Exploring novel mechanisms of pancreatic β–cell development, dysfunction and survival

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ABSTRACT

Diabetes has become a global health concern affecting more than 382 million people. It is ultimately a disease of the pancreatic β–cell, as associated cell death and dysfunction is necessary for the progression towards hyperglycemia. In the work presented here we explore new mechanisms involved in β–cell development, dysfunction and protection using a combination of cell culture techniques and *in vivo* work using frogs and mice as model organisms.

Using the frog *Xenopus laevis*, we show that transient overexpression of Neurogenin3 (Ngn3), the main determinant of endocrine cell fate in the embryonic pancreas, at distinct developmental stages was able to promote ectopic and early development of β–cells throughout the foregut. This was achieved by brief activation of Ngn3 after gastrulation and, most importantly, generated ectopic β-cells, but not α–cells. Using microarray analysis of endoderm tissue following Ngn3 overexpression, we identified novel genes expressed in the embryonic pancreas required for the creation of ectopic β–cells; including Tbx2, Mtg8 and Mtgr1. We propose these genes are important regulators of early β-cell fate specification and warrant further investigation in mammalian systems downstream of Ngn3.

Once β-cells are produced, they must maintain efficient function throughout their life cycle for adequate secretion of insulin and subsequent blood glucose control. Reduced expression of Peroxisome proliferator-activated receptor γ coactivators-1α and β (Pgc1-α/β), master regulators of mitochondrial biology, has been observed in several tissues associated with the pathogenesis of diabetes; in particular Pgc1-α was significantly reduced in islets of type II diabetics. To test whether this could contribute to β-cell dysfunction, we reduced the expression of Pgc1-α/β in adult β-cells of mice using a tamoxifen-inducible Cre-lox system, which lead

to impaired insulin secretion both *in vitro* and *in vivo,* but did not affect glucose homeostasis. Surprisingly, β–cells lacking Pgc-1 showed no significant change in mitochondrial respiratory capacity, despite reduced mitochondrial density and dysregulated expression of genes involved in mitochondrial dynamics. Inhibition of palmitate-potentiated insulin secretion, along with altered expression of key enzymes involved in acyl-glycerol metabolism, suggests the defect in secretion may be the result of dysregulated lipid metabolism.

During the course of our experimentation, we found that mice carrying the Mouse Insulin Promoter-CreERT (MIP-CreERT) transgene are protected against hyperglycemia caused by the β–cell specific toxin Streptozotocin (STZ). While control littermates were significantly hyperglycemic shortly following STZ administration, MIP-CreERT mice remained normoglycemic up to four weeks following treatment. These mice had increased blood insulin levels following an oral glucose tolerance test, yet surprisingly, their islets did not show protection against STZ-induced β–cell death. Further research into the underlying mechanism of protection in the model may reveal novel pathways to improve and protect β-cell from cellular stress and could improve β–cell physiology under hyperglycemic conditions. Taken together, our studies investigating mechanisms regulating β-cell development, metabolism, and response to cellular stress shed light on several aspects of β–cell biology that could contribute to the development of new therapies for both Type I and II diabetes.

RÉSUMÉ

Le diabète est actuellement un problème de santé publique majeur à l'échelle mondiale affectant plus de 382 millions d'individus. Cette maladie est associée à une dysfonction et ultérieurement à la mort des cellules β du pancréas entraînant l'hyperglycémie. Les recherches présentées dans cette thèse font la lumière sur de nouveaux mécanismes impliqués dans le développement des cellules β, leurs fonctions à l'aide de techniques de culture cellulaires et de travail *in vivo* chez la grenouille et la souris.

Dans le modèle de grenouille *Xenopus laevis*, nous avons montré que la surexpression transitoire dans l'endoderme de Neurogenin3 (Ngn3), qui est principalement impliqué dans la différenciation des cellules endocrines du pancréas, engendrait le développement prématuré et ectopique de cellules β au niveau de l'intestin antérieur de l'embryon. La brève activation de Ngn3 après la gastrulation permet la génération de cellules β ectopiques exclusivement (cellules alpha absentes). Une analyse par puce à ADN de l'endoderme, suite à la surexpression de Ngn3, a permis d'identifier de nouveaux gènes importants dans la création de cellules β ectopiques tels que Tbx2, Mtg8 et Mtgr1. Ainsi, nous proposons que ces gènes soient importants pour la régulation de la spécificité embryonnaire des cellules β et justifie des analyses plus approfondies des mécanismes dépendants de Ngn3.

À la suite du développement des cellules β, celles-ci doivent s'assurer de leur bon fonctionnement afin de bien réguler la sécrétion d'insuline pour maintenir les niveaux de glucose sanguin stables. Une diminution de l'expression du coactivateur du récepteur activé par les proliférateurs de peroxysomes-1α et β (Pgc-1α/β), qui sont des régulateurs importants de la mitochondrie, a été observée dans plusieurs organes associés avec une pathologie diabétique. En

effet, une diminution significative de Pgc-1α est présente dans les ilôts pancréatiques de patients atteints de diabète de type II. Afin de tester si cette diminution pourrait mener au dysfonctionnement des cellules β, nous avons réduit l'expression de Pgc-1α et Pgc-1β dans les cellules β de souris adultes à l'aide d'un système inductible au tamoxifène Cre-lox. Cette méthode induit une dérégulation de la sécrétion d'insuline tant *in vitro* qu' *in vivo* sans toutefois affecter l'homéostasie du glucose. Il est intéressant de noter que les cellules β n'avaient pas de défectuosité significative de la mitochondrie, mis à part une faible dérégulation de gènes impliqués dans la dynamique mitochondriale et une faible diminution de la densité des mitochondries. Finalement, l'inhibition de la sécrétion d'insuline en réponse à un traitement avec du palmitate, ainsi qu'une faible diminution de l'expression de certains gènes clés du métabolisme des acyl-glycérol suggèrent que le défaut de sécrétion d'insuline pourrait être dû à une dérégulation du métabolisme des lipides.

Pour finir, nous avons découvert que le transgène du promoteur de l'insuline chez la souris inductible au tamoxifène (MIP-CreERT) protège les souris contre l'hyperglycémie provoquée par 2 injections de la toxine spécifique aux cellules β streptozotocin (STZ). Même après 4 semaines suivant l'injection de la dernière dose de STZ, les souris MIP-CreERT ne deviennent pas hyperglycémiques en comparaison avec des souris WT de la même portée qui deviennent significativement hyperglycémiques en peu de temps. Nous avons remarqué que ces souris transgéniques ont un niveau d'insuline plus élevé dans le sang lors d'un test de tolérance au glucose. Cependant, les ilots isolés de ces souris ne montrent pas de signes protecteurs contre la mort cellulaire. Des études plus poussées nous permettront de déterminer la cause réelle de cette protection face à un traitement avec STZ et pourraient ainsi aider à la découverte de nouveaux mécanismes protecteurs pour les cellules β qui sont soumises à des conditions

d’hyperglycémie. En tout état de cause, les résultats obtenus par ces différentes études permettent de mieux comprendre plusieurs mécanismes au niveau du développement, du métabolisme et de la réponse au stress cellulaire des cellules β et ainsi promouvoir au développement de nouvelles thérapies pour le diabète de type I et II.

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PREFACE

This thesis is written in the traditional format in accordance with McGill University's "Guidelines for Thesis Preparation". The data presented in this thesis is the original work of the candidate, and part of it has been published in the following peer-reviewed articles:

Oropeza D, Horb M. Transient expression of Ngn3 in Xenopus endoderm promotes early and ectopic development of pancreatic beta and delta cells. Genesis. 2012. 50(3):271-85.

The candidate was the lead investigator for the work presented in this thesis, but would like to acknowledge the contributions of colleagues in the described work.

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LIST OF ABBREVIATIONS

Arx	Aristaless-related homeobox
Atp5b	ATP synthase subunit beta, mitochondrial
bHLH	Basic helix-loop-helix domain
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
COX5b	Cytochrome c oxidase subunit V b
COXII	Cytochrome c oxidase subunit II
CreERT	Cre recombinase fused to mutated ligand binding domain of estrogen receptor
CPT-1	Carnitine palmitoyl transferase I
CytoC	Cytochrome C
EGFP	Enhancer green fluorescent protein
ERRα	Estrogen-related recptor α
ESCs	Embryonic stem cells
ETC	Electron Transport chain
E10	Embryonic day 10
FAO	Fatty acid oxidation
FACS	Fluorescence activated cell sorting
Foxo1	Forkhead box protein O1
GFP	Green fluorescent protein
Glut2	Glucose transporter type 2
GR	Glucocorticoid receptor ligand binding domain
GSIS	Glucose stimulated insulin secretion
H&E	hematoxylin and eosin
Hes	Hairy and enhancer of split-related genes
HFD	High fat diet
iPSCs	Induced pluripotent stem cells
i.p.	intraperitoneal injection
Insm1	Insulinoma associated 1
KO	Knock-out
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene

MCAD	Medium chain acyl-coenzyme A dehydrogenase
MCK	Muscle creatine kinase
MEMFA	Mem salts plus formaldehyde
MMR	Marc's modified ringers
MIP	Mouse insulin promoter
Mtg8	Myeloid translocation gene on chromosome 8
Mtgr1	Mtg8 related protein 1
Mtg16	Myeloid translocation gene on chromosome 16
Myt1	Myelin transcription factor 1
NeuroD	Neuronal differentiation factor D
Ngn3	Neurogenin3
Nkx2.2	Nk2 homeobox 2
Nrf1	Nuclear respiratory factor-1
Nrf2	Nuclear respiratory factor-2
OGTT	Oral glucose tolerance test3
OXPHOS	Oxidative phosphorylation
Pax4	Paired box 4
PBS	Phosphate buffered saline
Pdx1	Pancreatic and duodenal homeobox 1
PGC-1α	Peroxisome proliferator activated receptor gamma co-activator 1-alpha
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
Ptf1a	Pancreas specific transcription factor 1a
Rfx6	Regulatory factor x 6
RIP	Rat insulin promoter
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SCAD	Short chain acyl-coenzyme A dehydrogenase
Si/shRNA	Short interfering/short hairpin RNA
STZ	Streptozotocin

Sur1	Sulfonylurea receptor 1
Tbx2	T-box 2
TID	Type I diabetes
TIID	Type II diabetes
Tfam	Mitochondrial transcription factor A
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tm4sf3	Transmembrane 4 super family 3
UCP-1	Uncoupling protein-1
UCP-2	Uncoupling protein-2
XPDI	Xenopus protein disulphide isomerase
WAT	White adipose tissue
WE	Whole endoderm
WEM	Whole endoderm plus mesoderm
WT	Wild type
Znt8	Zinc transporter 8

INTRODUCTION

Diabetes is a global epidemic and health concern

Diabetes has become one of the biggest global health concerns of this century. It is estimated that more than 382 million people suffer from this disease worldwide, with more than 5.1 million deaths resulting from it in 2013 (International Diabetes Federation). In countries such as Kuwait and Saudi Arabia the proportion of the population with diabetes can be as high as 23%. However, most alarming is the projected increase of cases, as it is estimated that by 2035 there will be more than 591 million people suffering from diabetes throughout the world (International Diabetes Federation). The health costs associated with these increasing numbers of diabetic patients are more than 540 billion dollars a year worldwide (International Diabetes Federation). In Canada, more than 3.3 million people have been diagnosed with diabetes, corresponding to 9% of the population, and by 2040 this number is likely to rise to 4.8 million (Canadian Diabetes Association). Total health costs are more than 13.5 billion Canadian dollars a year, but even more concerning is that despite a large investment of resources being dedicated towards treatment, it is estimated that one out of ten deaths of Canadian adults results from complications stemming from diabetes (Canadian Diabetes Association).

The ease of diagnosis allows for fairly accurate statistics. Diabetes is defined by fasting blood glucose above 7 mmol/L or by random blood glucose levels over 11 mmol/L with associated classic hyperglycemic symptoms such as polyuria or polydipsia (American Diabetes Association, 2010). Follow-up parameters confirm diagnosis, which include more than 6.5% glycated haemoglobin or blood glucose higher than 11 mmol/L two hours following an oral glucose challenge (American Diabetes Association, 2010). Hyperglycemia associated with diabetes causes damage to many different organs through various mechanisms. One of these mechanisms is the accumulation of toxic byproducts or intermediate metabolites that originate

from increased rates of glucose metabolism (Reviewed in Brownlee, 2005; Forbes and Cooper, 2013). One example of this is an increase in reactive oxygen species (ROS) originating from electron transport chain (ETC) activity as a result of glucose oxidation (Brownlee, 2005; Forbes and Cooper, 2013). This effect has been demonstrated in endothelial cells, which are more susceptible to damage as they are in direct contact with the high glucose in blood, and has been shown to promote cardiovascular complications (Brownlee, 2005). Nephropathy and neuropathy are other major complications, which may have severe consequences such as kidney failure, blindness and gastroparesis (Forbes and Cooper, 2013). In fact, diabetes accounts for 44% of new kidney failure cases in the US (United States Renal Data System).

There are several types of diabetes defined by different etiologies. In every type, extensive death or dysfunction of the insulin-producing pancreatic β-cell is necessary for the progression into hyperglycemia. The majority of cases are caused by Type I and II diabetes, ~10% and ~90 % respectively, and the remaining are attributed to more rare cases of monogenic diabetes affecting ~1-2% of patients.

Type I diabetes

Type I diabetes (TID) is caused by environmental and polygenic factors which activate an autoimmune attack on the β–cells, resulting in significant cell death, a dramatic decrease in serum insulin levels, and hyperglycemia at an early age (Rahier et al 1983; van Belle et al, 2011). There is limited knowledge about what environmental factors catalyze type I diabetes, but it is thought that diet, medications and infection play important roles. Viral and bacterial infection are the most studied environmental triggers of TID. They can activate autoimmune damage by causing direct release of autoantigens, presenting antigens that mimic autoantigens, or changing

the proportions of immune cells that infiltrate the islets (Reviewed in Lehuen et al, 2010). On the other hand, genetic components of the disease have been identified and confirmed using both linkage analysis and genome wide association studies. Perhaps not surprisingly, many associated genes are involved in regulation of immune cell function, including the human leukocyte antigen genes, which show the strongest association with type I diabetes (reviewed in Eisenbarth, 2009; Concannon et al, 2009). The insulin gene itself is an autoantigen with the second strongest association in genome wide studies (Eisenbarth, 2009; Concannon et al, 2009). However, in the best of cases, identified genes only account for ~60% of type I diabetes cases analyzed, pointing to the possible existence of other undiscovered genetic factors (Eisenbarth, 2009; Concannon et al, 2009).

The only available therapies for TID are islet transplantation and life-long supplementation with exogenous insulin. Islet transplantation is limited by the scarce number of donors, the need of a large amount of tissue per recipient and the need to provide life long immunosuppressive drugs, which can carry their own side effects and complications. While the administration of exogenous insulin is relatively simple and effective, even the most advanced methods of insulin administration cannot reproduce the delicate and precise regulation of insulin secretion that the β–cell can provide in response to physiological fluctuations in blood glucose levels. Thus, even though TID is currently a treatable disease with a long life expectancy, diabetic complications often arise later in life due to our inability to provide adequate long-term glucose regulation.

Type II diabetes

Type II diabetes (TIID) is also a polyfactorial disease with strong environmental and polygenic components that culminate in insulin resistance and defective β–cell function. Diet is a predominant environmental factor, as being overweight or obese exponentially increases the probability of developing TIID. Obesity promotes the incidence of insulin resistance through various mechanisms that include dysregulation of hormones including leptin and adiponectin and increasing concentration of cytokines and other inflammatory factors (reviewed in Kahn et al, 2006; Wellen and Hotamisligil, 2005). Extensive efforts using genome-wide association studies and large cohorts have uncovered dozens of TIID susceptibility loci (Gloyn et al, 2003; Grant et al, 2006; Sladek et al, 2007; Sandhu et al, 2007; Scott et al, 2013; SIGMA Type 2 diabetes consortium et al, 2014). Most loci include genes that regulate β–cell function or mass, but genes involved in the metabolism of glucose and lipids in peripheral organs have also been identified; such as the solute carrier SLC16A11, a transmembrane protein that regulates lipid metabolism in liver cells (SIGMA Type 2 diabetes consortium et al, 2014). In most cases, it is unclear how these genes are involved in the pathogenesis of TIID, in part because they do not appear to contribute to pathology in a strong way individually, but instead are thought to promote the incidence of TIID in a collective manner with other modifying genes (McCarthy et al, 2008).

