Understanding the Function of PGC-1α Isoforms in β-cell Survival and Diabetes

Sarah Sczelecki

Faculty of Medicine
Division of Experimental Medicine
McGill University
Montréal, Quebec, Canada

Supervisor: Dr. Jennifer L. Estall
Co-Supervisor: Dr. Woong-Kyung Suh
Unité de mécanismes moléculaires du diabète
Institut de recherches cliniques de Montréal (IRCM)

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Abstract

Peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1 alpha (PGC-1α) is a transcriptional co-activator responsible for mitochondrial biogenesis and oxidative metabolism. Many isoforms of PGC-1α have been described in the literature, most of which are shown to function similarly to the canonical PGC-1α protein. Recently, however, a novel isoform of PGC-1α was identified, PGC-1α4. It was shown to have a different, yet complementary function to canonical PGC-1α (PGC-1α1) in muscle. It is also expressed in other metabolically active tissues; however, it is unknown whether it has additional distinct tissue-specific functions. Furthermore, PGC-1α plays an important role in controlling metabolism in pancreatic β-cells and expression of the co-activator is decreased in diabetic islets; however, the role of PGC-1α isoforms in diabetes is unknown.

Our objective is to determine whether PGC-1α4 has a unique function in β-cells and whether it plays a role in the pathogenesis of diabetes. We show that stimulation with forskolin, exendin-4 and a cytokine cocktail of TNFα, IL-1β and IFNγ, induced specific PGC-1α isoforms in β-cells. Following over-expression of these isoforms in INS-1 cells, PGC-1α4 prevented the cleavage of caspase-3 in response to cytokines, suggesting that the novel isoform is uniquely anti-apoptotic. To assess whether PGC-1α isoforms play a role in β-cell survival *in vivo*, mice with a β-cell specific PGC-1α knockout of all isoforms were subjected to low-dose streptozotocin (STZ) treatment to induce β-cell apoptosis. Unexpectedly, knockout mice were protected from STZ induced hyperglycemia. However, there was no difference in percentage of cleaved caspase-3 positive cells in control versus knockout mice, suggesting no difference in apoptosis. Therefore, PGC-1α4 could be a novel factor important for β-cell survival and over-expression of this unique isoform may protect against the pathogenesis of diabetes.

Résumé

Peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1 alpha (PGC-1α) est un co-activateur transcriptionnel responsable de la biogenèse mitochondriale et du métabolisme oxydatif. De nombreux isoformes de PGC-1α ont été décrits dans la littérature, dont la plupart ont été démontrés comme fonctionnant de manière similaire à la protéine PGC-1α canonique. Récemment, cependant, un nouvel isoforme de PGC-1α a été identifié, PGC-1α4, possédant une fonction différente mais complémentaire de la protéine canonique (PGC-1α1) dans le muscle. PGC-1α4 est également exprimé dans d'autres tissus métaboliquement actifs, mais il n'est pas connu s'il démontre de nouvelles fonctions dépendantes du tissu. PGC-1α joue un rôle important dans la regulation métabolique des cellules β du pancreas; de plus l'expression du co-activateur est dérégulée dans les îlots de Langerhans de patients diabétiques.

Notre objectif est de déterminer si PGC-1α4 a une fonction unique dans les cellules β du pancréas et s'il joue un rôle dans la pathogenèse du diabète. Nous montrons que la stimulation avec la forskoline, l'exendine-4 et un cocktail de cytokines, TNFα, IL-1β et IFNγ, induit les isoformes de PGC-1α dans les cellules β. Suite à la surexpression des isoformes dans les cellules INS-1, PGC-1α4 empêche le clivage de la caspase-3 en réponse à des cytokines, suggérant qu'uniquement le nouvel isoforme est anti-apoptotique. Pour déterminer si les isoformes de PGC-1α jouent un rôle dans la survie des cellules β in vivo, les souris déficientes de tous les isoforms de PGC-1α, spécifiquequement dans les cellules β, ont été soumises à une faible dose de streptozotocine (STZ) provoquant l'apoptose des cellules β. De façon inattendue, les souris déficientes en PGC-1α ont été protégées contre l'hyperglycémie induite par la STZ. Cependant, il n'y avait pas de différence du pourcentage de cellules en apoptose chez les souris témoins par rapport aux souris déficientes. Donc, PGC-1α4 pourrait être un nouveau facteur important pour

la survie des cellules β du pancreas et la surexpression de cette isoform unique, peut protéger contre la pathogenèse du diabète.

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List of Abbreviations

AAV8	Adeno-associated virus 8
Ad-siPGC-1α	Adenoviral vector short interfering RNA against PGC-1α
ATMs	Adipose tissue macrophages
ATP5B	ATP synthase subunit β
ASO	Anti-sense oligonucleotides
BAT	Brown adipose tissue
bp	base pairs
BSA	Bovine serum albumin
BMI	Body Mass Index
cAMP	Cyclic adenosine monophosphate (AMP)
CBP	CREB binding protein
CMV	Cytomegalovirus
COXII	Cytochrome c oxidase subunit II
COXIV	Cytochrome c oxidase subunit IV
COXVb	Cytochrome c oxidase subunit V b
CPT-1	Carnitine palmitoyl transferase I
CREB	cAMP response element-binding protein
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
CVD	Cardiovascular disease
CytoC	Cytochrome C
DOX	Doxycycline
DPP-4	Dipeptidyl peptidase-4
ERRα	Estrogen-related recptor α

ETC	Electron transport chain
Ex-4	Exendin-4
FFA	Free fatty acids
FK	Forskolin
FOXO1	Forkhead box protein O1
G6pc	Glucose-6-phosphatase
GAD65	Glutamic acid decarboxylase 65
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
Glut2	Glucose transporter type 2
Glut4	Glucose transporter type 4
GSIS	Glucose stimulated insulin secretion
HAT	Histone acetyltransferase
HBSS	Hanks balanced salt solution
HLA	Human leukocyte antigen
HNF4α	Hepatocyte nuclear factor 4 alpha
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
I-A2	Insulinoma-associated antigen-2
IAP	Inhibitors of apoptosis
IDDM1	Insulin-dependent diabetes mellitus locus 1
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry

IL-1β	Interleukin-1beta
IL2RA	Interleukin 2 receptor A
IL6	Interleukin 6
IFNγ	Interferon gamma
iNOS	Nitric oxide synthase
INS-1	Rat insulinoma β-cell line
IP	Immunoprecipitation
ip	Intraperitoneal
kDa	Kilo Daltons
KO	Knock-out
LCAD	Long chain acyl-coenzyme A dehydrogenase
MCAD	Medium chain acyl-coenzyme A dehydrogenase
MCP-1	Monocyte chemoattractant protein 1
MEF2	Myocyte enhancer factor-2
MIP	Mouse insulin promoter
MSN	Myostatin 1
mtTFA	Mitochondrial transcription factor A
NOD	Non-obese diabetic
NRF1	Nuclear respiratory factor-1
NRF2	Nuclear respiratory factor-2
ns	Not significant
NT-PGC-1α	N-truncated PGC-1α
OGTT	Oral glucose tolerance test

Pck1	Phosphoenol pyruvate carboxykinase 1
PCR	Polymerase chain reaction
PEPCK	Phosphoenol pyruvate carboxykinase
PGC-1α	Peroxisome proliferator activated receptor gamma co-activator 1-alpha
PGC-1α1	PGC-1α isoform 1, canonical transcript
PGC-1α2	PGC-1α isoform 2
PGC-1α3	PGC-1α isoform 3
PGC-1α4	PGC-1α isoform 4
PGC-1α-b	PGC-1α isoform b
PGC-1α-c	PGC-1α isoform c
PGC-1β	Peroxisome proliferator activated receptor gamma co-activator 1-beta
PPARγ	Peroxisome proliferator activated receptor gamma
PPARα	Peroxisome proliferator activated receptor alpha
PRC	PGC-related coactivator
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
siRNA	Short interfering RNA
SRC-1	Steroid receptor coactivator-1
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TetO	Tetracycline-dependent promoter

Tfam	Mitochondrial transcription factor A
tTA	Tetracycline transactivator
TNFα	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UCP-1	Uncoupling protein-1
UCP-2	Uncoupling protein-2
WAT	White adipose tissue
WT	Wild-type
ZNT8	Zinc transporter 8

SECTION I:
INTRODUCTION

The Pancreas and β-cells

The pancreas is a mixed glandular organ consisting of both exocrine and endocrine cells. Exocrine cells are organized into pancreatic acini and secrete digestive enzymes, whereas the endocrine pancreas is organized into Islets of Langerhans, which secrete hormones (Collombat et al., 2010). These hormones include insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin, from the β-, α-, δ-, γ- and ε-cells, respectively (Collombat et al., 2010; Elayat et al., 1995). In humans, the different cells of the islet are interspersed throughout the whole structure, whereas in mice the α-cells (and δ-cells) surround the periphery and the β-cells are found primarily in the center of the islet (Kim et al., 2009). β-cells make up approximately 80% of the islet, whereas the other cell types comprise the other 20%. The main function of β-cells is to store and release insulin in response to glucose. Insulin is required in the blood to maintain glucose homeostasis and not surprisingly, β-cell dysfunction or death results in the development of diabetes. The main function of α-cells is to secret glucagon in times of starvation, to increase circulating glucose by initiating gluconeogenesis in the liver. Impaired glucagon secretion is also observed in type 2 diabetics (Del Prato and Marchetti, 2004). In type 2 diabetics, fasting plasma glucagon concentrations are elevated and there is impaired glucagon suppression after intake of a meal (Del Prato and Marchetti, 2004). These events contribute to hyperglycemia and eventual β-cell dysfunction, by maintaining hepatic glucose output when in the fed state. However, β-cell dysfunction and death remains the main cause of diabetes development and target for therapies.

Diabetes: A Global Pandemic

Both type 1 (T1D) and type 2 (T2D) diabetes mellitus are becoming a global concern. 90% of diabetics have T2D, which correlates with increased incidence of obesity, reduced physical behaviour and dietary changes. The remaining 10% of diabetics are classified as type 1. Currently, it is predicted that 347 million people worldwide are affected by diabetes (Danaei et al., 2011). By 2030, diabetes incidence is projected to increase by 50% and become the 7th leading contributor to increased mortality worldwide (Mathers and Loncar, 2006). Even though the prevalence of diabetes is higher in developed countries, the prevalence is rapidly increasing in developing countries (Forbes and Cooper, 2013).

Mortality associated with either type of diabetes is due to complications of the disease including cardiovascular disease and diabetic nephropathy, which are the leading causes of death for diabetic patients (Morrish et al., 2001). Globally, a total of 465 billion US dollars (USD) was spent on treating diabetes and its complications in 2011 and by 2030, this expense is projected to increase to 654 billion by 2030 (IDF, 2013).

T2D can be treated by lifestyle intervention if detected early on in disease progression, drastically improving symptoms and reducing the risk of developing complications. However, later stages of T2D cannot simply be treated with lifestyle changes and requires pharmacological intervention (Prentki and Nolan, 2006). Moreover, unlike T2D, T1D cannot be treated simply with lifestyle changes, but requires use of exogenous insulin and drugs to maintain euglycemia and to reduce the risk of diabetic complications. Overall, there is a need to further understand the underlying molecular basis of either disease to improve treatment efficacy to prevent diabetes associated mortality (van Belle et al., 2011).

Type 1 Diabetes

T1D is a multi-factorial disease, occurring due to a combination of environmental and polygenic factors (Forbes and Cooper, 2013) and described as insulin-dependent diabetes. It develops following immune mediated attack of the insulin producing β-cells in the Islets of Langerhans resulting in cell death (Padgett et al., 2013). The use of exogenous insulin controls glycemia; however, chronically elevated levels of glucose and insulin cause microvascular insults increasing the risk of cardiovascular disease (Reusch and Wang, 2011). Briefly, chronically high levels of circulating glucose cause an increase in glucose uptake in endothelial cells, which are unable to efficiently reduce glucose intake. This increases the amount of electron donors, such as NADH and $FADH_2$, being fed into the electron transport chain (ETC) (Brownlee, 2005). This inevitably generates electron radicals, which are able to bind oxygen to generate superoxide, a reactive oxygen species (ROS), which ultimately leads to endothelial cell dysfunction and death leading to microvascular disease (Brownlee, 2005).

The cause of type 1 diabetes is largely unknown; however, infectious insults or mutations in particular genes have been linked to the development of the disease. Many of the genes associated with the development of T1D are known and most are associated with immunological function. One class of genes mutated in T1D are the HLA (human leukocyte antigen) genes, HLA class I and II, located in a region known as the insulin-dependent diabetes mellitus locus (IDDM1) (van Belle et al., 2011). Other genes associated with the development of diabetes are (i) the insulin gene (Bell et al., 1984); (ii) PTPN22 (protein tyrosine phosphatase, non-receptor type 22), a negative regulator of T-cell function; (iii) IL2RA (interleukin 2 receptor A) , a Treg cell survival signal and; (iv) CTLA-4 (cytotoxic T-lymphocyte Antigen 4), a crucial molecule for the negative regulation of inflammatory signalling (reviewed in van Belle et al., 2011).

Not surprisingly, a majority of the genes associated with T1D relate to dysregulated inflammatory signalling, including loss of T-cell regulation and Treg cell function. Type 1 diabetes is characterized by immune infiltration of CD4+ and CD8+ T-cells around the islets, as well as the presence of macrophages, B-cells, NK cells and NKT cells (reviewed in Bending et al., 2011). Auto-immune attack of β-cells by these cell types is caused by a loss of tolerance to self-antigens, such as GAD65 (glutamic acid decarboxylase 65) (Karlsen et al., 1992), I-A2 (insulinoma-associated antigen-2), proinsulin, and ZnT8 (Zinc transporter 8) (van Belle et al., 2011). Presence of immune cells surrounding the islet exposes the β-cells to chronic levels of pro-inflammatory cytokines, such as TNFα (Tumor necrosis factor alpha), IL-1β (Interleukin 1 beta) and IFNγ (Interferon gamma) (Donath et al., 2003). Interestingly, acute exposure of β-cells to these cytokines can increase β-cell function; however, chronic exposure to pro-inflammatory cytokines can lead to β-cell death.

Immune infiltration of the islet leads to β-cells death via various pathways. In a chronic context, β-cells up-regulate pro-inflammatory pathways, such as NF-κB and JAK/STAT1, which are detrimental to β-cell health (van Belle et al., 2011). As well, β-cells increase the amount ROS and decrease pro-survival genes, like Bcl-2. It is thought that pro-inflammatory cytokines can increase iNOS (Nitric oxide synthase), leading to nitric oxide accumulation and eventually caspase activation and β-cell death. Another pathway involved in β-cell death is an increase in signalling of the death receptor, Fas. In a non-disease state, Fas receptor expression is negligible; however, upon exposure to pro-inflammatory cytokines, Fas expression increases and causes islets to be more susceptible to death (van Belle et al., 2011). Moreover, cell death promotes antigen presentation, perpetuating the auto-immune attack of the β-cells and further promoting their death (van Belle et al., 2011). The culmination of all these events, whether acting

simultaneously or individually, results in decreased β-cell mass, reducing circulating insulin and causing the severe hyperglycemia characteristic of type 1 diabetes.

Type 2 Diabetes

Type 2 diabetes is known as insulin-independent diabetes and is associated with insulin resistance and hyperglycemia. In early stages, type 2 diabetics exhibit insulin resistance in their peripheral tissues, but at this stage β-cells are able to compensate and increase secretion of insulin maintaining euglycemia. This compensation in rodents is due to an increase in β-cell mass (Steil et al., 2001) and function (Chen et al., 1994). The compensatory increase in β-cells mass also occurs in type 2 diabetics (Butler et al., 2003); however, the extent to which β-cells in humans can expand seems limited and the source of the expanded β-cell pool remains unclear and is highly debated. New β-cells may come from pre-existing β-cells (Dor et al., 2004) or are derived from the ductal epithelium (Inada et al., 2008; Lee et al., 2010), where β-cell stem-like cells are thought to reside. Regardless of the origin, this is an important mechanism to understand and can serve as a potential therapy for T2D, since over time the compensatory β-cell mass increase appears to be lost and type 2 diabetics begin to become hyperglycemic.

Many mechanisms contribute to the loss of β-cell function in T2D. Since T2D incidence relates to over-nutrition and inactivity, there is often an increase of both free-fatty acids (FFAs) and glucose in the blood, which are all processed by the mitochondria. Overuse of the mitochondria over time, increases ROS, due to more substrates entering the ETC, generating free electrons and eventually leads to mitochondrial dysfunction (Lowell and Shulman, 2005; Maestre et al., 2003). β-cells are more susceptible to ROS, as they have comparatively lower

amounts of detoxifying enzymes (reviewed in Prentki and Nolan, 2006). This inability to detoxify free radicals eventually leads to β-cell death via apoptosis (Maestre et al., 2003).

In addition to over-nutrition causing an overload on metabolic machinery, there is also an inflammatory component that contributes to the progression of the disease. However, these two processes are difficult to separate. Over-nutrition in its extreme form causes obesity, leading to increased fat storage in adipocytes and generating larger fat pads. This increase in fat mass also coincides with an increase in resident macrophages, known as adipose tissue macrophages (ATMs) (Weisberg et al., 2003; Xu et al., 2003). An increase of circulating FFAs activates the ATMs via TLR4 receptors (Huang et al., 2012; Nguyen et al., 2007), causing them to secrete a number of pro-inflammatory cytokines, such as, TNFα, IL-1β and IL-6 (interleukin-6) (Fain, 2006). High levels of circulating TNFα contributes to insulin resistance and poor glucose tolerance (Hotamisligil et al., 1993) and inflammatory markers are also predictive for type 2 diabetics. For example, IL1RA is elevated in obese patients before the onset of T2D as a compensatory mechanism to counteract the increase in IL-1β levels (Meier et al., 2002). An increase in circulating cytokines provided an environment for chronic exposure of the β-cells to these harmful stimuli, and similar to T1D, is thought to lead to β-cell death by apoptosis.

In later stages of T2D, there is also hyperglycemia caused by β-cell dysfunction and death. Similar to T1D, type 2 diabetics are also at risk to develop micro- and macrovascular complications, such as cardiovascular disease (CVD), retinopathy and renal failure (Murea et al., 2012). Thus both T1D and T2D, despite having different initial causes, are similar in their inflammatory aspects causing β-cell dysfunction and eventually death. The eventual outcome of both diseases is a loss of the insulin producing cells, causing a dysregulation of glucose homeostasis, which can only be managed by pharmacological intervention or islet transplantation

treatments. Therefore, understanding how to limit β-cell death can be applied therapeutically to both types of diabetes.

β-cell Death by Apoptosis

β-cell death in diabetes is linked to apoptosis. β-cell apoptosis can be mediated through an extrinsic or intrinsic pathway. Cell death via the extrinsic pathway occurs through Fas and FasL interaction (Peter and Kramer, 1998). Pro-inflammatory cytokine exposure of islets induces the expression of Fas on β-cells, making them more susceptible to FasL binding. Additionally, TNFα binding to its cognate receptor (TNFR) (Locksley et al., 2001) also activates the extrinsic pathway. These receptors bind their adaptor protein, FADD and TRADD, respectively, which cleave and activate caspase-8, ultimately leading to apoptosis through activation of caspase-3 (Figure 1) (Locksley et al., 2001; Peter and Kramer, 1998). Caspases, or cysteine-aspartic proteases, are first inactive pro-caspase proteins that are then cleaved into two pieces, which need to hetero-dimerize to become active to carry out their functions (Alnemri et al., 1996).

The intrinsic pathway also is an important arm of cell death by apoptosis. This is cell death based on non-receptor mediated activation of the apoptosis pathway and is initiated by the mitochondria (Elmore, 2007). Apoptotic cues such as loss of growth signals or exposure to cytokines causes inner mitochondrial membrane changes, leading to the release of pro-apoptotic proteins cytochrome-c (CytoC) (Garrido et al., 2006) and Smac/DIABLO (Du et al., 2000) from the inner membrane space to the cytosol. Once in the cytosol, these proteins activate caspase-9 activity or inhibit IAP (inhibitors of apoptosis), respectively, committing the cell to undergo apoptosis (Elmore, 2007). The mitochondrial apoptotic events are mediated by Bcl-2 family

members, such as Bcl-2 and Bcl-XL which are anti-apoptotic and Bax, Bid, and Bim, which are pro-apoptotic (Cory and Adams, 2002). Bcl-2 and Bcl-XL inhibit mitochondrial membrane permeabilization, effectively preventing the release of CytoC. Whereas, Bim, Bax and Bid, can either inhibit Bcl-2 and Bcl-XL, or promote membrane permeabilization, causing CytoC release (Cory and Adams, 2002). There is cross-talk between the extrinsic and intrinsic pathways, since the Fas pathway can cause mitochondrial damage leading to activation of Bid (Figure 1) (Elmore, 2007).

Both of these pathways converge on executioner caspases, such as caspase-3, -6, -7, which are ultimately responsible for the changes seen in apoptotic cells, including DNA fragmentation and membrane blebbing (Elmore, 2007; Cory and Adams, 2002). Caspase-3 can be activated by the most initiator caspases (caspase-8, -9 or -10) and once activated, leads to DNA fragmentation and formation of apoptotic bodies, irreversibly committing the cell to apoptosis (Elmore, 2007).

