

STRESS-INDUCIBLE MIG6 PROMOTES PANCREATIC BETA CELL
DESTRUCTION IN THE PATHOGENESIS OF DIABETES

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STRESS-INDUCIBLE MIG6 PROMOTES PANCREATIC BETA CELL

DESTRUCTION IN THE PATHOGENESIS OF DIABETES

Pancreatic insulin-secreting beta cell failure is central to the development of diabetes. Therapeutic applications targeted at understanding and manipulating beta cell destruction mechanisms should enhance the preservation of functional beta cell mass and prevent diabetes. To this end, we have demonstrated that diabetogenic assaults (e.g., endoplasmic reticulum stress, glucolipotoxicity, and pro-inflammatory cytokines) attenuate the activation of beta cell pro-survival signaling pathways via a stress-inducible molecule called Mitogen-inducible gene 6 (Mig6). We discovered that the overabundance of Mig6 exacerbates stress-induced beta cell apoptosis and inhibits insulin secretion. Conversely, the deficiency of Mig6 partially protected beta cells from DNA damage-induced cell death. Further, we established that Mig6 haploinsufficient mice retained islet integrity and function and exhibited greater beta cell mass recovery following treatment with multiple low doses of the beta cell toxin streptozotocin. These data suggest that Mig6 may be a therapeutic target for beta cell preservation in diabetes.

Patrick T. Fueger, Ph.D., Chair

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ABBREVIATIONS

ADA	American Diabetes Association
AGEs	Advanced Glycation End products
AR	Amphiregulin
AREs	Adenylate-uridylate-Rich Elements
ATF4	Activating Transcription Factor 4
BAX	Bcl-2-Associated X
BIRC	Baculoviral Inhibitors of apoptosis Repeat Containing
BMI	Body Mass Index
BTC	Betacellulin
C-lobe	Carboxy-terminal lobe
CA	Camptothecin
CHOP	C/EBP Homologous Protein
CRIB	Cdc42/Rac-Interaction and Binding
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
CMV	Cytomegalovirus
DI	Disposition Index
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFR-DN	EGFR kinase Dominant Negative
ENDIT	European Nicotinamide Diabetes Intervention Trial
ER	Endoplasmic Reticulum

ERAD	ER-Associated Degradation
ERK	Extracellular signal-Regulated Kinase
FFA	Free Fatty Acids
FPIR	First Phase Insulin Response
GAD65	Glutamate Decarboxylase
GFP	Green Fluorescent Protein
GLP-1	Glucagon-Like Peptide-1
GLT	Glucolipotoxicity
GSIS	Glucose-Stimulated Insulin Secretion
GSK3	Glycogen Synthase Kinase 3
GWAS	Genome-Wide Association Study
HB-EGF	Heparin-Binding EGF-like Growth Factor
HER	Human EGF Receptor
HLA	Human Leukocyte Antigen
HOMA	Homeostasis Model Assessment
HSP60	Heat Shock Protein 60
IA2	Insulinoma-associated Antigen 2
IAP	Inhibitor of Apoptosis
IAPP	Islet Amyloid Polypeptide
IFG	Impaired Fasting Glucose
IFN- γ	Interferon Gamma
IGRP	Islet-specific Glucose-6-phosphatase catalytic subunit-Related Protein
IGT	Impaired Glucose Tolerance

IκB	Inhibitor of Kappa B
IL-1β	Interleukin 1-Beta
IL2RA	Interleukin 2 Receptor Alpha-subunit
IRES	Internal Ribosomal Entry Sites
ITT	Insulin Tolerance Test
IVGTT	Intravenous Glucose Tolerance Test
JNK	c-Jun N-terminal Kinase
K _{ATP} Channel	ATP-sensitive potassium Channel
LRIG1	Leucin-Rich and Immuneoglobulin-like domains protein 1
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-activated protein/Extracellular signal-regulated kinase Kinase
MIG6	Mitogen-Inducible Gene 6
MLD	Multiple Low-Dose
mTOR	Mammalian Target of Rapamycin
N-lobe	Amino-terminal lobe
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGT	Normal Glucose Tolerance
NO	Nitric Oxide
NOD	Non-Obese Diabetic
NRG	Neuregulin
OGTT	Oral Glucose Tolerance Test
PDX-1	Pancreatic and Duodenal homeobox-1
PI3K	Phosphatidylinositol 3-Kinase

PTEN	Phosphatase and Tensin Homolog
PTP1B	Protein Tyrosine Phosphatase 1B
PTPN22	Protein Tyrosine Phosphatase, Non-receptor type 22
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
SEM	Standard Error of Mean
SHIP2	SH2-containing 5'-Inositol Phosphatase
SOCS	Suppressor of Cytokine Signaling
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Tg	Thapsigargin
TGF- α	Transforming Growth Factor Alpha
TNF- α	Tumor Necrosis Factor Alpha
TRIB3	Tribbles homolog 3
TRIGR	Trial to Reduce Incidence of Diabetes in a Genetically at Risk study
UTR	Untranslated Region
VNTR	Variable Tandom Repeats
XBP-1	X-box Binding Protein 1
ZNT8	Zinc Transporter 8

1 INTRODUCTION

1.1 Type 1 diabetes

1.1.1 Epidemiology and diagnosis

Diabetes mellitus is one of the most common non-communicable diseases, affecting more than 366 million people worldwide. By 2030, the prevalence of diabetes is predicted to rise to a staggering 552 million (1). There are two type of diabetes – type 1 and type 2 diabetes (T1D and T2D, respectively). T1D, classified by the presence of autoimmune-mediated beta cell destruction (2), accounts for approximately 5% of diabetes mellitus patients.

Historically, T1D has been considered a child- or juvenile-onset disease; however, age is not the defining factor. The main symptoms of T1D are polyuria, polydipsia, and polyphagia, and the clinical features are hyperglycemia, weight loss, and diabetic ketoacidosis. The diagnostic criteria include: a random plasma glucose concentration ≥ 11.1 mmol/L or a fasting plasma glucose concentration ≥ 7.0 mmol/L (3). Other diagnostic markers such as plasma autoantibody levels, C-peptide levels, and ketonuria are currently not recommended for routine diagnostic use (4-6).

1.1.2 Etiology of T1D

Most of our understanding about the pathogenesis of T1D comes from: 1) retrospective patients' serum samples and cadaveric pancreata analyses, and 2) T1D prevention or intervention trials. These studies demonstrate that T1D is an autoimmune-mediated disease, in which pancreatic beta cells are destroyed, resulting in insulin deficiency and hyperglycemia. The proposed causes of T1D include genetic predisposition and environmental factors.

Genetic predisposition is required for the development of T1D (7). Whereas early twin studies suggested that the risk of T1D for the monozygotic twins of patients with T1D is as low as 30%, a recent cumulative incidence analysis revealed that the concordance of twins exceeds 65% by age 60 (8), indicating strong genetic basis for T1D. The linkage and candidate gene studies, and recent genome-wide association study (GWAS) have revealed more than 50 loci associated with risk to T1D. Among them, human leukocyte antigen (HLA) class II DR and DQ alleles show the greatest risk (7, 9). HLA alleles encode a group of surface proteins that present antigen to T lymphocytes, and thus alterations in HLA alleles may modulate antigen epitope presentation (10), leading to disrupted central tolerance and autoimmune reactions (11-13). The second prominent T1D risk loci is located upstream of the insulin gene (INS, encoding pre-proinsulin peptide). The length of the variable number tandem repeats (VNTR) in that loci controls the thymic INS expression level, therefore regulating the immune tolerance toward insulin (14-16). Other frequent T1D risk loci are PTPN22 (encoding lymphoid protein tyrosine phosphatase, a suppressor of T cell activation) (17, 18), IL2RA (α -subunit of the IL-2 receptor, which controls self-tolerance) (19), and CTLA4 (T-cell-specific transmembrane co-receptor, negative regulator of T cell activation). The extensive collection of genetic studies highlight the immunological origin of T1D, that is, an incomplete self-tolerance to beta cell antigens triggers an adaptive immune response, disrupts innate immunity, and results in the destruction of beta cells. However, further genetic mapping and functional studies must be performed to gain a better understanding of the molecular mechanisms leading to the development of T1D.

Despite the influence of genetic susceptibility, the etiology of T1D cannot be fully explained by genetics because: 1) the pair-wise concordance of T1D in monozygotic twins is lower than expected if T1D was purely due to genetics (8), and the disease progression rates vary (20), 2) the proportion of subjects with high-risk HLA genotypes has decreased in the last few decades, whereas the low-risk HLA population has increased (21, 22), suggesting genetic predisposition's contribution to T1D is decreasing (23), 3) the T1D geographical distribution pattern (highly concentrated in Northern Europe) cannot solely be explained by genetic factors (24), and 4) migration studies suggest that the population from low incidence regions have increased T1D frequency when they move to high incidence regions (25), highlighting the environmental influences on T1D etiology. Although the identification of environmental factors is still in its early stages, viruses (26), bacteria (27), and diet (20) have been suggested to trigger and/or potentiate the development of T1D.

1.1.3 The natural history of T1D

In 1986, Dr. George Eisenbarth first proposed that T1D is a cell-mediated autoimmune disease (28). In his well-received T1D pathogenesis model, the natural history of T1D could be separated into different stages as illustrated in **Figure 1-1**.

First, genetic and environmental factors trigger the autoimmune response. Subsequently, the dysregulated immune system attacks pancreatic beta cells, leading to beta cell de-granulation and death. In this stage, the surviving beta cells are still able to produce sufficient insulin to meet the body's metabolic demands. Only when a critical amount of functional beta cells are destroyed, do the clinical symptoms (e.g., hyperglycemia and glucose intolerance) emerge and T1D is diagnosed.

Following diagnosis and the initiation of insulin therapy, most patients experience a transient period of beta cell recovery (known as the “honeymoon phase”). Ultimately though, most beta cells succumb to death after the disease onset. However, in some longstanding patients, low concentrations of C-peptide and scant numbers of beta cells are observed (29).

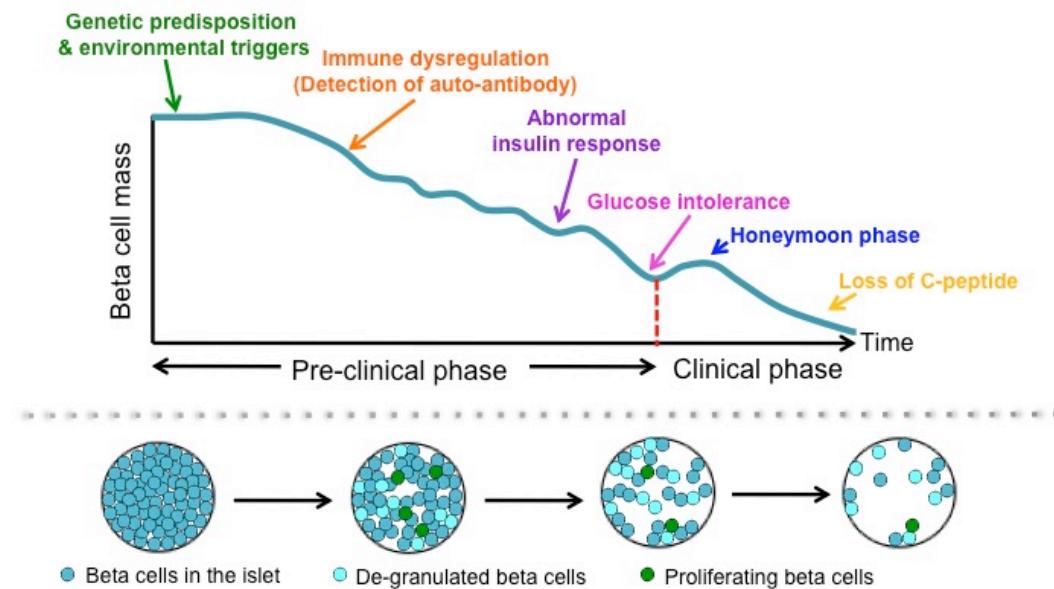


Figure 1-1

The natural history of T1D (Modified from Akirav et al. (30) and Von Herrath et al. (31)).

The already complex model for the natural history of T1D will undoubtedly become even more complex in the future. As new technology has emerged leading to additional discoveries, we are now starting to appreciate the complex cellular and molecular events of immune-mediated beta cell destruction in both the preclinical and clinical stages of T1D. Importantly, the prodrome stage of T1D may last from months to decades (32). The implications of this timeframe are that the therapeutic window to prevent the onset of T1D is much longer than what was originally thought decades ago.

Pre-clinical stage:**Immune-mediated attack**

Physiological beta cell injury or basal beta cell turnover/death are speculated to initiate homing of macrophages and dendritic cells (33). Infiltrated macrophages normally secrete chemokines and pro-inflammatory cytokines (such as interleukin-1 beta and tumor necrosis factor alpha) to attract dendritic cells and T cells. Yet under normal conditions, the antigen presenting cells do not present autoantigens to T cells, nor should the recruited T cells become active (34-36). However, T cells with high-affinity T cell receptors for self-peptide-MHC complexes escaping the selection by thymus or peripheral deletion can become autoreactive and attack the beta cells. Intriguingly, many studies suggest that islet inflammation is rarely observed in pre-onset (and even recently-onset) individuals, suggesting T1D to be a heterogeneous disease driven by extra-immunological causes (37-39). Unquestionably, compared to the non-obese diabetic mouse model (NOD, a spontaneous model of T1D), pre-onset humans often have less frequent infiltrating immune cells (mainly CD3⁺/CD8⁺ T cells, CD68⁺ macrophages, with few CD20⁺ B cells and CD3⁺/CD4⁺ T cells (38)) and develop diabetes more slowly. However, extrapolating data/conclusions based on limited pre-onset cadaveric samples should be interpreted with great caution. If more pancreas samples from pre-diabetic donors become available, it is very likely that more lymphocyte species will be identified and characterized (40). Additionally, in the near future, once the time-to-onset prediction substantially improves, more knowledge of how cell-mediated immunity contributes to T1D will be attained.

Apart from cell-mediated immunity, humoral immunity is also associated with the etiology of T1D (41). In humans, the presence of islet autoantibodies is the earliest indicator of pre-diabetes, and there is a strong association between the presence of multiple antibodies and the risk of developing T1D (42-44). However, the autoantibodies are considered by-products of B cell activation and therefore perceived as non-pathogenic (45-47). The primary way in which B cells contribute to T1D seems to be through their antigen presentation function (48). To date, multiple T1D autoantigens have been identified, including those targeting insulin (49, 50), proinsulin (51, 52), glutamate decarboxylase (GAD65) (53, 54), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (55), insulinoma-associated antigen 2 (IA2), and Zinc transporter 8 (ZNT8) (56). Interestingly, as the majority of identified targets of beta cell autoantigens traffic through the endoplasmic reticulum (ER) and become localized within the secretory granules, ER abnormalities are suspected to contribute to autoantigenicity (57, 58). Indeed, recent reports have demonstrated that multiple ER stress markers are elevated before the onset of T1D in rodent models (59, 60). It will be important to determine: 1) if the misfolded proteins become neo-antigens, 2) if the upregulation of the ER-associated degradation (ERAD) pathway allows autoreactive cells to escape the unfolded protein response (UPR)-mediated beta cell death, thus facilitating T1D pathogenesis (37, 58), and 3) whether ER stress in beta cells precipitates immunopathology of T1D in humans.

Beta cell turnover

As autoimmune-mediated beta cell destruction proceeds, the exposure of new autoantigens (either released from damaged beta cells or derived from dysregulated secretory pathways) may further precipitate the adaptive immune response and augment the killing of remaining beta cells (61). On the other hand, compensatory beta cell growth may already exist in autoantibody-positive subjects. The histopathological observations have demonstrated the presence of: 1) small- and medium-sized islets, 2) neogenesis of endocrine cells within the ductal epithelium, and 3) Ki67⁺ beta cells in the pancreas from pre-clinical individuals (39). Indeed, although the overall beta cell loss is linear during the progression of T1D, at any given time, the heterogeneous beta cells may differentially respond to the inflammatory microenvironment and metabolic demands. As such, the cellular regeneration or death programs are triggered according to the integrated cellular intrinsic and extrinsic signals (62).

Beta cell (dys)function

Regarding beta cell function, preclinical T1D subjects usually develop a reduced first-phase insulin response (FPIR) immediately after their seroconversion (e.g., when the first T1D autoantibody is detectable in the blood) (63, 64). Therefore, the reduced FPIR, along with the presence of autoantibodies, have been used to predict T1D in at-risk individuals (65, 66). Following the loss of FPIR, preclinical subjects remain normoglycemic and have stable C-peptide levels months to years, until impairments in arginine-stimulated insulin secretion, the incretin response, and glucagon suppression are observed (67, 68). Due to the difficulties in longitudinally assessing beta cell mass in T1D subjects (even with the aid of mathematical modeling), it is not known whether the

loss of insulin responses is the result of beta cell deficiency or beta cell dysfunction. Nevertheless, cumulative animal studies have suggested that the loss of acute phase glucose-stimulated insulin response is usually accompanied by marked changes in the beta cell phenotypes in T1D (69, 70). Additionally, much *in vitro* work has shown that the proinflammatory cytokines present in the T1D milieu clearly cause beta cell dysfunction (through disrupting beta cell calcium homeostasis, activating the unfolded protein response, and/or elevating nitric oxide stress (71, 72)). Based on above observations, it is proposed that beta cell dysfunction likely precedes beta cell destruction during the development of T1D.

Clinical stage:

Beta cell recovery

Soon after diagnosis, patients receive exogenous insulin replacement therapy to manage blood glucose and to avoid the life-threatening ketoacidosis. Interestingly, the administration of insulin often elicits a response that features near normal glucose metabolism and markedly decreased insulin requirements. Though this disease remission phase was first documented in 1940 (73), the underlying mechanism was not addressed until the 1970s. Based on the observation from a controlled clinical trial, Mirouze et al. proposed that the insulin treatment reduces the beta cells' secretory burden, leading to enhanced insulin secretion and beta cell viability (74). In addition, it was speculated that insulin therapy corrects glucotoxicity (observed around the time leading up to clinical diagnosis) and facilitates the functional recovery of beta cells. This concept of beta cell rest and recovery was finally experimentally tested in 1980s, when Pipeleers, Eizirik, and colleagues reported that beta cells and rodent islets undergo functional recovery after

experimentally-induced damage (75-78). They proposed that beta cells first become functionally impaired upon the autoimmune attack. In this phase, different cellular repair mechanisms are activated. However, depending on the context of challenge and the effectiveness of cellular repair mechanisms, beta cells might remain dysfunctional, die, or recover from assaults.

To provide translational evidence of the discoveries from rodent studies to the human disease, a recent report from Sherry et al. documented that human beta cells indeed become degranulated in T1D (79). This interesting observation raises several questions: 1) can human beta cells re-establish their insulin granules, 2) what have the degranulated beta cells become; are they considered de-differentiated or merely beta cells devoid of insulin, and 3) could beta cell recovery-enhancing agents be employed to promote the recovery of insulin granules and beta cell constitution? Indeed, hyperglycemia in rodents can provoke beta cell de-differentiation to progenitor-like cells (70, 80, 81). Additionally, beta cell de-granulation was observed in a primate model of T1D (82). Likewise, culturing adult human beta cells *in vitro* induces cell de-differentiation and subsequent proliferation of de-differentiated cells (83, 84). Reverting the de-differentiated cells back to *bona fide* beta cells can be achieved *in vitro* (85), but the extent to which beta cell degranulation/de-differentiation, and subsequent re-granulation/re-differentiation occurs during T1D is unclear. Therefore, it is essential to perform lineage-tracing experiments in T1D animal models with a well-characterized disease remission phase to address the questions described above. Also, re-examining whether or not the previously reported proliferating beta cells present in injury/diabetes models also have the de-differentiation markers (e.g., mesenchymal cell or early

progenitor cell markers), and rendering the cells transiently dysfunctional yet highly proliferative, is needed. Moreover, it is likely that the proliferating beta cells observed in T1D are originated from alpha or other islet cell types (trans-differentiation) because it has been shown that beta cell injury may induce phenotypic changes of adjacent cells, such as transforming alpha cells into less-mature pro-alpha cells, thereby making them capable to transiently express beta cell genes (86). Besides, in most T1D animal models, increases in alpha and delta cell numbers are often observed following the onset of autoimmune activation and/or beta cell damage (87, 88). It will be informative to examine whether or not these newly generated cells could trans-differentiate into beta cells. On the other end of spectrum, high-throughput analyses on pancreatic samples collected from patients in the honeymoon phase (or samples from chemically-induced diabetic primates receiving islet xenografts) may facilitate the identification of key genes and proteins involved in beta cell recovery. Once the functional relevance of the recovery genes is validated in knockout mouse models, strategies can be designed to foster the functional integrity of the remaining beta cells and promote the recovery of damaged beta cells, therefore prolonging the honeymoon phase (e.g., create a T1D remission).

From a clinical perspective, promoting beta cell recovery is suitable for therapeutic implementation because new-onset T1D patients still possess ~20% of their initial beta cell mass, and their insulin secretory responses to a mixed meal glucose tolerance test are about 50% of normal healthy control subjects (89). In addition, stimulated C-peptide levels from new-onset T1D patients remain clinically significant (29). As even slight preservation of insulin production can significantly improve

metabolic control (thereby reducing the risk of developing retinopathy, nephropathy, hypoglycemic episodes, and other diabetes complications) (90), it is extremely important to preserve the remaining beta cells. In short, promoting beta cell recovery and survival is the most straightforward and attainable approach deserving more attention experimentally.

Beta cell regeneration

During the progression of T1D, the inflammatory insults have been suggested to trigger beta cell proliferation or transdifferentiation in rodents (79, 87, 91). Yet, whether these phenomena occur in humans is still under debate. Butler et al. first reported that the beta cell replication rate is not increased in recent-onset T1D patients (92); whereas Willcox et al. recently documented that the increased islet cell (alpha and beta cells) proliferation observed in newly diagnosed T1D patients was associated with the presence of insulitis lesions. The later study also pointed out that beta cell proliferation, however, is not detectable in patients with longstanding T1D. The explanation of such observation is that beta proliferation is limited by age-related factors (93, 94). In other words, although beta cell replication rarely occurs in human adults, it is detectable in children and young adults (95, 96). A supportive study from In't Veld et al. also showed that younger patients with life support (which is correlated with higher levels of immune infiltration in pancreatic parenchyma) have higher beta cell proliferation rates (97). On the contrary, chemically-induced diabetic primates failed to display any beta cell replication, most likely because the monkeys used in those studies were adults (82, 98). However, small clusters of beta cells adjacent to ducts, as well as beta cells bearing other endocrine markers were found in the diabetic monkeys (82, 98). In short, whereas beta

cell proliferation possibly occurs in newly diagnosed T1D juveniles, beta cell transdifferentiation may be detectable in youth and adults. Future studies will need to address whether immune insults can promote beta cell proliferation and/or neogenesis in different age-groups. More importantly, if immune modulation suppresses beta cell regeneration, a combinatorial approach needs to be developed to simultaneously encourage the growth of beta cells.

Beta cell destruction

As T1D progresses, the immune infiltration, ambient hyperglycemia, and ER stress eventually accelerate the loss of beta cells. Among the destructive factors, the immune attack is the main contributor of beta cell death. Recently, a landmark study from Lebastchi et al. reported that the administration of an immune modulator (teplizumab, an anti-CD3 monoclonal antibody) decreased the rate of beta cell death in new-onset T1D patients (99). The authors suggested, for the first time, that an immune therapy reduces the decline in C-peptide and the need for exogenous insulin, possibly by decreasing beta cell death. This study nicely highlighted the importance of limiting beta cell death for attaining better glycemic control in T1D. In addition, the molecular signaling networks involved in the immune-mediated beta cell destruction have been extensively studied in animal and cultured cell systems (100). It is believed that under autoimmune challenges, beta cells are destroyed by direct contact with activated macrophages and T cells, and/or exposure to pro-inflammatory cytokines, nitric oxide, and reactive oxygen species (100). Such beta cell homicide may continue for years in humans (101). Meier et al. initially claimed that beta cell apoptosis is detectable in long-standing T1D patients (102). Later, the same group observed an increased beta cell

apoptosis rate in new-onset T1D patients (92); and this notion was supported by Akirav et al. via a novel methylation-specific PCR assay (103). Excitingly, this new methodology (e.g., detecting beta cell death events with assays using serum) enables researchers to measure beta cell death in a non-invasive way, thereby permitting us to study: 1) if beta cell death is halted by insulin therapy in the honeymoon phase, 2) do immune modulators other than teplizumab prevent beta cell death, and 3) could we use the apoptotic index, coupled with other biological markers, to predict the onset of T1D?

Apart from immune-mediated destruction, the hyperglycemic environment in T1D may directly destroy beta cells, and/or indirectly elevate ER stress that causes beta cell dysfunction and death (60, 104, 105). It has been suggested that ER stress genes such as ATF4 are increased in human islets collected at the onset of T1D (105). Interestingly, Engin et al. demonstrated that the UPR genes ATF4 and spliced XBP-1 are dysregulated in beta cells acquired from T1D donors. Further, the administration of the chemical chaperone taurourosodeoxycholic acid protects pre-diabetic NOD mice from developing diabetes (106). In other words, ER stress due to abnormal blood glucose fluctuations and immune insults may have been present before the initiation of beta cell death, which precedes the onset of T1D. As Dr. Eisenbarth suggested, the susceptibility of T1D is analogous to rocks on a hill, poised but requiring a push to determine which rock rolls down the hill (107). It is, therefore, critical to halt the disease progression at the early steps to prevent beta cell destruction and the subsequent development of T1D.

1.1.4 Treatments

Substantial advancements had been made in last 30 years in finding strategies to prevent, predict, and/or reverse T1D (108). Unfortunately, none of the tested therapies have persistent effects. Moreover, these therapies are less convenient to employ (compared to the currently available insulin therapies) and often come with significant side effects (109). With the support from well-organized clinical trial networks and registries, the field must better define disease heterogeneity and identify more practical and effective therapies to prevent or reverse T1D (61).

Because algorithms to predict T1D are improving, several large-scale clinical trial centers are investigating how to prevent (primary prevention) or delay (secondary prevention) the onset of diabetes.

Primary prevention trials target genetically high-risk individuals without islet autoimmunity. The most common primary prevention trials involve dietary intervention. For example, the Trial to Reduce Incidence of Diabetes in a Genetically at Risk study (TRIGR) investigates if T1D can be prevented by restricting infants' exposure to cow milk (110, 111), given: 1) diabetes-prone biobreeding rats and NOD mice fed a complex weaning diet are prone to develop diabetes (because the complex diet triggers islet autoimmune reactions); whereas amino acids- or extensively hydrolyzed casein-fed rodents are protected from the development overt diabetes, and 2) previous observations demonstrated that the early weaning to cow's milk-based formula is a diabetes risk factor (112). Unfortunately, based on recent reports (5- and 7- years follow-ups), a formula-based diet exhibited no protection against T1D (113, 114). Currently, the TRIGR study group is collecting more test results to determine if a formula-based diet protects against

T1D at the 10 year follow-up. Meanwhile, other small-scale trials such as the BABYDIET study (delaying gluten exposure) (115), and the Nutritional Intervention to Prevent T1D (NIP) study (docosahexaenoid acid/DHA supplementation in infant formula) (116), are still in the pilot study phase. To date, none of the dietary interventions yield improvements in preventing T1D development (117).

Secondary prevention trials are conducted in autoantibody positive individuals before their clinical diagnosis of T1D. The most famous study is the European Nicotinamide Diabetes Intervention Trial (ENDIT). The ENDIT study employed nicotinamide (which mediates cellular NAD⁺ and ATP production) as a cytoprotective agent to preserve beta cells in order to delay T1D development (118). Another large-scale study is the Diabetes Prevention Trial Type 1 (DPT-1). DPT-1 utilized oral insulin treatment (to metabolically and/or immunologically preserve beta cells) to delay the onset of T1D (119). However, neither of these two studies documented an improvement in delaying T1D development. Indeed, it is very difficult to identify universal therapies to prevent T1D because different individuals possibly respond to interventions differently.

Other than trying to prevent and/or delay the development of T1D, much work has been dedicated to finding a cure for T1D. Unfortunately, there is no permanent cure. Currently, immune-modulation and the pancreas/islet transplantation are the only strategies likely to be employed to reverse T1D.

The goals of reversal trials are to preserve the production of C-peptide and induce immune tolerance, thereby halting beta cell destruction. Past approaches concentrated on employing systemic immunosuppressants, which have notorious side effects. Recent approaches include non-antigen specific trials (e.g., CTLA-4 antibody, TNF- α antagonist,

HSP60, anti-CD3 antibody, anti-CD20 antibody, and autologous non-myeloablative hematopoietic stem cell transplantation) (61), and antigen specific trials (e.g., Alutamic acid decarboxylase vaccination (120)). Unfortunately, none of these approaches have long-lasting effects. The anti-CTLA-4 therapy was able to slow down the reduction of beta cell function for up to 2 years (121, 122), and HSP60 yielded only 1-year C-peptide preservation (123).