Insulin resistance can occur many years before the onset of diabetes, increasing the demand for insulin and causing hyperinsulinemia (Bergstrom et al, 1990; Haffner et al, 1990; Martin et al, 1992). β–cells are thought to initially compensate by increasing insulin secretion and mass (reviewed in Prentki and Nolan, 2006). Nonetheless, eventually β–cells cannot sustain the increased demand for insulin due to a number of factors, and insulin secretion is significantly decreased. This leads to dysregulation of glucose levels that promote a decline in β–cell mass

and hyperglycemia.

Impaired glucose stimulated insulin secretion (GSIS) is a hallmark of TIID. Decreased GSIS in diabetics is observed following a glucose tolerance test, as well as in islets isolated from diabetic patients (Pfeifer et al, 1998; Deng et al, 2004; Fukushima et al, 2004; Del Guerra et al, 2005; Ling et al, 2008). The primary causes of β–cell dysfunction are not fully understood, but there are clear environmental and genetic components that can affect GSIS. Increased concentrations of blood glucose and free fatty acid that are chronically present throughout the progression or insulin resistance to diabetes create a toxic environment that negatively impacts β–cell function. Prolonged incubation of human isolated islets in high concentrations of glucose (11 mM for seven days) or free fatty acids (1 mM of a mixture of free fatty acids for two days) significantly decreases GSIS (Eizirik et al, 1992; Lupi et al, 2002). Interestingly, if the islets are subsequently cultured in low glucose concentrations, there is a partial recovery of GSIS (Eizirik et al, 1992). Rat studies demonstrate that prolonged infusion of high glucose concentrations *in vivo* severely impairs GSIS in a time and concentration-dependent manner (Leahy et al, 1986; Leahy et al, 1988). Furthermore, polymorphisms in genes encoding proteins that compose the insulin secretory or insulin granule processing machineries have been associated with TIID (Gloyn et al, 2003; Sladek et al, 2007). In support of genetic factors contributing to β–cell dysfunction in TIID, normal glucose-tolerant relatives of type II diabetics also exhibit reduced total blood insulin levels following a glucose tolerance test, compared to individuals with no family history of diabetes (van Haeften et al, 1998).

There has been controversy on whether the decrease in GSIS observed in TIID is due only to β-cell dysfunction or whether it also requires a progressive decline in β–cell mass. More than 50 years ago it was shown that β–cell mass is significantly decreased in diabetics. In that

study, there was no distinction between TID and TIID, but the age of diabetes onset provides a good estimation of the type in each case (Maclean et al, 1955). When separated by age of onset, there is a clear decrease in β–cell mass in both early and late onset diabetics, yet no difference in α-cell mass (Maclean et al, 1955). Since then, a few studies have also shown no difference in β–cell mass in type II diabetics (Rahier et al, 1983; Kobayashi et al, 1997; Guiot et al, 2001). However, their conclusions may have been limited by small sample sizes (less than 10 patients) or histological analysis of only a small proportion of the pancreas per patient. Since then, most studies report a 40% to 60% decrease in β–cell mass in type II diabetics (Sakuraba et al, 2002; Butler et al 2003; Yoon et al, 2003b; Rahier et al, 2008). A seminal study showed a decrease in β–cell volume in both lean and obese diabetics as well as in obese individuals with impaired fasting glucose that were not yet diabetic (Butler et al, 2003). Thus, it remains a general consensus in the field that type 2 diabetes is associated with a decrease in β–cell mass.

The decrease in β–cell mass can be attributed to many mechanisms, including failure to proliferate and/or replenish cell populations or increased cell death due to cellular stress. Apoptosis is thought to be one of the main contributors to loss of β–cell mass in TIID. Apoptotic markers are increased in β–cells of both lean and obese type II diabetics (Butler et al, 2003). Isolated human islets exposed to chronic high glucose (16 mM for five days) or fatty acids (2 mM of a mixture of free fatty acids for two days) concentrations exhibit increased β–cell apoptosis, evident by changes in β-cell ultrastructure, as well as increased transcription of genes associated with apoptotic pathways (Lupi et al, 2002; Federici et al, 2001; Maedler et al, 2001). Different molecular and cellular activators of β–cells apoptosis in TIID have been identified, including pro-apoptotic signals coming from the mitochondria, endoplasmic reticulum stress and cytokine-activated signaling (reviewed in Donath et al, 2005). Nonetheless, in some instances

type II diabetics have decreased insulin staining without incidence of apoptosis (Butler et al, 2003). New mechanisms underlying the decline in β–cell mass independent of apoptosis have been proposed. Recent research shows that β–cell dedifferentiation, without any detectable apoptosis, occurs in islets of mice of with β–cell-specific knockout of Forkhead box protein O1 (Foxo1), a transcription factor associated with TIID and needed to maintain β–cell function (Talchai et al, 2012; Mussig et al, 2009).

The complex interplay between environmental and genetic factors that integrate to affect β–cell function is not fully understood. For example, mechanisms by which glucolipotoxicity is augmented by specific polymorphisms associated with TIID to potentiate β-cell death or dysfunction have yet to be identified. These types of studies will undoubted help to us understand how diabetes risk is augmented by the combined outcomes of our genetics, environment, and lifestyle choices.

As the primary cellular and molecular causes of TIID are not known, current therapies focus on improving glucose uptake and utilization in peripheral organs and promoting insulin secretion from β–cells. Incretin hormones (like glucagon-like peptide 1) or potentiators of insulin exocytosis (like the sulfonylureas) improve the secretory capacity of β–cells, whereas different combination of drugs can promote glucose uptake by muscle, reduce glucose output by liver, or reduce reabsorption by the kidney (Reviewed in Tahrani et al, 2011). In advanced cases where there is extensive β–cell loss or dysfunction, exogenous insulin needs to be administered to maintain glucose control. Yet, because the cellular and molecular causes of TIID and β–cell dysfunction are not targeted by these therapies, the best we can do is prevent or delay diabetic complications by controlling hyperglycemia and there is currently no cure for diabetes.

Monogenic diabetes

Monogenic diabetes is a collective term used to describe rare types of diabetes caused by mutations in various single genes that in most cases produce severe developmental or functional defects in β–cells (reviewed in Edghill and Hattersley, 2008; Schwitzgebel, 2014). This promotes the development of hyperglycemia, either neonatally or from an early age. Gene products essential for the insulin exocytosis machinery cause the majority of neonatal diabetes cases. While rare cases are caused by a significant impairment in the development of endocrine or pancreatic tissue, these are quite severe and can be associated with serious problems in other organs due to the pleiotropic function of the causative genes such as Regulatory factor x 6 (Rfx6) (Edghill and Hattersley, 2008, Schwitzgebel, 2014). Monogenic diabetes that arises in early childhood or early adulthood is termed Maturity onset diabetes of the young (MODY). It is usually autosomal dominant and caused by autosomal dominant mutations in genes involved in the regulation of GSIS (Edghill and Hattersley, 2008, Schwitzgebel, 2014). The most common cases are caused by severe mutations in transcription factors that are needed to maintain β–cell function and by mutations in the glucokinase gene, encoding an enzyme that is needed to secure shuttling of glucose into glycolysis and ATP production (Edghill and Hattersley, 2008).

Finally, there is a special kind of monogenic diabetes termed mitochondrial diabetes that arises from mutations in mitochondrial DNA. This type of diabetes is caused by severe defects in mitochondrial function and has nearly 100% penetrance (Kadowaki et al, 1994; Maassen et al, 2004). Mitochondrial diabetes is extremely rare and typically arises during middle age (between 30-40 years); however, the range of onset can be quite broad (Kadowaki et al, 1994; Maassen et al, 2004). Mitochondrial diabetes is most notably characterized by impaired insulin secretion as well as β–cell loss, which worsens over time (Kobayashi et al, 1997; Kadowaki et al, 1994;

Maassen et al, 2004). Due to these characteristics, it may be mistaken for TIID. Although maternal transmission is typically a strong indication, only genetic analysis can ultimately provide a definitive diagnosis.

The pancreas and the islet of Langerhans

The pancreas is an endocrine and exocrine organ composed of different cell types including exocrine, duct, endocrine, endothelial and neuronal cells. The majority of the pancreas is made up of exocrine and endocrine compartments. Exocrine cells, referred to as acinar tissue, make the bulk of the pancreas. They produce a wide variety of digestive enzymes that are secreted into the intestinal lumen to cleave proteins, nucleic acids, lipids and carbohydrates, and include amylase, elastase, trypsin and lipase. Duct cells make the lining of the ducts through which digestive enzymes are transported into the intestine and secrete bicarbonate to neutralize acids coming from the stomach.

The endocrine cells are grouped together into Islets of Langerhans that are highly vascularized micro-organs found embedded in the acinar tissue randomly throughout the pancreas. Islets make up about ~1-2% of pancreatic tissue. Endocrine cells are composed of β–cells that secrete insulin, α-cells that secrete glucagon, δ-cells that secrete somatostatin, PP cells that secrete pancreatic polypeptide and the more rare ε–cells that secrete ghrelin. The cell types are intimately linked and communicate with each other through cell-cell interactions and paracrine signaling. In mice, β–cells compose 80% of the islet, α-cells ~10-15% and the rest are a mix of the other endocrine cells. Murine β–cells are concentrated in the middle of the islet surrounded by a mantle of other endocrine cells (Brissova and Powers, 2008) On the other hand, in human islets the proportions change to ~50 % β–cells and ~30 % α–cells (Cabrera et al, 2006).

Human β–cells are generally more interspersed with the other endocrine cells throughout the islet, but there is still a clear segregation between β–cells and the rest. In islets of smaller diameter, the β–cells tend to be clumped more towards the middle, similar to murine islets (Cabrera et al, 2006; Bosco et al, 2010). This assembly of islet architecture (β–cells are mostly grouped in the center) is seen in the majority of vertebrates to different degrees, although some exceptions do exist, such as in horses where the center of the islet is composed mainly of α–cells (Steiner et al, 2010).

Pancreas and β-cell development

The main morphogenetic movements and transcriptional cascades that orchestrate pancreas and β–cell development are conserved between higher vertebrates. In mouse embryonic development, the pancreas forms from one ventral and one dorsal bud that evaginates from the foregut epithelium around embryonic day 9 and 10 (E9-10; reviewed in Pan and Wright, 2011; Gittes, 2009). The mechanism governing bud formation and morphology remain unclear; however, the key transcriptional steps that give rise to pancreatic progenitors are well characterized. Starting around E8 in mice, co-expression of the transcription factors Pancreatic duodenal homebox 1 (Pdx1) and Pancreas specific transcription factor 1a (Ptf1a) defines pancreas progenitors (Figure 1) and is largely responsible for activating the subset of genes that will ultimately generate all endocrine and exocrine tissues of the mature pancreas (Afelik et al, 2006; Gittes et al, 2009; Jonsoon et al, 1994; Kawaguchi et al, 2002; Krapp et al, 1998). Ptf1a promotes expression of genes involved in the formation of acinar as well as duct tissue (Arda et al, 2013; Gittes et al, 2009). Pdx1, in conjunction with other transcription factors, activates the transcriptional network of pancreatic endocrine progenitor cells (Arda et al, 2013; Gittes et al,

2009; Oliver-Krasinki et al, 2009).

One of the targets of Pdx1 is Neurogenin3 (Ngn3), a transcription factor that determines the identity of endocrine progenitors and is needed for the formation of all pancreatic endocrine cells (more detailed information on Ngn3 will be provided in the introduction for Chapter 1). Ngn3 sets in motion a series of transcriptional events that activate transcription factors involved in maintaining endocrine progenitor identity, as well as, upregulating genes that will determine each endocrine lineage. Genes encompassing the first group include Insm1, NeuroD, Rfx6 and Nkx2.2, which have been shown to be direct targets of Ngn3 (Arda et al, 2013) (Figure 1). These genes are co-expressed with Ngn3 and knockout studies have shown they are necessary to maintain optimal mass of the majority of endocrine cells (Gittes, 2009; Pan and Wright, 2011). While deemed essential, their specific roles in endocrine progenitors are not clear or how they are involved in integrating transcriptional information to direct the specification of each endocrine lineage.

Shortly after Ngn3 expression is detected, the first endocrine cells are specified (around E9) (Figure 1). A first wave of endocrine cell formation begins with a small cluster of cells being formed; glucagon is the first hormone to be expressed in mice, while in humans it is insulin (Gittes, 2009; Pan and Wright, 2011; Jennings et al, 2013). Endocrine cell number modestly increases up to E11.5 after which endocrine growth becomes somewhat quiescent up until E12.5 (Gittes, 2009; Pan and Wright, 2011). During this time period the pancreatic buds fuse and strong proliferation of pancreatic progenitors cause the branching out of epithelial tissue forming complex structures that will give rise to the mature pancreas. A burst in endocrine cell formation occurs from ~E12.5 to ~E15, during which the majority of the cells that will make the mature endocrine compartment are formed (Gittes, 2009; Pan and Wright, 2011).

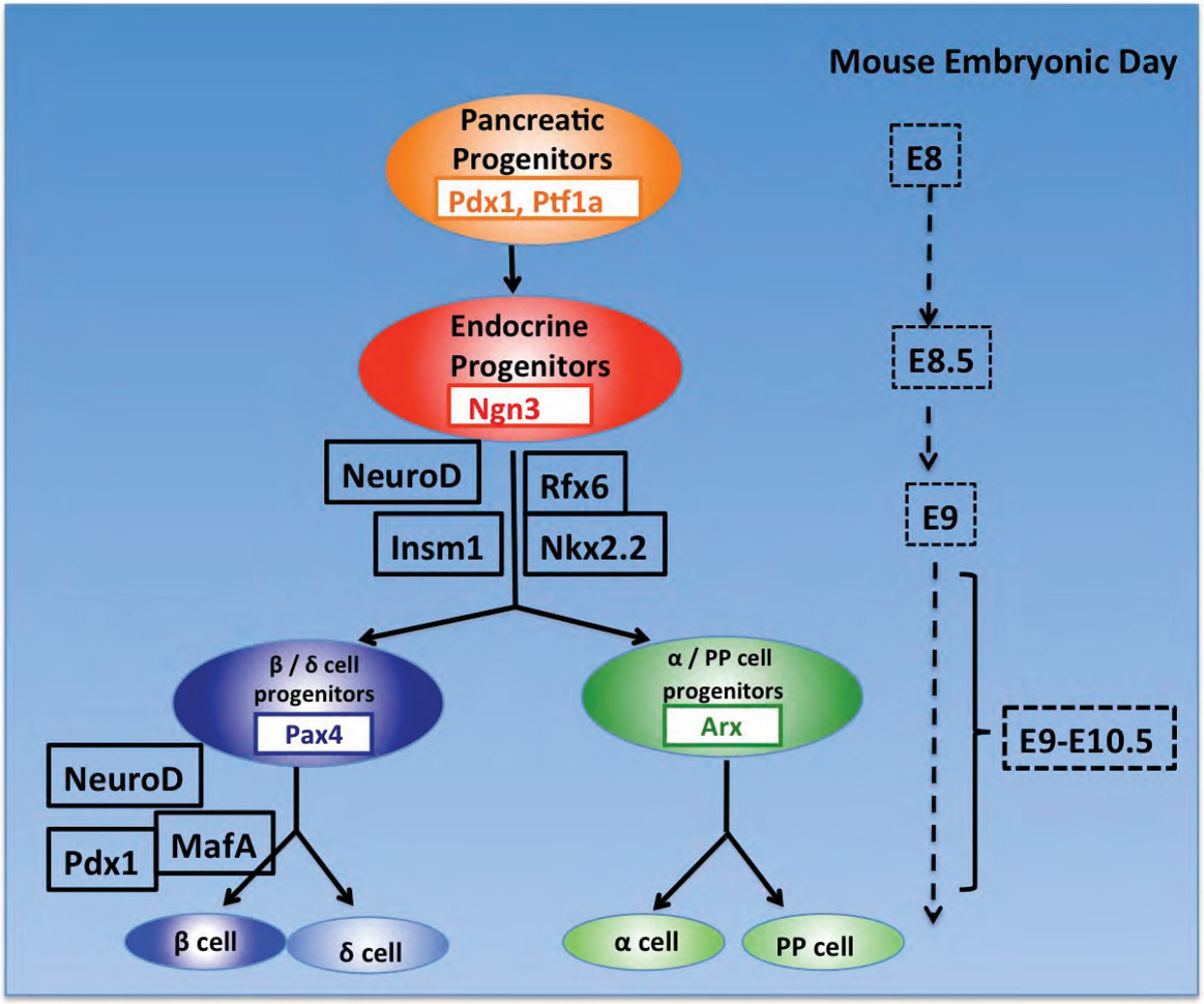

Figure 1 – Simplified diagram of the main transcription factors involved in embryonic β–cell development (boxes with solid frames) and approximate mouse embryonic stages corresponding to the first time expression of the transcription factors is detected.

