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Study of the Proliferation, Function and Death of Insulin-Producing Beta-Cells *in vitro*: Role of the Transcription Factor ZBED6

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Abstract

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A thorough understanding of beta-cell proliferation, function, death and regeneration under normal condition as well as in the progression of diabetes is crucial to the conquest of this disease. The work presented in this thesis aimed to investigate the expression and role of a novel transcription factor, Zinc finger BED domain-containing protein 6 (ZBED6), in beta-cells.

ZBED6 was present in mouse β TC-6 cells and human islets as a double nuclear band at 115/120 kDa and as a single cytoplasmic band at 95-100 kDa, which lacked N-terminal nuclear localization signals. Lentiviral shRNA-mediated stable silencing of ZBED6 in β TC-6 cells resulted in altered morphology, decreased proliferation, a partial S/G2 cell cycle arrest, increased expression of beta-cell specific genes, and higher rates of apoptosis. ChIP sequencing of human islets showed that ZBED6 binding was preferentially to genes that control transcription, macromolecule biosynthesis and apoptosis. We proposed that ZBED6 supported proliferation and survival of beta-cells, possibly at the expense of specialized beta-cell function, i.e. insulin production.

To further investigate the role of ZBED6 in beta-cells, ChIP sequencing and whole transcriptome analysis were performed using MIN6 cells. More than 4000 putative target genes of ZBED6 were identified, including Pdx1, MafA and Nkx6.1. ZBED6-silencing resulted in differential expression of more than 700 genes, which was paralleled by an increase in the content and release of insulin in response to a high glucose concentration. Altered morphology/growth patterns as indicated by increased cell clustering were observed in ZBED6 silenced cells. We found also that ZBED6 decreased the ratio between N- and E-cadherin. A lower N- to E-cadherin ratio may hamper the formation of three-dimensional beta-cell clusters and cell-to-cell junctions with neural crest stem cells, and instead promote efficient attachment to a laminin support and monolayer growth. Thus, by controlling beta-cell adhesion and cell-to-cell junctions, ZBED6 might play an important role in beta-cell differentiation, proliferation and survival.

Keywords: ZBED6, Pancreatic beta-cell, Proliferation, Insulin secretion, Apoptosis, Adhesion

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Wang X, Jiang L, Wallerman O, Engstrom U, Ameer A, Gupta RK, Qi Y, Andersson L, Welsh N. (2013) Transcription factor ZBED6 affects gene expression, proliferation, and cell death in pancreatic beta cells. *Proc Natl Acad Sci U S A*, 110:15997-16002
- II Jiang L,* Wang X,* Wallerman O, Klaesson A, Welsh N and Andersson L. ZBED6 negatively regulates insulin production, neuronal differentiation and cell aggregation in MIN6 cells. *Submitted* *Contributed equally
- III Wang X, Qi Y, Wallerman O, Andersson L, Vasylovska S, Koslova E, Welsh N. Knock-down of ZBED6 in insulin-producing cells promotes N-cadherin junctions between beta-cells and neural crest stem cells *in vitro*. *Manuscript*

Related paper by the author finished during the doctoral study

(Not included in the thesis)

Wang, X and Welsh N. Bcl-2-mediated protection against sodium palmitate-induced RINm5F cell death: Role of reactive oxygen species, mitochondrial membrane integrity and ER stress. *Submitted*

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Abbreviations

[Ca ²⁺] _i	Cytoplasmic Ca ²⁺ concentration
ATP	Adenosine-5'-triphosphate
Bim/BCL2L11	BCL-2-interacting mediator of cell death /Bcl-2-like protein 11
ChIP	Chromatin immunoprecipitation
CHOP	C/EBP homologous protein
DDHD2	DDHD domain containing 2
DETA/NO	DETA/NONOate
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FDR	False discovery rate
FFA	Free fatty acids
FoxO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLP1	Glucagon-like peptide 1
HGF	Hepatocyte growth factor
IGF2	Insulin-like growth factor 2
IRES	Internal ribosome entry site
kb	Kilobase
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
mRNA	Messenger RNA
NCSC	Neural crest stem cell
Nefm	Neurofilament, medium polypeptide
NeuroD1	Neurogenic differentiation factor 1
NeuroD2	Neurogenic differentiation factor 2
Neurog3	Neurogenin 3
Nkx6.1	Homeobox protein Nkx 6.1
NLS	Nuclear localization signal
NO	Nitric oxide
ORF	Open reading frame
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Pdx1	Pancreatic and duodenal homeobox 1
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PTB	Polypyrimidine tract binding protein
Ptf1a	Pancreas transcription factor 1 subunit alpha
ROS	Reactive oxygen species
RPKM	Reads per kilobase per million mapped reads
shRNA	Small hairpin RNA
TSS	Transcription start site
UTR	Untranslated region
ZBED6	Zinc finger BED domain-containing protein 6
ZC3H11A	Zinc Finger CCCH-Type Domain Containing 11A

Introduction

Diabetes mellitus is a global public health problem, which is characterized by hyperglycemia induced by an absolute or relative insulin deficiency. Functional failure of the insulin-producing beta-cell has been observed in both Type 1 and Type 2 diabetes. Therefore a thorough understanding of beta-cell proliferation, function, death and regeneration under normal condition as well as in the progression of diabetes is vital to the conquest of this disease.

ZBED6 is a recently discovered transcription factor restricted to and highly conserved among placental mammals. The intron-less ZBED6 has evolved from a domesticated DNA transposon and is located in one of the first introns of another gene, ZC3H11A, with which it has no sequence homology. ZBED6 belongs to the CCCH zinc-finger BEAT and DREF (BED) domain-containing family and contains two DNA-binding BED domains and one hobo-Ac-Tam3 (hATC) dimerization domain. ZBED6 has a broad tissue distribution in mammals. In muscle tissue of pigs, it has been observed that ZBED6 negatively regulates insulin-like growth factor-2 (IGF2) transcription. Silencing of ZBED6 in mouse C2C12 myoblast cells was associated with elevated IGF2 expression, increased cell proliferation and a faster wound healing process. Chromatin immunoprecipitation (ChIP) sequencing using C2C12 cells identified more than 2000 ZBED6 binding sites in the genome [1].

It is possible that ZBED6 controls not only IGF2 expression in muscle, but also the function of insulin-producing beta-cells. For example, genes coding for transcription factors crucial to the pancreatic beta-cell, such as Neurog3, Nkx6.1, NeuroD2, MafA, were found to be putative binding targets for ZBED6 in myoblast cells [1]. ZBED6-mediated control of IGF2 may be relevant to beta-cells as it has been observed that aberrant IGF2 production in embryonic pancreas preceded the subsequent beta-cell mass anomaly that develops in diabetic GK rats [2]. Thus, it is conceivable that the putative expression of ZBED6 in beta-cells influences or controls important events in beta-cell development and function, and, as a consequence, might be pertinent to the pathogenesis of various types of Diabetes mellitus.

Background

Diabetes mellitus

Diabetes mellitus is a global public health problem affecting more than 360 million individuals worldwide. Diabetes is characterized by hyperglycemia induced by an absolute or relative insulin deficiency. The disease is separated into two major groups: type 1 and type 2. Both types of diabetes are characterized by pancreatic beta-cell failure.

Type 1 diabetes (T1D), representing around 10%-15% of all cases, is the result of an autoimmune attack on the pancreatic beta-cell, leading to the destruction of the cells and insulin deficiency [3]. By the time of diagnosis, around 70%-80% of the beta-cells have been destroyed. The standard treatment for T1D is insulin injection. In severe cases, when insulin treatment failed to restore the glucose homeostasis, pancreas or islets transplantation has been shown to give promising clinical outcomes [4,5]. However, immune rejection, the shortage of donors, poor engraftment and revascularization limit the use of the treatment. Type 2 diabetes (T2D) is the most common form of diabetes, representing 80%-85% of all cases. T2D results from a reduced ability of the beta-cells to secrete enough insulin to stimulate glucose utilization by peripheral tissues in the face of insulin resistance. Both genetic causes [6] and environmental factors including energy dense diets rich in sugars and saturated fats and a sedentary life style have been identified as risk factors for developing T2D.

The islets of Langerhans

In the pancreas there are cell clusters named the islets of Langerhans, which are crucial to glucose homeostasis. The human pancreas contained about 1-2 million islets ranging from 30 to 300 μm in diameter and in each islet there are about 2-3000 endocrine cells. There are five major types of endocrine cells: 1: β -cells, constituting 60-80% of the islets cells, which secrete insulin and amylin; 2: α -cells, constituting 10-20% of the islets cells, which secrete glucagon leading to glycogen breakdown in the liver and increased glucose concentrations; 3: δ -cells, constituting 3-10% of the islets cells, which secrete somatostatin; 4: PP-cells, constituting 0.5-1% of the islets cells, which secrete pancreatic polypeptide, a hormone that has been shown to affect

hepatic glycogen levels and gastrointestinal secretion; 5: ϵ -cells, constituting less than 1% of the islets cells, which secrete ghrelin, a hormone that regulates appetite and promotes the release of growth hormone [7,8]. Except the endocrine cells, the islets contain fibroblasts, neurons, endothelial and macrophages cells.

Pancreas development and maintenance through life

Morphological changes during pancreas development and regulating factors

At embryonal day 9.5 (E9.5) in mouse, the pancreas starts to form from the foregut endoderm. Two pancreatic buds are generated at this stage: the dorsal bud, which arises first and generates most of the pancreas; and the ventral bud, which arises next to the bile duct and forms only part of the pancreas. Retinoic acid (RA), sonic hedgehog (SHH), fibroblast growth factor (FGF), and ACTIVIN signaling all regulate the patterning of the dorsal pancreatic endoderm. Bone morphogenetic protein (BMP) and FGF signaling affects the patterning of ventral pancreatic endoderm. Due to the subsequent rotation of stomach and duodenum, the two pancreatic buds become positioned next to each other and then fuse to form the pancreas [8].