Compared to immune modulating trials, pancreatic transplantation is considered the only cure for T1D, with a 5-year post-surgery survival of ~90%. The graft survival rate is ~60% at 5 years post-transplantation, with a half-life of 7-14 years (124). Compared to the effective pancreas transplantation, islet transplantation still remains an experimental procedure. Other than pancreas and islet transplantation, stem cell-based therapies have recently received considerable attention. However, many hurdles remain, including: how to generate functional beta cells (125, 126), how to vascularize the graft, where to transplant the graft (127), and so on. In short, better understanding of the pathogenesis of T1D is required for harnessing immune regulation and beta cell regeneration therapies to prevent/reverse T1D.

1.2 Type 2 diabetes

Type 2 diabetes (T2D) accounts for 90-95% of diabetes. Patients with T2D predominantly present with insulin resistance coupled with an insulin secretory defect or relative insulin deficiency (128).

1.2.1 Epidemiology and diagnosis

The global prevalence of T2D has been increasing rapidly in the last three decades, particularly in youth and children (129). By 2030, about 396 million people are expected to have T2D (130). This remarkably high prevalence of T2D has created an enormous public health burden.

Most patients with T2D are obese or have increased body fat distribution in the abdominal region. However, obesity itself is insufficient for causing diabetes as only 30% of people with obesity develop T2D. People ultimately developing T2D may remain undiagnosed for years, because their hyperglycemia develops slowly and largely unnoticed. The diagnostic criteria for diabetes are: 1) HbA1C $\geq 6.5\%$, 2) fasting plasma glucose $\geq 126 \text{ mg/dl}$, 3) plasma glucose level $\geq 200 \text{ mg/dl}$ at 2-h post oral glucose tolerance test, or 4) random plasma glucose $\geq 200 \text{ mg/dl}$.

People with intermediate plasma glucose levels, yet not clinically categorized as diabetics, are considered to have pre-diabetes; the ADA classifies these subjects as at a “High Risk State of Developing Diabetes”, and WHO prefers the term “Intermediate Hyperglycemia” instead of pre-diabetes (128, 131). The laboratory test results representing pre-diabetes are: 1) fasting plasma glucose of 100-125 mg/dl (impaired fasting glucose, IFG), 2) 2-h post-load glucose of 140-199 mg/dl (impaired glucose tolerance, IGT), or 3) A1C range from 5.7-6.4%.

1.2.2 Etiology of T2D

T2D, like T1D, is a multi-factorial disease dependent on a mix of genetic and environmental factors. T2D is driven by chronic fuel surfeit in genetically susceptible people (132). The factors contributing to the development of T2D are discussed below.

It has long been accepted that genetic factors contribute to the development of T2D. The first supporting observations arose from an early population study, in which specific ethnic groups were identified to have a higher prevalence of T2D (133). Later, heredity studies revealed that the T2D concordance rate in monozygotic twins was much higher than dizygotic twins (134-136), and the risk of developing T2D is greater if parents have T2D (137). These findings and others suggested that genetic predisposition could be a triggering factor of T2D. Recently, more than 60 common genetic variants associated with T2D were identified via GWAS, linkage analysis, and candidate gene approaches. Interestingly, many of the T2D susceptibility genes are, in fact, pancreatic beta cell genes. For example, *TCF7L2* (encoding transcription factor 7-like 2, regulates pro-glucagon gene expression levels) (138, 139) and *KCNJ11* (encoding the potassium channel KIR6.2, regulates beta cell insulin secretion) variants are highly prevalent and linked to high T2D risk (140). Other identified genes such as *KCNQ* and *PPARG* are related to insulin sensitivity; *MC4R* and *FTO* genes are related to obesity; *CAPN10* is related to glucose transport (141). Unfortunately, due to the polygenic nature of T2D, it is difficult to analyze a single gene's contribution to the increased risk for diabetes.

In addition to one's genetic composition, epigenetic regulation and early life events also control the development of T2D (142). For example, inadequate fetal nutrition increases the risk of developing T2D in adult life (143-145), and such risk could

be aggravated by subsequent nutrient over-exposure (146). In addition, individuals exposed to gestational diabetes *in utero* also have a greater T2D risk (147).

Other than the genetic components, the environmental factors such as the Western lifestyle (e.g., high-energy diet and decreased physical activity) is associated with diabetes development based on: 1) migration studies suggesting populations moving from rural to urban areas have increased obesity and T2D incidence (148, 149), 2) epidemiological studies demonstrating rapid lifestyle and dietary structure changes caused sharp increases in the prevalence of T2D in Asian countries (150, 151), and 3) studies suggesting a sedentary lifestyle is strongly associated with being overweight and developing diabetes (152, 153). In contrast, a hypocaloric diet and exercise can reduce T2D risk. Both dietary and lifestyle interventions have been well documented to improve metabolic parameters among people with impaired glucose tolerance, therefore reducing their T2D risk (154-156).

1.2.3 The natural history of T2D

T2D is a progressive disease characterized by beta cell dysfunction and insulin resistance-associated hyperglycemia. From the clinical perspective, the progression of T2D can be separated into 2 stages: pre-diabetes (pre-clinical) and frank diabetes.

Pre-clinical stage:

According to the ADA and a Chinese diabetes prevention trial, up to 70-90% individuals with pre-diabetes will eventually develop diabetes (131, 157). Thus, it is essential to identify individuals with pre-diabetes in a timely manner because early interventions can help prevent or delay the development of frank T2D and its complications (158). In the clinic, non-invasive risk factor assessments, together with

laboratory blood glucose measurements, are sufficient for identifying pre-diabetic individuals (159).

The first clinical abnormality observed in susceptible individuals is IGT, IFG, or both. Interestingly, people with IGT usually have different pathophysiological abnormalities compared to those with IFG (160). For example, individuals with IGT seem to have reduced muscle insulin sensitivity (yet their hepatic insulin sensitivity remains near-normal), reduced FPIR, and reduced second phase insulin release (161). Conversely, individuals with IFG usually have reduced hepatic insulin sensitivity and loss of FPIR. These pathological differences underlying IGT and IFG may reflect the chronological sequence of T2D pathogenesis. For instance, Prentki and Nolan have proposed a “fuel surfeit” etiology model of T2D, in which the pathogenesis of T2D starts with skeletal muscle insulin resistance (observed as IGT) (132, 162). The rationale behind this hypothesis is that, muscles of susceptible people often fail to contain excessive nutrients *in situ* (163-165), prior to diverting extra nutrients to adipose tissue and liver (166). Over time, the accumulation of lipid species in the plasma and tissues render muscle and liver insulin resistant (observed as IFG). However, insulin resistance is just a triggering factor of T2D. What drives the complete manifestation of T2D is the failure of pancreatic beta cells to secrete more insulin to fully compensate for systemic insulin resistance (167-170).

Defective insulin secretion

To maintain glucose homeostasis, insulin secretion is controlled by intricate cellular mechanisms. Briefly, glucose influx into the beta cell raises the cytoplasmic calcium concentration and triggers insulin-containing secretory granule exocytosis (171).

Normally, insulin secretion oscillates to exert maximal hypoglycemic effects (172); and the pattern of oscillations is regulated by nutrients, hormones, and neuronal signals (173-176). However, in first-degree relatives of T2D, the post-meal insulin secretion oscillations are deranged (177). Additionally, Polonsky et al. have reported inadequate glucose entrainment in IGT patients, signifying that the pre-diabetic individuals have lost the feedback loop between glucose and insulin secretion (178, 179). Moreover, recent reports suggested that the oscillatory insulin release in human islets, as a coordinated event of glucose-induced calcium-mediated intra-islet electrical signal transmission, is perturbed by diabetogenic insults, again emphasizing that the early defects in insulin secretion could drive the development of T2D (180-184).

Apart from the above-mentioned coordinated glucose-stimulated insulin secretion examinations, insulin secretion can be robustly assessed in the clinical settings via: 1) intravenous tests, including intravenous glucose tolerance testing (IVGTT) and hyperglycemic clamp techniques, or 2) an oral glucose tolerance test (OGTT). Overall, the cumulative clinical reports indicate that: 1) the loss of FPIR is apparent in patients with IGT and/or IFG (169, 185-191), and 2) total insulin response during an OGTT is significantly decreased in pre-diabetic subjects. Given that beta cell (dys)function cannot be evaluated based on insulin secretion without considering underlying insulin resistance, the disposition index (DI, the ratio of insulin increments to glucose increments divided by insulin resistance) is also employed to characterize beta cell function. Not surprisingly, the hyperbolic relationship between insulin secretion and insulin sensitivity is altered in pre-diabetic subjects (192, 193).

Interestingly, beta cells might be able to secrete more insulin to compensate for insulin resistance in the pre-diabetic stage (194, 195). In particular, Tabak et al. have drawn trajectory curves of fasting glucose, 2 h post-load glucose, insulin sensitivity, and beta cell function of the British civil servants during a 13-years time span in the Whitehall study (**Figure 1-2**) (131, 195). In this landmark report, the authors demonstrated an increased blood glucose level and decreased insulin sensitivity as early as 13 years before T2D diagnosis. However, these subjects' blood glucose levels were maintained within a normal range, suggesting that enhanced insulin secretion might have already existed during the early stages of disease. In fact, Ferrannini et al. have performed a cross-sectional study, and observed that insulin secretion is increased from lean NGT to obese NGT, plateaued in IGT, and decreased in T2D humans (192). However, in the same study, they showed that glucose sensitivity of beta cells is drastically decreased in IGT and T2D individuals. This report strongly supports the notion that beta cells are able to provide more insulin to meet metabolic demands, but they eventually become dysfunctional, thereby leading to the development and diagnosis of T2D. Of note, Tabak et al. documented a sudden increase in 2 h post-load glucose level and steep decline of insulin sensitivity prior to the drastic enhancement of beta cell function in the Whitehall study. The discrepancies in the sequence of events occurring during the pre-diabetic stage in the two reports mentioned above merely reflect the different methodologies used: Tabak and colleagues used a homeostasis model assessment (HOMA2-%B) to assess beta cell function, which might have limited resolution in assessing insulin secretion dynamics. Collectively, clinical observations in

past decades have clearly demonstrated that beta cell compensation followed by decompensation is inherent in T2D development.

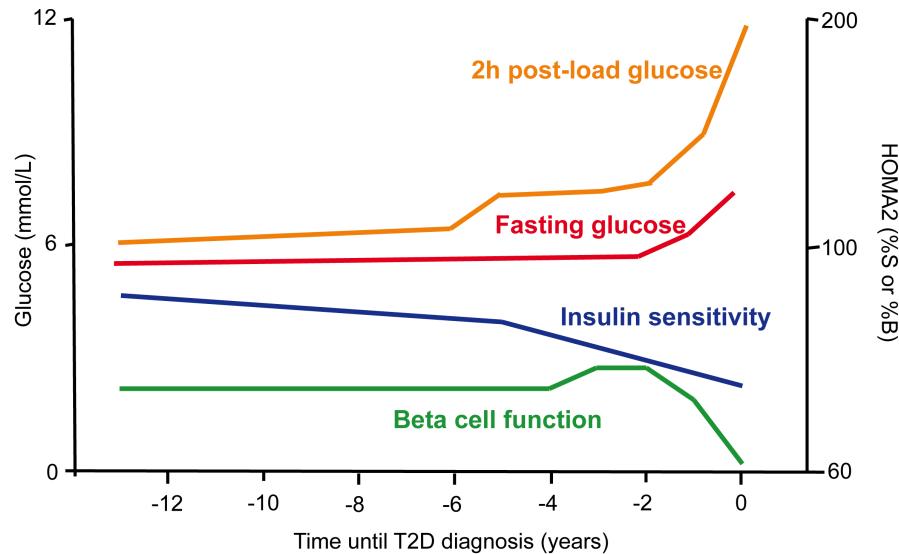


Figure 1-2

Fasting and 2 h post-load glucose, insulin sensitivity, and beta cell function trajectories before the diagnosis of diabetes (Adapted from Tabak et al. (195)).

Factors contributing to beta cell dysfunction

On the cellular level, the pathogenic factors and mechanisms responsible for defective insulin secretion are under intensive investigation. Currently, it is believed that the “susceptibility factors” first cause beta cell damage; and the “potentiation factors” such as glucotoxicity, lipotoxicity, islet amyloid polypeptide deposits, and inflammatory insults accelerate the disease progression (132).

Glucotoxicity

Although many believe that chronic hyperglycemia sabotages beta cell function, the supporting *in vivo* experimental evidence in humans is scarce. In fact, in one study a 24 h glucose infusion actually promoted insulin secretion in humans (196). As prolonged

glucose infusion is not well tolerated in humans, it is difficult to recapitulate the authentic glucotoxicity in an experimental setting (197). Fortunately, a chronic glucose infusion protocol is well established in rodent models. In most animal studies, continuous glucose infusion has been suggested to enhance beta cell insulin secretion and promote beta cell mass expansion (198-200), although in some cases glucose infusion led to beta cell dysfunction (201).

In contrast, *ex vivo* experiments yielded more unifying results – e.g., prolonged high glucose exposure damages isolated human islet function and integrity (202-205). Similarly, grafting human islets in hyperglycemic nude mice results in beta cell degranulation and rough ER expansion, and the grafted islets have reduced insulin content and impaired GSIS (203, 206). The potential molecular mechanisms behind glucotoxicity-associated beta cell dysfunction are ER stress and oxidative stress (207) because: 1) ER signaling cascades are deranged and oxidative stress markers are elevated in islets from T2D patients (208-210); 2) isolated human islets treated with the potassium channel blocker diazoxide, which blocks insulin secretion, are partially protected from high glucose pretreatment-impaired GSIS, indicating beta cells may suffer from overstimulation-induced stress, and beta cell rest might be beneficial for restoring insulin content and release; and 3) rodent studies suggested that the failure to activate UPR in response to obesity promotes diabetes progression; conversely, overabundance of ER chaperones in beta cells prevented mice from developing high-fat diet-induced glucose intolerance (211, 212).

The aforementioned experimental evidence indicates that chronic high glucose stimulation leads to detrimental effects in islets. However, it's important to note that

pronounced hyperglycemia does not develop until the later stages of pre-diabetes. In other words, other factors contribute to beta cell dysfunction before the manifestation of glucotoxicity.

Lipotoxicity

Chronic exposure of pancreatic islets to excess free fatty acids (FFA) in the pre-diabetic milieu is proposed to compromise beta cell function, based on the clinical observations reporting that: 1) plasma lipidome profiles in pre-diabetic subjects are strikingly similar to T2D patients (213), 2) saturated fatty acid composition in plasma is positively correlated with the incidence of T2D (214-216), and 3) pancreatic fat accumulation is increased in IFG and/or IGT individuals (217, 218). This hypothesis has been tested under both *in vivo* and *ex vivo* settings. For instance, elevated plasma FFA (via intralipid infusion) impairs beta cell function in obese non-diabetic humans as well as individuals with a family history of T2D (based on DI or absolute GSIS) (219-222). On the contrary, lowering plasma FFA has been proven effective in improving insulin secretion in the genetically predisposed individuals (223, 224). In addition, culturing human islets with FFA results in detrimental effects in beta cells, including: 1) accumulated intracellular lipid droplets and triglyceride (204, 225), 2) increased inflammation and oxidative stress (226, 227), 3) increased ER stress (228), 4) dysregulated circadian gene expression profiles (229), 5) modified lipid metabolism gene expression profiles (230), 6) decreased insulin content, 7) diminished FPIR (determined by perfusion assays) and GSIS (measured using static culture protocols) (203, 225, 231), and 8) increased cell death (232, 233).

Amyloid polypeptide deposits

Islet amyloid polypeptide (IAPP) deposit is already present in pre-diabetic primates (234, 235), and human adiposity (determined by BMI) is positively correlated with fasting plasma IAPP-like immunoreactivity (236). However, how IAPP fibrils accumulate in human islets is still unclear. Some suggest that IAPP is co-secreted with insulin, and the accumulation of IAPP may repress GSIS and promote beta cell death via a local inflammation-mediated machinery (237-239).

Inflammatory insults

Recent findings suggest that inflammatory markers are predictors of T2D development (240-242), and an inflammatory response may promote beta cell failure in T2D (243). For example, treatment with high glucose or free fatty acids in isolated human islets induces the release of IL-1 β and other proinflammatory factors (244-246). Conversely, blocking IL-1 β 's effects with the IL-1 receptor antagonist anakinra improves the DI of obese, insulin resistant, non-diabetic individuals (due to an improvement of beta cell function (247)). Yet, a conflicting report from the Insulin Resistance Atherosclerosis Study reported that inflammation is related to insulin resistance but not decreased insulin secretion in the pre-diabetic state (248).

Alternatively, histological examinations suggest that insulitis may exist in the pre-diabetic pancreas, as islet fibrosis (a hallmark of chronic inflammation) is detected in diabetic patients (249). Nevertheless, it would be extremely valuable if more pancreatic tissue samples from pre-diabetic subjects become available because no autopsy or gene/protein analysis studies to date have demonstrated the existence of infiltrating pro-inflammatory cells in the IGT or IFG individuals.

Beta cell mass in the pre-diabetic stage

Not only beta cell function, but also beta cell mass is dynamically modulated in pre-diabetic individuals. Based on cross-sectional autopsy studies, many studies indicate that beta cell mass initially increases slightly with obesity or insulin resistance, and then gradually decreases in the context of the diabetic milieu (250-257). Yet, despite years of intensive investigation, the main mechanisms promoting human beta cell mass expansion (e.g., proliferation, transdifferentiation, or neogenesis) and loss (e.g., cell death or de-differentiation) have not been definitively determined, and the factors regulating human beta cell mass dynamics remain poorly defined (258, 259).

Beta cell mass expansion

The dominant driving force of beta cell mass expansion in rodents is beta cell proliferation. In contrast, primates have a relatively limited beta cell regeneration capacity (260, 261); yet, beta cell neogenesis, rather than proliferation, has been reported to facilitate beta cell mass expansion in insulin resistant humans (256, 257, 262). The refractoriness of the mechanisms of beta cell mass expansion between rodents and primates could be attributed to age-related factors. For instance, young rodents typically do not develop spontaneous diabetes and are largely resistant to diet-induced diabetes, possibly because the young animals used in most T2D studies have a greater beta cell proliferation capacity; hence they acquire sufficient beta cell mass to compensate for insulin resistance. On the other hand, experimentally identified factors that prevent animals from developing experimental diabetes actually may have more subtle effects (e.g., improve beta cell function and survival), yet these effects are overshadowed by the massive enhancement of beta cell proliferation.

Another major hurdle in the field is to determine whether or not beta cell mass expansion occurs in pre-diabetic humans. Although obese individuals have higher beta cell mass compared to lean control individuals, it remains to be determined if such a phenotype is the result of compensatory beta cell mass expansion or from predetermined genetic traits (263). If human beta cell mass expansion occurs *in vivo*, the next grand challenge will be to identify the factors promoting such events. In fact, whereas the paradigm of nutrient- and hormone-induced beta cell proliferation has been comprehensively examined in rodents, few factors can efficiently induce human beta cell proliferation *in vitro*. In addition, it is essential to examine if the newly replicated and/or regenerated beta cells are fully functional and whether they remain viable in the pre-diabetic environment (264).

Beta cell death

At the other end of the spectrum, the factors leading to beta cell loss before the onset of diabetes are yet to be elucidated. Pioneered by the work from Butler et al., most consider beta cell death the main contributor of beta cell loss (250, 260, 265). This hypothesis has become so well accepted because numerous *in vitro* studies have successfully demonstrated that glucotoxicity, lipotoxicity, inflammatory mediators, or combinations of these insults promotes cultured beta cell and human islet destruction via apoptotic cell death pathways (205, 266). However, induction of hyperglycemia and diabetes in rodents and non-human primates by high fat feeding alone seems unable to evoke substantial beta cell death (267-269). The reason could be current technology is insufficient to capture the *in vivo* cell death events. Indeed, to date no clinical/histological reports document increased beta cell death in pre-diabetic individuals.

Nevertheless, a better understanding of stress-induced beta cell death mechanisms would promote the discovery of new therapeutic strategies in the field of transplantation and regenerative medicine. Until now, human beta cell transplants (either from cadaveric origin or stem cell-derived beta cells) quickly become dysfunctional and die shortly after transplantation. It is urgent to seek methods to foster the survival and function of the grafts. Indeed, several approaches (e.g., enhancing islet revascularization, eliminating blood-mediated inflammatory reactions, introducing antioxidants, resolving ER stress, and utilizing mesenchymal stem cell-derived factors (270, 271)) are currently under intensive investigation.

Beta cell de-differentiation

Recent thought-provoking reports from the Accili laboratory suggested that glucotoxicity perhaps contributes to beta cell de-differentiation, rather than apoptosis (272). Although the concept of the loss of beta cell identity (defined by decreased expression of genes characteristic of beta cells and a loss of glucose responsiveness) in the diabetic milieu is far from new (273), Accili's group revealed that beta cells lacking the transcription factor Foxo1 could turn into progenitor-like cells and subsequently adopt an alpha cell fate following physiologic stress. Interestingly, they did not observe beta cell death or beta cell self-renewal under their experimental settings (e.g., aging and pregnancy) (272). Supporting data from Nichols's groups suggested that beta cell K_{ATP} channel gain-of-function mice (reiterating features of human neonatal diabetes) develop diabetes due to beta cell inexcitability and subsequent de-differentiation. More strikingly, reversing hyperglycemia in these mice leads to re-differentiation of mature beta cells (274, 275). Apart from the above mechanistic studies, only a few immunohistological

studies have reported de-differentiation events in primates (256, 267, 273). Indeed, why beta cell de-differentiation, rather than beta cell death, should be a preferred mechanism in response to diabetic milieu is not clear. Possible explanations could be: 1) it may be more energy efficient to keep cells alive, so that the beta cells can strive to survive through stressful conditions and return to their original functional states, rather than undertaking the energy-expensive cell death and mitosis processes. 2) In the hyperglycemic milieu, beta cells release and sense the soaring level of insulin. As a first-line self-protective mechanism against the development of hyperinsulinemia-induced hypoglycemia, beta cells might transiently be re-programmed to glucagon secreting cells. In brief, although the de-differentiation hypothesis is groundbreaking, much work is still needed to establish the endocrine pancreas plasticity/hierarchy under pathological and/or regenerative settings to ultimately facilitate the discovery of therapeutic strategies to expand/maintain functional beta cell mass.

Overall, as current non-invasive technology to measure beta cell mass is still not available for humans, it remains difficult to determine the natural history of beta cell expansion and loss, not to mention to dissociate the contribution of beta cell dysfunction and beta cell loss, in the progression of T2D. Apart from human autopsy and biopsy studies, future research aimed at identifying early biomarkers and/or that using invasive large animal models will help elucidate the chronological order of beta cell failure events that lead to the development of T2D.

Clinical stage:

The majority of the pathological events initiated in the pre-diabetes stage only deteriorate with time following diagnosis. For example, 1) beta cell mass in the

diagnosed T2D patients is 40-50% less than healthy individuals, and beta cell mass in T2D patients is inversely correlated with the duration of the disease (254), 2) beta cell functional parameters (e.g., first phase insulin secretion (276), ultradian and high frequency insulin secretion oscillations (277-279), and glucose sensing (280, 281)) are markedly reduced in T2D patients, and 3) beta cell loss is associated with IAPP deposition in T2D patients (282); whereas islet amyloidosis severity is correlated with increased fasting plasma glucose and HbA1c as well as decreased beta cell mass in primates (283). The aforementioned beta cell dysfunction and destruction events in diagnosed T2D patients indeed implicate a loss of cellular protective mechanisms and deranged beta cell homeostasis. For instance, a recent study from Engin et al. suggests that the UPR markers are disturbed (instead of systematically up- or down-regulated) in cadaveric islets from T2D patients (210). Further, hypoxia-inducible factors are induced by acute hyperglycemia; however, chronic hyperglycemia curtails most of the hypoxia-responsive elements in beta cells (284). These observations strongly suggest that T2D as a disease of dysregulated metabolic homeostasis; and at the late-stage of diabetes, most adaptive responses likely have failed.

1.2.4 Treatments

Current anti-diabetic interventions aim to improve insulin sensitivity and restore beta cell function in different stages of the disease (285). In the pre-diabetic stage, lifestyle changes can increase baseline beta cell function and insulin sensitivity toward NGT (157, 286-288); and reversion to NGT is associated with significantly reduced T2D risk (289). In newly diagnosed patients, insulin therapy, an IL-1 β antagonist, and GLP-1 receptor agonists have been shown to temporarily restore beta cell function. Indeed,

there are many medications available for T2D patients to achieve and maintain closer-to-normal blood glucose levels (e.g., metformin, sulfonylureas, incretins, and insulin). With increasing therapeutic options, practitioners often utilize a patient-centered approach, together with consensus treatment initiation/adjustment algorithms to treat patients (290-293).

Ultimately, the goals of anti-diabetic therapy are to preserve the existing beta cells, and to restore/expand functional beta cell mass. Below, I discuss the potential applications of growth factor-related therapies.

1.3 Mitogenic signals control functional beta cell mass

To produce adequate amounts of insulin to guarantee tight glycemic control, functional beta cell mass increases and decreases in response to different (patho)physiological conditions. For instance, pregnancy induces beta cell mass expansion; yet after parturition, the beta cell mass declines (294). Conversely, pathological stimuli such as insulin resistance, hyperglycemia, and tissue injury also promote beta cell mass expansion (295, 296). On the molecular level, glucose, insulin, insulin-like growth factor, incretins, lactogens, hepatocyte growth factor, parathyroid hormone-related protein, epidermal growth factor (EGF), betatrophin, and liver-derived mitogenic signal have been suggested to promote beta cell mass expansion (199, 255, 297-300).

Unfortunately, despite work over the past three decades that has significantly increased our understanding of how mitogen signaling pathways promote beta cell proliferation in rodent models, the feasibility and translation of mitogen-derived therapies to humans remains poor. Some possibilities for the inability to translate these therapies are: 1) species differences, as rodents display a relatively large beta cell proliferation response in contrast to that found in humans, 2) age-related factors, as most diabetes studies use young rodents when the beta cells have a high replication capacity, and 3) the lack of understanding of how mitogenic signaling pathways are regulated during the diabetic conditions. Though several mitogenic signaling cascades have been shown to prevent T1D/T2D and reverse early-stage T1D, none of them have cured fully developed diabetes. In other words, mitogenic signaling pathways might be differentially regulated in the diabetic milieu. For instance, pathogenic stress may impose barriers that inhibit the

beneficial effects of the mitogen therapies. In order to close the knowledge gap and potentially use mitogens to treat diabetes, mitogen-mediated beta cell regeneration and recovery mechanisms must be examined in diabetic states.

1.3.1 ErbB receptor tyrosine kinase signaling pathways in the pancreas

The ErbB receptor family has four members: EGFR/Human epidermal growth factor receptor-1 (HER1)/ErbB1, Neu/HER2/ErbB2, HER3/ErbB, and HER4/ErbB4 (301). These ErbBs contain an extracellular ligand-binding domain, a trans-membrane domain, and an intracellular tyrosine kinase domain (with the exceptions that ErbB2 does not have a functional ligand-binding domain and ErbB3 does not contain an intracellular kinase domain) (302). Upon ligand binding (EGF, heparin-binding EGF-like growth factor/HB-EGF, transforming growth factor- α /TGF- α , amphiregulin/AR, betacellulin/BTC, epiregulin, neuregulin/NRG, or epigen), the receptors either homo- or hetero-dimerize to facilitate intracellular kinase activation, and then recruit adaptor proteins and activate the Extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways to control cell growth, differentiation, and survival (301).

EGFR and its ligands in the pancreas

In the neonatal developmental stages, EGFR is expressed throughout the gastrointestinal tract. After birth, EGFR becomes restricted in the pancreatic islets and pancreatic ducts (303). The significance of EGFR in controlling pancreatic development has been demonstrated by EGFR knockout mice: *EGFR*^{-/-} mice exhibit defective pancreatic branching morphogenesis, streak-like islets, and reduced beta cell mass (304). Not surprisingly, *EGFR*^{-/-} mice die soon after birth due to multi-organ failure. In order to

study the roles of EGFR in the pancreas beyond the developmental phases, pancreatic and duodenal homeobox-1 (Pdx-1)-driven EGFR kinase dominant-negative (EGFR-DN) mice were generated (305). Interestingly, the EGFR-DN mice have reduced beta cell proliferation rates, decreased beta cell mass, and developed spontaneous diabetes.

On the other hand, independently deleting EGF, AR, BTC, or TGF- α peptides has no observable effect on the development or maturation of pancreatic islets, indicating that high levels of functional redundancy exists among EGFR ligands (306). However, in the *in vitro* setting, BTC induces differentiation of embryonic explants towards a beta cell lineage (307, 308); whereas supplementing with EGF or TGF- α promotes ductal cell proliferation and differentiation in the isolated fetal pancreas (309-311).

ErbB2, ErbB3, ErbB4 and their ligands in the pancreas

Compared to EGFR, the other ErbB receptors (ErbB2, ErbB3, and ErbB4) are predominantly expressed in the pancreatic ducts during development (312, 313). The loss of ErbB2 or ErbB4 causes early (before pancreas formation) embryonic lethality, whereas ErbB3 knockout mice have an irregular pancreatic structure.

