, and further differentiation is necessary *in vivo* (after implantation of beta-like cells into mice) in order to achieve a more mature beta cell phenotype [16].

The precise knowledge of transcription factors that regulate the development of the embryonic pancreas from the endoderm germ layer into a fully mature pancreas with endocrine and exocrine functions became essential. From mouse models we know that there are four distinct phases of beta cell generation [30]. The primary endocrine cell phase (before embryonic day 13 in mice) during which a few endocrine cells are generated, which are mostly glucagon positive, with few insulin positive cells, that contain low levels of insulin, co-express glucagon, and lack mature beta cell markers, including transcription factors Nkx6.1, MafA and Pdx1 (detailed description of beta cell transcription factors is provided below) [31]. The next phase is called secondary transition (embryonic days 13-18 in mice), during which beta cell neogenesis from

ductal epithelium (tubular progentiors) accelerates dramatically, and the newly formed cells are more similar to mature beta cells. The process of differentiation of progenitor cells down the endocrine linage is initiated by the transient expression of transcription factor Ngn3 [32]. Simultaneously, exocrine acinar cells start to differentiate, and the progenitor cells became restricted to the ducts. The third phase of beta cell formation starts shortly before birth and lasts through the first few weeks of postnatal life [33]. This period is characterized by a significant expansion in beta cells due to proliferation. A similar expansion also occurs in human infants [34]. The final phase of beta cell generation occurs in adulthood, when replacement or expansion of beta cell mass happens in response to metabolic needs. This period is not well characterized, as signals that drive the process are not well known and the sources of new beta cells remained unidentified. Latest research suggests that in mice, adult beta cell regeneration mostly depends on proliferation of preexisting beta cells rather than neogenesis; whereas in humans, neogenesis from pancreatic ductal epithelium and beta cell proliferation probably contributes equally to the regeneration process [35].

Table 1. Transcription factors involved in endocrine pancreas development and implicated in diabetes.

Gene/protein affected	Phenotype
Monogenic diabetes	
Hnf4α	MODY1
Hnf1α	MODY3
Pdx1	MODY4
Hnf1β	MODY5
NeuroD1	MODY6
Polygenic contribution to diabetes	
Pax4	Late-onset diabetes
Isl1	Late-onset diabetes
Ngn3	Late-onset diabetes

Several transcription factors are now recognized to be critical regulators of pancreatic development based on mouse models in which specific genes have been deleted (gene

knock-outs) or inhibited. Remarkably, almost without exception, disruption of these genes has resulted in phenotypes of impaired development of the pancreas and consequent diabetes. Furthermore, lessons learned from the gene knock-outs in mice have been used to successfully identify mutations in several of the corresponding orthologous genes in individuals with familial monogenic type 2 diabetes (Table 1). Detailed description of some of the most crucial transcription factors is given below and a simplified transcription factor cascade is depicted in Figure 1., based on recent reviews [36]. Expression patterns of transcription factors limit the boundaries of the developing pancreas and determine the differentiation programs of individual cell lineages. Importantly, transcription factors appear to serve dual functions in determining early cellular development and later in maintaining the phenotype of terminally differentiated cells.

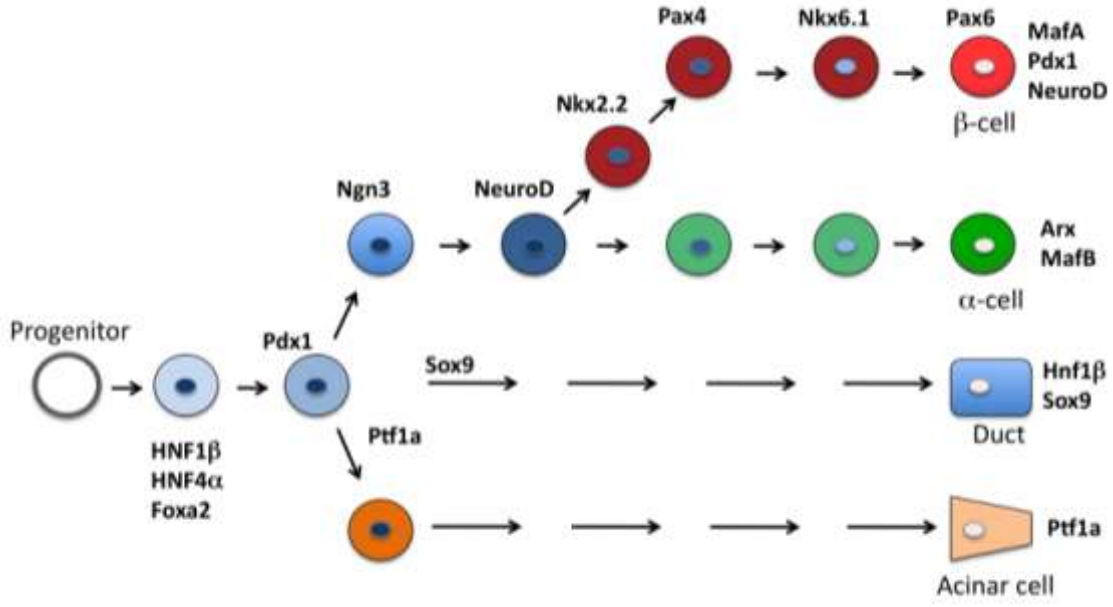

Figure 1. A simplified model of the transcription factor cascade during endocrine differentiation in the developing pancreas. Arx, aristaless related homeobox; Foxa2, forkhead box A2; Hnf1β, and 4α, hepatic nuclear factor 1β, and 4α; MafA, and B, musculoaponeurotic fibrosarcoma oncogene homolog A and B; NeuroD1, neurogenic differentiation 1; Ngn3, neurogenin 3; Nkx2.2, and 6.1, NK2 homeobox 2 and NK6 homeobox 1; Pax4, and 6, paired box 4, and 6; Pdx1, pancreatic and duodenal homeobox 1; Ptf1a, pancreas specific transcription factor 1a; Sox9: sex determining region Y box 9. The figure is a modified version of the model by German [36].

1.1.1. Pdx1

The pancreatic duodenal homeobox gene-1 (Pdx1) is a master regulator of both pancreatic development and the differentiation of progenitor cells into the beta cell phenotype [37]. Because Pdx1 is a pancreas-specific homeoprotein, it was cloned and identified as a beta and delta cell specific regulatory factor for transcriptional expression of insulin and somatostatin genes and has subsequently been shown to regulate the expression of other islet-specific genes, including Glut2 [38], and glucokinase [39]. In the differentiated beta cell, Pdx1 is a glucose-responsive regulator of insulin gene expression [40, 41]. The function of Pdx1 in response to glucose is regulated by both its phosphorylation [42] and nuclear translocation [43].

The patterns of expression of Pdx1 in the developing pancreas are maintained throughout development and provide both spatial and temporal contributions to the commitment of endoderm to a pancreatic phenotype. Based on mouse models, Pdx1 expression is first detected in murine cells in a narrow band of foregut endoderm, then is seen in both ventral and dorsal pancreatic buds, later Pdx1 is expressed throughout the developing ductal tree. As the exocrine pancreas appears and the islets begin to form into the hormone-producing cells, Pdx1 expression shifts to the endocrine compartment [44]. During the later stages of islet development, the expression of Pdx1 becomes mostly restricted to the mature beta cells of the endocrine pancreas [44]. In the adult pancreas, subpopulations of somatostatin-producing and pancreatic polypeptide-producing cells also express Pdx-1 [44].

Targeted disruption of the Pdx1 gene in mice results in agenesis of the pancreas [45]. Notably, a child born without a pancreas (pancreatic agenesis) was homozygous for an inactivating mutation in Pdx1 [46], underscoring the importance of this transcription factor in the development of the human, as well as the mouse, pancreas.

1.1.2. Ngn3, Notch signaling, and Hes

Notch signaling is an important factor in the somatogenesis of the vertebrate embryo [47], and there is evidence of Notch signaling in the developing pancreas, suggesting a primary role for governing cell fate [48, 49].

The basic concept of Notch signaling relies on lateral inhibition [47]. Percursor cells express extracellular ligands, such as Jagged, that activate Notch receptors on adjacent cells, and downstream signaling results in expression of Hes genes. The Hes genes encode basic helix-loop-helix (bHLH) factors that function to repress Ngn3 and other target genes, therefore prevents the differentiation of these cells adjacent to the original differentiating cell. In essence, the lateral inhibition model provides a mechanism by which the cell that will differentiate into the endocrine lineage (Ngn3 positive) inhibits its neighboring cells, forcing them to adopt a non-endocrine fate.

In the generation of the pancreas, Ngn3 is required for the development of all endocrine cell lineages [49] and has been designated as a marker of islet precursor cells. Ngn3 promoter contains binding sites for and is activated by upstream factors Hnf3β, Hnf1β, and Hnf6 (hepatic nuclear factors). Expression of Ngn3 starts around pancreatic bud formation from the foregut, and it peaks during the major wave of endocrine cell genesis. By birth, Ngn3 expression is greatly diminished and it is not detectable in the adult pancreas.

Significantly, mice homozygous for an Ngn3-null mutation failed to generate any endocrine cells or putative endocrine precursors during development [49]. These observations led to the assumption that Ngn3 expression is a functional marker of an islet cell precursor population in the developing pancreas. More recently, a case of permanent neonatal diabetes and congenital malabsorptive diarrhea secondary to enteroendocrine cell dysgenesis was reported, where severe deficiency of Ngn3 was confirmed due to biallelic mutations [50]. This finding also confirms the essential role of Ngn3 in humans.

1.1.3. NeuroD1/Beta2

The basic helix-loop-helix transcription factor NeuroD1 (also known as Beta 2) is a key regulator of both insulin gene transcription in pancreatic beta cells and the terminal differentiation of neurons, hence its name [51, 52]. NeuroD1 expression initiates slightly later than Ngn3 during pancreatic development. First, the expression appears within and in proximity to ductal epithelium, and later it becomes restricted to islets. NeuroD1 heterodimerizes with ubiquitously expressed E47 to regulate transcription of

the insulin gene and other beta cell-specific genes. Expression of the NeuroD1 gene is activated by Ngn3 [53].

Mice homozygous for a targeted disruption of the NeuroD1 gene survive to birth, but die within 3–5 days postpartum of severe hyperglycemia. The islets of these mice are dysmorphic and have markedly diminished numbers of endocrine cells arranged in streaks and irregular aggregates and reduced numbers of beta cells [54].

1.1.4. Pax4 and Pax6

Pax4 and Pax6 are paired box homeoproteins, whose expression is restricted to the endocrine pancreas and the central nervous system [55, 56].

Pax4 is expressed in the developing pancreas buds, and by the time of birth, expression is restricted to beta cells. Targeted disruption of Pax4 in mice results in a striking pancreatic phenotype; there is a virtual absence of beta and delta cells, but alpha cells are increased [55]. The Pax4-null mice survive birth, but die within 3 days of hyperglycemia and dehydration. Pdx-1 expression in Pax4-null pancreas is absent, a finding consistent with the absence of differentiated beta cells. Thus, Pax4 functions early in the development of islet cells to promote the differentiation of beta and delta cells.

Pax6 is expressed early in the epithelium of the developing pancreas, and later, contrary to Pax4, it is expressed widely in differentiated endocrine cells, including alpha, beta, delta and PP cells. A spontaneous mutation of the Pax6 gene in mice results in abnormal organization of the islets, with decreased number of alpha, beta, delta and PP cells, and reduced glucagon and insulin production [57]. Knock-out Pax6 nullizygous mice die within minutes of birth, and these animals fail to form islets [58].

These mouse models of gene knock-outs implicate both Pax4 and Pax6 as key regulators of the terminal steps in cellular differentiation of the endocrine pancreas.

1.1.5. Nkx2.2 and Nkx6.1

Members of the Nkx family of homeoprotein transcription factors, Nkx2.2 and Nkx6.1, are regulators of the differentiation of pancreatic endocrine cells [59]. Nkx2.2 is

expressed early in developing pancreatic buds and is later restricted to alpha, beta, and PP cells of islets. Nkx6.1 is expressed primarily in beta cells of adult islets.

Disruption of either the Nkx2.2 or Nkx6.1 gene in mice results in death soon after birth due to severe diabetes [60]. The pancreata of Nkx2.2 and Nkx6.1 knockout mice have no insulin-producing cells, and glucagon producing cells are diminished, although the exocrine pancreas is histologically normal [60, 61].

1.1.6. MafA and MafB

MafA and MafB are members of a large family of basic leucine zipper transcription factors. In the adult pancreas, MafA expression is restricted to mature beta cells [62], whereas MafB is typical of the mature alpha cells. Interestingly, during embryogenesis, MafB is expressed early in pancreatic endocrine cells (both in glucagon-positive and insulin-positive cells), where it regulates the expression of key factors that are required for the production of mature alpha and beta cells [63]. MafA is produced only in insulin-positive cells within the pancreas, with production first detected around secondary transition, during the principal wave of beta cell production [64]. At a late stage of differentiation, even after birth, beta cells undergo a switch from MafB to MafA, concomitant with the acquisition of glucose sensing and other characteristics of the mature beta cell [65].

MafA is an essential, glucose-responsive transcriptional activator in adult beta cells, due in part to regulating genes associated with cell identity, including insulin, Glut2 and prohormone convertase PC1/3. Adult islet MafA levels appear to be a sensitive barometer of beta cell function, since many key metabolic and cellular effectors, such as glucose [62], fatty acids [66], and insulin [67], greatly impact MafA expression.

It is noteworthy, that MafA null mutant mice have overall normal pancreatic development, but develop diabetes later in life [68]. This finding in accordance with the notion that MafA is not necessary for beta cell specification, but it plays a role in beta cell function in mature animals.

In contrast to MafA knock-outs, in MafB null mutants, there is delayed development of early insulin-positive and glucagon-positive cells, and a 50% reduction

in insulin-positive and glucagon-positive cells overall, with an abundance of hormone-negative cells that appear otherwise to be of the endocrine lineage. Thus, MafB appears to be a key regulator of alpha and beta cell formation [63].

1.2. Regulation of insulin gene expression

Increases in blood glucose levels stimulate insulin gene transcription and insulin secretion. Insulin gene transcription is mainly controlled by a 340 bp promoter region upstream of the transcription start site of the insulin gene. The insulin promoter region comprises cis-acting sequence motifs, which serve as binding sites for both beta cell-specific and ubiquitous transcription factors [69]. Much of the glucose responsiveness inherent to the insulin promoter is conferred by the A3, E1 and C1 sites, which are bound by the transcription factors Pdx1 [70], NeuroD1 [51] and MafA [71] respectively. These three transcription factors act in a coordinated and synergistic manner to stimulate insulin gene expression in response to increased glucose levels [69, 72] (Figure 2). Compared with humans, rodents (rat and mouse) have two insulin genes, of which the insulin II gene and its promoter is mostly similar to that of humans [73]. On the basis of the analysis of transcription factor-binding sites and spacing of the cis-regulatory elements, there are significant differences between rodent and human insulin gene promoters. However, a detailed comparative analysis of the insulin promoters from various species suggest that the all three regulatory elements (A3, E1 and C1), important for glucose regulation of insulin gene expression, are conserved between species with the A-box (A3) to which Pdx1 binds being the most highly conserved one [74].

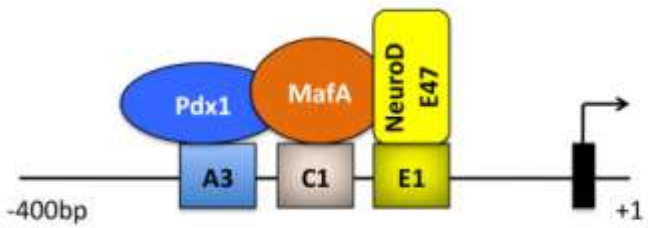

Figure 2. Insulin gene promoter is activated by the glucose-responsive, synergistic action of transcription factors Pdx1, MafA and NeuroD1.

Although Pdx1, NeuroD1 and MafA have been shown to be crucial for glucose regulation of insulin gene transcription, the exact mechanisms by which glucose modulates the function of these transcription factors remains to be established. In the case of Pdx1, it has been proposed that glucose modulates the function of Pdx1 by regulating the localization and DNA-binding activity of Pdx1 [75]. Several lines of evidence suggest that also NeuroD1 changes its localization in response to changing glucose levels. Under normal glucose conditions, NeuroD1 is mainly cytosolic, and exposure to high glucose causes its translocation into the nucleus [76]. MafA expression itself requires high glucose. Although expression of MafA is low under normal glucose conditions, MafA transcription increases drastically in response to high glucose [71, 77].

1.3. Glucose-stimulated insulin secretion

Beta cells are highly specialized cells that maintain blood glucose levels in the physiologically relevant range by rapid and precise changes in insulin secretion in response to nutrient secretagogues, particularly glucose [78]. Specialised metabolic pathways ensure the coupling of external nutrient stimuli to insulin secretion in adult beta cells [79] (Figure 3). During glycolysis in the adult beta cell, glucose is metabolized to pyruvate and the glucose-derived carbons are then oxidized in the mitochondria [80]. The very low expression of lactate dehydrogenase and monocarboxylate transporters results in negligible lactate production [81]. Glycolysis-derived cytosolic NADH is reoxidized in mitochondrial membrane shuttles, which include glycerol–phosphate, and the malate–aspartate, pyruvate–citrate and pyruvate–malate shuttles [82, 83]. In addition, beta cells show strong pyruvate carboxylase activity [83] that facilitates the flux of pyruvate into the pyruvate–malate and pyruvate–citrate shuttles. Glucose oxidation promotes the production of ATP, the key metabolic coupling factor for glucose-stimulated insulin secretion (GSIS). The closing of ATP-dependent K^+ channels results in depolarisation, opening of voltage-activated Ca^{2+} channels and subsequent exocytosis of the insulin granules. GSIS can also be stimulated by glucose metabolism, which exerts its effect independently of the ATP-

dependent K^+ channels, working through as yet undefined mechanisms [84]. In addition, there are other metabolic and neurohormonal amplifying pathways in beta cells that can potentiate insulin release.

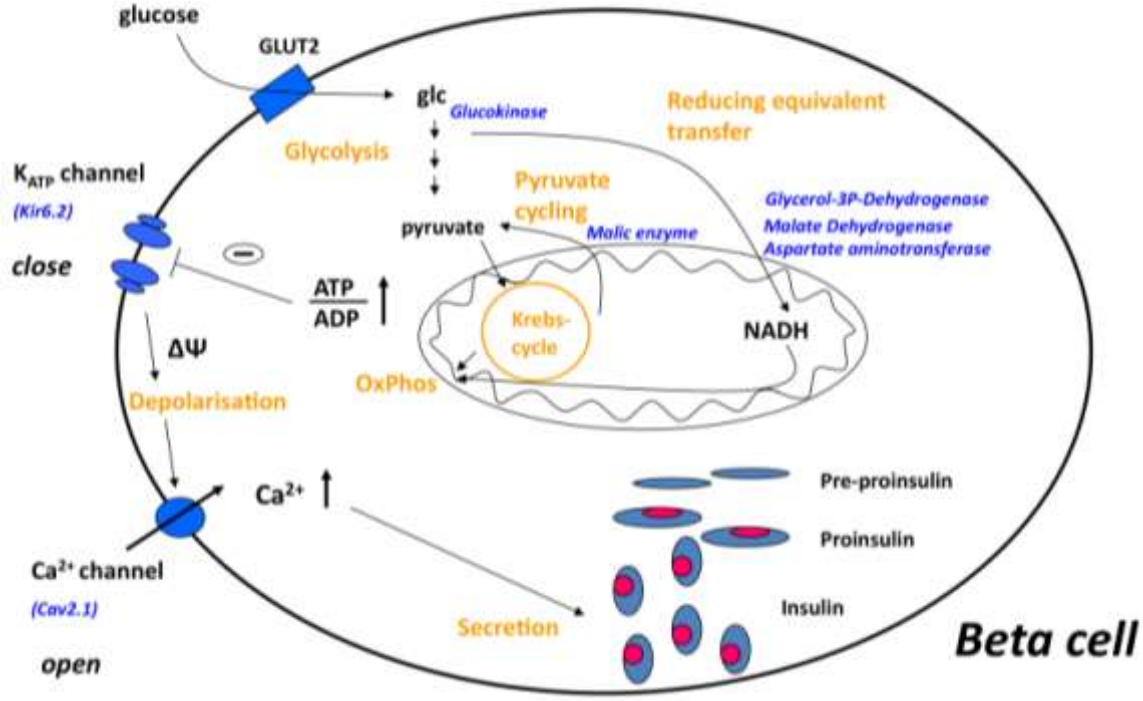

Figure 3. The current concept of glucose-stimulated insulin secretion in pancreatic beta cells, described in details in the text. Abbreviations: glc, glucose; OxPhos, oxidative phosphorylation; ΔΨ, membrane depolarisation.