One of the targets of Ngn3, the transcription factor Paired box 4 (Pax4), marks progenitors that will give rise to β and δ–cells while Aristalesss related homeobox (Arx), another transcription factor target of Ngn3, defines progenitors that produce α and PP cells (Figure 1, Arda et al, 2013). Pax4 and Arx inhibit the expression of each other, but the mechanisms

controlling which one will prevail under certain circumstances are still unclear (Collombat et al, 2003; Collombat et al, 2005). Repression of Arx is necessary to maintain the β–cell phenotype, while overexpression promotes formation of α-cells (Dhawan et al, 2011; Collombat et al, 2007).

The final step in endocrine development consists of the maturation of endocrine cells that primes them for efficient hormone secretion. This occurs up until the first few days after birth and through the expression of transcription factors that control hormone expression, processing and secretory machineries (Gittes, 2009; Pan and Wright, 2011). Interestingly, these transcription factors are comprised of genes that also control early endocrine and pancreas development including NeuroD and Pdx1, as well as, β–cell specific genes that only start to be expressed at the maturation step such as MafA (Figure 1).

Inactivation of Pdx1 in mouse β-cells, starting at the onset of insulin expression, results in normal islets and blood glucose levels at birth; but a progressive decrease in β-cell function and insulin secretion that causes hyperglycemia by five months of age (Alhgren et al, 1998). This dysfunction arises because Pdx1 directly regulates the expression of the insulin gene, as well as, mitochondrial genes needed to obtain optimal GSIS (Mcfarlane et al, 1994; Gauthier et al, 2009). Inhibition of NeuroD in either fetal or adult β-cells also causes β–cell dysfunction characterized by decreased expression of the insulin gene, a slight decrease in β-cell mass and impaired insulin secretion (Gu et al, 2010). However, unlike with β–cell-specific Pdx-1 inactivation, mice lacking NeuroD are severely glucose intolerant yet do not become hyperglycemic (Gu et al, 2010). MafA also directly activates the insulin promoter and acts synergistically with NeuroD and Pdx1 (Olbrot et al, 2002; Kataoka et al, 2002). The MafA whole body knockout mouse has normal endocrine cell mass at birth, but β–cell defects develop with age including decreased β–cell mass and strongly impaired insulin secretion both *in vitro* and *in vivo*; eventually leading to

hyperglycemia (Zhang et al, 2005c). This suggests MafA is not needed for the creation of β–cells, but is only required for their maturation. This was supported by results from MafA overexpression experiments using the Pdx1 promoter, which showed that it is not able to promote β-cell fate (Nishimura, 2009). MafA overexpression causes cell cycle arrest and inhibition of pancreatic progenitor proliferation, which severely obstructs the development of the pancreas (Nishimura et al, 2009).

De novo generation of β–cells for cell replacement therapy

One of the most promising therapies for both TID and TIID is replacement of healthy β-cells to compensate for those that have died or become dysfunctional. *De novo* β-cells can be generated by directed differentiation of pluripotent stem cells or by reprogramming adult cells of various endodermal origins (reviewed in Pagliuca and Melton, 2013). Key transcription factors and signaling molecules identified from the extensive research performed on pancreas endocrine development are used as markers and catalysts of β–cell differentiation. In the past ten years, great achievements have been accomplished, including *in vitro* generation of β-cells from human embryonic or induced pluripotent stem cells (ESCs, iPSCs; (Kroon et al, 2008; Maehr et al, 2009; Hrvatin et al, 2014). Furthermore, work in mice shows that it is possible to reprogram adult acinar tissue or fetal intestinal tissue into β–cells *in vivo* (Zhou et al, 2008; Talchai et al, 2012b).

Several problems have hindered progress towards cells that would be suited for transplantation. In most cases the efficiency of β–cell formation is low and all of the endocrine cell types are generated, with β–cells being only a minor subset (Reviewed in McKnight et al, 2010; Pagliuca and Melton, 2013). The β–cells produced often do not behave like endogenous β–

cells and may have lower insulin production, insufficient or dysregulated insulin secretion, and in some instances, dedifferentiate after prolonged culture (Kroon et al, 2008; Hrvatin et al, 2014; McKnight et al, 2010; Pagliuca and Melton, 2013).

Ideally, to assist the development of an effective therapy, a system is needed that would produce a homogenous population of functional β-cells in high enough numbers for transplantation. Apart from the associated technical limitations of current methods used to reprogram cells, one of the reasons why functional β–cells cannot be produced may be because the complete transcriptional program responsible for generating functional β–cells during embryonic development is still unknown. Key transcriptional steps may still be missing that are essential to reconstitute the β–cell phenotype. It has become clear that the sole expression of insulin and the secretory machinery is not sufficient to equip *de novo* β–cells with the tools needed for the delicate glucose-stimulated regulation of insulin secretion observed in endogenous β-cells. Emphasis should be put on early determinants of β-cell fate specification; for example, factors that determine if expression of Pax4 or Arx will prevail, since there is virtually no information available about what occurs immediately after Ngn3 expression to define β–cell fate. It is plausible that a gene or, more likely, a set of genes exists that are both necessary and sufficient to promote development of β–cells, but not the other endocrine cells.

CHAPTER I PREAMBLE

The following body of work encompasses distinct, yet intimately related aspects of β-cell biology. In order to develop effective and sustainable therapies for diabetes that would efficiently regulate insulin levels it is important to understand both the developmental pathways defining β–cell fate and the mechanisms that regulate and maintain function and insulin secretion in adult β-cells. This will aid in preserving function of *de novo* β–cells that are transplanted into diabetic patients. The first chapter delineates the temporal nature of Ngn3 function needed to generate ectopic β-cells during embryonic development and explores the function of transcription factors that have not been previously implicated in this process. The second chapter explores how dysregulation of key transcriptional regulators, the Pgc-1 genes, in mature β-cells leads to impaired function and insulin secretion. In the third chapter, we describe an unusual phenotype where mice with a transgene expressing a tamoxifen-inducible Cre-recombinase in β-cells are protected against Streptozotocin-induced hyperglycemia. To do this we used a combination of *in vitro* and *in vivo* techniques using β–cell lines and transgenic frogs and mice.

In the following chapter we manipulated Ngn3 function in discrete temporal windows throughout early endoderm development of the frog *Xenopus laevis* in an effort to promote β–cell development and investigate the important transcriptional regulators that are activated immediately after Ngn3 expression begins.

CHAPTER I

Transient overexpression of Ngn3 in *Xenopus* endoderm promotes early and ectopic development of pancreatic β and δ-cells

INTRODUCTION

Neurogenin3 is a master regulator of pancreatic endocrine development

Neurogenin 3 is a basic helix-loop-helix (bHLH) transcription factor part of a small family of transcription factors consisting of Neurogenin 1, 2 and 3 (Ngn1, Ngn2 and Ng3). Ngn1 was first identified from a PCR screen using degenerate primers targeting conserved sequences of the bHLH protein domain and cDNA from rat dorsal root ganglia (Ma et al, 1996). Ngn1 expression in *Xenopus laevis* embryos demarcates prospective regions of primary neurogenesis and subsequent overexpression causes ectopic neurogenesis (Ma et al, 1996). Following these results, another screen using degenerate primers for the bHLH protein domain was performed on a cell line made from neural crest stem cells (Sommer et al, 1996). Ngn2 and Nng3 were identified and are expressed in the developing mouse central nervous system in regions also destined for early neurogenesis (Sommer et al, 1996). Since their discovery, research in different systems has showed that the Neurogenin proteins are central in the determination of neural fates in the early stages of formation of the central nervous system (Guillemot, 2007).

Ngn3 is also expressed throughout the embryonic pancreas (Sommer et al, 1996). It is expressed in biphasic fashion in the epithelium of the developing mouse pancreatic buds (Villasenor et al, 2008). Ngn3 is first detected around E8.5, twelve hours after the start of Pdx1 expression; Ngn3 expression then increases until E10.5, mirroring the primary transition. Between E10.5 and E11.5, expression dramatically decreases, yet strongly reappears from E11.5 to E14.5 (Villasenor et al, 2008). This expression pattern also mirrors the spatiotemporal timeframe of the secondary transition. Ngn3 expression is observed right before the expression of endocrine hormones, but is not found to be co-expressed with them (Schwitzgebel et al,

2000). Early studies show no detectable or very scarce expression of Ngn3 after E14.5 (Apelqvist et al, 1999; Schwitzgebel et al, 2000). This biphasic expression pattern is also observed in human pancreas development (Lyttle, 2008). Using a mixture of transgenic reporters, immunoassays and more sensitive microscopy techniques expression of Ngn3 can be detected, albeit, at low levels in adult mouse β-cells as well as the other pancreatic endocrine cells (Wang et al, 2009).

Inhibiting Ngn3 function in the mouse demonstrates its importance in the development of pancreatic endocrine cells. Ngn3 knockout mice become hyperglycemic after two days of birth and die shortly after (Gradwohl et al, 2000). They lack expression of all pancreatic endocrine hormones, as well as, expression of transcription factors previously shown to regulate development of pancreatic endocrine development like Pax4 and NeuroD (Gradwohl et al, 2000). Apoptosis is not increased, thus pointing to the function of Ngn3 in early specification of the endocrine progenitors. This was confirmed by lineage tracing studies using tamoxifen-inducible Cre-recombinase protein knocked-in the Ngn3 locus (Gu et al, 2002). More recent work shows that Ngn3 is also needed in adult β–cells as inactivation of Ngn3 in adult mouse β-cells affects endocrine function, gene expression profiles, and renders mice glucose intolerant (Wang et al, 2009).

The previous work suggests Ngn3 genetic variation might be associated with monogenic forms of diabetes. A study of 91 primarily Caucasian families showed no association between three Ngn3 coding sequence polymorphisms and patients with autosomal dominant diabetes (Kim et al, 2000). However, Ngn3 mutations affecting transcriptional activity were identified in three patients with congenital malabsorptive diarrhea as a result of decreased numbers of gastric enteroendocrine cells, but without diabetes (Wang et al, 2002). Reduction of intestinal endocrine

cells following Ngn3 inactivation was also observed in mice (Lee et al, 2002). Only two cases have been reported where point mutations causing biologically inactive Ngn3 proteins were identified in patients with congenital malabsorptive diarrhea and neonatal diabetes (Pinney et al, 2011; Rubio-Cabezas et al, 2011). One of these patients did not have any other mutations in genes normally associated with neonatal diabetes, such as like Glucokinase and Pdx1 (Pinney et al, 2011), suggesting a possible causal link between the inactive Ngn3 protein and the associated diabetes. The lack of additional reported cases of neonatal diabetes might be because mutations severely affecting Ngn3 function are likely embryonically lethal due its role in early neurogenesis. Given these observations, Ngn3 is positioned at the top of the pancreatic endocrine differentiation program and is an attractive target for the promotion of ectopic endocrine cells.

Ngn3 *in vivo* overexpression studies

Despite the pivotal role of Ngn3 in the specification of β-cells during embryonic development, in most cases Ngn3 overexpression *in vivo* does not increase β–cell numbers. In transgenic mice, overexpression of Ngn3 under the Pdx1 promoter, which would target all pancreatic cell types, causes pancreatic hypoplasia due to forceful differentiation of pancreatic progenitors, primarily into the α–cell linage with no ectopic insulin detected (Gu et al, 2000; Apelqvist et al, 1999). Likewise, in the chick developing endoderm, electroporation of plasmids overexpressing Ngn3 into the gut epithelium causes an increase of glucagon and somatostatin, but not insulin, from the esophagus all the way to the yolk sac (Grapin-Botton et al, 2001). Liver-specific overexpression of Ngn3 using adenoviruses in adult mice rendered hyperglycemic by streptozotocin (STZ), a β-cell specific toxin, causes ectopic expression of insulin, as well as, glucagon, somatostatin and pancreatic polypeptide (Yechoor et al, 2009). It was determined that

these ectopic cells originate from transdetermination of adult liver progenitors, termed oval cells. However, it is unclear if this phenomenon only occurs under a hyperglycemic state or solely in the adult liver. While the reasons remain unknown, the consensus remains that Ngn3 alone cannot promote ectopic β–cell development *in vivo* (Puri and Hebrok, 2010). To demonstrate this, overexpressing Pdx1, Ngn3 and Mafa using adenoviruses targeting exocrine tissue reprogrammed acinar cells into β-cells that showed indistinguishable ultrastructural characteristics compared to endogenous β-cells (Zhou et al, 2008). These reprogrammed β–cells improve STZ-induced hyperglycemia; however, the efficiency of reprogramming is quite low, with only ~20% of cells infected are reprogrammed into β-cells and the amelioration of hyperglycemia was mild and transient (Zhou et al, 2008).

Further insight into the pro-endocrine function of Ngn3 came when it was shown that in mice it acts in restricted temporal competence windows to promote development of each endocrine lineage (Johansson et al, 2007). Rescue of Ngn3 expression in an Ngn3-null background starting at different time points during pancreatic development revealed the timing of development of each endocrine lineage. When Ngn3 was overexpressed at early developmental stages, α–cells were predominantly formed, while overexpression at later stages caused formation mainly of β and PP cells (Johansson et al, 2007). These results revealed essential information as to why constitutive Ngn3 overexpression might only promote ectopic α–cell development. It is possible that, instead of constitutive overexpression, transient Ngn3 overexpression in discrete temporal competence windows is needed to mimic what occurs during development. In mice, this is difficult to achieve because ligands often used to induce transient transgene expression *in vivo* remain active up to a week (i.e. tamoxifen), which would encompass most of the timeframe of pancreas endocrine development. Thus the question remains

whether Ngn3 can promote ectopic formation of β–cells *in vivo* and consequently, there is very little known about how transcriptional information is integrated immediately after Ngn3 expression begins to specify the β–cell fate.

Ngn3 *in vivo* microarray studies and gene targets

Identification of transcriptional mechanisms through which β–cell fate could be induced is of great interest. Given the pro-endocrine function of Ngn3, this transcription factor has previously been used as a marker of endocrine progenitors in several *in vivo* microarray studies and attempts have been made to identify gene targets of Ngn3 activity. Transgenic mice expressing an NGN3-green fluorescent protein (GFP) fusion protein under the Ngn3 promoter were used to analyze FACS-sorted GFP+ cells at E13.5 (Gu et al, 2004). As expected, expression of previously known targets like NeuroD increase compared to GFP- controls. The transcription factor Myt1 also significantly increases in GFP+ cells and is expressed in the embryonic pancreas in the same regions as Ngn3. Dominant negative constructs targeting Myt1 decreased the expression of insulin and glucagon in the mouse and chick embryonic pancreas (Gu et al, 2004). Myt1 was later shown to be a direct target of Ngn3 and to promote ectopic formation of α–cells but not β-cells; yet Ngn3-null animals maintained expression of Myt1 (Wang et al, 2008).