The activation of a complex network of transcription factors is critical for pancreatic patterning and specification. Pdx1 and Ptf1a are both necessary for the formation and growth of pancreatic buds [9,10]. The specification and differentiation of the five endocrine cell types in islets comes as 'two waves'. The first wave can be found as early as E9.5. During the first wave, the hormone-expressing cells, mostly glucagon-producing cells, are present in the pancreatic buds as single lying or in groups. Most of the endocrine cells are specified during the second wave, between E12.5 to E15.5 [8]. Using knockout mice models, many transcription factors essential for beta-cell differentiation have been identified. All endocrine cell lineages are derived from the Neurog3 positive endocrine progenitor cells, supported by the fact that Neurog3 null mice lack all endocrine cells [11]. As mentioned before, Pdx1 marks the pancreatic progenitor cells and is necessary for pancreatic bud formation. With progression of development, the expression of Pdx1 in the endocrine progenitor cells is down regulated and by later gestation is selectively maintained at high levels in the beta-cells [12]. Similar to Pdx1, Nkx6.1 is expressed early in the pancreas development and later becomes highly restricted to beta-cells. Loss of beta-cell precursors is induced by deletion of Nkx6.1 [13]. NeuroD1 null mice die perinatally from diabetes due to beta-cell apoptosis [14]. MafA deficient mice are viable but develop glucose intolerance due to beta-cell dysfunction after birth, which indicates

that MafA is required for maturation and not specification of pancreatic beta-cells [15].

Beta-cell proliferation and beta-cell mass maintenance through life

The beta-cell mass is determined by: 1, beta-cell proliferation rate; 2, beta-cell size; 3, beta-cell death; 4, beta-cell neogenesis from precursors. Beta-cells replicate at an extremely low rate, approximately 0.38% as assessed by the percentage of BrdU-positive beta-cells in 9 weeks rats, and an even lower replication capacity can be observed in adult human [16,17]. There are many inducers or regulators of beta-cell proliferation: the extrinsic inducers, such as glucose, insulin/IGF signaling, prolactin, GLP1, hepatocyte growth factor (HGF), growth hormone and extracellular matrix [18]; as well as the intrinsic regulators, such as cyclins, cyclin dependent kinases, cyclin dependent kinase inhibitors, E2F, retinoblastoma protein (Rb) and p53 [19]. The endocrine pancreas can respond to certain physiological or pathophysiological conditions with an increased beta-cell mass through changing the balance between beta-cell proliferation and apoptosis, as seen during pregnancy and obesity [20,21]. Currently, there is an increasing number of studies that support an important role of beta-cell neogenesis in the expansion of the beta-cell mass in adult life, for instance upon duct ligation and partial pancreatectomy [22,23]. Neogenesis from duct epithelium is the most documented process of differentiation of precursor cells into beta-cells. However, it is still debated whether precursor cells exist in duct epithelium in the adult. Nevertheless, a line of studies propose that there is a pool of resting precursor cells inside ducts [24]. Other studies indicate that all the ductal cells are potential precursors that are able to differentiate into beta-cells [25]. GLP1 has been reported to induce differentiation of Pdx1-positive pancreatic ductal cells into insulin-secreting cells [26]. Another resource of beta-cell neogenesis is the transdifferentiation of acinar cells [27], intra-islet precursor cells [28], alpha-cells [29] and even the extra-pancreatic tissues such as hepatocyte precursor cells [30].

Insulin gene expression

Insulin is encoded by the INS gene in humans and Ins1 and Ins2 in mice. A variety of mutant alleles with phenotypic effects have been identified. INS-IGF2, a read-through gene, overlaps with INS at the 5' region and with the IGF2 gene at the 3' region. A 340 base pair promoter region, to which several beta-cell specific transcription factors, such as Pdx1, MafA, Pax4 and NeuroD1, bind, controls insulin gene transcription. These transcription fac-

tors collectively activate insulin gene transcription. Although multiple factors have been identified to affect insulin gene expression, glucose is still considered the most important regulator. In the case of glucose-Pdx1-mediated regulation of insulin gene expression, changes in glucose levels will affect the phosphorylation, by the p38/SAPK, PKC, MAPK and PAS kinases, and the subcellular localization of Pdx1 and its interaction with co-regulators. At low or normal glucose conditions, Pdx1 is mainly localized to the nuclear periphery where it interacts with a histone deacetylase. In response to high glucose, Pdx1 becomes phosphorylated and translocates into the nucleoplasm and interacts with the histone acetyltransferase p300 and the histone methyltransferase Set7/9 to stimulate insulin gene expression [31]. MafA binds to the C1 element within the insulin promoter in a glucose-dependent manner [32]. Low glucose conditions down-regulate MafA expression by decreasing MafA transcription. In response to a high glucose concentration FoxO1, FoxA2, Nkx2.2, Pdx1 and NeuroD1 all bind to the MafA promoter and stimulate its transcription [33,34]. The fraction of the newly synthesized MafA that escapes ubiquitination and degradation in a proteasome-dependent manner translocates to the nucleus and participates in the transcription of the insulin gene.

Besides insulin gene transcription, there are other steps which are also crucial to insulin biosynthesis, such as correct splicing of insulin pre-mRNA [35,36], insulin mRNA stability [37], endoplasmic reticulum synthesis and folding of insulin. It has been shown that at low glucose concentration, the half-time of insulin mRNA is between 29 hours while at high glucose concentration, the half-time is extended to 77 hours [38]. The results indicate the effect of high glucose on insulin mRNA stability. An increased expression of polypyrimidine tract binding protein (PTB) has been shown to parallel the glucose-induced increase of insulin mRNA. PTB binds to the pyrimidine-rich sequence at insulin mRNA 3'-untranslated region (UTR) and stabilizes insulin mRNA [39]. Glucose also increases the translation of insulin mRNA either by up-regulating the translation rate globally or by interacting with the 5' UTR of the preproinsulin mRNA specifically [40].

Regulation of insulin secretion

It has been known for long time that glucose-stimulated insulin secretion is a biphasic process with an initial and transient peak followed by a less pronounced but sustained secretory phase [41]. Each beta-cell contains more than 10,000 secretory granules, which belong to two different pools. A small portion of the insulin granules (around 1%) constitute a readily releasable pool which can be triggered by a rise of Ca^{2+} and are responsible for the first phase of insulin secretion. While the majority of the granules (around 99%)

belong to a reserve pool and need to be activated for release by a series of ATP and Ca^{2+} dependent signals. The second phase insulin release recruits the granules from the reserve pool [42,43]. Due to periodic depolarizations and oscillations of intracellular messenger molecules, the second phase secretion is pulsatile [44,45].

Glucose is transported into the beta-cell by glucose transporters and then becomes metabolized to ATP through glycolysis and mitochondrial oxidation. An elevated ATP to ADP ratio closes the ATP-regulated K^+ -channels in the plasma membrane and causes membrane depolarization. When the membrane potential reaches the threshold for activation of L-type voltage dependent Ca^{2+} channels, a rapid Ca^{2+} influx triggers exocytosis of insulin granules [46]. The glucose-induced elevation of $[\text{Ca}^{2+}]_i$ in beta-cell is preceded by an initial lowering due to uptake of the ion into the ER by the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump [47]. The subsequent elevation of $[\text{Ca}^{2+}]_i$ is often oscillatory. Several types of Ca^{2+} oscillations have been reported in islets, either depending on periodic depolarization of the plasma membrane [48] or Ca^{2+} release from intracellular stores [49]. Both types of Ca^{2+} oscillations induce pulsatile release of insulin.

Beta-cell death in type 1 and 2 diabetes

Progressive beta-cell loss in T1D patients is mediated by infiltrating immune cells (insulinitis). Immunostaining of the pancreases of non-obese-diabetic (NOD) mice, an animal model of T1D, showed that during the progression of T1D macrophages were the first immune cells to invade the islets, followed by T-lymphocytes (CD4^+ and CD8^+) and B-lymphocytes [50]. The direct contact with the infiltrating immune cells and/or exposure to cytotoxic mediators, such as pro-inflammatory cytokines (interleukin- 1β , tumor necrosis factor- α and interferon- γ) and Fas ligand, is thought to induce beta-cell apoptosis and necrosis [3] [51] [52] [53]. In rodent islets the toxic effect of cytokines has been shown to be induced by augmented nitric oxide (NO) production. Interleukin- 1β , the main factor responsible for inflammation-induced islet toxicity, activates NF- κB , leading to transcription of inducible nitric oxide synthase (iNOS) and subsequent NO production [54]. The notion that elevated level of NO contributes to beta-cell destruction is supported by observations that iNOS inhibition blocks cytokines-induced beta-cell death *in vivo* and *in vitro* in rodent islets [55,56], and that iNOS knockout mice are protected from streptozotocin-induced diabetes [57]. Human islets are less sensitive than rodent islets to the toxic level of NO, possibly due to a higher scavenging capacity of free radicals and increased expression of heat shock protein 70 [58,59].