1.4. Immature glucose-stimulated insulin secretion from fetal/neonatal islets

Although the metabolism of adult beta cells has been characterised extensively, that of fetal/neonatal beta cells is less well understood. Both human and rodent fetal and rodent neonatal islets [85-90] lack glucose responsiveness despite adequate insulin reserves [91], but secrete insulin in response to amino acids, particularly leucine [92, 93]. When newborn (P1) Sprague–Dawley rat islets were tested in a dose–response experiment using different secretagogues, insulin secretion was impaired in response to all stimuli; insulin secretion in response to different secretagogues started to rise at 1

week of age [93, 94]. The insulin secretion in response to glucose of perifused islets of different-aged rats [95] was small compared with adult islets at postnatal day (P) 7, with a slight increment at P14 and a doubling at P21; however, even at P21, the response was less than half of that of adult islets (see Figure 4, adapted from Bliss et Sharp). A variety of mechanisms focusing on individual genes have been proposed to account for immature insulin secretion, including lack of inhibition of fatty acid oxidation by glucose metabolism [95], lack of increased ATP content in response to glucose stimulation [96], and low levels and activity of mitochondrial glycerol-3-phosphate dehydrogenase and mitochondrial malate dehydrogenase, both NADH shuttle enzymes [97].

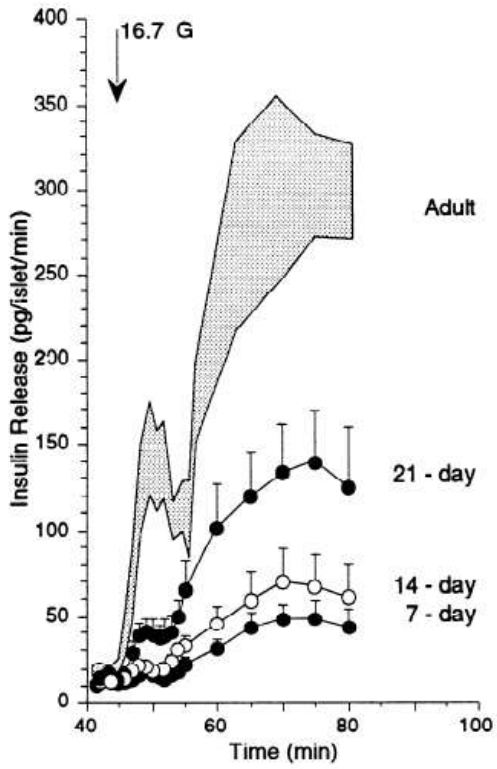

Figure 4. Insulin release from islets from rats of different ages in response to 16.7 mM glucose (G). Adapted from Bliss et Sharp, 1992 [95].

My primary objective was to study the mechanisms responsible for the immaturity of glucose-stimulated insulin secretion in rat beta cells by comparing gene expressional profiles of neonatal (P1) and adult beta cells. Beta cell immaturity proved to be a complex phenomenon, involving lower expression of many key metabolic enzymes

[98]. During the first weeks of postnatal life, expression of these genes increased in islets as insulin secretory responsiveness matured. Thus, the generalised low expression of key metabolic enzymes, may account for the poor glucose-responsiveness of neonatal beta cells [98].

1.5. Role of transcription factors in the maturation of beta cells

With many genes differentially expressed in the neonatal beta cell, a possible role of differential expression of key beta cell-enriched transcription factors must be considered. Pdx1 and MafA are most likely to be involved in the maturation process, because their expression is turned on after that of insulin during development [65]. Target genes of both Pdx1 [99] and MafA [100] have been reported, with many exocytotic genes regulated by Pdx1, and genes of insulin synthesis and secretion regulated by MafA. As discussed before, Pdx1 is crucial in pancreas formation [45, 101] and beta cell differentiation [38, 102, 103]. A role for MafA in maturation is supported by: (1) several of its putative target genes (insulin 2, prohormone convertase 1/3 (Pcsk1) and glucagon-like peptide 1 receptor (Glp1r) [100]) have reduced expression in neonatal beta cells [98]; (2) the switch from MafB+MafA–Insulin+ to MafB–MafA+Insulin+ cell state is a late stage event in beta cell differentiation [65]; and (3) the MafA-deficient mice appear normal at birth but later develop impaired secretagogue-stimulated insulin secretion and glucose intolerance [68]. In addition, insulin-expressing MafB+ cells derived from human embryonic stem cells lacked glucose-stimulated insulin secretion *in vitro* [15, 104] but acquired both glucose responsiveness and MafA production after transplantation into mice [16].

My secondary objective was to assess whether maturation of neonatal beta cells into glucose-responsive, mature insulin secreting cells is regulated by Pdx1 and/or MafA, by overexpressing these factors in neonatal islets.

1.6. Role of MafA in mature beta cell function

MafA is unique to the beta cells and appears to be essential for maturation [105], rather than being a developmental factor and playing a role in cell commitment [68]. MafA has been shown to be critical for adult beta cell function, because mice lacking MafA become glucose intolerant and develop diabetes, although insulin content of islets *per se* is not affected [68]. In addition, MafA knock-down experiments in cell lines suggested that MafA has a function beyond regulating insulin transcription. Several beta cell important genes were progressively down-regulated with the loss of functional MafA in cell lines [100]. The confirmation of these putative MafA target genes, including Glut2 and Pcsk1, is awaited in primary cells and in *in vivo* experiments.

There are natural and pathological conditions where beta cells are "dysfunctional" and do not respond to glucose with insulin secretion.

One such example is the previously discussed neonatal period, which is not really impairment but rather an immature state, characterized by the lack of glucose-responsive insulin secretion. In my secondary objective, we studied the effects of enhanced MafA expression in neonatal beta cells as we speculated that it could drive the acquisition of glucose-responsive insulin secretion [105].

Another dysfunctional beta cell state develops when adult beta cells are exposed to chronic glucotoxicity. MafA levels were reported to be lower and GSIS is impaired in glucotoxic beta cells, along with the down-regulation of several important genes of the beta cell metabolism [106]. Normalization of glucose homeostasis reconstitutes MafA expression and restore glucose-stimulated insulin secretion [106].

My tertiary objective was to establish the causal relationship between MafA expression and functional integrity of adult beta cell; and to study MafA targets besides insulin with the inhibition of MafA function in adult rat beta cells.

My final objective was to investigate the ability of MafA over-expression in a diabetic model to restore glucose-stimulated insulin secretion.

2. Aims

The general objective of my PhD thesis is to provide a molecular description of the evolving beta cell phenotype; from the immature neonatal period, through the mature, glucose-responsive stage, to the development of glucotoxicity and a dysfunctional beta cell phenotype (Figure 5).

AIM 1. To characterize the underlying mechanisms responsible for the immature, glucose-unresponsive phenotype of neonatal beta cells.

Postulating that the mechanisms responsible for neonatal beta cell immaturity are complex, we used microarray analysis to compare the gene expression profile of neonatal (postnatal day P1) and adult beta cells excised by laser-capture microdissection. Sets of genes with differential expression were confirmed with quantitative realtime PCR (qPCR) and immunostaining. Moreover, expression pattern of these genes were followed during the first weeks of postnatal life, as the insulin secretion of the islets matures.

AIM 2. To assess whether maturation of neonatal beta cells into glucose-responsive, mature insulin secreting cells is regulated by transcription factor MafA and/or Pdx1.

We characterized the expression of MafA and Pdx1 and that of other key beta cell genes during the first 4 weeks of postnatal life. We hypothesized that enhancing the expression levels of these factors in neonatal beta cells could drive the acquisition of glucose-responsive insulin secretion.

AIM 3. To characterize the importance of MafA in regulating beta cell function in adult rat islets.

We hypothesized that there is a causal relationship between MafA expression and functional integrity of adult beta cell; and we studied MafA targets besides insulin with the inhibition of MafA function in adult rat beta cells.

AIM 4. To test the role of MafA in dysfunctional beta cells.

We investigate the ability of MafA overexpression in a diabetic model to restore glucose-stimulated insulin secretion.

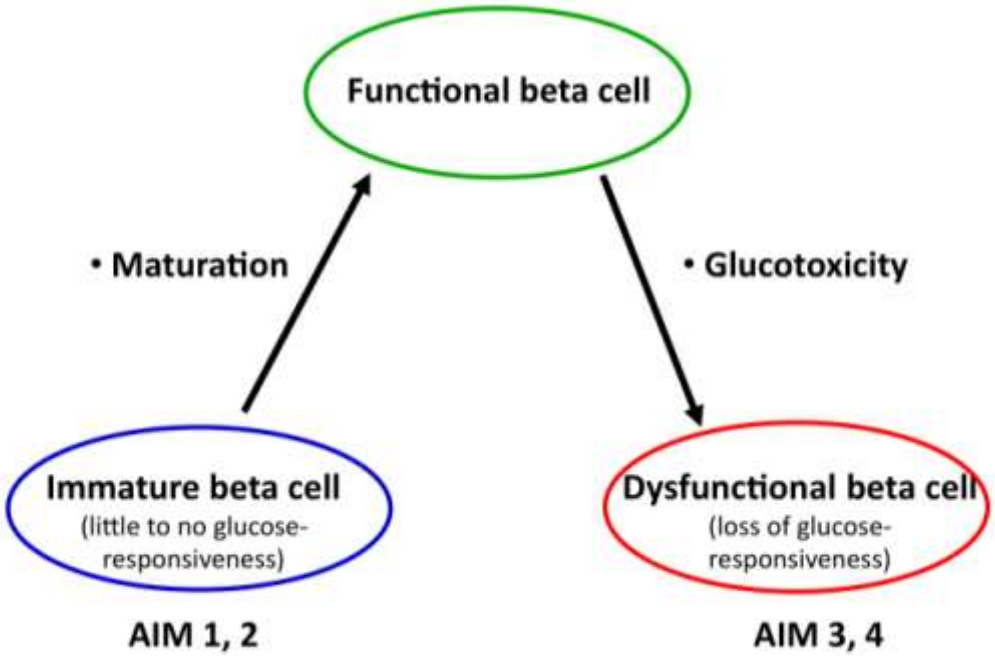

Figure 5. The paradigm of beta cell. The aims of the current thesis are described in the text.

3. Methods

3.1. Animals

Adult Sprague-Dawley rats (male and female), female Goto-Kakizaki (GK) rats (12 weeks old) and age-, gender-matched Wistar-Kyoto rats were purchased from Taconic Farms (Germantown, NY). In order to have P1 animals (P0 as day of birth) pregnant animals were purchased and checked daily until delivery. Animals were kept under conventional conditions in climatized rooms, with free access to tap water and standard pelleted food. Fed glucose levels and body weight of the adult animals were monitored weekly. All procedures were approved by the Joslin Institutional Animal Care and Use Committee. Neonatal pups were nursed until they were killed at P1, P2, P7, P9, P11, P13, P15, P21 or P28. For immunostaining, excised pancreases (n=3 for P2, P7 and adult) were fixed by immersion in 4% paraformaldehyde, and stored in PBS until paraffin embedding and sectioning. For laser capture microdissection (LCM), excised pancreas immersed in TissueTek OCT medium (VWR Scientific Products, San Diego, CA) was rapidly frozen in chilled isobutane and immediately stored at -80°C.

Adult male rats were anaesthetized with Nembutal for islet isolation by collagenase digestion according to the method described by Gotoh [107] with rodent Liberase RI (Roche, Indianapolis, IN). Islets from two adults were pooled as one sample. For neonatal islets, rats were decapitated and pancreases from a litter (10–12 pups) were pooled, finely minced and digested with Liberase solution. Adult and neonatal islets were purified by gradient separation using Histopaque-1077 (Sigma, St Louis, MO), and after overnight culture were handpicked under a stereomicroscope to ensure high purity. Islets were then further processed for *in vitro* experiments, or were put in Buffer RLT reagent (Qiagen, Germantown, MD) for RNA extraction and stored at -80°C.

3.2. Laser-capture microdissection

Frozen sections (8 μm) on uncoated slides were quickly processed for LCM: first in 70% ethanol for 10 sec, haematoxylin for 40 sec, dehydrated for 10 sec each in 70%

and 95% ethanol, stained with eosin for 10 sec and finally dehydrated for 3 min in 100% ethanol followed by xylene for a minimum of 2 min. Once sections were air-dried, beta cell-enriched cores of islets were microdissected using a PixCell II LCM system (Arcturus, Mountain View, CA). For each sample, at least 4,000 'hits' from two to five islets per section were excised from 10–20 sections. Total RNA from each sample was extracted using a modification of the RNA microisolation protocol as described [108-110]. T7-based RNA amplification was carried out using the RiboAmp kit (Arcturus) according to the manufacturer's recommendations. To obtain enough amplified RNA for microarray, a second round of RNA amplification was performed [111].

3.3. Microarray hybridization

Biotinylated cDNA (BioArray HighYield RNATranscript Labeling kit; Enzo Life Sciences, Farmingdale, NY, USA) of four adult and four neonatal beta cell-enriched samples were run on Affymetrix GeneChip Rat Genome U34A (Affymetrix, Santa Clara, CA; adult by MGH Cancer Center DNA Microarray Core Facility; neonatal by Joslin DERC Genomic Core). Data have been deposited in a MIAME-compliant database (GEO series accession number GSE24790). Analysis was performed using a DNA-Chip Analyzer (dChip; Harvard School of Public Health, Boston, MA, USA; www.dchip.org). This software uses the model-based expression analysis, which allows probe-level analysis on multiple arrays. As the LCM RNA samples were amplified before labeling, we followed the manufacturer's recommendation of using 50% masked data for analysis. Principal component analysis for sample classification with dChip software revealed that one of the adult samples was an outlier; therefore this array was excluded from further analysis. Data from multiple arrays are normalized to a common baseline array having the median overall brightness. Thus, by pooling information across multiple arrays, it is possible to assess the standard errors for the expression indices and to calculate confidence intervals for fold changes. Lower confidence boundary (LCB) and p values (<0.050) were used to assess differentially expressed genes.

Differentially expressed genes were classified into functionally related clusters using Database for Annotation, Visualization and Integrated Discovery (DAVID) software [112, 113]. The functional annotation tool of DAVID using high-classification stringency identified enriched functionally related gene groups; the enrichment p values are reported.

3.4. Adenovirus infection

Using the AdEasy system with a CMV promoter (Stratagene, La Jolla, CA) we generated recombinant adenoviruses expressing: AdMafA, expressing the full human MafA coding sequence; AdDN-MafA, containing a dominant negative mutant of MafA lacking the N-terminal transcriptional activation domain [100]; and AdNeuroD1, expressing the hamster NeuroD1 coding sequence; an adenovirus encoding green fluorescent protein alone (AdGFP) served as control. Adenovirus containing the coding sequence for Pdx1 (AdPdx1) was a kind gift from Dr. D. Melton [22]. All adenoviruses were amplified in 293Ad cells purchased from Stratagene (La Jolla, CA), and were purified with the Vivapure AdenoPACK 100 kit (Sartorius Stedim Biotech, Gottingen, Germany). Virus titers were established based on quantification of plaque formation.

After overnight culture in RPMI 1640 (11 mM glucose with 10% Fetal Bovine Serum [FBS] and 1% Penicillin-Streptomycin), islets were dispersed to single cells using trypsin (>10000 U/mg, from Sigma, St Louis, MO; final concentration 1 mg/mL) and DNAse (from Roche, Indianapolis, IN; final concentration 60 U/mL). Dispersed islet cell numbers were established and was calculated for the independent virus infections. Dispersed islets cells were incubated with adenoviruses at a multiplicity of infection (MOI) of 1 to 5 for 24 hours at low (5 mM) glucose RPMI 1640 (low 5 mM glucose with 10% FBS and antibiotics) on ultra-low attachment hydrophobic plates (Corning, Corning, NY), which allowed islet cell reaggregation. After the virus incubations, the media was changed to high (20 mM) glucose RPMI 1640 with the same supplements. The switch from low to high glucose concentration was chosen because it enables the strongest activation of important glucose-sensitive beta cell transcription factors including MafA, Pdx1 and NeuroD1.

3.5. RNA extraction from isolated islets and reverse transcription

Total RNA from isolated islets or reaggregated islet samples after various times of culture was extracted using RNeasy Plus Mini Kit (Qiagen, Germantown, MD). After quantification by spectrophotometry, 500 ng total RNA from each sample was used as starting material for cDNA preparation. Reverse transcription was done in 25 mL reaction solution containing 5 mL Superscript buffer, 0.1 M dithiothreitol (DTT), 50 ng random hexanucleotide primers, 10 mM dNTP, 200 units of RNaseOUT, and 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription reactions were incubated for 10 min at 25°C, 60 min at 42°C, and 10 min at 95°C. The resulting cDNA was diluted 1: 2 in nuclease-free water, resulting in 10 ng/mL final concentration and stored at -20°C until analysis.

Table 2. Sequences of oligonucleotide primers used for real-time PCR.

Gene Name	Size (bp)	5' oligonucleotide	3'oligonucleotide
Carnitine palmitoyltransferase 1	295	GAGACACCAACCCCAACATC	GTCTCTGTCCTCCCTTCTCG
Carnitine palmitoyltransferase 2	196	GCAGATGAACCACAACATCC	TTCCAAGCACTTCTGGACAC
Glutamate oxalacetic transferase 1	158	TGACCGGATTCTGACCATG	AGATGTGCTTCTCGTTGACC
Glycerol-3-phosphate dehydrogenase 2	107	TGAGTTTCTGCAGCTGATGA	TTGGAACTCTACGGTCCAAGT
Malate dehydrogenase 1	149	ACTCGTTCCCTGTCGTGATC	TCATGCGGAGGAGAGAAAC
Malic enzyme 1	159	TTAGCCCACAGACTCATGGG	AGGACTTTCTCAGCAGCAGG
Pyruvate carboxylase	167	TTGAAGGATGTGAAGGGCC	ACCTTTCGGATAGTGCCCTC
Pyruvate kinase, L-type	248	AGCCGTGACCCAACTGAG	TCCACATCATCTGCCCAG
Ribosomal protein L32	82	CAATGTGTCCTCTAAGAACCGAAA	CCTGGCGTTGGGATTGG

3.6. Quantitative real-time PCR (qPCR) for confirmation of differential expression of genes

qPCR with SYBR Green detection was performed using the ABI7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with primers (Table 2) designed

using Primer Express (Applied Biosystems). Oligonucleotide specificity was computer tested (BLAST, NCBI), and later confirmed by dissociation curve analysis and resolving the PCR products in agarose gel electrophoresis. Each primer set displayed approximately equal efficiency for amplification of target cDNA. The reactions were set by mixing 10 µl SYBR Green Master Mix (Applied Biosystems) with 1 µl of each 5′ and 3′ oligonucleotides (10 pmol/µl) and 1 µl cDNA sample (10 ng/µl). To exclude genomic DNA contamination, RT-negative samples were run for each cDNA sample. After normalization of the gene of interest to a control ribosomal gene (L32 or S25) [114], the comparative C_T (threshold cycle) method was used to calculate relative gene expression levels. Adenoviral-hMafA mRNA was determined by comparison of two different sets of primers: one recognizing the coding sequence (CDS) of both endogenous (rat) and adenoinfected (human) MafA, and the other recognizing the 3′ untranslated (UTR) sequence of MafA only present in the endogenous transcript.

3.7. Insulin secretion *in vitro*

Insulin secretion of the islet cell aggregates was measured with sequential static incubations 24, 36, 48 and 72 hours after adenoviral infections. Aggregates were preincubated for 1 hour in low (2.8 mM) glucose Krebs-Ringer bicarbonate buffer (KRBH, with 16 mM HEPES and 0.1% Bovine Serum Albumin, pH 7.4) to establish basal conditions. The buffer was then replaced for fresh low glucose KRBH for 1 hour, followed by KRBH containing various reagents stimulating insulin secretion (16.7 mM glucose, low glucose with 10 mM leucine and 4 mM glutamine, low glucose with 10 mM arginine, or low glucose with 30 mM KCl). Media aliquots were collected at the end of the low glucose incubation and after stimulation and stored at -20°C until insulin analysis with Insulin Rat EIA kit (ALPCO Diagnostics, Salem, NH). Aggregate lysates after sonication were assayed for DNA content (CyQUANT kit, Invitrogen, Carlsbad, CA) and insulin content.

3.8. Insulin secretion *in vivo*: intra-peritoneal glucose tolerance test

For intraperitoneal glucose tolerance tests (IPGTT), rats were fasted with free access to

water only, for 8–12 h. Rats were then injected intraperitoneally with 10% glucose solution at a dose of 2 g/kg body weight. Blood glucose readings were performed from the tail vein at 0, 30, 60, 90 and 120 minutes post injection, using OneTouch Ultra blood glucose meter (LifeScan, Milpitas, CA).

3.9. Western Blot analysis

20 μg of total protein extract of sonicated islets were boiled for 5 min in the presence of β-mercaptoethanol and resolved on 10% SDS-PAGE (polyacrylamide gel electrophoresis), transferred to PVDF membranes and probed with either MafA antibody (1:2000, [65]) or HSV antibody (1:5000, Abcam, Cambridge, MA), which detects the HSV-tagged DN-MafA protein. GFP was used as a loading control. Primary antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 (TBST). Membranes were washed in TBST and incubated with either anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA). The blots were visualized with chemiluminescence SuperSignal West Dura reagent (Pierce, Thermo Fisher Scientific, Waltham, MA).