Two studies using microarray analysis of embryonic pancreatic tissue at E13, E15 or E18 of Ngn3-null mice also identified known Ngn3 targets including NeuroD and Insm1 (Juhl et al, 2008; Petri et al, 2006). The transcription of two transcription factors identified from the microarray results, Iriquois-class homeodomain 1 and 2 (Irx1 and Irx2), is inhibited in the Ngn3-null mice and activated in the chick following *in vivo* Ngn3 overexpression (Petri et al, 2006).

Irx1 and Irx2 were only expressed in mouse α-cells and were not detected in β-cells. In the second study, one of the genes that was decreased in the microarray, the zinc transporter 8 (Znt8) is expressed in α- and β–cells during mouse embryonic development (Juhl et al, 2008). Subsequent research showed Znt8 is necessary for glucose stimulated insulin secretion (Davidson et al, 2014), thus being a marker of mature β–cells and probably not of β–cell progenitors.

Previous microarray studies analyzed tissue many hours after endogenous Ngn3 expression begins. To analyze tissue within a small timeframe following the initial activation of Ngn3 expression, transgenic mice with a time-activated fluorescent protein under the Ngn3 promoter were generated (Miyatsuka et al, 2009). This transgene changes fluorescence from GFP to RFP within a few hours of being expressed, allowing for analysis of cells that have recently activated expression of Ngn3. Cells from E17.5 were FACS sorted, analyzed with microarrays and organized in chronological order of Ngn3 activation. This allowed for the identification of genes that are sequentially activated after Ngn3. One candidate, Rfx6, is necessary for the creation for all endocrine cells in the pancreas and is a marker for human neonatal diabetes (Smith et al, 2010). It remains unclear if Rfx6 is directly regulated by Ngn3 or if is capable of promoting ectopic β–cell development.

Pax4 was identified as a target in most of the Ngn3 microarray studies. Pax4 is a marker of β and δ-cell progenitors and Ngn3 directly regulates its transcription (Smith et al, 2003). Pax4 knockout mice have dramatically decreased β–cell mass, yet still express insulin, while α–cell mass remains unaffected (Sosa-pineda et al, 1997); pointing to a role of Pax4 in proliferation or maintenance of β–cell progenitors rather than specification. Indeed, transgenic overexpression of Pax4 in mice using the Pdx1 or Ngn3 promoters caused a massive increase in β–cell mass

(Collombat et al, 2009). Islets were up to 4 fold larger in size, with no ectopic insulin expression outside of the islet.

In summary, the majority of microarray studies using Ngn3 as marker of endocrine progenitors have been largely unsuccessful at identifying genes involved in the early determination of the β–cell fate over other endocrine cell types. This would require microarray analysis of mRNA expression in cell populations destined to generate ectopic β–cells following Ngn3 overexpression. Thus, the development of an *in vivo* system where Ngn3 overexpression promotes ectopic β-cell formation would facilitate detailed analysis of genetic pathways essential for determining this endocrine cell fate.

Advantages of using the *Xenopus laevis* system for the study of embryonic development

The African clawed frog *Xenopus laevis* is an ideal system for the study of early development. The basis of cellular reprogramming knowledge that laid the groundwork for discoveries such as induced pluripotent cells was mostly obtained using *Xenopus* as a model. *Ex vivo* development of *Xenopus* embryos allows for simple manipulation of unfertilized eggs and embryonic tissues. Each *Xenopus* female can lay hundreds of eggs in a day allowing for genetic screenings with large experimental sample sizes. Targeted knockdown and overexpression studies can be performed quickly by injecting antisense morpholinos or mRNA into blastomeres, whose lineage derivatives are known from well-established fate maps (Chalmers and Slack, 2000). Additionally, transgenic frogs can also be produced with ease by injecting mRNA at the one cell stage. *Xenopus* embryos develop faster than mammalian embryos, for example E12.5 in mice corresponds to Stage 40 (66 hours post-fertilization) in the frog, thus, functional genetic studies can be achieved in a matter of weeks. Finally, cultures of embryonic explants and

transplantations between two embryos can be performed to analyze development avoiding signals from other tissues and contributions from different genetic backgrounds, respectively. In *Xenopus* it is quite easy to perform transient overexpression of genes because embryos develop in culture media. In theory, ligands that activate overexpression can be washed out of the embryos by transferring into fresh media without any ligand, effectively causing the ligand to diffuse out of the cells.

Xenopus laevis pancreas development

In general, morphogenetic movements and transcriptional pathways involved in mammalian endocrine pancreas development are conserved in *Xenopus*. The *Xenopus* pancreas also develops as two buds, dorsal and ventral, that eventually fuse at Stage 40 (Kelly and Melton, 2000). However, insulin is the first endocrine marker expressed in the pancreas, first detected by *in situ* hybridization in the dorsal bud at Stage 32 (40 hours post-fertilization, Pearl et al, 2009). Glucagon and somatostatin are expressed after the buds fuse and initially only in the stomach at Stage 41; they are both then expressed throughout the pancreas at Stage 45 (Pearl et al, 2009). During Stages 44-46, endocrine cell numbers dramatically increase, analogous to the secondary transition in mammals (Pearl et al, 2009). Another main difference in the *Xenopus* pancreas is the formation of islets. In mammals, endocrine cells cluster together in an organized fashion, yet, in *Xenopus*, this does not occur and instead endocrine cells are dispersed in separate small groups (Kelly and Melton, 2000).

The transcriptional cascade of factors involved in pancreas development is also conserved; in fact Pdx1 was initially identified in *Xenopus* (Wright et al, 1989; Ohlsson et al, 1996). Pdx1 and Ptf1a expression precedes insulin expression and follows similar expression

patterns as seen in mice (Afelik et al, 2006; Horb and Slack, 2001; Pearl et al, 2009). Ngn3 expression is observed throughout the pancreas at Stage 44, concurrent with the burst of endocrine cell formation (Pearl et al, 2009). Insm1 has also been shown to work downstream of Ngn3 and share a similar role in endocrine progenitors as in mice (Horb et al, 2009). The plasticity observed in the mouse pancreas is also conserved in Xenopus as overexpression of Pdx1 and Ptf1a in the endoderm, separately or in conjunction, can promote ectopic pancreatic endocrine development, including β-cells (Afelik et al, 2006; Jarikji et al, 2009; Horb et al, 2003).

RATIONALE AND HYPOTHESIS

The transcriptional mechanisms necessary for the formation of β–cell during embryonic pancreas development are not fully understood. In particular, the genes needed after Ngn3 expression starts to specify β–cell progenitors are largely unknown. The lack of understanding of this key transcriptional step, along with others, might be one of the reasons why it is still not possible to create functional β–cells *in vitro*.

Constitutive Ngn3 overexpression increases the number of α–cells, but not β-cells (Gu et al, 2000; Apelqvist et al, 1999; Grappin-botton et al, 2003). In general, transient Ngn3 overexpression, that would mimic endogenous expression, is difficult to perform *in vivo*. Consequently, identification of Ngn3 targets that promote β–cell fate *in vivo* has been difficult. We utilized *Xenopus laevis* to perform controlled, transient overexpression of Ngn3 at early stages of endoderm development in an effort to activate ectopic β-cell development. Furthermore, *ex vivo* development of *Xenopus* embryos provides easy access to embryonic tissue for analysis shortly after initiation of Ngn3 overexpression.

We hypothesized that transient Ngn3 overexpression in the *Xenopus laevis* anterior endoderm would increase the number of β-cells in the foregut and microarray analysis of the endoderm would identify genes involved primarily in the determination of the β-cell fate by Ngn3.

RESEARCH DESIGN AND METHODS

Construction of inducible Ngn3-GR clones. The full-length *Xenopus laevis* ngn3 ORF (GenBank accession no. NM_001134785) was cloned from wild type stage 42 cDNA into the pCS2+ vector. The ORF was then subcloned into a pCS2+ vector containing the human glucocorticoid receptor ligand-binding domain (GR) with primers SP6 5'-GATTTAGGTGACACTATAG-3' and Ngn3GrRevClaI 5'-AAGCTATAGGCAT CGATAC AAGAACTCTA-3'. The GR was fused to the C-terminus of the *Xenopus* Ngn3 ORF and referred to as Ngn3-GR. The full-length mouse ngn3 ORF (NM_009719) was subcloned from a pCS2+ vector into a pCS2+ vector containing the human glucocorticoid receptor ligand-binding domain (GR) with primers SP6 5'-GATTTAGGTGACACTATAG-3' and mNgn3GrRevBamHI 5'- ACAGGTCGGATCCCAAGAAGTCTGA – 3'. The GR was fused to the C-terminus of the mouse Ngn3 ORF and referred to as mNgn3-GR.

Embryo manipulations. Embryos were obtained by *in vitro* fertilization and incubated in 0.1 X Marc's Modified Ringers (MMR). Antisense morpholino oligonucleotides and mRNA were injected into embryos in the two dorsal vegetal blastomeres at the eight-cell stage. GFP mRNA or fluoresceins were used for lineage tracing. Embryos were injected in 1X MMR 2% Ficoll and raised to the desired stage in 0.1X MMR.

Whole mount *in situ* hybridization. Embryos at the desired stage were fixed in MEMFA (50 ml of 10X MEM salts, 50 ml Formaldehyde, and 400 ml distilled water) and stored in EtOH. Whole mount *in situ* hybridization was performed as previously described using BM purple (Horb et al, 2003). Samples were rehydrated by incubating at room temperature in 5

minute washes of 100% methanol, 75% methanol/25% water, 50% methanol/50% water, 25%methanol/75% PBSTween (1XPBS, 0.1% Tween20), 100% PBSTween. Samles were then incubated for 15 minutes in 10μg/ml proteinase K. 0.1M trisethanolamine was then used to rinse the samples twice for 5 minutes and then they were incubated in acetic anhydride (12.5 μls of 100% liquid acetic anhydride in 1 ml of solution ,Sigma). Samples were then fixed in 4% paraformaldehyde, washed five times in PBStween and incubated in 1 ml of hybridization buffer with 1μg of the desired probe at 60° C for 18 hours. The next day samples were sequentially washed in 2X and 0.2X saline-sodium citrate buffer at 60° C and maleic acid buffer (MAB) at room temperature. One hour incubations in MAB/2%BMB blocking solution and MAB/2%BMB/20% goat serum were performed before α-dioxygenin antibody (1:2000 dilution, Roche) was added and incubated overnight at 4° C. Following five MAB washes color reaction was carried out with 0.5 ml of BM purple AP substrate (Roche).

Antisense morpholino and mRNA injections. Antisense morpholino oligonucleotides were designed and produced by Gene Tools LLC. Tbx2, mtg8, mtgr1 and mtg16 morpholinos were designed to the translation start. The sequences of the antisense morpholinos are tbx2 5'-GGTGCATACACAAATCCAGCAGGGA-3', mtg8 5'-GGTGCTTGAGATTCCCACCATT CGC-3', mtgr1 5'-GATAGGGTCCTGGAATCCCAACCAT-3' and mtg16 5'- TGAGTCTG CATTGTAAACGCTGTA -3'. For mRNA injections, all mRNA was synthesized *in vitro* (Ambion mMessage) and coinjected with 400pg GFP mRNA to verify targeting by observing fluorescence in whole embryos or dissected whole guts. To activate the Ngn3-GR protein, injected embryos were cultured in dexamethasone (Dex) in a final concentration of 10μM in 0.1X MMR.

Antisense RNA Probes. Antisense probes for *Xenopus* mtg8, mtgr1, tbx2, geminin,

hes3.1 and oct25 were prepared using the full-length ORFs cloned from wild type stage15 cDNA into pCRII-TOPO (Invitrogen) based on sequence from GenBank and confirmed by sequencing. The GenBank Accession Number for mtg8, mtgr1, tbx2, geminin, hes3.1 and oct25 are NM_001095596, NM_001086057, NM_001086520, NM_001088403, NM_001088503 and NM_001093992, respectively. Digoxigenin (Dig)-labelled antisense RNA probes were transcribed *in vitro* using the Dig RNA labelling mix (Roche).

RT-PCR. Whole embryos or explants were harvested and immediately stored in RNAlater (Ambion). Total RNA was extracted with Trizol (Invitrogen). First strand cDNA was synthesized with Superscript III kit (Invitrogen). The following primer pairs and cycles were used:

Gene	Forward Primer	Reverse Primer
Insulin: ~350 bp, 28 cycles.	atg agt tgg atg gaa tgc agc	cct gta gca tta caa tag cga
Twist: ~250bp, 25 cycles.	aga aac tgg agc tgg atc	ggc ttc aaa ggc acg act
Foxf1: ~300 bp, 25 cycles	aac ctc tgt cct cca gcc	ggt tag tgg aat gac taa ctt
Ef1α: ~250 bp, 20 cycles	gca agc aat gtg agc agt gt	atg cac cat gaa gcc ctt ac

Embryological Dissections. Stage 15 whole endoderm plus mesoderm (WEM) explants were made by cutting the embryo throughout the neural plate to expose the endoderm and removing the lateral, posterior and anterior parts of the embryo leaving only the ventral endoderm and the surrounding ventral mesoderm and ectoderm. For whole endoderm (WE) explants mesodermal and ectodermal layers were manually removed from WEM explants. Explants were grown until stage 26 by comparison to non-injected controls. Groups of five

explants were pooled for RT-PCR.

Microarray Analysis. Embryos injected with Ngn3-GR mRNA were incubated in Dex for 4 h at Stage 12, and then all dorsal structures were removed, leaving only the ventral portion of the embryo. Sample sets (4 sets, 10 embryos/ set) were stored in RNAlater (Ambion) until RNA extraction with TRIzol (Invitrogen), followed by cleanup with the RNeasy Kit (Qiagen). RNA quality analysis, cDNA synthesis and hybridization to the Affymetrix GeneChip *Xenopus laevis* Genome 2.0 Array were performed by Genome Quebec (McGill University, Montreal). Microarray results were analyzed using the expression analysis software FlexArray V1.4.1 (Genome Quebec, Montreal, Canada). Data were normalized with Robust Multi-Array Average (RMA) and the algorithm EB (Wright and Simon) was used for statistical analysis (p-value cut off 0.05). Microarray data were deposited in NCBI's Gene Expression Omnibus under accession number GSE29017. All data entries for transcribed locus were removed from Table 1.

RESULTS

Transient Ngn3 overexpression promotes β-cell fate over the α-cell fate.

To transiently overexpress Ngn3 we constructed a dexamethasone-inducible Ngn3 protein by fusing the ligand-binding domain of the glucocorticoid receptor (GR) to the C-terminus of Ngn3 (Ngn3-GR). This domain sequesters the protein in the cytoplasm and it is only after the binding of Dex that Ngn3-GR translocates into the nucleus and becomes transcriptionally active. We co-injected Ngn3-GR and GFP mRNAs into vegetal blastomeres of *Xenopus laevis* embryos. It is known from fate maps that these blastomeres give rise to the anterior endoderm, which in turn will form the foregut. The foregut then develops into the liver, stomach, pancreas and duodenum. We were able to confirm this targeting by GFP fluorescence. This allowed us to activate Ngn3-GR in cells that make up the anterior endoderm and in all their progeny that would later form the organs in the anterior gut.