The ligands of ErbB3 and ErbB4 are HB-EGF, BTC, and NRG. Among them, NRG not only stimulates differentiation of pancreatic delta cells, but also supports the development of alpha cells in embryonic pancreas explants (307). Whereas the underlying mechanisms are not clear, this observation indicates that the ErbB receptors and their ligands are fundamental in controlling pancreas development and progenitor cell fate determination.

1.3.2 EGF and EGFR control nutrient-induced beta cell mass expansion

Functional beta cell mass is precisely controlled by nutrient availability and systemic insulin sensitivity. Whereas 7-d high-fat feeding enhances insulin secretion and beta cell proliferation in 2-month-old mice (269), 72 h glucose and lipid infusion promotes beta cell mass expansion through EGFR and mammalian target of rapamycin (mTOR) signaling pathways in 6-month-old rats (314). However, compensatory beta cell mass expansion is not observed in 1-year-old high fat-fed mice (315). Whether or not chronic nutrient oversupply abrogates EGFR signaling cascades (e.g., by reducing EGF/EGFR levels or dampening EGFR activation) and/or facilitates the beta cell decompensation remains unknown.

EGF levels in the diabetic pancreas

Indeed, systemic EGF deficiency has been associated with diabetes mellitus, as diabetic rodents and humans have: 1) decreased EGF production/secretion from salivary glands (316-320), and 2) increased EGF excretion by the kidney (321, 322). At the level of the pancreas, decreased EGF uptake by pancreatic cells is observed in diabetic NOD mice (323). Yet, three important questions remain: 1) are pancreatic EGF and EGFR levels/uptake reduced in human T1D and T2D, 2) does the circulating or pancreatic EGF level correlate with the activation status of EGFR, and 3) does EGF-induced EGFR activation promote beta cell survival and/or regeneration in diabetic states?

EGF as a potential treatment for diabetes

Because the plasma EGF concentration is reduced in diabetic states, it is intuitive to consider using EGF as a treatment to promote beta cell recovery and regeneration, given its mitogenic potential. In fact, activation of EGFR signaling cascades has been

suggested to facilitate tissue remodeling and repair (324). For instance, EGF therapy has demonstrated positive effects in treating necrotising enterocolitis and high-grade diabetic foot ulcers (325) and has been used to facilitate skin wound healing and gastrointestinal ulcer healing in phase I-IV and double-blind randomized-controlled clinical trials (326-329). Most importantly, EGF-related therapies have been tested for potentially improving glycemic control. Beginning in 2007, Transition Therapeutic Inc. (later partnered with Eli Lilly & Co.) reported a series of trials combining a gastrin analog (gastrin has been shown to induce EGF production (330)) and a GLP-1 analog (GLP-1 has been suggested to trans-activate EGFR in the beta cells (331)) to treat both T1D and T2D patients. These clinical assessments were terminated in 2010 because the company's latest randomized, double-blind, placebo-controlled study failed to meet the efficacy endpoints. If the reasons of such ineffectiveness could be identified, EGF-related therapies may then be used to target diabetes because: 1) EGF is very well tolerated in humans, 2) the only reported side effect of long-term EGF treatment (even at supra-physiological concentrations) is epithelial hyperplasia and such symptoms are reversed by withdrawal of EGF, and 3) administration of EGF does not induce carcinogenesis in mice and humans (332-334). Future mechanistic studies focusing on EGFR signaling cascades will encourage the clinical translation of EGF.

EGFR signaling cascades regulate beta cell mass expansion in animal models of diabetes

Many groups have established that EGFR is central to compensatory beta cell mass expansion. Otonkoski's laboratory has demonstrated: 1) pancreatic specific EGFR kinase-negative (EGFR-DN) mice failed to acquire high-fat diet- and pregnancy-induced

beta cell mass expansion, due to insufficient beta cell proliferation, and 2) constitutively active EGFR transgenic mice are protected against streptozotocin-induced diabetes, as the activation of EGFR protects beta cells from cytokine-mediated cell death (335, 336). On the other hand, Rabinovitch and others have shown that EGF and gastrin treatments: 1) increase beta cell mass and reverse hyperglycemia in NOD or STZ/alloxan-induced diabetic mice (337-340), 2) increase implanted human beta cell mass and improve beta cell function in immunodeficient nonobese diabetic-severe combined immune deficiency (NOD-SCID) mice (341), and 3) delay autoimmune diabetes recurrence in NOD mice transplanted with syngenic islets (342). Recently, Baeyens et al. further demonstrated that treatment with the combination of EGF and ciliary neurotrophic factor partially restores beta cell mass in STZ-induced chronic hyperglycemic mice via reprogramming acinar cells into beta cells (87).

However, overt diabetes could not be cured by EGF therapy. Wang et al. recently suggested that in addition to EGF, a concomitant immune-modulation should be employed to reverse hyperglycemia in late-stage NOD mice (343). In this innovative study, the authors stated that the diabetogenic stress (autoimmune assault) has to be abrogated to allow EGF to exert its maximal cytoprotective and regenerative effects on beta cells. Indeed, the activation of EGFR could be hindered by various pathological stimuli (344, 345). For example, EGFR is subject to advanced glycation end product (AGE) modification (the addition of glucose to a protein without involvement of an enzyme) and inactivation during hyperglycemia, and such modifications are associated with delayed epidermal wound healing in diabetic mice (346-348). Contradicting this, acute stress stimuli induce ligand-independent EGFR activation, resulting in non-

canonical intracellular receptor trafficking and aberrant molecular actions in various cancer cell types (349-351). It would be interesting to determine: 1) if acute and chronic stress initiate different EGFR activation networks, 2) whether primary and transformed cells have different reactions to stress stimuli in the context of EGFR activation/inactivation, 3) how EGFR activation status dictates tissue recovery *in vivo*, 4) what are the molecular mechanisms controlling EGFR dynamics under diabetic stress conditions in the pancreatic beta cells, and 5) are stress-responsive EGFR negative regulators involved in these processes.

1.3.3 Negative regulators of EGFR signaling pathways

Fortunately, many studies have led to the identification of stress-induced regulators of EGFR and its downstream signaling. As a master regulator of cell fate, the EGFR signaling cascade is one of the most comprehensively studied molecular pathway (302). EGFR activation begins with the ligand-induced conformational changes of the EGFR juxtamembrane domains, leading to activation of one of the intracellular EGFR kinase domains. The carboxy-terminal lobe (C-lobe) of the activated kinase molecule then binds to and stimulates the amino-terminal lobe (N-lobe) of the second kinase molecule. Once dimerized and fully activated, EGFR undergoes endocytosis and propagates downstream signals. Gradually, the EGFR signalosome is disintegrated and deactivated before trafficking to the lysosomes for degradation. To ensure the proper cellular signal transmission, EGFR activity is controlled by tiers of negative regulators (301).

The first tier of negative regulators consists of pre-existing components that trigger rapid inhibitory responses (e.g., controlling receptor tyrosine kinase (RTK)

phosphorylation and EGFR endocytosis). RTK phosphatase SH2-containing 5'-inositol phosphatase (SHIP2) and protein tyrosine phosphatase 1B (PTP1B) are two such negative regulators implicated in promoting EGFR dephosphorylation and degradation (352, 353). RTK endocytic machinery could be central in maintaining cell integrity, and diabetic stress conditions have been shown to disrupt RTK endocytosis. For example: 1) deregulated RTK endocytosis is observed in cells derived from diabetic mice (354), and 2) the perturbation of EGFR plasma-to-early endosome trafficking is associated with elevated intracellular cholesterol (355). Interestingly, the RTK phosphatase SHIP2 gene and protein expression levels are elevated in the beta cells of diabetic animals. In addition, SHIP2 and PTP1B have been reported to control beta cell proliferation and insulin secretion (356-358).

The second (delayed) tier negative regulators undertake the spatial and temporal compartmentalization of EGFR, thereby shaping the cellular phenotypic responses. This group of negative regulators includes: 1) the scaffold proteins that become inhibitory only after being modified by activated EGFR, 2) transcriptionally induced molecules that directly bind to EGFR and repress its RTK activity, and 3) inducible inhibitors of the mTOR, PI3K/Akt, and RAF/MEK/ERK signaling pathways. Among the second tier regulators, increasing attention has been paid to characterize the transcriptionally induced EGFR inhibitors. To date, four inducible feedback inhibitors have been identified in mammals: leucine-rich and immunoglobulin-like domains protein 1 (LRIG1), suppressor of cytokine signaling 4 and 5 (SOCS4 and SOCS5), and mitogen-inducible gene 6 (Mig6). Among them, Mig6 is the only EGFR feedback inhibitor that has been implicated in metabolic diseases.

1.3.4 Mitogen-inducible gene 6

The human Mig6 (also called receptor-associated late transducer/RALT, ERBB receptor feedback inhibitor 1/Errfi1, and gene-33) gene is located on chromosome 1p36. It has 5 exons, encodes a 3144 bp mRNA, which is translated into a 50 kDa cytoplasmic protein containing an EGFR binding domain, a Cdc42/Rac-interaction and binding (CRIB) domain, a 14-3-3 protein binding motif, and a Grb2 binding motif.

Mig6 inhibits EGFR activity via the aforementioned first- and second-tier mechanisms. At the first tier (immediately after ligand binding and EGFR activation), Mig6 acts as an adaptor protein that recruits AP-2 and intersectins that facilitates clathrin-mediated EGFR endocytosis, and engages syntaxin 8 that directs EGFR toward the late endosome-to-lysosome degradation pathways (359, 360). The second tier of Mig6-mediated inhibition involves an allosteric inhibition of EGFR kinase activity (361). Based on crystallography and biochemical binding studies, Mig6 binds to the C-lobe of the EGFR kinase domain and interrupts the formation of asymmetric dimers (361, 362). It is likely that, in the EGFR activation programs, pre-existing Mig6 first disrupts the EGFR signalosome by enhancing the lysosomal routing. Later, *de novo* mitogen-induced Mig6 further prevents the activation of plasma membrane EGFR. Taken together, Mig6 provides robust and lasting inhibitory functions to repress EGFR activity.

Transcriptional and translational regulation of Mig6

Mig6 transcription is rapidly induced by mitogens (e.g., EGF, insulin, hepatocyte growth factor, platelet-derived growth factor, and transforming growth factor alpha (363)) and stress stimuli (e.g., cytokines, hypoxia, osmotic stress, and mechanical strain (364, 365)). Because Mig6 is an effective EGFR inhibitor, cellular Mig6 mRNA and

protein levels are tightly controlled. For instance, epigenetic modifications alter *Mig6* expression levels in human lung cancer and melanoma cell lines (366), and the transcription of *Mig6* is controlled by MAPK signals in response to growth and stress stimuli (367-369). Post-transcriptionally, *Mig6* degradation is likely facilitated by microRNA species against *Mig6* (e.g., miR148a, which has been implicated in controlling insulin biosynthesis) (370, 371), as well as the mRNA-degrading enzymes, as the 3'-UTR of *Mig6* contains many AU-rich elements (AREs) (372, 373). Interestingly, *Mig6* is possibly translated during stress conditions when general protein translation is halted. The ability of *Mig6* to avoid translational inhibition is likely because the *Mig6* 5'-UTR region bears putative internal ribosomal entry sites (IRES) that allows for atypical translation initiation.

Studies examining the post-translational modifications of *Mig6* are emerging. Some have suggested that mitogen stimulation triggers *Mig6* phosphorylation (at Ser 251, Tyr 394, Tyr 395, Tyr 458, and Ser 461 sites), leading to decreased *Mig6*-EGFR binding (374-379). However, based on the protein sequence alignments, it is more likely that the phosphorylation modifications change *Mig6* and other proteins' association patterns. For example, the Ser 251 site of *Mig6* is located within the predicted 14-3-3 protein association domain, and the Ser 461 site is adjacent to the SH3 binding region. Perhaps the post-translationally modified *Mig6* regulates mitogenic signaling cascades via an EGFR binding-independent mechanism.

The clearance of *Mig6* protein is controlled by the proteasomal degradation systems, as *Mig6* is modified by poly-ubiquitin and is subsequently targeted to the 26S proteasome (369). However, the pressing question is whether or not *Mig6* is subject to

mono-ubiquitinylation. For example, the mono-ubiquitin tagged Mig6 might tether EGFR toward lysosomes, and Mig6 has been shown to haul EGFR toward lysosomes in an EGFR ubiquitinylation-independent manner (360). In addition, Mig6 might bind to ubiquitin-associated and SH3 domain-containing protein B (Ubash3b, better known as Sts-1). Sts-1 is known to promote EGFR stabilization by interfering with Cbl-mediated down-regulation and degradation of EGFR. It will be interesting to examine the interplay between Mig6, Sts-1, and EGFR, and its relation to controlling EGFR lysosomal degradation programs.

Mig6 controls organ development and tissue homeostasis

Mig6 is expressed in multiple tissues and organs (e.g., breast, lung, brain, liver, uterus, heart, kidney, and pancreas) with a distinctive temporal expression patterns. For example, Mig6 is barely detectable in the livers of the rat fetus; yet its expression becomes abundant in the newborn (380). Mig6 expression level is also elevated in the window of metanephric mesenchyme to nephron epithelium conversion during kidney development (381). Finally, Mig6 is differentially expressed in the endometrium in the stages of pregnancy. In fact, the Mig6 expression pattern nicely mirrors the gestational estrogen and progesterone expression patterns, suggesting estrogen- and progesterone-induced Mig6 likely serves as feedback inhibitor to regulate the maintenance of pregnancy (382, 383).

The critical developmental and physiological roles of Mig6 are demonstrated by Mig6 knockout (*Mig6*^{-/-}) mice. *Mig6*^{-/-} mice have 50% embryonic lethality, and the surviving *Mig6*^{-/-} mice were first found to have altered lung architecture (384). Gradually, the mice developed chronic obstructive pulmonary disease, degenerative joint disease,

skin hyperplasia, endometrial hyperplasia, and spontaneous tumors in the skin, biliary ductal epithelia, mammary gland, and gastric mucosa (385-390). To further investigate the roles of Mig6 in different organs, conditional Mig6 knockout animals were generated. Thyroid-specific Mig6 knockout mice displayed altered EGFR membrane distribution in the thyrocytes, and developed thyroid tumors (391, 392). Limb-targeted deletion of Mig6 resulted in elevated expression of EGFR signaling and progenitor markers (Sox9, notch1, beta-catenin, and transforming growth factor beta mediators) in articular chondrocytes, enhanced cartilage matrix degradation, and thickened articular cartilage in mice (393). Similarly, deficiency of Mig6 in the chondrocytes leads to an osteoarthritis-like disorder, due to excessive proliferative activity of the articular cartilage (394). In the uterine epithelium-specific Mig6 knockout mice, Notch1 expression in the epithelial cells is increased, which promotes endometrial hyperplasia and the onset of estrogen-dependent endometrial tumors (395).

Mig6 also modulates tissue homeostasis and regeneration in experimental models of injury. For instance, *Mig6*^{-/-} mice displayed higher hepatocyte proliferation after a partial hepatectomy (396). Likewise, ovariectomized *Mig6*^{-/-} treated with progesterone and estrogen had endometrial hyperplasia and developed endometrial adenocarcinoma. Surgical removal of knee ligament and meniscus in *Mig6*^{-/-} mice leads to rapid and increased joint damage (397). In addition, *Mig6*^{-/-} mice are susceptible to the formation of chemically induced skin tumors (385). Overall, the published reports define Mig6 as an inhibitor of cell proliferation, and Mig6 deficiency in mice results in penetrant phenotypes related to hyperproliferation of injured tissue types. With that being said, tissue regeneration and remodeling are extremely complicated processes. How

temporally regulated stress- and cytokine-sensitive Mig6 interplays with mitogenic signaling cascades in damaged tissue types remains unknown. The studies described in **Chapter 2, 3, and 4** address this critical question.

Mig6 controls tissue destruction

Mig6 is induced by *in vitro* stress stimuli including: lipid agonists, synthetic glucocorticoids, hypoxia, *Staphylococcus Aureus* infection, and mechanical stretch (364, 398-404). Because the above reactions are strongly associated with inflammatory responses and cell death, it is conceivable that Mig6 might not only inhibit cell proliferation, but also promote cell destruction. Indeed, Mig6 can induce transcriptional activation of the master stress mediator nuclear factor κB (NF-κB) by sequestering the inhibitor of κB-α (IκB-α) via Mig6's Cdc42 binding domain (365, 405, 406). Additionally, Mig6 has been reported to activate another well-characterized cellular stress regulator, c-Jun N-terminal kinase (JNK) (407, 408). The pro-apoptotic features of Mig6 have recently been determined, for example: 1) overexpression of Mig6 promotes non-small cell lung cancer cell apoptosis, whereas Mig6 knockdown prevented cancer cell death (409), 2) Mig6 reduces Akt and ERK signaling, and Mig6 is required for hypoxia-induced cardiomyocyte death (410), 3) uterine epithelial cells lacking Mig6 have higher Baculoviral IAP repeat containing 3 (Birc3, also called inhibitor of apoptosis protein 1) expression levels (395), whereas progesterone positive cells lacking Mig6 and Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) have higher Baculoviral IAP repeat containing 1 (Birc1) levels, and showed significantly decreased uterine epithelium cell apoptosis (411), and 4) Mig6 deletion causes impaired apoptosis and luminal filling of mouse mammary ducts (390).

Chronic pathological stimuli, such as diabetic nephropathy, also induce Mig6 (407, 412). But whether such induction is sustainable, and what are the pathophysiological consequences of Mig6 elevation under prolonged stress conditions, are not known. From examining developmental biology, Mig6 could possibly attenuate mitogenic signaling cascades and facilitate cell differentiation at the final stage of stress/injury-induced tissue regeneration/remodeling (380, 381, 413, 414). Reverse evidence comes from cancer biology, where Mig6 has been reported to modulate epithelium-to-mesenchyme transition (415). Apart from a role in facilitating cell maturation, Mig6 could be essential in restraining cell expansion in post-injury tissue because Mig6 expression levels are elevated during cellular senescence and Mig6 overexpression leads to pre-mature senescence (416, 417). The bottom line is, in most circumstances, restricting mitotic cell (e.g., epithelial, stromal, and vascular cells) expansion is central to tissue homeostasis. As a proof of concept, *Mig6*^{-/-} mice exhibited accelerated and increased joint fibrosis after ligament and meniscus trauma, and cardiac specific Mig6-overexpressing transgenic mice are protected from maladaptive cardiac hypertrophy due to decreased tissue inflammation and fibrosis (387, 397, 418). Finally, the most interesting question is, whether or not the post-mitotic cells (e.g., neurons and pancreatic beta cells) could benefit from Mig6 modification. For instance, disruption of Mig6 might contribute to protection against cell apoptosis.

The metabolic roles of Mig6

Mig6 was initially identified as an insulin-responsive gene in the rat liver (380). Ironically, the metabolic roles of Mig6 are largely unknown. Recently, Ku et al. suggested that Mig6 controls cholesterol homeostasis and bile acid synthesis, and mice

with Mig6 deficiency specifically in the liver developed hepatomegaly and fatty liver (419). However, the detailed molecular mechanisms are not fully defined.

Mig6 has been suggested to inhibit pancreatic islet proliferation. Recently our laboratory reported that Mig6 is induced by a synthetic glucocorticoid, dexamethasone; and Mig6 is responsible for glucocorticoid-induced beta cell proliferation blockade (420). In the work to follow, I will address the roles of Mig6 as a stress-responsive factor in mediating beta cell dysfunction and death. I hypothesized that Mig6 will be induced by various diabetogenic stress stimuli; and I predicted that Mig6 would promote beta cell death via inhibiting EGFR signaling cascades. The specific aims of my research are to determine if Mig6 is a stress-inducible factor that controls beta cell apoptosis (**Chapter 2**), to determine the extent to which Mig6 controls T1D progression *in vivo* (**Chapter 3**), and to determine if Mig6 attenuates EGFR signaling cascades and facilitates beta cell death in the T2D milieu (**Chapter 4**).

2 INDUCIBLE GENE 6 TRIGGERS APOPTOSIS AND EXACERBATES ER STRESS-INDUCED BETA CELL DEATH

2.1 Synopsis

The increased insulin secretory burden placed on pancreatic beta cells during obesity and insulin resistance can ultimately lead to beta cell dysfunction and death and the development of type 2 diabetes. Mig6 is a cellular stress responsive protein that can negatively regulate the duration and intensity of EGFR signaling and has been classically viewed as a molecular brake for proliferation. In this study, we utilized Mig6 heterozygous knockout mice (*Mig6^{+/−}*) to study the role of Mig6 in regulating beta cell proliferation and survival. Surprisingly, the proliferation rate of *Mig6^{+/−}* pancreatic islets was lower than wild-type islets despite having comparable beta cell mass and glucose tolerance. We thus speculated that Mig6 regulates cellular death. Using adenoviral vectors to overexpress or knockdown Mig6, we found that caspase 3 activation during apoptosis was dependent on the level of Mig6. Interestingly, *Mig6* expression was induced during ER stress and its protein levels were maintained throughout ER stress. Using polyribosomal profiling, we identified that Mig6 protein translation was maintained whereas the global protein translation was inhibited during ER stress. In addition, Mig6 overexpression exacerbated ER stress-induced caspase 3 activation *in vitro*. In conclusion, Mig6 is transcriptionally up-regulated and resistant to global translational inhibition during stressed conditions in beta cells and mediates apoptosis in the form of caspase 3 activation. The sustained production of Mig6 protein exacerbates ER stress-induced beta cell death. Thus, preventing the induction, translation, and/or function of Mig6 is warranted for increasing beta cell survival.

2.2 Introduction

T2D is an endemic disease that greatly impacts the healthcare and financial systems in both developed and developing countries (421). T2D is characterized by tissue insulin resistance and pancreatic beta cell failure (422). During the development of T2D, insulin resistance is initially compensated for by increased insulin secretion by the beta cells and beta cell mass expansion. However, the body's insulin demands eventually exceed the beta cell secretory capacity thus placing an insurmountable burden on the ER; the unmitigated protein synthesis/folding stress in the ER of beta cells finally initiates an apoptotic response (423, 424).

The execution mechanism of ER stress-induced apoptosis remains an area of intensive study. Multiple signaling pathways tightly control a cell's life and death decisions. Whereas the activation of CHOP and JNK signaling pathways and their direct connections to the mitochondrial apoptotic program are considered to be the classical ER stress-induced cell death mechanism (425-427), the cross-talk between the ER stress responsive factors and canonical cell proliferation and survival signaling pathways is an emerging field. For example, Tribbles homolog 3 (TRIB3), an inhibitor of the Akt signaling pathway, was recently found to respond to stress stimuli in beta cells. The up-regulation of TRIB3 leads to cell apoptosis by promoting translocation of Bcl-2-associated X (BAX) to the mitochondria (428-430). Here, we identified Mig6 as an ER stress-responsive protein, which modulates beta cell apoptosis.

Mig6 was identified as a feedback inhibitor of EGFR signaling (413, 431). By binding to EGFR, Mig6 controls the temporal and spatial continuity of EGFR signaling cascades (359, 360, 362). Interestingly, Mig6 expression is induced by many stress

stimuli including hypoxia, osmotic stress, mechanical strain, and LPS-induced infections in various cell types (369, 400, 410, 432, 433). Nevertheless, the role of Mig6 in response to ER stress in beta cells has not been investigated.

ER stress not only initiates adaptive responses by transcriptionally up-regulating stress-responsive proteins but also directly controls the protein translation machinery. Under normal physiological conditions, cap- and scanning-dependent translation is the default translation mechanism. In contrast, during stressed conditions such as those that occur during the development of type 2 diabetes, global protein translation is halted to conserve resources and mitigate the unfolded protein stress (434). Meanwhile, cells enhance cap-independent translation to generate proteins that are involved in stress alleviation or apoptosis induction. Intriguingly, Mig6 is resistant to global translation inhibition under hypoxic stress in a prostate cancer cell line (435). Thus, we speculate that Mig6 is a stress-responsive molecule that modulates cellular apoptosis in diverse disease states including diabetes.

During our characterization of the proliferation and survival of beta cells from mice lacking one Mig6 allele in the present study, we hypothesized that Mig6 is an inducer of apoptosis. In addition, we speculated that Mig6 would be induced during ER stress. Ultimately, we have identified a new role for Mig6 as a mediator of ER stress-induced apoptosis in pancreatic beta cells. Thus, Mig6 becomes a potential therapeutic target to preserve pancreatic beta cell mass and prevent the irreversible pathogenesis of diabetes.

2.3 Results

2.3.1 Mig6 heterozygous knockout mice have similar glucose tolerance and pancreatic beta cell area, but decreased islet proliferation

Previous reports showed that deletion of Mig6 in mice leads to hyperactivation of EGFR signaling pathways and the development of tumors in the skin, lungs, gall bladder, and bile duct (436, 437). Mice with both *Mig6* alleles nullified also had increased embryonic lethality (438), and in our breeding facility we rarely obtain *Mig6* homozygote knockout mice (3 out of 234 pups; 1.3% observed from heterozygous matings). We, therefore, utilized *Mig6*^{+/−} and their wild-type control littermates (*Mig6*^{+/+}) to study the function of Mig6 in regulating glucose homeostasis and the proliferation of beta cells *in vivo*. Methods are described in detail in **Chapter 5**. Intraperitoneal glucose tolerance tests were performed on 8- to 10-week-old mice following a 5 h fast. Blood glucose concentrations in *Mig6*^{+/+} and *Mig6*^{+/−} mice were not different at fasting or throughout the glucose tolerance test (**Figure 2-1A**; all figures for the data chapters are located at the end of their respective chapter). We then harvested the pancreata from the same animals to study the islet morphology and beta cell cross-sectional area. Similarly, *Mig6*^{+/+} and *Mig6*^{+/−} showed comparable beta cell cross-sectional area and islet morphology (**Figure 2-1B and 2-1C**). Surprisingly, islet proliferation of *Mig6*^{+/−} mice was lower than *Mig6*^{+/+} mice, as measured by the numbers of beta cells positive for phosphorylated histone H3 *in vivo* (**Figure 2-1D**) and tritiated-thymidine incorporation in isolated islets *in vitro* (**Figure 2-1E**).

2.3.2 Mig6 regulates caspase 3-mediated beta cell apoptosis

Because the regulation of beta cell mass involves the balance between processes that both increase and decrease the numbers of beta cells (260), we hypothesized that

Mig6^{+/−} mice islets must have decreased apoptosis because they have comparable beta cell area and decreased beta cell proliferation. To study the role of Mig6 in regulating beta cell apoptosis, we first employed an adenovirus carrying an shRNA against rat *Mig6* mRNA to knockdown Mig6 in rat insulinoma INS-1-derived 832/13 cells (75% knockdown efficiency, **Figure 2-6A**). Following the knockdown, cell apoptosis was induced by etoposide (a topoisomerase toxin that causes DNA double strands break). The activation of cellular apoptosis was measured by the amount of cleaved caspase 3 by western blotting (**Figure 2-2A and 2-2B**), and a caspase 3 activity assay (**Figure 2-2C**). Indeed, knocking down Mig6 protected beta cells from etoposide-induced apoptosis as indicated by decreased caspase 3 activity following treatment with etoposide (Fig. 2-2C). In addition, adenoviral-mediated overexpression of Mig6 (**Figure 2-6B**) significantly exacerbated etoposide-induced apoptosis (**Figure 2-2D and 2-2E**). These results suggested that Mig6 regulates caspase 3-mediated beta cell apoptosis.

2.3.3 Mig6 exacerbates ER stress-induced beta cell death

As chronic hyperglycemia and hyperlipidemia in diabetes disrupt pancreatic beta cell ER homeostasis and lead to beta cell death (439), we further investigated the apoptosis-regulating function of Mig6 during ER stress. To induce ER stress, we utilized a pharmacological ER calcium channel blocker thapsigargin to the compromise protein folding capacity of the ER. As hypothesized, overexpression of Mig6 in 832/13 cells remarkably exacerbated thapsigargin-induced beta cell apoptosis (**Figure 2-3A and 2-3B**). The phosphorylation of eIF2 α and the induced CHOP protein expression served as hallmarks of the UPR in an ER stress environment (**Figure 2-3A, 2-3C, and 2-3D**). Interestingly, overexpression of Mig6 did not influence the magnitude and induction of

the UPR, suggesting that the apoptosis-promoting mechanism of Mig6 is downstream and/or independent of the UPR pathways.

To verify that caspase 3 cleavage was indicative of apoptosis and ultimately cell death, PI staining was performed on cells transduced with control or Mig6-overexpressing adenoviruses. As both adenoviruses express GFP, cell death was measured in only those cells successfully transduced by adenoviruses (e.g., GFP positive cells). As indicated by PI staining, Mig6 overexpression induced cell death in cells treated with DMSO or thapsigargin (**Figure 2-3E**).