3.10. Immunostaining

Paraffin sections were blocked for endogenous peroxidase, microwaved in 10 mmol/l citrate buffer pH 6.0 for 15 min at 20% power and then incubated overnight at 4°C with anti-pyruvate kinase antibody (1:100 goat-anti rabbit, US Biologicals, Swampscott, MA), then with biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA) 1 hour, with ABC reagent 1 hour and visualized with VIP (Vector VIP substrate kit for peroxidase, Vector Labs). Incubations with anti-rabbit glycerol-3-phosphate dehydrogenase (1:100, the kind gift of M. MacDonald, Department of Pediatrics, University of Wisconsin, WI), donkey biotinylated anti-rabbit IgG (1:400), were followed by streptavidin-conjugated Alexafluor Green (1:400). Sections were double stained for insulin (guinea pig anti-human, 1:200, Linco Research, St Charles, MO) with Texas Red-conjugated Affinipure donkey anti-guinea pig IgG (1:400) as secondary antibody. Images were taken with an Olympus BH2 or, in confocal mode, a

Zeiss 410 or 710 LSM microscope. Sections of different ages were stained and photographed in parallel using the same settings, so the relative intensities reflect the protein levels. For beta cell composition, pancreatic sections double stained with anti-insulin and a cocktail of anti-non-beta cell hormones were imaged by tile scan collection, and then the insulin-positive area of all clusters at least 35 μm diameter were quantified as proportion of total islet area (Table 5).

3.11. Data analysis

For statistical analysis, unpaired Student's t test was used. To see differences among groups, ANOVA was used followed by post hoc analysis (Tukey's). A p value <0.050 was considered statistically significant.

4. Results

Characterization of immature, glucose-unresponsive neonatal beta cells

Microarray analysis revealed different mRNA expression patterns in neonatal compared with adult beta cells

Using dChip analysis on 50% masked probes and the high stringency LCB cutoff of 2 and $p<0.050$, we found 217 genes/ 38 expressed sequence tags (ESTs) higher in neonatal compared with adult beta cell-enriched cores and 345 genes/33 ESTs lower in neonatal; with a false-discovery rate of 4.9%. Hierarchical clustering analysis of differentially expressed genes showed a clear separation between neonatal and adult control samples (Figure 6). Differentially expressed genes ranged widely across biochemical functions, including metabolism, cell cycle, ion channels and cell surface proteins.

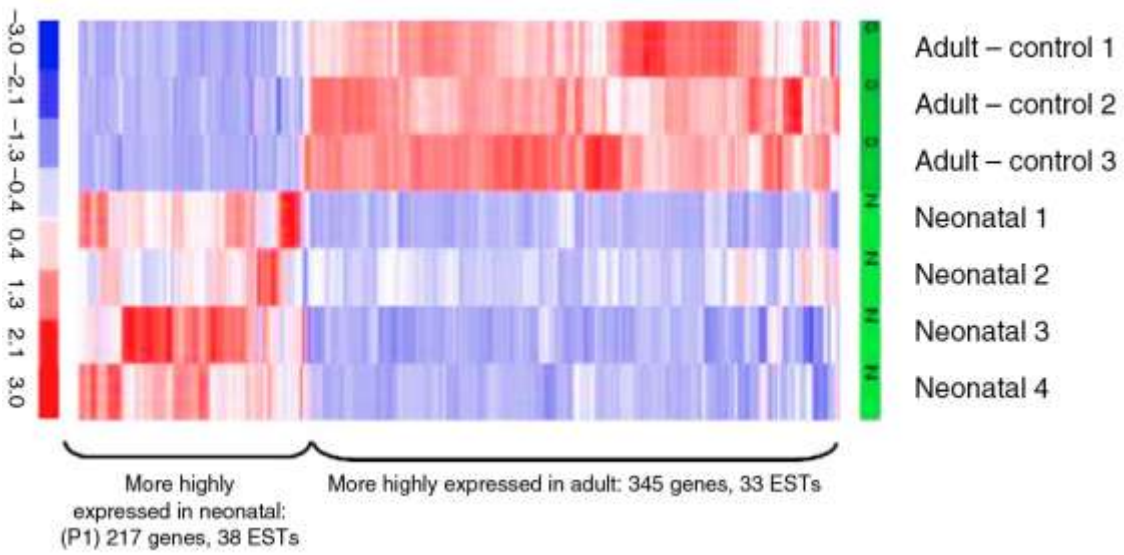

Figure 6. Heat map display of the differentially expressed genes in laser captured enriched beta cells from neonatal (P1) and adult pancreas. dChip analysis with LCB cutoff of 2 and $p<0.050$.

For an unbiased analysis of the differentially expressed genes in neonatal beta cells (LCB>2, $p<0.050$), DAVID functional annotation clustering [112, 113] was used.

The functional annotation clustering algorithm of the DAVID program systematically analyzes gene lists according to gene ontology terms and statistically highlights the most overrepresented (enriched) biological annotation as clusters of biological processes [112]. Here the top 15 clusters for higher and lower expressed genes in the neonatal compared to adult beta cells are presented (Figure 7.). Bars represent enrichment scores, which are calculated as the negative logarithm of the geometric mean of the individual over-representation P value in each cluster. The number of genes and corresponding percent of each biological process relative to a total number of genes in a specific comparison are shown to the right of the bars.

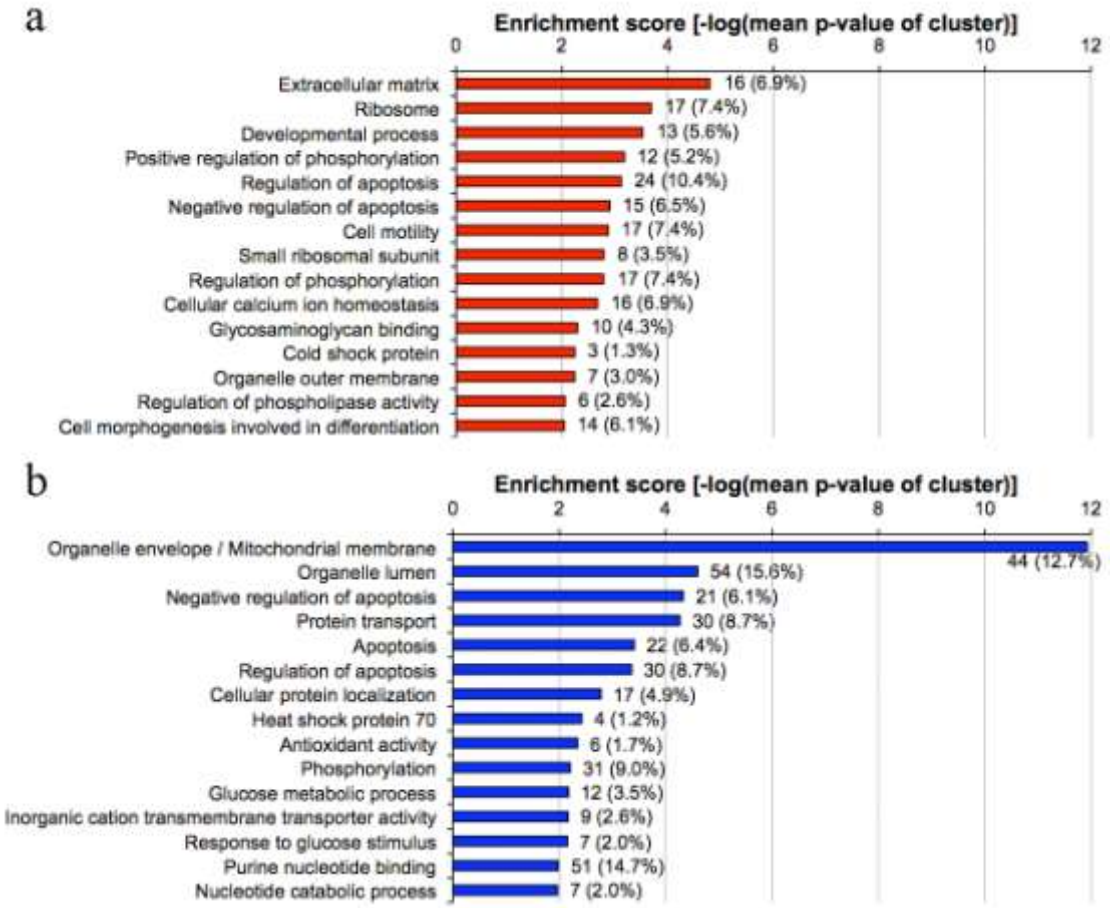

Figure 7. Gene ontology analysis of genes expressed significantly higher (panel a), in red, lower confidence boundary >2) or lower (panel b, in blue, lower confidence boundary <-2) in neonatal versus adult beta cell-enriched islet cores.

The three most highly enriched clusters from the genes expressed higher in the neonatal beta cell samples (extracellular matrix, ribosomes and developmental process) might be expected for newly differentiated tissues. The enrichment score of the organelle/mitochondrial membrane genes (11.92, which is more than double that of other categories) indicates a very strong over-representation. This robust enrichment suggests a systemic lower expression of mitochondrial genes, especially those for the membrane bound proteins and shuttle enzymes. Clusters that might also be expected for immaturity of beta cell function, such as calcium handling and membrane-bound vesicles, had enrichment scores of 1.5 but were only ranked as clusters 30 and 31 respectively; regulation of hormone secretion cluster showed no enrichment (enrichment score 0.67).

In contrast the three most highly enriched clusters for the genes with lower expression in the neonates were organelle/mitochondrial membrane, organelle lumen and negative regulation of apoptosis. Of interest, clusters of regulation of apoptosis/cell death are enriched in both neonatally higher and lower differentially expressed genes, even though the same genes are not represented in both. The frequency of apoptosis is about 3-fold higher in neonatal beta cells than adults [115].

Less stringent criteria (LCB cutoff of 1.2 and $p<0.050$) were used to selectively analyze genes important in the beta cell phenotype. The number of genes higher in neonatal samples increased to 363 probe sets for genes/54 ESTs and those with reduced gene expression increased to 685 genes/ 68 ESTs. It is striking that a high percentage of genes in the mitochondrial shuttles and fatty acid metabolism were differentially expressed. We focused on differentially expressed genes of the beta cell-specialized metabolic pathway, grouping them into functional clusters (Table 3 and 4).

Table 3. Expression of metabolic genes in beta cells grouped into functional categories (Glycolysis, Krebs-cycle, Mitochondrial shuttles). Genes of the various pathways are listed with the mean±SEM, fold change (FC), lower confidence boundary (LCB) and p-value as determined with dChip. Where multiple probe sets were on the microarray, the more stringent _at set is listed; if no_at probe set, the set with highest expression is listed. Microarray signals of adult (n=3) and neonatal (n=4) samples. Those with significance ($p<0.050$, fold change of 1.5 or greater, LCB of 1.2, and signal mean difference of at least 100) are marked with asterisks.

Probe set	Gene	Adult	Neonate	FC	LCB	p-value
Glycolysis						
L28135_at	*Slc2a2 (Glut2)*	16679±1942	7253±3157	-2.30	-1.28	0.055
D63834_at	*Slc16a1 (Mct1)*	49±17	286±79	5.84	2.74	0.055
rc_AI012593_at	*Hxk1*	1535±427	4245±1415	2.76	1.16	0.150
M68971_at	*Hxk2**	1169±2	3278±642	2.80	1.90	0.046
X53588_at	*Gck (Hxk4)*	1751±172	1847±519	1.05	0.56	0.871
X58865mRNA_at	*Pfkl**	1290±157	538±74	-2.40	-1.77	0.024
U25651_at	*Pfkm**	6665±228	3140±601	-2.12	-1.60	0.006
L25387_at	*Pfkp*	99±61	144±71	1.45	0.24	0.653
M12919mRNA#2_at	*Aldoa*	96±38	488±245	5.07	0.85	0.207
X02284_at	*Aldob*	524±42	481±66	-1.09	-0.85	0.604
X06984cds_s_at	*Aldoc*	277±129	1416±731	5.11	0.73	0.218
rc_AI104399_at	*Tpi1*	6406±1864	3446±1261	-1.86	-0.84	0.263
M17701_s_at	*Gapdh*	800±142	320±113	-2.49	-1.40	0.055
rc_AA892797_at	*Pgk1**	2088±276	900±341	-2.32	-1.34	0.042
S63233_at	*Pgam1*	4582±574	3728±695	-1.23	-0.86	0.387
Z17319_at	*Pgam2*	306±69	3505±1440	11.46	3.60	0.113
X02610_at	*Eno1*	160±49	287±145	1.80	0.30	0.457
X07729exon#5_s_at	*Eno2*	2034±28	1187±615	-1.71	-0.92	0.262
rc_AA851223_at	*Eno3*	1036±307	61±47	-16.86	-5.83	0.083
X05684_at	*Pklr*	1871±624	363±127	-5.16	-2.08	0.132
rc_AA818951_at	*Pkm2*	40625±2773	59823±17780	1.47	0.75	0.361
M54926_at	*Ldha*	3265±865	20975±9144	6.42	1.75	0.148
Krebs-cycle						
rc_AA799598_at	*Pdha1*	2536±193	1487±393	-1.71	-1.16	0.071
Z18878cds_at	*Pdha2*	79±48	93±66	1.17	0.00	0.876
rc_AA892828_at	*Pdhb*	4343±713	1945±649	-2.23	-1.30	0.060

D10655_at	*Dlat*	6328±1563	4950±1480	-1.28	-0.66	0.552
L22294_at	*Pdk1*	1600±385	154±47	-10.38	-5.45	0.062
U10357_g_at	*Pdk2**	1955±379	222±91	-8.80	-4.61	0.038
AF034577_at	*Pdk4*	39±8	34±9	-1.14	-0.68	0.699
U32314_g_at	*Pc**	8758±940	1431±654	-6.12	-3.37	0.004
rc_AI638990_at	*Aco2*	221±88	6294±3764	28.53	0.46	0.205
rc_AA892314_at	*Idh1*	3938±237	9800±1925	2.49	1.67	0.055
rc_AA892808_at	*Idh3g*	2024±83	2702±293	1.34	1.09	0.100
D90401_at	*Dlst*	161±69	161±64	-1.00	-0.27	0.999
J03621_at	*Suclg1**	1796±268	724±170	-2.48	-1.61	0.033
rc_AA800250_at	*Sdha*	5729±1571	4941±570	-1.16	-0.62	0.675
J04473_at	*Fh1*	11073±1611	6447±1837	-1.72	-1.07	0.117
rc_AI010480_at	*Mdh2**	2727±467	712±293	-3.83	-2.07	0.027
Mitochondrial shuttles						
AB002558_at	*Gpd1**	263±20	142±27	-1.86	-1.37	0.016
U83880UTR#1_g_at	*Gpd2**	9746±1415	2508±1187	-3.89	-2.04	0.015
AF093773_s_at	*Mdh1**	65299±9280	4245±1499	-15.38	-9.03	0.020
rc_AI010480_at	*Mdh2**	2727±467	712±293	-3.83	-2.07	0.027
J04171_at	*Got1**	7296±439	1341±246	-5.44	-4.09	0.001
M18467_at	*Got2**	6162±417	4096±323	-1.50	-1.27	0.016
rc_AI008020_at	*Me1*	10982±2349	1255±328	-8.75	-5.00	0.051
J05210_at	*Acly*	6679±824	4700±1741	-1.42	-0.84	0.360
L12016_at	*Slc25a1 (Cic)**	2674±375	1128±154	-2.37	-1.70	0.038
AJ223355_at	*Slc25a10 (Dic)*	185±17	210±47	1.13	0.70	0.646

Beta cell monitoring of circulating glucose concentrations is made possible by the co-expression of GLUT2 glucose transporter and high Km glucokinase [116]; neither of these genes was differentially expressed. However, hexokinase 2, which is expressed at very low levels in functional adult beta cells [117], was more highly expressed in neonatal beta cells (p=0.046). Gene expression of other glycolytic enzymes was unchanged (Table 3) except two genes that were lower in neonatal beta cells: phosphoglycerate kinase (p=0.042) and phosphofructokinase L and M types, which have been implicated in oscillatory insulin release [118], (p=0.024 and 0.006, respectively). Lactate dehydrogenase A and monocarboxylate transporter 1 (also known as Slc16a1), minimally expressed in adult beta cells [117], were present at higher levels in neonatal samples, which however did not reach statistical significance

in part because of variability.

Glucose is metabolized to pyruvate, and pyruvate provides the carbon skeleton to the Krebs cycle. Interestingly, while genes of the pyruvate dehydrogenase enzyme complex were not differentially expressed, pyruvate carboxylase (Pc), the key anaplerotic enzyme in mitochondria, was markedly lower in neonatal beta cells ($p=0.004$). The anaplerotic mechanism of pyruvate and pyruvate cycling is closely correlated with GSIS in beta cells [82, 83, 119, 120]. It was striking that a number of mitochondrial shuttle enzymes, so key to the efficient generation of ATP and pyruvate cycling in the adult beta cell, had less than 20% of adult expression in neonatal beta cells (Table 3). Shuttle genes with lower neonatal expression included malate dehydrogenase 1 (Mdh1; 15-fold lower, $p=0.020$) and 2 (Mdh2; $p=0.027$), aspartate aminotransferase 1 (Got1; $p=0.001$) and 2 (Got2; $p=0.016$), glycerol-3-phosphate dehydrogenase 1 (Gpd1; $p=0.016$) and 2 (Gpd2; $p=0.015$). Other pyruvate-shuttling genes with neonatally lower expression included Slc25a1 (mitochondrial citrate/isocitrate carrier; $p=0.038$) and cytosolic malic enzyme (Me1), a critical component of pyruvate-malate shuttle, which had almost ninefold difference in expression but just missed statistical significance ($p=0.051$).

Notably, gene expression levels of Krebs cycle enzymes were not statistically different between adult and neonatal beta cells, except for succinate-CoA ligase 1 (also known as succinyl-CoA synthetase; Suclg1) and Mdh2, which is also a key enzyme of the pyruvate–malate shuttle (Table 3).

Of the 53 genes present on the microarray encoding subunits of the electron transport chain complexes I–IV and the ATP synthase, ten were differentially lower in neonatal beta cells ($p<0.050$; Table 4). Only Nd3, the only mitochondrial DNA-encoded electron transport chain gene on the microarray, and Cox8a were more highly expressed in the neonatal beta cells ($p=0.029$ and 0.042, respectively).

With regard to lipid metabolism genes, fatty acid synthase was significantly upregulated in neonatal beta cells ($p=0.009$). Carnitine palmitoyl transferase 1 (Cpt1a), which transfers acyl groups from the cytosol to the mitochondria for beta-oxidation, was also upregulated whereas the inner mitochondrial membrane Cpt2 was eightfold lower in neonatal beta cells ($p=0.046$ and 0.021, respectively). There is a switch in fatty acid transporters with Slc27a5, being four to five times higher in the neonate beta cell

(p=0.032), and Slc27a1, eight times higher in the adult (p=0.001). Expression levels of genes encoding mitochondrial enzymes of beta-oxidation were largely unchanged, except for certain subunits of the hydroxyacyl-CoA dehydrogenase complex (Table 4).

For genes implicated in insulin exocytosis, very few expression differences were found. Synaptotagmin 5, Vamp2 and Stxbp1 had lower expression in neonatal beta cells compared with adult (p=0.002, 0.041, and 0.002, respectively); while synaptotagmin 6 and 8 were higher (p=0.017 and 0.011, respectively). However, genes encoding key SNARE proteins of the exocytotic machinery Snap25 and syntaxin 1A (Stx1a) did not differ. Similarly, those encoding ATP-sensitive K^+-channel subunits Kir6.2 (also known as Kcnj11), Sur1 (also known as Abcc8), delayed rectifier K^+ channel Kv2.1 (also known as Kcnb1) and voltage-dependent Ca^{2+}-channels Cav1.2 (also known as Cacna1c) and Cav1.3 (also known as Cacna1d) did not differ. It is noteworthy that insulin 2 gene expression was almost identical in neonatal and adult beta cells as well.

A simplified diagram of the investigated metabolic pathways implicated in glucose-stimulated insulin secretion is shown in Figure 8.

Table 4. Expression of metabolic genes in beta cells grouped into functional categories (Electron transport chain, Fatty acid synthesis and beta oxidation, Insulin secretion). Genes of the various pathways are listed with the mean±SEM, fold change (FC), lower confidence boundary (LCB) and p-value as determined with dChip. Where multiple probe sets were on the microarray, the more stringent *_at set* is listed; if no_*at* probe set, the set with highest expression is listed. Microarray signals of adult (n=3) and neonatal (n=4) samples. Those with significance (p<0.050, fold change of 1.5 or greater, LCB of 1.2, and signal mean difference of at least 100) are marked with asterisks.