The dysfunction of the beta-cell in T2D is also accompanied by increased apoptosis, which causes further progression of the disease [60]. The beta-cell apoptotic stimuli in T2D are mainly free fatty acids (FFA), such as palmitate, (lipotoxicity) and high glucose (glucotoxicity) [61]. When beta-cells are exposed to FFA for prolonged time periods, apoptosis can be observed [62]. The exact mechanisms that underlie FFA-induced pancreatic beta-cell apoptosis are still unclear. It may be that FFA induces several different events, including endoplasmic reticulum (ER) stress [63], mitochondrial perturbations [64], generation of reactive oxygen species [64], ceramide and NO [65] and altered Ca^{2+} homeostasis [66] that together or individually promote beta-cell death. Prolonged or repeated exposure to supra-physiological glucose concentration induces deleterious effects on beta-cells *in vitro* and *in vivo* [67,68], which is known as glucotoxicity. The mechanisms of glucotoxicity include: beta-cell overstimulation, which leads to impaired glucose stimulated insulin secretion (GSIS) and elevation of the ratio of proinsulin to insulin [69,70]; ER stress [71]; oxidative stress [72]; protein glycation, which induces structural alternation of intracellular proteins and consequently reduced GSIS and increased beta-cell apoptosis [73]; amyloid deposition in islets [74]; proinflammatory cytokines and chemokines released by adipose tissue under hyperglycemic conditions [75] and hypoxia induced by acceleration of mitochondrial metabolism, elevated ATP consumption and limited oxygen supply [76,77].

Extracellular matrix and intercellular junctions of beta-cells

In islets, beta-cells are in direct contact with the basement membranes (BMs) of capillary endothelial cells. BMs are the thin layer of specialized extracellular matrix (ECM) composed mainly of type IV collagen and laminin. It has been shown that laminins are the functionally most important components of BMs in islets, and laminin-511 has been identified as the only laminin isoform present in the human islet endocrine BM. Laminins are trimeric glycoproteins composed of an α , β and γ chain and the laminin molecules are named according to their chain composition, for instance laminin-511 for $\alpha 5\beta 1\gamma 1$. In human islets, between the blood vessel endothelial cells and endocrine islet cells, there are two separate sheaths of BMs: endothelial BM (composed of laminin-511 and laminin-411) and endocrine BM (composed of laminin-511) [78]. On the surface of islet cells, there are several receptors for laminin-511, such as integrin $\alpha 3\beta 1$ and Lutheran glycoprotein [79]. Integrin receptors are Ca^{2+} dependent heterodimeric transmembrane adhesion molecules composed of α and β subunits, which mediate adhesion to extracellular matrix and transduce signals from extracellular

matrix to the interior of the cell. Perturbation of integrin $\alpha 3$ function in human islets and INS-1 cells induces significant loss of beta-cell function, such as impaired adhesion, spreading, insulin secretion, proliferation and Pdx1 expression [80]. Integrin $\beta 1$ -mediated activation of FAK/ERK signaling has been shown to enable differentiation and survival of human fetal islet cells [81]. Besides integrins, there is another type of Ca^{2+} dependent adhesion molecules named cadherins. Cadherins are homophilic glycoproteins that mediate cell-to-ECM and cell-to-cell interactions. E-cadherin has been shown to be expressed in both alpha and beta-cells of human islets, whereas N-cadherin is preferentially expressed in beta-cells. E and N-cadherins are involved in the maintenance of beta-cell viability and secretion of insulin [82,83]. As insulin secretion is a multi-cellular synchronized event, cadherins, as well as other adhesion/junction molecules, are crucial for the maintenance of normal beta-cell function.

The zinc finger BED domain-containing protein 6

The zinc finger BED domain-containing protein 6 (ZBED6) is a novel transcription factor in placental mammals. The protein is encoded by an intronless gene located in intron 2 of *Zc3h11a*, a gene encoding a poorly characterized zinc-finger CCCH type containing protein. ZBED6 contains two BED domains and a hATC dimerization domain and has no significant sequence similarity to ZC3H11A. There are two possible translation start sites for ZBED6, which produce two isoforms of ZBED6 (around 122 and 116KD in murine C2C12 myoblast cells). ZBED6 regulates IGF2 expression and muscle growth. A G to A transition in intron 3 of IGF2 in pigs abrogates the binding site for ZBED6 and leads to a 3-fold up-regulation of IGF2 in skeletal muscle. Silencing of ZBED6 in mouse C2C12 myoblast cells is associated with elevated IGF2 expression, increased cell proliferation and a faster wound healing process which is consistent with the phenotypic effects in mutant pigs. ZBED6 has a broad tissue distribution and the protein is highly conserved among placental mammals. Chromatin immunoprecipitation (ChIP) sequencing using C2C12 cells has identified more than 2000 ZBED6 binding sites in the genome. Genes associated with ZBED6 binding sites show a highly significant enrichment for certain gene ontology classifications, including development and transcriptional regulation [1].

Neural crest stem cell

Neural crest stem cells (NCSCs) are transient, multipotent, migratory cells, which are derived from ectoderm. It has been shown that NCSCs play a role

in pancreatic development and beta-cell maturation. The embryonal pancreas starts to form at embryonal day 9.5 (E9.5) in the mouse and NCSCs enter the pancreatic mesenchyme at approximately E10.0. They intermingle with pancreatic progenitors, as the epithelium branches into the surrounding mesenchyme, and eventually differentiate into pancreatic nerve and glia cells. Ablation of NCSCs by deletion of *Foxd3* induces elevated proliferation of insulin-expressing cells and expansion of the insulin-positive area in the developing pancreas. However, the functional maturation of these beta-cells is significantly impaired as decreased expression of *Pdx1*, *MafA* and *Glut2* is observed. The presence of abnormal insulin granules is also detected by transmission electron microscopy [84]. Although NCSC migration to pancreas [85] and NCSC-mediated regulation of the beta-cell mass [86] has been partially investigated, much of the exact role of NCSCs in embryonic beta-cell maturation remains to be determined.

Aims

The general aims of this thesis were to investigate the expression pattern of ZBED6 in insulin-producing beta-cell and to study the roles of ZBED6 in beta-cell proliferation, insulin secretion, adhesion and death.

The specific aims for the different papers were:

- I To investigate the expression pattern of ZBED6 in mouse β TC cells, mouse pancreas and human islets and to study the role of ZBED6 on pancreatic beta-cell morphology, expression of specific genes, proliferation, cell-cycle distribution and viability.
- II To identify ZBED6 putative target genes in mouse MIN6 cells by ChIP sequencing, and to further study the role of ZBED6 in beta-cells with regard to differentiation, calcium mobilization and insulin release.
- III To investigate the role of ZBED6 in beta-cell cell-to-cell interaction with neural crest stem cells.

Methodology

Cell culture and islets isolation

Murine β TC6 cells, purchased from ATCC, at passage numbers 20-40 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS) (Sigma Chemicals), 2 mM L-glutamine, streptomycin (0.1 mg/ml) and benzylpenicillin (100 U/ml). Murine MIN6 cells (passage 20-35) were maintained in DMEM with 15% FCS, 2 mM L-glutamine, 70 μ M 2-mercaptoethanol, streptomycin (0.1 mg/ml) and benzylpenicillin (100 U/ml). Human PANC-1 cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, streptomycin (0.1 mg/ml) and benzylpenicillin (100 U/ml).

Human islets were pre-cultured for 3-6 days free-floating in Sterilin dishes in CMRL 1066 medium (ICN Biomedicals) containing 5.6 mM glucose, 10% fetal calf serum and 2 mM L-glutamine. All cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

Generation of stable ZBED6 silencing cell lines

Short-hairpin sequences against the ZBED6 gene and the scrambled shRNA sequences were cloned into the lentiviral vector pLKO.1-TRC (Addgene). The target sequences selected are: ZBED6-sh1: 5'-CTTCAACACTTCAACGACA -3'; ZBED6-sh2: 5'- TGTGGTACATGCAATCAAAA -3'; shMock: 5'-GAACCCTATATCCAACACT-3'. Viral particles were obtained by co-transfection of HEK-293FT cells with the pLKO.1-shRNA, envelope (pMD2.G) and packaging (psPAX2) vectors. β TC-6, MIN6 and PANC-1 cells were transduced with the shRNA lentiviral particles (10 MOI) and cells with stable expression of shZBED6 were selected by incubation in a medium containing puromycin (5-10 μ g/ml) for at least 2 weeks. ZBED6 mRNA expression levels were determined by real-time RT-PCR and protein expression was confirmed by immunoblotting.

RNA isolation and cDNA synthesis

Cells were washed once with PBS and total RNA was isolated either by Ultraspec™ RNA Isolation system (Biotech Laboratories) or RNeasy mini Kit (Qiagen) according to the instructions of the manufacturers. The cDNA synthesis was performed using M-MuLV reverse transcriptase Rnase H- (FINNZYMES) and random nonamer primers. The synthesis reaction was terminated by heating at 95°C for 5 min.

Semi-quantitative real time RT-PCR

The mRNA transcripts were measured by semi-quantitative PCR analysis using the SYBR Green Taq Readymix (Sigma) on a Light Cycler 2.0 instrument (Roche). The value is then normalized against the value of GAPDH. To ensure the function of GAPDH as a housekeeping gene, GAPDH levels were compared with those of β -actin and 18S rRNA. GAPDH mRNA levels were unaffected by all treatments, which verifies that GAPDH is a suitable housekeeping gene. The PCR products were analyzed by a 2% agarose gel to ensure that the fragments had the correct sizes.