2.3.4 *Mig6* mRNA expression is induced and stabilized by ER stress

The transcription of *Mig6* was induced by ER-stress in a time-dependent manner in 832/13 cells (**Figure 2-4A**), suggesting that *Mig6* is a *bona fide* stress-responsive protein (432, 440, 441). Importantly, *Mig6* gene expression was also induced in primary rat islets exposed to thapsigargin for 6, 16, and 24 h (**Figure 2-4B**). In contrast, the mRNA level of *pdx-1* was unchanged in thapsigargin-treated 832/13 cells (**Figure 2-4A**), and notably was decreased in thapsigargin-treated rat islets at all time-points studied (**Figure 2-4B**). Despite the elevated *Mig6* mRNA level during ER stress, *Mig6* protein expression was unchanged (**Figure 2-4C and 2-4D**). In contrast, Pdx-1 protein level decreased in the stressed environment.

Mig6 is categorized as an immediate early gene, which are usually induced by p38 and JNK/stress-activated protein kinases (SAPKs) (442). We thus investigated the participation of three different mitogen-activated protein kinases (MAPKs) in regulating *Mig6* transcription. Pharmacological MEK (UO126), p38 (PD169316), or JNK (SP600125) inhibitors did not block the thapsigargin-induced *Mig6* mRNA expression

(Figure 2-7A). However, the combination of JNK and p38 inhibitors hindered the ER stress-stimulated *Mig6* expression by 40% (Figure 2-7B).

Sequence analysis of the *Mig6* mRNA reveals putative AREs in the 3'-untranslated region (443). ARE-containing mRNAs are translated into proteins that normally control cell survival and are usually labile (444). In response to stimuli, ARE-containing mRNAs associate with different ARE-binding proteins thus yielding increased or decrease stability. We analyzed the half-life of *Mig6* during ER stress using actinomycin D (an antibiotic that causes a transcription initiation blockade). We discovered that *Mig6* mRNA was stabilized by ER stress (Figure 2-4E).

2.3.5 *Mig6* translation is maintained during ER stress

During ER stress, general protein biosynthesis is decreased through a phospho-eIF2 α -dependent manner as a protective mechanism to conserve cellular energy and prevent protein-folding overload (445). However, some UPR adapter proteins are translated continuously during ER stress. Most of the alternatively translated mRNAs contain upstream open reading frames or IRES (446). Not surprisingly, the 5'-untranslated region of *Mig6* mRNA is rich in GC content (443), which might form secondary structures and possibly serve as an IRES. To test if *Mig6* is alternatively translated during ER stress, we employed polyribosome analysis to examine the polyribosome association pattern of *Mig6* mRNA. After stimulating 832/13 cells with thapsigargin (or DMSO as an unstressed control), sucrose gradient layered-cell lysates were fractionated, and the isolated mRNAs from the fractions were analyzed by qRT-PCR. Upon thapsigargin treatment, the overall polyribosome level was significantly reduced whereas monoribosome levels were robustly elevated (Figure 2-5A). The *Mig6*

mRNAs distribution in the ribosome profiles showed the same occupancy in control and stressed conditions (**Figure 2-5B and Table 2-1**). Under normal conditions, 76% of *Mig6* mRNAs were associated with translating polysomes (which was defined as fractions 6 to 10). During ER stress, 60% of *Mig6* mRNA remained associated with polysomes (the difference between control and thapsigargin-mediated polysomal occupancy was not statistically significant). In this report, *ATF4* served as positive control (**Figure 2-5C and Table 2-1**) because *ATF4* mRNA associates with translating ribosomes under stress conditions (447). On the other hand, *cyclinD1* was used to demonstrate general protein translation blockade (448). The polysome-associated *cyclinD1* mRNA level shifted from 87% in control group to 57% in ER-stress conditions (**Figure 2-5D and Table 2-1**). These findings suggested that *Mig6* translation was maintained, whereas global translation was attenuated during ER stress.

2.4 Discussion

The hyperglucolipidemic milieu present during the pathogenesis of diabetes compromises the integrity of the ER in pancreatic beta cells. The perturbed ER eventually initiates a series of molecular pathways to trigger cell death. Our work presented here highlights a new mechanism linking ER stress to beta cell apoptosis through an adaptor protein, Mig6. During our characterization of *Mig6*^{+/−} mice we speculated that Mig6 could also be a regulator of beta cell death. Consequently, we demonstrated that Mig6 exacerbates ER stress-induced cell apoptosis. Interestingly, *Mig6* transcript was both induced and stabilized by ER stress. Finally, we showed that the translation of Mig6 was preserved during ER stress whereas global protein translation was attenuated.

Mig6 was initially characterized as an immediate early gene induced by glucocorticoids and hormones (443, 449). More recently, Mig6 was identified as a negative regulator of EGFR in multiple tissues (437, 450, 451). Much of the research regarding Mig6 has focused on its role as an anti-proliferative tumor suppressor in various tissues (362, 385, 431, 436). On a molecular level, Mig6 inhibits EGFR activation through a two-tiered mechanism: suppressing EGFR kinase and directing EGFR degradation (359). Correspondingly, a loss of Mig6 is associated with murine and human tumorigenesis (385, 436, 452, 453). Nevertheless, increasing evidence suggests that Mig6 responds to diverse pathophysiological stimuli, including mechanical stress, tissue injury, and hypoxia (396, 410, 432). However, the detailed stress-sensing mechanism and the consequences of Mig6 induction in different tissues remains to be fully characterized. Our study suggests that Mig6 regulates pancreatic beta cell apoptosis during ER stress. This finding not only provides a new drug target to prevent ER stress-associated beta cell death in diabetes, but also indicates a possible role of Mig6 as a pro-apoptotic molecule in treating cancers.

Because Mig6 is an inhibitor of EGFR, we hypothesized that haploinsufficiency of Mig6 would result in increased pancreatic beta cell mass. However, *Mig6*^{+/-} mice had the same glucose tolerance and beta cell mass compared to *Mig6*^{+/+} mice under normal chow diet-fed conditions. Surprisingly, islet proliferation from *Mig6*^{+/-} mice was lower than that in *Mig6*^{+/+} mice. How islets from *Mig6*^{+/-} mice respond to a challenge that requires an expansion of beta cell mass, such as following a partial pancreatectomy or during high fat feeding remains to be determined.

As beta cell mass is regulated by the balance between cell proliferation and death, we speculate that *Mig6*^{+/−} islets have lower levels of apoptosis. Conversely, we hypothesized that elevated levels of Mig6 could induce cell death. To address this hypothesis, we used the INS-1-derived rat insulinoma cell line 832/13 to identify whether or not Mig6 regulates apoptosis. We observed that whereas Mig6 overexpression exacerbated beta cell apoptosis and cell death through caspase 3-mediated pathways, silencing Mig6 mitigated apoptosis. During chemical-induced ER stress, Mig6 overexpression also led to increased beta cell apoptosis and death. Our results suggest that Mig6 most likely regulates beta cell death during ER stress independent of the canonical CHOP-mediated pathway. Instead, Mig6 possibly compromises traditional growth factor receptor-mediated cell survival signals, such as Akt, ERK, and their downstream signaling molecules (454, 455).

Intriguingly, ER stress induced *Mig6* mRNA expression, partially through the activation of p38 and SAPK pathways. Being a classical immediate early gene, the *Mig6* mRNA contains AU-rich elements, which are targets for fast degradation (*Mig6* t_½ = 34.64 min, see **Figure 2-4E**). However, *Mig6* mRNAs were, in fact, stabilized under ER stress conditions (*Mig6* t_½ > 120 min, see **Figure 2-4E**). Typically, translating mRNAs are degraded rapidly during stress conditions. By clearing the transcription and translation load, the ER is freed to correct unfolded/misfolded proteins (456). For example, proinsulin mRNAs are unstable in stressed beta cells (457, 458). Yet our work presented here suggested an enhanced stability of *Mig6* mRNA during ER stress. We speculate that beta cells conserve *Mig6* transcripts during stress conditions should the need to initiate the apoptotic program arise.

In adaptation to ER stress, global protein synthesis is inhibited to prevent the accumulation of misfolded proteins in the ER lumen. The profound protein translation attenuation is correlated with the activation of PERK and the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2 α). Phosphorylated eIF2 α binds to eIF2B, whereby it interferes with the assembly of the translation initiation complex, and thus cap-dependent translation initiation stalls (459). However, the genes essential for alleviating the stress or triggering apoptosis escape this translational blockade. Many of the preferentially translated genes contain upstream open reading frames or internal ribosomal entry sites in the 5'-untranslated regions, which allows for cap-independent translation initiation to occur during cellular stress (447, 460-463). *Mig6* should be added to the list of genes that evade complete translational blockade during stress as its 5'-untranslated region has secondary structures that possibly serve as the internal ribosomal entry site (449). From our observation, *Mig6* protein level was maintained during ER stress, whereas other protein levels were decreased. The polyribosome analysis in 832/13 cells revealed that *Mig6* mRNAs remained associated with actively translating polyribosomes during ER stress. In agreement with our work, Thomas et al. reported that *Mig6* mRNAs continue to associate with polyribosomes during hypoxic stress (435). On the other hand, mRNAs of the classically induced ER stress gene *ATF4* were shifted from untranslating monoribosomes to translating polyribosomes upon ER stress in beta cells. In contrast, *cyclinD1* mRNAs were redistributed from polyribosomes to monoribosomes in the stressed conditions. Indeed, translation inhibition in the UPR is not limited to cyclin D1. However, the drastic depression of cyclin D1 translation might slow down

cell cycle progression, thus permitting beta cells to either re-establish ER homeostasis or proceed to apoptosis (464).

This study demonstrates that Mig6 is a stress-responsive protein mediating beta cell death. *Mig6* transcripts are induced, stabilized, and remain translated during ER stress. Additionally, Mig6 regulates beta cell apoptosis through a caspase 3-dependent pathway. Further studies must be undertaken to understand on the molecular level how Mig6 bridges growth factor signaling and ER stress-induced apoptosis. This work highlights that Mig6 should be considered as a target for alleviating ER stress and beta cell death for the prevention and treatment of diabetes.

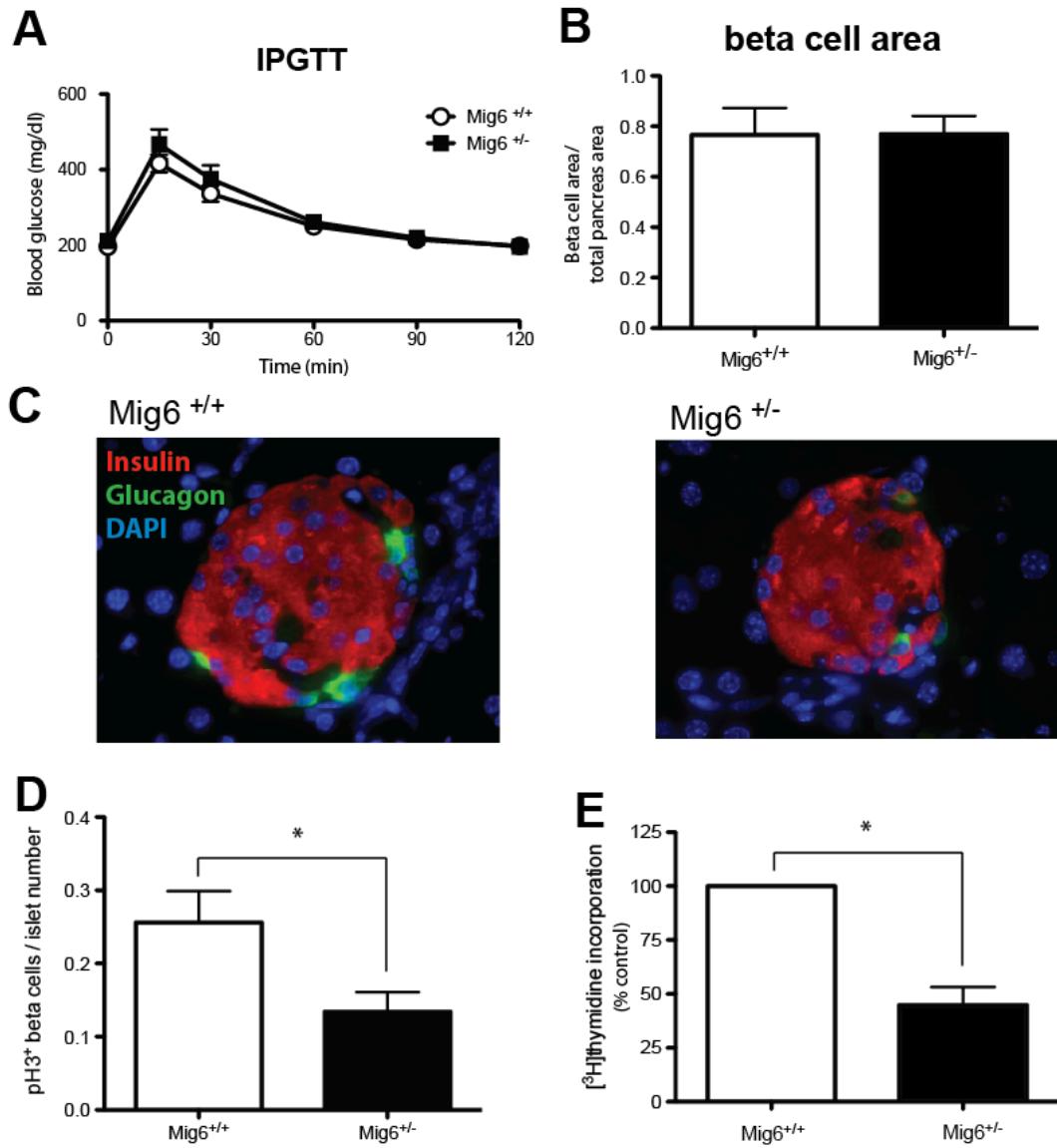


Figure 2-1

***Mig6^{+/+}* and *Mig6^{+/-}* mice have same similar glucose tolerance and, islet morphology, but different islet proliferation rates.**

Eight- to ten-week-old *Mig6^{+/+}* and *Mig6^{+/-}* mice ($n = 6-8$ per group) were submitted to (A) intraperitoneal glucose tolerance testing and pancreata were assessed for (B) beta cell cross-sectional area and (C) islet morphology (insulin: red, glucagon: green, and nuclei: blue). (D) Relative beta cells proliferation rates were determined *in vivo* by counting phospho-histone H3 (green) and insulin (red) stained cells normalized to total islet numbers. (E) Islet proliferation rates were measured *in vitro* by [³H]-thymidine incorporation assays (islets from the same genotype were pooled, $n = 3$). *, $p < 0.05$ between *Mig6^{+/+}* and *Mig6^{+/-}*.

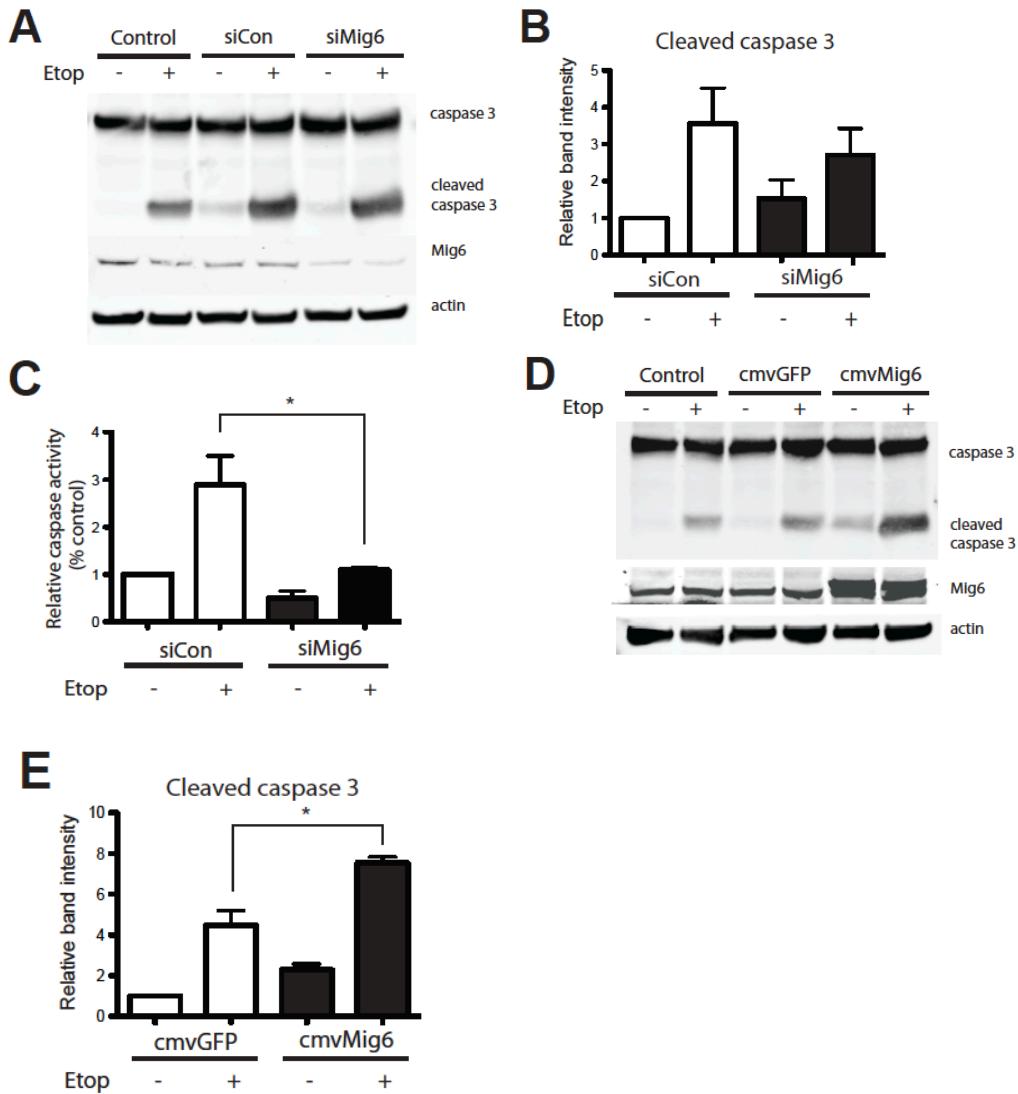


Figure 2-2

Knockdown of Mig6 attenuates etoposide-induced apoptosis, whereas overexpression of Mig6 exacerbates apoptosis.

INS-1-derived 832/13 cells were transduced with adenoviruses producing either a scrambled control RNA (siCon) or a small interfering hairpin RNA sequence against Mig6 (siMig6). After 4 hours of 50 nM etoposide treatment, immunoblotting was performed to determine the cleaved caspase 3 levels as an indicator of apoptosis (**A, B**), or cell lysates were used for a caspase 3 activity assay (**C**). INS-1 cells were transduced with adenoviruses carrying CMV-promoter driven GFP (cmvGFP) or Mig6 (cmvMig6). After treating with 50 nM etoposide, cell lysates were collected for immunoblotting (**D, E**). Data are presented as representative immunoblots and means \pm SEM; $n = 3-4$. *, $p < 0.05$ between etoposide-treated siCon and siMig6; #, $p < 0.05$ between etoposide-treated cmvGFP and cmvMig6.

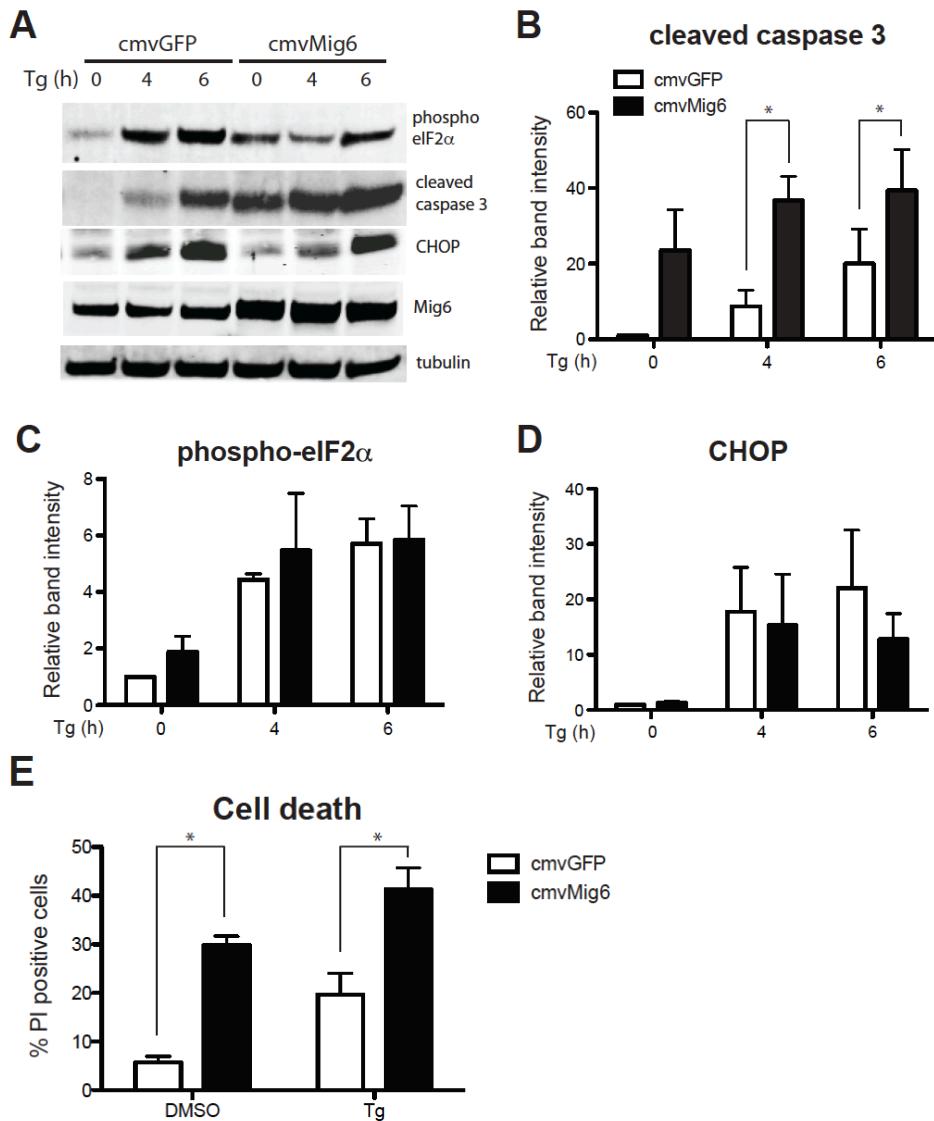


Figure 2-3

Overexpression of Mig6 exacerbates apoptosis.

INS-1-derived 832/13 cells were transduced with adenoviruses carrying cmvGFP or cmvMig6. After 48 h, cells were treated with 1 μ M thapsigargin for 0, 4, or 6 h. (A) Cell lysates were collected for immunoblotting to determine cleaved caspase 3 (B), phosphorylated eIF2 α (C), and CHOP (D) levels. Data are presented as representative immunoblots and means \pm SEM; $n = 3-4$. *, $p < 0.05$ between cmvGFP and cmvMig6 groups at both 4 and 6 h thapsigargin treatment. (E) Cell death was measured by propidium iodide (PI) staining in 832/13 cells transduced with adenoviruses carrying cmvGFP or cmvMig6 and treated with DMSO or 1 μ M thapsigargin for 6 h. Data represent means \pm SEM; $n = 5$. *, $p < 0.05$ between cmvGFP and cmvMig6 groups.

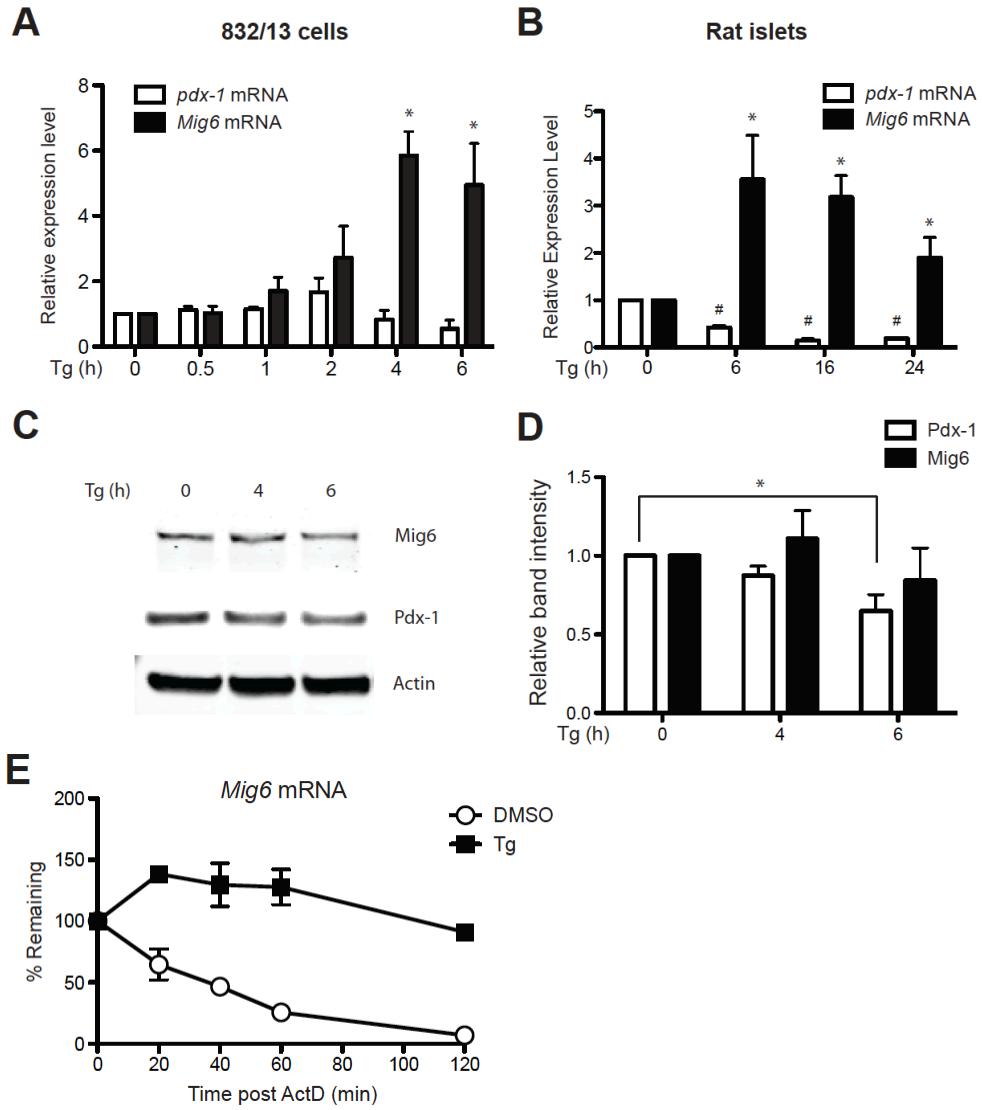


Figure 2-4

Thapsigargin-induced *Mig6* mRNA expression.

(A) INS-1-derived 832/13 cells were treated with 1 μ M thapsigargin for the indicated times. Isolated mRNA was subjected to qRT-PCR analysis with *Mig6* and *Pdx-1* primers and probes. (B) Isolated rat islets were treated with 1 μ M thapsigargin for the indicated times and collected for qRT-PCR analysis. (C, D) INS-1-derived 832/13 cells were treated with 1 μ M thapsigargin for 4 and 6 h. Cell lysates were collected to determine *Mig6* protein level. (E) INS-1-derived 832/13 cells were pre-treated with 1 μ M thapsigargin for 2 h, followed by 5 μ g/ml actinomycin D treatment for 0, 20, 40, 60, 120 min, and subjected to qRT-PCR analysis. Data are presented as means \pm SEM; $n = 3-4$. *, $p < 0.05$ between the indicated groups.

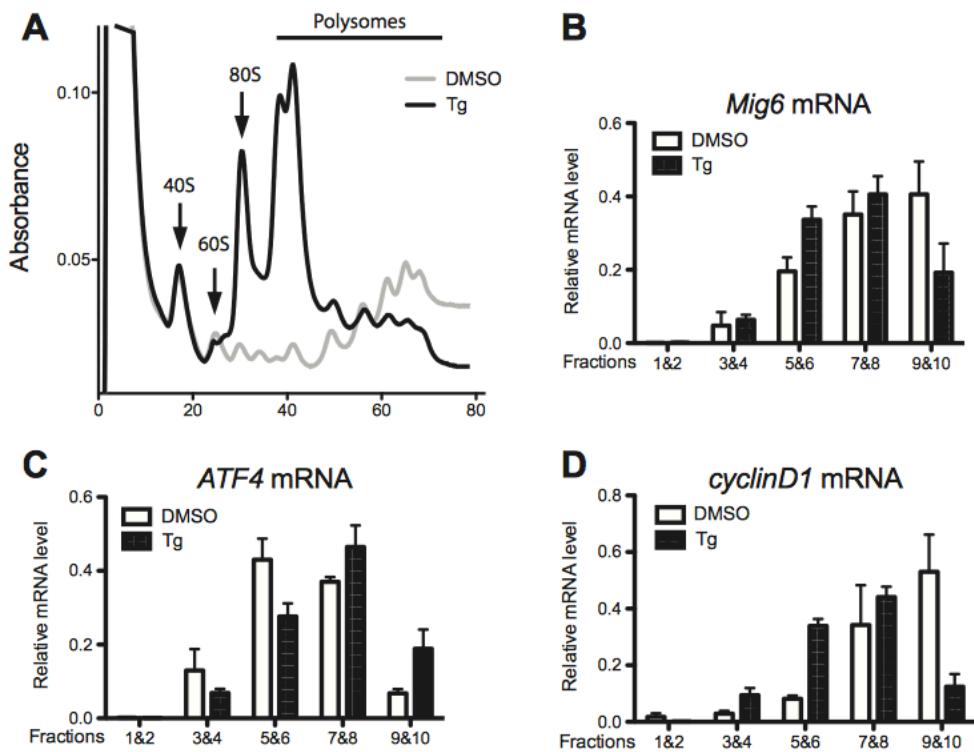


Figure 2-5

Translation (polyribosome association) of *Mig6* is maintained during ER stress.
(A) INS-1-derived 832/13 cells were treated with DMSO or 1 μ M thapsigargin for 6 h. Cell lysates were separated on a sucrose gradient. Ten fractions were collected while absorbance at 254 nm was continuously monitored to indicate the 40S ribosome subunits, 60S ribosome subunits, 80S monosomes and polysomes. Samples from adjacent 2 fractions were pooled and subjected to RT-PCR analysis. Fractions 1-4 represent the monosomes, whereas fractions 5-10 represent the polysomes. *Mig6* (**B**), *ATF4* (**C**), and *cyclinD1* (**D**) mRNA in the fractions were presented as percentage of unfractionated inputs. Data are presented as means \pm SEM; $n = 4$.