Probe set	Gene	Adult	Neonate	FC	LCB	p-value
Electron transport chain						
rc_AA945152_s_at	*Nd3**	147675± 27942	282517± 10180	1.91	1.45	0.029
D86215_at	*Ndufa5*	451±179	695±367	1.54	0.20	0.581
rc_AA819547_at	*Ndufa6**	6317±223	3014±730	-2.10	-1.49	0.016
rc_AI232012_at	*Ndufa8*	735±111	5104±2097	6.95	2.22	0.128
rc_AA799525_at	*Ndufa9*	3202±33	7111±3602	2.22	0.37	0.357

AA799336_at	*Ndufab1*	636±79	490±142	-1.30	-0.82	0.415
rc_AI112237_at	*Ndufb2**	16803±1347	2690±567	-6.25	-4.49	0.004
rc_AA799499_at	*Ndufb3*	48072±3275	55745±8754	1.16	0.85	0.460
rc_AA859957_at	*Ndufb4*	7915±813	4165±1298	-1.90	-1.20	0.061
rc_AA893185_at	*Ndufb5*	10594±2248	4380±2413	-2.42	-1.11	0.119
rc_AI104528_at	*Ndufb6*	1011±241	515±282	-1.96	-0.87	0.240
rc_AA964320_at	*Ndufb8*	4030±621	3834±773	-1.05	-0.70	0.851
rc_AI171542_at	*Ndufb9**	15754±2020	1912±839	-8.24	-4.53	0.011
rc_AA893690_at	*Ndufb11*	221±102	134±83	-1.64	-0.34	0.545
rc_AA891171_s_at	*Ndufc2*	17267±3945	21008±3859	1.22	0.75	0.529
AA686031_at	*Ndufs1*	87±14	444±296	5.12	0.00	0.314
rc_AI013297_at	*Ndufs4*	327±54	466±50	1.43	1.05	0.122
rc_AI009390_at	*Ndufs5b**	5230±28	699±67	-7.48	-6.46	0.000
rc_AA875268_at	*Ndufs7**	3924±753	944±255	-4.16	-2.45	0.046
AA685112_at	*Ndufs8**	254±9	91±25	-2.80	-1.92	0.004
M22756_at	*Ndufv2*	3908±421	6436±3000	1.65	0.38	0.463
AB000098_at	*Ndufv3l*	57±14	276±204	4.82	0.00	0.363
rc_AA800250_at	*Sdha*	5729±1571	4941±570	-1.16	-0.62	0.675
rc_AI235358_at	*Uqcrc2*	7374±380	11458±3700	1.55	0.73	0.351
M24542cds_at	*Uqcrfs1*	256±47	212±107	-1.21	-0.60	0.725
rc_AA799474_at	*Cyc1*	16248±2825	9843±1747	-1.65	-1.08	0.137
K00750ex#2-3_at	*Cycs*	2679±358	3778±1206	1.41	0.66	0.438
rc_AA818226_s_at	*Cox4i1*	34187±6021	20453±2045	-1.67	-1.15	0.139
rc_H31232_at	*Cox4i2*	4291±1107	8284±2255	1.93	0.97	0.183
rc_AA800179_at	*Cox4nb*	163±50	232±77	1.42	0.59	0.488
X15030_at	*Cox5a**	3592±433	1042±562	-3.45	-1.75	0.016
rc_AI229620_s_at	*Cox5b*	7364±563	4516±2637	-1.63	-0.82	0.363
X72757_at	*Cox6a1*	400±73	545±119	1.36	0.81	0.351
X72758mRNA_at	*Cox6a2*	38±17	317±226	8.43	0.00	0.304
M27467_at	*Cox6c*	1004±228	18359±8029	18.29	5.00	0.119
M20183_at	*Cox6c1*	461±30	614±106	1.33	0.94	0.248
rc_AA819708_s_at	*Cox7a2*	4138±522	7368±1086	1.78	1.27	0.052
rc_AI232307_at	*Cox7a2l*	40545±1891	22020±6643	-1.84	-1.22	0.064
rc_AA866477_at	*Cox7b*	2433±510	1903±739	-1.28	-0.67	0.581
rc_AI102505_at	*Cox8a**	6556±23	15336±2576	2.34	1.69	0.042
X64827cds_s_at	*Cox8h*	1009±165	474±263	-2.13	-1.03	0.149
X56133_at	*Atp5a1*	2430±268	2758±855	1.14	0.55	0.735
rc_AI105050_at	*Atp5b*	16597±949	12260±3164	-1.35	-0.94	0.268
L19927_at	*Atp5c1*	3967±1277	4230±1983	1.07	0.23	0.916
U00926_at	*Atp5d*	2053±169	1333±275	-1.54	-1.11	0.079
rc_AI171844_at	*Atp5e*	869±165	1405±526	1.62	0.60	0.392
rc_AA799778_at	*Atp5f1*	14331±1989	13459±3682	-1.06	-0.68	0.844
D13123_s_at	*Atp5g1**	13527±1104	3075±1152	-4.40	-2.66	0.001

D13124_s_at	*Atp5g2**	8443±714	3732±1145	-2.26	-1.46	0.019
D13120_s_at	*Atp5h*	282±170	132±78	-2.14	-0.02	0.484
X54510_at	*Atp5j*	1035±311	298±168	-3.47	-1.35	0.124
D13127_at	*Atp5o*	112±14	7147±4316	63.74	0.43	0.202
rc_AA799829_at	*Atp5s**	269±3	114±44	-2.36	-1.45	0.038
X03894_at	*Ucp1*	77±22	137±78	1.77	0.10	0.510
AB010743_at	*Ucp2*	16699±1068	14011±1692	-1.19	-0.96	0.240
AF035943_at	*Ucp3*	389±33	810±581	2.08	0.00	0.521
D12770_s_at	*Slc25a4 (Ant1)*	10444±532	10931±2050	1.05	0.72	0.831
D12771_s_at	*Slc25a5 (Ant2)*	10987±2144	4415±1115	-2.49	-1.48	0.071

Fatty acid synthesis and beta-oxidation

J03808_at	*Acaca*	169±73	410±184	2.42	0.58	0.294
X13527cds_s_at	*Fasn**	788±77	1487±137	1.89	1.51	0.009
L07736_at	*Cpt1a**	819±84	3961±962	4.83	2.84	0.046
D43623_at	*Cpt1b*	2622±255	2344±509	-1.12	-0.79	0.649
J05470_at	*Cpt2**	1583±241	185±73	-8.58	-4.79	0.021
rc_AA800120_at	*Slc25a20 (Cac)*	1009±305	2506±1369	2.48	0.25	0.358
X05341_at	*Acaa2*	4664±627	4737±1231	1.02	0.56	0.960
rc_AA945583_at	*Hsd17b10 (Hadh2)**	2230±121	852±214	-2.62	-1.83	0.003
D16478_at	*Hadha*	7288±1791	18748±7502	2.57	0.85	0.225
D16479_at	*Hadhb**	9390±1452	1958±1035	-4.80	-2.39	0.015
rc_AA891362_at	*Hadh (Hadhsc)*	569±97	249±117	-2.29	-1.18	0.088
X15958_at	*Echs1*	2378±495	697±151	-3.41	-2.04	0.066
D00569_g_at	*Decr1*	12903±1860	6058±2040	-2.13	-1.27	0.056
U89529_at	*Slc27a1**	1303±110	166±81	-7.86	-4.27	0.001
D85100_at	*Slc27a2*	326±18	1563±449	4.79	2.52	0.070
rc_H31236_at	*Slc27a5**	66±18	312±67	4.69	2.65	0.032

Insulin release

M25584_at	*Ins1/Ins2*	2076±71	4709±1200	2.27	1.31	0.116
D86039_at	*Kcnj11 (Kir6.2)*	41452±9132	38550±5092	-1.08	-0.66	0.798
L40624_at	*Abcc8 (Sur1)*	3975±944	2399±560	-1.66	-0.92	0.237
AF051526_at	*Cacna1a (Cav2.1)*	122±36	121±53	-1.01	-0.43	0.987
AF055477_at	*Cacna1b (Cav2.2)*	1414±280	1230±267	-1.15	-0.70	0.656
M59786_at	*Cacna1c (Cav1.2)*	1290±611	337±107	-3.83	-0.82	0.257
rc_AI639212_at	*Cacna1d*	474±40	1331±731	2.81	0.27	0.325

	(Cav1.3)					
M32867_at	*Kcna4 (Kv1.4)*	582±180	447±200	-1.30	-0.53	0.637
X76723_at	*Kcnab3 (Kvbeta3)*	38±5	262±242	6.86	0.00	0.423
X16476cds_at	*Kcnb1 (Kv2.1)*	54±3	43±8	-1.25	-0.93	0.304
rc_AI639467_at	*Syt1*	292±18	1177±507	4.04	1.18	0.179
M64488_at	*Syt2*	82±24	207±74	2.52	0.97	0.191
D28512_at	*Syt3*	703±111	782±67	1.11	0.84	0.582
U14398_at	*Syt4*	61±25	48±8	-1.28	-0.41	0.653
U26402_at	*Syt5**	2119±83	1257±109	-1.69	-1.45	0.002
U20105_at	*Syt6**	96±31	298±48	3.10	1.84	0.017
U20106_at	*Syt7*	130±68	282±109	2.16	0.67	0.295
U20110cds_at	*Syt8**	38±12	511±87	13.42	7.82	0.011
U20108_at	*Syt9*	98±18	299±206	3.06	0.00	0.402
U85513_at	*Syt10*	61±20	134±84	2.22	0.00	0.448
AF000423_at	*Syt11*	276±86	303±88	1.10	0.51	0.835
U71294_at	*Syt12*	12±4	678±527	57.75	0.00	0.295
X06889cds_at	*Rab3a*	56±16	79±43	1.41	0.14	0.643
D17352_at	*Rab27a*	171±8	985±321	5.77	2.67	0.085
D10392_i_at	*Stx1a*	4668±824	6568±1853	1.41	0.72	0.401
M24105_at	*Vamp2**	1520±43	924±179	-1.65	-1.24	0.041
AB003991_at	*Snap25*	119±58	116±79	-1.02	-0.18	0.978
rc_AI102079_at	*Stxbp1* (Munc18-1)*	40384±3492	7184±2219	-5.62	-3.62	0.002
U20283_at	*Stxbp2 (Munc18-2)*	82±29	116±52	1.42	0.34	0.597
U92072_at	*Stxbp5 (Tomosyn)*	285±71	74±57	-3.86	-1.44	0.078
rc_AI176308_at	*Cdc42*	10045±262	12191±2976	1.21	0.73	0.524

Confirmation of microarray data with qPCR

Differential expression seen in microarray data (Figure 8) was confirmed with qPCR on RNA from isolated neonatal P2, P7 and adult islets. The percentage of beta cells/islets did not differ significantly among P2, P21 and adult islets; only at P11 was it significantly lower (20%) than adult (Table 5). Even so, neonatal islets expressed only 10–20% of adult levels of the metabolic genes: Pc, Me1, Mdh1, Got1, Gpd2 and Cpt2.

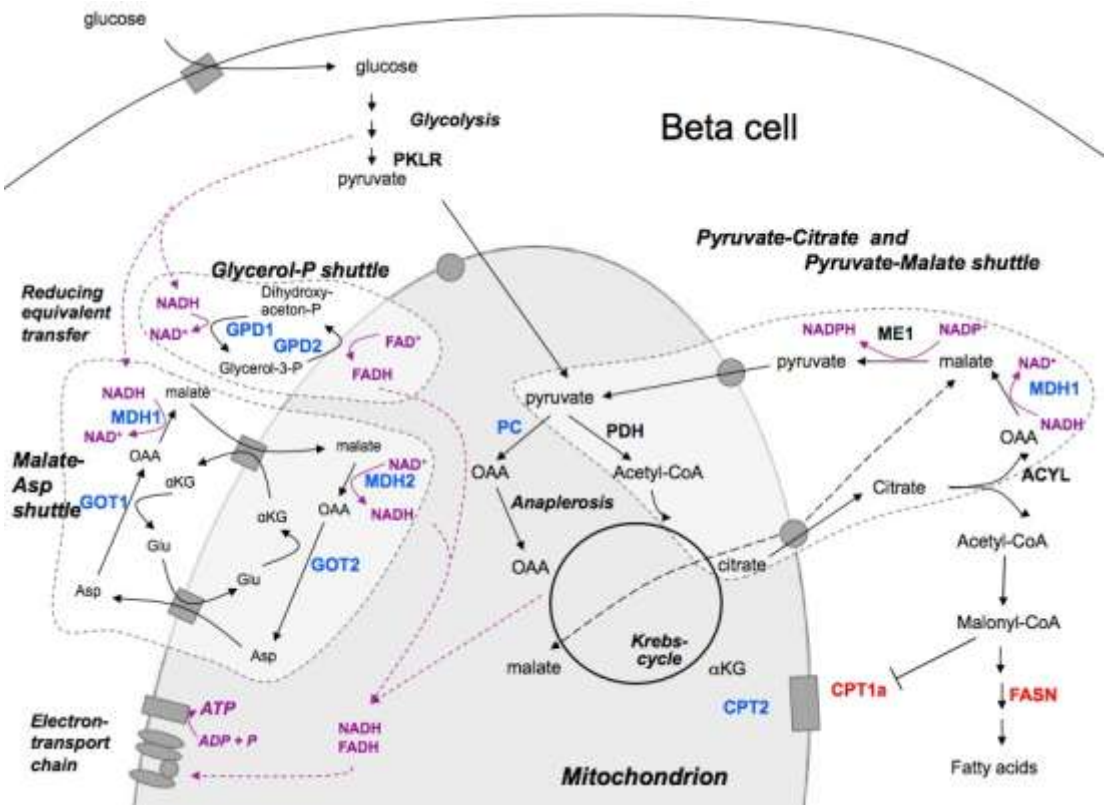

Figure 8. Metabolic pathways involved in glucose-stimulated insulin secretion in beta cells. Based on dChip analysis of microarray data of enriched beta cell samples from neonatal versus adult samples, genes with lower expression in the neonates (adult/neonatal ≤-1.2) are in blue and those with higher expression in neonates (neonatal/adult ≥1.2) are in red; unchanged in black. Abbreviations: GPD1 and 2, cytosolic and mitochondrial glycerol-3-phosphate dehydrogenase; MDH1 and 2, cytosolic and mitochondrial malate dehydrogenase; GOT1 and 2, cytosolic and mitochondrial glutamate oxalacetate transaminase (also known as aspartate aminotransferase); PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PK, pyruvate kinase; ME1, cytosolic malic enzyme; CPT1 and 2, carnitine palmitoyltransferase; ACLY, ATP citrate lyase; FASN, fatty acid synthase; OAA, oxalacetic acid; αKG, α-ketoglutarate; Glu, glutamate; Asp, aspartate.

Table 5. Beta cell composition of islets at postnatal day P1 or P2, P11, P21 and adult time points. Immunfluorescently stained pancreas sections using both anti-insulin and a cocktail of anti-non beta endocrine hormone antibodies were quantitated for the proportion of the islet that was insulin positive. All islets/clusters greater than 35 mm diameter corresponding to the islet size obtained by handpicking after islet isolation were included. * $p<0.05$ compared to adult.

Age	Number of animals (total islets)	% insulin-positive cells (mean±SEM)
P1-2	5 (107)	64±2
P11	3 (68)	55±4 *
P21	3 (56)	58±3
Adult	3 (55)	70±4

In contrast, Cpt1 levels at P2 did not differ from adult but at P7 decreased to 45% of adult (Figure 9). Metabolic genes encoding pyruvate kinase (PK) and glycerol-3-antibody does not distinguish between PK muscle isoform (PKM) and PK liver and red blood cell isoform (PKLR) isoforms. Both enzymes had low-intensity staining in P2 islets but had strong staining in adult islets (Figure 10). At P7, PK protein was intermediate between neonatal and adult islets. In contrast GPD2, with little to no staining at birth, was clearly produced at P7 with similar pattern and intensity as adult islets.

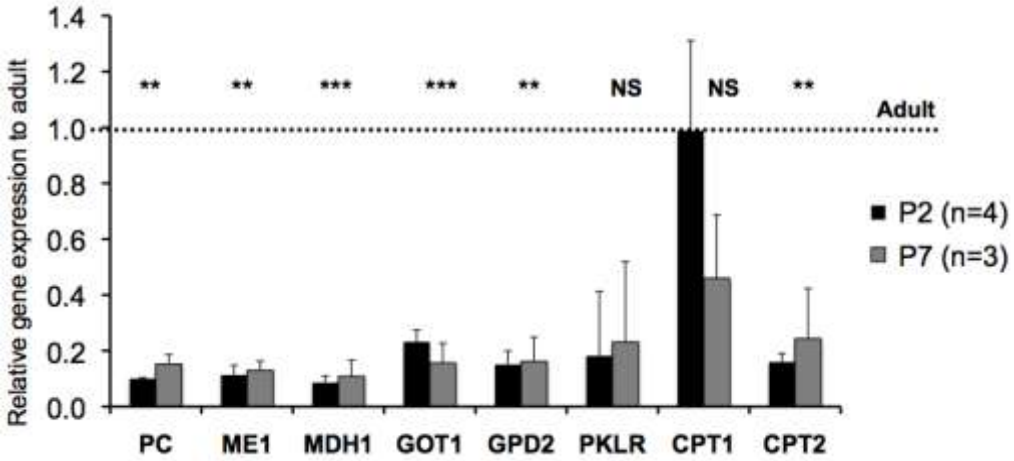

Figure 9. QPCR confirmation of selected metabolic genes. The differential expression of selected metabolic genes was confirmed by quantitative PCR in islets isolated from neonatal P2 (black bars, n=4 samples of islets pooled from a litter) and P7 rats (grey bars, n=3 samples of islets pooled from a litter); data are expressed relative to isolated adult islets (dotted line, n=5). One-way ANOVA with Tukey post hoc analysis. ** $p<0.01$; *** $p<0.001$; N.S.= not significant.

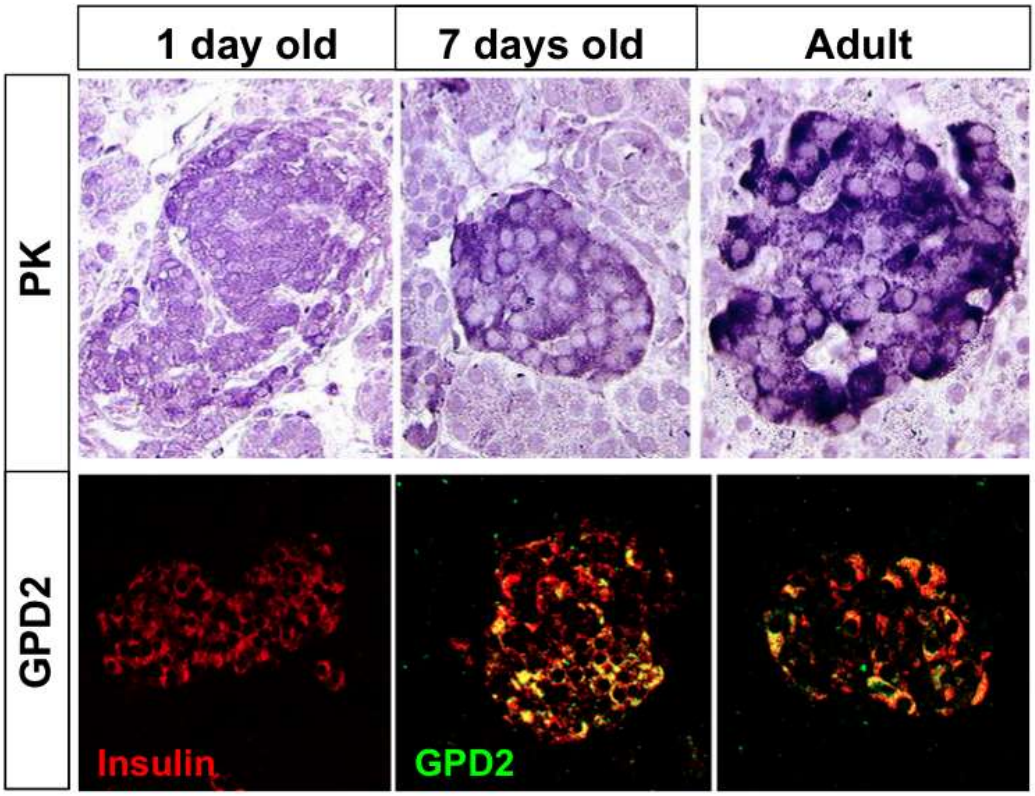

Figure 10. Immunostaining of pyruvate kinase (PK) and mitochondrial glycerol-3-phosphate dehydrogenase (GPD2). By immunostaining, the protein levels of PK (top panels, VIP as chromagen) and GPD2 (bottom panels, insulin-red, GPD2-green, overlap yellow) reflect the mRNA expression through the neonatal time period.

To study the development of the characteristic metabolic profile of adult beta cells, we examined expression of these genes over the first postnatal month (Figure 11). Several different expression patterns were found, perhaps reflecting different regulation. Me1 and Pc had low expression even at P7 but with sharp increases at P9; Me1 had another sharp increase at P28 while Pc gradually increased from P9 onwards (Figure 11a). In contrast, Mdh1, Got1 and Gpd2 (Figure 11b) had stable low expression with a moderate increase only at P28. While Cpt2 expression followed a pattern similar to that of Mdh1, Got1 and Gpd2, Cpt1 decreased further to 20% of adult by P9 (Figure 11c). For Pk (primers selective for Plkr) there was a sharp increase at P9, reaching levels that were highly variable but consistently high as or higher than adult through P28 (Figure 11d).