ChIP-seq analysis

Human islets (1000 islets/sample) and MIN6 cells were crosslinked with 1% formaldehyde for 10 min, quenched with glycine and stored at -80°C. After thawing and treatment with cell lysis buffer, chromatin was sonicated in RIPA buffer using a BioRuptor. Two separate ChIPs were prepared using 20 μ l protein A and G beads with 2 μ g ZBED6 antibody. Illumina libraries were prepared using NEXTflex adaptors (BIOO Scientific) and enzymes from Fermentas (Fast End Repair, 25 μ l for 15 min, 1 μ l Klenow exo-minus DNA polymerase for 30 min at 37°C, 0.5 μ l fast ligase for 15 min). Sequencing was done using Illumina HiSeq 2000 instruments. For human islets, 16.8 million read pairs could be aligned to the hg18 human reference (BWA v0.5.9). Read pairs aligned with a distance of less than 500 bp and a combined mapping quality of at least 60 were used to calculate overlaps of the corresponding ChIP fragments in order to identify peaks of enrichment. A minimum of 12 unique overlapping fragments was required and the list of peaks was filtered to remove recurrent false positive regions such as Satellite repeats. For MIN6 cells, all reads were aligned to the mouse mm9 assembly using BWA version 0.5.9 at default settings. SAMtools were used to remove alignments with low alignment quality (< 20) and the MACS peak caller (version 1.41) was used to identify enriched peaks and create wiggle tracks for visualization.

Motif analysis was done with MEME-ChIP on the 200 bp sequences centered on the peak summits for the 500 highest and 500 lowest peaks. Default settings were used except that the search space was limited to motifs of 6 - 20 bp length.

RNA sequencing

Total RNA was isolated using the RNeasy mini kit (Qiagen) and were further enriched for mRNA using the MicroPoly(A) Purist kit (Ambion). The quality and quantity of the mRNA was evaluated with a Bioanalyzer 2100 (Agilent) and 0.5 to 1 µg mRNA for each sample was used for RNA sequencing (SOLiD5500XL system for human islets and Illumina HiSeq 2000 platform for MIN6 cells). The fragment library was constructed according to the manufacturer's protocol. For human islets, alignment of reads to the human reference sequence (hg19 assembly) and splicing analysis were performed using LifeScope software 2.1. For MIN6 cells, TopHat 2.0.4 was used to align all the reads to the mouse genome (mm9) and Cufflink 2.0.2 was used to assemble transcripts, estimate gene expression levels and identify genes with differential expression.

Gene Ontology analysis

The official gene symbols of the differentially expressed genes were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/>) for the functional annotation chart analysis. The default setting was used for the GO analysis. The criteria of FDR-corrected $P < 0.05$ and fold enrichment > 2.5 were used to identify significantly enriched GO categories.

Preparation of cytosolic and nuclear fractions

Human islets (100/group) or βTC-6 cells were washed once in cold PBS and resuspended in solution A (10 mM HEPES, PH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM Dithiothreitol, protein inhibitor cocktail) and incubated on ice for 10 min. Following a brief centrifugation, the cells were again resuspended in solution A and lysed with an electric homogenizer. The lysates were then centrifuged for 5 min at 3200 rpm at 4°C and proteins in the supernatant fraction were precipitated using 70% acetone for 10 min on ice followed by a 5 min centrifugation at 13000 rpm at 4°C. After removal of all acetone, the precipitated proteins were resuspended in SDS-sample buffer

and used as cytosolic fractions. The pelleted fractions of the lysates were resuspended in SDS-sample buffer and used as nuclear fractions.

Immunoblot analysis

Cells were washed in cold PBS and lysed on ice in SDS-sample buffer (2% SDS, 0.15 M Tris, pH 6.8, 10% glycerol, 5% β -mercaptoethanol, bromophenol blue and 2 mM phenylmethylsulfonyl fluoride). The samples were boiled for 5 min and separated on 9% or 12% SDS-PAGE gels. Proteins were transferred to Hybond-P membranes (GE Healthcare). The membranes were pre-blocked in 2.5% BSA for 1h and then incubated with specific antibodies. The bound antibodies were visualized with the Kodak Image Station 4000 MM using ECL plus (GE Healthcare). The band intensities were calculated using Carestream Molecular Imaging Software 5.0.6. Total protein loading and transfer onto the membranes was visualized by amidoblack staining.

Immunoprecipitation

Cells (10^8) were washed three times in ice-cold PBS and resuspended in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Igepal CA-630, supplemented with PMSF and Halt protease and phosphatase inhibitor cocktail (Thermo Scientific)) on ice for 30 min. The lysed cells were cleared by centrifugation and remaining extracts were first precleared with 50 μ l Protein A Sepharose (GE Healthcare) and then incubated with 5 μ g anti-ZBED6 antibody or 5 μ g of rabbit serum (control) for 1h on ice. Immune complexes were purified by binding to 50 μ l Protein A Sepharose for 30 min on ice and thereafter washed 3 times with RIPA SDS buffer and once with H₂O. The sepharose beads were resuspended in SDS-sample buffer and immunoprecipitates were loaded to Mini-PROTEAN precast gel (4-20%) (Bio-Rad) and separated by electrophoresis.

Identification of proteins by mass spectrometry

After silver staining, the relevant bands were cut out and treated for in-gel digestion as follows: after removing the silver stain by Farmer's reagent (50 mM sodium thiosulphate/15 mM potassium ferricyanide), and extensive washing with water, gel plugs were treated with 50 mM ammonium bicarbonate (ambic) and then dried by neat acetonitrile (ACN). Porcine trypsin (modified, sequence grade from Promega) was added and incubation continued at 37°C overnight. Digestion was terminated by using 10% trifluoroac-

tic acid and peptide retrieval was facilitated by mechanical vortex and sonication. The samples were desalted and concentrated using a micro-C18 Zip-Tip (Millipore) and eluted directly onto the target plate using alpha-cyano-4-hydroxycinnamic acid in 75% ACN as matrix. Mass spectra for peptide mass fingerprinting were acquired in positive reflectron mode on an Ultraflex III TOF/TOF (Bruker Daltonics). Searches for peptide identities were done via the engine ProFound (The Rockefeller University and National Centre for Research Resources) and according to the following conditions: taxonomy mouse with mass tolerance 0.03 Da. Alternatively, the target proteins were inserted in GPMAW (Lighthouse Data) and analyzed with regard to the obtained peptide masses.

Insulin release and total insulin content measurement

Cells (1×10^5 cells/well, 24-well plate) were seeded and precultured in DMEM for 2 days. The cells were washed once with HEPES-balanced Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl₂, 1.19 mM MgCl₂, 1.19 mM KH₂PO₄, 25 mM NaHCO₃, and 10 mM HEPES, pH 7.4) and then harvested and homogenized by sonication in 200 μ l distilled water. A fraction of the homogenate was mixed with acid-ethanol and insulin was extracted overnight at 4°C. Samples were then stored at -20°C until insulin analysis. For insulin release determinations, the cells were preincubated for 30 min in HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.2% BSA and 1.67 mM D-glucose and then incubated for 1 h with 1.67 or 16.7 mM glucose or 30 mM KCl combined with 1.67 mM glucose. Insulin concentrations were measured by Mercodia Insulin ELISA Kit (Mercodia AB, Sweden). The amount of insulin was normalized by cell number, as assessed by flow cytometry.

Determination of cell viability by flow cytometry

Cells were exposed to 0.5 mM palmitate (0.5% BSA, 10% FCS) for 16h; or incubated with 100 U/ml IL-1 β and 1000 U/ml IFN- γ for 24h; or treated with 1 mM DETA/NO for 24h. Then cells were vital stained with 10 μ g/ml propidium iodide (PI) for 10 min at 37°C. Free-floating cells and cells attached to the culture dish were then washed separately with PBS, pooled and examined by flow cytometry using the FACSCalibur (BD Biosciences). In each experimental group 10000 cells were examined and gated by PI intensity and FSC signal. The percentage of dead cells was analyzed using CELLQUEST software (BD).

Cell proliferation and cell cycle analysis

The proliferation properties of stable ZBED6-shRNA β TC-6 and PANC-1 cells and control cells (shMock) were investigated using flow cytometry. 10000 cells of each type of cell clones were seeded to 24-well culture plate and number of cells in each well was counted by flow cytometry at day 1, 2, 3, and 4. For cell cycle analysis, β TC-6 cells were trypsinized, washed with cold PBS, fixed in ice-cold 70% ethanol, and stored at -20°C until analysis. Before analysis, cells were subsequently washed twice with PBS, incubated for 30 min at room temperature with RNase A (100 $\mu\text{g}/\text{ml}$), and stained with propidium iodide (2.5 $\mu\text{g}/\text{ml}$) for 30 min. Flow cytometry was performed with the FACSCalibur instrument and data were analyzed with the ModFit LT V3.3.11 software.

Immunohistochemistry, immunofluorescence and confocal microscopy

Cells were cultured on poly-lysine coated coverslips before staining. Intact human islets were cyto-spinned (1200 rpm for 2 min) to poly-lysine microscopy slides. In some experiment islets were trypsinized into cell clusters before cyto-spinning. Cells were fixed in 4% PFA for 10 min at room temperature, permeabilized with 0.2% Triton X-100 on ice for 10 min, and blocked with 5% FCS for 30 min in PBS. Cells were incubated for 1h with primary antibody at room temperature and then washed four times with PBS to remove unbound antibodies and then treated with fluorescent secondary antibodies (20 $\mu\text{g}/\text{ml}$ each) (Life Technologies) for 1h. Cells were washed four times with PBS and mounted with VECTASHIELD Hard Set mounting medium with DAPI (Vector Laboratories) or Fluoromount-G (Southern Biotechnology) and inspected with a Nikon Eclipse fluorescence/confocal C1/TE-2000U microscope or a Zeiss 780 confocal microscope.