To define the competence window of ectopic β-cell creation by Ngn3, we activated Ngn3-GR (prior to its peak of endogenous expression at stage 44) at different times and for limited duration. Continuous activation of Ngn3-GR after gastrulation in the anterior endoderm (Stages 12–44, 80 h) promoted ectopic expression of insulin, somatostatin, and glucagon (Figure 1b,e,h). However, activation of Ngn3-GR for only 4 hours after gastrulation (Stages 12–15) resulted in ectopic expression of only insulin and somatostatin but not glucagon (Figure 1c,f,i). In both cases, ectopic expression of insulin was observed throughout the liver, stomach, and duodenum (black arrowheads, Fig. 1b,e). In rare instances, we observed ectopic expression of insulin quite posteriorly in the intestine (Figure 1-l). Although the morphology of the

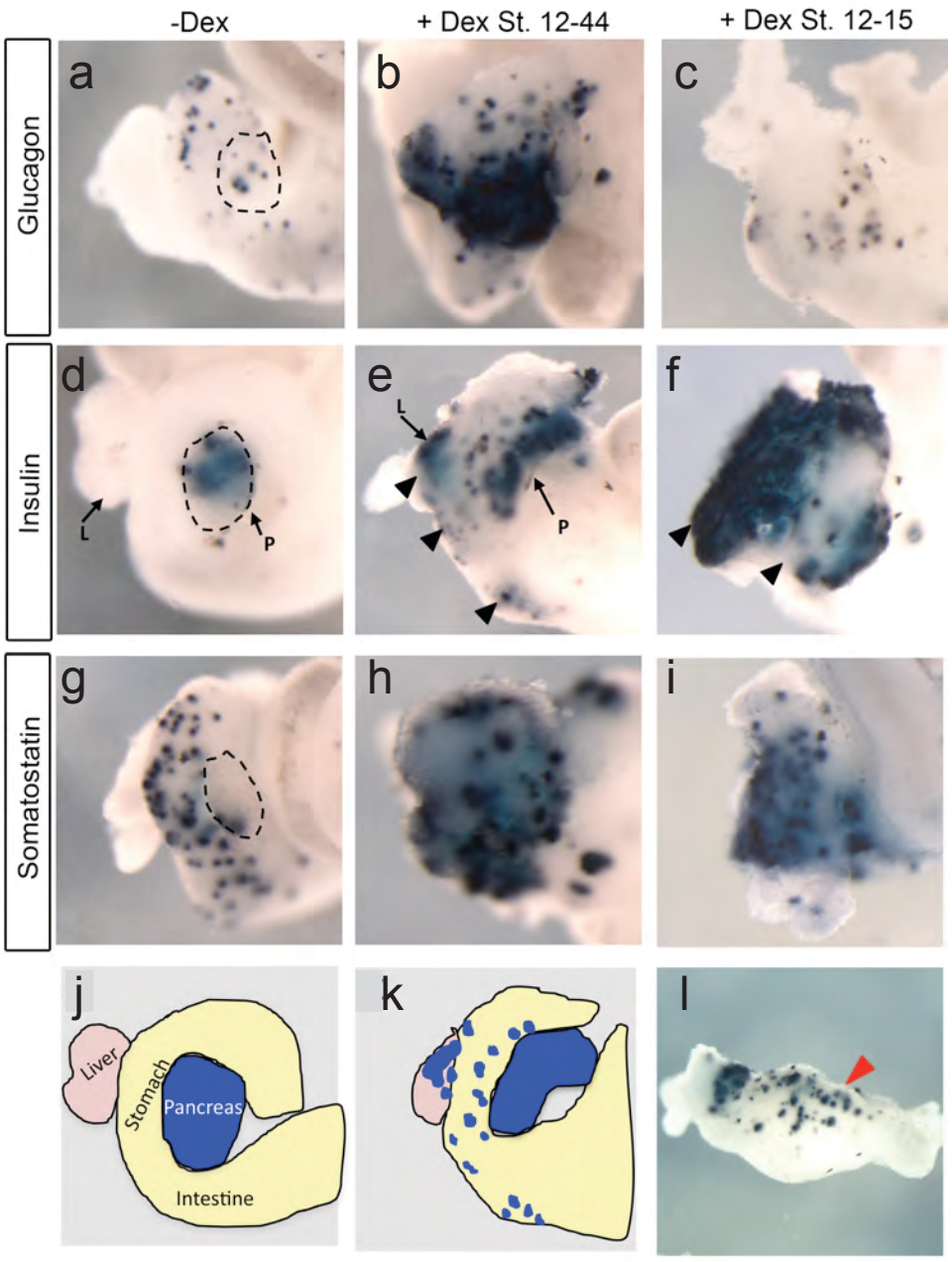

Figure 1. Differential effects of Ngn3-GR temporal activation. Whole mount in situ hybridization of Stage 44 whole guts from embryos injected with 1,800 pg of Xenopus Ngn3-GR mRNA at the eight-cell stage. (a,d,g) Control embryos injected with Ngn3-GR mRNA but not treated with dexamethasone. (b,e,h) Dexamethasone treatment from Stages 12 to 44 increased expression of glucagon (n=14/20), insulin (n= 23/23), and somatostatin (n=10/10). Arrowheads indicate ectopic insulin expression in the liver, stomach, and duodenum. (c,f,i) Dex treatment for 4 h from Stages 12 to 15 increased insulin (n= 55/55) and somatostatin (n=22/22) but not glucagon (n= 24/24). (j,k) Schematics illustrating organs in the whole gut of panels a and b (blue-insulin). (l) Insulin expression was detected in rare instances (n=2/50) in posterior areas of the intestine. L: liver, P: pancreas.

pancreas was disturbed, there was only a mild decrease in expression of other acinar pancreatic markers, protein disulphide isomerase (XPDI) and elastase, in the tissue that remained (Fig. 2a–d); suggesting imparied fusion of the pancreatic buds due to problems in the development of the pacnreas. In contrast, expression of stomach and liver markers, cathepsin E and hex was strongly reduced (Fig. 2e–h). As we expressed Ngn3-GR in the anterior endoderm (targeting stomach and liver in addition to pancreas), these data show that Ngn3-GR can promote cell fate conversion to a β or δ–cell phenotype over other endodermal lineages including the α–cell fate. To provide more evidence for cell fate conversion, we looked at markers of the anterior endoderm at Stage 15 following four hours of Ngn3-GR activation. Both hex and frp5 were dramatically decreased (Figure 3). Thus, these data show that transient Ngn3 overexpression in the Xenopus naïve endoderm can promote β–cell fate specifically over α–cell fate, while decreasing early and late endoderm markers. Unless specified, in all subsequent experiments we activated Ngn3-GR for 4 h at Stage 12.

To ascertain if the stage of Dex addition was most critical in defining the different effects of Ngn3, we tested short-term activation of Ngn3-GR at later stages. Activation for one hour at Stage 15 (until Stage 18) resulted in increased expression of insulin, glucagon and somatostatin as previously seen with continuous activation (Figure 4). To determine the minimal time of Ngn3 activity required for ectopic promotion of β-cells, we activated Ngn3-GR for different time periods after gastrulation, and found that activation for as short as 1 hour (at either Stage 12 or 14) was sufficient to promote ectopic insulin and somatostatin expression over glucagon expression (Figure 4, 10a). These results show that the competence of endodermal cells is indeed important for the ability of Ngn3 to promote one endocrine cell type over another and only a transient increase in Ngn3 activity (one hour) is sufficient to program cells towards β–cell fate.

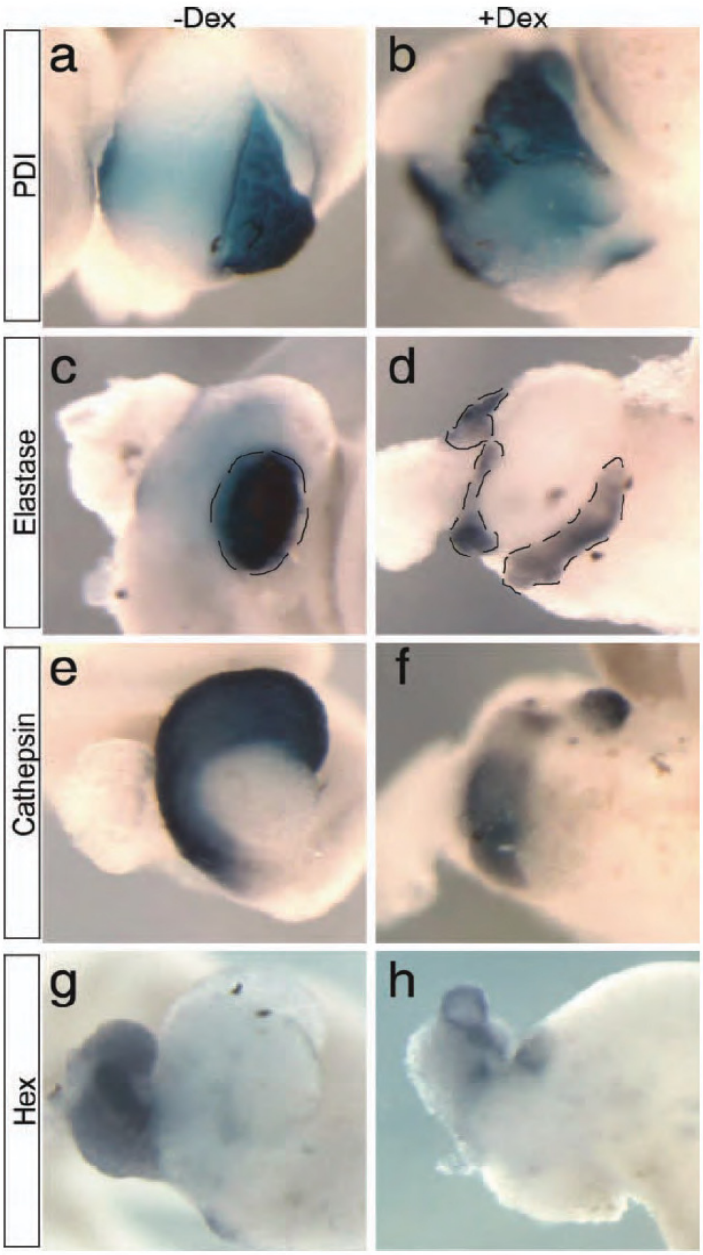

Figure 2. Ngn3-GR activation with dexamethasone for four hours after gastrulation (stages12-15) affects anterior gut markers at stage 44. (a–d) Exocrine pancreatic markers PDI and elastase are slightly decreased (n=16/16 and n=14/15, respectively). (e,f) Stomach marker cathepsin E is strongly reduced (n=14/14). (g,h) Liver and duodenum marker hex is slightly decreased (n=7/7). Pancreas is outlined in panels (c) and (d).

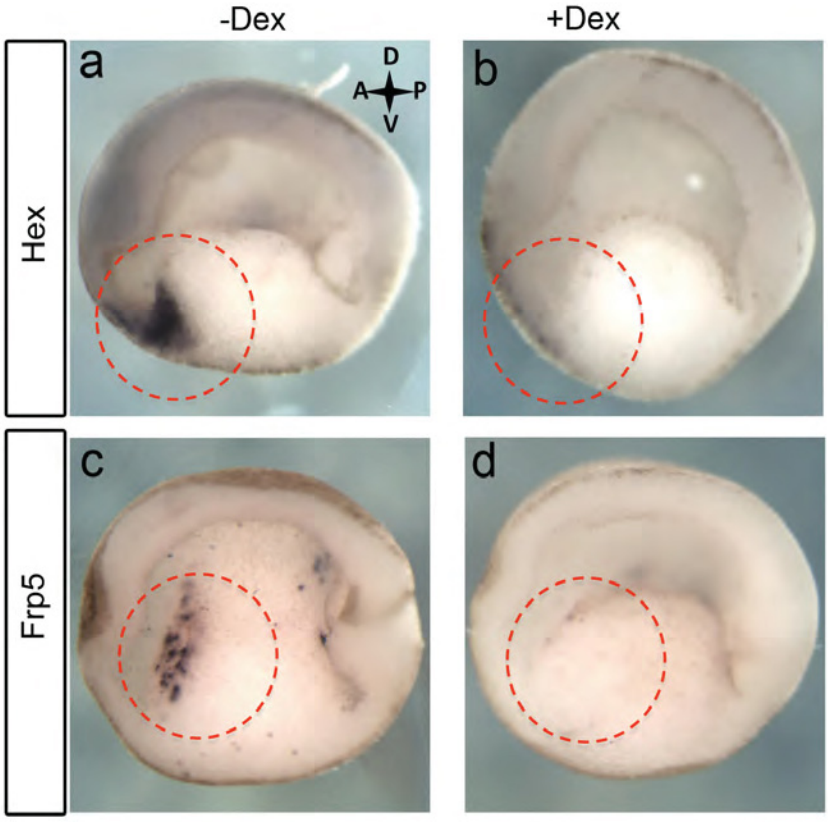

Figure 3 . Ngn3-GR activation at stage 12 abolishes expression of anterior endoderm markers at stage 15 . Sagittal section of embryos at stage 15 following activation of Ngn3-GR at stage 12 for four hours. **(**a,b) *hex* (n=14/15). (c,d) *frp5* (n=19/21).

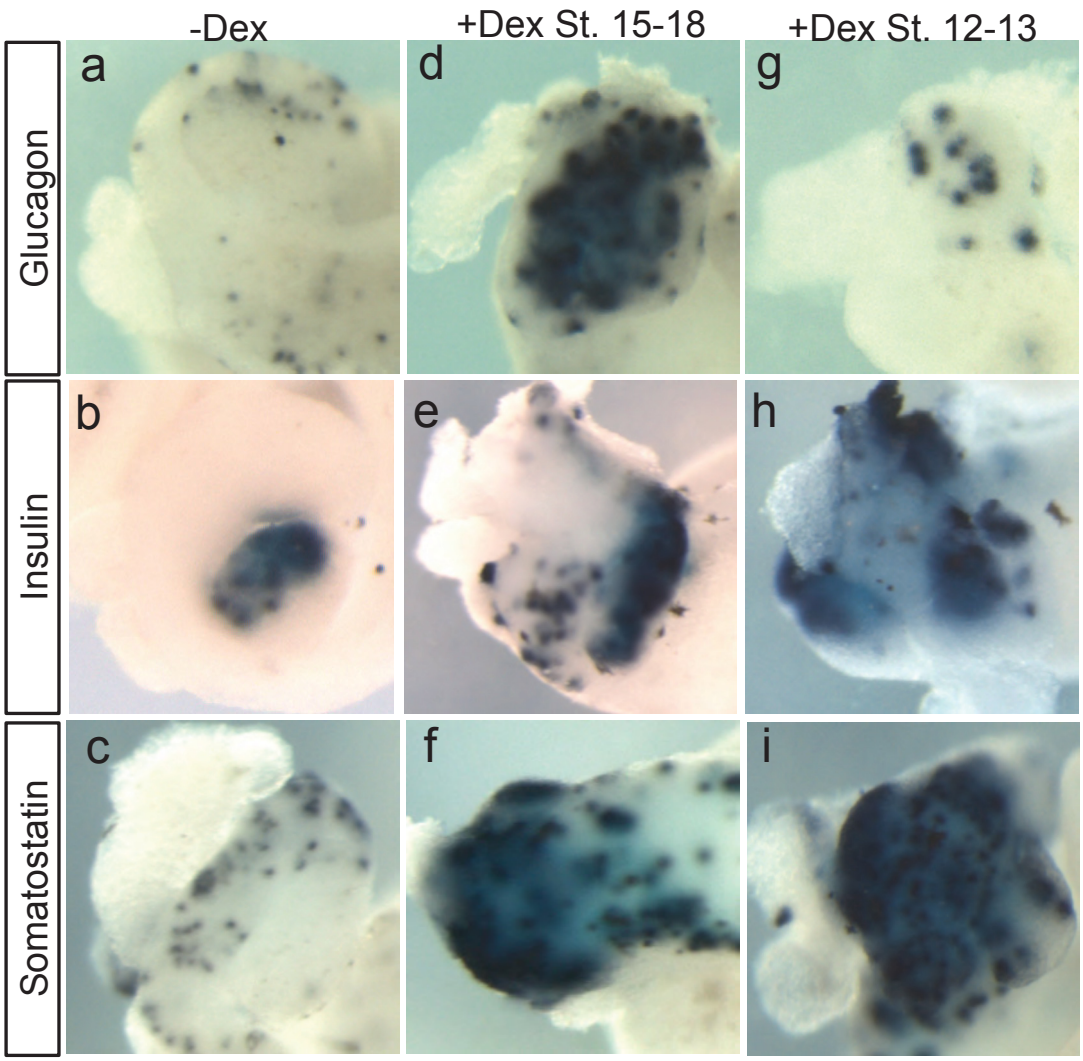

Figure 4. Short tem activation of Ngn3-GR is sufficient to promote ectopic expression of endocrine hormones. Whole mount in situ hybridization of Stage 44 whole guts from embryos injected with Xenopus Ngn3-GR mRNA at the eight-cell stage. (a-c) Control embryos injected with Ngn3-GR mRNA but not treated with dexamethasone. (d-f) Dex treatment for one hour from Stages 15 to 18 increased expression of glucagon (n=7/9), insulin (n= 11/11), and somatostatin (n=8/8). (g-i) Dexamethasone treatment for one hour at Stage 12 increased insulin (n= 9/9) and somatostatin (n=8/8) but not glucagon (n=8/8).