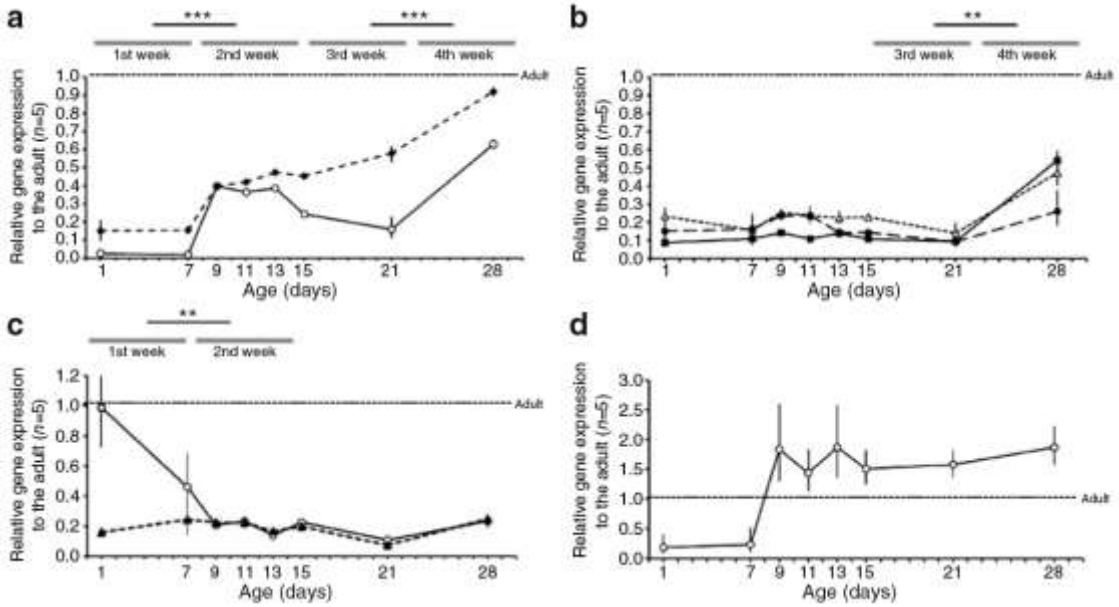

Figure 11. Time course of expression of selected metabolic genes over the first postnatal month by quantitative real time PCR. The metabolic enzymes have different patterns of expression through the first postnatal month. All but Cpt1 express at 20% or less than adult levels during the first week. Data expressed relative to pooled isolated adult islets (n=5). Student's t-test for comparison of expression between weeks. * p <0.05, ** p <0.01, *** p <0.001. ANOVA for repeated measures throughout the time course, p <0.001 in all comparisons, except for pyruvate kinase.
(a) pyruvate carboxylase (♦); malic enzyme 1 (○, cytosolic).
(b) malate dehydrogenase 1 (■, cytosolic); glutamate oxalacetate transaminase 1 (Δ, cytosolic); glycerol phosphate dehydrogenase 2 (●, mitochondrial).
(c) carnitine palmitoyltransferase 1 (○); carnitine palmitoyltransferase 2 (▲).
(d) pyruvate kinase (○, Pklr isoform).

Assessment of neonatal beta cell maturation

Expression of beta cell transcription factors during the postnatal period

The reduced expression of many key beta cell genes in neonatal beta cells suggests that regulated expression of transcription factors may drive beta cell maturation. To examine the expression of these factors, quantitative RT-PCR was performed on neonatal islets isolated over the first postnatal month. Key beta cell transcription factors (Figure 12a) had very low (3–12%) expression at P2, with sharp increases between P7 and P9. By P7 both Pdx1 and NeuroD1 mRNA levels no longer differed from adult levels and from P9 to P13 were transiently higher than adult (Pdx1 was increased in all samples but did not reach significance). Nkx6-1 mRNA was expressed at levels lower

than adult only through P7. By contrast, MafA expression increased from 7% at P7 to 43% of adult at P9 but remained significantly lower than adult until P28. We further explored the role of Pdx1 and MafA in functional maturation because both are major regulators of insulin gene expression. MafA expression during the neonatal period seemed independent of Pdx1 expression. Interestingly, the pattern of insulin mRNA mirrored more closely that of MafA than either Pdx1 or NeuroD1 (Figure 12a, b).

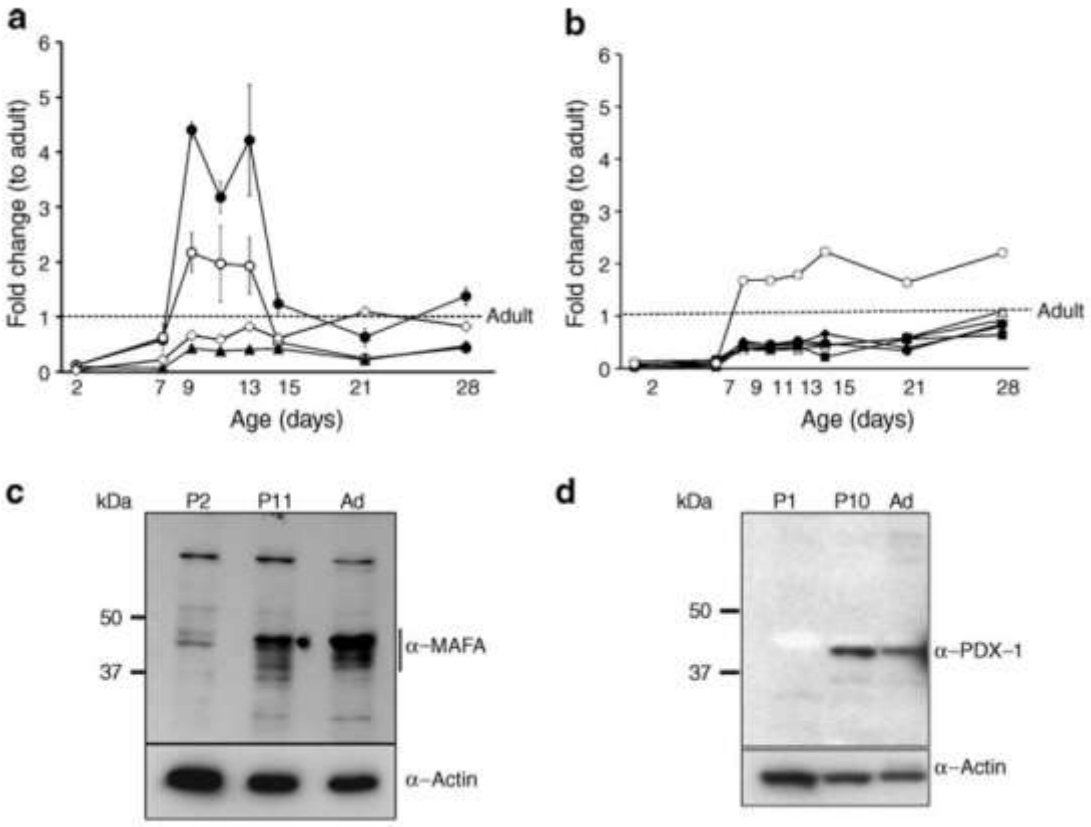

Figure 12. Expression pattern of beta cell transcription factors during the first postnatal month.
(a) Beta cell transcription factors NeuroD1 (●), Pdx1 (○), MafA (▲), Nkx6-1 (◊).
(b) Other key genes: Glp1r (♦), Pc (□), Pcsk1 (Δ), insulin (●), Glut2 (■) and Gck (○) mRNA show different patterns of expression over the neonatal period as measured by quantitative RT-PCR. Data expressed as fold change with respect to adult using S25 as internal control gene. Mean ± SEM, n=4–6 isolated samples per age, each pooled from three to ten animals.
(c) By western blot, MafA protein; and
(d) Pdx1 protein are low at birth (P2). MafA increases at P11 but is still low compared with adult. Pdx1 increased at P11 and decreased in adult islets in a pattern similar to that of mRNA levels at the same ages. Representative gels of three independent samples. Ad, adult

MafA protein levels showed age-dependent increases. In western blots, MafA protein (Figure 12c) reflected the RNA levels with very low levels in P2 islets and increases in P11. Pdx1 protein, by western blot (Figure 12d) showed comparable levels at P11 and adult, whereas mRNA levels tended to be higher at P11 (Figure 12a). Putative MafA targets (insulin, Glut2, Gck, Glp1r and Pcsk1) had an expression pattern similar to that of MafA, with very low expression at P2, and significant increases from P7 to P9; however, unlike MafA, these genes reached adult mRNA levels by P28 (Figure 12b).

Previous quantification of the beta cell proportion showed isolated neonatal islets did not significantly differ from adult in % beta cell (Table 5). Thus the changes in gene expression are likely to be due to changes in phenotype rather than proportion of beta cells. The findings that Pdx1 expression reached adult levels already by P7 whereas MafA expression remained significantly lower than adult even through P21 suggest MafA as the factor that drives final maturation of beta cell function and enhances glucose-stimulated insulin secretion.

Adenoviral overexpression of Pdx1 and MafA in P2 islets

To determine the effect of Pdx1 and MafA on the maturation of the neonatal beta cells more directly, we overexpressed each separately using adenoviruses in P2 islets and assessed expression of putative target genes, and glucose-responsive insulin secretion.

Pdx1 overexpression achieved a 2.4-fold increase in mRNA (Figure 13a), with significant increases in MafA, NeuroD1 and Gck mRNA, but no change in Nkx6.1, cMaf, MafB, Glp1r, Glut2, Ins2, preproinsulin or pyruvate carboxylase mRNA. Insulin secretion was significantly increased at both 2.8 and 16.8 mmol/l glucose (Figure 14a) in AdPdx1-infected cells compared with AdGFP controls. However, there was no increase in fold change because the responses to low and high glucose did not differ (Figure 14b). This lack of glucose responsiveness was seen even though insulin content significantly increased after Pdx1 overexpression (Figure 14c).

MafA overexpression was adjusted to obtain a modest 50% increase of MafA levels at 72 h compared with control AdGFP-infected cells; this is comparable to the level seen in P9-P28 islets. This change was due to increased exogenous MafA as the

amount of PCR product for 3′ UTR message (endogenous gene, see Methods) was unchanged (Figure 13b). With this overexpression, transcription of NeuroD1, Nkx6.1 and Gck were significantly upregulated, as was Glp1r. However, Ins2, Pdx1, Glut2, various metabolic genes and channels were not significantly changed. There was no change in MafB, whereas cMaf was significantly reduced.

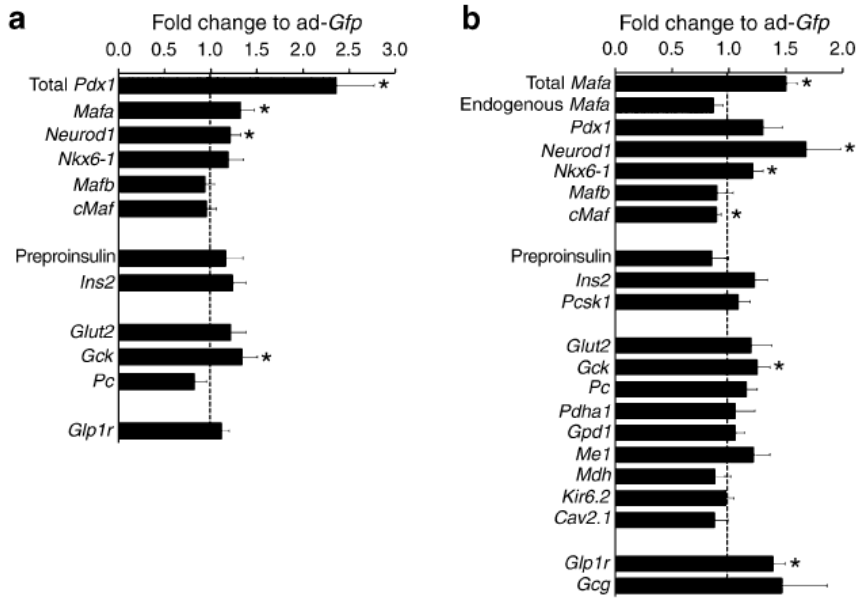

Figure 13. Effect of adenoviral-mediated increase of Pdx1 (a) and MafA (b) on genes important for beta cell function. After 72 h culture adenoviral-mediated overexpression of Pdx1 or MafA in postnatal day P2 islets had significant increases of total Pdx1 mRNA (a) and total MafA mRNA (b). Overexpression of Pdx1 upregulated MafA, NeuroD1 and Gck whereas overexpression of MafA had upregulated NeuroD1, Nkx6.1, Gck and Glp1r. Quantitative RT-PCR; expression compared to AdGFP infected cells (equal to 1, dotted line). Mean ± SEM, n=4–6 independent experiments; *$p<0.05$.

Importantly, MafA overexpression induced glucose stimulated insulin secretion. In static incubations AdMafA-infected cells significantly decreased basal insulin secretion at 2.8 mmol/l glucose while significantly increasing insulin secretion in 16.8 mmol/l glucose compared with AdGFP cells (Figure 14a). Insulin secretion increased fourfold from low to high glucose (Figure 14b), a change comparable to the fivefold increase of cultured control AdGFP-infected adult cells. It is striking that adult glucose responsiveness was nearly achieved in MafA-infected P2 cells that still had lower insulin expression (Figures 12b, 13b) and content (Figure 14c) than adult cells treated

and cultured under the same conditions. Considering that only about 50% of the cells were infected, the glucose responsiveness may be underestimated in these experiments. It is also important to note that these Ad-infected P2 isolated islets were cultured for 5 days and thus cannot be compared with freshly isolated P2 islets.

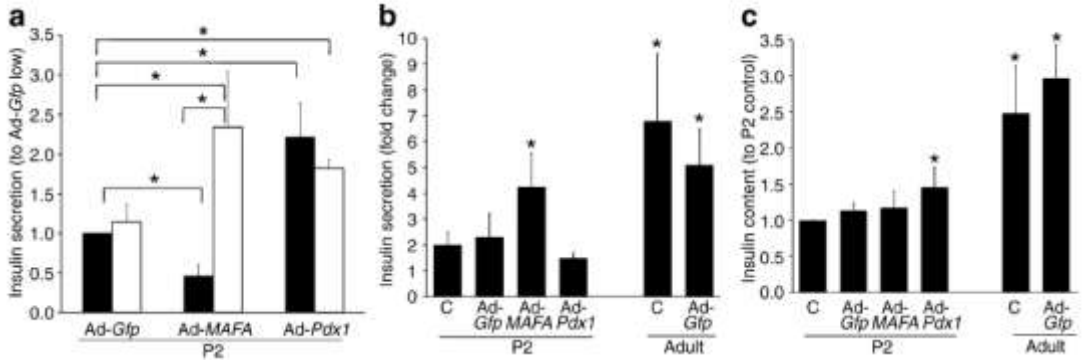

Figure 14. Insulin secretion and insulin content after AdPdx1, AdMafA and AdGFP infection.

(a) Black bars represent low glucose (2.6 mM), white bars represent high glucose (16.8 mM) conditions. Insulin secretion in response to 16.8 mM glucose increased in both AdPdx1 and AdMafA infected P2 cells compared with AdGFP infected control cells after 5 days culture. However, insulin secretion from AdMafA infected cells in 2.6 mM glucose significantly decreased while that from AdPdx1 increased almost as much as with high glucose. *p<0.05 in marked comparisons.

(b) Insulin secretion expressed as fold change in response to glucose stimulation reflects the glucose responsiveness of the cultured cells. As previously shown [87, 95], neonatal (P2) islets have little glucose responsiveness when compared with adult islets similarly cultured. Only AdMafA increased the glucose responsiveness of the P2 cells; the uninfected, AdGFP and AdPdx1 infected cells had little to no response to the increased glucose concentration.

(c) There was no change in insulin content (pg/ng DNA) in AdMafA infected P2 islets and cultured control islets (untreated or AdGFP infected), but insulin content was significantly higher in AdPdx1 compared with cultured uninfected cells. Mean ± SEM; n=4 independent experiments in duplicate; C, control. *p<0.05 compared with untreated, control cultured P2 cells.

Characterization of the role of MafA in regulating beta cell function

As both neonatal and dysfunctional adult beta cells have impaired glucose-responsive insulin secretion and decreased expression of functionally important genes, we hypothesized that low levels of MafA may be the culprit. In order to unequivocally establish the role of MafA in the mature beta cell function, we decreased functional MafA protein levels by adenovirus-mediated dominant-negative MafA (DN-MafA).

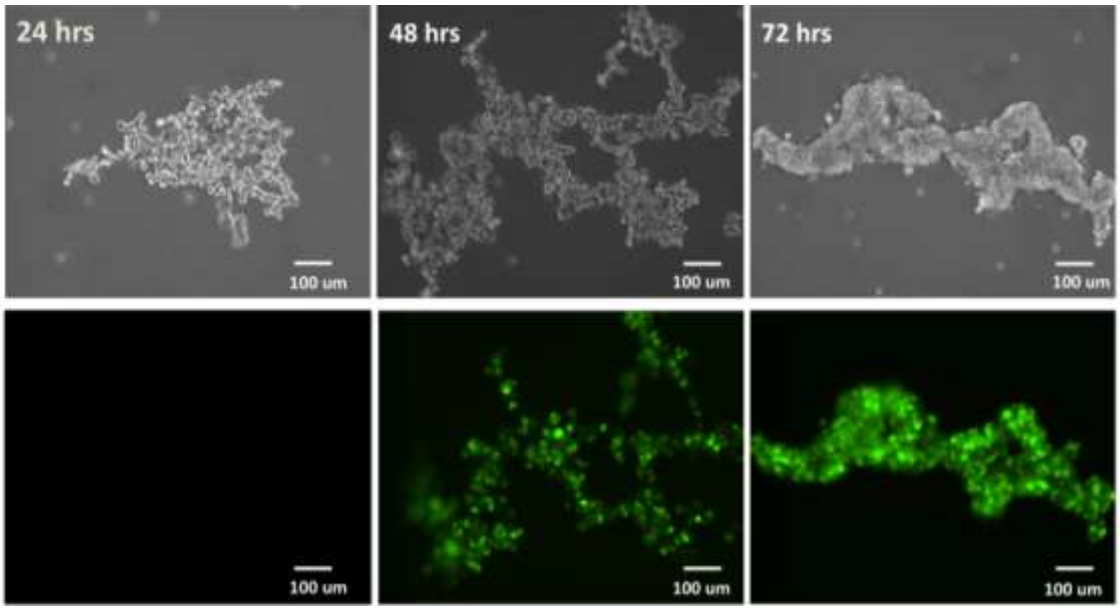

Figure 15. AdGFP infected islet cell aggregates after different culture times. Isolated, handpicked Sprague-Dawley rat islets were cultured over-night and subsequently dispersed to facilitate optimal adenovirus infection. Islet cells progressively reaggregated on low attachment plates by 72 hours. A significant expression of GFP from the transduced gene became visible by 48 hours. Upper row represents islet aggregates studied by light microscopy at 24, 48, and 72 hours after dispersion; lower row displays respective images taken with fluorescent microscopy.
Induction of DN-MafA protein was confirmed by Western blot analysis of islet cell aggregates 48 hours after infection with AdDN-MafA (Figure 16). This observation suggests that MafA expression itself is down-regulated in an autoregulatory process.

DN-MafA down-regulates expression of endogenous MafA and targets

The function of transcriptional factor MafA was inhibited in adult, mature rat beta cells by expressing DN-MafA. After the dispersed islets were infected with AdDN-MafA or the control AdGFP vector, the cells reaggregated on hydrophobic plates (Figure 15). By 72 hours the aggregates looked compact and similar to intact islets except for their

irregular shapes. No central necrosis was observed in the aggregates. The GFP signal became detectable under UV light 48 hours after infection and showed homogeneous distribution through the islet cell aggregates, suggesting an almost 100% infection rate (Figure 15).

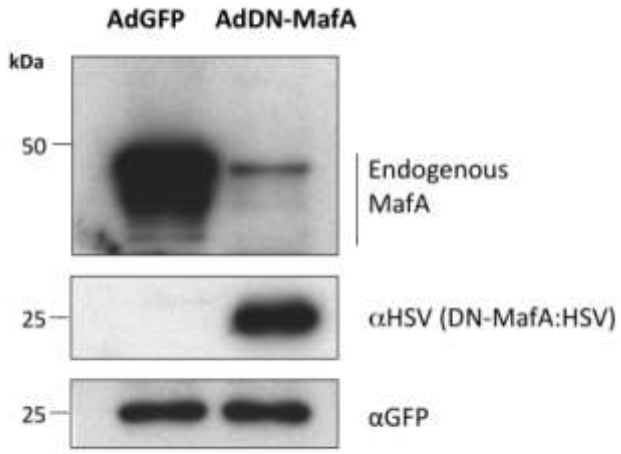

Figure 16. A Western blot analysis of endogenous MafA protein levels after Adeno-GFP or Adeno-DN-MafA infection in islet cell aggregates. Protein extracts (20μg) from islet cell aggregates, cultured for 48 hours after Adenovirus infection, were resolved in 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with an anti-MafA, ant-HSV and anti-GFP antibody. The anti-MafA antibody is directed against the N-terminal region of MafA and was used to detect the native, endogenous MafA band at 49 kDa. The HSV-tagged DN-MafA protein, lacking the N-terminal domain of the transcription factor, was detected with the anti-HSV antibody, as a faster migrating band at 25 kDa, only in cells infected with AdDN-MafA. The endogenous MafA protein is detectable much weaker (80% less) in the presence of DN-MafA than in control AdGFP-infected islet cells. Anti-GFP immunoblotting was used as loading controls. Representative immunoblots are shown from at least three independent experiments.