Pancreata from 6.5 weeks old C57BL/6 mice were fixed in 10% formalin and embedded in paraffin. Sections, 5 μm thick, were cut and deparaffinized. After antigen retrieval, the sections were washed with PBS and incubated for 10 min with freshly prepared 3% H_2O_2 in methanol. Consecutive sections were blocked using the blocking solution (TSA Biotin System, Perkin-Elmer Life Sciences) for 30 min and incubated with primary antibody for 1h. The sections were subsequently stained for 30 min with biotinylated anti-rabbit IgG (Dako) or biotinylated anti-mouse IgG (Vector Laboratories), followed by incubation for 30 min with Streptavidin–horseradish peroxidase (Perkin-Elmer). Peroxidase activity was revealed using the AEC peroxidase substrate kit (Vector Laboratories). Alternatively, a tyramide amplification step

(TSA-Biotin System, Perkin-Elmer) was included. Microscopy was done using an inverted Nikon Eclipse C1/TE-2000U.

Plate coating and cell attachment assay

The culture plates or cover slips were coated with 10 µg/ml mouse laminin (Invitrogen) or 10 µg/ml and 1 µg/ml human laminin 511 (BioLamina) in PBS over night at 4°C. After laminin coating, the remaining uncoated surfaces were blocked with BSA for 1 hour at 37°C. The culture plates and cover slips were washed 3 times with PBS before use.

Cells were harvested by Accutase (Millipore). Serum-containing DMEM was used to inactivate Accutase. Cells were then washed with serum free DMEM, counted and allowed to recover in suspension for half an hour at 37°C. For cell attachment assay, cells (3×10^4 cells per well) were seeded onto the laminin coated 96-well plates and incubated for 4 hours. After removing the non-attached cells, the attached cells were fixed with 96% ethanol for 10 minutes and stained with crystal violet (0.1% in water) for 20 minutes. After extensive washing, the stained protein was solubilized with 0.5% SDS and the absorbance was measured at 600 nm.

Statistical analysis

Data are presented as means \pm S.E.M. Statistical significance for pairwise comparisons was analyzed using Student's t-test. One-way ANOVA on ranks followed by the Student-Newman-Keul method was used for multiple comparisons.

Results and discussion

Paper I

Transcription factor ZBED6 affects gene expression, proliferation, and cell death in pancreatic beta cells. *Proc Natl Acad Sci U S A*, 110:15997-16002

Differential expression pattern of ZBED6 in β TC6 cells and human islets

Immunoblot analysis of mouse β TC6 cell lysates using an antibody directed against the N-terminal ZBED6 BED domains revealed several bands: a double band with the approximate molecular weights of 115/120 kDa and a single, weaker 95 kDa band (Fig.1A). ZBED6 subcellular localization study in β TC6 cells revealed that the large 115/120 kDa ZBED6 forms were mainly localized to the nuclear fraction, whereas the small 95 kDa form was instead cytosolic (Fig.1C). To further characterize the observed ZBED6 120, 115 and 95 kDa bands, we immunoprecipitated ZBED6 from β TC6 cells using the same anti-mouse ZBED6 N-terminal antibody. Five bands with molecular weights between 125 and 70 kDa were cut out and analyzed by mass spectrometry. The upper two bands, 125 kDa and 110 kDa respectively, were identified with high confidence as ZBED6. The two bands represent the two isoforms of ZBED6 (predicted molecular weight 109 kDa and 104kDa), and that unknown post-translational modifications enhance the apparent molecular weight. These two bands were further verified by tandem mass spectrometry (ms/ms). The result is consistent with the previous study performed in mouse C2C12 myoblasts [1]. The explanation for the smaller size of the second band is not known, but the decreased size may correspond to an alternative translation start site at the second methionine at amino acid 46, which generates a predicted 5 kDa smaller protein. Interestingly, the third band (100 kDa double band) was identified with lower probability as ZBED6 using the GPMW (General Protein/Mass Analysis for Windows) software based on four peptides starting at amino acid 257 and ending at 659. Ms/ms analysis of the AVPQLYDSVR (459-468) peptide gave also some support for the ZBED6 identity. The 95-100 kDa size of the third band therefore corresponds to a N-terminal truncation somewhere before amino acid 257. This would generate a predicted 80-85 kDa protein that migrates somewhat slower due to post-translational modifications. Such a N-terminal

truncated form of ZBED6 fits well with the observed cytoplasmic localization of the 95 kDa band as the ZBED6 nuclear localization signals are located at amino acids 61–80 and 231–248 [1]. However, the third band was not pure as also the DDHD domain containing 2 protein (DDHD2) was identified in the same double band. It is not clear whether DDHD2 immunoprecipitated with ZBED6, or whether the ZBED6 antibody cross-reacted with DDHD2. The remaining two bands did not generate any ZBED6 protein identification.

The ZBED6 expression in human islets was visualized using an anti-human ZBED6 C-terminal antibody. Analysis of islet lysates revealed three bands with anticipated molecular weights. The upper two corresponded well in size with the 115/120 bands and the third was slightly larger (100 kDa) than the 95 kDa ZBED6 band observed in β TC6 cells. Interestingly, the 100 kDa band was more prominent in human islet cells, whereas the 115/120 kDa bands were the most prominent in the β TC6 cell line (Fig.1B). In human islet cells, the 115-120 kDa ZBED6 forms were predominantly located to the nuclei and the smaller 100 kDa form was mainly cytoplasmic as assessed by nuclear and cytosolic fractionation study (Fig.1D).

To further verify the differential expression pattern of ZBED6 in β TC6 cells and human islets, we immunostained β TC6 cells, mouse pancreas and human islets. Immunostaining with the anti-mouse ZBED6 antibody revealed a strong nuclear and weak cytoplasmic staining in β TC6 cells (Fig.1E). This corroborates our immunoblotting results showing that β TC6 cells express high levels of nuclear ZBED6. In mouse pancreas ZBED6 immunohistochemical staining was stronger in islets than in surrounding exocrine tissue. The signal was mainly cytoplasmic and was present in both insulin- and glucagon-positive cells, as assessed by staining in consecutive sections. In pancreatic duct cells, however, ZBED6 staining was mainly nuclear. ZBED6 immunoreactivity in isolated human islets was mainly cytoplasmic in both insulin- and glucagon-positive islet cells (Fig.1F). Cytokeratin 19 positive human duct cells displayed stronger ZBED6 immunoreactivity than the endocrine cells, and in some cases clear nuclear ZBED6 staining was observed.

We next attempted to understand the mechanisms by which three different ZBED6 isoforms are produced from inside the ZC3H11A gene. One possible explanation is alternative splicing of pre-mRNA. As the ZBED6 coding sequence is located to intron 2 of ZC3H11A, we mapped RNA sequencing reads of human islet samples to the entire ZC3H11A gene region. Numerous reads were uniformly distributed from the start of exon 1 to the end of exon 4 of ZC3H11A, consistent with frequent retention of introns 1-3. Few reads covering the first splice site (intron 1) was found, indicating incomplete splicing of intron 1. No reads indicated alternative splicing in intron 2 or 3 that could explain the presence of the three isoforms of ZBED6

detected in this study. This suggests that intron 2 containing ZBED6 is retained in most ZC3H11A transcripts in human islets and that there is only one major transcription start site (TSS) for both genes in these cells. Remaining introns (4-19) of the ZC3H11A transcript were efficiently spliced. As no TSS or alternative splicing were observed in intron 2 from the current investigation, it seems unlikely that the N-terminal truncated ZBED6 isoform is generated by alternative splicing of pre-mRNA or by another TSS.

The RNA sequencing data also allows us to have a closer look on the transcription of ZBED6 from the surrounding gene ZC3H11A. As available data indicate that ZC3H11A and ZBED6 are transcribed from a common promoter located upstream of ZC3H11A exon 1, it is likely that ZBED6 expression is dependent on the retention of the intron containing the ZBED6 coding sequence in ZC3H11A transcripts. Furthermore, an unknown mechanism of alternative splicing must determine whether ZC3H11A transcripts with or without the ZBED6 sequence are produced. As the ZBED6 ORF is located upstream of the translation start site for ZC3H11A, ZBED6 intron retention would result in translation of ZBED6 and not of ZC3H11A. Comparing the human islet RNA sequencing results with the C2C12 RNA sequencing results, we found that at least 9.2% ZBED6 transcripts were spliced out of the ZBED6/ZC3H11A transcripts in human islets and at least 25% ZBED6 transcripts were spliced out in C2C12 cells. The ZC3H11A transcript without ZBED6 is translated to ZC3H11A protein, which is supported by our immunoblot data showing that C2C12 cells express both ZBED6 and ZC3H11A. To summarize, we propose that human islet cells predominantly express the bi-cistronic ZBED6/ZC3H11A transcript that results in the translation of the full-length ZBED6 protein and that ZC3H11A and ZBED6 are produced from separate mRNA molecules.

Another possible explanation of the generation of the three ZBED6 forms in insulin-producing cells (Fig.1G) is proteolytical processing or the use of alternative translation start sites. Indeed, the first three methionines of the ZBED6 open reading frame correspond well to the sizes of the three ZBED6 forms, supporting the notion that the three forms may represent alternative translational start sites. Alternatively, differentiated beta-cells may express specific proteases that cleave ZBED6 at specific sites so that the N-terminal region is removed. For example, using the GPS-UUP 1.0 software, a high score calpain proteolytical site was predicted at amino acid 241. Calpains are calcium-activated proteases, and a previous study reported that Calpain-3 removes the nuclear localization signal peptide from the enzyme dUTPase [87], thereby controlling the subcellular localization of the enzyme. Calpain-10 was identified as a susceptibility gene for common Type 2 diabetes [88]. Enhanced expression/activity of calpains might increase the cleavage of ZBED6 at specific sites; disturb the NLS at the N-terminal; increase the level of the cytosolic form of ZBED6 and finally impede the effect of

ZBED6 as a transcription factor. Further studies are required to unravel how beta-cells control ZBED6 size, its subcellular localization and effects on transcriptional regulation.