Glucagon-like Peptide 1 (GLP-1) and Diabetes

The discovery that β-cell death is an integral part of the pathogenesis of diabetes lead to great interest in finding chemical entities, both endogenous and synthetic, that could prevent β-cell damage and apoptosis. Glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists are a class of pharmacological intervention therapies that promote survival of β-cells and are already being used to treat diabetes. GLP-1 is encoded in the proglucagon gene and is a posttranslational proteolytic product of the proglucagon precursor. It is secreted from L-cells found in the more distal regions of the intestine. Primarily, GLP-1 secretion is post-prandial, particularly in

response to a meal high in fats and carbohydrates. One of the main functions of the hormone is to enhance glucose stimulated insulin secretion from β-cells (Baggio and Drucker, 2007). The half-life of GLP-1 is under 2 minutes due to rapid inactivation by DPP-4 (dipeptidyl peptidase-4) (Deacon et al., 1995), limiting this use of the native form of the peptide in a clinical setting.

GLP-1 binds to its cognate receptor, GLP-1R, which is a seven pass transmembrane GPCR receptor. Interestingly, GLP-1R can also bind other agonists such as Exendin-4. Exendin-4 was originally isolated from the saliva of the *Heloderma suspectrum*, and is a long-lasting analog of GLP-1, which can activate mammalian GLP-1R and functions the same as native GLP-1 (Baggio and Drucker, 2007). For example, agonist binding of GLP-1R on β-cells activates adenylyl cyclase to stimulate intracellular cyclic AMP (cAMP) production (Figure 2). Activation of cAMP in β-cells increases insulin secretion and promotes β-cell survival. Cyclic AMP increases insulin secretion in β-cells by ultimately increasing intracellular calcium concentrations, promoting insulin release. Moreover, cAMP signalling in the β-cell confers GLP-1's cyto-protective effect either through activation of CREB (cAMP response element-binding protein) leading to Bcl-2 and Bcl-XL expression or suppression of caspase-3 activation, following Akt-PKB activation (Figure 2) (Baggio and Drucker, 2007; Jhala et al., 2003; Wang and Brubaker, 2002).

Defects in insulin secretion and β-cell death are hallmarks of the pathophysiology of diabetes. GLP-1 can potentiate insulin secretion and is cyto-protective, making GLP-1R agonists effective treatments for type 2 diabetes. In fact, the incretin response is deficient after food intake in type 2 diabetic patients (Nauck et al., 1993). However, because GLP-1 has a short half-life, long lasting agonists are used, such as Exendin-4. Indeed, Exendin-4 is more potent than GLP-1 in lowering blood glucose in diabetic animal models (Young et al., 1999), and in diabetic

patients (Fineman et al., 2003), using the pharmacological analog, Exenatide. An alternative drug, Liraglutide, is more stable than Exenatide and just as efficient. Because of its increased stability, only one injection daily of Liraglutide is needed as compared to two for Exenatide (Degn et al., 2004). Alternatively, drug therapy to inhibit the action of DPP-4 is also effective to prevent the rapid degradation of secreted and circulating native GLP-1. Drugs to inhibit DPP-4 action, such as Vildagliptin and Sitagliptin, increase plasma concentrations of incretin hormones including GLP-1, and enhance GSIS and β-cell function (Baggio and Drucker, 2007). Even though GLP-1R agonists or DPP-4 inhibitors are being used to treat T2D, their effects may also be beneficial for type 1 diabetics. Exenatide improves insulin secretion in patients with transplanted islets; however, there is no evidence to suggest any change in β-cell survival or proliferation (Ghofaili et al., 2007).

GLP-1R agonists have been therefore shown to improve glucose homeostasis in diabetic patients mostly by potentiating insulin secretion. There is limited evidence to suggest that GLP-1R agonists and DPP-4 drugs can impact β-cell growth and survival in patients. Therefore, understanding the molecular mechanisms responsible for diabetes and potentiating β-cell growth and survival pharmacologically could enhance the efficacy of this therapeutic option (Lovshin and Drucker, 2009).

Mouse Models of Diabetes

To better understand the molecular mechanisms of human diabetes and pharmacological interventions, mouse models of diabetes are often utilized. Common mouse models to mimic type 1 diabetes mouse models are streptozotocin (STZ) treated mice or non-obese diabetic

(NOD) mice. Popular models of type 2 diabetes are the *ob/ob*, *db/db* genetic models, or mice fed a high-fat diet to cause diet induced obesity and diabetes (Sakata et al., 2012). Type 2 rodent diabetes models are more related to an increased incidence of insulin resistance. Moreover, initially β-cells in type 2 mouse models are able to proliferate to compensate for the insulin resistance occurring in the periphery (Prentki and Nolan, 2006) so β-cell death is not immediate. Therefore, to study β-cell death early on, type 1 diabetes models may be more relevant.

STZ is a chemical toxin similar in structure to glucose and is therefore able to enter cells using the Glut2 glucose transporters (Schnedl et al., 1994; Szkudelski, 2001). In the islet, only β-cells express Glut2, thus STZ selectively enters this cell type (Zhang et al., 2012) and only results in β-cell depletion leaving other islet cell types intact. Additionally, other cells of the islet, such as the α-cells proliferate after STZ treatment further demonstrating the specificity of STZ to the β-cells (Li et al., 2000). STZ treated mice develop insulitis from β-cell death and survive 1 – 2 months after the onset of hyperglycemia. However, the type of cell death differs depending on the dose of STZ administered. β-cell death can either occur by necrosis or apoptosis by two mechanisms; (i) a direct toxic effect on the cell and; (ii) a delayed immune infiltration of the islet. To cause β-cell death by apoptosis, multiple low dose STZ injections are administered. Concentrations vary from 40 – 50 mg/kg and are administered daily for 5 days (Li et al., 2003; O'Brien et al., 1996). Generally, apoptotic cell number is highest between the last day of STZ injections and 48 hours afterwards (Li et al., 2003; O'Brien et al., 1996). Additionally, a second peak of apoptosis 6 days after the last STZ injection is associated with β-cell death a peak of immune infiltration (O'Brien et al., 1996). Initially, the low dose of STZ increases in DNA fragmentation (Morgan et al., 1994) and nitric oxygen radicals (Szkudelski, 2001), eventually causing DNA damage and cell death. Damaged and dying cells then present self-antigens, which

the immune system are not tolerant to causing immune mediated β-cell death (O'Brien et al., 1996). Conversely, single high dose STZ injections, approximately between 100 – 200 mg/kg, cause β-cell death by necrosis through direct cytotoxicity of STZ. These mice become hyperglycemic within 48 hours of the last injection (Wu and Huan, 2008), whereas multiple low dose STZ injections causes hyperglycemia within 1 – 5 days after the last injection (varies depending on mouse age, gender and strain) (O'Brien et al., 1996)

The pathology in non-obese diabetic mice is initiated by immune infiltration of the islets by T-cells, B-cells and lymphocytes causing insulitis, eventually leading to β-cell death (Leiter et al., 1987; Sakata et al., 2012). These mice progressively develop diabetes, between 16 – 20 weeks for females and 21 – 28 weeks for males (Leiter et al., 1987). These mice are able to survive without exogenous insulin 1 – 2 months after detection of hyperglycemia (Leiter et al., 1987). Additionally, the progression of the disease in this model closely resembles the pathogenesis of human type 1 diabetics because of similar immune infiltration, and is therefore a popular model to investigate the mechanisms behind this disease. This model is more physiologically relevant in terms of human disease, because β-cell death is spontaneous and primarily caused by immune mediated cell death, rather than exposure to a toxin. However, STZ, although not identical to the pathogenesis in human disease, is considered an appropriate model to investigate the role of genes in the context of diabetes.

Peroxisome proliferator activated receptor gamma (PPARγ) co-activator 1-alpha (PGC-1α)

PGC-1α is a nuclear transcriptional co-activator that is responsible for oxidative metabolism and mitochondrial biogenesis (Handschin and Spiegelman, 2006). PGC-1α is of

interest to study in diabetes because β-cell function relies greatly on functional mitochondria. Mitochondria are necessary to metabolize glucose and promote insulin secretion (Maechler et al., 2010). In fact, mitochondrial dysfunction is associated with the progression of the disease (Sivitz and Yorek, 2010). PGC-1α was discovered in brown adipose tissue (BAT), by a yeast-two-hybrid screen to identify PPARγ interacting proteins (Puigserver et al., 1998). PGC-1α is robustly induced in BAT upon cold exposure, can be activated by β-adrenergic signalling in brown fat, and regulates the expression of UCP-1 (uncoupling protein-1; a classical brown fat marker and regulator of heat dissipation) (Puigserver et al., 1998). In addition to PGC-1α, two other family members have been described; PGC-1β (Lin et al., 2002a) and PRC (PGC-related co-activator) (Andersson and Scarpulla, 2001). PGC-1α has been associated with increased risk of developing diabetes (Ek et al., 2001; Hara et al., 2002) and is dyregulated in the muscle and islets of type 2 diabetics (Mootha et al., 2003; Olsson et al., 2011).

PGC-1α is approximately a 113 kilo Dalton (kDa) protein and contains an activation domain, repression domain, arginine and serine (RS) rich domain and a RNA binding domain (Figure 3) (Lin et al., 2005). PGC-1α's co-activator properties arise from its ability to bind histone acetlytransferase (HAT) containing proteins such as CBP (CREB binding protein), p300 and SRC-1 (steroid receptor coactivator-1) in the activation domain (Puigserver et al., 1999). These interactions open heterochromatin, allowing for active transcription of genes. Furthermore, PGC-1α can bind subunits of the mediator complex (in the RS and RNA binding domain), which associates with RNA polymerase II, thus assisting in transcriptional initiation (Wallberg et al., 2003). Moreover, through its LXXLL motifs, PGC-1α is able to bind a variety of different nuclear receptors (Puigserver et al., 1998), such as PPARγ (Puigserver et al., 1998), ERRα (estrogen-related receptor α) (Mootha et al., 2004) and NRF-1,2 (nuclear respiratory

factor-1,2) (Mootha et al., 2003; Wu et al., 1999). In addition, PGC-1α binds and activates transcription factors that are not nuclear receptors, such as MEF2 (myocyte enhancer factor-2) (Lin et al., 2002b) and FOXO1 (forkhead box protein O1) (Puigserver et al., 2003). Finally, PGC-1α can be phosphorylated in its repression domain by Sirt1, a protein de-acetylase, to repress its coactivator functions (Lin et al., 2005).

Co-activation of ERRα and NRF-1,2 by PGC-1α regulates mitochondrial biogenesis and mitochondrial gene expression (Evans and Scarpulla, 1990; Mootha et al., 2004; Schreiber et al., 2004; Virbasius et al., 1993). NRF-1 and -2 are able to bind a variety of mitochondrial genes, including cytochrome c oxidase subunit 4 (COXIV), β-ATP synthase (ATP5B), CytoC and mitochondrial transcription factor A (Tfam) (Evans and Scarpulla, 1990; Virbasius et al., 1993). PGC-1α is a potent co-activator of NRF-1 and -2, and when ectopically expressed in muscle, gene expression of these nuclear transcription factors increases and activates their downstream targets (Wu et al., 1999). Therefore, PGC-1α is an important regulator of mitochondrial gene expression. Additionally, PGC-1α's ability to increase mitochondrial biogenesis is blocked by NRF-1 inhibition (Wu et al., 1999). Furthermore, co-activation of ERRα by PGC-1α also initiates mitochondrial biogenesis (Mootha et al., 2004; Schreiber et al., 2004) and this interaction acts upstream of NRF-1 activation (Mootha et al., 2004).

Due to its potential role in regulating mitochondrial function, PGC-1α was first identified as a master regulator of adaptive thermogenesis in BAT. Ectopic expression of PGC-1α in murine BAT cells causes an increase of UCP-1, mitochondrial respiratory enzyme subunits, COXII (cytochrome oxidase subunit II) and COXIV, and stimulates mitochondrial biogenesis as indicated by an increase in mitochondrial DNA content (Puigserver et al., 1998). Additionally, expression of PGC-1α in white adipose tissue (WAT) increases expression of UCP-1 and

mitochondrial biogenesis, creating a brown-fat-like phenotype of the WAT (Fisher et al., 2012; Puigserver et al., 1998).

The ability of PGC-1α to bind a variety of transcription factors allows it to have different physiological functions in a number of different tissues. PGC-1α is also induced upon fasting in the liver. During fasting, it is critical to increase gluconeogenesis and shift fuel utilization from glucose to fatty acids in order to maintain glucose homeostasis. PGC-1α expression regulates these metabolic responses in the liver by activating hepatic transcription factors, such as FOXO1 (Puigserver et al., 2003) and HNF4α (hepatocyte nuclear factor 4 alpha) (Rhee et al., 2003) to promote gluconeogenesis and PPARα to promote fatty acid oxidation (Vega et al., 2000). PGC-1α interaction with FOXO1 increases the expression of Pck1 (phosphoenolpyruvate carboxykinase 1) and G6pc (Glucose-6-phosphatase) (Puigserver et al., 2003); both critical enzymes in the gluconeogenic pathway. Additionally, PGC-1α also acts via HNF4α to induce expression of G6pc, promoting expression of another gluconeogenic gene, PEPCK (Phosphoenolpyruvate carboxykinase) (Rhee et al., 2003). Furthermore, Vega et al. observe that PGC-1α and PPARα cooperatively induce fatty acid oxidation genes, such as CPT1 (Carnitine palmitoyltransferase I), MCAD (medium chain acyl-coenzyme A dehydrogenase) and LCAD (long chain acyl-coenzyme A dehydrogenase) (Vega et al., 2000). Therefore, PGC-1α plays a critical role in the liver to activate metabolic programs to maintain the amount of circulating glucose and switch fuel consumption to fatty acids to ensure glucose homeostasis during a period when energy intake is limited.

Furthermore, PGC-1α regulates metabolism in skeletal muscle. Here, PGC-1α is induced during exercise and by β-adrenergic agonists (Baar et al., 2002). Expression in muscle can

promote the switch of muscle fibers from a fast-twitch phenotype, to a slow-twitch fiber phenotype by binding to MEF2. These fibers are more oxidative and are associated with endurance training (Lin et al., 2002b; Millay and Olson, 2013). PGC-1α serves as a sensor for external cues to adapt skeletal muscle to exercise and its metabolic needs.

Additionally, PGC-1α plays an important role in the heart, brain and intestines. PGC-1α is expressed abundantly in the heart, likely due to the heart's great demand for ATP. Over-expression of PGC-1α in cultured cardiomyocytes induces expression of mitochondrial genes (MCAD and CPT-1) and stimulates mitochondrial biogenesis (Lehman et al., 2000). Conversely, loss of PGC-1α in the heart leads to cardiac dysfunction (Arany et al., 2005; Leone et al., 2005). The role of PGC-1α in the brain is less understood; however, a whole body knock out of PGC-1α causes behavioural abnormalities including hyperactivity (Leone et al., 2005). These defects are due to axonal degradation in the brain (Lin et al., 2004), which is thought to be caused by an increase of ROS due to mitochondrial dysfunction (Lin et al., 2005). Finally, PGC-1α is an important regulator in intestinal epithelium. Ectopic expression of PGC-1α induces expression of key mitochondrial genes, such as Tfam, MCAD and ATP5B and an increase in mitochondrial biogenesis as seen by an increase in mitochondria DNA copy number (D'Errico et al., 2001). An increase in mitochondrial activity in the intestinal epithelium is necessary to maintain their constant turnover, so PGC-1α is important in this process and also regulates cell fate (D'Errico et al., 2011). Even though PGC-1α confers different functions in a variety of tissues, almost all of these ascribed functions are related to an increase in metabolic activity of the tissue, an increase in mitochondrial function and number, and are important for sensing changes (i.e. nutrient availability) in the extracellular environment.

PGC-1α and β-cell Biology

While one family member, PGC-1α, was shown to be expressed in β-cells, less is known about the role of PGC-1α in β-cells. Interestingly, PGC-1α is increased in β-cells of rodents in the context of diabetes (Yoon et al., 2003). Previous studies investigating PGC-1α function in β-cells have used anti-sense oligonucleotides (ASOs) or short-interfering RNA (siRNA) to knock-down co-activator expression or adenovirus for over-expression (De Souza et al., 2005; De Souza et al., 2003; Kim et al., 2009; Yoon et al., 2003). There is only one genetic study, which utilizes a tetracycline-dependent inducible gene system *in vivo* expression to enable PGC-1α over-expression specifically in β-cells (Valtat et al., 2013).

Targeting PGC-1α by ASOs achieved an 80% knock down, which decreased blood glucose in a diet-induced diabetes model in mice. Additionally, these mice exhibited an increase in serum insulin levels, were more glucose tolerant and more insulin sensitive (De Souza et al., 2005). The same group demonstrated that PGC-1α inhibition (using ASOs) reversed cold-inhibition of insulin secretion and prevented UCP-2 expression in islets upon cold exposure (De Souza et al., 2003). However, it is important to note that ASOs *in vivo* also target other organs (i.e. liver and adipose tissue) very efficiently and it is impossible to dissociate the contribution of PGC-1α knock-down in these other organs to the phenotype.

The role of PGC-1α in glucolipotoxicity-associated β-cell dysfunction was evaluated by siRNA. Glucolipotoxic (high glucose, high lipid) environments decrease β-cell insulin content and increase endogenous expression of PGC-1α (Kim et al., 2009; Zhang et al., 2005). Insulin content was decreased by both glucolipotoxicity and over-expression of PGC-1α using adenovirus (Ad-PGC-1α) in cultured rat islets. This effect on insulin content was reversed by

adenoviral expression of siPGC-1α (Ad-siPGC-1α) (Kim et al., 2009). Since decreasing PGC-1α was beneficial for β-cell health *in vitro*, they also investigated whether reduced PGC-1α expression protects against β-cell dysfunction *in vivo*. Using 90% pancreatectomized mice to induce hyperglycemia, they delivered Ad-siPGC-1α to the islets by the celiac artery. Mice injected with Ad-siPGC-1α are moderately more glucose tolerant, have lower fasting glucose and higher fasting insulin levels (Kim et al., 2009). Consistent with this study, over-expressing PGC-1α by adenovirus in cultured mouse islets *in vitro* negatively effects glucose stimulated insulin secretion and these islets cannot rescue STZ induced hyperglycemia through transplantation (Yoon et al., 2003). These studies demonstrate that reduced PGC-1α expression in rodent islets positively impacts β-cell function, whereas PGC-1α over-expression is detrimental for functionality. However, adenoviral delivery via the celiac artery is not β-cell specific and therefore the effects of PGC-1α knock-down or over-expression can be confounded by off target effects. These caveats demonstrate the need to manipulate PGC-1α expression in genetic models specifically in β-cells.

Mice expressing PGC-1α downstream of a tetracycline-dependent promoter (TetO) crossed with mice expressing the tetracycline transactivator (tTA) under the Insulin1 gene promoter allows β-cell specific over-expression of PGC-1α that can be turned off in the presence of doxycycline (DOX). Over-expression of PGC-1α in β-cells during mouse development and throughout life causes hyperglycemia, lower fed insulin serum levels, glucose intolerance and altered glucose-stimulated insulin secretion (GSIS) in 6-month old mice. These mice also have reduced β-cell mass and reduction of insulin content. In mice with increased β-cell PGC-1α only during adulthood (given DOX from birth until 4 months of age to prevent PGC-1α over-expression) have normal glucose tolerance and GSIS at the same age, suggesting that the

effects of PGC-1α on β-cell function are caused by neonatal over-expression of the gene (Valtat et al., 2013). This demonstrates that PGC-1α over-expression *in vivo* specifically in β-cells negatively impacts β-cell function only when over-expressed neonatally.

The literature for PGC-1α function in β-cells is limited; however, these rodent studies complement each other. Simply these studies have shown that a knock-down of PGC-1α in β-cells increases β-cell function and over-expression impairs it. This data suggests that PGC-1α expression could be a contributing factor to diabetes and inhibiting it could be a possible therapy by improving β-cell function. However, PGC-1α expression is decreased in human type 2 diabetic patients (Ling et al., 2008), raising the possibility that loss of PGC-1α a may have yet unidentified pathological effects on β-cell health. The recent discovery of novel PGC-1α isoforms adds and additional layer of complexity making it of interest to elucidate any potential roles of these isoforms in β-cell physiology.

PGC-1α Isoforms

In addition to the canonical PGC-1α transcript (herein referred to as PGC-1α1), there recently has been the emergence of new isoforms. The first isoforms published were PGC-1α-b and PGC-1α-c (Figure 4) (Miura et al., 2008). These differed from canonical PGC-1α only in their N-terminus, with a 16 amino acid difference due to alternative splicing of exon 1 (Miura et al., 2008). This was the first report of a novel exon1 of PGC-1α, found approximately 14 kilobases (kb) upstream of the canonical exon 1. This novel exon is alternatively spliced to a shared exon 2 giving rise to two new isoforms (Miura et al., 2008).