Table 2-1 Association of mRNAs to the translating polysomes (as a percentage of total mRNA)

	<i>Mig6</i>	<i>ATF4</i>	<i>Cyclin D1</i>
DMSO	0.76 ± 0.07	0.44 ± 0.00	0.87 ± 0.02
Tg	0.60 ± 0.03	0.65 ± 0.04 ^a	0.57 ± 0.01 ^a

Table 2-1

Quantitative results of Figure 2-5 B-D.

Tg, thapsigargin. Data are presented as means ± SEM; $n = 4$. ^a, $p < 0.05$ vs. DMSO.

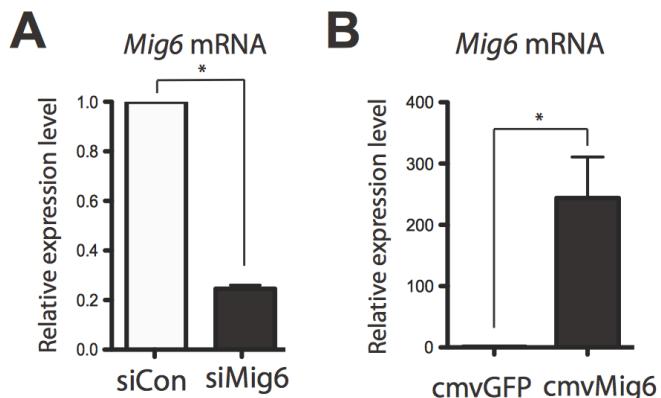


Figure 2-6

Mig6 expression.

qRT-PCR analysis of *Mig6* knockdown (**A**) and over-expression efficiency (**B**). *, $p < 0.05$ between control and *Mig6* knockdown or *Mig6* overexpressing groups.

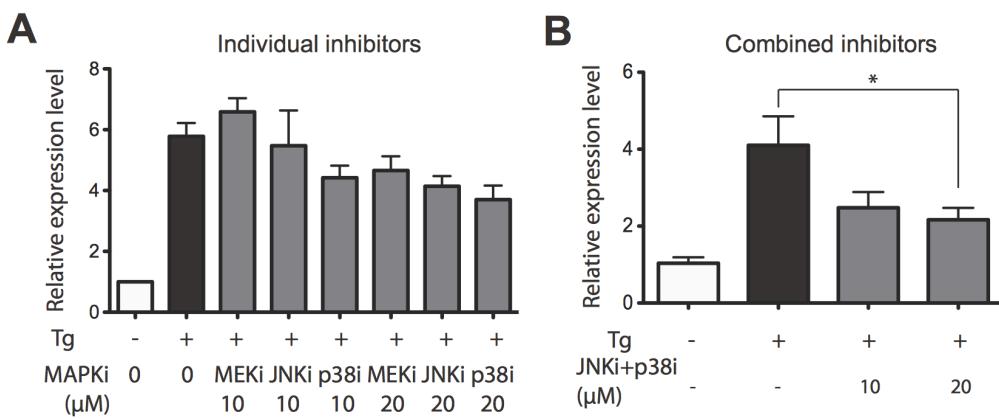


Figure 2-7

Mig6 transcription and translation is partially mediated by MAPKs.

(A) INS-1-derived 832/13 cells were pre-treated with a MEK inhibitor (UO126), p38 inhibitor (PD169316), or JNK inhibitor (SP600125) at the indicated dose for 1 h, followed by 4 h of 1 μM thapsigargin treatment. *Mig6* mRNA was determined by qRT-PCR. (B) Indicated inhibitor cocktails were used to pre-treat INS-1-derived 832/13 cells for 1 h following 1 μM thapsigargin treatment for 4 h. *Mig6* mRNA was determined by qRT-PCR. Data are presented as means ± SEM; n = 4. *, p < 0.05 between thapsigargin-treated and MAPKi-treated groups.

Portions of the text in this chapter are reproduced from: Chen Y-C, Colvin ES, Maier BF, Mirmira RG, Fueger PT. (2013) Mitogen-inducible gene 6 triggers apoptosis and exacerbates ER stress-induced β-cell death. Mol Endocrinol. 27(1):162-71.

3 MIG6 HAPLOIN SUFFICIENCY PROTECTS MICE AGAINST STREPTOZOTOCIN-INDUCED DIABETES

3.1 Synopsis

EGF and gastrin co-administration is effective in correcting hyperglycemia and pancreatic beta cell mass deficiencies in various T1D rodent models, yet the failure in clinical translation of this treatment implies that EGF-mediated tissue repair is a complicated process warranting further investigation. In this study, we aimed to determine if EGF receptor feedback inhibition by Mig6 limits the effectiveness of EGF, and contributes to the development of T1D. We hypothesized that Mig6 haploinsufficiency, and hence heightened EGFR signaling, would abrogate the development of diabetes in mice. To this end, we treated *Mig6^{+/−}* and *Mig6^{+/+}* with multiple low doses of streptozotocin (STZ) to induce beta cell death and diabetes. We found that whereas STZ-treated *Mig6^{+/+}* mice became diabetic, STZ-treated *Mig6^{+/−}* mice remained fully glucose tolerant. Concurrently, STZ-treated *Mig6^{+/−}* mice exhibited preserved levels of circulating insulin following a glucose challenge. As insulin sensitivity was similar between *Mig6^{+/−}* and *Mig6^{+/+}* mice, the preserved glucose tolerance in STZ-treated *Mig6^{+/−}* mice is likely the result of preserved beta cell function. Conversely, Mig6 overexpression in isolated islets compromises glucose-stimulated insulin secretion. Studies in 832/13 INS-1 beta cells suggested that Mig6 hinders EGF receptor activation, thereby inhibiting EGF-mediated DNA damage repair. Finally, STZ-treated *Mig6^{+/−}* mice have greater beta cell mass recovery at 20 days post-STZ. These data demonstrate that a reduction in Mig6 promotes beta cell damage repair, thus

abating the progression of diabetes. Our work suggests that Mig6 may be a novel therapeutic target for beta cell preservation in T1D.

3.2 Introduction

T1D is a progressive disease characterized by autoimmune-mediated destruction of the insulin-secreting beta cells of the pancreas (31). To cure T1D, most research efforts and clinical trials have focused on understanding and modulating immunological responses. Unfortunately, to date none of the therapies targeting the immune system have proven to be efficacious in the long-term (465). As an alternative to immune modulation, cellular therapies promoting the recovery and survival of beta cells could be of value for treating T1D (30, 466, 467). Based on the "honeymoon phase" (a disease remission period often seen in newly diagnosed T1D patients after initiation of insulin therapy), human beta cells appear to have finite potential to recover from assaults (90, 468), and this notion is supported by multiple experimental studies (79, 98, 469). Additionally, the beta cell recovery/survival capacity could be enhanced *in vivo* by introducing antioxidant enzymes and trophic factors (470-472). For example, EGF and gastrin treatments have been shown to prevent/reverse the development of diabetes in various rodent models (337, 339, 340, 343). However, the lack of understanding in molecular events underlying such effects impedes the clinical translation of EGF as an anti-diabetic agent. In the current study, we aimed to investigate if EGFR is desensitized in the T1D milieu by an endogenous EGFR feedback inhibitor; and if such inhibitor modulates functional beta cell mass recovery in a model of beta cell destruction.

Playing pivotal roles in diverse intracellular programs, EGFR signaling cascade is fine-tuned by multiple feedback regulators, including Mig6. Following EGFR activation by ligand binding and dimerization, Mig6 is rapidly induced to: 1) suppress EGFR kinase activity, and 2) facilitate EGFR endocytosis and degradation (361, 362, 473, 474). Interestingly, Mig6 expression is also induced by physiological stress stimuli, suggesting that Mig6 possibly plays crucial roles in integrating stress responses and growth factor signaling to maintain tissue homeostasis (454, 475, 476). If stress persists, however, the sustained up-regulation of Mig6 might lead to pathological consequences. In fact, we have shown that glucocorticoids and endoplasmic reticulum stress up-regulate Mig6 in the pancreatic beta cells, leading to cell cycle arrest and apoptosis, respectively (420, 477). Because beta cell fate dictates the progression to diabetes, it is essential to understand if stress-inducible Mig6 counteracts EGFR signaling pathway, leading to beta cell dysfunction and destruction in the pathogenesis of diabetes. In the present study, we investigated whether haploinsufficiency of Mig6, hence heightened EGFR signaling, protects mice against the development of diabetes. To this end, we treated wild-type and Mig6 heterozygous knockout mice with multiple low doses of streptozotocin to elicit beta cell death and an immune response that mimics human T1D in mice and then performed metabolic assessment and morphological analyses. Additionally, we used rat islets and 832/13 INS-1 derived beta cells to examine the molecular signaling events mediated by Mig6 that are associated with beta cell dysfunction and destruction in the T1D milieu.

3.3 Results

3.3.1 Mig6 is induced by proinflammatory cytokines

Originally characterized as an immediate-early response gene (478), *Mig6* is induced by various growth factors and cellular stress stimuli to regulate tissue homeostasis(363). We sought to determine whether *Mig6* is also induced by T1D-associated pathological stimuli, henceforth mediating the progression of diabetes. Because the proinflammatory cytokines are a major contributor of beta cell dysfunction and death in T1D, we treated isolated human islets and rat 832/13 INS-1-derived beta cells with a cytokine cocktail containing IL-1 β , TNF- α , and IFN- γ . We observed that *Mig6* expression is induced in both human islets and rat beta cells (**Figure 3-1**).

Interestingly, in human islets, *Mig6* and *Ins* expression level are inversely correlated, thereby suggesting *Mig6* might control beta cell homeostasis and function.

To identify the cellular and molecular mechanisms controlled by *Mig6* in the development of diabetes, we exposed 832/13 beta cells to proinflammatory cytokines to induce a condition that mimics the islet inflammation present in developing T1D. As shown in **Figure 3-2**, we discovered that chronic cytokine treatment blunted EGFR phosphorylation/activation in beta cells. As *Mig6* is a *bona fide* EGFR inhibitor and *Mig6* expression is induced by proinflammatory cytokines, we speculated that the cytokine-induced *Mig6* is responsible for the inactivation of EGFR. Indeed, siRNA-mediated suppression of *Mig6* restores the cytokine-induced attenuation of EGFR and ERK1/2 activation.

3.3.2 Mig6 haploinsufficiency protects against STZ-induced metabolic dysfunction

To determine the pathophysiological roles of Mig6 during the development of T1D, we challenged *Mig6^{+/−}* and *Mig6^{+/+}* mice with multiple low doses of STZ (MLD-STZ). Heterozygous rather than homozygous Mig6 knockout mice were used because: 1) the homozygous knockouts have been reported to have higher embryonic lethality rate (438), and they are not viable in our facility, and 2) under normal conditions, heterozygous mice exhibit physiologic and metabolic features similar to wild-type mice(477). MLD-STZ administration is known to provoke hyperglycemia by: 1) a direct DNA-damaging effects in beta cells, and 2) an indirect inflammatory effect that facilitates islet dysfunction and death (479). Hence, we employed MLD-STZ treatment as an experimental model to mimic the beta cell destruction in human T1D. We previously demonstrated that *Mig6^{+/−}* and *Mig6^{+/+}* mice exhibit comparable islet architecture and glucose tolerance under normal chow-fed conditions (477). In the current study, we treated 10-week-old *Mig6^{+/−}* and *Mig6^{+/+}* mice with MLD-STZ, and performed metabolic and histological analyses on various days following STZ treatments (**Figure 3-3**). Whereas STZ-treated *Mig6^{+/+}* mice developed profound glucose intolerance, STZ-treated *Mig6^{+/−}* mice (hereafter referred to as STZ-*Mig6^{+/−}* mice) remained glucose tolerant compared to saline-treated control mice at the time of measurement (**Figures 3-4**). Additionally, STZ-*Mig6^{+/−}* exhibited fasting hyperglycemia compared to STZ-*Mig6^{+/−}* mice (223.4 ± 15.42 vs. 184.0 ± 10.19 , $p < 0.05$). Concurrently, STZ-*Mig6^{+/−}* mice had higher serum insulin concentrations following a glucose injection (**Figures 3-5**). As *Mig6^{+/−}* and *Mig6^{+/+}* mice treated with or without STZ have comparable insulin tolerance, the preserved glucose tolerance in STZ-*Mig6^{+/−}*

mice is likely the result of preserved beta cell function. In fact, pancreatic islets from STZ-*Mig6*^{+/−} mice have higher *Pdx-1* and *IRS2* mRNA expression levels comparing to islets from STZ-*Mig6*⁺⁺ mice. Taken together, these data suggest that Mig6 is required for the destructive events leading to glucose intolerance and the loss of islet beta cell integrity following STZ treatment.

3.3.3 Mig6 regulates beta cell integrity and function

The findings above suggest Mig6 participates in the beta cell destruction and/or dysfunction in this rodent diabetes model. To further examine a role for Mig6 in beta cell (dys)function, we treated rat islets with an adenoviral vector containing Mig6 and demonstrated elevated Mig6 expression significantly reduces glucose-stimulated insulin secretion (**Figure 3-6**). Additionally, because ERK signaling has been implicated in beta cell integrity and function, and Mig6 suppressed EGFR signaling (including phosphorylation of ERK), we determined the contribution of Mig6 in suppressing downstream effectors of ERK activation. Elevated Mig6 expression indeed reduced ERK target gene expression (e.g., *Pdx1* and *Ins1*). Collectively, our findings suggest that cytokine-induced Mig6 abrogates EGFR and ERK signaling cascades in beta cells, and the elevated Mig6 compromises beta cell function.

3.3.4 Mig6 haploinsufficiency does not affect STZ- and cytokine-induced islet inflammation

As mentioned earlier, MLD-STZ treatment provokes glucose intolerance and hyperglycemia and mimics human T1D through the activation of islet inflammation. To determine if Mig6 regulates islet inflammation, we examined the degree of immune cell

infiltration in STZ-*Mig6*^{+/−} and *Mig6*^{+/+} mice. As indicated in **Figure 3-7**, *Mig6*^{+/−} and *Mig6*^{+/+} mice showed comparable, low insulitis scores. Furthermore, islets from *Mig6*^{+/−} and *Mig6*^{+/+} mice exhibited similar cytokine-responsiveness, as both produce similar amounts of nitric oxide. Taken together, these data suggest that whereas Mig6 likely does not modulate immunity in this model, it is crucial in controlling beta cell function and integrity during a diabetogenic assault.

3.3.5 *Mig6*^{+/−} mice have improved beta cell mass recovery following STZ treatment

To determine if Mig6 regulates beta cell regeneration following MLD-STZ treatment, we examined the histomorphology of isolated *Mig6*^{+/−} and *Mig6*^{+/+} mouse pancreata. Whereas *Mig6*^{+/+} exhibited a sustained decrease in insulin-positive cross-sectional area, beta cell mass recovered by 21 days post-STZ in *Mig6*^{+/−} mice to a level comparable to saline-treated mice (**Figure 3-8**). To identify whether the observed regeneration occurred by replication, we performed pH3 staining to evaluate the beta cell mitosis rate. Interestingly, STZ-*Mig6*^{+/−} and *Mig6*^{+/+} mice had essentially identical beta cell replication rates, suggesting another factor(s) contributes to Mig6 haploinsufficiency-enhanced beta cell mass recovery.

3.3.6 Mig6 mediates DNA damage-induced beta cell apoptosis

We speculated that the enhanced recovery of beta cells observed in STZ-*Mig6*^{+/−} mice is due to elevated beta cell survival through enhanced repair/recovery mechanisms. We again used 832/13 cells to analyze these cellular events. First, we observed that diabetogenic factors such as proinflammatory cytokines (**Figure 3-9**) and reactive oxygen species (data not shown) induce beta cell DNA damage/repair, as indicated by the Ser-15

phosphorylation and accumulation of p53 protein, the hallmarks of activated DNA and cellular repair. Next, we examined if a reduction in Mig6 would heighten EGFR signaling and promote beta cell survival. Importantly, siRNA-mediated suppression of Mig6 in 832/13 cells elevated camptothecin-induced p53 protein expression, and partially protected beta cells from DNA-damage induced apoptosis, as measured by caspase 3 cleavage.

3.4 Discussion

T1D is caused by the autoimmune destruction of insulin-secreting beta cells. To date, the only cures for T1D are pancreas or islet transplantation, which require long-term immunosuppression. Despite their successes, these cures are profoundly hindered by the availability of suitable cadaveric organs and islets for transplantation. Given these limitations, novel therapeutic approaches aimed at promoting beta cell regeneration and/or survival to combat the autoimmune attack are necessary for patients with T1D. Several groups have utilized EGF and gastrin co-administration to increase beta cell mass and reverse hyperglycemia in various T1D animal models (87, 337, 339, 340, 343, 480). Unfortunately, this approach has failed in clinical translation, suggesting that the downstream signaling of ligand-mediated receptor activation is complicated and warrants further investigation.

We and others (335) have become interested in the anti-diabetogenic effects of EGFR signaling, and specifically for our work, the impact of the EGFR feedback inhibitor Mig6. In current study, by employing Mig6 haploinsufficient mice, we exposed this anti-diabetic capacity of EGFR signaling. Mig6 limits EGFR signaling by blocking receptor dimerization and promoting its degradation, leading to anti-mitogenic

effects (361, 362, 390). Interestingly, recent studies have demonstrated that *Mig6* transcription is induced by stress stimuli (363), and *Mig6* regulates cellular apoptosis to maintain tissue homeostasis during development and in pathological conditions (390, 454, 481). These aspects of *Mig6*'s biology compelled us to determine if *Mig6* limits the therapeutic potential of EGF, facilitates beta cell death, and promotes the progression to diabetes.

By employing *Mig6* haploinsufficient mice, we were able to examine the roles of *Mig6* in the progression of T1D. We treated *Mig6*^{+/−} and *Mig6*^{+/+} mice with multiple low doses of STZ to induce diabetes. We observed that *Mig6*^{+/−} mice are resistant to developing STZ-induced diabetes, as they have preserved post-prandial glycemia as well as glucose tolerance and serum insulin levels following a glucose load. The insulin sensitivities (before and after STZ treatment) are comparable between *Mig6*^{+/−} and *Mig6*^{+/+} mice, suggesting that the improved glucose homeostasis in STZ-*Mig6*^{+/−} mice is likely the result of preserved beta cell function. Nevertheless, we recognize that the systemic *Mig6* haploinsufficiency might alter immunity, angiogenesis, or nutrient utilization in other tissues. However, we established that *Mig6* modulates beta cell function and integrity *in vivo* and *in vitro*. Whereas half of the beta cells in *Mig6*^{+/−} and *Mig6*^{+/+} were ablated by the STZ treatment, the remaining islets from *Mig6*^{+/−} mice have higher *Pdx-1* and *IRS2* expression levels. In contrast, elevated *Mig6* expression compromises glucose-stimulated insulin secretion in isolated rat islets. As *Mig6* has been shown to inhibit ERK1/2 activation in beta cells (420), we examined other beta cell genes that are transcriptionally regulated by ERK1/2 in the context of adenovirally-mediated *Mig6* overexpression. We found that *Pdx-1* and *Ins1/2* mRNA levels are reduced in

832/13 INS-1 cells with elevated Mig6 expression. Of exceptional importance, Mig6 possibly compromises beta cell function in T1D humans, as we demonstrated that proinflammatory cytokine-induced *Mig6* mRNA expression is inversely correlated with *preinsulin* mRNA levels in isolated human islets.

We utilized 832/13 cells to study the consequences of Mig6 induction in relation to EGFR signaling in a T1D milieu. We established, for the first time, that proinflammatory cytokines impair EGF-induced EGFR activation. Excitingly, siRNA-mediated suppression of Mig6 partially restored the cytokine-mediated attenuation of EGFR activation. Because proinflammatory cytokines promote nitric oxide (NO) production and cause beta cell damage (100), it will be interesting to ultimately determine if NO is the mediator of EGFR unresponsiveness as implicated in other cell types (482-484). In addition, it remains to be determined if cytokine-induced NO participates in the induction of negative regulators of EGFR such as Mig6. Regardless, our discovery brings a new level of complexity to traditional growth factor-based anti-diabetic therapies. We suggest that in pathological conditions, intracellular and extracellular stress can directly inactivate mitogenic signaling pathways via stress-inducible negative regulators. Particularly, we propose that before employing growth factors as anti-diabetic therapies or in regenerative medicine, cellular stress should be eliminated. Excitingly, a recent study from Wang et al. presented the same concept (343). They reported that a combinatorial therapy of anti-CD3/CD8 immunotherapy (to eradicate inflammatory assaults) and gastrin/EGF (to augment beta cell regeneration) reverses *late-stage* diabetes in NOD mice.

Previous reports have suggested that EGF/gastrin co-treatment increases beta cell mass via promoting beta cell proliferation (339, 343), transdifferentiation(87, 340, 480), and neogenesis (343). In the current study, we used *Mig6*^{+/−} mice as an alternative model to examine the beta cell regeneration mechanisms regulated by EGF. We showed that STZ-*Mig6*^{+/−} mice had improved beta cell mass recovery at 20 days following STZ treatment. However, beta cell replication is not likely the mechanism for increasing beta cell mass, as *Mig6*^{+/−} and *Mig6*^{+/+} mice exhibited similar STZ-stimulated beta cell proliferation rates. Given this, we infer that the recovery of insulin-positive area is attributed to other sources such as transdifferentiation or restoring of insulin positivity (e.g., beta cell recovery) (79). However, we do not consider the duct-to-beta cell transdifferentiation the main contributor of increased beta cell mass in STZ-*Mig6*^{+/−} mice, because CK19/Ins positive cells are extremely rare at the time points where our histological studies were performed (data not shown).

As an alternative to the above mechanisms, we were compelled to examine if *Mig6* haploinsufficiency facilitates beta cell survival because: 1) newly generated beta cells are more susceptible to cell death (485), 2) we and others have demonstrated that *Mig6* promotes cell apoptosis (481, 486), and 3) EGFR has been associated with promoting DNA damage repair and facilitating cell survival in various cell types (487, 488). We revealed that proinflammatory cytokines induce DNA damage in 832/13 cells, as indicated by elevated levels of phosphorylated- and total p53 protein. In addition, we identified that siRNA-mediated suppression of *Mig6* partially protected beta cells from DNA damage-induced apoptosis, associated with elevated p53 expression levels. However, we cannot rule out the possibility that *Mig6* mediates beta cell death through

EGFR-independent mechanisms as suggested by Hopkins et al. (390). Further, EGFR facilitates cell survival through various molecular actions, such as promoting PI3K/Akt- and Ras/ERK-mediated transcription of DNA repair genes(489, 490), and activating nuclear proteins involved in damage repair (491, 492). Thus, future investigations should elucidate through which signaling pathways Mig6 regulates EGFR activity and cell survival.

Taken together, we demonstrated that whereas Mig6 deficiency does not enhance STZ-stimulated beta cell proliferation, it likely allows the beta cell to mount a more robust damage-repair response, leading to superior recovery of beta cells and insulin positivity. Our discovery highlights the largely overlooked beta cell repair mechanisms as potential therapeutic targets for T1D (466). Indeed, accumulated cadaveric studies reported that eleven long-term T1D patients have minimal levels of c-peptide and preserved beta cells (albeit degranulated and scattered as small clusters) (29, 102, 493), and human islets are capable of recovering from attacks (468, 469, 494, 495). These findings point out that patients with T1D certainly have beta cells that could likely be repaired if provided the proper stimulation. We suggest that targeted inhibition of Mig6 could be of value in treating T1D, as we have shown that Mig6 compromises beta cell functional integrity and facilitates beta cell death.

In summary, our data demonstrated that cytokine-induced Mig6 negatively controls EGFR signaling activation, compromises beta cell function and identity, and inhibits recovery of beta cells in a chemically-induced form of T1D. This study indicates that using growth factors alone as anti-diabetogenic therapies might be ineffective, as pathological stimuli can directly turn-off mitogenic signaling pathways via negative

regulators such as Mig6. Further, we propose that targeting the beta cell recovery machinery may represent a promising therapeutic strategy to prevent or reverse the progression of T1D.

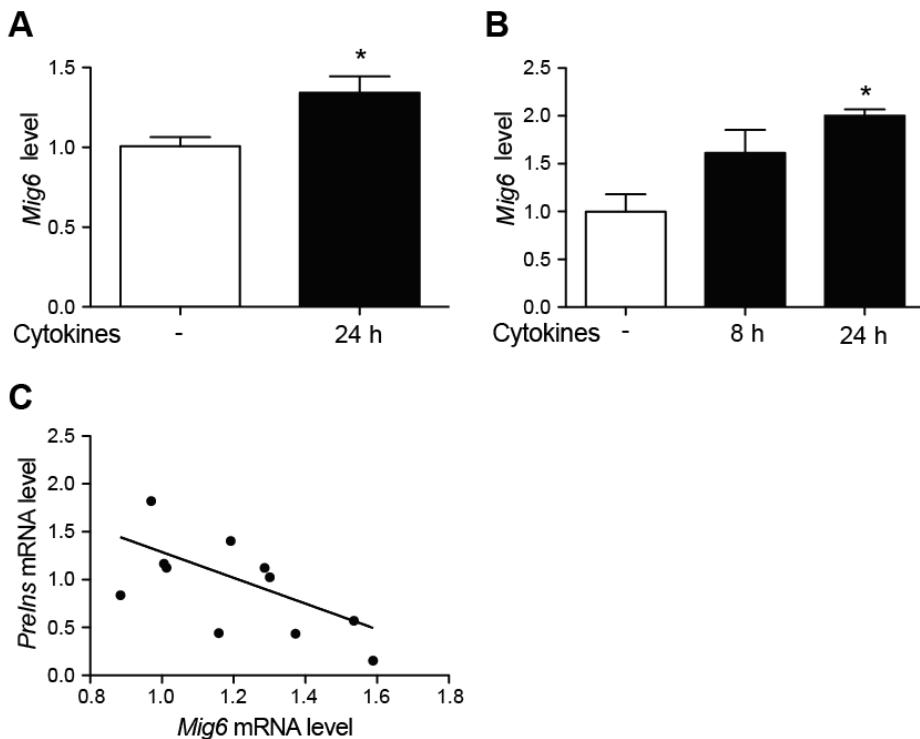


Figure 3-1

Proinflammatory cytokines induce *Mig6* expression.

Human islets (**A**) or 832/13 cells (**B**) were treated with cytokines, and *Mig6* expression was determined by qRT-PCR analysis. *Mig6* mRNA levels are reported as fold induction relative to non-cytokine-treated samples. Data are means \pm SEM; n = 4. *, p < 0.05. (**C**) *Preinsulin* mRNA levels in human islets (\pm cytokines) were plotted against *Mig6* mRNA levels ($r = -0.64$, p < 0.05).