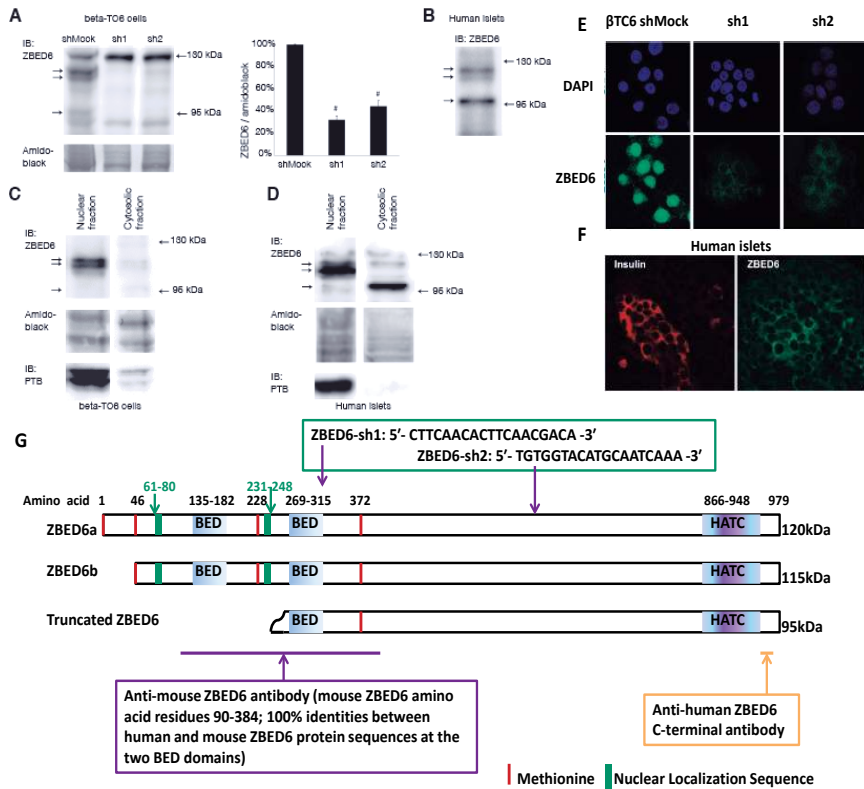


Figure 1. ZBED6 expression, knock-down and subcellular localization in β TC6 cells and human islets. (A) Expression of ZBED6 protein in β TC6 cells after stable transduction with ZBED6-targeting shRNA. ZBED6 immunoreactivity was obtained using the anti-mouse ZBED6 antibody. Arrows indicate the positions of the three ZBED6 immunoreactive bands. Results are means \pm SEM for three observations. # denotes $P < 0.01$. (B) Expression of ZBED6 proteins in human islets detected using the anti-human ZBED6 antibody. (C) β TC6 cytosolic and nuclear fractions were analyzed by immunoblotting using the anti-mouse ZBED6 antibody. The subcellular fractionation procedure was verified by immunoblot analysis of PTB, a nuclear RNA-binding protein. (D) Human islet nuclear and cytosolic fractions were analyzed by immunoblotting using the anti-human ZBED6 antibody. (E) Staining of shMock, sh1 and sh2 β TC6 cells with anti-mouse ZBED6 antibody and DAPI. (F) Staining of human islet cells for ZBED6 and insulin. (G) Schematic presentation of three human ZBED6 forms with the molecular weights 120 kDa, 115 kDa and 95 kDa. Putative translation start sites (methionine at residues 1, 46, 228 and 372 in the ZBED6a isoform) are indicated together with the localization of the BED1 (residues 135-182), BED2 (residues 269-315) and HATC (residues 866-948) domains, and putative nuclear localization signals (NLS) at residues 61-80 and 231-248; the two NLS sequences were identified in mouse C2C12 cells but the second NLS in human ZBED6 (KKHDKSASDALRAERGRFL) differs at two positions from the mouse sequence (KKRDKSASDALRAKRGRFL), and it is therefore uncertain whether the sequence is active as an NLS in human ZBED6. Epitope regions for mouse and human ZBED6 antibodies as well as shRNA target sites are depicted.

Stable silencing of ZBED6 in β TC6 cells

Stable silencing of ZBED6 in β TC6 cells was achieved by transduction with lentiviral vectors expressing shRNAs that specifically target ZBED6 mRNA. Two shRNA sequences targeted to different sites of ZBED6 mRNA were used and the transduced β TC6 cells were named ZBED6-sh1 and ZBED6-sh2. A lentiviral vector carrying a scrambled shRNA sequence was used to create transduced control cells (shMock). Down-regulation of ZBED6 was confirmed by real-time RT-PCR showing reductions of ZBED6 mRNA levels by 75% (sh1) and 59% (sh2). In sh1 and sh2 cells, the three isoforms of ZBED6 were clearly weakened resulting in a 60-70% decrease in ZBED6 intensity when expressed per total amidoblack staining (Fig. 1A).

ChIP-sequencing analysis revealed more than 350 putative target genes of ZBED6 in human islets, which are enriched for transcription, transcription regulation, apoptosis, positive regulation of cellular biosynthetic process and transmembrane genes

ChIP-sequencing of human islet cells revealed ZBED6 binding to 351 genes, using 19 overlapping reads as threshold. The 351 genes were significantly clustered into categories dealing with transcriptional regulation, apoptosis, biosynthetic processes and transmembrane proteins. Most of the peaks had a match to the ZBED6 binding sequence GCTCGC in the peak center, which is in consistent with the previous study in C2C12 cells. Most of the binding sites containing the motif located closed to TSS, indicating that ZBED6 mainly affects gene expression by binding to proximal regulatory elements.

ZBED6 binding to the MafA, Neurog3 and NeuroD2 genes has been reported in C2C12 cells [1], but was not observed in human islet cells. It may be that ZBED6 acts as a repressor of these genes in non-beta-cells and that ZBED6 binding to the same genes is abolished in beta-cells.

ZBED6 knockdown in β TC6 cells enhanced the expression of beta-cell specific genes and affected cell morphology

Knockdown of ZBED6 in β TC6 cells increased the expression of Igf2, which is in line with a previous study reporting that ZBED6 acts as an Igf2 repressor [1]. The mRNA levels of insulin, PDX1, glucokinase and MafA were all significantly increased in sh1 cells. A similar pattern was observed in sh2 cells, but the effect was not as pronounced as in sh1 cells, indicating that the sh2 sequence is less efficient in knocking down ZBED6. The increased expression of PDX1 and insulin was further verified by immunostaining and immunoblot analysis. Different cell morphology was observed in the ZBED6 knock-down cells, which rounded up and formed 3-dimensional islets-like clusters, whereas the control cells to a higher degree spreaded out and covered the surface as a monolayer.

ZBED6 knock-down cells proliferated at a lower rate due to a partial inhibition of the S/G2 phase of the cell cycle

We observed that sh1 and sh2 β TC-6 cells proliferated at a lower rate than controls, and that the cells transduced with a combination of sh1 + sh2 proliferated the slowest. The sh1 and sh2 both exhibited a lowered fraction G1 cells. In addition, sh1 cells had an increased percentage S-phase cells. These results indicate that ZBED6 knock-down cells proliferate at a lower rate due to a partial inhibition of the S/G2 phase of the cell cycle.

Higher cell death rates were observed in ZBED6 knock-down cells when treated with palmitate

Sodium palmitate, the nitric oxide donor DETA/NO and the cytokines IL-1 β + IFN- γ are commonly use in *in vitro* studies to mimic the pathological stimuli of beta-cells in T2D and T1D. At basal conditions sh1 and sh2 β TC-6 cells displayed similar cell death rates as control cells. When stressed by over-night exposure to sodium palmitate, however, the ZBED6 knock-down resulted in augmented cell death rates as compared to the Mock cells. Also in response to DETA/NO and cytokines an increased cell death was observed, although statistical significances were in some cases not obtained. The increased sensitivity to palmitate was paralleled by an augmented level of the pro-apoptotic protein CHOP and increased phosphorylation of Bim (BCL2L11).

The β TC6 cell line was established from insulinomas derived from transgenic mice carrying a hybrid insulin-promoted SV40 tumor antigen gene [89]. The immortalize cell line has been used in various *in vitro* studies as a pancreatic beta-cell line. However, it can never compete with the fully differentiated primary beta-cell. ZBED6 silencing in β TC6 cells induced a

more pronounced beta-cell phenotype, as supported by increased expression of beta-cell specific genes; altered morphology; decreased proliferation with a partial S/G2 cell-cycle arrest and higher sensitivity to pro-apoptotic stimuli. Staining of β TC6 cells revealed that β TC6 cells expressed high levels of nuclear ZBED6 and that knockdown affected mainly this form of ZBED6 (Fig.1E). Considering the differential expression pattern of ZBED6 in β TC6 cells and human islets in that the nuclear ZBED6 is the predominant form in rapidly proliferating β TC-6 cells, but not in more differentiated human islet cells, we propose that the nuclear ZBED6 supports proliferation and survival of beta-cells, possibly at the expense of specialized beta-cell function. Future studies are warranted to clarify how ZBED6 controls its transcriptional regulatory effect by deciding its subcellular localization. Finally, the putative function of the cytoplasmic form of ZBED6 is not understood, but this truncated ZBED6 protein may participate in cytoplasmic functions of the fully mature and non-proliferating beta-cell.