Recombinant PGC-1α-b and PGC-1α-c are functional as transcriptional co-activators *in vitro* and *in vivo*. Fusion of the isoform cDNA to Gal4-DB (DNA binding domain) constructs and transfection into HEK293 cells activated a UAS-luciferase reporter (Miura et al., 2008). Furthermore, transgenic mice over-expressing PGC-1α-b and PGC-1α-c in muscle, show increased expression of classical PGC-1α regulated genes, such as Tfam, COXII, COXIV and MCAD. Additionally, after injection of mouse muscle with clenbuterol (a β-adrenergic activator) or exercise, total PGC-1α expression increased, which induces, PGC-1α1, PGC-1α-b and PGC-1α-c (Miura et al., 2008). It was later shown that PGC-1α-b is induced upon exercise in human muscle, and constitutes approximately 10% of the total PGC-1α transcripts after 2 hours of exercise (Norrbom et al., 2011).

PGC-1α-b and PGC-1α-c were also identified in liver and brown adipose tissue (BAT) using primers that detect only the exon 1 sequence. Unlike PGC-1α1, these isoforms are not induced in the liver upon fasting; however, they are moderately increased upon cold exposure in the BAT, like PGC-1α1.

Similar to PGC-1α-b and PGC-1α-c, two other isoforms were recently published, PGC-1α2 and PGC-1α3 (Figure 4) (Chinsomboon et al., 2009; Ruas et al., 2012). PGC-1α2 and PGC-1α3 have the same upstream exon 1 as PGC-1α-b- and PGC-1α-c, respectively (Figure 4); however, this group identified a novel alternative promoter. This promoter lies approximately 14kb upstream of the proximal promoter, similar to the distance where the alternative exon 1 is found for PGC-1α-b and PGC-1α-c (Miura et al., 2008). While PGC-1α-b and PGC-1α-c were not originally described as being transcribed from this new alternative promoter, with its discovery it is likely that these isoforms are regulated by this site. However, PGC-1α-b / PGC-1α-c and PGC-1α2 / PGC-1α3 differ in the majority of their exons downstream of the novel

exon 1 (Figure 4). Chinsomboon et al. noted that PGC-1α2 and PGC-1α3, similar to PGC-1α-b and PGC-1α-c, were expressed relatively abundantly in muscle and BAT, but absent from other metabolically active tissues, such as the liver (Chinsomboon et al., 2009). In contrast to PGC-1α-b and PGC-1α-c, they also demonstrated that after exercise and β-adrenergic activation, the total amount of PGC-1α expressed is attributed only to isoforms from the alternative promoter. Since Miura et al. used primers specific for exon 1 to characterize PGC-1α-b and PGC-1α-c, which also recognize PGC-1α2 and PGC-1α3 respectively, it is possible that expression changes reported could be in part due to changes in PGC-1α2 and PGC-1α3. Regardless of the differential expression, the functions of PGC-1α2 and PGC-1α3 are unknown and those of PGC-1α-b and PGC-1α-c are closely related to that of canonical PGC-1α1.

Another recently identified isoform, NT-PGC-1α, is transcribed from the proximal (or canonical) promoter and encodes a truncated version of full-length original PGC-1α (Zhang et al., 2009). This isoform was discovered while cloning full length PGC-1α from mouse BAT cDNA library. It was found to have a 31 basepair (bp) inclusion in intron 6 causing an in frame premature stop codon in the transcript, resulting in translation of a truncated version of PGC-1α (Figure 4). NT-PGC-1α is induced in BAT upon cold exposure and the liver upon fasting. NT-PGC-1α induces expression of UCP-1 and CPT1 in cultured adipocytes and mitochondrial biogenesis in cultured brown adipocytes (Zhang et al., 2009). Alternative splicing of intron 6 does not favour one transcript over the other in white adipose tissue (WAT), spleen or heart; however, splicing appears to favour NT-PGC-1α expression in the brain due to an unknown mechanism (Zhang et al., 2009). Authors also note that, although mRNA levels are similar in tissues examined, protein expression of NT-PGC-1α is more robust compared to PGC-1α1. This is attributed to decreased susceptibility for proteosomal degradation. Although NT-PGC-1α

seems physiologically regulated similarly to PGC-1α1 at the level of transcription, it has unique properties such as increased protein stability (Zhang et al., 2009).

Up to this point, all biologically active PGC-1α isoforms regulate the same transcriptional pathways as PGC-1α1 and have closely related functions surrounding mitochondrial metabolism. However, in 2012, Ruas et al. published the first report of a novel isoform, PGC-1α4, having a distinct function to PGC-1α1 in muscle (Ruas et al., 2012). Like NT-PGC-1α, PGC-1α4 is truncated due to the introduction of an in frame stop codon in intro 6, however, PGC-1α4 is transcribed from the alternative promoter (Figure 4) and thus differs in its exon 1 sequence (Figure 5).

A microarray screen of myotubes adenovirally over-expressing PGC-1α1, PGC-1α2, PGC-1α3, and PGC-1α4, revealed that PGC-1α1 and PGC-1α4 regulate only 98 genes similarly, with the majority of genes regulated by either isoform being unique. From this screen, they discovered that PGC-1α4 does not regulate classical PGC-1α genes, such as CytoC, COXVb (cytochrome c oxidase subunit V b), Glut4 (glucose transporter type 4), but does regulate genes involved in cell growth and proliferation of muscle, such as IGF1 (insulin-like growth factor 1) and MSN (myostatin). Furthermore, *in vitro* adenoviral over-expression of PGC-1α4 causes muscle fiber hypertrophy and knock-down of PGC-1α4 prevents this action in response to clenbuterol. Additionally, mice over-expressing PGC-1α4 in muscle either by adenovirus, plasmid injection or in a transgenic model, have increased muscle size and strength or decreased muscle wasting in limb suspension and cancer cachexia models (Ruas et al., 2012).

In muscle, PGC-1α1 is responsible for mitochondrial biogenesis and oxidative metabolism in response to exercise, while PGC-1α4 is responsible for muscle hypertrophy and

strength (Millay and Olson, 2013). PGC-1α4 transcripts have been identified in multiple metabolically active tissues and is the only PGC-1α isoform thus far shown to have a unique function.

PGC-1α and Disease

PGC-1α dysregulation is linked to diseases particularly in regards to mitochondrial dysfunction. Neurogenerative diseases such as Parkinson's, Alzheimer's and Huntington's, are associated with a decrease in mitochondrial function and oxidative phosphorylation (OXPHOS) genes (Schon and Manfredi, 2003). Low PGC-1α is also linked to heart disease (Arany et al., 2005) and hepatic porphyrias in humans (Handschin et al., 2005). Furthermore, loss of PGC-1α expression in mice causes hepatic steatosis in the liver (Estall et al., 2009; Leone et al., 2005) and PGC-1α expression is decreased in the livers from patients with varying degrees of non-alcoholic fatty liver disease (NAFLD) (Westerbacka et al., 2007). Moreover, a common PGC-1α glycine to serine substitution polymorphism is correlated to NAFLD in children (Lin et al., 2013).

PGC-1α dysregulation is also strongly correlated to type 2 diabetes; however, it is currently debated whether loss of PGC-1α is a cause or consequence of the disease. PGC-1α dysregulation in muscle has been linked to being both a cause (Schuler et al., 2006) and consequence (Shiff et al., 2009) of diabetes, demonstrating the lack of consensus in the field. In human type 2 diabetics, OXPHOS gene expression is decreased in muscle from these patients, which correlates with a 20% decrease in PGC-1α transcripts (Mootha et al., 2003). Decreased OXPHOS gene expression also correlates with mitochondrial dysfunction in diabetic patients

and is speculated as a potential cause of insulin resistance in the muscle (Mootha et al., 2003). OXPHOS genes are also decreased in islets of type 2 diabetics (Olsson et al., 2011) and PGC-1α expression is low in comparison to control healthy islets (Ling et al., 2008). Moreover knock-down of PGC-1α in human islets prevents insulin secretion (Ling et al., 2008). Interestingly, these findings in human β-cells contradict both the knock-down and over-expression studies in mouse models of PGC-1α. This could be attributed to the fact that PGC-1α is up-regulated in β-cells of obese rodents (Yoon et al., 2003), whereas in diabetic humans it is down-regulated (Ling et al., 2008). This illustrates the differences between rodents models of diabetes and humans and why it is important to eventually study genes of interest in human tissues.

Interestingly, a polymorphism of PGC-1α is also associated with an increased risk in developing type 2 diabetes. A Gly482Ser amino acid substitution is found in various populations, including Danish (Ek et al., 2001) and Japanese populations (Hara et al., 2002); however, this mutation is ethnicity specific, since there was no correlation between this polymorphism and incidence of diabetes in French or Austrian populations (Lacquemant et al., 2002; Oberkofler et al., 2004). Even though the Gly482Ser PGC-1α variant is associated with type 2 diabetes (Ek et al., 2001), it has not been associated with body mass index (BMI), fasting glucose or fasting insulin (Barroso et al., 2006). Currently it is unclear whether PGC-1α dysfunction in diabetes is restricted to the canonical PGC-1α1 transcript or also involves the function of other PGC-1α isoforms. However, it is clear that the dysregulation of PGC-1α can contribute to the pathogenesis of diabetes.

While most assays to characterize reduced PGC-1α expression (typically qPCR) in human disease collectively measure all isoforms of PGC-1α, until recently, characterization of

the role of PGC-1α in disease only focused on the canonical full-length transcript. In mice, PGC-1α over-expression in muscle protects against declining mitochondrial function and increased oxidative damage during aging and improves insulin sensitivity (Wenz et al., 2009). Over-expression of canonical PGC-1α1 inhibited insulin secretion in rodent β-cells (Valtat et al., 2012), while reduced levels seen in diabetic islets from humans include all isoforms (Ling et al., 2008). Furthermore, whole body PGC-1α transgenic mice have hepatic insulin resistance, but improved muscle insulin sensitivity, lower ROS and NF-κB signalling in muscle (Liang et al., 2009). In terms of PGC-1α isoforms, only PGC-1α4 has been investigated within the context of disease and can protect against cancer-induced cachexia in muscle (Ruas et al., 2012).

Objectives

β-cell dysfunction and death are hallmarks of both type 1 and type 2 diabetes. PGC-1α dysregulation is associated with diabetes. OXPHOS genes controlled by PGC-1α are down in muscle (Mootha et al., 2003) and islets (Olsson et al., 2011) and a polymorphism of PGC-1α is associated with an increased risk of developing T2D (Ek et al., 2001; Hara et al., 2002). PGC-1α transcripts are decreased in islets of diabetic humans (Ling et al., 2008); however, it is still unknown whether PGC-1α isoforms play a role in the pathogenesis of diabetes.

We set out to investigate whether PGC-1α isoforms transcribed from the alternative promoter played a role in the pathogenesis of diabetes. Our main objectives were; (i) to determine whether PGC-1α isoforms are differentially regulated in β-cells; (ii) investigate whether these isoforms have unique molecular functions and; (iii) determine whether their dysregulation contributes to the pathogenesis of diabetes.

SECTION II:
MATERIALS AND METHODS

Cell Culture

INS-1 parental cells, between passage 8 to 25, were maintained in RPMI 1640 (Wisent) with 10% FBS (Wisent), 1% penicillin and streptomycin (Wisent), and 1x supplement (10mM HEPES, 1mM sodium pyruvate, 50μM β-mercaptoethanol). INS-1 cells were seeded into 6-well plates at a density of $0.45x10^6$ cells/ml the evening prior to experimentation, unless otherwise stated. Primary islets isolated from wild-type male and female C57Bl/6J mice of various ages, were cultured in RPMI 1640 with 10% FBS and 1% penicillin and streptomycin. Islets were cultured for 2 days prior to experimentation to ensure the death of extraneous acinar tissue and immune cells. All were maintained at 37°C with 5% CO_2.

Islet Isolation

Pancreata from male and female C57Bl6 mice, between 20 – 30 g, were perfused with 0.4 U/mL of Liberase TL enzyme (Roche). Pancreata were digested at 37˚C for 30 minutes. They were disrupted by vigorous shaking by hand 45 times, then mixed with HBSS solution (Wisent) with 0.1% bovine serum albumin and 0.02 M HEPES and centrifuged for 3 minutes at 500 g at 4˚C. After 4 washes in HBSS solution, samples were resuspended in 9 mL Histopaque (density 1.077gml, Sigma), and layered with RPMI 1640 without glucose (Wisent). To separate the islets based on this density gradient, samples were centrifuged for 30 minutes at 400 g at 4˚C. Supernatant was decanted and total volume was completed with HBSS without BSA and HEPES. Samples were centrifuged for a final time for 3 minutes at 500 g at 4˚C, resuspended in 10 mL of RPMI 1640 with 10% FBS and 1% penicillin and streptomycin and picked into untreated plates.

RNA Extraction and cDNA Synthesis

Total RNA from INS-1 cells was extracted using Trizol reagent (Invitrogen), as indicated by the manufacture's protocol. Total RNA from primary isolated islets (minimum 100 islets) was extracted using the RNeasy Micro Kit (Qiagen), as indicated by the manufacturer's protocol.

For cDNA synthesis, first, 1 µg of RNA from INS-1 cells and 400 ng of RNA from isolated islets, were incubated with 1 U/mL DNase at 37˚C for 15 minutes, followed by 15 minutes at 65˚C for DNase heat inactivation. Then, total RNA in a total volume of 20 µl was reverse transcribed with 50 U MultiScribe™ reverse transcriptase (Life Technologies) and 20 U RNase Inhibitor (BioBasic). cDNA was synthesized at 25˚C for 10 minutes, 37˚C for 120 minutes and 85˚C for 5 minutes. 80 µl of water (1:5 dilution) was added to each sample and was stored at -20˚C. cDNA samples were assessed by qPCR.

Quantitative Real-Time PCR

cDNA was subjected to two amplifications, one for the gene of interest and one for the endogenous control hypoxanthine-guanine phosphoribosyltransferase (HPRT). Each condition was in triplicate, in 5 µl reactions in a 384-well plate using Power SYBR® Green PCR master mix (Life Technologies). The cycling program was in two steps, a polymerase activation step for 2 minutes at 50˚C and 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 95˚C and 1 minute of 60˚C, using the Viia7 system from Life Technologies. Data was normalized to the endogenous control and relative mRNA expression was determined using the $\Delta\Delta C_t$ method. Graphpad Prism was used for graphing results and performing statistical analysis.

Protein Analysis

Cells were lysed in RIPA buffer (50mM Tris, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) plus protease cocktail inhibitor (Calbiochem) and protein concentrations were estimated by DC Assay. Equal amounts of total protein (40-60 µg) were resolved by SDS-PAGE on 10% poly-acrylamide gels for PGC-1α immunoblots and 12% gels for cleaved caspase-3 immunoblots, and were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). PGC-1α isoforms were detected using the anti-PGC-1α mouse 4C1.3 antibody (1:1000, Millipore™) and activation of caspase-3 was detected using a cleaved caspase-3 antibody (1:500, Cell Signalling). A β-actin (1:50000) antibody was used as a loading control. Both, PGC-1α and cleaved caspase-3 primary antibodies were diluted in 2% milk and membranes were incubated at 4˚C overnight. The primary β-actin antibody was diluted in Tris buffered saline with 0.1% Tween (TBST) and incubated for 1 hour at room temperature. Secondary antibodies used were goat anti-mouse IgG antibody conjugated to horseradish peroxidise (HRP) (1:10000, GenScript) and goat anti-rabbit IgG antibody conjugated to HRP (1:5000, GenScript), for the PGC-1α and cleaved caspase-3 antibodies, respectively. Signal detection was performed using an ECL detection system (GE Healthcare©). Films were exposed for 1 hour, except for β-actin, which was exposed for 1 minute or less.

Genotyping

Genotyping of mice with PGC-1α floxed alleles with or without the cre transgene was performed on DNA prepared from tails of 3 week old mice. DNA was extracted by boiling the tails at 100˚C in 200 µl of 50 nM sodium hydroxide (NaOH) for 20 minutes. After 20 minutes,

20 µl of 1M TRIS pH 6.8 was added to the tail digested and vortexed for 15 seconds. They were frozen and stored at -20˚C.

PGC-1α floxed alleles were genotyped using forward (5'- TCCAGTAGGCAGAGATTT ATGAC -3') and reverse (5'- TGTCTGGTTTGACAATCTGCTAGGTC -3') primers which flank the 5' loxP site. Genotyping was performed by polymerase chain reaction (PCR) for 35 cycles at 96˚C for 1 minute, 58˚C for 1 minute and 72˚C for 1 minute. Expected band patterns are; one band of 400 bps for the mutant; 1 band each of 400 bps and 360 bps for a heterozygote and; 1 band of 360 bps for WT (Figure 6A). Cre recombinase positive alleles were genotyped using a forward (5'- TAAGGGCCCAGCTATCAATGGGAA -3') primer in the mouse insulin promoter and a reverse (5'- GTGAAACAGCATTGCTGTCACTT -3') primer in the Cre transgene. Genotyping was performed by PCR for 35 cycles at 94˚C for 30 seconds, 60˚C for 1 minute and 72˚C for 1 minute. The expected band pattern is 1 band at 800 bps for Cre positive mice (Figure 6B). Both genotyping reactions were visualized on 2% agarose gels stained with Ethidium Bromide.

Induction of PGC-1α Isoforms

INS-1 and primary isolated islets were treated with forskolin (FK) (10µM) and Exendin-4 (Ex-4) (50nM) for 2 hours (for RNA isolation) or 4 hours (for protein isolation, to allow sufficient time for mRNA transcription and protein synthesis), or DMSO or water as the vehicle, respectively. Additionally, INS-1 cells were treated with cytokines (TNFα (50ng/ml), IFNγ (50ng/ml) and IL-1β (10ng/ml)) or water as the vehicle, for 12 hours prior to harvesting cells for RNA or protein isolation. qPCR was performed using isoform specific primers to identify the

relative mRNA expression of each and protein analysis using an anti-PGC-1α antibody (Millipore).

Cleaved Caspase-3 Assay

INS-1 cells were infected with adenoviruses expressing GFP, PGC-1α1 and PGC-1α4, previously described in (Ruas et al., 2012), with a titer of 0.625 x 10^7 ifu/ml for 8 hours, after which the virus media was changed for fresh media. 30 hours post infection, cells were treated with cytokines for 18 hours, for a total of 48 hours. Total protein was isolated in RIPA buffer and the same protein lysates were resolved in 2 separate SDS-PAGE gels, one to visualize PGC-1α isoform expression and the other for cleaved caspase-3.

PGC-1α Knock Out and Streptozotocin *in vivo* Experiment

PGC-1α floxed/floxed MIP-Cre (PGC-1α$^{fl/flCre+}$) and PGC-1α floxed/floxed (PGC-1α$^{fl/fl}$) (age/gender-matched littermate controls) mice, on a mixed background, were gavaged with tamoxifen (100mg/kg, Sigma Aldrich) for 10 days, separated by a 2 day rest in between. After 2 weeks recovery to allow complete gene excision and elimination of tamoxifen, mice were injected intraperitoneally (ip) with Streptozotocin (STZ) (Bioshop) in 0.1mM sodium citrate pH 4.5, at a dose of 50mg/kg everyday for 5 days. Random fed blood glucose measurements were taken bi-weekly after STZ injections to monitor blood glucose over time. 1 day post-final STZ injection, select mice were sacrificed (n=7-8) and the pancreata were harvested and fixed in 4% PFA for histology, for PGC-1α$^{fl/fl}$, PGC-1α$^{fl/flCre+}$, WT and MIP-Cre only mice, to control for possible effects of the cre-recombinase transgene. These samples were embedded in paraffin and sectioned (5 μm) for immunohistochemical staining for cleaved-caspase-3. 11 days post-STZ

injection, an early OGTT (n=9 PGC-1α$^{fl/fl}$, n=8 PGC-1α$^{fl/flCre+}$) was performed following an overnight fast and gavaged with 1g/kg of glucose. 18 days post-final STZ injections, glucose measurements and blood samples were taken via tail vein following an overnight fast and then after re-feeding for 2 hours. Lastly, a late OGTT (n=6 PGC-1α$^{f/lfl}$, n=5 PGC-1α$^{fl/flCre+}$) was performed, 32 days after the last STZ injection following an overnight fast and were gavaged with 1g/kg of glucose. All glucose measurements were taken using the FreeStyle Lite glucometer (Abbot Diabetes Care). Mice were sacrificed one week after and pancreata were fixed in 4% paraformaldehyde. Mice were maintained on a chow diet throughout the duration of the experiments. Mice were housed in a mouse-specific pathogen-free (SPF) facility, with a 12 hour light and dark cycle. All mouse experiments were approved by the Institut de recherches cliniques de Montréal Animal Care Committee (protocol number 2011.14) and complied with the Canadian Council of Animal Care rules.