In AdDN-MafA infected islet cells expression of some putative MafA target genes were down regulated compared to AdGFP-infected controls measured by qPCR (Figure 17). As with MafA protein, endogenous MafA mRNA level became progressively down-regulated after DN-MafA expression ($p<0.001$ at 72 hours, compared to AdGFP controls). Insulin message was unchanged, which was not surprising considering the abundance and long half-life of the insulin mRNA. However, insulin pre-mRNA level, which reflects acute changes of insulin gene

transcription [121] were significantly lower as expected in the absence of functional MafA. In addition, marked reductions in the mRNA of other putative targets Glp1r, pyruvate carboxylase (Pc) and prohormone convertase 1 (Pcsk1) were observed ($p<0.001$ for each gene at 72 hours compared to AdGFP controls). Importantly to this being a specific effect, glucagon expression was unchanged and lactate dehydrogenase (Ldh) mRNA was significantly elevated ($p<0.01$) after 72 hours of infection with AdDN-MafA (Figure 17). Ldh is usually present at very low or undetectable levels in mature beta cells [117], and its up-regulation may suggest a loss of beta-cell phenotype.

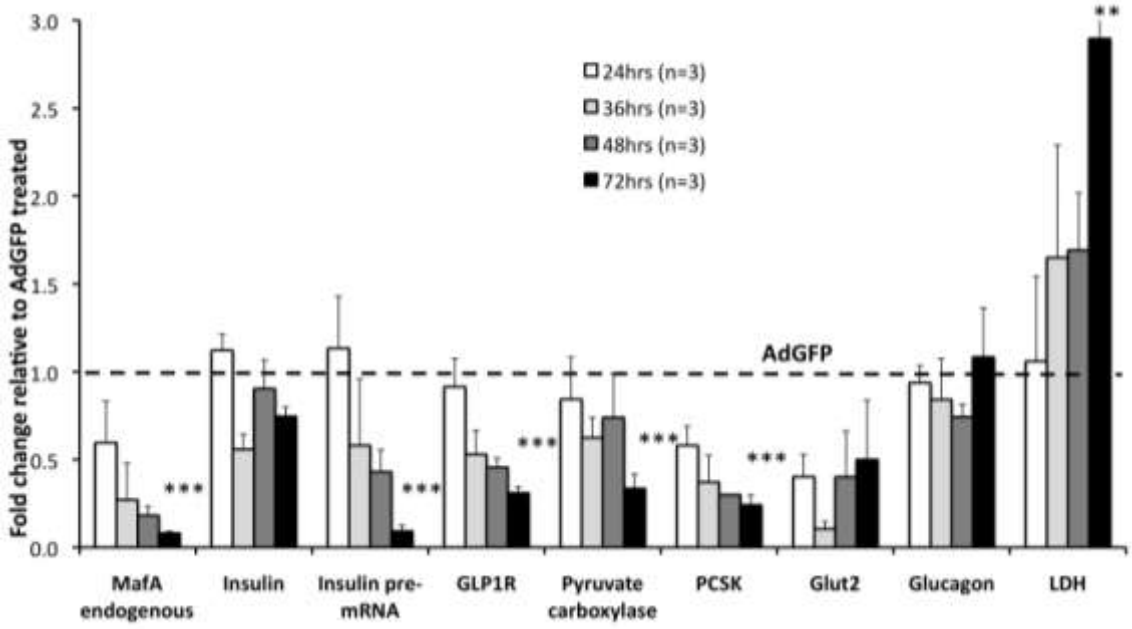

Figure 17. Effect of DN-MafA infection on genes important for beta cell function after various times in culture. DN-MafA very efficiently down-regulates expression of MafA and its target genes in rat islets, whereas glucagon expression is not affected. Interestingly LDH is up-regulated, suggesting a loss of beta cell phenotype. Quantitative real-time PCR analysis after 24, 36, 48 and 72 h of infection with AdDN-MafA. Expression is compared to AdGFP infected islets from the same animals (equal to 1, dashed line). Mean ± SEM, n=4–6 independent experiments; **$p<0.01$, ***$p<0.001$.

Several metabolic genes important for the specialized beta cell phenotype were down-regulated already 48 hours after infection with AdDN-MafA, including glucokinase (Gck) and the components of the mitochondrial membrane shuttles: malic

enzyme (Me1), malate dehydrogenase (Mdh1), glutamate oxalacetate transaminase (Got1) and glycerol-3P-dehydrogenase (Gpd2) (Figure 18). Additionally, the ATP-sensitive K^+ channel subunit Kir6.2 and Ca-channel Cav2.1, both implicated in insulin secretion, were down-regulated by 48 hours after AdDN-MafA infection (Figure 18).

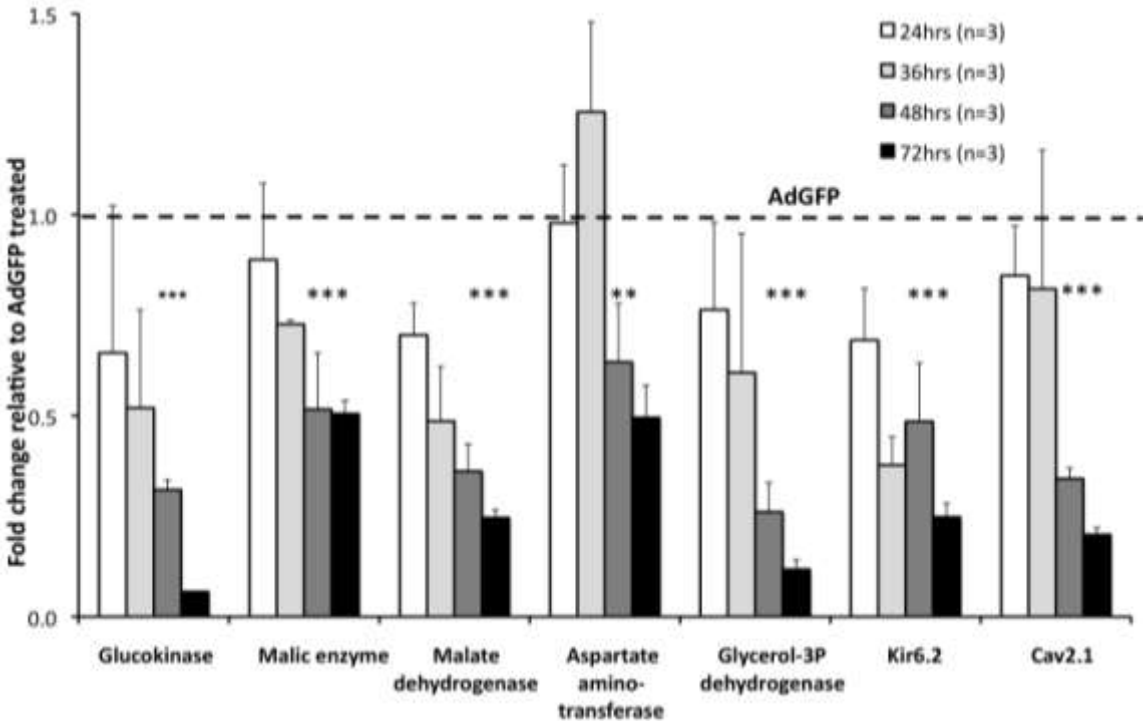

Figure 18. Effect of DN-MafA infection on genes implicated in glucose-stimulated insulin secretion, after various times in culture. DN-MafA down-regulates the expression of several metabolic genes, including genes of the mitochondrial membrane shuttles, as well as ion channels indispensable for the insulin secretory process. Quantitative real-time PCR analysis after 24, 36, 48 and 72 h of infection with AdDN-MafA. Expression is compared to AdGFP infected islets from the same animals (equal to 1, dashed line). Mean ± SEM, n=4–6 independent experiments; **p<0.01, ***p<0.001.

Since many beta cell important genes were down-regulated coincident with the inhibition of MafA transcriptional activation, we determined the expression level of the other key beta cell transcription factors, Pdx1 and NeuroD1, as well as MafB, which is known to be alpha cell specific in the adult rat pancreas. Surprisingly, by 24 hrs after infection with AdDN-MafA, Pdx1 and NeuroD1 mRNAs were down-regulated, reaching around 20% of control by 36 hrs (Figure 19). These data suggest strong

feedback regulation between MafA, Pdx1 and NeuroD1 since only MafA function was perturbed by the DN-MafA. However, DN-MafA also inactivates MafB as seen by the 50% decrease in MafB mRNA. Decreased expression of the three important transcription factors MafA, Pdx1 and NeuroD1 could explain the loss of beta-cell phenotype with AdDN-MafA infection.

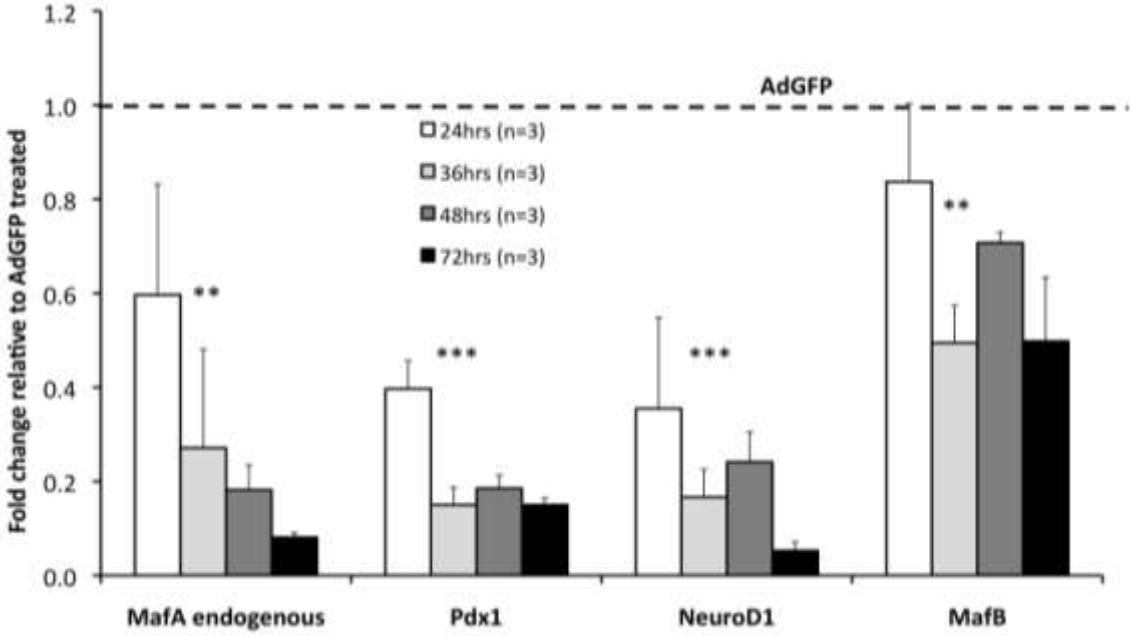

Figure 19. Effect of DN-MafA infection on beta cell important transcription factors, after various times in culture. With the inhibition of MafA function, MafA expression is down-regulated, and coincidently, the expression of Pdx1 and NeuroD1 is also diminishing, suggesting a feedback regulation between MafA, Pdx1 and NeuroD1. Quantitative real-time PCR analysis after 24, 36, 48 and 72 h of infection with AdDN-MafA. Expression is compared to AdGFP infected islets from the same animals (equal to 1, dashed line). Mean ± SEM, n=4–6 independent experiments; **p<0.01, ***p<0.001.

DN-MafA blunts glucose-stimulated insulin secretion (GSIS) in adult rat islets

Insulin secretion from untreated control samples, AdGFP infected and AdDN-MafA infected aggregates were compared after 24, 36, 48 and 72 hours of infection. In static incubations at low (2.8 mM) and high (16.7 mM) glucose concentrations, aggregates infected with AdGFP, the control adenovirus, secreted approximately 50% less insulin at high glucose stimulus than the uninfected aggregates, thus showing that adenovirus infection itself dampened insulin secretion (Figure 20). Even so, the expression of DN-MafA further blunted GSIS from 36 hours onwards in a time-dependent manner

(AdGFP versus AdDN-MafA infection at high glucose, p<0.001). By 72 hours, when important beta cell gene mRNAs were expressed at levels 20-40% of controls, GSIS was lost (Figure 20). The DN-MafA-treated islet cells also had elevated insulin release at basal, non-stimulatory conditions (approximately 3-fold increase at 72 hours, 1.43 vs 4.44 ng/mg insulin, AdGFP vs AdDN-MafA-infected, respectively). Together the increased basal insulin release and the blunted insulin secretion in response to high glucose stimulus suggest that beta cells become dysfunctional with the loss of functional MafA.

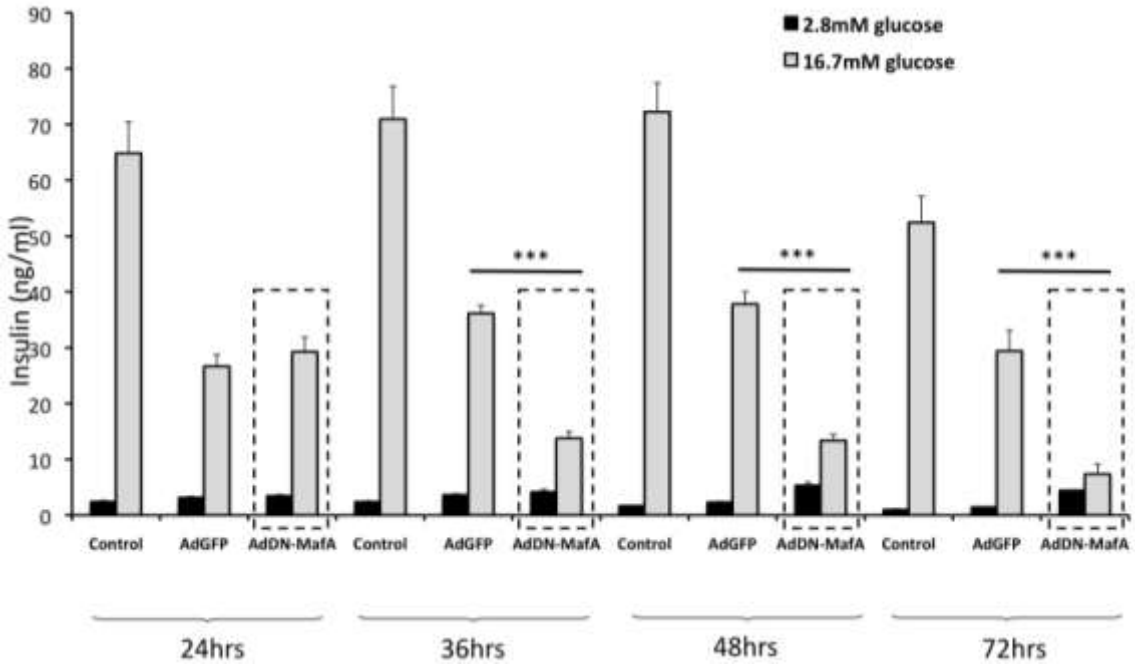

Figure 20. Glucose-stimulated insulin secretion from islet cell aggregates after various time in culture, either untreated or infected with AdGFP or AdDN-MafA. Static incubations at low glucose (2.8 mM, black bars) and high glucose (16.7 mM, grey bars) revealed that adenovirus infection itself inhibits insulin secretion, but DN-MafA further blunts GSIS in a time-dependent manner (marked with dashed rectangles). By 72 hours, when the expression of the important beta cell genes are down to 20-40% of controls, glucose-stimulated insulin secretion is fully inhibited. Mean ± SEM of 3 experiments, done in duplicate for each time point. P values represent comparison between AdGFP and AdDN-MafA infected islet aggregates; *** p<0.001.

Overexpression of NeuroD1 or Pdx1 could not rescue GSIS from AdDN-MafA treated islets

Significant down-regulation of NeuroD1 and Pdx1 upon AdDN-MafA infection may

contribute to the loss of glucose responsiveness. To explore this possibility, additional adenoviruses (AdNeuroD1 and AdPdx1) were used to over-express transcription factors NeuroD1 and Pdx1 in AdDN-MafA infected islets. The adenoviral infections were carefully titrated to reconstitute normal levels of Pdx1 and NeuroD1 expression based on qPCR analysis. Glucose-stimulated insulin secretion at 72 hours after infection demonstrated that with AdNeuroD1 or AdPdx1 alone, islets were glucose responsive similarly to control AdGFP infected islets (Figure 21). However, even co-infection with AdNeuroD1 or AdPdx1 could not rescue GSIS in DN-MafA expressing islets even though NeuroD1 and Pdx1 mRNAs were reconstituted to normal levels as measured in AdGFP islets (Figure 21).

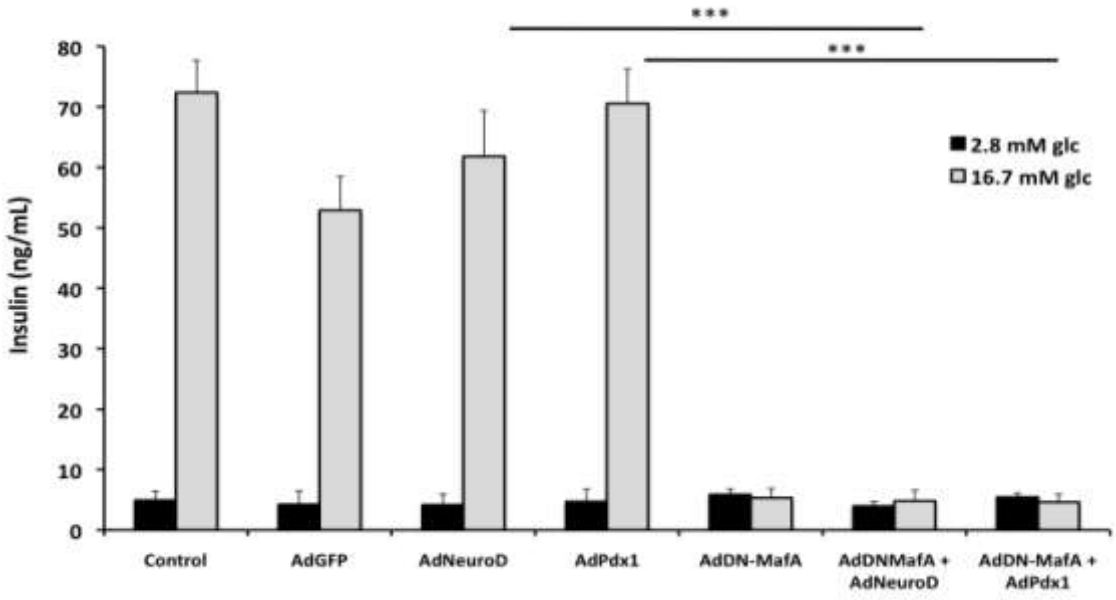

Figure 21. Glucose-stimulated insulin secretion from islet cell aggregates after infection with AdGFP, AdNeuroD1, AdPdx1, or AdDN-MafA alone, and in combination. Static incubations after 72 hours of culture at low glucose (2.8 mM, black bars) and high glucose (16.7 mM, grey bars) revealed that dominant-negative MafA resulted in complete inhibition of glucose-stimulated insulin secretion that could not be rescued by overexpression of Pdx1 or NeuroD1. Mean ± SEM of 3 experiments, done in duplicate for each condition. P values represents comparison between insulin release at high glucose from AdNeuroD1 alone vs AdNeuroD1 plus AdDN-MafA co-infected islet aggregates; and similarly, AdPdx1 alone vs AdPdx1 plus AdDN-MafA co-infected islet aggregates; *** $p<0.001$.

The total insulin content of islet aggregates after 72 hours in culture were approximately the same for untreated islets and adenovirally infected islets, irrespective of the over-expressed genes (Figure 22). The DN-MafA expressing, glucose-

unresponsive islets had normal insulin stores, yet had decreased expression of key beta-cell metabolic genes and blunted GSIS, so we conclude that the stimulus-secretion coupling mechanism becomes dysfunctional with the inhibition of MafA function.