To examine whether promotion of ectopic insulin expression by transient Ngn3 activation is conserved within species and not limited to *Xenopus*, we performed experiments using mouse and human Ngn3 sequences. The *Xenopus laevis* protein sequence is only 53% identical to the mouse and human Ngn3 sequence, mainly at bHLH DNA binding domain, while mouse and human Ngn3 sequences are 73% identical. Transient overexpression of either mouse or human Ngn3-GR were sufficient to promote ectopic β-cell fate similar to *Xenopus* Ngn3 (Figure 5). The only difference was in the amount of mRNA required; human and *Xenopus* Ngn3 required the same amount of mRNA while 50 times less mRNA was required for mouse Ngn3. These results suggested that the protein domains required for Ngn3's ability to promote β-cell differentiation are conserved between the three species.

Ngn3-GR prematurely activates insulin expression via Insm1 and Rfx6 function.

To establish the earliest time point when ectopic insulin expression appeared upon Ngn3-GR misexpression, we determined at what stage in development insulin becomes detected. Since endogenous insulin mRNA expression is initially detected in the dorsal pancreas at stage 32 (Pearl et al, 2009), we examined Ngn3-GR injected embryos at stages 28, 30, and 32 for ectopic expression of insulin. By *in situ* hybridization, we found insulin to be expressed 8 hours earlier (Stage 28) than endogenous in Ngn3-GR injected embryos treated with Dex (Fig. 6e). In addition to the timing differences, we also found insulin to be expressed more posterior and ventrally in the endoderm (Fig. 6h). By RT-PCR, we were able to detect ectopic insulin expression in Dex treated embryos as early as Stage 24, which is about 12 hours after activation of Ngn3-GR (Fig. 7a). To eliminate the possibility that the results were due to ectopic transcription of the insulin without true β-cell differentiation, we determined whether other β-cell markers were also expressed. The Sulfonylurea receptor 1 (Sur1) is a mature pancreatic β-cell

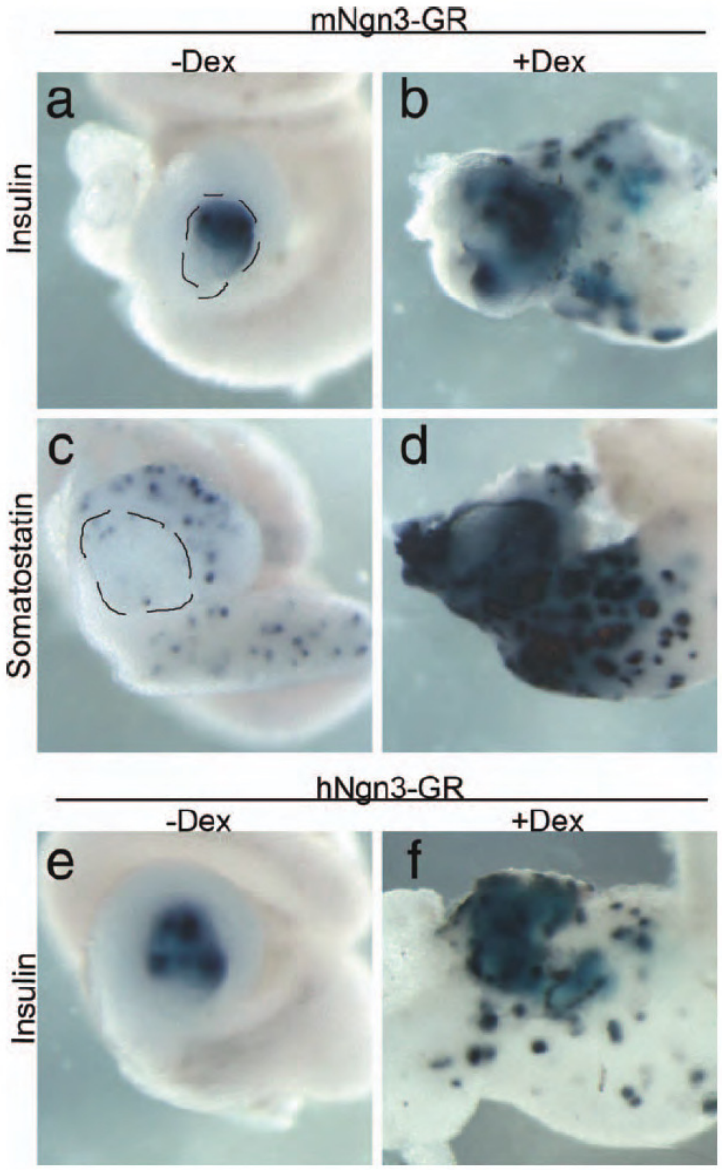

Figure 5. Transient activation of mouse Ngn3-GR and human Ngn3-GR also promoted increased insulin expression. (a–d) Embryos injected with 15 pg of mouse Ngn3-GR and activated with dexamethasone for 4 h at Stage 12 showed increased insulin (n=10/10) and somatostatin (n=7/7) expression. (e,f) Injection of 1,800 pg of human Ngn3-GR also caused increased insulin expression (n= 8/8). Pancreas is outlined in panels (a) and (c).

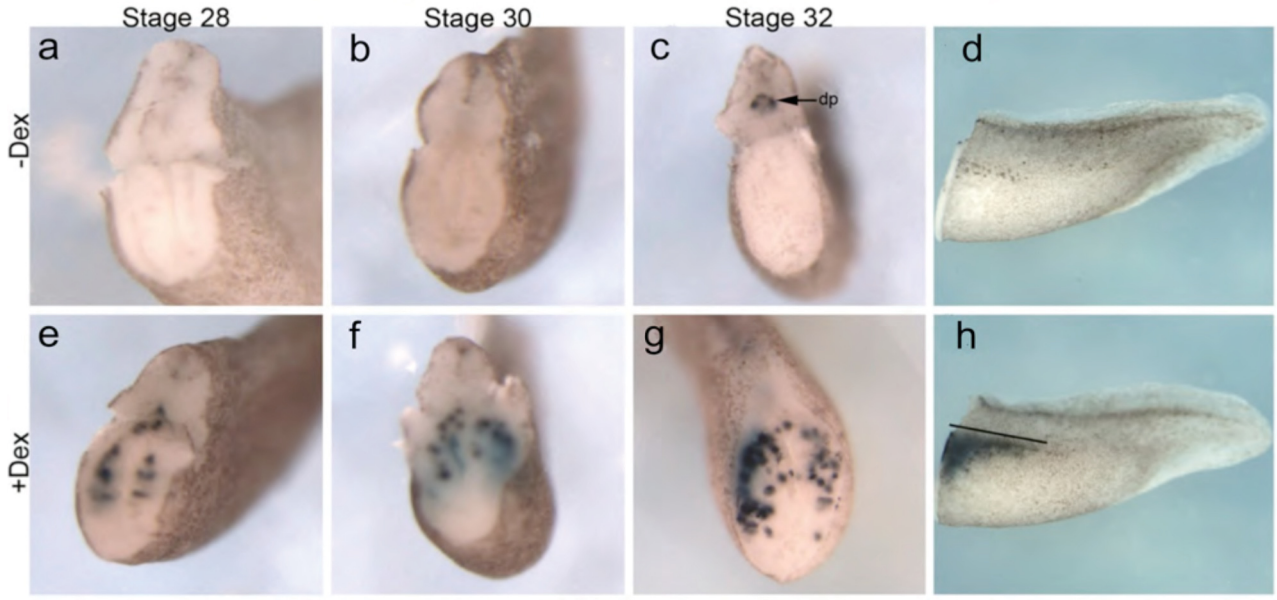

Figure 6. Ectopic and early induction of insulin by Ngn3-GR. Insulin expression in tadpoles that have been sectioned through the dorsal pancreas, with the head removed. (a–d) Control injected embryos. Endogenous insulin expression is first detected at Stage 32 in a small dorsal domain. Endogenous insulin expression in dorsal pancreas (dp) is labeled by arrow in panel (c). (e–h) Ngn3-GR injected embryos treated with dexamethasone for 4 h at Stage 12 showed increased insulin expression at Stage 28 (n =14/18), Stage 30 (n=24/30), and Stage 32 (n=46/56). (h) Side view of Stage 34 tadpole treated with dexamethasone showing the extent of ectopic insulin expression (line).

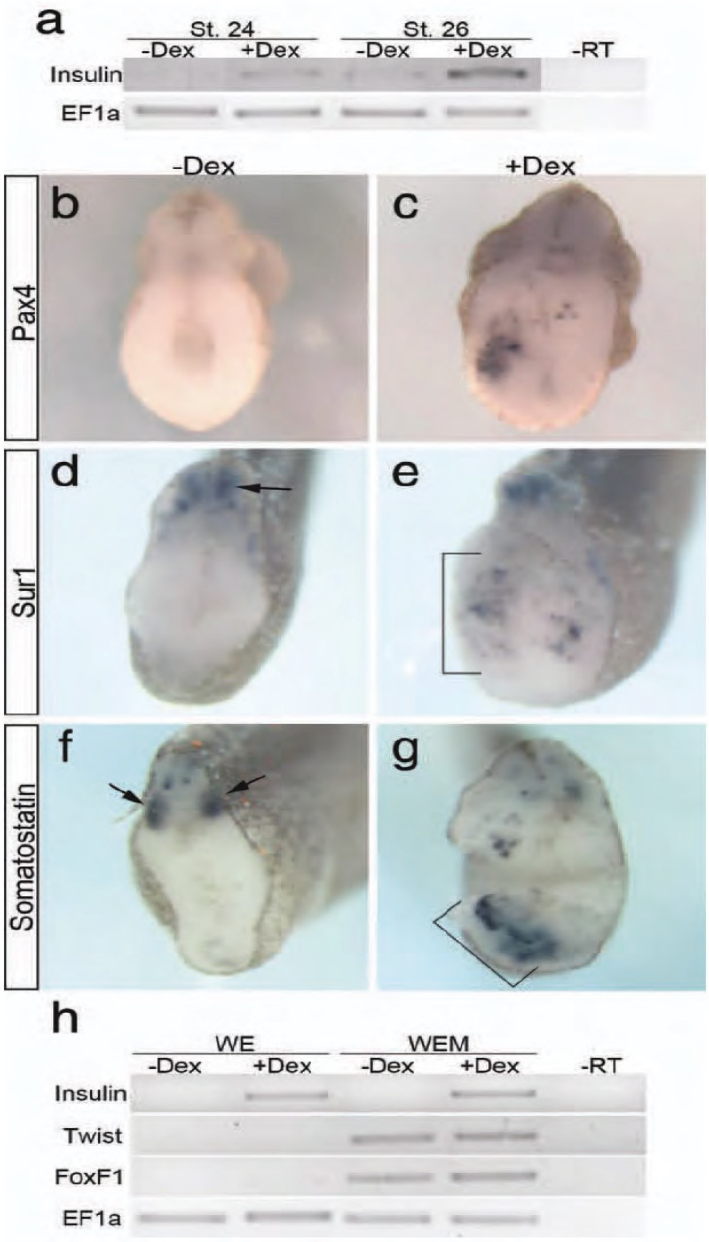

Figure 7. Ngn3-GR promotes β-cell differentiation. (a) RT-PCR for insulin of whole embryos at Stages 24 and 26. (b,c) Pax expression in dexamethasone treated embryos at Stage 32 (n=15/17). (d,e) Sur1 expression at Stage 32 (n=19/31) in dexamethasone treated embryos. In panel (e), bracket denotes ectopic expression in endoderm. (f,g) Increase in somatostatin at Stage 32 (n=12/27) in dexamethasone treated embryos. In panel (g), bracket denotes ectopic expression in endoderm. (h) RT-PCR at Stage 26 of whole endoderm (WE) and whole endoderm plus mesoderm explants (WEM). Explants were dissected at Stage 15 following 4h of Ngn3-GR activation, cultured until Stage 26.

ATP-sensitive K+ channel critical for proper insulin secretion (Bennett et al, 2010). We found ectopic and early expression of both Pax4 and Sur1 throughout the endoderm at Stage 32 (Fig. 7b–e). In addition, we also found increased expression of the endocrine specific δ-cell marker somatostatin at Stage 32 (Fig. 7f,g). These results demonstrate that Ngn3-GR misexpression was sufficient to promote premature and ectopic differentiation of β and δ-cells within the early endoderm.

Previous results in *Xenopus* have shown that endoderm–mesoderm interactions are critical for differentiation of pancreatic β-cells (Horb and Slack, 2001). We therefore examined whether Ngn3-GR promoted ectopic β-cell differentiation independent of signals from the mesoderm. We isolated explants of either endoderm alone (WE) or endoderm plus mesoderm (WEM) from Ngn3-GR injected embryos at Stage 15, grew them until Stage 26 and examined for ectopic insulin expression by RT-PCR. In agreement with our hypothesis, ectopic insulin expression was detected in Dex treated explants of endoderm alone (Fig. 7h). These results show that Ngn3 misexpression in the early endoderm directly promoted β-cell differentiation independent of signals from the mesoderm, though we cannot exclude the remote possibility of earlier mesoderm interactions during the short Dex treatment.

Since we ectopically expressed Ngn3, using a hormone inducible construct at an earlier time point and in locations ectopic to those that occur during normal development, it was possible that the pathway activated by Ngn3-GR was not the same as that utilized by endogenous Ngn3. To ascertain whether this promotion of early and ectopic β-cell differentiation by Ngn3 was indeed acting through the characterized pathway of β-cell development, we examined whether this activity was dependent on the proper function of the two endocrine specific

transcription factors Insm1 and Rfx6. Both these proteins have been shown to act downstream of Ngn3 during pancreatic endocrine cell development (Gierl et al, 2006; Horb et al, 2009; Mellitzer et al , 2006; Pearl et al, 2011; Smith et al, 2010; Soyer et al, 2010). Injection of the antisense Insm1 morpholino abolished endogenous insulin expression in controls embryos not treated with Dex (-Dex controls) as well as ectopic insulin expression in Dex treated tadpoles at Stage 32 (Fig. 8c,d).

Similarly, knockdown of Rfx6 also inhibited the ability of Ngn3-GR to promote ectopic β-cell differentiation as well as endogenous insulin expression (Fig. 8g,h). Similar results were also observed for both the Insm1 and Rfx6 morpholinos when insulin expression was analyzed at Stage 44 (Figure 9). These results suggest that Ngn3 promotion of ectopic and early β-cell differentiation occurred through recognized pathways of β-cell differentiation.

Tbx2, Mtg8, Mtg16, and Mtgr1 are needed for ectopic β-cell development.