Insulin Enzyme-Linked Immunosorbent Assay (ELISA)

Serum insulin concentration was determined using an ultrasensitive ELISA (Alpco), according to the manufacturer's protocol. Serum was collected from the mice via the tail vein, into 0.4 U/mL of heparin. Serum was separated from blood cell fraction by centrifugation at 15,000 rpm for 5 minutes. 25 µl of serum was used per well for the insulin ELISA. Standards and controls recommended for 25 µl reactions were also used. Samples were incubated with 100 µl of the detection antibody at room temperature for 2 hours, shaking at 800 rpm. Samples were read at 450 and 650 nm, to determine OD of the samples and of the background, respectively. Data was analyzed using GraphPad Prism.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5 µm thick paraffin sections. Before staining, sections were de-waxed in Xylene 4 times for 5 minutes, followed by hydration in decreasing concentrations of alcohol, from 100 to 70 percent, for 3 minutes. Sections were quenched in endogenous peroxidase and antigen retrieval was performed using Rodent Decloaker (Biocare) for 40 minutes at 85˚C, and blocked with Rodent Block M (Biocare) for 20 minutes. Sections were incubated with the cleaved caspase-3 antibody (Cell Signalling) diluted 1:150 in diluent solution, and were incubated overnight at 4˚C. The next day, the sections were incubated with rabbit HRP polymer secondary antibody (Biocare) for 20 minutes followed by colour developing using the DAB Chromogen kit (Biocare) for 3 minutes. Sections were counterstained using Mayer's hematoxylin, dehydrated using increasing concentrations of alcohol from 70 to 100 percent and were rinsed in Xylene and mounted in mounting media.

Cleaved caspase-3 Quantification

One section for each mouse was stained for cleaved caspase-3 following the immunohistochemistry protocol described above. Photos were taken using a Leica Axiophot MZ12 with a 10x objective magnification. For each islet in the section the number of positively and negatively stained cells were counted. Percentage of positive cells was calculated by dividing the number of positive cells, by total number of cells in the islet multiplied by 100.

Statistical Analysis

Statistical analysis was calculated using GraphPad Prism. Results were expressed as means ± SD for cell experiments and ± SEM for animal experiments and two-tailed student's t-test was used to determine p values, unless otherwise stated. Statistical significance was defined at $p < 0.05$.

SECTION III:
RESULTS

PGC-1α isoforms are differentially expressed in a rodent β-cell line and primary islets

PGC-1α is an important modulator of mitochondrial function in metabolically active tissues. Recently, Ruas et al., described the function of PGC-1α isoforms found in muscle and reported that they are also expressed in other metabolically active tissues; however, it is unknown whether these isoforms have other tissue-specific functions (Ruas et al., 2012). Interestingly, total PGC-1α levels are decreased in islets of type 2 diabetic patients, but the contribution of different PGC-1α isoforms to diabetes has not been reported, making it of interest to elucidate whether differential expression or function of these isoforms occurs in β-cells and contributes to the pathogenesis of the disease.

Since the expression of these novel isoforms has not been investigated in β-cells, the first step in determining their importance in β-cell biology was to measure basal expression levels of each of the isoforms identified in muscle, in an immortalized β-cell line (INS-1) and primary islets isolated from mice. In particular, we wanted to investigate isoforms regulated by the alternative promoter because one isoform from this promoter, PGC-1α4, has a unique function from PGC-1α1. Since PGC-1α2 and PGC-1α3 are also transcribed form this promoter these isoforms potentially also have novel functions in β-cells. Because of sequence similarity between different isoforms, the primers used to detect PGC-1α2 and PGC-1α3 also detect PGC-1α-b and PGC-1α-c, respectively, and PGC-1α4 primers can also detect NT-PGC-1α. So, all the isoforms are measured at first to narrow down the isoforms of interest. For simplicity sake, however, only PGC-1α1, PGC-1α2, PGC-1α3 and PGC-1α4 will be referred to, but the primers or antibody cannot distinguish them at this time.

To determine whether the isoforms, PGC-α1 (the canonical transcript), PGC-α2, PGC-α3 and PGC-α4, were endogenously expressed in β-cells and can be regulated under physiological

conditions, INS-1 cells and primary islets were treated with 10 μM Forskolin, an activator of adenylyl cyclase (linked to GPCR signalling) known to induce expression of the canonical PGC-1α (Cowell et al., 2008), for 2 hours or with DMSO as the vehicle control. Under these conditions, PGC-1α1, PGC-1α3 and PGC-1α4 were detected at the mRNA level in INS-1 cells and primary islets (Figure 7A and 7B); however, PGC-1α2 expression was undetected by qPCR, in either model, and the induction of PGC-1α3 by forskolin in islets did not reach statistical significance (Figure 7B).

While PGC-1α3 mRNA was detected by qPCR only PGC-1α1 and PGC-1α4 proteins were visualized by Western blotting after a 4 hour treatment with Forskolin, in both INS-1 cells (Figure 7C) and primary isolated islets (Figure 7D). There was approximately a 5-fold and 12-fold induction of PGC-1α1 and PGC-1α4 in INS-1 cells, respectively (Figure 7E), but only a ~3-fold induction of these isoforms in primary islets (Figure 7F). For PGC-1α4, there was a more dramatic induction in INS-1 cells after forskolin treatment (Figure 7E), but only a moderate induction in islet cells (Figure 7F). The exact fold increase of PGC-1α4 in islets is difficult to determine due to the high variability. Since islets are a more physiological model of β-cell biology, it is likely that PGC-1α4 expression is more closely related to levels observed in islets rather than INS-1 cells. However, since PGC-1α4 is expressed and regulated similarly in both islets and INS-1 cells, they are a useful and relevant culture model to study isoform function.

These results show that, of the isoforms investigated, PGC-1α1 and PGC-1α4 are endogenously expressed in β-cells, and can be regulated by cAMP in an isoform dependent manner.

Cytokines modulate the expression of PGC-1α1 and PGC-1α4

The biological function of canonical PGC-1α1 has been extensively studied in multiple cell types where it was found to be a master regulator of mitochondrial function and biogenesis. However, only one function for PGC-1α4 has been described in muscle, that being induction of muscle cell growth, a function not shared by the related isoform PGC-1α1.

We next set forth to determine whether PGC-1α1 and PGC-1α4 have unique functions in β-cells. Unpublished microarray data from our lab using hepatocytes over-expressing either PGC-1α1 or PGC-1α4 demonstrated that in the presence of an inflammatory stimulus (TNF-α) PGC-1α4 differentially regulated genes involved in inflammatory pathways, compared to PGC-1α1 and the control vector. This preliminary data in liver cells suggested that inflammatory stimuli could affect the expression of the PGC-1α isoforms and the genes that they regulate. Inflammatory signalling is known to have dramatic effects on the biology of β-cells and plays an important role in the pathogenesis of diabetes. Since our preliminary screen suggested that the differential gene regulation between PGC-1α1 and PGC-1α4 could be revealed upon exposure to inflammatory stimuli, we next determined whether cytokines impacted isoform expression in β-cells.

To determine whether activation of inflammatory signalling also regulates PGC-1α isoform expression in β-cells, INS-1 cells were treated with a cytokine cocktail of TNF-α, IL-1β and IFNγ, which have been shown to cause β-cell dysfunction with chronic exposure (Donath et al., 2003). Indeed, the expression of PGC-1α1 and PGC-1α4, but not PGC-1α3, were induced at the mRNA level in a time dependent manner (Figure 8A). MCP-1 is an early target of NF-κB activity and its mRNA was measured as a positive control for the activation of inflammatory signalling pathways (Figure 8A). PGC-1α2 was also measured; however, it was again

undetectable by qPCR. Expression of these two isoforms was most robust at 12 hours (~8 fold induction for both PGC-1α1 and PGC-1α4) and then began to decline, to only a 5-fold and 6-fold induction after 24 hours of cytokine stimulation, for PGC-1α1 and PGC-1α4, respectively (Figure 8A). Interestingly, the induction of the PGC-1α isoforms was most robust after early markers of inflammatory signalling began to decline, as determined by MCP-1 gene expression. This suggests that the induction of PGC-1α1 and PGC-1α4 may not result from initial and direct activation of inflammatory signalling pathways (i.e. NK-κB activation) in INS-1 cells, but may increase as a compensatory response to the inflammatory reaction. This induction is unique for PGC-1α1 and PGC-1α4, since PGC-1α2 expression was undetectable by qPCR and PGC-1α3 expression trended downward at all time points observed as compared to the control; however, this was not statistically significant (Figure 8A). Additionally, an increase in PGC-1α4 protein expression by 20-fold (Figure 8B, 8C), was observed. While PGC-1α1 mRNA was increased, the protein was not detected by Western blotting. However, we could not rule out a meaningful induction of PGC-1α1 at the protein level (Figure 8B) because the antibody is quite poor and PGC-1α1 protein is known to be relatively unstable, with a half-life of 2-3 hours (Fernandez-Marcos and Auwerx, 2011; Sano et al., 2007). Cytokine induction of the isoforms in islets proved to be technically challenging. Cytokine responsiveness of the islets was different compared to INS-1 cells, so concentrations and time-points still need to be optimized.

These results reveal that the expression of both PGC-1α1 and PGC-1α4 can be induced in a time dependent manner in cultured β-cells in response to an inflammatory stimulus, but it is likely due to an indirect, rather than direct mechanism. These findings indicate that PGC-1α isoforms may play differential roles in the response to inflammatory cytokines in β-cells.

PGC-1α4 prevents the cleavage of caspase-3 in response to cytokines

The next step was to determine the physiological implications of cytokine regulation of the PGC-1α isoforms. It is known that chronic exposure of β-cells to cytokines, particularly TNFα, IL-1β and IFNγ, leads to β-cell dysfunction and death by apoptosis (Donath et al., 2003). Cytokine signalling is also thought to mediate β-cell death in both type 1 and type 2 diabetics. Since the basal expression level of PGC-1α3 was very low and the protein was not visible under basal or Forskolin treated conditions we focussed on determining whether PGC-1α1 and PGC-1α4 have an impact on in β-cell health and survival in response to cytokines.

Although many apoptotic pathways exist, the activation of caspase-3, also known as an executioner caspase, is a classical marker of cell death by apoptosis. Caspase-3 is activated via proteolytic cleavage at conserved aspartic residues, generating a large and a small subunit, which consequently dimerize to form the active enzyme (Elmore, 2007). Therefore, caspase-3 activation is measured by cleaved caspase-3 protein, as detected by Western blotting. To assess whether the isoforms can affect the activation of caspase-3 in response to cytokine-mediated apoptosis, INS-1 cells were transduced with adenoviruses over-expressing PGC-1α1, and PGC-1α4 or GFP as a control. 30 hours post-infection (allowing sufficient isoform expression), cells were treated with a cytokine cocktail of TNFα, IL-1β and IFNγ, for 18 hours (to promote cell death).

First, to confirm over-expression of the isoforms, a Western blot for PGC-1α was performed (Figure 9A). This revealed that PGC-1α1 and PGC-1α4 were over-expressed to similar levels and higher than the GFP-infected control. Interestingly, cytokine treatment reduces protein expression of over-expressed PGC-1α1 and PGC-1α4; however, the underlying mechanism is unknown. TNFα is known to decrease expression of PGC-1α in cardiac cells

(Palomer et al., 2008) and may be the cause for the decrease in PGC-1α1 and PGC-1α4 seen in these blots. Another plausible explanation could be that the cytokines disrupt the viral or CMV-promoted protein over-expression from the vector. Additionally, PGC-1α1 and PGC-1α4 seem to be induced by the expression of the other (Figure 9A). For this reason, it cannot be determined whether the protective effect is from PGC-1α4 alone, or a combination of actions from the two. To directly address this, over-expression would have to be induced with the combination of short-hairpin RNA (shRNA) to prevent the endogenous induction of the opposite isoform. Moreover, regardless of the mutual up-regulation of PGC-1α1 and PGC-1α4, protein expression of each of the isoforms was more highly expressed as compared GFP, allowing a comparison between the control and each isoform to be made in terms of impact on cell death pathways.

To determine whether cytokines have an effect on the apoptotic profile of INS-1 cells, the activation of caspase-3 was investigated by a Western blot for cleaved caspase-3, using the same protein lysates used for the PGC-1α Western blot (Figure 9B). There is some degree of cleaved caspase-3 present in control samples without cytokine treatment, probably due to cytotoxicity from using virus for over-expression. However, in the presence of cytokines, there is a more robust increase of caspase-3 cleavage when observing protein in the GFP control lanes. There was an evident decrease of cleaved caspase-3 in the INS-1 cells over-expressing only PGC-1α4 in the presence of cytokines (Figure 9B and 9C). PGC-1α1 may also prevent caspase-3 cleavage, to an intermediate degree; however, the decrease compared to the control was not statistically significant (Figure 9C). Additionally, this moderate decrease may be due to the up-regulation of endogenous PGC-1α4 in the sample. Furthermore, the basal levels of cleaved caspase-3 decreased in the presence of over-expressed PGC-1α1 and PGC-1α4 as compared to GFP, only

the difference between GFP and PGC-1α4 was statistically significant (Figure 9C); however, there caspase-3 cleavage in the presence of PGC-1α1 is markedly decreased. These results suggest that PGC-1α4, and possibly to a lesser degree PGC-1α1, may be anti-apoptotic. To convincingly conclude whether PGC-1α1 and PGC-1α4 are anti-apoptotic in β-cells, other experiments, such as protection from nucleosome fractionation, will need to be performed, since active caspase-3 also has non-apoptotic functions (Aispuru et al., 2008; D'Amelio et al., 2011; Fujita et al., 2008; McComb et al., 2010; Woo et al., 2003). What also remains unclear is how these isoforms regulate this function at the molecular level, and if they do so in a similar or unique manner.

PGC-1α1 and PGC-1α4 expression is regulated by GLP-1

The actions of PGC-1α4 are strikingly reminiscent to the anti-apoptotic effects of glucagon-like peptide-1. Binding of GLP-1 to its receptor increases intracellular cAMP levels, which has been shown to mediate GLP-1's action on postprandial glucose-stimulated insulin secretion. Interestingly, GLP-1 also has significant cyto-protective properties and can promote β-cell survival in the presence of a cytotoxic chemical, Streptozotocin (STZ), ER-stress or cytokine exposure (Cunha et al., 2009; Li et al., 2005; Riedel et al., 2010; Yusta et al., 2006). GLP-1 regulates its cyto-protective actions by activation of cAMP, followed by activation of CREB by phosphorylation. GLP-1 can decrease pro-apoptotic proteins, such as cleaved caspase-3 and increase pro-survival factors such as Bcl-2 and Bcl-xL (Baggio and Drucker, 2007). However, how GLP-1 regulates its anti-apoptotic effects is still not well understood. PGC-1α4 expression is induced by cAMP, which is downstream of GLP-1 signalling and PGC-1α4 is capable of decreasing the activation of caspase-3 in the presence of cytokines,

similar to GLP-1. Therefore, we hypothesized that the anti-apoptotic actions of GLP-1 may be mediated through activation of PGC-1α4.

The first step to determine whether PGC-1α4 mediates the actions of GLP-1 is to assess whether GLP-1 receptor activation lies upstream of PGC-1α4 expression in β-cells. This was accomplished by investigating the expression of PGC-1α1 and PGC-1α4 after treatment of INS-1 cells with Exendin-4 (Ex-4), a long lasting GLP-1 analog. After 2 hours, expression of PGC-1α4 mRNA was increased by ~6-fold and PGC-1α1 was moderately increased, by ~3.5-fold (Figure 10A). Both PGC-1α1 and PGC-1α4 induction is clear at the mRNA level, however, only PGC-1α4 expression is apparent after immunoblotting with an anti-PGC-1α antibody (Figure 10B). PGC-1α4 protein is expressed ~3-fold higher in comparison to the untreated cells (Figure 10C). The protein expression of PGC-1α4 goes from being undetectable in the control, lanes to being expressed upon treatment with Ex-4. Since Ex-4 can increase expression of PGC-1α4 in INS-1 cells, it is plausible that PGC-1α4 acts downstream of GLP-1 to mediate the hormone's anti-apoptotic effects.

Knock-down of PGC-1α isoforms, prevents streptozotocin induced hyperglycemia *in vivo*.

Since PGC-1a4 appears anti-apoptotic *in vitro,* we hypothesized that loss of PGC-1α isoform expression would increase the susceptibility of β-cells to cytokine-mediated apoptosis. To address this question, we turned to an *in vivo* β-cell specific knockout of PGC-1α and its isoforms. PGC-1α floxed mice were crossed with mice carrying a MIP (mouse insulin promoter)-CreER transgene, to allow for inducible, β-cell specific deletion of PGC-1α and its isoforms, herein named PGC-1α$^{fl/flCre+}$. Both PCG-1α$^{fl/flCre+}$ and age- and weight-matched

littermate controls, PGC-1α$^{fl/fl}$, were genotyped using a PCR strategy, and crossed until the floxed allele was homozygous and mice were either positive or negative for MIP-CreER.

To understand how PGC-1α1 and PGC-1α4 regulate β-cell survival in a physiological context of diabetes, a steptozotocin (STZ) model of diabetes was utilized. The experimental plan was as described in Figure 11. Two weeks after PGC-1α$^{fl/flCre+}$ and PGC-1α$^{fl/fl}$ mice were gavaged with tamoxifen, to induce ablation of all PGC-1α isoforms in pancreatic β-cells, they were subjected to multiple low-dose streptozotocin (STZ) injections to induce hyperglycemia and β-cell apoptosis. One group of mice (n=7-8) was sacrificed 1 day after the final STZ injection to quantify the amount of activated caspase-3 occurring in the β-cells at this early time point. This was to address the question of whether the loss of PGC-1α isoforms modulates the levels of apoptosis *in vivo*. Since STZ also impacts β-cell function and is a model of type 1 diabetes, we also evaluated the impact of PGC-1α loss on glucose homeostasis, 11, 18 and 32 days post the last STZ injection, by performing an early oral glucose tolerance test (OGTT), a fasted/refed experiment, and a late OGTT, respectively. Furthermore, to evaluate the effect of STZ on the development of hyperglycemia, random blood glucose was taken bi-weekly after the first injection of STZ.

Interestingly, PGC-1α$^{fl/flCre+}$ mice were protected against STZ induced hyperglycemia over the course of the experiment (Figure 12A). PGC-1α$^{fl/flCre+}$ mice trended to have lower glycemia as compared to the controls; however, only the last blood glucose measurement was significantly different. Furthermore, the early OGTT revealed that PGC-1α$^{fl/flCre+}$ mice, 11 days after the final STZ injection, were more glucose tolerant than the controls (Figure 12B), as determined by a 2way ANOVA on the glucose curves and area under the curve (Figure 12B). However, 32 days after the last STZ injection, both PGC-1α$^{fl/flCre+}$ and PGC-1α$^{fl/fl}$ mice had the

same glucose tolerance (Figure 12E). Additionally, all test mice at this late time point had high fasting glucose levels, and could not clear their glucose back to fasting levels. This could be because maximal β-cell death had occurred in both groups after this extensive period of time, and differences in their glucose response are too subtle to detect or are out of detection range by the glucose meters used.

Furthermore, STZ treated PGC-1α$^{fl/flCre+}$ mice tend to have lower glucose after an overnight fast and 2 hours after re-feeding, in comparison to controls (Figure 12C). As well, PGC-1α$^{fl/flCre+}$ mice tend to have higher circulating insulin after fasting and re-feeding, partially explaining their lower glucose levels (Figure 12D). These results, however, did not reach statistical significance, due to high variability but are similar to results shown in hyperglycemic mice following siRNA-mediated knock-down of islet PGC-1α (Kim et al., 2009).

The mechanism to explain the protection of PGC-1α$^{fl/flCre+}$ mice from STZ induced hyperglycemia remains elusive and could be unrelated to the extent of β-cell death, yet instead related to effects of PGC-1α isoforms on regulation of β-cell metabolism or insulin secretion. Thus, to address the role of PGC-1α1 and PGC-1α4 in β-cell apoptosis, and whether it contributes to the protection against STZ induced hyperglycemia in this model, pancreas samples from mice 1 day post-STZ injections were stained by IHC for cleaved caspase-3 (Figure 13A). Since our hypothesis is that PGC-1α4 (and potentially PGC-1α1) is anti-apoptotic, we predicted that the KO mice would have more activation of cleaved caspase-3. Unexpectedly, the there was no difference in caspase-3 immunostaining between these two groups visually (Figure 13A). This was confirmed by quantification of the percentage of positive cells for cleaved caspase-3 staining in islets (Figure 13B). Approximately 30 – 35% of cells in each islet were positive for caspase-3 in both groups and there was no statistical difference between the two groups (Figure

13B). This suggests that the protection against STZ induced hyperglycemia is not due to a difference in β-cell apoptosis and points to a difference in β-cell function. It also indicated that loss of PGC-1α isoforms in β-cells does not impact cell survival in a streptozotocin model of β-cell apoptosis.