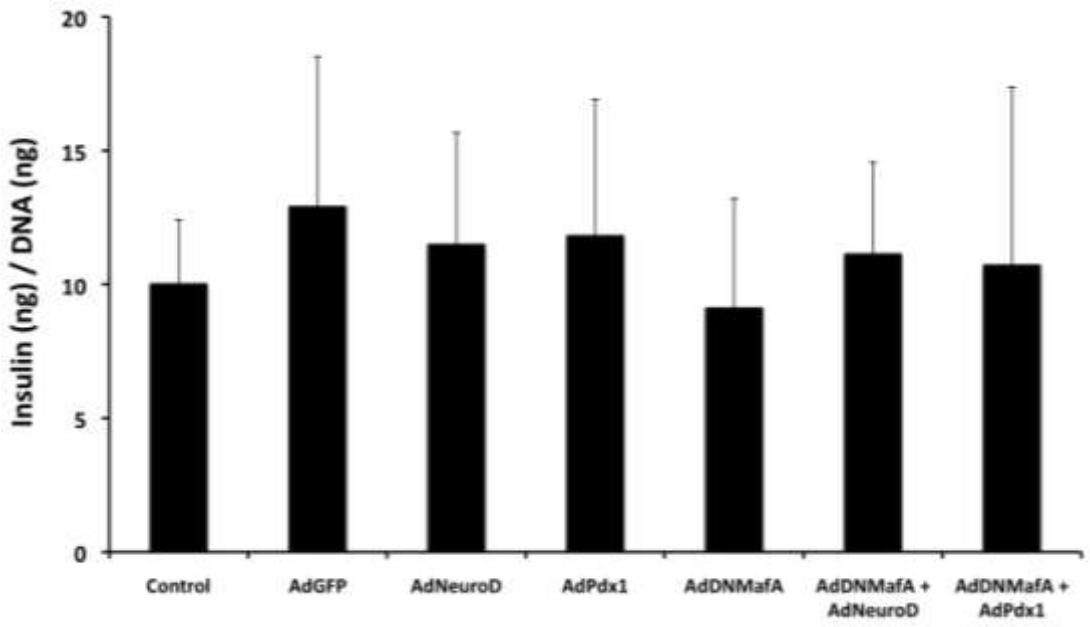

Figure 22. Insulin content of islet cell aggregates after various adenovirus infections. Total insulin content of beta cells are not affected by DN-MafA or overexpression of NeuroD1 and Pdx1 during the 72 hour culture period. Insulin content is expressed as ng/ ng DNA. Mean ± SEM of 3 experiments, done in duplicate for each condition. P values are not significant in any comparison.

As the beta cells lacking functional MafA have normal insulin stores, we wondered if they could respond to other insulin secretagogues. The responsiveness of the DN-MafA infected islets to amino acids and depolarizing agent stimuli was tested in a separate set of experiments 72 hours after AdDN-MafA infection (Figure 23). As before, the islet cell aggregates displayed blunted insulin secretory response to high glucose. Similarly, stimulation with 10 mM leucine + 4 mM glutamine was unable to elicit insulin secretion. However, 10 mM arginine, and to a lesser extent 30 mM KCl, at low glucose (2.8 mM), stimulated insulin secretion from AdDN-MafA infected islets ($p<0.001$ compared to glucose stimulated insulin secretion) (Figure 23).

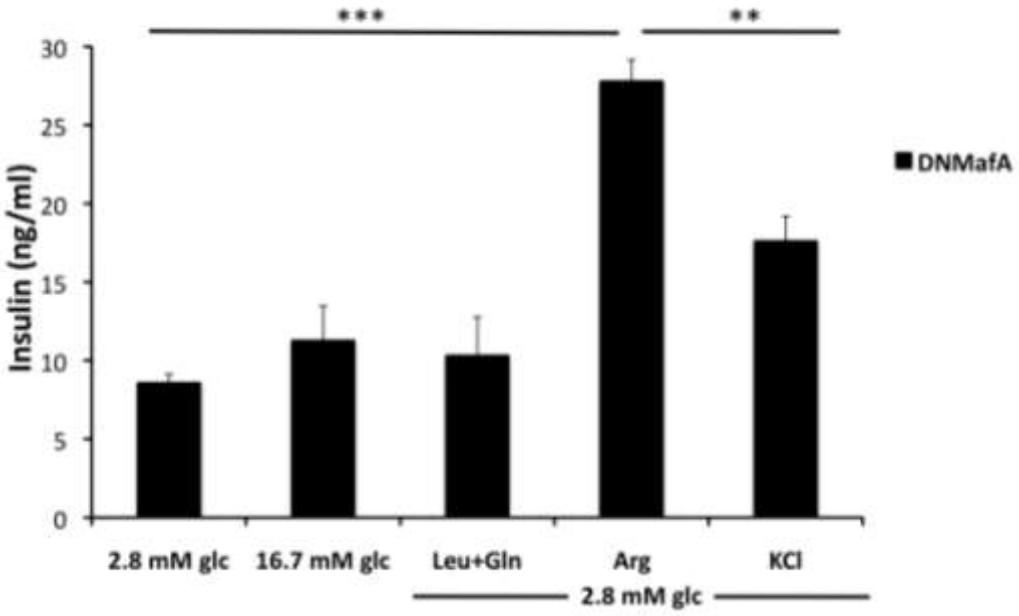

Figure 23. Insulin secretion from AdDN-MafA infected islet cell aggregates in response to different secretagogues (16.7 mM glucose, 10 mM leucine + 4 mM glutamine, 10 mM arginine and 30 mM KCl), after 72 hours in culture. Non-glucose secretagogues were used in the presence of low glucose concentration. Only depolarizing agents arginine and KCl elicit insulin secretion from DN-MafA treated cells. Mean ± SEM of 3 experiments, done in duplicate for each time point. P values represent comparison between static incubations with arginine vs other secretagogues; ** $p<0.01$; *** $p<0.001$.

Characterization of the role of MafA in dysfunctional beta cells

Dysfunctional GK rat islets express less insulin and MafA mRNA but can be partially rescued

Since the loss of MafA led to dysfunctional beta cells, we wondered if over-expression of MafA could rescue dysfunctional beta cells that had low MafA expression and impaired GSIS. We identified the GK rat model as a possible model for this experiment since it is considered as a "prototype" for the diseased human beta cell with poor insulin secretory response [122] and their dysfunctional phenotype remains even after several days of culture [123]. At 16 weeks age, female GK rats, which were not overtly diabetic compared to Wistar-Kyoto (WKY) controls, had higher random blood glucose values and lower body weights (136±5 vs 111±3 mg/dl, p=0.003; and 229±4 vs 276±3 gramm, p<0.001; GK vs WKY, respectively). The GK rats have poor glucose tolerance and insulin secretion by IPGTT compared to WKY controls (Figure 24).

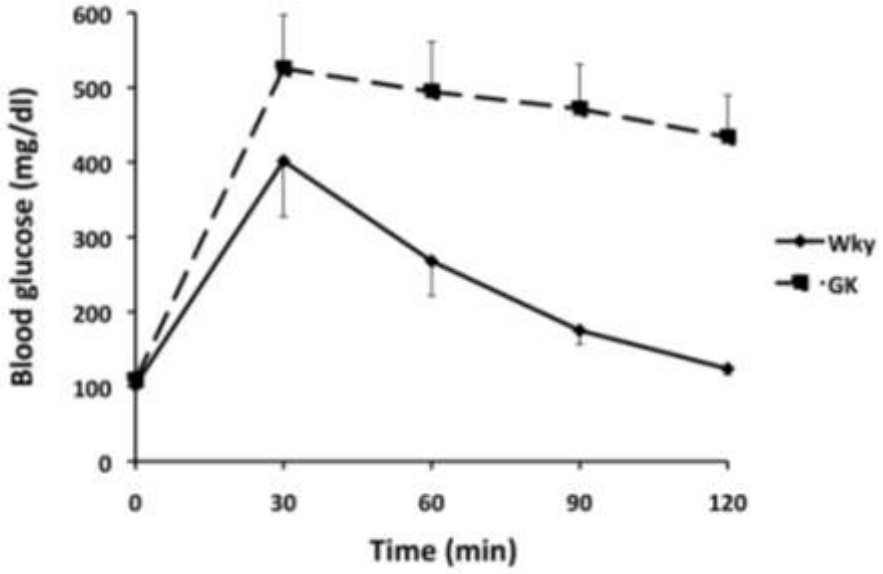

Figure 24. Intraperitoneal glucose tolerance tests (IPGTT) in GK and WKY control rats. Rats were fasted overnight, then injected intraperitoneally with 10% glucose solution at a dose of 2 g/kg body weight. Blood glucose readings were performed from the tail vein at 0, 30, 60, 90 and 120 minutes post injection. Measurements revealed that GK animals have normal fasting blood glucose, but display a diabetic post-challenge curve. N=5 in each group.

By quantitative real-time PCR (Figure 25) isolated GK islets had decreased insulin and MafA mRNA levels compared to WKY islets (41%, $p<0.001$ and 57%, $p=0.021$, respectively), unchanged NeuroD1 and Pdx1 mRNA levels and 51% higher glucagon expression in the GK islets ($p=0.022$).

Next, adenovirus mediated MafA overexpression was used to reconstitute MafA levels in dysfunctional GK rat islets. MafA over-expression ameliorated glucose-stimulated insulin secretion from these dysfunctional islets. Uninfected GK islet cell aggregates after 3 days of culture responded only modestly to the glucose stimulus control whereas WKY islet cell aggregates elicited a robust insulin secretory response ($p<0.001$, Figure 26).

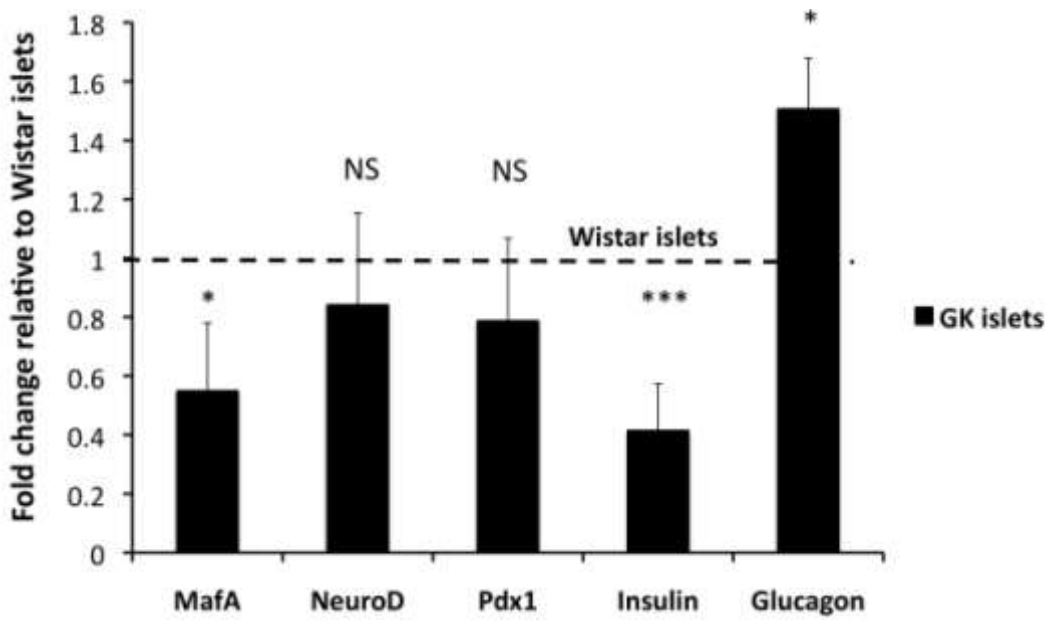

Figure 25. Expression levels of important beta cell transcription factors, insulin and glucagon in dysfunctional GK islets compared to control WKY islets. Isolated GK islets have decreased insulin and MafA mRNA levels compared to WKY islets, whereas NeuroD1 and Pdx1 levels are similar, and glucagon expression is significantly higher. Quantitative real-time PCR, data expressed as fold change with respect to WKY (marked as 1, dashed line) using S25 as internal control gene. Mean ± SEM, n=3 isolated samples. P values represent comparison between expression levels of GK and WKY islets; * $p<0.05$; *** $p<0.001$.

Comparing the insulin release in response to high glucose of AdGFP infected and uninfected islets of both strains, we again found that adenoviral infection impaired

somewhat the insulin response. When MafA over-expression was titrated carefully to obtain near physiological MafA levels in GK islets, comparable to that of the WKY islets, glucose responsiveness of GK islets was enhanced compared to AdGFP infected GK controls ($p<0.001$). In AdMafA-infected GK islets the fold change of insulin secretion between low and high glucose stimulus was 8.1, compared to the 5.6 fold change in uninfected GK islets, which corresponds to a 43% increase. MafA over-expression seemed to influence primarily the amount of insulin released at basal non-stimulatory conditions (at 2.8 mM glucose AdMAfA infected: 1.8 ng/ml insulin; AdGFP infected: 3.3 ng/ml insulin; uninfected GK islets: 2.8 ng/ml insulin at low glucose). WKY control untreated islets secreted similarly low 1.1 ng/ml insulin during low glucose incubations. Thus, MafA reconstitution seems to ameliorate the dysfunctional beta cell phenotype of the GK rat islets.

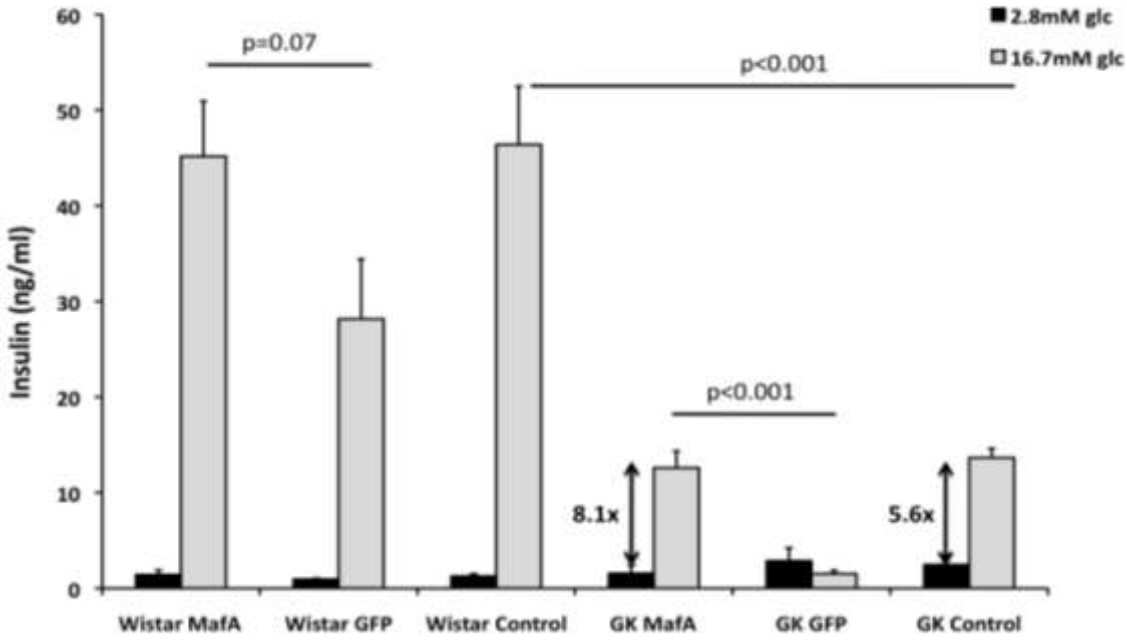

Figure 26. Effect of MafA over-expression on glucose-stimulated insulin secretion from WKY and GK islet cell aggregates. WKY and GK islets were infected with AdMafA, AdGFP, or remained untreated. Static incubations after 72 hours of culture at low glucose (2.8 mM, black bars) and high glucose (16.7 mM, grey bars) revealed that MafA over-expression in GK islets improves the fold change of insulin release (8.1x), compared to control (5.6x) GK islets. As observed before, adenovirus infection itself inhibits insulin secretion compared to control islet aggregates. Mean ± SEM of 3 animals, done in duplicate for each condition. P values represent comparison between insulin secretion in response to high glucose stimuli, after different treatments.

5. Discussion

The physiological experiments of Grill et al. [94] and Bliss et al. [95] showed a continuum in maturation of insulin secretion from birth to adult in response to glucose and to different secretagogues [94]. In both studies glucose-stimulated insulin secretion was minimal at P7 and even at 3 weeks age it was not as robust as in the adult islets. To address the mechanisms of this immature insulin secretion, we performed gene expression studies using beta cell-enriched samples and microarray technology. Our microarray results were confirmed for a number of genes by qPCR analysis on isolated rat islets. While changes in the proportion of beta cells in isolated islets could have affected the results, the proportion of beta cell/islet did not significantly differ among P2, P21 and adult islets and so could not explain the gene expression results (Table 5).

Comparison of the transcriptosomes of neonatal and adult beta cells showed a number of differentially expressed genes, particularly those critical to the specialized beta cell metabolism. Pyruvate enters the Krebs cycle via decarboxylation by the pyruvate dehydrogenase complex or via carboxylation and conversion to oxaloacetate by pyruvate carboxylase (Figure 8). In adult rat islets approximately 50% of the glucose-derived carbon enters the Krebs cycle via carboxylation, which is correlated with glucose metabolism and insulin release [83]. Adult beta cells show strong pyruvate carboxylase activity [83] that facilitates anaplerotic flux into the Krebs cycle that leads to the generation of other putative stimulus–secretion coupling factors, such as NADPH [82, 83] or malonyl-CoA [124]. Knocking-down pyruvate carboxylase resulted in impaired glucose-stimulated insulin secretion in INS-1 cells [125, 126] but not in isolated rat islets, in which compensatory increased enzyme activity was detected [127]. Another unique feature of the adult beta cell is the presence of mitochondrial membrane shuttles, including the glycerol phosphate, malate–aspartate, pyruvate–citrate and pyruvate–malate shuttles, through which glycolysis-derived cytosolic NADH is reoxidized [82, 83, 128].

However, in neonatal beta cells pyruvate would be handled differently. Pyruvate carboxylase, the key anaplerotic enzyme gene, was expressed significantly lower through the third postnatal week. Both lactate dehydrogenase A (LdhA) and monocarboxylic transporter 1 (Slc16a1) were elevated in the neonatal microarray data

but missed significance. However, by qPCR, both were significantly elevated at P2 (seven- and fourfold; p=0.040 and 0.056, respectively) and P7 (17- and 7-fold, respectively; both p=0.001), and by P15 LdhA was still higher than adult (9.5- fold; p=0.004) but not Slc16a1 [129]. Their expression would divert pyruvate away from the mitochondria towards lactate production.

Both the glycerol phosphate and malate–aspartate shuttles had been reported to have lower expression in fetal rat islets compared with adult, with induced overexpression of Gpd2 in fetal islets improving glucose-stimulated insulin secretion [97]. Here, we show that not only the genes encoding the enzymes of the two main NADH shuttle systems (the mitochondrial Gpd2 of the glycerol phosphate shuttle, and the cytosolic Got1 and Mdh1 of the malate–aspartate shuttle) had differentially lower expression in neonatal beta cells, but by qPCR so did cytosolic malic enzyme, a key enzyme in the pyruvate–malate shuttle; mRNA for all four enzymes had less than 40% expression of adults through P21 and had not reached adult levels by P28. While previous studies have shown that the impairment of any of these enzymes could lead to diminished glucose-stimulated insulin secretion *in vitro* [119, 126] or *in vivo* [97, 130], we found all three shuttles and pyruvate carboxylase had very low gene expression during the neonatal period. Overall, the metabolic specialization found in adult beta cells for amplifying the ATP-derived from glycolysis is missing in neonatal beta cells.

A limitation of our study is that while we mainly investigated gene expression levels of enzymes, the activity of enzymes can be regulated at many levels. The assumption that low mRNA levels correlate with low enzymatic activity is reasonable, and our immunostaining data on PK and GPD2 proteins provide additional assurance. Both Pk and Gpd2 had low mRNA expression in P2 islets and were equally low even at 1 week after birth. However, increased protein production of both of these two enzymes at 1 week is consistent with physiological results showing a slight glucose-responsive insulin secretion in P7 islets [94].

Interestingly, Cpt1 and Cpt2, encoding carnitine palmitoyl transferases 1 and 2, which are involved in the transport of long-chain acyl-CoA molecules from the cytosol

through the mitochondrial membrane, were differentially expressed. In neonates Cpt1 was highly expressed while Cpt2 has lower expression by array data. By qPCR, Cpt1 mRNA in P2 neonatal islets did not differ from adult; however, Cpt2 was indeed expressed at low levels (20%). A natural inhibitor of Cpt1 is the glucose-derived malonyl-CoA, thus fatty acid oxidation is suppressed when glucose is in abundance and oxidized in the cell. Consequently, fatty acids diverted from oxidative pathway flux into various esterified products, such as diacylglycerol and phospholipids, which have been suggested as triggering factors in glucose-stimulated insulin secretion [131]. In neonatal beta cells, a possible consequence of high Cpt1 expression could be high enzymatic activity that was not fully inhibited by malonyl-CoA, suggesting that lipids may be diverted to beta-oxidation and are potentially a more important fuel in fetal/neonatal beta cells than in adults. Interestingly, over-expression of Cpt1 has been demonstrated to diminish GSIS *in vitro* [132]. Considering the immaturity of other mitochondrial shuttles and the scarcity of pyruvate-cycling based coupling factors, abundant lipids in fetal and neonatal beta cells might act as important coupling factors regulating insulin secretion.