Paper II

ZBED6 negatively regulates insulin production, neuronal differentiation and cell aggregation in MIN6 cells. *Submitted*

ChIP sequencing analysis in MIN6 cells identified more than 4000 putative ZBED6 target genes, including Pdx1, Nkx6.1 and MafA

Immunofluorescence staining showed weak nuclear and strong cytoplasmic staining of ZBED6 in MIN6 cells. To identify the target genes of ZBED6 in insulin-producing MIN6 cells, we performed ChIP sequencing using the anti-mouse ZBED6 antibody. Using a cut-off at 30 reads to reduce the number of false positive peaks, we found 4700 significant peaks of ZBED6 bindings. In agreement with our previous results from mouse C2C12 myoblast cells and human islet cells (Paper I), the majority of peaks were found close to transcription start sites (TSS). Analysis of the peaks within one kb of TSS detected in the three ChIP-seq analyses (C2C12 [1], human islets [Paper I] and MIN6) showed that 79% peaks identified in C2C12 cells overlapped with the same peak in MIN6 cells. The overlap between putative binding sites in MIN6 cells and human islets were not high (39%). The low overlap rate might be due to a lower affinity of the anti-mouse ZBED6 antibody to human ZBED6, or a low nuclear expression of ZBED6 in human islets. Nevertheless, the data suggest that the overlap in ZBED6 binding sites is higher between cell types within species than within cell type between species.

The same ZBED6-binding motif sequence GCTCG was identified in MIN6 cells. This motif was present in 82.4% of the peaks above the thresh-

old, compared to 9% in background regions. When the peaks with no GCTCG match were searched for motifs, a longer motif containing two GCTC sequences was found to be overrepresented. The high enrichment of ZBED6 motifs indicates that most of the significant peaks are locations of direct ZBED6 binding.

Interestingly, Pdx1, Nkx6.1 and MafA, three transcription factors crucial to beta-cell maturation, all had ZBED6 peaks at the TSS, with peaks for Pdx1 and Nkx6.1 well above our stringent cut-off.

Stable Zbed6-silencing in MIN6 cells and whole transcriptome analysis

Stable silencing of Zbed6 in MIN6 cells was achieved by lentiviral-mediated shRNA. To decrease the possibility of off-target effects, two different Zbed6 shRNA sequences (sh1 and sh2) were used. Zbed6 silencing was confirmed by semi-quantitative real-time PCR and immunoblotting. Immunofluorescence staining showed that both the nuclear and cytoplasmic staining of ZBED6 was dramatically decreased. Whole transcriptome analysis of sh1 and shMock cells was carried out to further investigate the role of ZBED6 in MIN6 cells. The sh1 and shMock samples showed a similar overall distribution of gene expression. We detected 12,352 (50.5%) out of 24,468 annotated RefSeq genes. Changes in gene expression, expressed as Log₂ fold changes of shMock and FDR adjusted P values, between sh1 and shMock samples were calculated. The criterion of an FDR smaller than 5% was used to identify differentially expressed (DE) genes. We found 731 DE genes after Zbed6-silencing in MIN6 cells.

Gene ontology analysis indicated that genes associated with neuronal differentiation and cell adhesion were significantly over-represented among DE genes after Zbed6 silencing

The DE genes were submitted for enrichment analysis of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the Database for Annotation, Visualization and Integrated Discovery (DAVID). These genes were significantly enriched in several GO categories including neuronal differentiation and projection, cell projection, regulation of neuronal differentiation, generation of precursor metabolites, axon guidance molecules, MAPK signaling pathway and cell adhesion.

As mentioned in the introduction part, beta-cell attachment to ECM is vital to beta-cell proliferation and insulin secretion. It is also well known that beta-cells resemble neurons as both cell types are excitable and capable of regulated secretion. Indeed, a recent investigation reported that 15% of conserved beta-cell markers were shared with neurons, and that these genes were mainly involved in regulating synaptic vesicle transport [90]. Therefore, we decided to focus on neuronal differentiation and cell adhesion. A group of genes belonging to these two categories were verified by quantitative PCR and the results supported the notion that ZBED6 affects the expression of genes that control neuronal differentiation and cell adhesion.

Silencing of Zbed6 affected cell morphology/growth patterns and promoted the formation of neurofilament-positive and axon-like protrusions

To further study the effects of ZBED6 on neuronal differentiation and cell adhesion, we analyzed cell morphology during culture with or without laminin coating. Interestingly, clear morphological differences were observed after a 5-day culture period in the absence of laminin coating. The sh1 and sh2 cells were more aggregated and formed three-dimensional structures, whereas the control cells to a higher extent spread out as a traditional monolayer. This indicates that ZBED6 is required for monolayer growth on a plastic support. The result is also in line with the morphological changes observed in β TC6 cells after knockdown ZBED6 (Paper I). In the case of laminin coating, after 5 days of culture, the three-dimensional cell clusters were still evident in the sh1 and sh2 cell lines. In addition, thin and extended axon-like protrusions were observed to originate from the Zbed6-silenced cells. Interestingly, sh1 and sh2 cells were strongly stained with anti-neurofilament (Nefm) antibody, whereas shMock cells were only weakly stained for Nefm, with some cells showing no Nefm immunoreactivity at all. Using a higher magnification, we observed in sh1/sh2 MIN6 cells that the dense neurofilament fibers were present in the axon-like protrusions. The increased expression of Nefm in sh1 and sh2 cells was also confirmed by immunoblot.

Nefm is one of the DE genes identified by whole transcriptome analysis and it also has ZBED6 binding site. It has been observed that a MIN6 subline with an intact glucose-induced insulin release expressed more neurofilament than a MIN6 subline with an impaired insulin release [91]. Thus, it is likely that increased beta-cell neuronal differentiation underlies an improved insulin release and beta-cell function.

Similar morphological changes were observed in both β TC6 and MIN6 cells after silencing Zbed6. The aggregated and three-dimensional clusters indicate more intercellular interactions, which are important for the development and maintenance of the beta-cell phenotype. As it has been reported that the function of MIN6 cells improves when the cells aggregate into three-dimensional islet-like structures, known as pseudoislets [92].

ZBED6 knockdown increases beta-cell transcription factor expression, insulin content, glucose-induced insulin release and cytoplasmic Ca²⁺ mobilization

Silencing of Zbed6 in MIN6 cells increased the expression of Pdx1, Nkx6.1 and MafA, which all have ZBED6 binding sites. As the up-regulation of these three genes is compatible with a more mature beta-cell phenotype, we also analyzed the content and release of insulin in these cells. Immunostaining of insulin indicated increased accumulation of insulin granules in the cytoplasm of most of the sh1 cells and some of the sh2 cells. The insulin

content was significantly higher in sh1 MIN6 compared with mock cells. The insulin release of both sh1 and sh2 cells at a high glucose concentration was significantly higher than in mock cells. The release of insulin in response to potassium chloride was not significantly different in sh1/sh2 cells as compared to mock cells, indicating that depolarization-mediated insulin release is not affected by silencing of Zbed6. The increased glucose-stimulated insulin secretion in the ZBED knockdown cells was accompanied by a more pronounced glucose-induced rise of the cytoplasmic Ca^{2+} concentration in the sub-plasma membrane space.

The current results indicate that the expression of these three important beta-cell transcription factors, Pdx1, MafA and Nkx6.1, is coordinatively suppressed by ZBED6, possibly via direct binding of ZBED6 to these three genes. The fact that Zbed6-silenced MIN6 cells express elevated levels of beta-cell transcription factors, contain more insulin and release more insulin in response to high glucose supports the notion that ZBED6 acts as an anti-beta-cell-maturation factor.

Paper III

Knock-down of ZBED6 in insulin-producing cells promotes N-cadherin junctions between beta-cells and neural crest stem cells *in vitro*. *Manuscript*

Zbed6-silenced β TC6 and MIN6 cells displayed an altered morphology *in vitro*

Plating of an equal number of shMock, sh1 or sh2 cells to culture dishes resulted in an altered organization and morphology of the cells after 3 days of culture. We observed that shMock cells spreaded out and formed traditional monolayers, whereas sh1/2 cells in some cases formed three-dimensional cell clusters.

Zbed6-silenced β TC6 cells attached less efficiently to a laminin-coated support, which was paralleled by weaker FAK phosphorylation at sites close to the laminin support

A significantly lower attachment of sh1 cells to mouse laminin support during a 4-hour incubation period was observed. Similar tendency was also found in sh2 cells but this did not reach statistical significance. Next, we studied attachment to human laminin 511, a specific isoform which has been demonstrated to interact with integrin $\alpha 3\beta 1$ of human islet beta-cells and convey proliferation and plasticity of these cells [93]. Also in this case sh1 and sh2 cell adhesion was impaired at 1 μ g/ml.

Immunoblot analysis indicated that neither total FAK levels, nor the total FAK phosphorylation was affected by ZBED6-silencing in cells growing on

a mouse laminin support. However, confocal analysis revealed that the distribution of phospho-FAK sites was different in sh1/2 cells as compared to shMock cells. Intercellular phospho-FAK sites were frequent in sh1/2 cells, but not in shMock cells. On the other hand, phospho-FAK sites were frequent and prominent close to the laminin support in shMock cells, but not in sh1/2 cells. Thus, the weaker adhesion to the laminin support of Zbed6-silenced cells is associated with a weaker FAK activation at this site.

A decreased attachment to laminin of Zbed6-silenced cells could be induced by disturbance of the integrin $\alpha3\beta1$ expression on the cell surface. It has been reported that glucose-induced activation of FAK is mediated by $\beta1$ integrin [94]. Therefore, a decreased expression of integrin $\beta1$ might link the poor attachment to laminin and the weak FAK activation close to laminin support together. Further studies are warranted to investigate the expression of intergrin $\beta1$ in Zbed6-silenced cells as well as other possible adhesion molecules.