There are many reports that expression of certain cre-recombinase transgenes alone in cells can impact cell function dependent of target gene excision (Lee et al., 2005). Therefore it was important to assess whether the MIP-CreER transgene itself was contributing at all to the glycemic phenotype or β-cell death. As a control for potential effects of cre-transgene expression alone, a group of WT (wild-type) and MIP-Cre alone mice (n=7) were gavaged tamoxifen and given the same STZ dose as all experimental mice, following the same experimental plan as the KO mice up until the STZ injections. These mice were sacrificed 1-day post-STZ injections to determine whether the cre transgene influences the phenotype. Again, there was no difference in cleavage of caspase-3 between the WT and MIP-Cre only mice either visually (Figure 14A) or following quantification (Figure 14B). Approximately 25 – 30% of the cells in the islet stained for caspase-3; however there was also no statistical difference between the WT and MIP-Cre only controls (Figure 14B). This demonstrated that the presence of the cre transgene does not impact STZ-induced β-cell apoptosis in our experiment.

SECTION IV:
DISCUSSION

Summary of Results

In this study, we set forth to determine whether PGC-1α isoforms are endogenously expressed and regulated in pancreatic β-cells. This study is the first attempt to understand the function of different PGC-1α isoforms in β-cells. We report the novel finding that PGC-1α1 and PGC-1α4 are induced at the mRNA and protein level by cAMP signalling, a cytokine cocktail and Exendin-4, in either INS-1 cells or primary murine islets. To understand the biological consequence of isoform induction by cytokines, we investigated whether PGC-1α1 and PGC-1α4 play a role in cytokine-mediated apoptosis. We demonstrated that PGC-1α4 over-expression in INS-1 cells prevented the activation of caspase-3 to a greater extent than PGC-1α1, suggesting differential regulation of apoptosis. We also showed that PGC-1α4 expression is promoted by Ex-4, a long lasting GLP-1 analog, at the mRNA and protein level, suggesting a potential for PGC-1α4 to act downstream of GLP-1 action in β-cells. Finally, to understand the function of β-cell expression of PGC-1α1 and PGC-1α4 within a physiological context *in vivo*, PGC-1α isoforms were knocked out specifically in mouse β-cells and mice were subjected to low-dose STZ to cause β-cell death by apoptosis. Unexpectedly, these mice were protected against STZ mediated hyperglycemia and more glucose tolerant compared to the controls at an earlier time-point. Immunohistochemistry of pancreatic sections from mice 1 day after the last STZ injection revealed that there was no difference in activation of caspase-3 in control versus KO mice. This suggests differences in β-cell function rather than apoptosis as a possible mechanism for the protection against STZ induced hyperglycemia in β-cell specific PGC-1α KO mice.

Regulation and Function of PGC-1α Isoforms in β-cells

It is clear from this data that PGC-1α1 and PGC-1α4 are expressed and likely have a significant physiological role in β-cells; however, expression of other published isoforms remains inconclusive or untested at this time. Our focus was on the isoforms transcribed from the alternative promoter, since PGC-1α4, was found to have a distinct function from PGC-1α1 in muscle (Ruas et al., 2012). Although, PGC-1α-b, PGC-1α-c, PGC-1α2, and PGC-1α3 (Chinsomboon et al., 2009; Ruas et al., 2012) were not found to have unique functions in muscle, we also investigated their expression in β-cells since they are also transcribed from the alternative promoter and may have a unique function in β-cells.

PGC-1α2 / PGC-1α-b was not expressed in β-cells and while PGC-1α3 / PGC-1α-c mRNA was detectable, its low mRNA expression level, and undetectable protein expression, suggests that it might not play a significant role in β-cell function. However, to rule out the possibility that the detection level of our assays is not sensitive enough to detect low, yet biologically relevant expression and regulation, immunoprecipitation can be performed to enrich and concentrate PGC-1α proteins.

In contrast to these isoforms, we demonstrated that PGC-1α1 and PGC-1α4 were induced by forskolin (a cAMP activator), a cytokine cocktail, and the GLP-1 analog, exendin-4. With forskolin treatment, induction of PGC-1α1 and PGC-1α4 is variable between INS-1 and islets, since expression of the isoforms as determined by qPCR are more robust in INS-1 cells compared to islets. This is a caveat of using immortalized cell lines versus primary cell culture. Since primary isolated islets are more physiologically relevant, we expect that the induction of

PGC-1α1 and PGC-1α4 are better reflected in the primary islet, rather than INS-1. However, both models support that PGC-1α1 and PGC-1α4 are expressed and inducible in β-cells.

Even though mRNA expression of both PGC-1α1 and PGC-1α4 was induced for all of the stimuli studied, PGC-1α1 protein expression was detected only after forskolin treatment, and was not visible following exposure of β-cells to cytokines or Ex-4. PGC-1α4 is known to be more stable than PGC-1α1 (Ruas et al., 2012), since PGC-1α1 is rapidly degraded by the proteosome (Zhang et al., 2009). PGC-1α1 can be stabilized by p38 phosphorylation, downstream of cAMP signalling, on residues in its repression domain (Cao et al., 2001; Fan et al., 2004). This suggests that treatment with forskolin may have stabilized the PGC-1α1 protein in β-cells. Interestingly, cytokines (TNFα, IL-1β and IL-1α) can also stabilize PGC-1α in muscle (Puigserver et al., 2001); however, our data suggest that cytokines and exendin-4 do not have the same stabilizing effect on the PGC-1α1 protein in β-cells. Again, to exclude the possibility that the protein is low to detect using available antibodies, an IP can be performed to enrich and concentrate the protein or pulse chase experiments can be performed with radioactivity to increase sensitivity of detection. Cells could also be treated with a proteosomal inhibitor MG132 (Zhang et al., 2009) to stabilize the protein, making it visual by Western blotting. In contrast, it is clear PGC-1α4 mRNA and protein is abundant after treatment with cytokines or Exendin-4. Our data suggests that PGC-1α4 may play a significant role in the intracellular response to these stimuli, but at this time it cannot be determined whether PGC-1α1 plays a physiologically significant function under these conditions.

Interestingly, unlike INS-1 cells, PGC-1α4 expression was undetectable by qPCR in unstimulated islets. Only upon treatment with forskolin did PGC-1α4 mRNA become detectable. Currently, we have only investigated induction of these isoforms with forskolin stimulation in

islets, and Ex-4 and cytokine experiments are planned to determine if a similar induction of PGC-1α4 is observed in islets as with INS-1 cells. Regardless of differences in unstimulated expression of PGC-1α4 between INS-1 and primary islets, PGC-1α4 was significantly induced at the mRNA and protein level in both *in vitro* and *ex vivo* models by extracellular signals, suggesting dynamic regulation of this molecule in response to external stimuli.

Increased levels of circulating pro-inflammatory cytokines, particularly TNFα, IL-1β and IFNγ are hallmarks of both type 1 and type 2 diabetes (Donath et al., 2003; Fain, 2006). Chronic stimulation by pro-inflammatory factors causes β-cells dysfunction and death. Since PGC-1α4 protein is induced after stimulation with a cytokine cocktail *in vitro*, this suggests that *in vivo* PGC-1α4 may also be induced in β-cells during the progression of diabetes. Ultimately, this could be tested by determining the relative expression of PGC-1α4 in islets from diabetic patients. Additionally, our data suggests that PGC-1α4 may be anti-apoptotic through prevention of caspase-3 activation, so PGC-1α4 could potentially be induced when β-cells are exposed to chronic levels of circulating cytokines, as a mechanism to protect against β-cell. Moreover, PGC-1α4 is induced by cAMP signalling and exendin-4, which promote β-cell survival (Jambal et al., 2003; Jhala et al., 2003; Li et al., 2005; Li et al., 2003). These findings support our hypothesis that PGC-1α4 is anti-apoptotic in β-cells, since the co-activator can act downstream of these cyto-protective factors, and thus may, in part, mediate their anti-apoptotic effects. This is therapeutically relevant as GLP-1 receptor agonists, such as Exenatide and Liraglutide, are current pharmacological interventions to treat type 1 and type 2 diabetes (Lovshin and Drucker, 2009), but their complete mechanism of action on β-cells is not yet understood. Therefore, PGC-1α4 induction by GLP-1 may be a mechanism that mediates or improves the effectiveness of these drugs to treat disease through promotion of β-cell survival. To test this theory one could

knock down PGC-1α4 in INS-1 cells and islets and determine whether exendin-4 or cAMP still promotes β-cell survival in response to cytotoxic signals such as pro-inflammatory cytokines in the absence of co-activator expression. If the anti-apoptotic function of GLP-1 or cAMP is lost when PGC-1α4 is knocked down, this would suggest that these factors are dependent on PGC-1α4 expression to exert their pro-survival functions.

A caveat of our *in vitro* experiments is the efficiency of infection by the adenoviruses. To achieve detectable levels of expression while maintaining health of the cultures, we could only obtain an infection efficiency of 50-60%. Thus, cells that were not infected may have diluted out the biological responses of the cells that we were measuring. Therefore, prevention of caspase-3 activation by PGC-1α4 could actually be a more robust response than we observed. In addition, it was evident from our experiments that simple transduction of INS-1 cells with viral vectors activated apoptotic pathways, as shown by the detection of cleaved caspase-3 in untreated samples. It is possible that the virus primes the cells for cell death, amplifying or modulating the cytoprotective effects of PGC-1α isoform over-expression. To eliminate this variable, stable lines over-expressing PGC-1α1 and PGC-1α4 were generated and will be used to test this theory in future experiments. PGC-1α1 and PGC-1α4 were cloned into the multiple cloning site of pCDNA, downstream of a CMV promoter and with a N-terminal FLAG tag. INS-1 cells were transfected with linear plasmid and following successful integration of the linearized plasmid, cells were selected for using G418 antibiotic. These cells will be used to circumvent the use of virus to over-express PGC-1α1 and PGC-1α4. Additionally, this model will ensure approximately 100% of the cells are over-expressing their respective isoform and ameliorate the poor infection efficiency of the viruses.

To confirm PGC-1α4's anti-apoptotic function, more conclusive assays can be performed that directly assess cell death, as cleaved caspase-3 is also known to have apoptosis-independent functions. Caspase-3 has been shown to be a critical factor in; (i) erythroid cell expansion and survival (Aispuru et al., 2008); (ii) $CD8^+$ T-cell expansion (McComb et al., 2010); (iii) stem cell differentiation (Fujita et al., 2008); (iv) inhibition of B-cell proliferation through loss of repression of a cell cycle promoter, Cdkn1a (Woo et al., 2003) and; (v) dendrite synaptic dysfunction (D'Amelio et al., 2011). Although these functions are very cell-type specific, it confirms that activation of caspase-3 does not necessarily lead to an increase of apoptosis. So, when studying caspase-3 as a marker for apoptosis, it is critical to confirm whether an increase in caspase-3 activation truly corresponds to an increase in apoptosis. One such assay is a nucleosome ELISA that measures the amount DNA fragmentation, which is indicative of cells undergoing apoptosis. The effects of isoform over-expression on apoptosis could also be determined with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, which labels DNA fragmentation (Gavrieli et al., 1992). Even if we are limited by transduction or transfection efficiency, this assay allows us to determine the percentage of cells positive for GFP (co-expressed with PGC-1α isoforms) and negative for TUNEL, revealing which cells are protected against cell death. Another method could be to sort cells by flow cytometry. Cells undergoing cell death by apoptosis are positive for Annexin V. If PGC-1α4 and/or PGC-1α1 protect INS-1 cells from apoptosis, GFP positive cells will be Annexin V negative, which will indicate whether cells directly expressing the isoform are protected against apoptosis. These experiments will help to convincingly determine whether over-expression of PGC-1α4 in INS-1 cells protects cells against apoptosis and if this is a function unique from PGC-1α1.

It has proven technically difficult to measure cleaved caspase-3 in primary islets; however addressing our hypothesis in this model would better support an anti-apoptotic role *in vivo*. It is substantially more difficult to transduce whole islets, compared to INS-1 cells with adenovirus. The virus fails to penetrate the inner cells of the islet, most of which are the β-cells. Additionally, it has been difficult to induce apoptosis in whole islets by cytokines. These conditions are being optimized and different methods to infect islets (such as using dispersed cells rather than whole islets) and conditions to induce apoptosis (such as high concentrations of cytokines, STZ, fatty acids or a combination or all three) are being tested.

Therefore, our data suggests that PGC-1α4 has the potential to be anti-apoptotic in β-cells, which is a novel function for this protein. However, further studies are needed to elucidate the relevance of this function *in vivo* and within the context of β-cell disease.

Isoforms and the Pathogenesis of Diabetes

Since promoting β-cell survival is an attractive diabetes therapy, we were interested in the role of PGC-1α isoforms within a physiological context. Using readily available tools, we first chose to use a knockout of all PGC-1α isoforms. Since PGC-1α is important in metabolically active tissues (Lin et al., 2005) and is a significant regulator of mitochondrial function, it is surprising that β-cell specific PGC-1α KO mice were protected against STZ induced hyperglycemia. However, our results are consistent with studies investigating the role of PGC-1α in rodent β-cells. PGC-1α1 over-expression in mouse β-cells causes glucose intolerance and decreases insulin secretion, whereas knock-down causes the inverse (De Souza et al., 2005; De Souza et al., 2003; Kim et al., 2009; Valtat et al., 2013; Yoon et al., 2003). These results suggest that constitutive PGC-1α1 expression may be detrimental to β-cell function. These findings

seemingly contradict that of data in human islets, since OXPHOS genes and PGC-1α expression are decreased in islets from diabetic patients and knock-down of PGC-1α in human islets decreases insulin secretion (Ling et al., 2008; Olsson et al., 2011). The techniques used to manipulate PGC-1α gene expression *in vivo* and *in vitro* in rodent models, may contribute to the inability of rodent models of diabetes to recapitulate the human etiology and underscores the importance of repeating experiments with human tissues and/or cell lines. Additionally, these studies fail to differentiate between potential differences in PGC-1α isoform function in β-cells in the context of human disease, which adds complexity in understanding the role of PGC-1α in the pathogenesis of diabetes. Our *in vitro* studies implicate PGC-1α4 as a protective factor in β-cells and is therefore a potential target to prevent β-cell death in diabetic patients. However, this effect must be further studied *in vivo*. Since the *in vivo* KO is of all isoforms, PGC-1α4's specific contribution to the phenotype remains unclear. Elucidating the effect of PGC-1α4 *in vivo*, by rescue experiments, can confirm whether PGC-1α4 is also protective in a physiological context. This will provide additional evidence to support targeting specific isoforms, namely PGC-1α4, to improve β-cell function or survival.

To determine whether a change in the apoptotic status of β-cells is a contributing factor to the glycemic phenotype of the beta-cell specific PGC-1α KO, mice were sacrificed soon after the final STZ injection and pancreas sections were analyzed by IHC for a marker of apoptosis. IHC analysis revealed that there was no difference in activation of caspase-3 between the knock-outs and controls. There was also no difference in the amount of caspase-3 activation between control mice expressing only the MIP-Cre transgene, confirming that there are no non-specific effects of cre recombinase expression on caspase-3 activation. These data suggest that the protection from STZ induced hyperglycemia in β-cell specific PGC-1α KO mice is due to a difference in β-cell

function rather than a change in the rate of cell death. However, since activation of caspase-3 does not absolutely determine whether a cell is undergoing apoptosis, an alternative method of quantify apoptotic β-cell death is required, such as TUNEL or Annexin V staining *in vivo*, to confirm these results.

A caveat to knocking out all PGC-1α isoforms is the difficulty in differentiating the contribution of each isoform to the phenotype. To generate a floxed mouse where PGC-1α4 is individually knocked out in β-cells is difficult because targeting any portion of PGC-1α4 will also affect the expression of other isoforms or full length PGC-1α. Therefore, to understand the role of PGC-1α1 and PGC-1α4 individually in an *in vivo* context, these isoforms could be rescued separately in our beta-cell specific PGC-1α KO mice prior to STZ injections. This could be achieved by utilizing AAV8 (adeno-associated virus 8)-MIP adenovirus (Rehman et al., 2008). AAV8 is a viral vector that specifically targets and infects all cells within the pancreas. The inclusion of the mouse insulin promoter in the vector restricts the over-expression of a gene of interest to the β-cell (Rehman et al., 2005; Wang et al., 2006). A double stranded AAV vector carrying PGC-1α1 or PGC-1α4 cDNA under the regulation of the mouse insulin promoter could be generated to rescue PGC-1α1 and PGC-1α4 *in vivo* (in the absence of other isoforms) in order to discern their individual roles in rodent models of diabetes.

Streptozotocin as a model of type 1 diabetes has limitations. It is a pharmacological toxin and inducer of β-cell death, but may also affect any other cell that expresses a functioning Glut2 receptor. In addition, it is not known whether the mechanism by which β-cells die in response to STZ is similar to the β-cell apoptosis observed in type 1 diabetic patients. To further delve into the role of PGC-1α4 (and other isoforms) in the pathogenesis of type 1 diabetes, studies with the NOD mouse may be more informative and address an alternative mechanism of β-cell mediated

death *in vivo* and will complement the studies using streptozotocin. To determine whether there are protective functions of PGC-1α1 and PGC-1α4 against β-cell death in an alternative model of diabetes that better mimics the disease pathogenesis, NOD mice can also be injected with the AAV8 viruses over-expressing PGC-1α1 and PGC-1α4 after birth and after the disease has progressed. This will investigate whether direct delivery of these isoforms to the β-cells of mice before and after the development of diabetes can prevent the progression of the disease and β-cell *in vivo*. This model can provide more direct evidence that PGC-1α4 is capable of preventing the pathogenesis of diabetes and whether this is a unique function from PGC-1α1 in β-cells. The NOD mouse develops diabetes by immune infiltration and since genes regulated by PGC-1α4 are revealed upon TNFα signalling in primary hepatocytes (unpublished), the NOD mouse may be a more direct *in vivo* model of PGC-1α4 because of the pro-inflammatory environment.

Even though there is an apparent disconnect between human and rodent data, studying mouse islets is still relevant to understand the underlying mechanisms of PGC-1α's involvement in diabetes. Mouse islets are used tools to understand complex processes in humans. Although data from mice may not identically recapitulate the human condition, they are useful to elucidate mechanisms since human tissue is difficult to obtain. Furthermore, discrepancies between rodents and humans may be related to the methods chosen to manipulate gene expression. For example, in the rodent studies investigating PGC-1α function in β-cells, PGC-1α knock-down includes all isoforms, whereas over-expression is restricted to PGC-1α1, which could explain the differences in rodent and human data. Additionally, there have not been any studies that genetically knock-down only PGC-1α1 *in vivo*, which will more closely mimic the human condition. Furthermore, the studies do not address the potential contribution of other PGC-1α

isoforms in diabetes, since their functions are not very well understood in β-cells. Therefore, as this project develops it is essential to evaluate the role of PGC-1α isoforms, particularly the differences between PGC-1α1 and PGC-1α4 in human tissues. Since these isoforms may play a critical role in rodent β-cells, it will be important to determine whether these isoforms are also important in human β-cells. As the function of PGC-1α4 is elucidated in β-cells, investigating whether these functions are conserved between different species will be relevant to human disease.

Differential Role of PGC-1α1 and PGC-1α4 in β-cells

Published data demonstrates that PGC-1α1 and PGC-1α4 have unique functions in muscle and PGC-1α4 does not regulate classical PGC-1α1 genes (Ruas et al., 2012). Our results also suggest that PGC-1α1 and PGC-1α4 have differential functions in β-cells in terms of apoptosis. We determined that PGC-1α4 is more effective at preventing the activation of caspase-3, suggesting that it is a more effective anti-apoptotic factor than PGC-1α1. However, this may be confounded by mutual up-regulation of each of the isoforms when either is over-expressed (Figure 9A). Moreover, although we only see increased PGC-1α4 protein following cytokine treatment, it cannot be confirmed whether PGC-1α4 is the only isoform induced and regulated by cytokines and Ex-4 in INS-1 cells, since the presence of PGC-1α1 protein expression under these conditions is inconclusive due to limitations of the antibody and protein instability.

Unpublished data from our lab in hepatocytes (a cell type that co-ordinately controls whole body metabolism with β-cells and are derived from similar lineages), demonstrate that differential gene expression between PGC-1α1 and PGC-1α4 is revealed in the presence of an

inflammatory stimulus, TNFα. We expect that the induction of PGC-1α1 and PGC-1α4 by cytokines in β-cells also results in differential gene regulation and function in β-cells. Since this is only speculation, it will be important to characterize differential gene expression patterns in a β-cell line or primary islets. To do this, INS-1 cells and isolated islets can be transduced with adenovirus expressing each isoform and treated with cytokines to investigate different inflammatory pathways by qPCR of candidate mediators. However, this is a biased approach. An unbiased approach would be to transduce INS-1 cells or islets with the different isoforms and subject them to microarray analysis (or second generation genomics/deep sequencing), to reveal all genes differentially regulated between PGC-1α1 and PGC-1α4. This would be a preliminary study to determine whether different pathways are regulated in response to an inflammatory stimulus, thus revealing potential differences in functional output between PGC-1α1 and PGC-1α4 in β-cells. This experiment could also indicate the molecular pathways regulating the cyto-protective properties of PGC-1α4.