To identify Ngn3 downstream targets involved in the formation of ectopic β-cells, we performed microarray analysis of the anterior endoderm immediately following activation of Ngn3-GR (Fig. 10b). Briefly, we activated Ngn3-GR injected embryos with Dex for 4 hours at Stage 12, collected embryos at Stage 15 and removed all dorsal structures, which included neural tissue and ectoderm (Fig. 10c). The results of this analysis yielded 162 genes upregulated by more than 1.4 fold in the +Dex samples (see Table 1 for a partial list). Several transcription factors that were previously identified as downstream of Ngn3 in other microarray studies were also identified in our screen, including Insm1 (2.51 fold), Neurogenic Differentiation 4 (NeuroD4; 2.12 fold), Zinc Finger Protein 238 (Zfp238; 2.65 fold), and Hairy and Enhancer of Split 5 (Hes5; 1.75 fold). In addition, we also found increased expression of several other

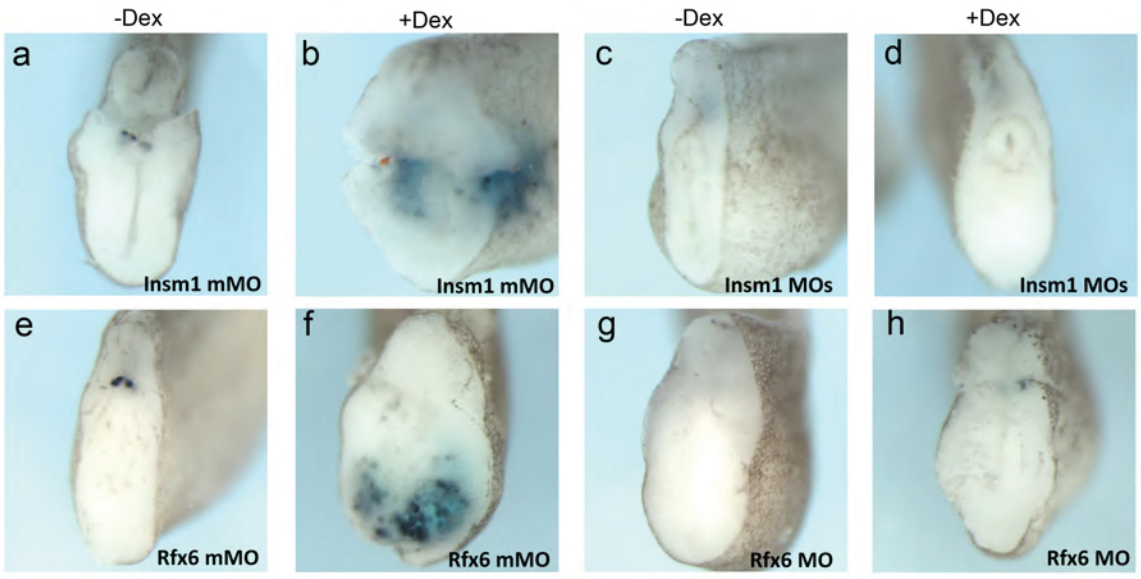

Figure 8. Insm1 and Rfx6 are necessary for ectopic β-cell promotion by Ngn3-GR. In situ hybridizations for insulin expression in all panels. Ngn3-GR mRNA was coinjected with a control mismatch morpholino (mMO) or morpholinos targeting insm1 or rfx6 mRNA, activated with dexamethasone for 4 h and fixed at Stage 32. (a,b) Insm1 mMO (20 ng) in either –dex (n=8/8) or +dex (n=12/17) treated embryos. (c,d) Injection of Insm1 MOs (2 morpholinos, 20 ng each) in –dex embryos (n=7/7) and +dex embryos (n=15/15). (e,f) Rfx6 mismatch morpholino (25 ng) in –dex (n=9/9) or +dex embryos (n=19/41). (g,h) Antisense Rfx6 morpholino (25 ng) in -dexamethasone embryos (n=33/33) and +dex embryos (n=46/46).

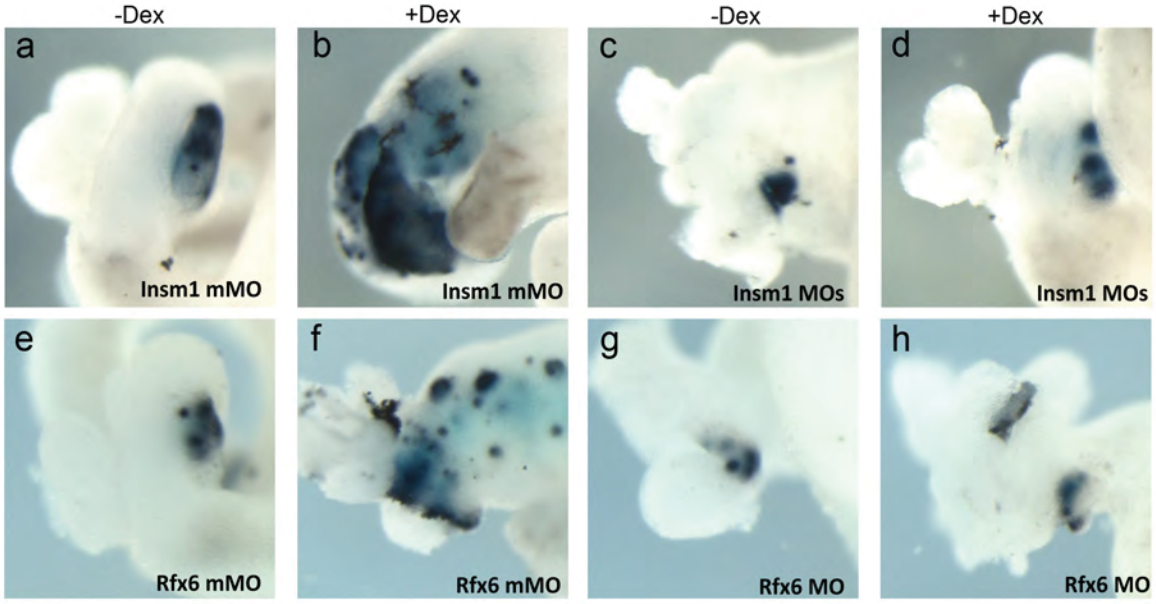

Figure 9. Insm1 and Rfx6 are necessary for ectopic β-cell promotion by Ngn3-GR. In situ hybridizations for insulin expression in all panels. Ngn3-GR mRNA (1,800 pg) was coinjected with a control mismatch morpholino (mMO) or morpholinos targeting insm1 or rfx6 mRNA, activated with dexamethasone for 4 h and fixed at Stage 44. (a,b) Insm1 mMO (20 ng) in either –dexamethasone (n=14/14) or +dexamethasone (n=20/21) treated embryos. (c,d) Injection of Insm1 MOs (2 morpholinos, 20 ng each) in –dexamethasone embryos (n=8/8) and +dexamethasone embryos (n=30/30). (e,f) Rfx6 mismatch morpholino (25 ng) in –dexamethasone (n=10/10) or +dexamethasone embryos (n=11/12). (g,h) Antisense Rfx6 morpholino (25 ng) in -dexamethasone embryos (n=10/11) and +dexamethasone embryos (n=13/13).

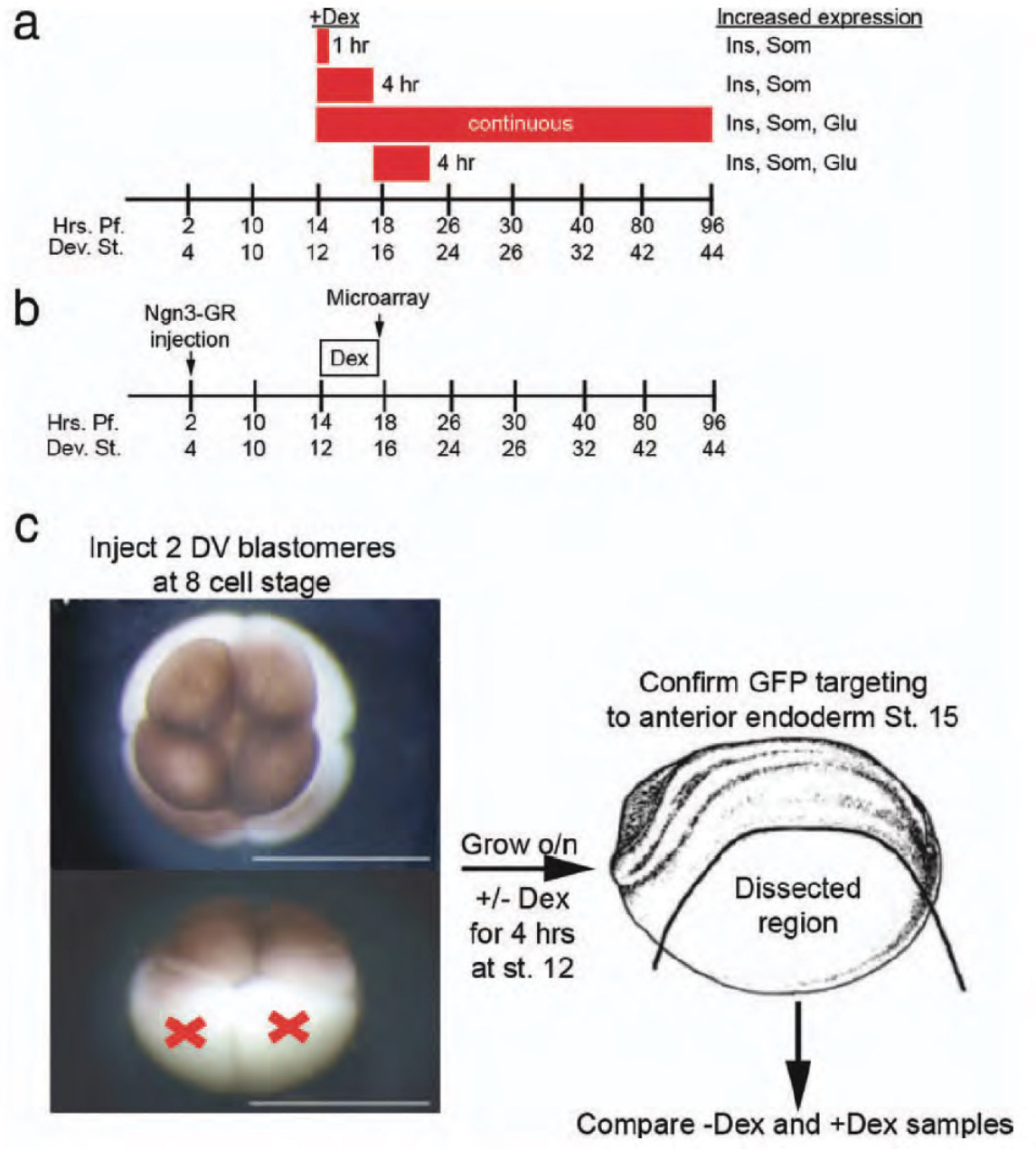

Figure 10. Summary of results and schematic diagram of microarray experiment. (A) Ectopic expression of endocrine markers. Red boxes indicate time of Dex treatment. Activation of Ngn3-GR for 1 or 4 h beginning at Stage 12 resulted in increased insulin and somatostatin expression, whereas continuous activation beginning at Stage 12 or a 4-h activation beginning at Stage 15 resulted in increased expression of insulin, somatostatin, and glucagon. (b,c) Diagram of microarray experiment. Ngn3-GR mRNA was injected in the two dorsal vegetal blastomeres at the 8-cell stage. Ngn3-GR was activated with dexamethasone for 4 h and confirmed targeting to the anterior endoderm at Stage 15 with GFP fluorescence. All dorsal structures were removed and RNA was extracted immediately after. Four replicates of ten embryos were used to hybridize the Affymetrix Xenopus laevis GeneChip 2.0.

Table 1

Partial list of genes upregulated in St. 15 endoderm after 4 hours of Ngn3-GR activation

Gene Symbol	Unigene ID	Gene Title	Fold change
GC115198	Xl.16704	Hypothetical protein MGC115198	3.93
Aif1l	Xl.10091	Allograft inflammatory factor 1-like	3.77
Hba3	Xl.1125	Hemoglobin alpha 3 subunit	3.02
Slc16a3	Xl.2852	Solute carrier family 16, member 3	2.80
Ankrd37	Xl.8033	Ankyrin repeat domain 37	2.67
Btg-x	Xl.49497	B-cell translocation protein x	2.67
Znf238.3	Xl.47150	Zinc finger protein 238.3	2.65
LOC100158417	Xl.17761	Hypothetical protein LOC100158417	2.59
Insm1	Xl.14733	Insulinoma-associated 1	2.51
Oct25	Xl.48605	POU class V protein oct-25	2.48
Rfesd	Xl.29449	Rieske (Fe-S) domain containing	2.47
Slc3a2	Xl.3242	Solute carrier family 3 member 2	2.47
Pdk4	Xl.1717	Pyruvate dehydrogenase kinase, isozyme 4	2.42
Brn3	Xl.701	POU class 4 homeobox 1	2.25
Hes3.1	Xl.585	Hairy and enhancer of split 3, gene 1	2.23
Twsg1	Xl.502	Twisted gastrulation homolog 1	2.21
Hes3.3	Xl.12067	Hairy and enhancer of split 3, gene 3	2.18
Mtg16	Xl.14852	Myeloid translocation gene 16	2.18
Neurod4	Xl.1263	Neurogenic differentiation 4	2.12
Ptgs2	Xl.33329	Prostaglandin-endoperoxide synthase 2	2.11
Mtg8	Xl.24705	Myeloid translocation gene 8	2.09
MGC68579	Xl.34749	Hypothetical protein MGC68579	2.08
Cer1	Xl.368	Cerberus 1	2.07
MGC131136	Xl.14925	Hypothetical protein MGC131136	2.05
Dbndd1	Xl.47316	Dysbindin domain containing 1	2.02
Tsc22d3	Xl.12378	TSC22 domain family, member 3	2.01
Mtgr1	Xl.4960	Myeloid translocation gene Related 1	2.01
Fgf13	Xl.13859	Fibroblast growth factor 13	1.98
Chn1	Xl.9113	Chimerin	1.92
MGC68858	Xl.16456	Hypothetical protein MGC68858	1.90
Fam101b	Xl.56544	Family with sequence similarity 101, member B	1.88
Bmp7	Xl.3326	Bone morphogenetic protein 7	1.87
Dll1	Xl.14759	Delta-like 1	1.85
Elavl3	Xl.1035	ELAV (embryonic lethal, abnormal vision)-like 3	1.84
Zfand2a	Xl.48357	Zinc finger, AN1-type domain 2A	1.82
Stmn3	Xl.21810	Stathmin-like 3	1.81

Ndrg4-b	Xl.9099	Protein NDRG4-B	1.81
Hoxd1	Xl.53491	Homeobox D1	1.81
Esr10-A	Xl.9271	Enhancer of split related 10	1.80
Chst10	Xl.8239	Carbohydrate sulfotransferase 10	1.80
Atoh7-a	Xl.176	Atonal homolog 5a	1.79
Dlc1	Xl.47179	Deleted in liver cancer 1	1.79
Rgs1	Xl.31883	Regulator of G-protein signaling 1	1.76
Irf-6	Xl.1273	Interferon regulatory factor 6	1.76
Sgk223	Xl.3085	Tyrosine-protein kinase SgK223	1.76
Hes5.1	Xl.48575	Hairy and enhancer of split 5, gene 1	1.75
Foxa4-a	Xl.441	Forkhead box A4	1.75
Sat1	Xl.24275	Spermidine/spermine N1-acetyltransferase 1	1.74
Pik3ip1	Xl.49469	Phosphoinositide-3-kinase interacting protein 1	1.73
Esr10-A	Xl.9271	Enhancer of split related 10	1.72
Igf2	Xl.47110	Insulin-like growth factor 2	1.72
LOC100158359	Xl.55698	Hypothetical protein LOC100158359	1.71
Pgam1	Xl.11442	Phosphoglycerate mutase 1	1.70
Hey1	Xl.469	Hairy/enhancer-of-split related with YRPW motif	1.69
Gadd45g	Xl.12125	Growth arrest and DNA-damage-inducible	1.69
Stripy	Xl.9206	Stripy	1.69
Unc119b-A	Xl.16769	Protein unc-119 homolog B-A	1.68
LOC496170	Xl.13938	Hypothetical LOC496170	1.68
MGC53782	Xl.8440	Similar to enhancer of split related	1.68
Ccnb1	Xl.4297	Cyclin B1	1.68
LOC100036864	Xl.50224	Hypothetical protein LOC100036864	1.68
Ptgs2	Xl.33329	Prostaglandin-endoperoxide synthase 2	1.67
Mospd1	Xl.26524	Motile sperm domain containing 1	1.67
Kcnk5	Xl.16485	Potassium channel, subfamily K, member	1.63
Egln3	Xl.12606	Egl nine homolog 3	1.61
Unc93a	Xl.48437	Unc-93 homolog A	1.60
Hes9.1-b	Xl.12444	hes9.1-b hairy and enhancer of split 9, gene 1	1.60
Frzb	Xl.55584	Frizzled-related protein	1.60
Dbn1	Xl.49084	Drebrin 1	1.60
Ppp1r14a	Xl.24383	Protein phosphatase 1, regulatory subunit 14A	1.60
Tob1	Xl.6421	Transducer of ERBB2, 1	1.59
Foxa4-b	Xl.1082	Forkhead box A4	1.59
Slc7a8	Xl.15844	Solute carrier family 7, member 8	1.59
Dlc	Xl.68	Putative ortholog of delta-like protein C precursor	1.58
Mespb	Xl.53943	Mesoderm posterior homolog B	1.58
Gramd3	Xl.7613	GRAM domain containing 3	1.57
Hspc159-a	Xl.13396	Galectin-related protein A	1.56
Ggt1	Xl.18682	Gamma-glutamyltransferase 1	1.56
Mxi1	Xl.50498	Max Interactor 1	1.56