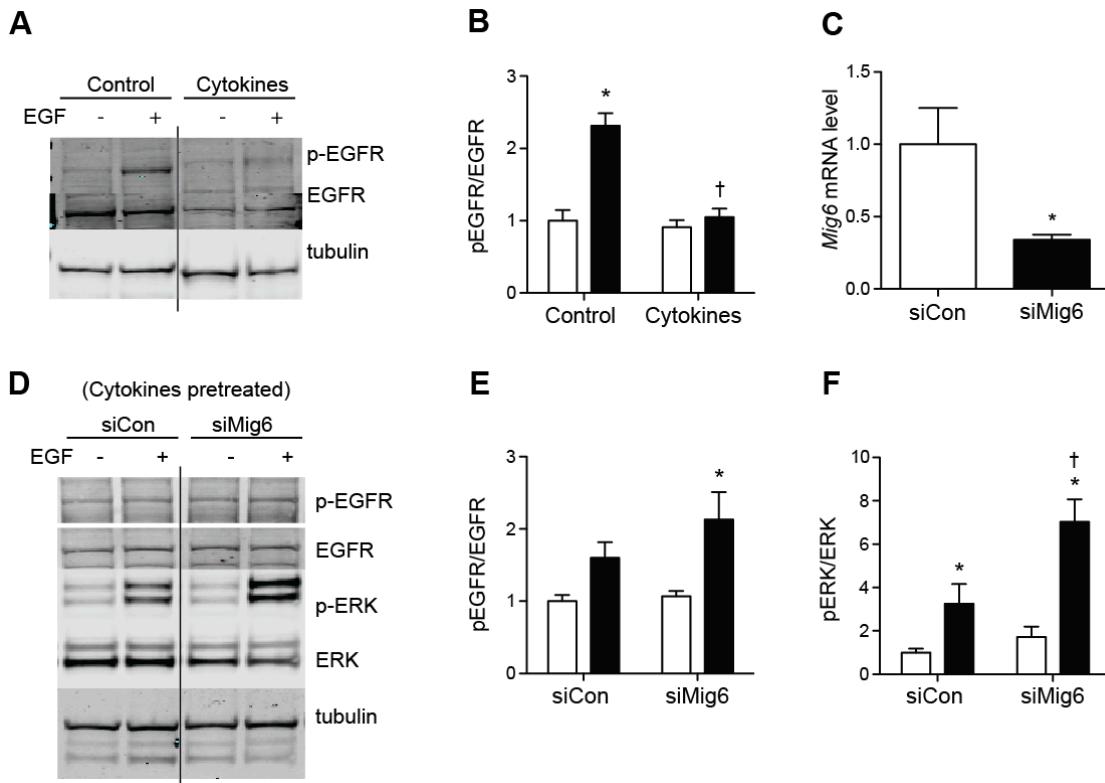


Figure 3-2

Mig6 suppression rescues cytokine-inhibited EGFR activation.

(A) 832/13 cells were pretreated with cytokines for 16 h, starved for 2 h, and stimulated with 10 ng/ml recombinant rat EGF for 5 min. Cell lysates were collected for western blot analysis to determine p-EGFR, EGFR, and tubulin levels. (B) Quantified p-EGFR protein levels are shown. Data are reported as fold induction relative to non-cytokine, non-EGF-treated group. n = 3. *, p < 0.05 non-treated vs. EGF-treated; †, p < 0.05 non-cytokine EGF-stimulated vs. cytokine EGF-stimulated. The solid line indicates cropped lanes within the same experiment. (C) 832/13 cells are transduced with adenoviral vectors carrying either a scrambled control RNA (siCon) or shRNA sequence against Mig6 (siMig6). *Mig6* knockdown efficiency was determined by qRT-PCR. n = 3. *, p < 0.05. (D) Following transduction, cells are treated with cytokines and EGF as described above. Protein levels of p-EGFR, EGFR, p-ERK, ERK, and tubulin were determined by western blot analysis. (E-F) Quantified p-EGFR and p-ERK protein levels are shown. Data are reported as fold induction related to cytokine-treated, non-EGF-stimulated group. n = 3. *, p < 0.05 non-treated vs. EGF-treated; †, p < 0.05 siCon EGF-stimulated vs. siMig6 EGF-stimulated.

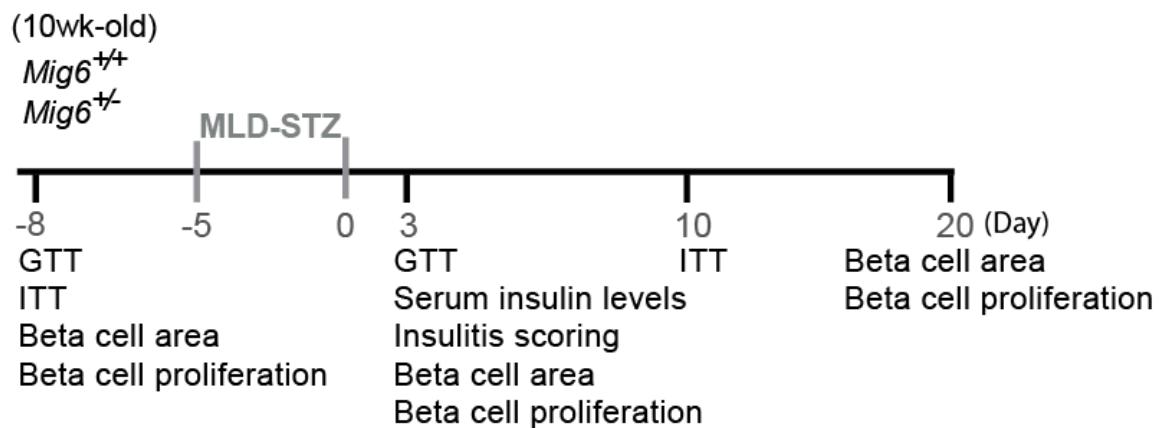


Figure 3-3

Schematic representation of the experimental timeline.

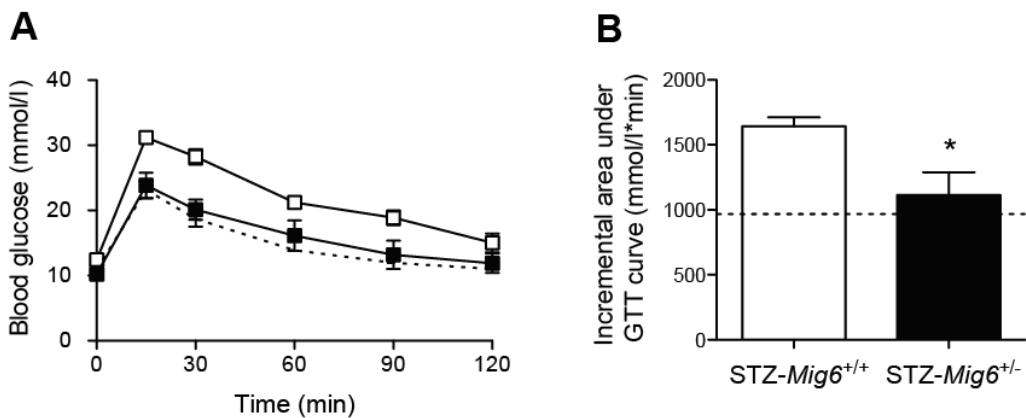


Figure 3-4

***Mig6^{+/}* mice are protected from STZ-induced glucose intolerance.**

(A) Glucose tolerance testing was performed at day 3 post-STZ injection (B) Time course data as well as area-under-the-curve (AUC) are reported. n = 5. *, p < 0.05.

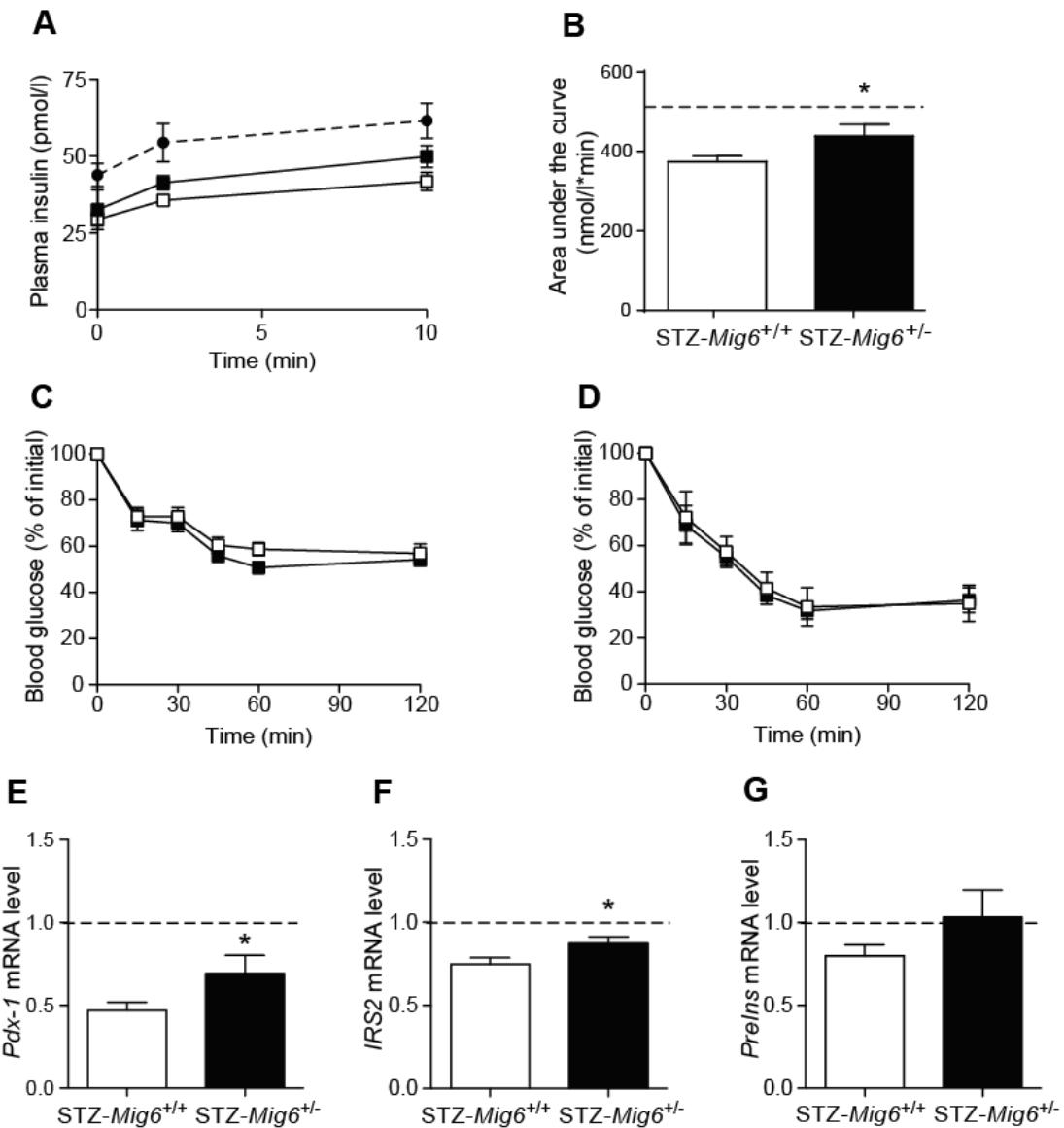


Figure 3-5

STZ-Mig6^{+/+} mice have preserved beta cell function.

(A-B) Circulating insulin levels during a glucose challenge, also presented as (B) area-under-the-curve. n = 8. *, p < 0.05. (C-D) Blood glucose levels during insulin tolerance test pre- and post-STZ treatment. (E-G) mRNA levels of *Pdx-1*, *IRS2*, and *Preinsulin* in islets isolated from saline-injected *Mig6^{+/+}*, STZ-treated *Mig6^{+/+}*, and STZ-treated *Mig6^{+/-}* mice. Target gene expression levels were determined by qRT-PCR and standardized to the saline-injected (dotted line) group. n > 5. *, p < 0.05.

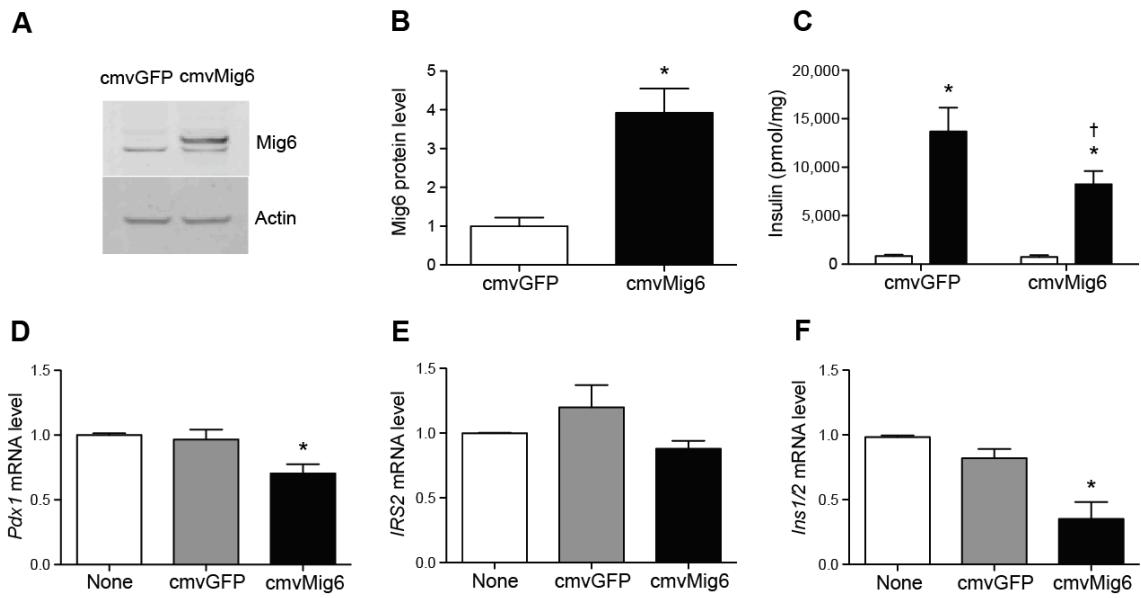


Figure 3-6

Overexpression of Mig6 compromises beta cell integrity and islet function.

(A-C) 832/13 cells were either not transduced or transduced with adenoviral vectors carrying cmvGFP or cmvMig6. *Pdx-1*, *IRS2*, and *Ins1/2* mRNA levels were determined by qRT-PCR and are reported as fold differences relative to non-transduced samples. n = 5. *, p < 0.05. (D) Isolated rat islets were transduced with adenoviral vectors carrying cmvGFP or cmvMig6, and then glucose-stimulated insulin secretion (GSIS) was assessed. n = 3. *, p < 0.05 low vs. high glucose; †, p < 0.05 high glucose stimulated-cmvGFP vs. cmvMig6).

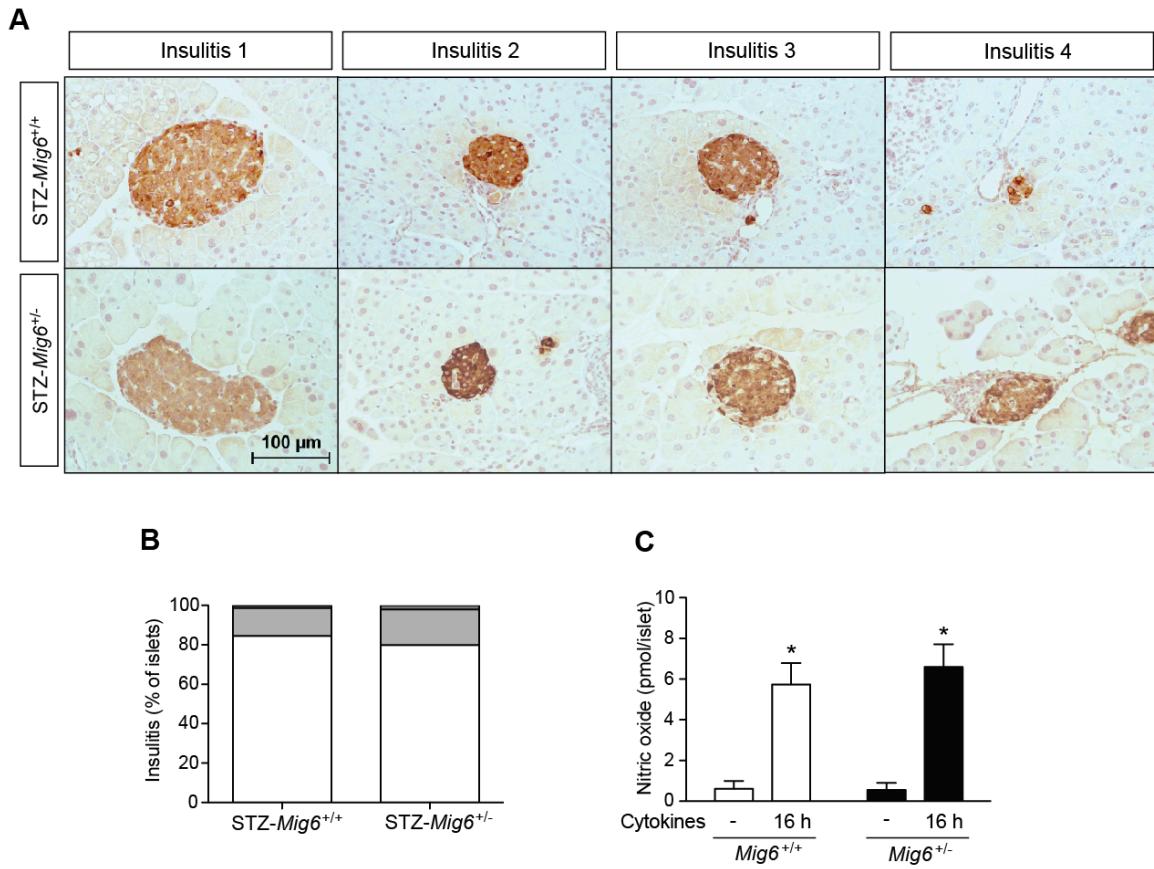


Figure 3-7

Mig6 dose not modulate cytokine- or STZ-induced immune response.

(A-B) Insulitis scoring (defined in Methods) of STZ-*Mig6^{+/-}* and -*Mig6^{+/+}* mouse pancreas. Shown are representative images of islets and quantified results (white, 0; light gray, 1; dark gray, 2; black, 3). (C) Islets from *Mig6^{+/-}* (white bars) and *Mig6^{+/+}* (black bars) were treated with cytokines and nitric oxide levels in the islet culture were measured. n = 3. *, p < 0.05 vs. non-treated.

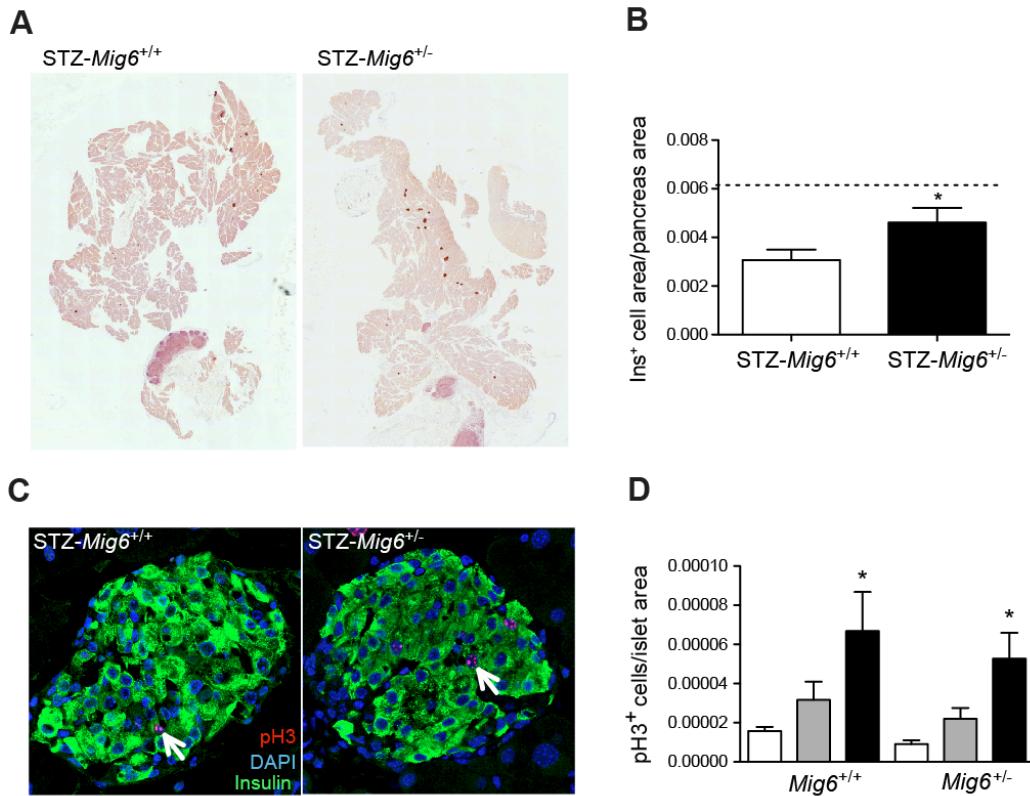


Figure 3-8

STZ-*Mig6*^{+/-} mice have enhanced beta cell mass recovery.

(A-B) Beta cell area was measured and expressed relative to pancreas area. The dotted line indicates beta cell area of non-STZ treated mice. n = 8. *, p < 0.05 vs. STZ-*Mig6*^{+/+}.

(C-D) The percentage of beta cells positive for pH3 were quantified in saline controls (white bars) and at 3 and 20 days post-STZ (gray and black bars, respectively). Arrows indicate proliferating beta cells. n > 5. *, p < 0.05 vs. non-STZ. Representative images are shown in A and C.

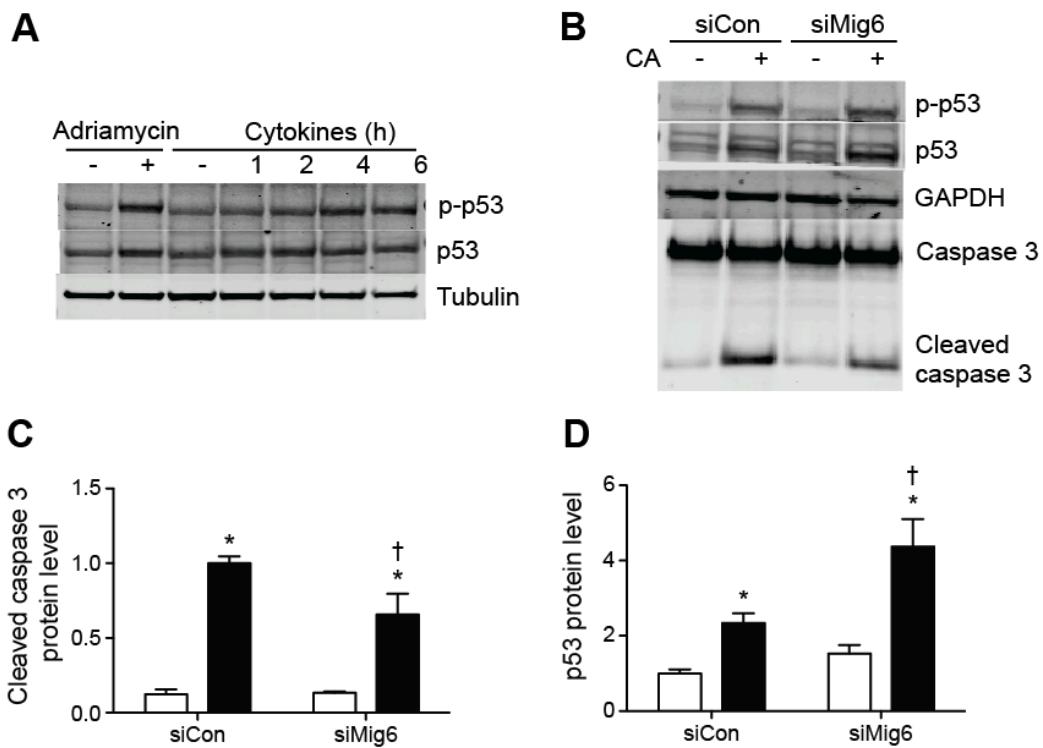


Figure 3-9

Mig6 promotes DNA damage-induced beta cell death.

(A) 832/13 cells were treated with adriamycin for 6 h or cytokines for the indicated times. p-p53, p53, and tubulin protein levels were determined. (B-D) 832/13 cells were transduced with siCon or siMig6 adenoviral vectors, and treated with vehicle (white bars) or 1 μ M camptothecin (CA; black bars). p-p53, p53, caspase-3, and Gapdh protein levels were determined. n = 3. *, p < 0.05 vs. non-treated; †, p < 0.05 vs. siCon + CA.

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Griffin KE, Maier BF, Fueger PT. (2014) Mig6 haploinsufficiency protects mice against streptozotocin-induced diabetes. Diabetologia 57(10):2066-75.

4 GLUCOLIPOTOXICITY-INDUCED MIG6 DESENSITIZES EGFR SIGNALING AND PROMOTES PANCREATIC BETA CELL DEATH

4.1 Synopsis

RTK signaling cascades have been considered the primary promoters of beta cell mass preservation and expansion. Interestingly, the distal effectors of RTKs are typically inhibited under diabetogenic stress conditions. Therefore, we hypothesized that RTKs are inhibited in the diabetic milieu, which then leads to decreased downstream signaling of RTKs and beta cell loss. To this end, we investigated a well-characterized RTK, EGFR, in beta cells during GLT. We discovered that the activation of EGFR is inhibited by stress stimuli present in T2D (e.g., GLT and ER stress). Moreover, we demonstrated that the stress-inducible EGFR negative regulator Mig6 is a major contributor to EGFR inhibition during GLT. Mig6 primarily controls EGFR, but not other RTKs signaling cascades, in the pancreatic beta cells. In addition, Mig6 mediates beta cell survival and death. Because Mig6 overexpression facilitates JNK activation, it also exacerbates GLT-induced beta cell apoptosis. Conversely, silencing Mig6 enhances expression of the pro-survival factor Birc5 in GLT-treated beta cells. Importantly, we discovered that *Mig6* is elevated in human islets from T2D donors. In summary, we established that T2D and GLT induce Mig6 in pancreatic beta cells. The elevated Mig6 desensitizes EGFR signaling and exacerbates beta cell death. Our findings suggest that Mig6 could be a novel therapeutic target for T2D, as blocking Mig6 could possibly enhance mitogenic signaling cascades in a diabetic milieu to promote beta cell survival and prevent beta cell death.

4.2 Introduction

To ensure proper, robust insulin release, beta cell insulin secretion capacity (e.g., beta cell function) and insulin stores (dependent on beta cell mass) are tightly regulated. For example, during pregnancy and obesity, functional beta cell mass is increased to allow for optimal glycemic control. Studies have suggested that the growth factor receptor signaling pathways, such as EGFR and insulin like growth factor 1 receptor signaling cascades are primary contributors of beta cell mass expansion. On the contrary, persistent hyperglycemia and hyperlipidemia (glucotoxicity and lipotoxicity, hereafter jointly referred as GLT) accentuate beta cell failure. Interestingly, the activation of proximal signaling molecules of growth factor receptors (e.g., Akt and Glycogen synthase kinase 3/GSK3) is inhibited under stress conditions (455, 496, 497). Such observations prompted us to speculate whether the activation of the growth factor receptors themselves are inhibited in the pathological states. In other words, we hypothesized that the diabetogenic stress stimuli repress the activation of beta cell surface receptors, thereby leading to the reduced activation of proximal signaling molecules of the receptors, and contributing to beta cell loss.

In this work, we primarily focused on how damped EGFR signaling cascades contribute to beta cell loss in a diabetic milieu because EGFR signaling is central to preserving beta cells *in vivo* and *in vitro*. Mice carrying pancreatic specific dominant-active EGFR alleles are protected against the development of beta cell toxin-induced diabetes, in that their beta cell apoptosis is inhibited (498). Conversely, blocking EGFR downstream Raf-1 signaling via a pharmacological inhibitor induces beta cell death (499). It is crucial to note that EGFR has to remain excitable to help maintain beta cell

homeostasis, because mice bearing an inactive form of EGFR in the pancreas failed to acquire compensatory beta cell expansion during pregnancy and obesity (336, 500). Interestingly, we have established (see **Chapter 3**) that pro-inflammatory cytokines inhibit the activation of EGFR via a stress-inducible EGFR inhibitor, Mig6 (501). We speculated that metabolic stress stimuli could alter EGFR activity via Mig6. Additionally, because Mig6 has been implicated as a regulator of beta cell proliferation and death (420, 481), we examined the beta cell pro-survival/pro-apoptosis signaling pathways modulated by Mig6 in a T2D milieu.

4.3 Results

4.3.1 GLT and ER stress attenuates EGFR activation in pancreatic beta cells

To study if GLT compromises EGFR activation, we pre-exposed 832/13 INS-1 cells to medium containing high glucose and palmitic acid and investigated the activation of EGFR. We found that GLT treatment prevents EGF-mediated EGFR phosphorylation (**Figure 4-1A and 4-1B**). Notably, the total cellular abundance of EGFR is not changed by GLT (**Figure 4-1C**), indicating that the attenuated EGFR phosphorylation and activation are likely the consequences of EGFR kinase interruption. Because GLT imposes ER stress on beta cells and triggers deleterious effects such as beta cell death (**Figure 4-1D and 4-1E**), we examined whether the induction of ER stress is sufficient to attenuate EGFR activation. We found that pretreatment with thapsigargin (a pharmacological ER stress inducer) significantly inhibits EGFR activation in 832/13 cells (**Figure 4-1F and 4-1G**). The above findings suggest that the pathological stress stimuli present in T2D compromise the activation of EGFR.