PGC-1α4 versus NT-PGC-1α

Since both NT and PGC-1α4 are similarly spliced (Figure 4), have almost identical sequences (Figure 5), and are approximately the same molecular weight (Ruas et al., 2012; Zhang et al, 2009), it is difficult to differentiate these two proteins at the mRNA level with qPCR primers or by Western blotting. The only difference between these isoforms is that NT-PGC-1α is transcribed from the proximal promoter and PGC-1α4 is transcribed from the alternative promoter, resulting in different exon 1 sequences. Primers to the alternative promoter and the shared exon 1 can differentiate between NT-PGC-1α and PGC-1α4, but also recognize PGC-1α2. Similarly, primers to the proximal promoter and the shared exon 1 can also differentiate between

NT-PGC-1α and PGC-1α4, but also recognize PGC-1α1. In addition, the available antibody recognizes all known isoforms of PGC-1α and cannot differentiate between NT-PGC-1α and PGC-1α4 because of their similar molecular weight. The similarity between these two isoforms potentially means that the mRNA and protein expression we observe in our experiments is actually NT-PGC-1α and not PGC-1α4.

An alternative means of differentiating between NT-PGC-1α and PGC-1α4 expression in β-cells is to clone the full-length mRNA sequences by PCR. Using cDNA generated from INS-1 cells or isolated islets and primers specific for each isoform, full-length products can be amplified. These PCR products would be subjected to sequencing analysis to confirm the identity of the amplicons and relative expression levels compared by qPCR. This method will determine the absolute expression of PGC-1α4 and NT-PGC-1α and demonstrate whether either or both isoforms are expressed and inducible in β-cells. Again it will be critical to also determine the relative abundance of both PGC-1α4 and NT-PGC-1α in human tissues. Although both are expressed in many human tissues (Ruas et al., 2012; Zhang et al., 2009) some tissues appear to have preference for one isoform over another and it is unknown which are expressed in human islets. Additionally, even if they are both expressed in human β-cells they may not be similarly regulated. Using rodent muscle as an example, over-expression of PGC-1α4 and NT-PGC-1α via electroporation, allows accumulation of over-expressed PGC-1α4, but not NT-PGC-1α, protein (Ruas et al., 2012). So even though these two proteins are similar, the distinct N-terminus of PGC-1α4 is enough to cause differential regulation of these isoforms in muscle. Furthermore, although they have very similar protein structures, only NT-PGC-1α regulates aspects of mitochondrial function and does not have the myotrophic effects of PGC-1α4 (Ruas et al., 2012), demonstrating that determining the precise identity of the protein in islets will be important when

trying to elucidate function. Therefore, it is plausible that PGC-1α4 and NT-PGC-1α may be differentially regulated in human islets, as well as mouse islets.

Perspectives and Conclusions

PGC-1α4 is potentially a novel factor important for β-cell survival in the context of diabetes. Pro-inflammatory cytokines induce expression of this protein, which could be a mechanism *in vivo* to prevent cytokine induced β-cell death in diabetes. We have identified a cyto-protective function for PGC-1α4 in β-cells; however, since PGC-1α4 is expressed in multiple tissues, this function may be shared in other cells types. Since we only investigated this one physiological outcome of PGC-1α4 biology, it is unclear whether PGC-1α4 has additional unique function from PGC-1α1 in β-cells and whether they regulate different gene pathways. We can speculate that this may be the case, because PGC-1α1 and PGC-1α4 regulate different gene sets in muscle (Ruas et al., 2012) and in hepatocytes (our unpublished data). If these isoforms do have different functions in β-cells, we would expect that even if they were similarly regulated by forskolin or cytokines, for example, their functional output could be unique. A microarray comparing mRNA expression patterns in PGC-1α1 and PGC-1α4 expressing INS-1 cells or islets will direct us towards more potential functional differences between PGC-1α1 and PGC-1α4.

Since β-cells are highly dependent on mitochondria and PGC-1α1 is a master regulator of mitochondrial function, it will be important to determine whether PGC-1α4 also has important roles in mitochondrial functions in β-cells. It is probable that PGC-1α4 will not regulate mitochondrial genes classically regulated by PGC-1α1 in β-cells, as this is the case in muscle (Ruas et al., 2012). However, it is still important to determine whether this is tissue specific or a general characteristic of PGC-1α4. This information can also be gleaned from microarray data

following over-expression of PGC-1α1 or PGC-1α4 in INS-1 cells or primary islets or classical analysis of using qPCR, Western blot analysis, and ETC activity assays.

Although up-regulation of PGC-1α in obese mouse models is associated with β-cell dysfunction (Yoon et al., 2003), this was limited to *in vitro* adenoviral over-expression of PGC-1α in isolated islets and may not reflect accurately the consequence of PGC-1α endogenous up-regulation *in vivo.* Therefore, PGC-1α induction in obese mice may be a reason why they do not develop frank diabetes and β-cell apoptosis. It is possible that both PGC-1α1 and PGC-1α4 can contribute to this phenomenon by increasing metabolism and protecting against cell death in β-cells, respectively. This would complement human studies, which demonstrate that knock-down of PGC-1α decreases β-cell function (Ling et al., 2008), rather than increasing β-cell function (De Souza et al., 2003; De Souza et al., 2005; Kim et al., 2009).

Since there was no difference in caspase-3 activation in the pancreatic sections of PGC-1α knock-outs and littermate controls, this suggests that the modest protection from STZ-induced hyperglycemia observed in β-cell specific PGC-1α KO mice is due to β-cell dysfunction rather than a difference in apoptosis. It is surprising that a knock-out of PGC-1α caused an increase in insulin secretion, since mitochondrial function is necessary for insulin secretion. Yoon et al., attributed this to an imbalance in metabolic gene expression, such as inappropriately increased glucose-6-phosphatase and glucokinase expression (Yoon et al., 2003). Likely the change in insulin secretion is due to an effect of PGC-1α1, as PGC-1α4 has not been associated with mitochondrial function (Ruas et al., 2012). However, investigating whether the rescue of PGC-1α4 with AAV8 adenoviruses *in vivo* can alter mitochondrial function or affect insulin secretion would address this question. If PGC-1α4 does affect mitochondrial function or genes,

this would be a novel function of PGC-1α4 in β-cells and it would demonstrate that PGC-1α4 has unique tissue specific properties.

PGC-1α4's function in β-cells appears to be a pro-survival factor. Thus, PGC-1α4's potential anti-apoptotic effects could be beneficial for the treatment of diabetes. Type 1 diabetes is caused by autoimmune attack of the β-cells. The simplest and most common method of treating T1D is by insulin injections to compensate for the loss of β-cell mass. In the past decade, islet transplantation has become a more attractive option, since patients can become insulin independent for 1 – 3 years (Bellin et al., 2008; Shapiro et al., 2000). However, islets that are transplanted into patients eventually succumb to autoimmune attack resulting in β-cell death, even if immunosuppressant drugs are administered (Monti et al., 2008). To improve islet transplantation therapy, it is critical to identify factors that can improve the health and survival of the transplanted islets to increase the efficacy of this treatment option. PGC-1α4's potential anti-apoptotic effects could be beneficial in this regard, as expression may improve the efficacy of islet transplantation by protecting islets from cell death caused by immune-mediated attack. The benefits of preventing β-cell death are not limited to T1D. T2D in later stages is also associated with high levels of circulating pro-inflammatory cytokines and immune-mediated β-cell death. We also show that both PGC-1α1 and PGC-1α4 are downstream of GLP-1 receptor signalling in β-cells, implicating these isoforms in the actions of GLP-1 (which are generally beneficial). Determining the role of each isoform in GLP-1 action and, in particular, whether PGC-1α4 mediates the cyto-protective properties of GLP-1 could improve the understanding of GLP-1 action in β-cells and be applied to improve the efficacy of GLP-1R agonist drugs.

Therefore, we showed that PGC-1α isoforms are expressed and inducible in β-cells, particularly PGC-1α1 and PGC-1α4. PGC-1α4 may be anti-apoptotic *in vitro* and this may be

applicable to β-cell survival *in vivo*. If PGC-1α4 is found to prevent the pathogenesis of diabetes *in vivo*, it is potentially a novel diabetic treatment either as a factor to protect β-cell death in islet transplantation or to improve current pharmaceutical interventions.

SECTION V:
FIGURES

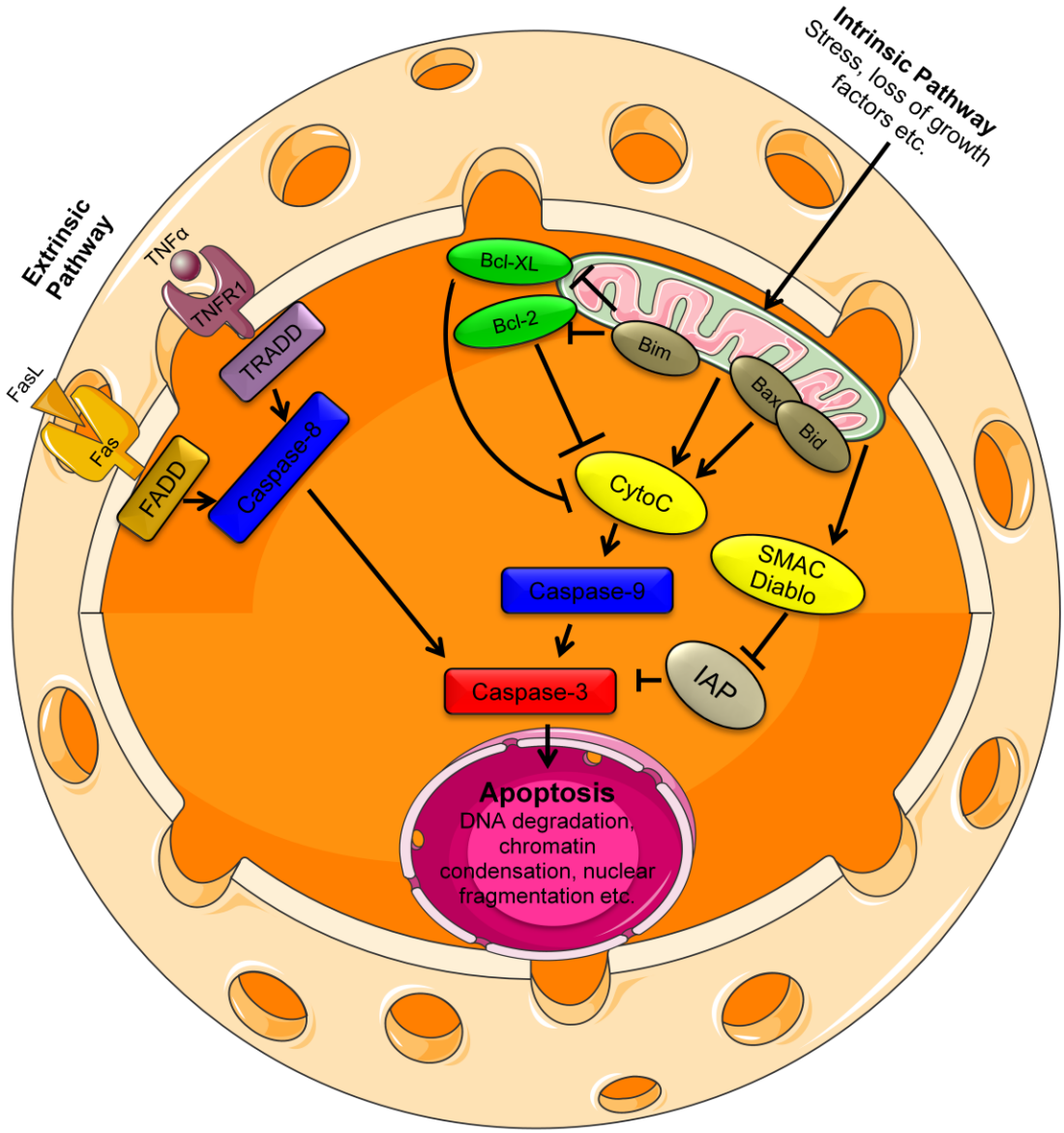

Figure 1. Summary of extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway works through binding of FasL and TNFα, to their cognate receptors. This action recruits adaptor proteins which then activates caspase-8 by proteolytic cleavage. This initiator caspase can then activate caspase-3, the executioner, to cause apoptosis. External stresses activate the intrinsic pathway causing permeability changes of the the mitochondrial membrane. This allows proteins such as cytochrome C and SMAC/DIABLO to be released by activation of caspase-3 or inhibition of IAP, respectively. Anti-apoptotic Bcl-2 family members, including Bcl-2 itself and Bcl-XL inhibit mitochondrial membrane permeabilization, effectively preventing the release of CytoC. Pro-apoptotic Bcl-2 members, Bim, Bax and Bid, can either inhibit Bcl-2 and Bcl-XL, or promote membrane permeabilization, causing cytochrome C release. Both the extrinsic and intrinsic pathways converge on caspase-3 activation to commit the cell to apoptosis. (Adapted from: Elmore, 2007)

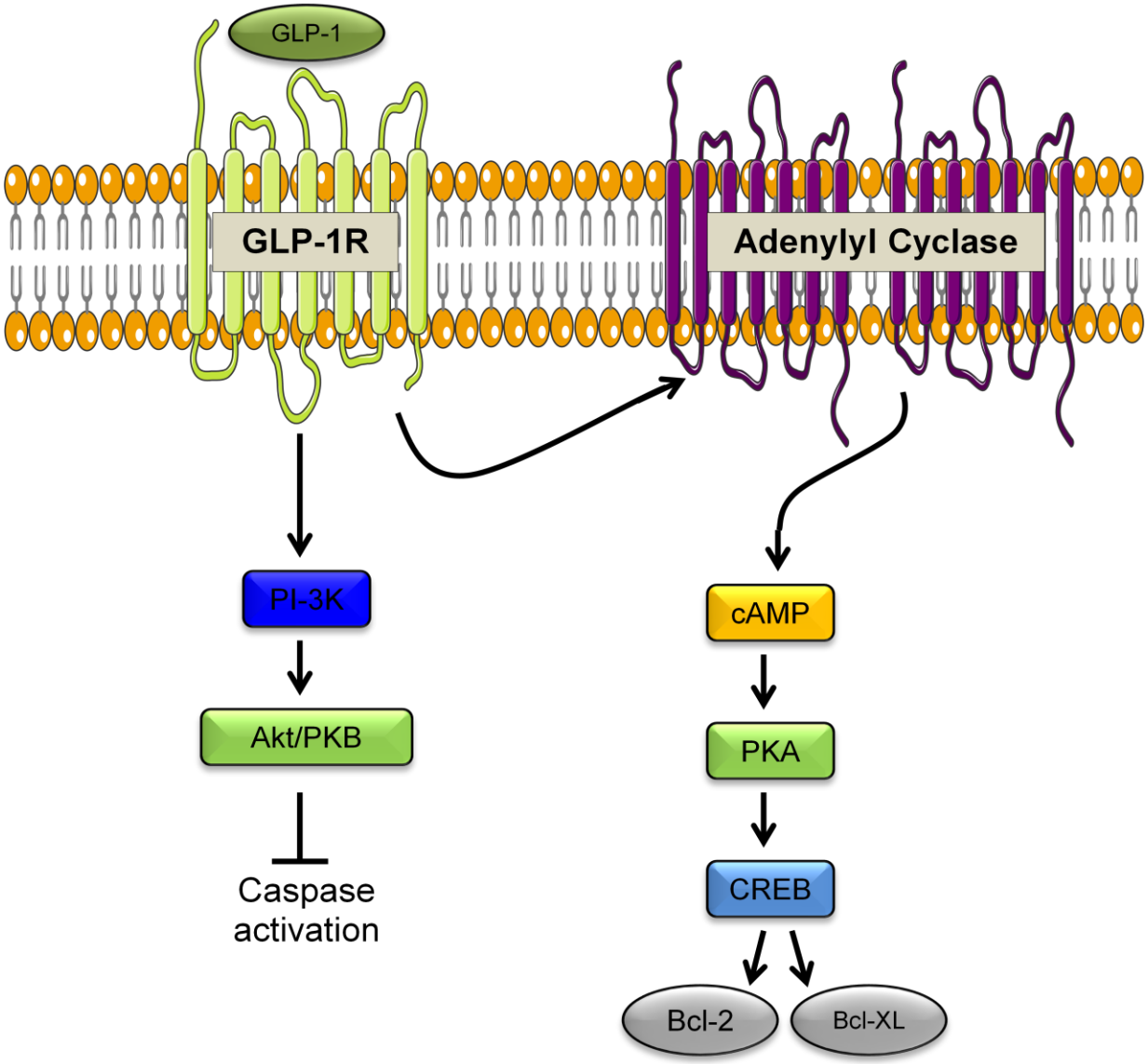

Figure 2: Anti-apoptotic actions of GLP-1. GLP-1 can promote cell survival either through adenylyl cyclase – CREB axis, or PI-3K – AKT axis. These pathways either prevent apoptosis by activating the expression of Bcl-2 and Bcl-XL, or preventing the activation of caspases. (Adapted from: Baggio and Drucker, 2007)

Figure 3. Functional domains of PGC-1α. PGC-1α has the following domains: activation, transcriptional repression, arginine and serine (RS) rich domains, and RNA binding domain. These domains can bind the HAT complex (in the activation domain) and the mediator complex (in the RS and RNA binding domain). LXXLL are nuclear receptor binding sites. Adapted from Lin et al., 2005.

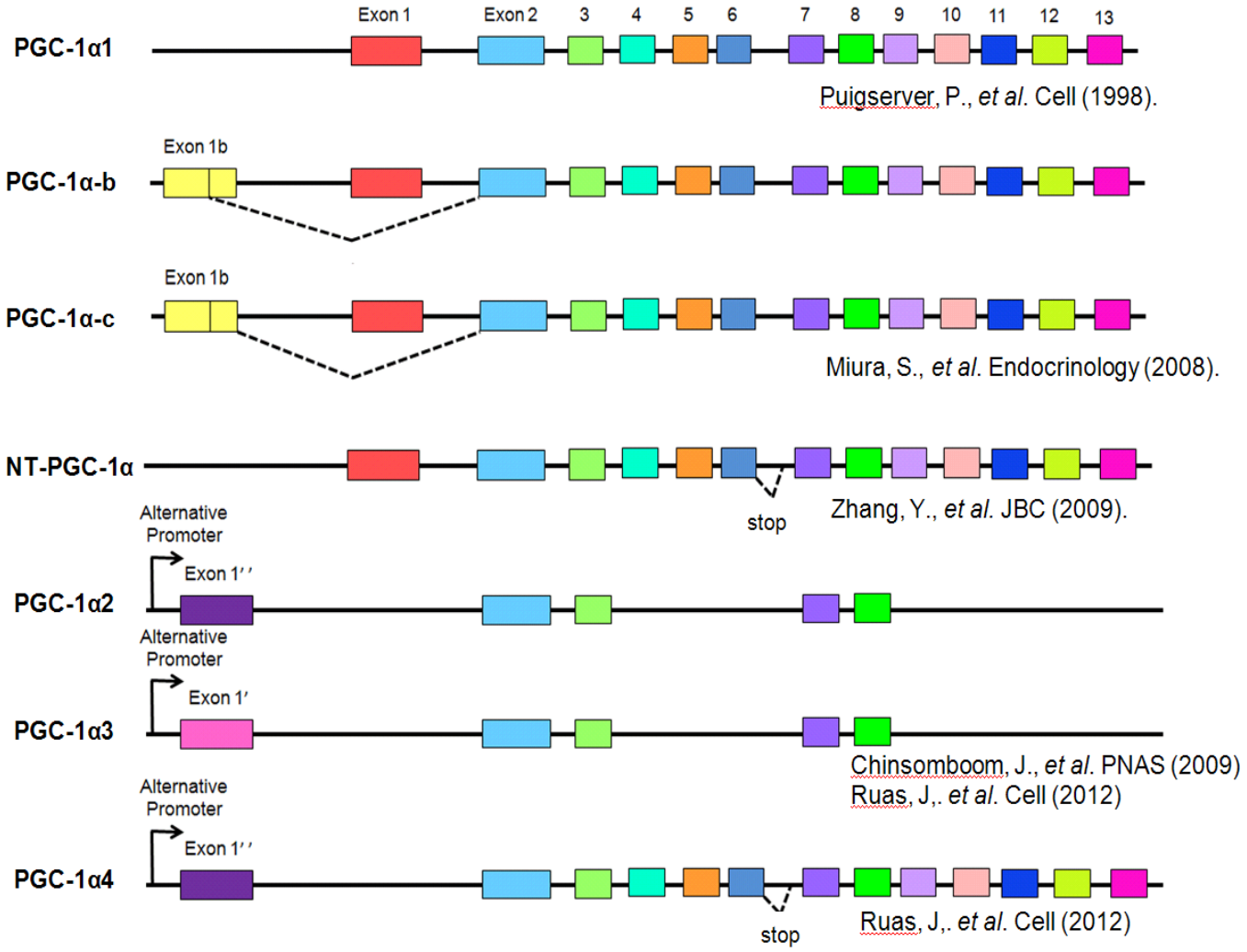

Figure 4. Summary of PGC-1α isoform transcripts. Each colour represents different exons as labelled for PGC-1α1. The canonical exon 1 is spliced out of PGC-1α-b and PGC-1α-c. Alternative splicing of intro 6 in NT-PGC-1α and PGC-1α4 introduces an in frame stop codon. PGC-1α2, PGC-1α3 and PGC-1α4 are transcribed from the alternative promoter approximately 14 kb upstream of the canonical exon 1. Out of the three, PGC-1α3 has a unique exon 1.