Dietary factors markedly differ during the neonatal period and adults [133], so the lack of glucose-responsive insulin secretion is not really an impairment but an evolutionary adaptation to neonatal fuel. In neonates fatty acid and glycerol levels are higher and only after weaning decrease to the adult fed values [134]. In addition, there is the switch from solute carrier family 27 (fatty acid transporter), member 5 (Slc27a5) to member 1 (Slc27a1) fatty acid transporters. Slc27a1 is a major insulin-sensitive long-chain fatty acid transporter, whereas Slc27a5 expression is exquisitely liver-specific in adulthood and its over-expression in other mammalian cells mediates long-chain fatty acid uptake [135]. We speculate that since neonatal beta cells lack the major fatty acid transporter Slc27a1, they use the transiently produced Slc27a5 transporter for lipid uptake. Blood glucose levels rise after birth and by P7 are similar to the adult fed state [134]. While these physiological variables may drive the expression of some of the beta cell-specific metabolic enzymes, the different patterns of gene expression that we found suggest complex and varied regulatory networks. The coincidence of generalized low expression of many key beta cell genes in neonatal islets and the lack of glucose

responsiveness suggests the existence of a critical regulator of functional maturity. Not all key beta cell transcription factors were represented on the microarrays used so determination of such a maturation factor by microarray was not possible. In subsequent experiments [105], we explored the role of MafA and Pdx1 in neonatal beta cell maturation.

We tested two of the three (Pdx1, MafA, NeuroD1) suggested transcription factors essential for beta cell specificity and insulin gene expression [69]. We demonstrated a close relationship between the expression patterns of MafA and some of its putative targets, including insulin, during this neonatal period (Figure 12). As MafA expression from P2 through P7 was less than 10% of adult, reconstitution of MafA at P2 provides means to unequivocally evaluate effects of MafA on beta cell functional development. In such reconstitution experiments, overexpressing MafA in neonatal cells led to their acquisition of an insulin-secretory profile similar to adult beta cells, with a greater insulin secretion in response to high glucose concentrations. Thus, increased MafA expression may represent a mechanism responsible for enhancing the beta cell functional maturity.

By contrast, Pdx1 reached adult levels by P7 and had a postnatal expression pattern that differed from the tested beta cell genes including insulin, Glut2 and Gck. Importantly, Pdx1 reconstitution in P2 beta cells failed to increase glucose responsiveness within the experimental timeframe. However, Pdx1 overexpression up-regulated MafA mRNA expression, increased insulin content and increased insulin secretion in both low and high glucose concentrations.

The expression patterns of MafA and Pdx1 during the neonatal period were distinct even though both have been implicated in regulating expression of the other [100, 136]. The parallel expression of insulin and MafA, the up-regulation of MafA expression by Ad-Pdx1, and previous observations that PDX1high is expressed before MAFA in insulin+ cells during development [65] are consistent with Pdx1 being an upstream regulator of MafA. Even though the induction of glucose responsiveness was not achieved by Pdx1 over-expression within the timeframe of our experiments, we expect that with longer incubation Pdx1-infected P2 cells would acquire glucose

responsiveness.

A previous report [100] had suggested Gck, Pcsk1 and Glp1r as putative MafA targets. As MafA knockout mice did not show any embryonic phenotype, it was not surprising that a recently published paper [137] failed to detect these genes as being directly under the control of either MafA or MafB in E18.5 MafB null mice. However, our data of parallel expression of Glp1r, insulin, Glut2 and Pcsk1 with MafA support their being MafA targets postnatally. Gck expression was up-regulated by both Pdx1 and MafA over-expression, yet the time course of its expression differed significantly from both. In addition to MafA and Pdx1, physiological stimuli, such as thyroid hormones [138], may regulate Gck expression.

A previous report showing developing insulin+ cells switching from MafB+MafA–Ins+ to MafB–MafA+Ins+ cells [65], suggested that MafA may be important for the postnatal functional maturation of beta cells. Our current results strongly indicate such a role. While global MafA knockout mice showed embryonic MafA expression was not essential for islet architecture at birth, that study did not address its function in maturation of beta cells [68]. A modest increase in fasting glucose at 4 weeks was the earliest defect reported in the MafA knockout mice, and this was followed by impaired glucose-stimulated insulin secretion at 8 weeks. There was no analysis of whether this impairment was due to a lack of maturation, an intrinsic defect or glucotoxicity. Neonatal rodent beta cells are analogous to a loss-of-function model owing to their very low MafA expression at birth, and we suggest that the acquisition with mature beta cell function after reconstitution of MafA expression to levels comparable with adult beta cells essentially represents a functional rescue. Demonstration of physiological signals regulating MafA expression during the neonatal period will underscore its importance as an essential transcription factor for functional maturation of beta cells.

Maternal hyperglycemia leads to precocious maturation and hyperplasia of fetal beta cells [93, 139], and chronic *in vitro* exposure of fetal beta cells to elevated glucose levels induced maturation of glucose-stimulated insulin secretion [93]. Both MafA and

Pdx1 are glucose-responsive transcription factors and so could have enhanced expression. However, increased expression of MafA, but not Pdx1, converted immature neonatal beta cells into glucose-responsive cells within the timeframe studied, supporting the more direct role of MafA in the maturation process.

In the second part of our experiments, we have focused our attention on adult, mature beta cells, and investigated the role of transcription factor MafA in mature beta cell phenotype. We have demonstrated that in adult rat islets, DN-MafA suppresses the function of endogenous MafA at the same time as down-regulating its expression and that of important beta cell genes, including insulin, GLP1R, metabolic enzymes and transcription factors.

Normally, MafA forms homodimers to initiate target gene expression, i.e. insulin, by binding to "mare" (MafA responsive element) promoter region. MafA protein consists of two domains: the N-terminal transcriptional activation domain and the C-terminal DNA-binding and dimerization domain [77]. In case of the DN-MafA construct, the N-terminal domain is missing, however the C-terminal domain is preserved, therefore it can bind to the endogenous full MafA protein, and can very efficiently block its transcriptional activation [65]. DN-MafA has been shown to inactivate MafA transcriptional function in insulin promoter luciferase assays [65]. Furthermore, DN-MafA blunts glucose-stimulated insulin secretion from beta cell lines [100], but had not been extended to primary cells before our study. MafA is a key activator of insulin gene but has also been implicated to control genes of the insulin biosynthesis and secretion. The N-terminally deleted DN-MafA was shown to reduce the transcript levels of Glp1r, Pdx1, Nkx6.1 and Pcsk in the INS1 cell line [100]. We have confirmed these putative MafA target genes in primary rat beta cell cultures, and we went further to demonstrate a systematic down-regulation of metabolic genes implicated in insulin secretion, upon infection with DN-MafA. Our results suggest that MafA either directly or indirectly regulates many important genes in beta cells to ensure glucose-responsive insulin secretion.

Among the genes down-regulated with the loss of MafA function in adult beta cells

were key transcription factors Pdx1 and NeuroD1 (Figure 19). MafA and Pdx1 were shown to regulate the expression of the other [100, 136], but to the best of our knowledge, this is the first report to show that MafA can also regulate NeuroD1. Further research is warranted in order to describe the intricate positive- and negative feedback mechanisms between these critical beta cell transcription factors.

The inhibition of MafA expression and function resulted in impaired glucose-stimulated insulin secretion, which could not be rescued by over-expression of other key transcription factors, such as Pdx1 and NeuroD1. However, islets lacking functional MafA had normal insulin content and were still secreting insulin in response to depolarizing agents. Considering the decreased expression of key beta-cell metabolic genes, we suggest that the stimulus-secretion coupling mechanism becomes dysfunctional with the inhibition of MafA function.

A strength of our study is a novel dissociation-reaggregation protocol developed for adenovirus infection of islet cells. The consistent and very high efficiency of infection of primary beta cells using this protocol is clearly advantageous to previous non-dissociation adenoviral techniques. In addition, the method prevents beta cell loss due to central necrosis of intact islets in culture. In previous studies our estimates of infection were only 50-60% of the total islet with much of the core of islets not infected [140], and infections occurring only in the mantle region.

With the inhibition of MafA function in adult beta cells, we managed to create a dysfunctional beta cell model, similar to neonatal beta cells. Neonatal beta cells and our model of DN-MafA induced dysfunctional beta cell have many features in common: (1) decreased expression of functionally important genes; (2) disrupted stimulus-secreting coupling; (3) impaired glucose responsiveness. Furthermore, based on our results, dysfunctional or immature beta cells lack MafA function. We established that overexpression of MafA in neonatal islets contributes to the acquisition of glucose responsiveness in neonatal beta cells. MafA also induced the expression of metabolic genes implicated in insulin secretion, including Gck and Glp1r.

Conversely, inhibition of MafA function in adult rat islet resulted in the loss of glucose

responsiveness, and diminished the expression of a set of metabolic genes, including the previously identified two MafA targets: Gck and Glp1r.

It is noteworthy, that the loss of MafA mRNA and protein expression occurs in dysfunctional beta cells from rats 4 weeks after 90% partial pancreatectomy and from 12-week-old hyperglycemic db/db mice [106]. In addition, a globally decreased expression of beta cell important genes has been reported in the partial pancreatectomy model of hyperglycemic rats [141, 142]. Based on these observations, we hypothesize that decreased levels of transcription factor MafA is the culprit.

In order to corroborate the critical role of MafA in normal beta cell function, we sought to perform a rescue experiment with adenovirus mediated MafA over-expression, in a diabetic condition where endogenous MafA level is lower and GSIS is impaired. The over-expression of MafA in DN-MafA infected cells was not feasible due to the mechanism of action of the MafA mutant. Therefore, we have identified the GK rat model as a valuable tool to study MafA over-expression in dysfunctional beta cells. The GK rat beta cell is considered to be a "prototype" for the diseased human beta cell [122] with poor insulin secretory response. To date, the expression profile of beta cell important transcription factors has not been characterized in the GK islets. Importantly, unlike most other models, the GK islets preserve their dysfunctional phenotype even in culture [123] rendering them suitable for an *in vitro* experiment.

When MafA over-expression was titrated to obtain near physiological MafA levels in GK islets, insulin secretion as fold change improved compared to control and Ad-GFP-infected GK islets (Figure 26). The amelioration of insulin secretion was mostly attributable to decreased basal insulin release from the GK islets. Of note, immature beta cell also release high amount of insulin in non-stimulatory conditions, and regulated insulin secretion was only achieved by MafA over-expression (Figure 14). Thus, we concluded that MafA is a critical regulator of mature beta cell function.

Although much emphasis has been put on elucidating the transcription factors involved in the embryonic differentiation (reviewed by Jensen [143]), and dysfunctional transformation of beta cells, the identification of factors that drive functional

maturation is also essential to harvest the true potential of human embryonic stem cells. Currently, *in vitro* differentiated insulin-positive cells lack glucose-stimulated insulin secretion and still express MafB [15, 104]. These human embryonic stem cell-derived insulin+ cells were shown to become glucose responsive and start to express MafA only several months after transplantation [16]. Thus, identification of the neonatal physiological stimuli that regulate MafA and Pdx1 expression will be important for developing *in vitro* strategies to convert stem-cell derived insulin-expressing cells into mature, fully functional beta cells for cell-based therapies for diabetes.

Furthermore, MafA as a potent activator of insulin and other beta cell important genes, and a critical regulator of beta cell function serves as a novel therapeutic target for diabetes.

In conclusion, we have characterized a spectrum of beta cell phenotypes with varying function, from the newly differentiated, immature cell, through the mature, glucose-responsive state, to the dysfunctional beta cell. We propose that transcription factor MafA is a master regulator in the molecular mechanisms underlying the beta cell spectrum of function (Figure 27).

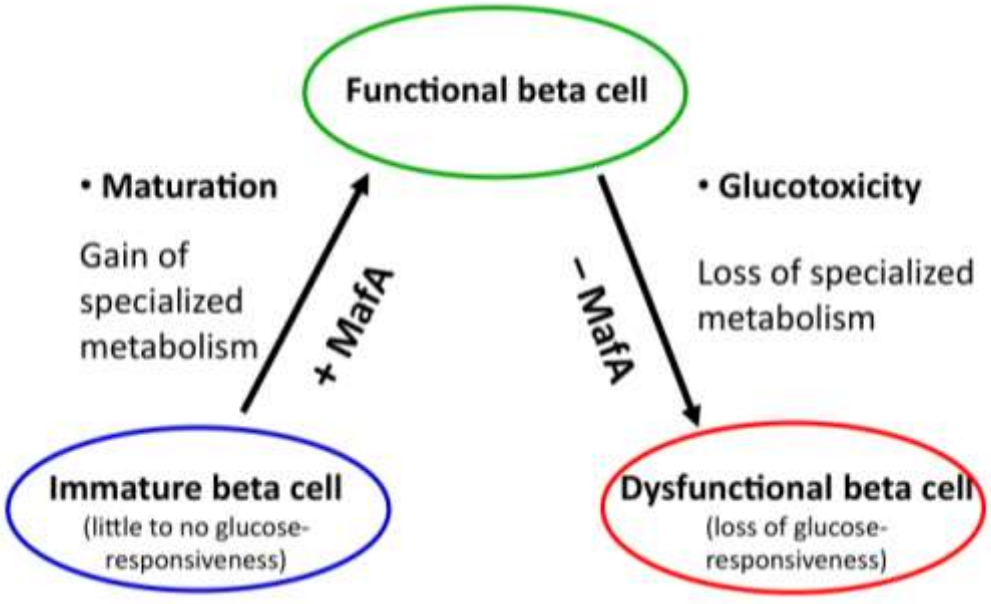

Figure 27. Paradigm of the beta cell, with MafA as a critical regulator of beta cell phenotype.

6. Conclusions

1. The lack of glucose responsiveness in neonatal islets is likely to be due to a generalized immaturity of the metabolic specialization of pancreatic beta cells.

2. In the process of functional maturation, to acquire glucose-responsive insulin secretion, neonatal beta cells undergo a coordinated gene expression program in which MafA plays a crucial role.

3. MafA knock-down leads to an immature phenotype with decreased expression of functionally important beta cell genes and selectively impaired glucose-stimulated insulin secretion in mature beta cells of primary rat islets. MafA plays a critical role in adequate beta cell function.

4. MafA over-expression in dysfunctional beta cells of GK rat improved insulin secretion by decreasing basal insulin release.

7. Summary

Beta cells are highly differentiated cells that maintain blood glucose levels in the physiologically relevant range by rapid and precise changes in insulin secretion. Specialised metabolic pathways ensure the coupling of external nutrient stimuli to insulin secretion in adult beta cells. Although the metabolism of adult beta cells has been characterised extensively, that of neonatal beta cells is less well understood. Neonatal beta cells lack glucose responsiveness despite adequate insulin reserves.

We postulated that the mechanisms responsible for this immaturity are complex, and performed a microarray analysis to compare gene expressional profiles of neonatal and adult beta cells. Key metabolic enzymes characteristic of beta cell phenotype were among the genes with differentially low expression in neonatal beta cells, and these findings were confirmed by quantitative real-time PCR and immunohistochemistry. Thus, the generalised low expression of key metabolic genes may account for the poor glucose-responsiveness of neonatal beta cells. In subsequent experiments, the role of MafA transcription factor was explored in neonatal beta cell maturation. MafA is a glucose-responsive transcription factor selectively expressed in adult beta-cells, and previously it has been implicated in both beta cell maturation and regulation of insulin synthesis and secretion. By enchancing the expression levels of MafA in primary rat neonatal beta cells, we have established that MafA contributes to the acquisition of glucose responsiveness during beta cell maturation.

Next, to characterize the importance of MafA in regulating beta cell function in adult rat islets, we knocked-down MafA function by adenoviral-mediated expression of dominant negative form of MafA. The lack of MafA in primary adult rat islet cells resulted in decreased expression of functionally important beta cell genes and selectively impaired glucose-stimulated insulin secretion. We concluded that MafA is necessary for the mature beta cell phenotype.

Finally, to test the role of this transcription factor in dysfunctional beta cells, we studied isolated islets from Goto-Kakizaki (GK) rats with impaired glucose tolerance.

In GK islets, insulin and MafA mRNA were decreased and when MafA over expression was titrated to obtain near physiological MafA levels, the insulin secretion was improved. Thus, glucose-responsiveness of the mature beta cells is dependent on adequate MafA function.

Based on our results, MafA may serve as a potential candidate for drug development for treating patients with type 2 diabetes.

8. Összefoglalás

A béta-sejtek a vércukor háztartás szabályozásáért felelős, jól differenciált sejtek, melyek glukóz stimulusra inzulin szekréciós válasszal reagálnak, és fiziológiás körülmények között a vércukorszintet normális tartományban tartják. A glukóz-stimulált inzulin szekréció finom szabályozását a béta-sejtben számos metabolikus út más sejtektől eltérő expressziója biztosítja. A béta-sejtek metabolikus folyamatai érett, egészséges sejtekben jól karakterizáltak, azonban az éretlen, glukózra nem reagáló, neonatális béta-sejtek metabolizmusa kevéssé ismert. Hasonló módon hiányosak ismereteink a diabeteses béta-sejtek metabolizmusának szabályozásáról. Látszólag az éretlen, neonatális béta-sejtek, valamint a diabeteses béta-sejtek nagyon eltérőek, azonban fenotípusuk megegyező: glukóz stimulusra nem reagálnak megfelelő inzulin szekrécióval. Kutatómunkám során a béta-sejt fenotípusának (éretlen – érett – diabeteses) változásaival foglalkoztam.

Kutatásaim első fázisában az éretlen, neonatális béta-sejtek génexpressziós mintázatát tanulmányoztam. Eredményeim szerint a glukóz stimulusra adott elégtelen inzulinszekréciós válasz hátterében egy komplex génexpressziós mintázat áll. A patkány béta-sejtek érését követve az élet első 4 hetében a vizsgált fehérjék expressziós szintje fokozatosan emelkedik, a glukóz-stimulált inzulin szekréció megjelenésével párhuzamban. A génexpressziós mintázat összehangolt változása egy regulátor fehérje működését feltételezi, és az ismert béta-sejt specifikus, inzulin gént szabályozó transzkripciós faktorok közül a MafA (musculoaponeurotic fibrosarcoma oncogene homolog A) fehérje expressziós mintázata hasonlított legjobban a vizsgált metabolikus enzimekére. Kísérleteim második szakaszának eredményei szerint, az exogén módon, adenovírus infekció segítségével bejuttatott MafA kifejeződése a neonatális béta-sejtekben fontos metabolikus enzimek indukciójához, és a glukóz-stimulált inzulin szekréció fokozódásához vezet. Kísérleteim harmadik szakaszában a MafA regulációs szerepének megismerését tűztem ki célul, és érett béta-sejtekben a MafA funkciót szelektíven kikapcsoltam egy “domináns negatív” konstrukcióval (DNMafA). Az alacsony MafA szint számos metabolikus enzim, béta-sejt specifikus gén és egyéb transzkripciós faktor expressziójának csökkenését eredményezte, valamint funkcionális

vizsgálatok szerint csökkent a glukóz-stimulált inzulin szekréciós válasz is. Eredményeim szerint a béta-sejtek a MafA kikapcsolásával egy éretlenebb állapotba jutnak, mintegy dedifferenciálódnak, és a neonatális, értelen sejtekhez hasonló fenotípust vesznek fel. Kísérleteim negyedik részében arra kerestem választ, hogy diabeteses patkányokból izolált, alulműködő béta-sejtekben a MafA expresszió növelésével javítható-e a glukóz-stimulált inzulin szekréció. Az adenovírus infekció segítségével bejuttatott MafA az inzulinszekréciós választ várakozásainknak megfelelően javította.

Eredményeimet összefoglalva megállapítható, hogy az alacsony MafA szint számos fontos béta-sejt gén expressziójának csökkenésével és a glukóz-stimulált inzulinszekréció gátlásával jár. Ez az állapot jellemzi a neonatális, éretlen, valamint a diabeteses, dedifferenciálódott béta-sejteket. A MafA funkció helyreállításával az inzulin szekréciós válasz javul, vagyis a MafA jelenléte elengedhetetlen az érett béta-sejtek működéséhez, a fiziológiás glukóz-stimulált inzulin szekrécióhoz. A MafA transzkripciós faktor potenciálisan gyógyszerfejlesztések célpontja lehet, ugyanis egy MafA expressziót vagy fehérje stabilitást fokozó hatóanyag a 2-es típusú diabetesben szenvedő betegekben a béta-sejt dedifferenciációt megelőzheti, és javíthatja az inzulinszekréciós kapacitást.

9. References

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10. Bibliography of the candidate's publications

10.1. Publications related to the thesis:

Jermendy Á, Toschi E, Aye T, Koh A, Aguayo-Mazzucato C, Sharma A, Weir GC, Sgroi D, Bonner-Weir. Neonatal beta cells lack the specialized metabolic phenotype of mature beta cells. Diabetologia 2011; 54:594-604.
IF: 6.814

Aguayo-Mazzucato C, Koh A, El Khattabi I, Li WC, Toschi E, **Jermendy Á**, Juhl K, Mao K, Weir GC, Sharma A, Bonner-Weir S. Mafa expression enhances glucose-responsive insulin secretion in neonatal rat beta cells. Diabetologia. 2011; 54:583-93.
IF: 6.814

10.2. Publications not related to the thesis:

Molvarec A, **Jermendy Á**, Kovács M, Prohászka Z, Rigó J Jr. Toll-like receptor 4 gene polymorphisms and preeclampsia: lack of association in a Caucasian population. Hypertens Res 2008; 31: 859-64.
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exocrine regeneration but no evidence of β-cell regeneration. Endocrinology 2013; 154: 4493-4502.

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