4.3.2 EGFR feedback inhibitor Mig6 is elevated in GLT-treated rat beta cells and T2D human islets

To identify the factors associated with EGFR inactivation during GLT, we examined a set of well-defined, inducible EGFR inhibitors (502). We discovered that Mig6, an adaptor protein that blocks EGFR activation, is induced by T2D and GLT in human and rodents islets, respectively (**Figure 4-2**). To further study the specific effects of glucotoxicity and lipotoxicity on the induction of Mig6, we treated the 832/13 cells with high levels of glucose and/or palmitic acids and measured *Mig6* expression levels (**Figure 4-3A to 4-3E**). We established that high glucose induced *Mig6* in both a dose- and time-dependent manner (**Figure 4-3B and 4-3C**). However, palmitic acid alone did not induce *Mig6* (**Figure 4-3D and 4-3E**). In addition, because high glucose stimulates insulin secretion in the beta cells, we speculated that the autocrine or paracrine effects of insulin could promote *Mig6* expression. However, exogenous insulin treatment did not alter *Mig6* expression levels (**Figure 4-3F**). These data suggested that diabetogenic stress, especially glucotoxicity, induces Mig6 in beta cells.

4.3.3 GLT-attenuated EGFR activation is rescued by siMig6

Because GLT hinders EGFR activation and the inhibitor of EGFR, Mig6, is induced by GLT, it is intuitive to speculate that stress-inducible Mig6 controls EGFR inactivation during GLT. Thus, we used a RNA interference approach to examine the functional significance of Mig6 during GLT (**Figure 4-4**). After silencing Mig6, GLT-attenuated EGFR and ERK signaling cascades are restored (50% restoration vs. BSA/EGF-treated, **Figure 1A**), implying Mig6 facilitates GLT-induced EGFR inactivation.

4.3.4 Elevated Mig6 facilitates GLT-mediated beta cell death

Previously, we demonstrated that Mig6 not only exacerbates ER stress-mediated beta cell death, but also inhibits insulin secretion in isolated rodent islets (481, 501). Hence, we sought to determine if Mig6 controls beta cell fate during GLT. In particular, because it has been reported that Mig6 facilitates stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation (475), and JNK is a critical mediator of GLT-induced beta cell death (**Figure 4-5A and 4-5B**), we investigated if Mig6 promotes JNK activation and induces GLT-mediated beta cell death. We found that in the overabundance of Mig6, JNK and caspase 3 are markedly activated at 4 h post-GLT treatment; whereas control GFP-overexpressed samples showed the activated JNK and caspase 3 at 8 h post-GLT treatment (**Figure 4-5C and 4-5E**). Therefore, we concluded that Mig6 overexpression aggravated GLT-induced JNK activation and expedited beta cell death. Conversely, knockdown of Mig6 partially prevented JNK phosphorylation and activation during GLT (**Figure 4-5F**). However, limiting the expression of Mig6 alone is not sufficient to inhibit GLT-mediated beta cell apoptosis (**Figure 4-5G**).

4.3.5 Mig6 controls EGF- but not IGF1- nor HGF-mediated pro-survival signaling pathways

Mig6 not only modulates pro-apoptotic signaling cascades, but also controls multiple growth factor receptors, thereby mediating multiple pro-survival signaling pathways. Based on the information from sequence alignments and structural studies, Mig6 likely regulates several RTKs. First, we established that Mig6 is a *bona fide* EGFR inhibitor in pancreatic beta cells (**Figure 4-6A to 4-6C**). Next, we examined if Mig6 modulated HGF signaling because HGF is essential in compensatory beta cell mass

expansion (503-505), since Mig6 has been implicated in regulating HGF-mediated neurite outgrowth (359). However, our data suggested that Mig6 does not influence HGF mitogenic signaling in beta cells (**Figure 4-6D** and **4-6E**). In addition, we demonstrated that Mig6 does not alter IGF-1 signaling in beta cells (**Figure 4-6F** and **4-6G**). These findings indicate that Mig6 modulates RTKs in a cell context-specific fashion; and Mig6 primarily regulates EGFR signaling in pancreatic beta cells.

Finally, we examined if Mig6 modulates pro-survival factors downstream of EGFR signaling pathways during GLT. We identified that a family member of the inhibitor of apoptosis (IAP), *IAP5*, is a target of Mig6 regulation. We report that silencing Mig6 significantly elevated *Birc5* levels in GLT-treated beta cells (**Figure 4-6H**).

4.4 Discussion

Although genetic manipulation of pancreatic EGFR in mice leads to the acceleration or prevention of diabetes, the natural history of EGFR kinase activity in different phases during the progression of diabetes remain unknown (305, 336, 498, 500). In regards to the beta cell destruction phase prior to the onset of diabetes, the extent to which diabetogenic stress stimuli alter EGFR activity and impact beta cell life/death decisions is unclear. It has been reported that diabetic stressors could compromise the activation and propagation of RTK signaling cascades in pancreatic beta cells (356, 357). For example, GLT and cytokine challenges hinder the activation of insulin receptor and downstream PI3K, hence prevent the cytoprotective effects of insulin in beta cells (496, 497, 506). However, the molecular mechanisms responsible for this stress-mediated RTK inactivation remain to be defined. It is likely that there are stress-responsive factors that crosstalk with RTK signaling machinery in pathological conditions.

In this study, we established that glucolipotoxicity and ER stress attenuate EGFR activation in pancreatic beta cells via the stress-responsive EGFR inhibitor, Mig6. Mig6 was initially characterized as an endogenous EGFR feedback inhibitor but has also been suggested to impair other RTKs. After mitogen stimulation, Mig6 is activated to abolish EGFR signaling transmission via a two-tiered mechanism: 1) Mig6 binds to the EGFR intracellular kinase domain and inhibits kinase dimerization and activation, and 2) Mig6 facilitates EGFR endo-lysosomal sorting and degradation. However, the role of Mig6 as a stress-induced modulator has only been revealed recently. Makkinje et al. first reported that mechanical stress in diabetic nephropathy is sufficient to induce Mig6; and the transient expression of Mig6 results in selective activation of JNK (475). Later, Mabuchi et al. further suggested that Mig6 is able to bind to I κ B- α , resulting in NF- κ B activation (406). Recently, Hopkins et al. demonstrated that ligand deprivation promotes Mig6-mediated c-Abl activation and cell death (390). Furthermore, as described in the previous chapters, we identified that ER stress and pro-inflammatory cytokines both induce Mig6; and haploinsufficiency of Mig6 prevents mice from developing an experimentally-induced form of T1D (481, 501).

Here, we established that Mig6 overexpression facilitates GLT-induced JNK activation and beta cell apoptosis. Conversely, silencing Mig6 promotes the expression of an EGFR downstream pro-survival molecule Birc5 during GLT. deleterious Mig6-mediated effects could be EGFR independent or dependent. Mig6 activates pro-apoptotic JNK via its Cdc42/Rac interactive binding domain, representing an EGFR independent response (407). On the other hand, Mig6-promoted Birc5 is transcriptionally and post-translationally regulated by EGFR signaling cascades. However, 60% knockdown of

Mig6 in 832/13 cells did not prevent GLT-induced beta cell apoptosis. One possible reason could be that Mig6 controls a particular type of cell death. As we have found that the suppression of Mig6 prevents DNA damage-, but not ER stress- and GLT-induced beta cell death. Alternatively, the existence of functionally redundant proteins may compensate for the effects of Mig6 deficiency. There are likely other stress-mediated factors controlling EGFR inactivation. For instance, cell surface EGFR could be modified and inhibited by advanced-glycation precursors present in GLT; and cellular stress-activated phosphatases could also inactivate EGFR (345, 346, 349). Lastly, we performed our cell death studies using transformed cells, which may have different cell death kinetics compared to post-mitotic islet beta cells. The physiological levels of GLT *in vivo* could create more prolonged assaults in long-lived primary beta cells. Therefore, silencing Mig6 (thus enhancing EGFR signaling) *in vivo* might better promote beta cell damage-repair, given that the primary beta cells are given a longer time to remediate the stress. Prospective studies of our laboratory include the generation of beta cell-specific Mig6 knockout mouse model and functional characterization of Mig6 in the experimental forms of T2D.

This work presented a possible rationalization for defective beta cell proliferation and survival observed in the state of chronic over-nutrition. It is known that short-term intralipid infusion enhances beta cell proliferation via EGFR and mTOR signaling pathways in adult rodents (314), but chronic nutrient overload (e.g., high-fat diet feeding) does not promote beta cell mass expansion (315). As Mig6 is elevated in islets derived from T2D patients, we speculated that Mig6 perhaps contributes to the dampening of EGFR signaling activation during the progression of T2D.

In summary, we discovered that GLT attenuates EGFR activation via Mig6; and Mig6 modulates GLT-induced beta cell apoptosis machinery. We propose that Mig6 might be a suitable therapeutic target to foster beta cell proliferation and survival in preventing and treating T2D.

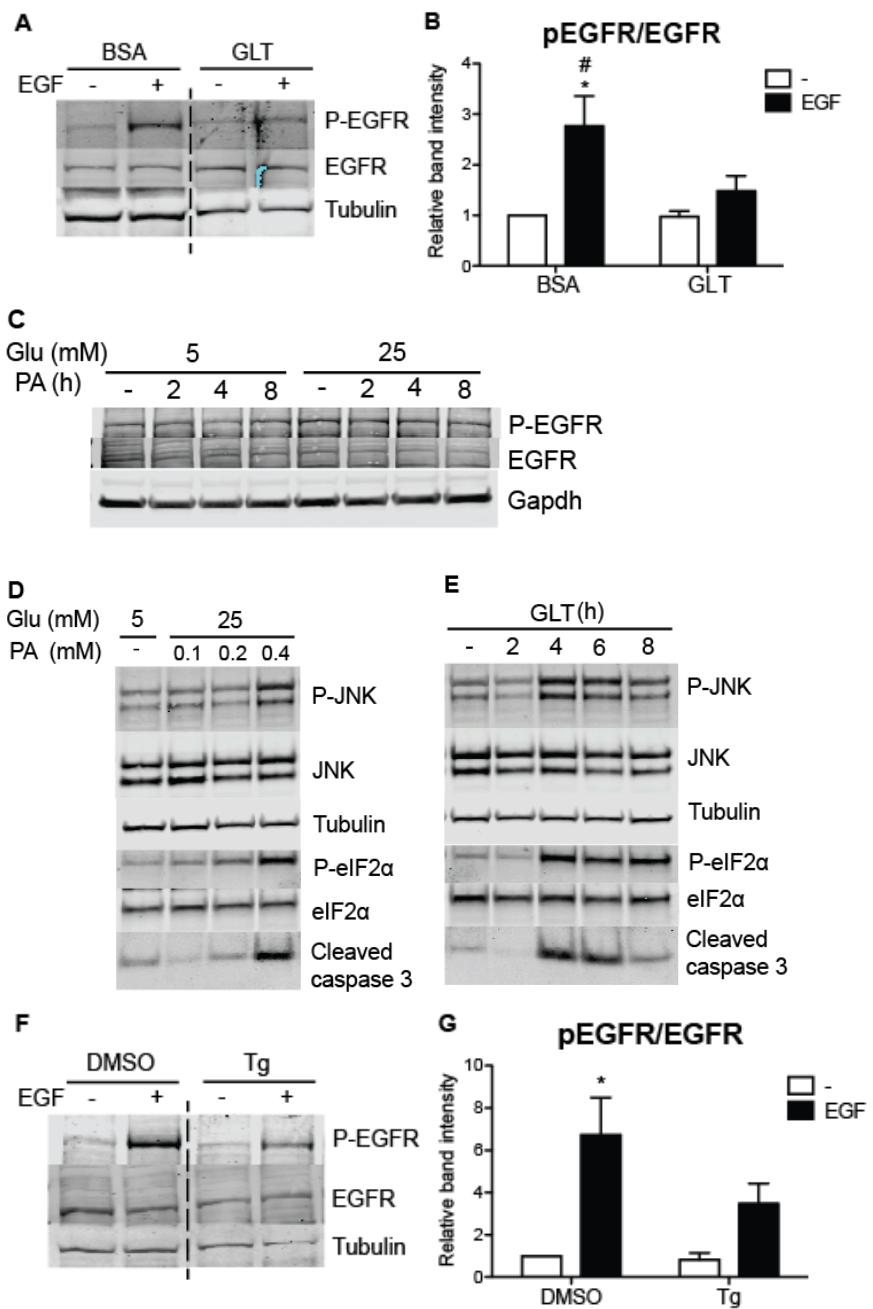


Figure 4-1

GLT and ER stress desensitize the EGFR signaling pathway in pancreatic beta cells. (A, B) 832/13 cells were treated with 5 mM glucose and BSA, or 25 mM glucose and 400 μ M palmitic acid (glucolipotoxicity, GLT) for 8 h, followed by a starvation in 5 mM glucose medium for 2 h, then stimulated with recombinant rat EGF (rrEGF) 10 ng/ml for 5 min. Protein levels of p-EGFR, EGFR, and tubulin were analyzed by western blotting. Data are reported as fold induction related to BSA non-stimulated samples. (C-E) 832/13 cells were treated with GLT at the times and concentrations as indicated. Protein levels of p-EGFR, EGFR, p-JNK, JNK, p-eIF2 α , eIF2 α , gapdh, tubulin, and cleaved caspase 3 were analyzed by immunoblotting. (F) 832/13 cells were treated with DMSO or 1 μ M

thapsigargin (Tg, a pharmacological ER stress inducer) for 4 h, followed by starvation and rrEGF stimulation. Protein levels of p-EGFR, EGFR, and tubulin were analyzed by western blotting. All blots are the representative of $n \geq 3$ experiments. *, $p < 0.05$ vs. BSA non-stimulated; #, $p < 0.05$ vs. BSA EGF-stimulated.

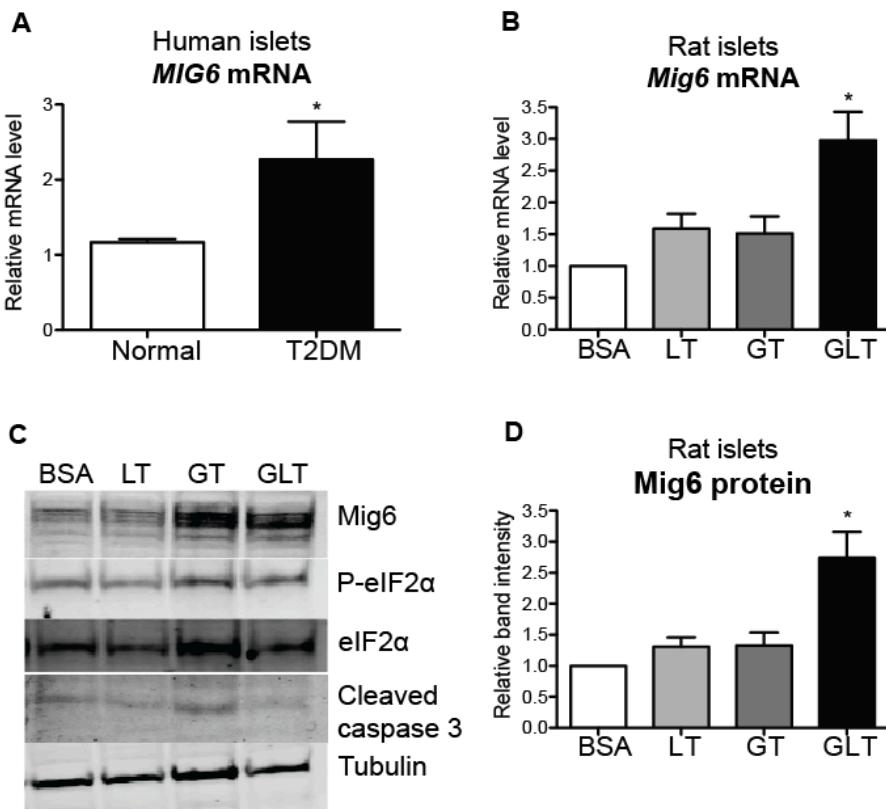


Figure 4-2

Mig6 is induced in T2D human and GLT-treated rat islets.

(A) Isolated human islets from healthy individuals or patients with T2D were collected. *Mig6* mRNA levels were determined by qRT-PCR ($n = 4$; *, $p < 0.05$). (B, C) Isolated rat islets were treated with BSA, 400 μ M palmitic acid (lipotoxicity, LT), 25 mM glucose (glucotoxicity, GT), or both (GLT) for 48 h. *Mig6* mRNA levels were determined by qRT-PCR. Mig6, p-eIF2 α , eIF2 α , cleaved caspase 3, and tubulin protein levels were determined by immunoblotting. $n = 4$. *, $p < 0.05$ vs. BSA.

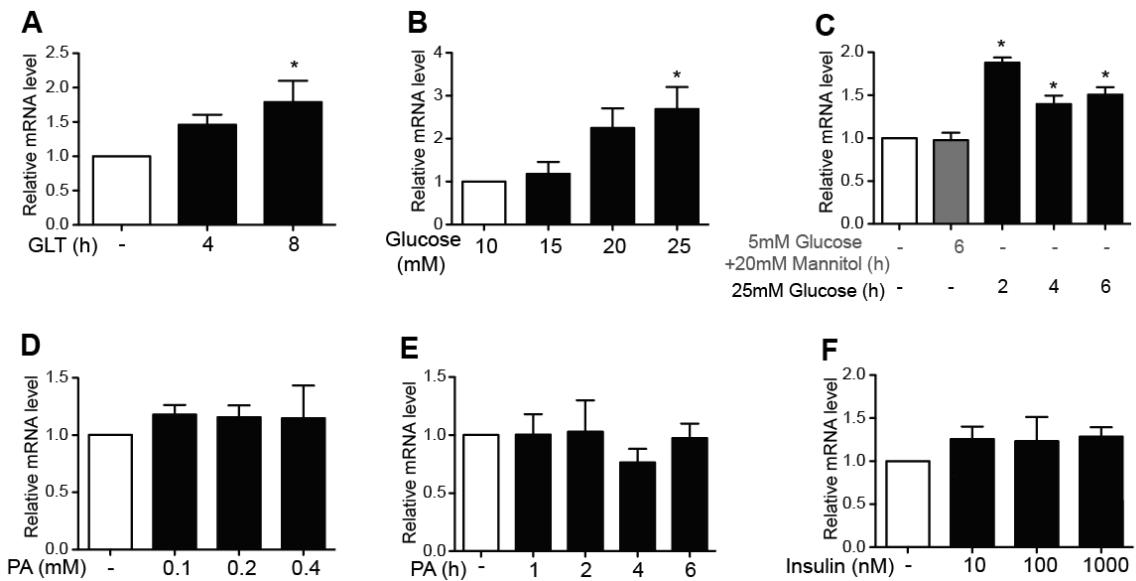


Figure 4-3

***Mig6* is induced by glucose but not insulin nor palmitic acid.**

832/13 cells were treated with (A) GLT for the indicated times, (B) 5, 10, 15, 20, or 25 mM glucose for 4 h, (C) 25 mM glucose or 5 mM glucose + 20 mM mannitol (as an osmotic stress control) for 0, 2, 4, or 6 h, (D) BSA, 100, 200, 400 μ M palmitic acid for 4 h, (E) 400 μ M palmitic acid for the indicated times, or (F) 0, 10, 100, or 1000 nM recombinant human insulin for 4 h. *Mig6* mRNA levels were determined by qRT-PCR. $n \geq 3$ experiments. *, $p < 0.05$ vs. BSA + 5 mM glucose + 20 mM mannitol.

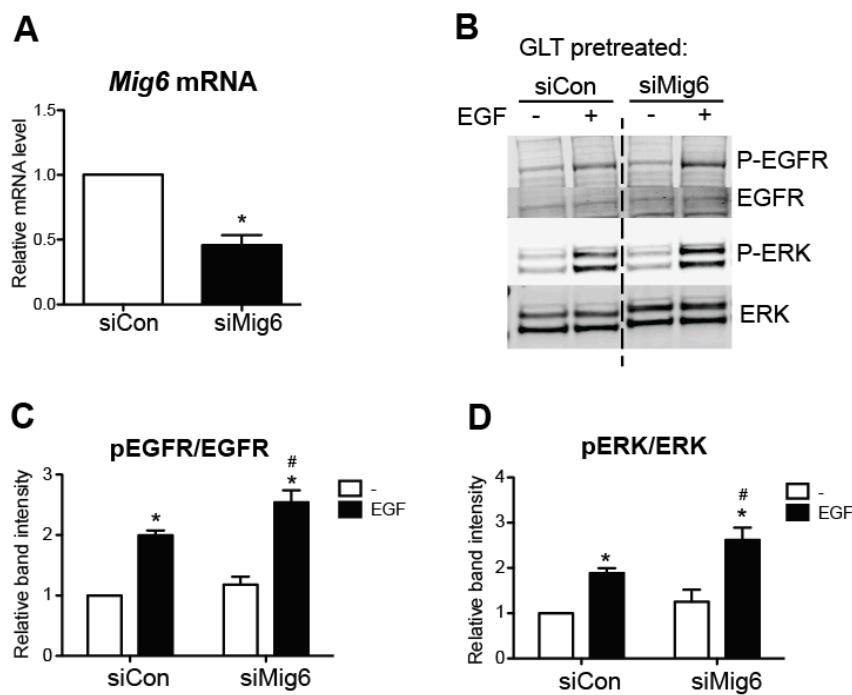


Figure 4-4

Down-regulation of Mig6 partially corrects GLT-induced desensitization of the EGFR signaling pathway.

(A) 832/13 cells are transduced with adenoviral vectors carrying either a scrambled control siRNA (siCon) or shRNA sequence against Mig6 (siMig6). *Mig6* mRNA levels were determined by qRT-PCR ($n = 4$; *, $p < 0.05$). (B-D) Transduced cells were treated with GLT and EGF as described. Protein levels of p-EGFR, EGFR, p-ERK, ERK, and tubulin were determined by immunoblotting. Data are reported as fold induction related to the GLT-treated, non-EGF-stimulated group. $n \geq 3$. *, $p < 0.05$ vs. EGF-treated; #, $p < 0.05$ vs. siCon EGF-stimulated.

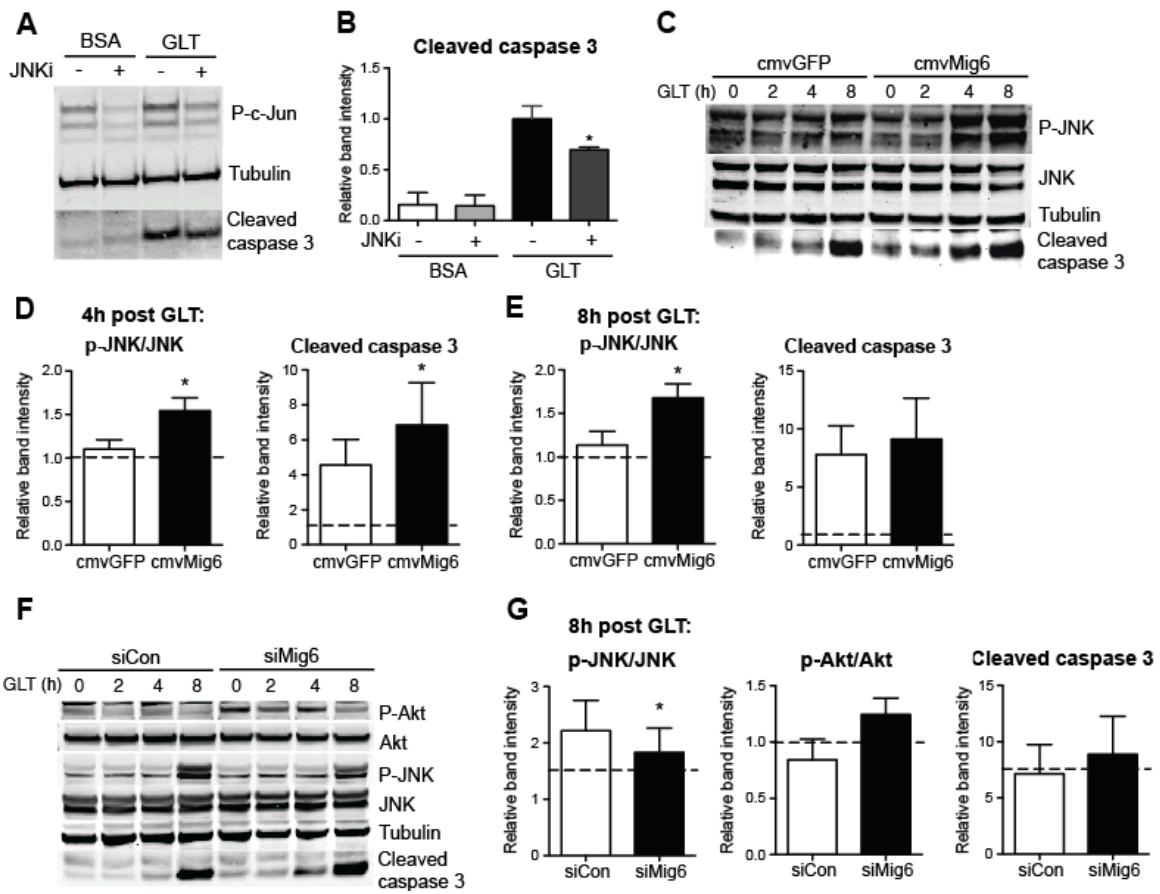


Figure 4-5

Mig6 facilitates GLT-induced apoptosis by potentiating JNK activation.

(A-B) 832/13 cells were pre-treated with 10 μ M SP600125 (JNK inhibitor) or DMSO for 2 h, following GLT treatment for 0 or 4 h. p-c-Jun, tubulin, cleaved caspase 3 protein levels were determined by western blotting. n = 3 experiments. *, p < 0.05 vs. GLT. **(C-E)** 832/13 cells were transduced with adenoviruses carrying cmvGFP vs. cmvMig6. Post transduction, cells were treated with GLT for 0, 2, 4, or 8 h. p-JNK, JNK, cleaved caspase 3, and tubulin protein levels were analyzed by western blotting. **(F-G)** 832/13 cells were transduced with adenoviruses carrying siCon vs. siMig6. Post transduction, cells were treated with GLT for 0, 2, 4, or 8 h. p-JNK, JNK, p-Akt, Akt, cleaved caspase 3, and tubulin protein levels were analyzed by western blotting. n = 5. *, p < 0.05 vs. siCon.

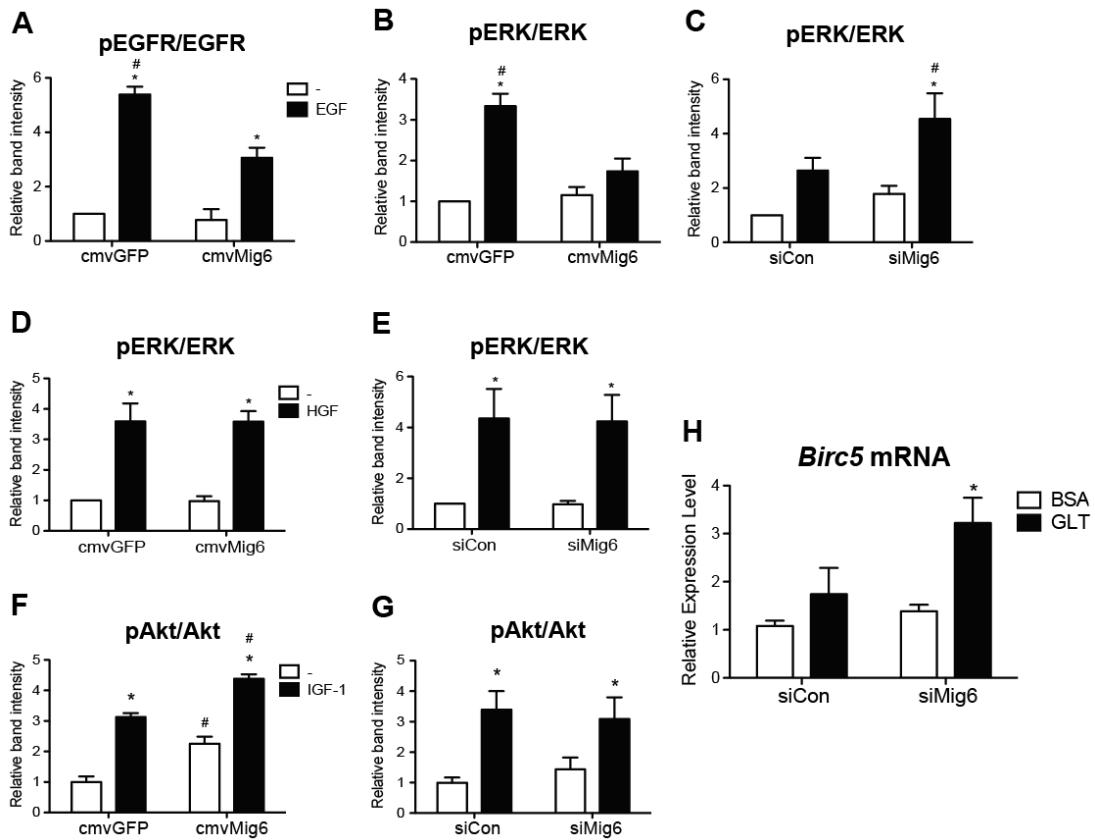


Figure 4-6

Mig6 control EGF but not IGF1 or HGF pro-survival signaling pathways.