NT-PGC-1α

A

ATGGCTTGGGACATGTGCAGCCAAGACTCTGTATGGAGTGACATAGAGTGTGCTGCTCTGG
TTGGTGAGGACCAGCCTCTTTGCCCAGATCTTCCTGAACTTGACCTTTCTGAACTTGATGTGA
ATGACTTGGATACAGACAGCTTTCTGGGTGGATTGAAGTGGTGTAGCGACCAATCGGAAATCA
TATCCAACCAGTACAACAATGAGCCTGCGAACATATTTGAGAAGATAGATGAAGAGAATGAGG
CAAACTTGCTAGCGGTCCTCACAGAGACACTGGACAGTCTCCCCGTGGATGAAGACGGATTG
CCCTCATTTGATGCACTGACAGATGGAGCCGTGACCACTGACAACGAGGCCAGTCCTTCCTC
CATGCCTGACGGCACCCCTCCCCCTCAGGAGGCAGAAGAGCCGTCTCTACTTAAGAAGCTCT
TACTGGCACCAGCCAACACTCAGCTCAGCTACAATGAATGCAGCGGTCTTAGCACTCAGAACC
ATGCAGCAAACCACACCCACAGGATCAGAACAAACCCTGCCATTGTTAAGACCGAGAATTCAT
GGAGCAATAAAGCGAAGAGCATTTGTCAACAGCAAAAGCCACAAAGACGTCCCTGCTCAGAG
CTTCTCAAGTATCTGACCACAAACGATGACCCTCCTCACACCAAACCCACAGAAAACAGGAAC
AGCAGCAGAGACAAATGTGCTTCCAAAAAGAAGTCCCATACACAACCGCAGTCGCAACATGCT
CAAGCCAAACCAACAACTTTATCTCTTCCTCTGACCCCAGAGTCACCAAATTTGTTTTTATAA

B

MAWDMCSQDSVWSDIECAALVGEDQPLCPDLPELDLSELDVNDLDTDSFLGGLKWCSDQSEIISN
QYNNEPANIFEKIDEENEANLLAVLTETLDSLPVDEDGLPSFDALTDGAVTTDNEASPSSMPDGTPP
PQEAEEPSLLKKLLLAPANTQLSYNECSGLSTQNHAANHTHRIRTNPAIVKTENSWSNKAKSICQQQ
KPQRRPCSELLKYLTTNDDPPHTKPTENRNSSRDKCASKKKSHTQPQSQHAQAKPTTLSLPLTPES
PNLFL

PGC-1α4

C

ATGTTGGGATTGTCATCCATGGATTCAATTTTGAAATGTGCTGCTCTGGTTGGTGAGGACCAG
CCTCTTTGCCCAGATCTTCCTGAACTTGACCTTTCTGAACTTGATGTGAATGACTTGGATACAGA
CAGCTTTCTGGGTGGATTGAAGTGGTGTAGCGACCAATCGGAAATCATATCCAACCAGTACAAC
AATGAGCCTGCGAACATATTTGAGAAGATAGATGAAGAGAATGAGGCAAACTTGCTAGCGGTC
CTCACAGAGACACTGGACAGTCTCCCCGTGGATGAAGACGGATTGCCCTCATTTGATGCACTG
ACAGATGGAGCCGTGACCACTGACAACGAGGCCAGTCCTTCCTCCATGCCTGACGGCACCCCT
CCCCCTCAGGAGGCAGAAGAGCCGTCTCTACTTAAGAAGCTCTTACTGGCACCAGCCAACACT
CAGCTCAGCTACAATGAATGCAGCGGTCTTAGCACTCAGAACCATGCAGCAAACCACACCCAC
AGGATCAGAACAAACCCTGCCATTGTTAAGACCGAGAATTCATGGAGCAATAAAGCGAAGAGC
ATTTGTCAACAGCAAAAGCCACAAAGACGTCCCTGCTCAGAGCTTCTCAAGTATCTGACCACAA
ACGATGACCCTCCTCACACCAAACCCACAGAAAACAGGAACAGCAGCAGAGACAAATGTGCTT
CCAAAAAGAAGTCCCATACACAACCGCAGTCGCAACATGCTCAAGCCAAACCAACAACTTTATC
TCTTCCTCTGACCCCAGAGTCACCAAATTTGTTTTTATAA

D

MLGLSSMDSILKCAALVGEDQPLCPDLPELDLSELDVNDLDTDSFLGGLKWCSDQSEIISNQYNNEP
ANIFEKIDEENEANLLAVLTETLDSLPVDEDGLPSFDALTDGAVTTDNEASPSSMPDGTPPPQEAEEP
SLLKKLLLAPANTQLSYNECSGLSTQNHAANHTHRIRTNPAIVKTENSWSNKAKSICQQQKPQRRPC
SELLKYLTTNDDPPHTKPTENRNSSRDKCASKKKSHTQPQSQHAQAKPTTLSLPLTPESPNLFL

Figure 5. Nucleotide and protein sequence similarities between NT-PGC-1α and PGC-1α4. Only the nucleotide sequence of exon 1 is unique between (A) NT-PGC-1α and (C) PGC-1α4, highlighted in green. This corresponds to different N-terminal ends of the protein sequence, seen highlighted in blue in (B) and (D).

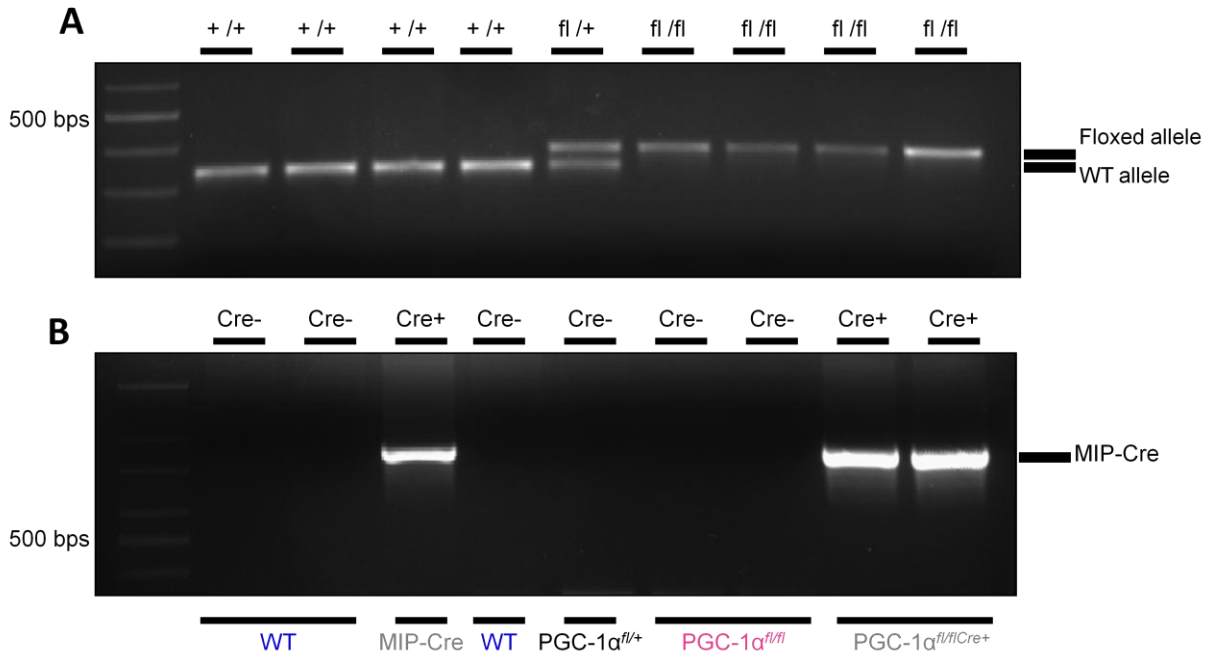

Figure 6. Genotyping of mouse tail crude DNA. (A) Floxed and WT alleles were genotyped using primers flanking the 5' loxP site, generating the upper band for alleles with the loxP site and a lower band for the WT allele. (B) MIP-Cre positive mice were genotyped using a forward primer in the mouse insulin promoter and reverse primer in the cre recombinase transgene. The combination of *fl/fl* alleles and *Cre+* are PGC-1α knockout mice and *fl/fl* alleles and *Cre-* were used as their littermate controls.

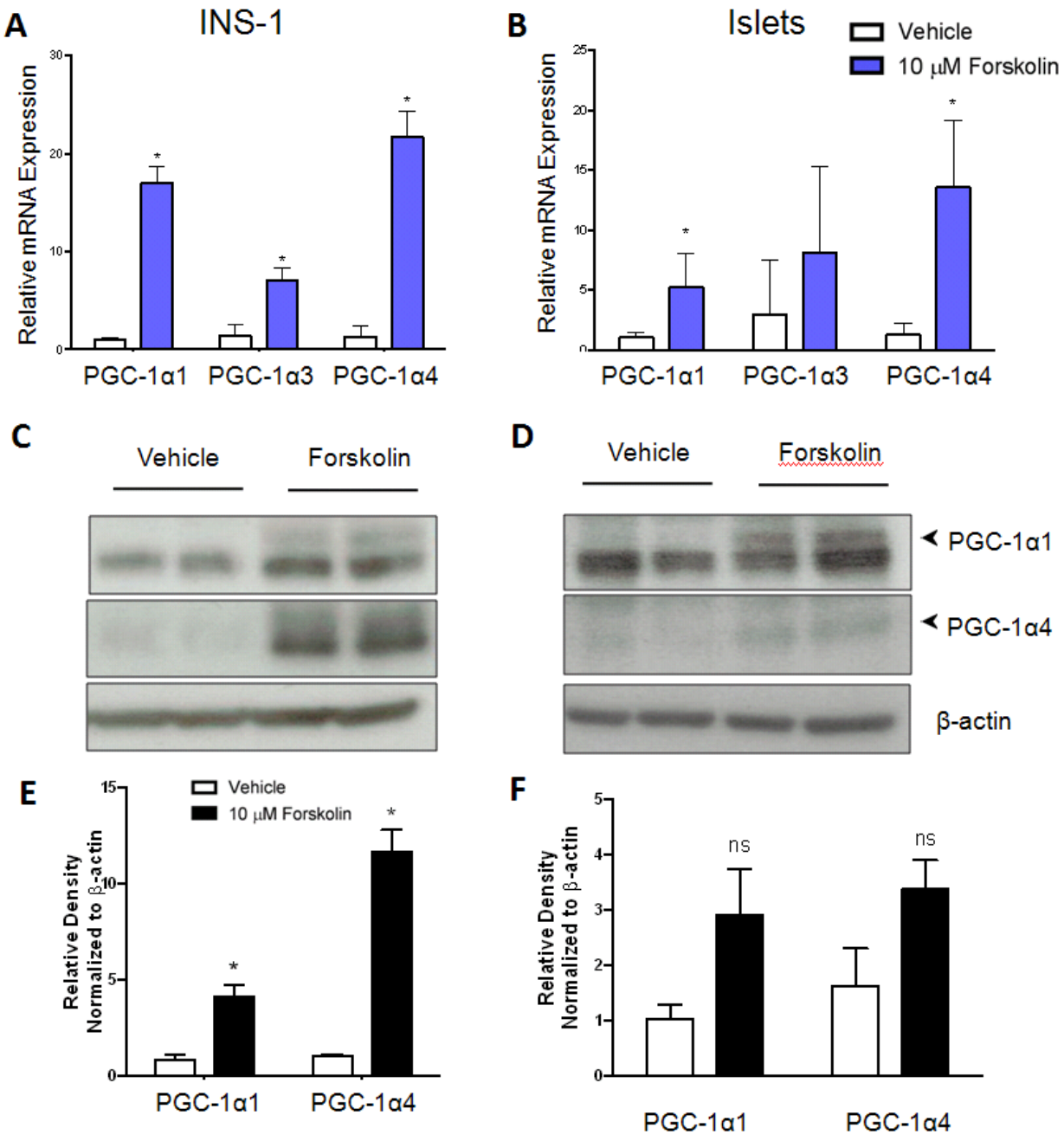

Figure 7. Endogenous expression of isoforms in a β-cell line and primary murine islets. Cells were treated with either DMSO (vehicle) or 10μM Forskolin for 2 hours for mRNA quantification and 4 hours for samples processed for total protein. (A) Analysis of gene expression for isoforms expressed in INS-1 cells and (B) isolated murine islets. Gene expression was analyzed by quantitative real-time PCR by using primers specific for individual isoforms. Protein lysates from (C) INS-1 cells and (D) isolated islets were immunoblotted for PGC-1α, using an antibody which recognizes all isoforms. Western blots for (E) INS-1 and (F) islet PGC-1α isoform expression were quantified using ImageJ software. Bars depict mean values, and error bars represent SD. *$p<0.05$ between indicated group and control. ns = not significant.

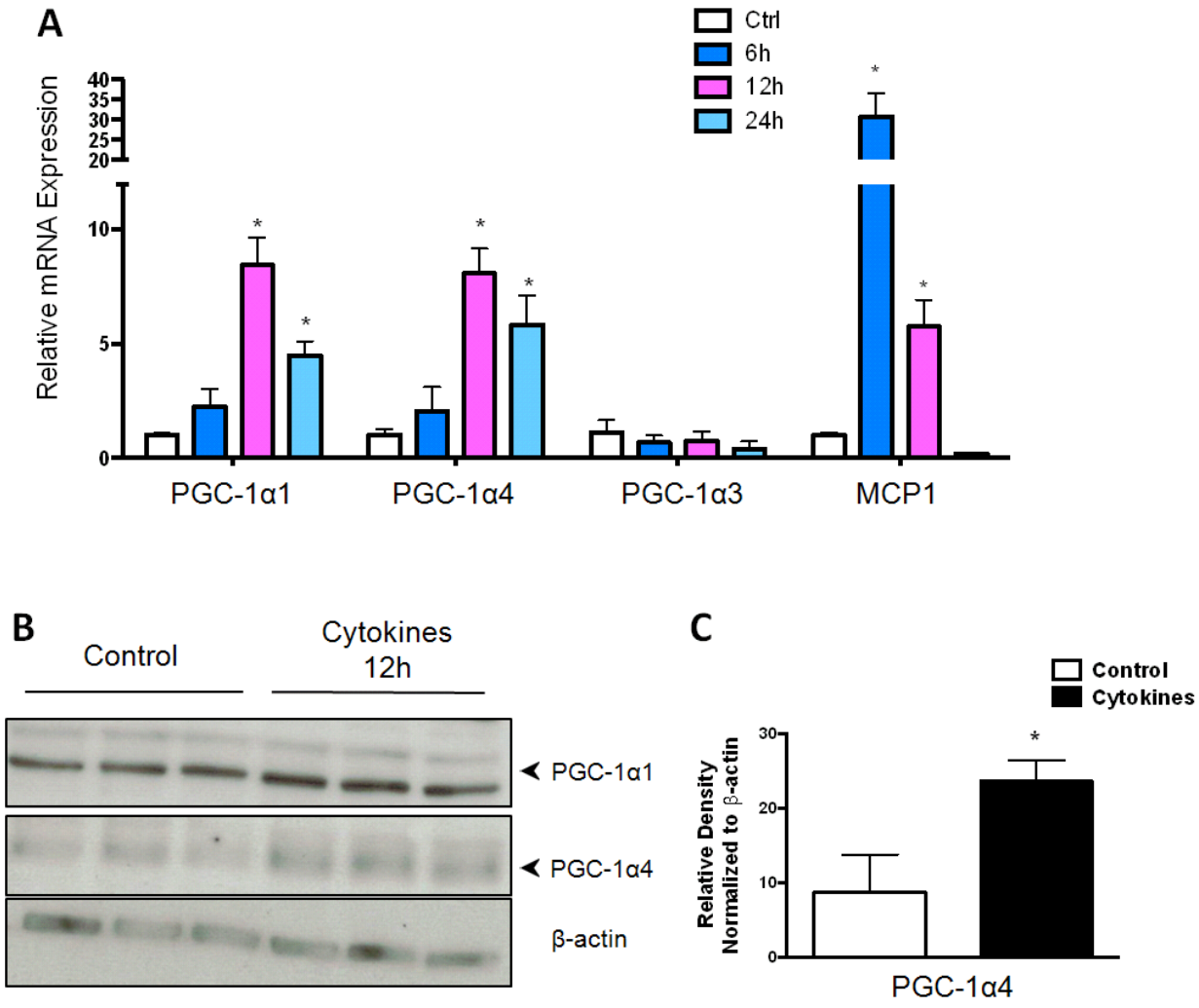

Figure 8. Incubation with a cytokine cocktail regulates the expression of PGC-1α1 and PGC-1α4 in a time-dependent manner. INS-1 cells were incubated without a cocktail of cytokines, TNFα (50ng/ml), IFNγ (50ng/ml) and IL-1β (10ng/ml), for 6, 12 and 24 hours. (A) Gene expression was analyzed by quantitative real-time PCR by using primers specific for indicated genes. (B) Total protein was harvested from INS-1 cells treated with or without a cytokine cocktail for 12 hours. Immunoblot of PGC-1α reveals PGC-1α4 expression in the cells treated with cytokines. (C) Western blot quantification of PGC-1α4 using ImageJ software. Bars depict mean values, and error bars represent SD. *$p<0.05$ between indicated group and control.

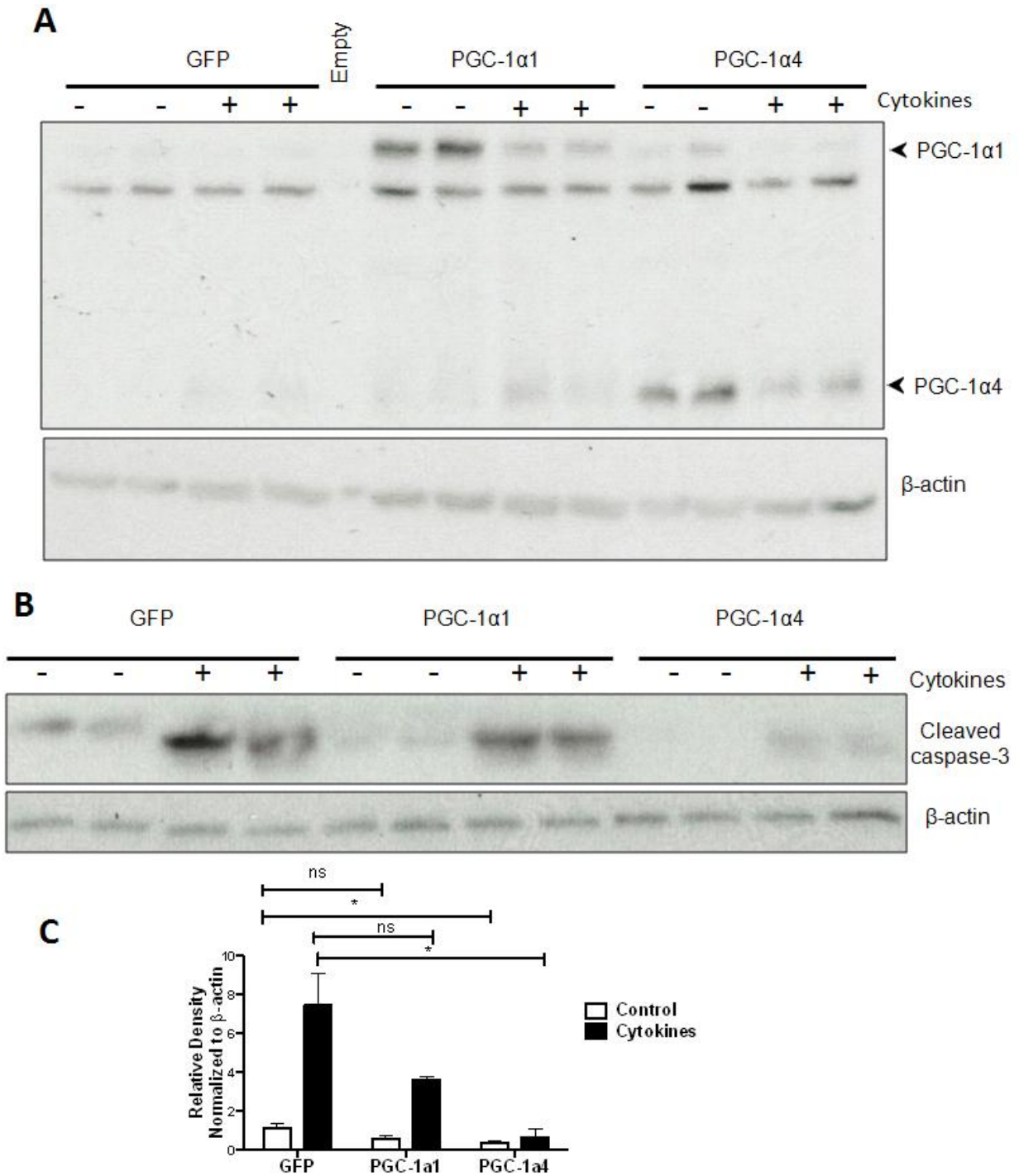

Figure 9. Overexpression of PGC-1α1 and PGC-1α4 reduces cleavage of caspase-3. INS-1 cells were infected with adenovirus expressing PGC-1α1, PGC-1α4 and GFP as a control. 30 hours post-infection, cells were treated with or without a cocktail of cytokines, TNFα (50ng/ml), IFNγ (50ng/ml) and IL-1β (10ng/ml). After an 18 hour treatment with cytokines, cells were processed for total protein. (A) Immunoblot for PGC-1α show overexpression PGC-1α1 and PGC-1α4. (B) Immunoblot with the same protein lysates for cleaved caspase-3. Presence of PGC-1α4, and PGC-1α1 to a lesser extent, reduces the amount of cleaved caspase-3. (C) Quantification of western blot in (B) using ImageJ. Bars depict mean values, and error bars represent SD. *p<0.05 between indicated group and control.