Zbed6-silenced β TC6 cells formed weaker E-cadherin but stronger N-cadherin cell-to-cell junctions and the stronger N-cadherin cell-to-cell junctions were necessary for the three-dimensional growth pattern of β TC6 cells

Insulin-producing cells are known to express both E-cadherin and N-cadherin [82]. Immunoblot analysis and confocal microscopy showed weaker E-cadherin but stronger N-cadherin at cell-to-cell junctions in Zbed6-silenced β TC6 cells. In addition to this, the formation of the three-dimensional clusters in Zbed6-silenced β TC6 cells was blocked by using a N-cadherin neutralizing antibody, which indicates that the stronger N-cadherin cell-to-cell junctions were necessary for the three-dimensional growth pattern.

Zbed6-silenced MIN6 cells formed stronger N-cadherin cell-to-cell junctions, possibly via a direct transcriptional effect of ZBED6 on the N-cadherin gene

Also in MIN6 cells ZBED6 knockdown resulted in increased N-cadherin expression at cell-to-cell junction. ChIP sequencing analysis revealed a strong ZBED6 binding peak approximately 300 bp upstream of the TSS. This indicates that ZBED6 directly represses N-cadherin gene transcription.

N- and E-cadherin are calcium-dependent glycoproteins that mediate homophilic cell-to-cell contacts, which are important for differentiation, tissue organization, motility, cell polarity, proliferation and survival [95]. A line of studies show that E-cadherin is the predominant form of the cadherins in mature beta-cells, and that N-cadherin expression is considerably weaker [95]. However, a recent study reported that N-cadherin expression is low and dispensable for beta-cell embryonic development, but that it in adult beta-cells is necessary for insulin granule turnover and a normal insulin release [96]. Thus, a gradual decrease in ZBED6, which may occur when beta-cells

differentiate and proliferate more slowly, will lead to a relief of the ZBED6-mediated inhibition of N-cadherin gene transcription, and ensure normal adult beta-cell function. This notion is supported by the present observation that neutralization of N-cadherin cell-to-cell contacts, using a N-cadherin antibody, counteracted the formation of three-dimensional structures often observed during culture of sh1/2 cells. Three-dimensional growth and the formation of pseudo-islet structures is known to enhance beta-cell function as compared to dispersed or mono-layer cells [97].

Junctions between beta-cells and NCSC bodies and processes stained more strongly for N-cadherin when sh1/2 cells were used during *in vitro* co-culture

Co-culture of GFP-positive mouse NCSCs with sh1/2 cells promoted an increase in GFP-positive cell processes, as compared to shMock cells. These processes radiated from NCSC bodies and projected into the surrounding mass of non-GFP positive β TC6 cells. In addition, the GFP-positive NCSC processes stained for N-cadherin to a higher extent when surrounded by sh1/2 cells as compared to shMock cells. Also the borders between NCSC bodies and β TC6 cells were more intensely stained for N-cadherin when sh1/2 cells were used during co-culture.

As mentioned in the Introduction, NCSCs play an important role in the embryogenesis of the pancreas and beta-cells, possibly via direct cell-to-cell contacts. Removal of NCSCs resulted in increased endocrine proliferation, but also a loss of beta-cell functional maturation [84]. The exact mechanism by which NCSCs regulate embryonic beta-cell maturation is still unclear. The current investigation with N-cadherin might offer a clue to this mystery in that strong N-cadherin cell-to-cell junctions between beta-cell and NCSCs stimulate the formation of NCSC processes and improve the contacts between these two types of cells, thereby slowing down beta-cell proliferation and instead enhancing beta-cell function/maturation.

Conclusions

The main conclusions of this thesis are:

- ZBED6 is expressed in mouse β TC-6 cells and human islets as a double nuclear band at 115/120 kDa and as a single cytoplasmic band at 95-100 kDa, which lacks N-terminal nuclear localization signals.
- ZBED6 supports proliferation and survival of beta-cells, possibly at the expense of specialized beta-cell function.
- ZBED6 represses the expression of Pdx1, Nkx6.1 and MafA in MIN6 cells.
- ZBED6 negatively regulates insulin production and release, neuronal differentiation and cell aggregation in MIN6 cells.
- ZBED6 decreases the ratio between N- and E-cadherin. A lower N- to E-cadherin ratio may hamper the formation of three-dimensional beta-cell clusters and cell-to-cell junctions with NCSC, and instead promote efficient attachment to a laminin support and monolayer growth.

Future prospects

Role of ZBED6 *in vivo* using ZBED6 global and beta-cell specific knockout mice

The work presented in this thesis indicates an important role of ZBED6 in beta-cell proliferation, function, death and neogenesis. It will be interesting to combine our present *in vitro* study with *in vivo* models for ZBED6 deficiency. In this case, ZBED6 global and beta-cell specific knockout mice will serve as critical resources. Global ZBED6 knockout mice have already been generated and beta-cell specific ZBED6 knockout mice will soon be available. It is noteworthy that there are many studies showing conflicting *in vitro* and *in vivo* results. However, in the case of ZBED6, knockout mice will offer more information on the role of ZBED6 in different tissues and whether other genes substitute for ZBED6 function during embryonic development. The knockout mice model will also provide more convincing results to support a putative role of ZBED6 in key events in the pathogenesis of diabetes. To study this, glucose homeostasis of the two types of knockout mice will be investigated at basal conditions and at conditions of glucose intolerance. Beta-cell mass and insulin content will be analyzed. In the case with the global knockout model, other tissues, which are also affected by diabetes, such as liver, skeletal muscle and adipose tissue, are of great interest for further ZBED6 studies, for example by employing RNA sequencing. Using the beta-cell specific knockout mice, in addition to the glucose homeostasis study, a closer investigation of pancreas development and endocrine cell maturation during the embryonic stage will provide more clues to ZBED6's role in beta-cell development, which will be discussed in detail in the next paragraph.

Study the effects of ZBED6 on pancreatic islet development

ChIP sequencing using C2C12 cells has identified more than 2000 ZBED6 binding sites in the genome. Genes associated with ZBED6 binding sites show a highly significant enrichment for development regulation [1]. In our present study using a mouse pancreatic beta-cell line, MIN6, more than 4000

putative ZBED6 target genes, including Pdx1, Nkx6.1 and MafA, were detected. RNA sequencing as well as real time PCR results indicated a repressive effect of ZBED6 on these genes (paper II). As mentioned in the introduction, Pdx1, Nkx6.1 and MafA are crucial transcription factors in pancreas development and endocrine cells maturation. It is conceivable that ZBED6, via modulating Pdx1, Nkx6.1 and MafA transcription, controls important events in pancreas and beta-cell development. To study this, human embryonic H9 stem cells will be differentiated to pancreatic progenitors and different islet precursor cells *in vitro*. The expression of ZBED6 at the different stages will be determined by immunoblotting and immunostaining. ZBED6 will be knocked down at different differentiation stages using the lentiviral approach and the effect on pancreatic stem cell differentiation will be studied.

Summary in Swedish

Insulinproducerande beta-celler slutar att fungera som de ska vid både typ-1 och typ-2 diabetes. Det är därför viktigt att bättre förstå vad som styr beta-cellens tillväxt, funktion, död och återbildning, både under normal förhållanden och vid diabetes. ZBED6 är en nyligen upptäckt transkriptionsfaktor som finns bara hos däggdjur, och det är möjligt att denna transkriptionsfaktor påverkar beta-cellens olika öden. Målsättningen med denna avhandling har därför varit att närmare studera ZBED6 i beta-celler.

ZBED6 finns i både en beta-cellslinje från mus (β TC-6) och i humana öceller. Proteinet kan endera förekomma som ett kärnprotein med storleken 115/120 kDa eller som ett cytoplasmiskt protein med storleken 95-100 kDa. I snabbt växande celler dominerar kärnformen medan i långsamt växande celler är cytoplasmiska formen mera vanlig. Tystar man ZBED6-genen i β TC-6 celler växer cellerna långsammare, Cellerna stannar upp i S/G2 fasen och uttrycket av insulin och Pdx1 ökar. Behandlar man celler med nedtystad ZBED6-gen med faktorer som är typiska för diabetes, nämligen cytokiner, kväveoxid och fria fettsyror, ökar celldöden. ZBED6 binder i humana öceller till gener som kontrollerar transkription, biosyntes av makromolekyler samt celldöd. Dessa resultat tyder på att ZBED6 upprätthåller celltillväxt och överlevnad, troligtvis på bekostnad av specialiserad beta-celfunktion, d.v.s. insulinproduktion.

För att bättre förstå betydelsen av ZBED6 i beta-celler analyserades vilka ställen i genomet ZBED6 binder till och effekter på transkription i MIN6-celler. Vi fann att ZBED6 troligtvis binder till ca 4000 gener och att bland dem finns de viktiga beta-cells-generna Pdx-1, MafA och Nkx6.1. Cirka 700 geners transkription förändrades som svar på tystning av ZBED6, och samtidigt ökade innehåll och frisättning av insulin. Vi observerade även att cellernas utseende förändrades så att cellerna bildade aggregat snarare än att sprida ut sig på odlingsplattan. Denna förändring i cellernas utseende kan möjligtvis förklaras av att tystning av ZBED6 orsakade en ökad kvot mellan N- och E-cadherin. En ökad kvot är förenlig med stimulerad bildning av cellaggregat, både innehållande bara beta-celler eller beta-celler tillsammans med neurallistceller. Den ökade förmågan att bilda cellaggregat kan samtidigt förklara den minskade förmåga till att binda till underlaget och där växa som ett platt cellskikt. Sammantaget tyder detta på att ZBED6 kan spela en viktig roll i beta-cellens differentiering, tillväxt och överlevnad.

Vi hoppas att detta arbete i förlängningen kan leda till ny kunskap av betydelse för framtida behandling av diabetessjukdomen, och då framförallt när det gäller beta-cellens nybildning och motståndskraft mot destruktion vid diabetes.

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