(A-C) 832/13 cells were transduced with adenoviruses carrying cmvGFP vs. cmvMig6 or siCon vs. siMig6. Post transduction, cells were starved in 5 mM glucose and 0.1% BSA medium for 2 h, followed by 10 ng/ml recombinant rat EGF stimulation for 5 min. (D, E) After adenoviral transduction and starvation as in A & B, 832/13 cells were treated with 10 nM recombinant human IGF-1 for 10 min. (F, G) After adenoviral transduction and starvation as in A & B, 832/13 cells were treated with recombinant human HGF for 5 min. Protein levels of p-EGFR, p-ERK, ERK, p-Akt, Akt, and tubulin were analyzed by western blotting. $n \geq 3$. *, $p < 0.05$ vs. stimulated; #, $p < 0.05$ vs. cmvMig6 EGF-stimulated. (H) 832/13 cells were transduced with adenoviruses carrying siCon vs. siMig6. Post transduction, cells were treated with GLT for 0, 2, 4, or 8 h. *Birc5* mRNA levels were determined by qRT-PCR. $n = 3$. *, $p < 0.05$

5 MATERIALS AND METHODS

5.1 Animal studies

5.1.1 Animals and streptozotocin treatments

All animals were maintained and used according to protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. C57Bl/6J mice lacking one *Mig6* allele (*Mig6*⁺⁻) were obtained from Dr. Vande Woude, bred with wild type C57Bl/6J mice (*Mig6*⁺⁺), and genotyped as previously described (438). Extract-N-Amp tissue PCR kit (Sigma-Aldrich) was used for genotyping. Mice were kept in a standard light-dark cycle with free access to water and a standard rodent chow diet.

For streptozotocin (STZ) treatments, ten-week-old male wild type C57Bl/6J mice were intraperitoneally injected with 35 mg/kg body weight or 55 mg/kg body weight STZ (Sigma-Aldrich) for five consecutive days. A separate group of animals was injected in the same manner with vehicle (saline) as a control. Glucose tolerance tests (see below for details) were performed 3 days post STZ injection (**Table 5-1** and **Figure 5-1**). We selected the dosage of 35 mg/kg body weight for injecting *Mig6*⁺⁻ and *Mig6*⁺⁺ mice.

5.1.2 Metabolic studies

Glucose tolerance test

For glucose tolerance testing (GTT), 1.5 g/kg body weight D-glucose (Sigma-Aldrich) was injected intraperitoneally into 5 h-fasted control or STZ-treated mice. Blood was sampled from a tail vein at the indicated time points, and blood glucose was

measured using an AlphaTRAK glucometer (Abbott Laboratories). Serum insulin was assayed with an ultra-sensitive mouse insulin ELISA kit (Crystal Chem).

Insulin tolerance test

For insulin tolerance testing (ITT), 0.75 U/kg body weight recombinant insulin (Eli Lilly & Co.) was injected intraperitoneally into 5 h-fasted pre- and post-STZ treated mice. Blood glucose was determined at the indicated time points.

5.1.3 Histological studies

Immunostaining of pancreatic sections was performed as described previously (481). In brief, mouse pancreatic sections (5 μ M) were deparaffinized with xylene, and rehydrated through a series of graded ethanol solutions. Afterward, antigen retrieval was performed by microwaving slides with Antigen Unmasking Solution (Vector Labs) followed by blocking (Serum Free Blocking Reagent, Dako) for 30 min.

Immunohistochemistry staining

For immunohistochemistry staining, slides were incubated with guinea pig anti-insulin antibody overnight at 4°C (all antibodies used are listed in **Table 5-2**). The next day, immunodetection was performed with a peroxidase conjugated anti-rabbit IgG antibody (ImmPRESS, Dako) and peroxidase substrate (VECTOR NovaRED, Vector Labs). Finally, slides were counterstained with hematoxylin and mounted. Digital images were acquired using an Axio-Observer Z1 inverted microscope (Zeiss) equipped with an AxioCam color camera. The area of the insulin-positive cells (calculated using Axio-Vision Software) was divided by the total pancreatic area to obtain the beta cell cross-sectional area as a percentage of total pancreatic area.

Insulitis scoring

Insulitis scoring was performed by grading islets as follows: 0, no infiltration; 1, mild peri-insular mononuclear infiltration; 2, moderate (25–50%) infiltration; 3, massive (>50%) infiltration (503).

Immunofluorescence staining

For immunofluorescence staining, slides were incubated with anti-insulin, anti-glucagon, and anti-phospho-histone H3 overnight at 4°C followed by incubation with Alexa Fluor 488 or Alexa Fluor 555-conjugated secondary antibodies for 1 h at room temperature. Cells were counterstained with DAPI to visualize the nuclei and then imaged using an Axio-Observer Z1 inverted fluorescent microscope (Zeiss), equipped with an Orca ER CCD camera (Hammamatsu).

5.1.4 Islet experiments

Human islet experiments

Cadaveric human islets were obtained from Beta-Pro, LLC, the National Disease Research Interchange, or the Integrated Islet Distribution Program. Islets from 4 donors with T2D and 4 donors without diabetes were analyzed to determine *MIG6* expression levels. A separate set of islets from four healthy donors was treated with a pro-inflammatory cytokine cocktail (50 U/ml interleukin-1 β , 1000U/ml tumor necrosis factor- α , and 1000U/ml interferon- γ ; Prospec Ltd.) or phospho buffered saline vehicle for 24 h in DMEM containing 5.5 mM glucose.

Rodent islet experiments

Primary pancreatic islets were also collected from male Wistar rats weighing approximately 250 g (507, 508) or collected from *Mig6^{+/+}* or *Mig6^{+/-}* mice. After collagenase digestion, islets were hand-picked and cultured in 5 mM glucose RPMI medium (supplemented with 10% fetal bovin serum, 50 units/ml penicillin, and 50 µg/ml streptomycin) overnight before drug treatments. For the virus transduction experiments, rat islets were transduced with adenoviral vectors expressing Mig6 or green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter, immediately after isolation.

Glucose-stimulated insulin secretion assay

Glucose-stimulated insulin secretion assays were performed 48 h post- adenoviral vector transduction as previously described (509). Insulin content in the buffer was determined by insulin radioimmunoassay (Coat-A-Count Insulin RIA; Siemens Medical Solutions), and data were expressed relative to total islet protein concentration.

[³H]-thymidine incorporation assay

Islet proliferation was assessed by measuring the incorporation of [³H]methyl-thymidine into genomic DNA (510). [³H]methyl-thymidine was added to groups of 100 islets at a final concentration of 1 µCi/ml medium for 16 h. Groups of 30 islets were picked in triplicate and washed twice with PBS. The DNA was precipitated in 500 µl of cold 10% tricholoroacetic acid and solubilized by the addition of 80 µl of 0.3 N NaOH. The amount of [³H]thymidine incorporated into DNA was measured by liquid scintillation counting and normalized by total cellular protein.

Nitrite assay

Groups of 50 islets from *Mig6^{+/+}* or *Mig6^{+/-}* mice were cultured in 100 µl RPMI medium or RPMI medium containing the cytokine cocktail for 24 h. Supernatants were removed to measure nitrate/nitrite concentrations as an index of nitric oxide with the Griess assay (Promega) according to the manufacturer's protocol (511).

5.2 Cell experiments

INS-1-derived 832/13 rat insulinoma cells were cultured as described (512). A starvation medium (RPMI 1640 containing 2.5 mM glucose and 0.1% BSA) was used for EGF stimulation experiments. Gene overexpression and knockdown were performed as previously described (481).

5.2.1 Apoptosis assays

To induce apoptosis, cells were treated with 50 nM etoposide (BioVision) for 4 h, 1 µM thapsigargin (Sigma) for 6 h, or 1 µM adriamycin or camptothecin (BioVision Inc.) for 6 h. Cells were then lysed with RIPA buffer (Santa Cruz). Caspase 3 enzyme activity in the cell lysate was determined using a Caspase 3 Fluorometric Substrate Assay (Upstate) and a SpectraMax M5 microplate reader (Molecular Devices). Protein concentration was measured using the BCA Protein Assay kit (Pierce) to normalize caspase 3 activity.

5.2.2 Pharmacological inhibitors

Inhibition of MAPK pathways by pharmacological agents was achieved via pre-treating 832/13 cells with 10 µM of the MEK inhibitor UO126, the p38 inhibitor

PD169316, or the JNK inhibitor SP600125 (Sigma) for 1 h. Cells were then treated with thapsigargin.

5.2.3 EGF and cytokines treatments

For cytokine plus EGF stimulation experiments, 832/13 cells were pretreated with cytokines for 6 h, starved for 2 h, and treated with 10 ng/ml rat recombinant EGF (R&D Systems) for 5 min.

5.2.4 Gluclipotoxicity experiments

Palmitic acid-BSA complex solution was prepared according to the literature (513). In brief, sodium palmitate was first dissolved in 0.1 M NaOH buffer at 70°C. Next, the palmitic acid solution was mixed with 5% fatty acid-free bovine serum albumin (BSA) solution at 37°C to yield a 5 mM palmitic acid-BSA complex stock solution (the molecular ratio of fatty acid to BSA was 6: 1). The resulted clear palmitic acid-BSA complex solution and BSA control solution were store at -20 °C.

For lipotoxicity experiments, palmitic acid-BSA complex was diluted into serum free RPMI culture medium to obtain various concentrations of palmitic acid ranging from 0.1 mM to 0.4 mM. Equal volumes of BSA solution were used as carrier controls.

For glucotoxicity experiments, 25 mM glucose serum free RPMI culture medium supplemented with 0.1% BSA was used as high glucose treatment, and 5 mM glucose plus 20 mM D-mannitol (osmotic control) was used as low glucose control.

For gluclipotoxicity experiments, 0.4 mM palmitic acid plus 25 mM glucose medium was used to create gluclipotoxicity; and BSA plus 5 mM glucose medium was used as control.

5.2.5 Immunoblot analysis

Cells were lysed in 1% IGEPAL reagent supplemented with 10% glycerol (Sigma), 16 mM NaCl (Sigma), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma), 60 mM n-octylglucoside (Research Products International Corp.), phosphatase inhibitor cocktails (PhosSTOP tablets, Roche) and phosphatase inhibitor cocktails (EDTA-free cOmplete tablets, Roche). Lysates were resolved on a 10% NuPAGE Bis-Tris Gel (Invitrogen), transferred to an Immobilon-FL Transfer Membrane (Millipore), and incubated with antibodies (all antibodies used are listed in **Table 5-3**). Subsequently, membranes were incubated with IRDye 800 or 700 fluorophore-labeled secondary antibodies from LI-COR. Protein bands were visualized using the Odyssey System (LI-COR) and quantified with Image J software (NIH).

The phosphorylated protein levels presented in bar graphs were normalized by total protein levels, and the total (e.g., non-phosphorylated) protein levels were normalized to tubulin or GAPDH protein levels.

5.2.6 Quantitative RT-PCR analysis

RNA from 832/13 cells, mouse, rat, and human islets was isolated using RNeasy Mini or Micro kits (Qiagen). Reverse transcription was completed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The threshold cycle methodology was used to calculate the relative quantities of the mRNA products of *Mig6*, *Pdx-1*, *Ins1/2*, *NeuroD*, *IRS2* (TaqMan assays; Applied Biosystems), and pre-insulin (SYBR Green methodology; primer sequences were described previously (514)). PCR reactions were performed in triplicate for each sample from at least three independent experiments and were normalized to *Gapdh* or *beta-actin* gene expression levels.

5.2.7 Polyribosome analysis

Polyribosome analysis was performed as described previously (59, 460). Briefly, 832/13 cells were cultured with or without 1 µM thapsigargin for 6 h. 10 minutes prior to harvesting, cells were treated with 50 µg/ml cycloheximide to block translation (Sigma). Cells were washed and lysed in a buffer of 20 mM pH 7.5 Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, and 0.4% NP-40 supplemented with 50 µg/ml cycloheximide. Cell lysates were homogenized by passing through a 25-gauge needle and incubated on ice for 10 minutes. The cell lysate was pre-cleared by centrifugation (9000 × g for 10 minutes at 4°C) and transferred onto a 10–50% sucrose gradient solution made with the above lysis buffer. A portion of unfractionated lysate was saved and used as the “input” to determine steady-state mRNA levels of *Mig6*, *ATF4*, and *cyclinD1*. The gradients were then centrifuged in a Beckman SW-41Ti rotor for 2 h at 35,000rpm at 4°C. 10 gradients were fractionated using a Biocomp Gradient Station and collected by a Fraction Collector (Bio-Rad). Meanwhile, absorbance of RNA at 254 nm was recorded using an ECONO UV Monitor (Bio-Rad). RNA was isolated as described above using the RNeasy Mini Kit (Qiagen) from each fraction. The samples from two adjacent fractions were pooled, and subjected to qRT-PCR analysis to determine *Mig6*, *ATF4*, and *cyclinD1* transcripts levels.

5.3 Statistical analysis

All data are reported as means ± SEM. Protein and mRNA data were normalized to control conditions and were presented as relative expression. Student's *t*-test, Pearson correlation, or ANOVA (with Bonferroni *post-hoc* tests) were performed using GraphPad Prism software to detect statistical differences (*p* < 0.05).

Minutes	Saline				35mg/kg STZ			55mg/kg STZ		
	1	2	3	4	1	2	3	1	2	3
0	175	200	198	171	224	195	250	197	238	258
15	405	340	413	403	424	324	523	501	490	562
30	369	250	282	297	441	302	391	563	523	512
60	255	190	195	206	336	192	271	388	469	515
90	219	191	209	208	327	225	300	387	504	481
120	201	212	230	189	251	228	298	352	461	403

Table 5-1

Different diabetogenic responses to moderate doses of streptozotocin.

Ten-week-old male wild type C57Bl/6J mice were intraperitoneally injected with 35 mg/kg body weight or 55 mg/kg body weight STZ for five consecutive days ($n = 3$ each group). A separate group of animals was injected in the same manner with saline as a control ($n = 4$). Glucose tolerance tests were performed at 3 days post STZ injection.

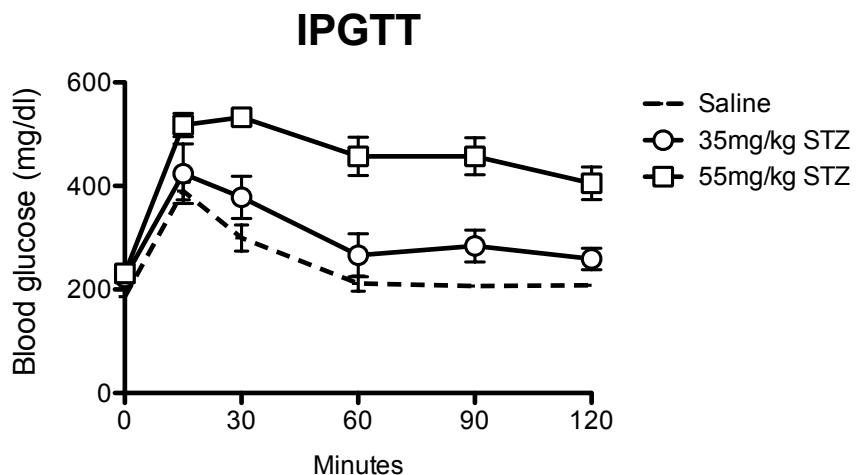


Figure 5-1

Different diabetogenic responses to moderate doses of streptozotocin.
Above figure was generated from data presented in **Table 5-1**.

Name	Vendor, lot number	Dilution
Anti-glucagon	Sigma, #G2654	1: 1000
Anti-insulin	Invitrogen, #180067	1: 250
Anti-phospho-histone H3	Millipore, #06-570	1: 1000
Alexa Fluor-conjugated antibodies	Invitrogen	1: 1000

Table 5-2

List of antibodies used for immunostaining.

Name	Vendor, lot number	Dilution
Anti-Actin	MP Biomedicals, #691002	1: 5000
Anti-Akt	Cell Signaling, #2920	1: 1000
Anti-caspase 3	Cell Signaling, #9662	1: 1000
Anti-CHOP	Santa Cruz, #7351	1: 250
Anti-EGFR	Sigma-Aldrich, #E3138	1: 1000
Anti-eIF2α	Cell Signaling, #5324	1: 1000
Anti-ERK1/2	Cell Signaling, #4696	1: 1000
Anti-γ-tubulin	Sigma-Aldrich, #T6557	1: 5000
Anti-Gapdh	Abcam, #Ab9483	1: 5000
Anti-Mig6	Santa Cruz, #D-1	1: 250
Anti-p53	Cell Signaling, #2524	1: 1000
Anti-Pdx1	Millipore, #07-696	1: 2000
Anti-phospho-Akt	Cell Signaling, #4056	1: 1000
Anti-phospho-EGFR	Cell Signaling, #3777	1: 1250
Anti-phospho-eIF2α	Cell Signaling, #3398	1: 1000
Anti-phospho-ERK1/2	Cell Signaling, #4370	1: 2000
Anti-phospho-p53	Abcam, #Ab1431	1: 1250
IRDye 800 or 700 fluorophore-conjugated antibodies	LI-COR	1: 10000

Table 5-3

List of antibodies used for immunoblotting.

6 DISCUSSION

6.1 Summary of the current studies

The loss of functional beta cells is the final and irreversible step towards the development of frank diabetes. To prevent or cure diabetes, many factors have been identified to preserve beta cells. Among them, growth factors have been considered as premier candidates for such an approach because growth factors facilitate cell cycle progression and inhibit the mitochondrial apoptotic machinery. However, the activation of growth factor signaling cascades are usually attenuated by chronic pathological stress conditions via unknown mechanisms.

In this document, I have suggested that the negative regulator of EGFR, Mig6, plays an important role in controlling beta cell fate. Previously, our group discovered that Mig6 is induced by synthetic glucocorticoids, and Mig6 contributes to glucocorticoid-mediated beta cell cycle arrest (420). We extended this work by examining the roles of Mig6 in regulating beta cell proliferation *in vivo*, and unexpectedly discovered that Mig6 also controls beta cell death (**Chapter 2**). We first established that *Mig6^{+/−}* and *Mig6^{+/+}* mice have normal glucose clearance and comparable beta cell mass. Yet, we found that beta cells of *Mig6^{+/−}* mice (bearing enhanced EGFR activity) surprisingly have lower proliferation rates compared to *Mig6^{+/+}* mice. Because steady-state beta cell mass is controlled by the rates of beta cell proliferation and cell death, the decreased beta cell proliferation observed in *Mig6^{+/−}* mice could be a secondary effect of decreased beta cell death. In other words, we speculated that the beta cells of *Mig6^{+/+}* mice are not required to proliferate as much because they have lower rate of death, which is sufficient for maintaining adequate beta cell mass and glucose

homeostasis. We then tested our hypothesis and examined the molecular events initially using 832/13 INS-1 derived beta cells. We found that Mig6 controls DNA-damage induced beta cell apoptosis; and overexpression of Mig6 exacerbates ER stress-induced cell death. Another significant aspect of this study is that we discovered that Mig6 is induced by stress stimuli. Furthermore, we found that Mig6 belongs to a group of specialized molecules that are resistant to stress, as Mig6 mRNA is more stable, and is preferentially translated under stress. The roles of stress-inducible Mig6 in diabetes were described in **Chapter 3** and **Chapter 4**.

We examined the roles of Mig6 in the T1D milieu because previous animal studies have demonstrated that EGFR is essential in preventing and reversing T1D. Yet, the EGF-related therapies failed to achieve long-term effects in humans, indicating that EGFR signaling pathways are complicated and warrant further investigation. In **Chapter 3**, we discovered that EGFR activation is attenuated by pro-inflammatory cytokines via Mig6. Notably, we reported that Mig6 mediates the progression of T1D, as *Mig6^{+/+}* mice are protected from developing experimentally-induced hyperglycemia and diabetes. This study highlighted the significance of stress-inducible negative regulators in blocking growth factor signaling pathways in a diabetic milieu. Based on this work, we propose that the diabetic stress stimuli should be eliminated to allow for maximal efficacy of growth factor therapy.

In the last data chapter (**Chapter 4**), we described how Mig6 controls beta cell fate in T2D. We demonstrated that Mig6 is a contributor of EGFR inactivation during ER stress and GLT; and that Mig6 controls the activation of beta cell survival signaling cascades. The broader implication of this work is that Mig6 may be a factor limiting beta

cell mass expansion in states of nutrient excess because EGFR and mTOR signaling pathways have been shown to regulate acute lipid infusion-induced beta cell mass expansion in adult rodents. Yet, chronic dietary nutrient oversupply failed to do so. Therefore, we think that the prolonged diabetogenic assaults could activate inducible negative regulators (e.g., *MIG6* is elevated in T2D islets), which inhibit beta cell regeneration and survival. Our future studies will reveal how Mig6 and other stress-inducible factors modulate growth factor receptor signal transduction cascades during the development of T2D.

6.2 Future studies

The previous chapters defined Mig6 as regulator of beta cell proliferation and apoptosis. However, how Mig6 protein is dynamically regulated in (patho)physiological settings remains unclear. For example, whereas various stress stimuli induces *Mig6* mRNA expression, Mig6 protein expression levels often remain unchanged. We suspected that Mig6 is being transported with EGFR toward the endo-lysosomal degradation system; and Mig6 protein turnover rates are possibly altered following mitogen and stress stimuli. Alternatively, we considered that it is the qualitative but not quantitative features of Mig6 regulating beta cell fates. For instance, Mig6 could be post-translationally modified or differentially distributed within the cell to facilitate its binding to different molecules. It is important to determine whether Mig6 and EGFR association patterns are changed under different circumstances. Future studies focusing on characterizing Mig6 functional domains and post-translational modification patterns will greatly assist the identification of anti-diabetic drug targets against Mig6.

The physiological roles of Mig6 in beta cells require further investigations. So far, we have demonstrated that *Mig6*^{+/−} whole body heterozygote knockout mice are protected from STZ-induced diabetes, most likely because they have preserved beta cell function and enhanced beta cell mass recovery. However, we are aware that the global disruption of Mig6 could modify the pancreatic progenitor/vasculature/immune cell repertoires. Therefore, we plan to generate Mig6 conditional knockout mice to examine the tissue-specific effects of Mig6. Additionally, the roles of Mig6 in human beta cells need to be explored. With the advancement at cell engineering technology, it is feasible to study the functional significance of Mig6 in iPS/hESC-derived beta cells (515, 516). Besides, the recently-developed adult human beta cell line could serve as an additional platform to examine the extent to which Mig6 controls human beta cell proliferation, senescence, and death (517).

We have demonstrated that Mig6 attenuates EGFR activity under stressful conditions *in vitro*. Yet, EGFR activity during the progression of diabetes is not known. The temporal examination of: 1) systemic/pancreatic EGF family ligands/receptors expression levels, 2) EGF cellular uptake efficiency, and 3) EGF ligands ectodomain shedding process (518), will facilitate the identification of remedial windows to promote EGF-related therapies. Plausibly, the shedding products of transmembrane proteins (e.g., growth factor precursors/receptors, cytokine/cytokine receptors, and adhesion molecules) could serve as early biomarkers to detect or monitor diabetes.

The early detection and early treatment are critical in the battle against diabetes because once hyperglycemia and other systemic distress are established, it becomes very difficult to stop and reverse diabetes. For instance, the growth factor therapies failed to

resurrect beta cells and reverse full-blown diabetes because the beta cell intrinsic/extrinsic environments have posed barriers against the activation of growth factor signaling cascades. Excitingly, we have identified Mig6 as a modulator of these processes. Future studies exploring other inhibitors are warranted. In particular, we suggest that the signaling pathways activated by the acute pathological stimuli (e.g., short-term cytokine and palmitic acid treatments) are the best candidates to be investigated because beta cells usually respond to acute stress stimuli by activating compensatory growth and insulin secretion mechanisms (91, 269, 315, 519). However, these pathways are dysregulated with prolonged stress exposure. It will be significant to find the molecular switches of these processes to benefit the identification of therapeutic targets to foster beta cell proliferation/survival signaling cascades in adverse conditions.

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Advisor: Dr. Patrick Fueger

- Investigated a novel stress-induced epidermal growth factor receptor inhibitor, Mig6, in regulating pancreatic beta cell function and survival in the progression of type 1 and type 2 diabetes *in vivo* and *in vitro*.

University of Connecticut, Department of Molecular and Cell Biology

August 2008 to May 2009

Advisor: Dr. Juliet Lee

- Studied cell-cell contact interaction and the generation of traction force in guiding cell movements.

Taipei Medical University, College of Medical Science and Technology

July 2007 to May 2008

Advisor: Dr. Horng-Mo Lee

- Screened and identified herb extracts that inhibit hepatic gluconeogenesis.

Department of Laboratory Medicine, Taipei Medical University Hospital

June 2007 to April 2008

- Performed laboratory tests in clinical hematology, biochemistry, immunology, and histology labs.

Publications

1. **Chen Y-C**, Colvin ES, Griffin KE, Maier BF, Fueger PT. (2014) Mig6 haploinsufficiency protects mice against streptozotocin-induced diabetes. *Diabetologia* (In press).
2. Hernandez AM, Colvin ES, **Chen Y-C**, Geiss SL, Eller LE, Fueger PT. (2013) Upregulation of p21 activates the intrinsic apoptotic pathway in beta cells. *Am J Physiol Endocrinol Metab.* 304(12):E1281-90.
3. **Chen Y-C**, Fueger PT, Wang Z. (2013) Depletion of PAK1 enhances Ubiquitin-mediated Survivin degradation in pancreatic β -cells. *Islets.* 1:5(1).
4. Colvin ES, Ma H-Y, **Chen Y-C**, Hernandez AM, Fueger PT. (2013) Glucocorticoid-induced suppression of beta cell proliferation is mediated by Mig6. *Endocrinology* 154(3):1039-46.
5. **Chen Y-C**, Colvin ES, Maier BF, Mirmira RG, Fueger PT. (2013) Mitogen-inducible gene 6 triggers apoptosis and exacerbates ER stress-induced β -cell death. *Mol Endocrinol.* 27(1):162-71.
6. Fueger PT, Hernandez AM, **Chen Y-C**, Colvin ES. (2012) Assessing replication and beta cell function in adenovirally-transduced isolated rodent islets. *J Vis Exp.* 25(64): 4080.
7. **Chen Y-C**, Colvin ES, Lutkewitte AL, Kono TM, Evans-Molina C, Fueger PT. Glucolipotoxicity-induced Mig6 desensitizes EGFR signaling pathway and promotes pancreatic beta cell death. (2014) (Manuscript in preparation)

Fellowships and Awards

2012 to 2014	DeVault Fellowship, Indiana University School of Medicine
2014	Erica Daniel Kepner Award, Indiana University School of Medicine
2014	Best Oral Presentation Award, Indiana Physiological Society Annual Meeting
2014	Caroline tum Suden/Frances Hellebrandt Professional Opportunity Award, American Physiological Society
2014	Virendra B. Mahesh Award of Excellence in Endocrinology, Endocrinology and Metabolism Section, American Physiological Society
2014	Graduate Student Traveling Award, Indiana University School of Medicine
2014	Educational Enhancement Grant (for Research Consumable Supplies), IUPUI
2014	Stier Award, Department of Cellular and Integrative Physiology, Indiana University School of Medicine
2013	Educational Enhancement Grant (Traveling Award), IUPUI
2009 to 2010	University Fellowship, Indiana University School of Medicine
2004 to 2008	Shin-Kong Life Scholarship, Shin-Kong Life, Taipei, Taiwan

Professional Affiliations

- 2012 to Present American Physiological Society
2010 to 2014 Indiana Physiological Society
2010 to 2014 Islet Biology Journal Club

Presentations and Conferences Attended

- 2014 Experimental Biology Annual Meeting
 Talk: Mig6 haploinsufficiency protects mice against STZ-induced diabetes
- 2014 Indiana Physiological Society Annual Meeting
 Talk: Mig6 haploinsufficiency protects mice against STZ-induced diabetes
- 2013 Experimental Biology Annual Meeting
 Poster: Mitogen-inducible gene 6 potentiates glucolipotoxicity-induced pancreatic beta cell death.
- 2012 Center of Diabetes Research, Indiana University School of Medicine
 Talk: The role of Mitogen-inducible gene 6 in pancreatic beta cell destruction
- 2012 University of Indianapolis
 Lecture: Diabetes therapy targeting pancreatic beta cells
- 2011 The Midwest Islet Club 5th Annual Meeting
 Poster: Induction of Mitogen-inducible gene 6 during endoplasmic reticulum stress exacerbates beta cell apoptosis
- 2011 71st Scientific Session American Diabetes Association
 Poster: Transcriptional regulation of Mig-6 by PPAR γ in beta cells
- 2011 The Midwest Islet Club 4th Annual Meeting
 Talk: Transcriptional regulation of Mig-6 by PPAR γ in beta cells

Volunteer Experience

- 2010 to 2014 Activity Assistant of Molecular Medicine in Action (MMIA)
 - Assisted with preparing hands-on histological staining workshops for high school students and teachers.
- 2011 to 2013 Student Ambassador of Indiana University School of Medicine graduate program campus recruits
 - Presented current research to the prospective students, and assisted with recruiting events.

Licensure

Medical Laboratory Scientist License, Taiwan