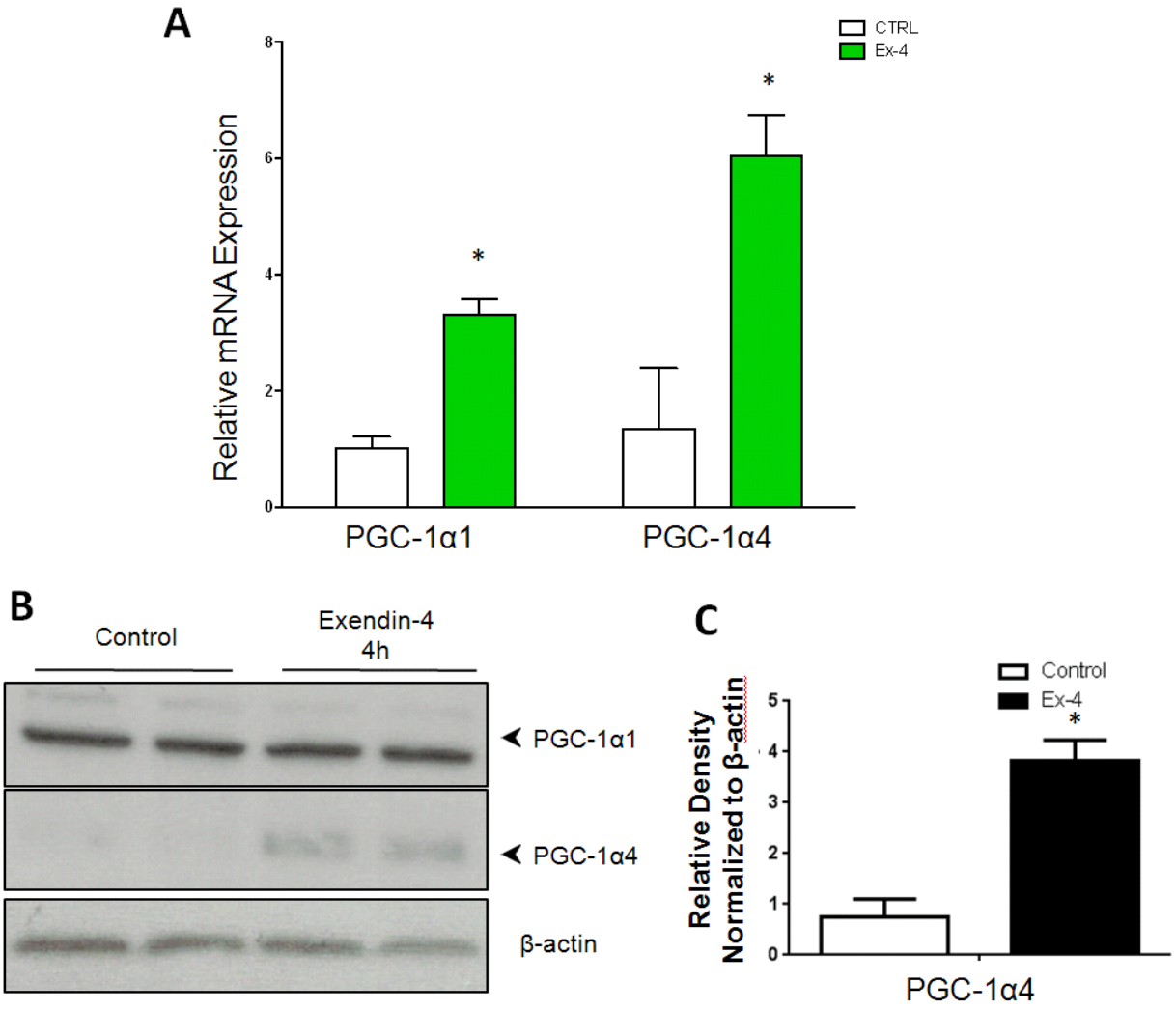

Figure 10. Treatment of INS-1 cells with Exendin-4 induces the expression of PGC-1α1 and PGC-1α4. INS-1 cells were treated with exendin-4 for 2 hours for mRNA quantification and 4 hours for total protein isolation. (A) Gene expression analysis was performed using qPCR for indicated genes. Exendin-4 induces expression of PGC-1α1 and PGC-1α4. (B) Immunoblot for PGC-1α shows an induction of PGC-1α4 protein. (C) Western blot quantification of PGC-1α4 using ImageJ software. Bars depict mean values, and error bars represent SD. *$p<0.05$ between indicated group and control.

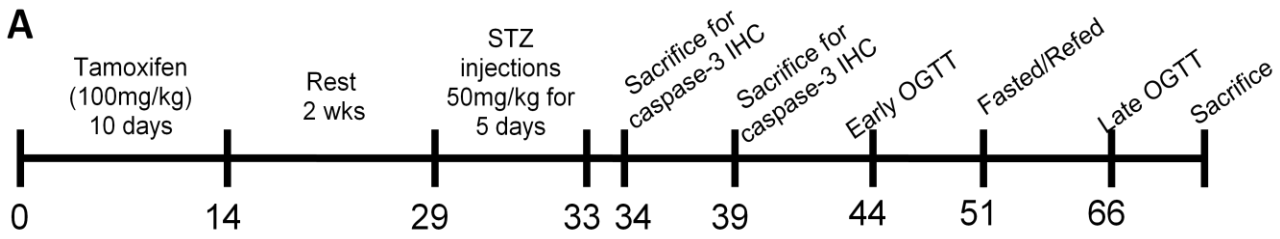

Figure 11. Experimental plan of PGC-1α KO with low-dose STZ injections. (A) Mice were gavaged 100mg/kg tamoxifen for 10 days. After 2 weeks, mice were injected with 50mg/kg of STZ. Blood glucose was taken bi-weekly after the first STZ injection. Two groups of mice (n=3) were sacrificed 1- and 6-days post STZ injections for histology of the pancreas. Then, 11, 18 and 32 days post STZ injections, an early OGTT (n=9 PGC-1α$^{fl/fl}$, n=8 PGC-1α$^{fl/flCre+}$), fasted/refed (n=9 PGC-1α$^{fl/fl}$, n=8 PGC-1α$^{fl/flCre+}$), and late OGTT (n=6 PGC-1α$^{fl/fl}$, n=5 PGC-1α$^{fl/flCre+}$) were performed, respectively.

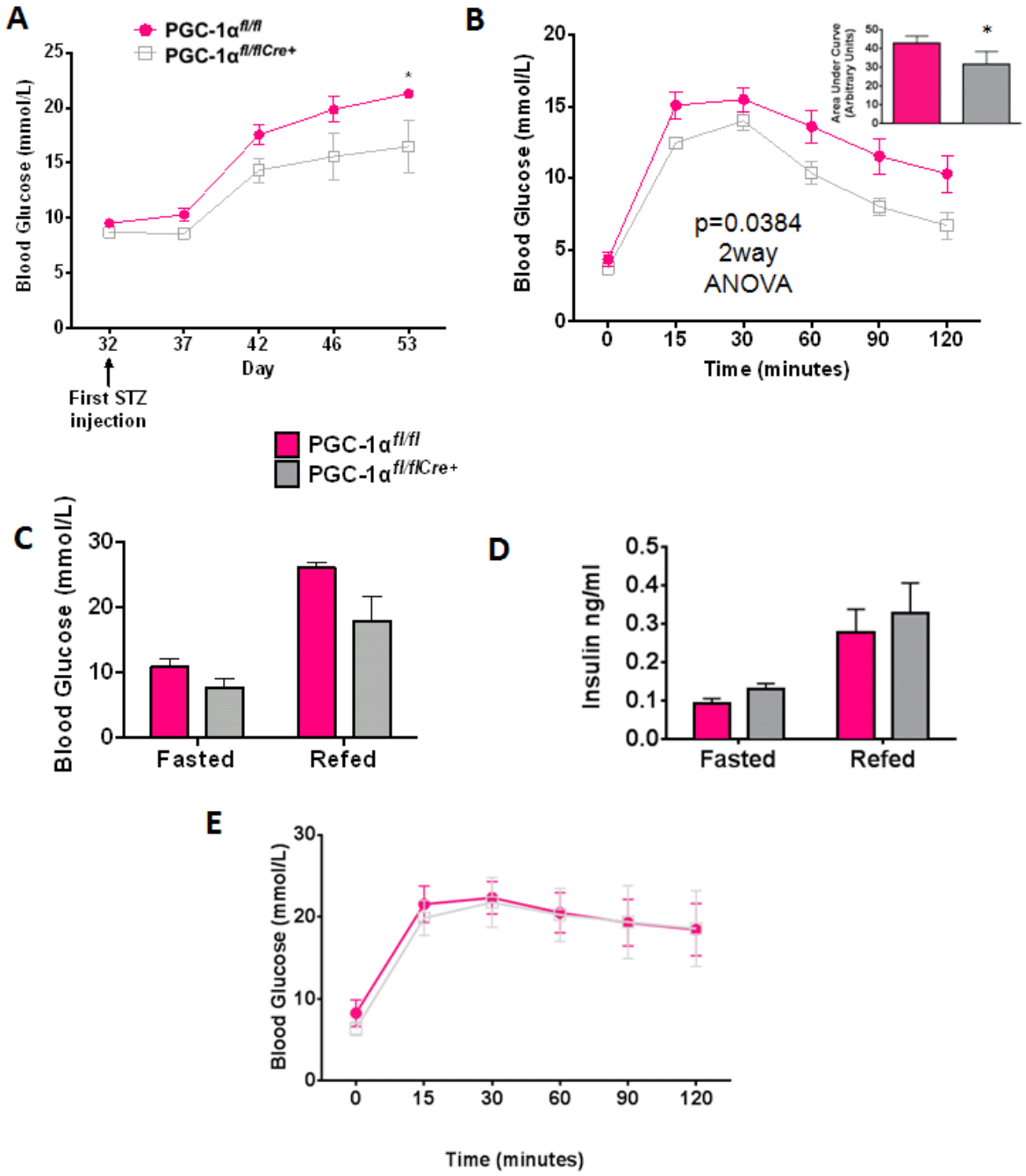

Figure 12. Knockout of PGC-1α and its isoforms *in vivo*. (A) Random fed glucose measurements of PGC-1α knockout mice, n=10 compared to littermate controls, n=11, starting from the first day of streptozotocin injections until the end of the experiment . (B) Early oral glucose tolerance (OGTT) test between PGC-1α knock out mice and littermate controls (n=8 and n=9, respectively). Significance by 2way ANOVA. OGTT is quantified by area under the curve. (C) Fasted/refed experiment (n=5). (D) Serum insulin content from fasted/refed experiment in (C). (D) Late OGTT between PGC-1α knock out mice and littermate controls (n=6 and n=5, respectively). Data for (A) and (B) are representative of 2 independent experiments. Bars and points on the curve depict mean values, and error bars represent SEM. *p<0.05 between indicated group and control, unless otherwise stated.

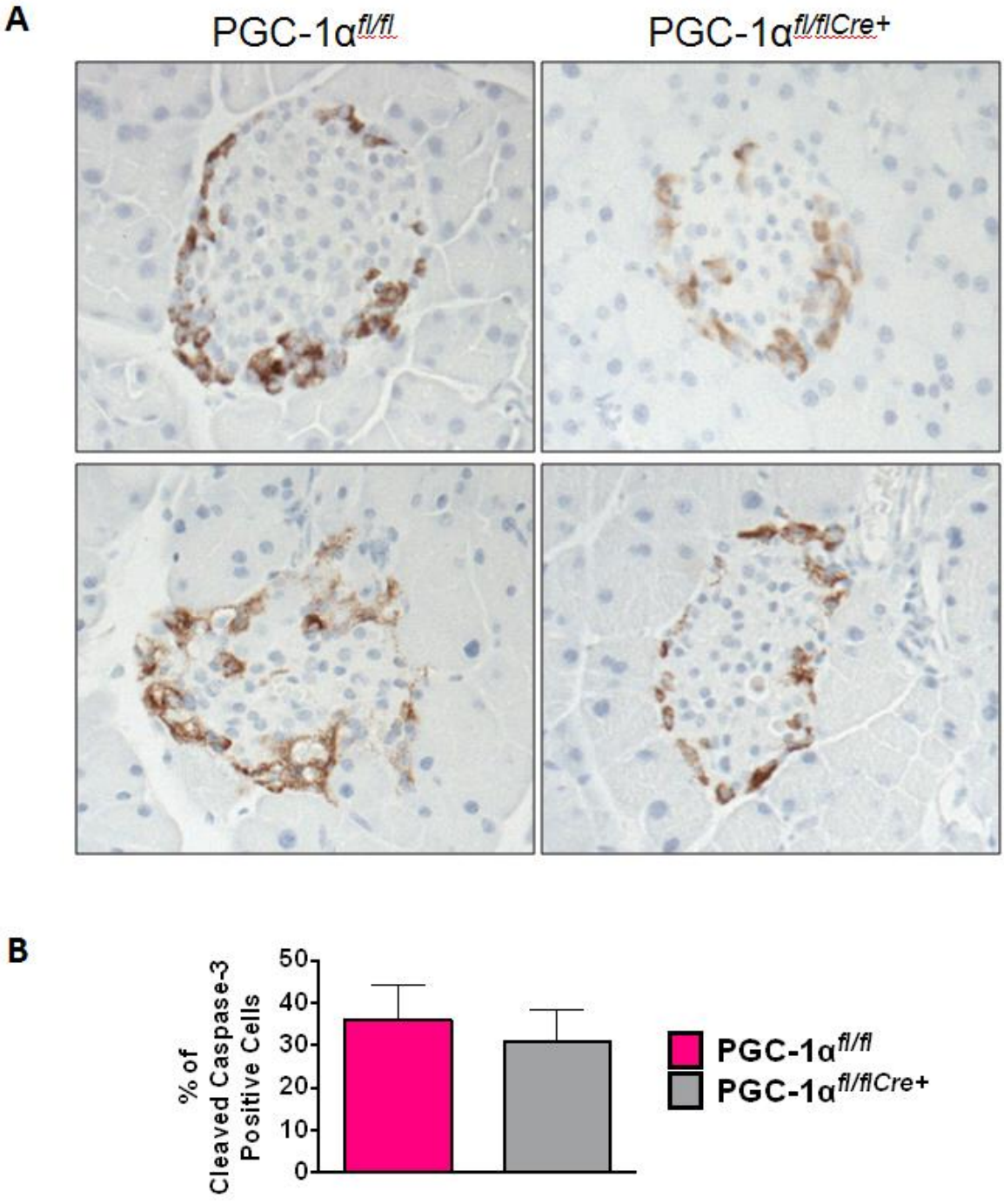

Figure 13. Cleaved caspase-3 expression in islets 1 day post-STZ injections of WT vs. KO mice. (A) Visually, cleaved caspase-3 staining by immunohistochemistry was not different between PGC-1α$^{fl/fl}$ (n=8) and PGC-1α$^{fl/flCre+}$ (n=7) groups. (B) Cleaved caspase-3 positive cells were not statistically different between the groups when quantified. Error bars represent SD. 10x magnification.

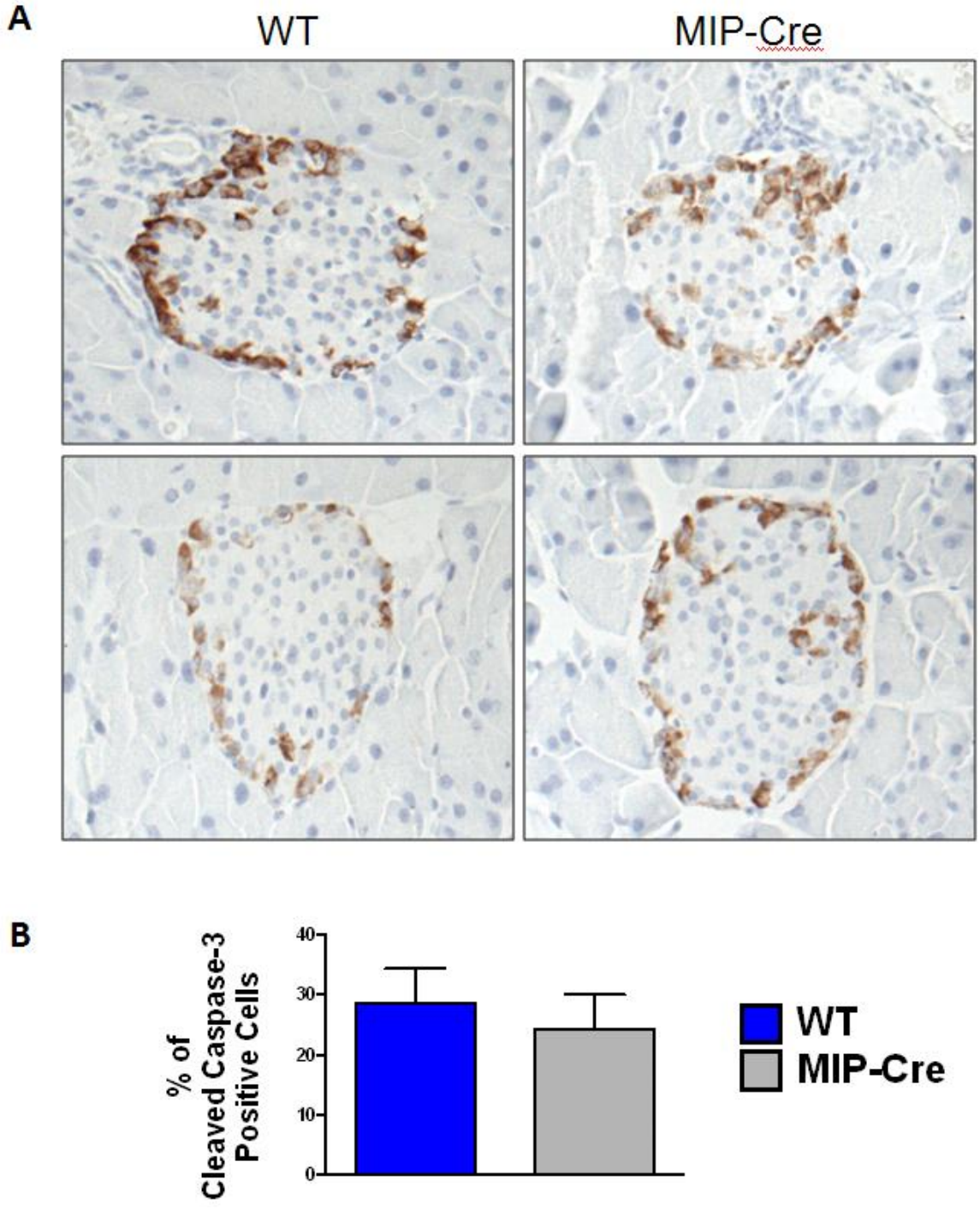

Figure 14. Cleaved caspase-3 expression in islets 1 day post-STZ injections of WT vs MIP-Cre control mice. (A) Visually, cleaved caspase-3 staining by immunohistochemistry was not different between WT (n=7) and MIP-Cre only controls (n=7). (B) Cleaved caspase-3 positive cells were not statistically different between the groups when quantified. Error bars represent SD. 10x magnification.

SECTION VI:
TABLES

Table 1: List of primers used for PCR

Name	Sequence (5'-- 3')
Alpha floxed	For: TCCAGTAGGCAGAGATTTATGAC Rev: TGTCTGGTTTGACAATCTGCTAGGTC
MIP-Cre	For: TAAGGGCCCAGCTATCAATGGGAA Rev: GTGAAACAGCATTGCTGTCACTT

Table 2: List of primers used for qPCR

Name	Sequence (5'-- 3')
PGC-1α1	For: GGACATGTGCAGCCAAGACTCT Rev: CACTTCAATCCACCCAGAAAGCT
PGC-1α2	For: CCACCAGAATGAGTGACATGGA Rev: GTTCAGCAAGATCTGGGCAAA
PGC-1α3	For: AAGTGAGTAACCGGAGGCATTC Rev: TTCAGGAAGATCTGGGCAAAGA
PGC-1α4	For: TCACACCAAACCCACAGAAA Rev: CTGGAAGATATGGCACAT
MCP-1	For: TGCTGTCTCAGCCAGATGCAGTTA Rev: TACAGCTTCTTTGGGACACCTGCT

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