LOC100036879	Xl.16140	Hypothetical protein LOC100036879	1.55
Znf238	Xl.23642	Zinc finger protein 238	1.55
Chst10	Xl.8239	Carbohydrate sulfotransferase 10	1.54
Ppm1k	Xl.17302	Protein phosphatase, Mg2+/Mn2+ dependent, 1K	1.53
Asb5	Xl.48897	Ankyrin repeat and SOCS box-containing 5	1.52
Ventx1.1	Xl.203	Vent Homeobox 1 gene 1	1.51
Mcoln2	Xl.1707	Mucolipin 2	1.50
Nudt22	Xl.6817	Nudix -type motif 22	1.50
LOC100158350	Xl.54372	Hypothetical protein LOC100158350	1.50
MGC115510	Xl.53440	Hypothetical protein MGC115510	1.50
MGC53717	Xl.7620	Hypothetical protein MGC53717	1.50

transcription factors or regulators that have not been shown to function in β-cell development. These include the POU class V protein Oct-25 (Oct25; 2.48 fold), as well as all three Myeloid translocation gene family members (Mtg), Mtg8 (2.09 fold), Mtg16 (2.18 fold), and Mtgr1 (2.01 fold). MTG proteins are known to be important for neural and gut development and they act (to repress transcription) as mediators of multiprotein transcriptional networks to promote repression of target genes (Amann et al., 2005; Calabi et al., 2001; Koyano-Nakagawa and Kintner, 2005; Rossetti et al., 2004). We also found increased expression of other transcription factors, including several Hairy and enhancer of split-related genes, Hes3 (2.2 fold), Hes9 (1.6 fold) and Hey1 (1.69 fold), and the T-box gene Tbx2 (1.4 fold). Surprisingly, there was also a significant increase of the enzyme Pyruvate dehydrogenase kinase 4 (Pdk4, 2.4 fold) that is involved in regulating fuel flexibility by altering fatty acid and glucose oxidation (reviewed in Zhang et al, 2014).

We next sought to confirm differential expression of selected target genes. We examined the spatial expression of specific candidate genes in bisected embryos following 4 hours of Ngn3-GR activation at two developmental time points, one immediately after activation for 4

hours at Stage 15 and the other at Stage 20. At Stage 15, we confirmed increased expression of tbx2, mtg8, hes3, and geminin in the anterior ventral endoderm (Fig.11a–h dashed circles and arrows). Geminin and tbx2 were chosen as candidates representative of genes found increased in the microarray, but below 1.5 fold. For oct25 we detected increased expression in the dorsal endoderm of the roof of the archenteron (Fig. 11ij dashed line). We also confirmed increased expression of the second MTG family member, Mtgr1 at Stage 20 (Fig. 11k,l dashed circles). Preliminary data also confirmed increased expression of Pdk4 in the anterior endoderm at Stage 15 following four hours of Nng3-GR activation (Figure 12)

We chose four of these genes (Mtg8, Mtg16, Mtgr1, and Tbx2) and, using antisense morpholinos, examined whether they are involved in β–cell development and if they function downstream of Ngn3 to promote ectopic β-cells. We first looked at their expression in the wild type embryonic pancreas at Stage 44, a time point of robust endocrine cell development and Ngn3 expression. There was endogenous expression of Tbx2, Mtg8 and Mtgr1 specifically in the pancreas at stage 44. Tbx2 and Mtg8 showed weak punctate expression compared to strong Mtgr1 expression throughout the whole pancreas, furthermore, only Tbx2 also showed strong expression in the liver (Figure 13). We did not perform the analysis for Mtg16 and at this point we do not know if these four genes are co-expressed in the same cells that express Ngn3. Thus we can only conclude from this data that Tbx2, Mtg8 and Mtgr1 are expressed in the pancreas at the same time as Ngn3. Using morpholinos, knockdown of Tbx2, Mtg8, Mtgr1, or Mtg16 in the presence of Ngn3-GR completely abolished promotion of ectopic β-cells by Ngn3 at Stage 32 (Fig. 14c,g,k,o). Further, injection of Tbx2, Mtg8, or Mtg16 morpholinos alone strongly reduced endogenous insulin expression at Stage 32, whereas the Mtgr1 morpholino alone had no effect (Fig. 14d,h,l,p). Analysis at Stage 44 showed similar results (Figure 15), except for Mtgr1, which

only partially inhibited the promotion of β–cell fate, as there was still some ectopic insulin expression in the stomach and duodenum although not as widespread as in the +Dex positive controls.

To ensure that the results with these morpholinos were specific and not due to off-target effects of morpholino injections, we examined the effects of knocking down Tm4sf3, which was previously shown to regulate pancreatic bud fusion and α–cell development but not β–cell development (Jarikji et al, 2009). In the majority of embryos injected, the antisense Tm4sf3 morpholino did not affect Ngn3-GR promotion of ectopic β-cells (Fig. 16). These data demonstrate that all four candidates identified in the microarray were indeed required for the ectopic promotion of β-cells by Ngn3, whereas only Tbx2, Mtg8, and Mtg16 were required for endogenous β-cell development.

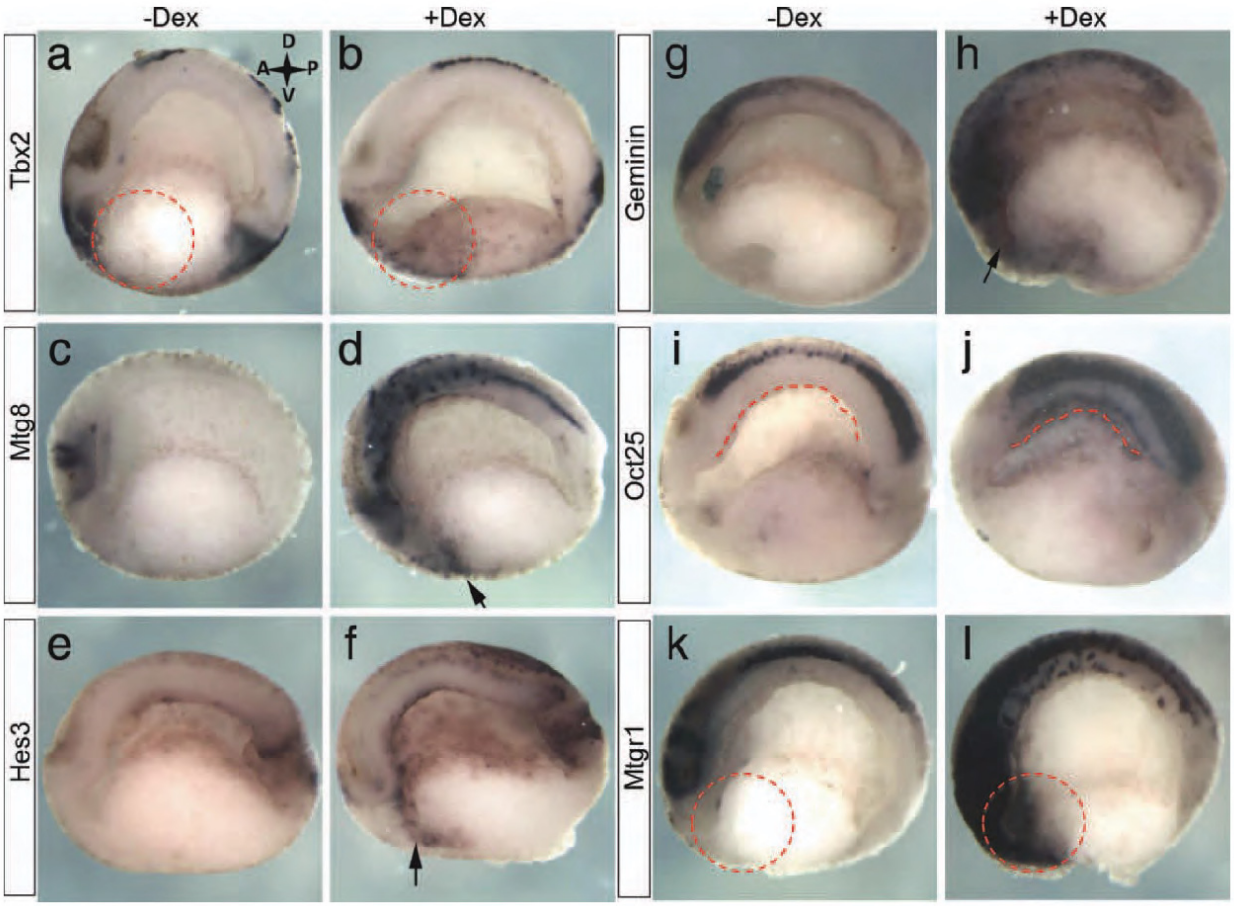

Figure 11. Validation of microarray data. Following 4 h of Ngn3-GR activation at Stage 12 candidate genes from the microarray analysis were increased in the endoderm at Stage 15: (a,b) tbx2 (n=24/24). (c,d) mtg8 (n=21/21). (e,f) hes3 (n=14/42). (g,h) geminin (n =13/44). (i,j) oct25 (n =13/13). And at Stage 20: (k,l) mtgr1 (n=9/13). Dashed circles and arrows indicate ectopic expression in the anterior endoderm. Dashed line indicates ectopic expression in the dorsal endoderm.

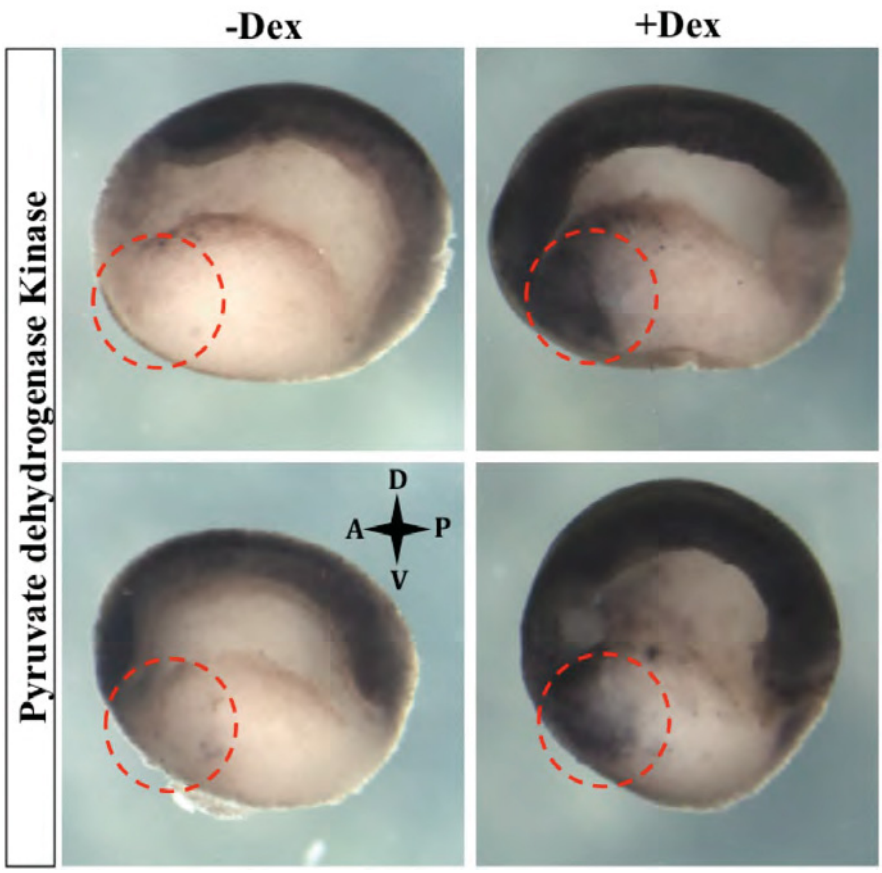

Figure 12. Pyruvate dehydrogenase kinase is increased in anterior endoderm by Ngn3-GR. *In situ* hybridization of *Xenopus* embryos (St.15) following 4 hours of Ngn3-GR activation at St.12 (n=7/7).

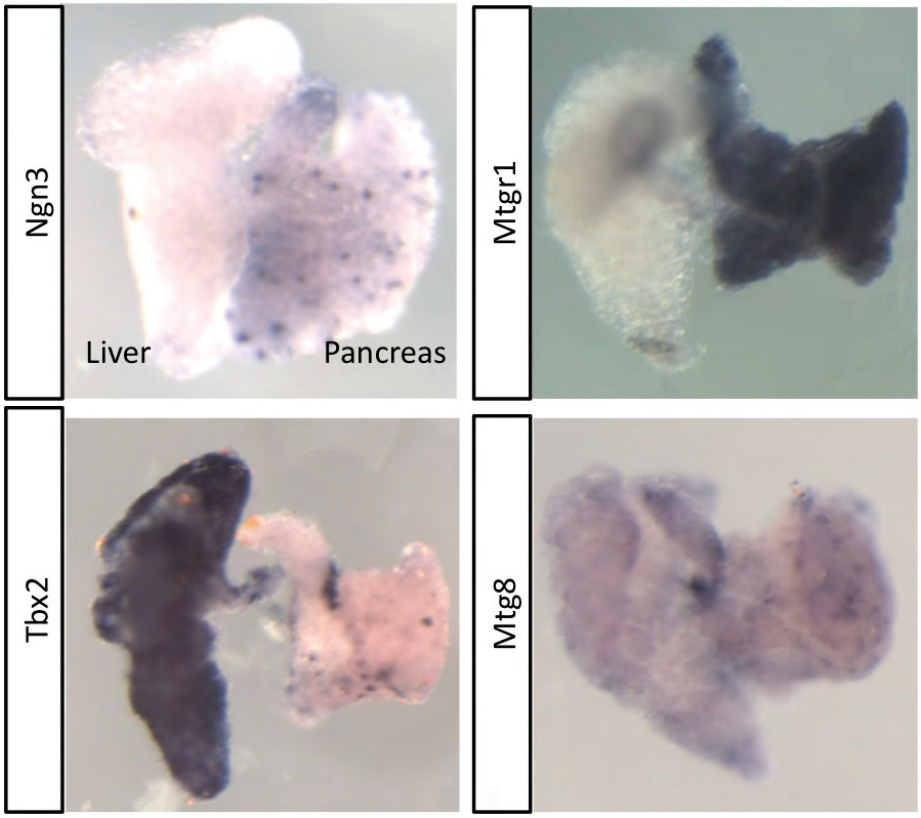

Figure 13. Tbx2, Mtgr1 and Mtg8 are expressed in the pancreas at the same time and same regions as Ngn3. Endogenous expression in isolated pancreas and liver at Stage 44 of ngn3 (n=5/5), mtgr1 (n=6/6), tbx2 (n=5/5) and mtgr8 (n=4/4).

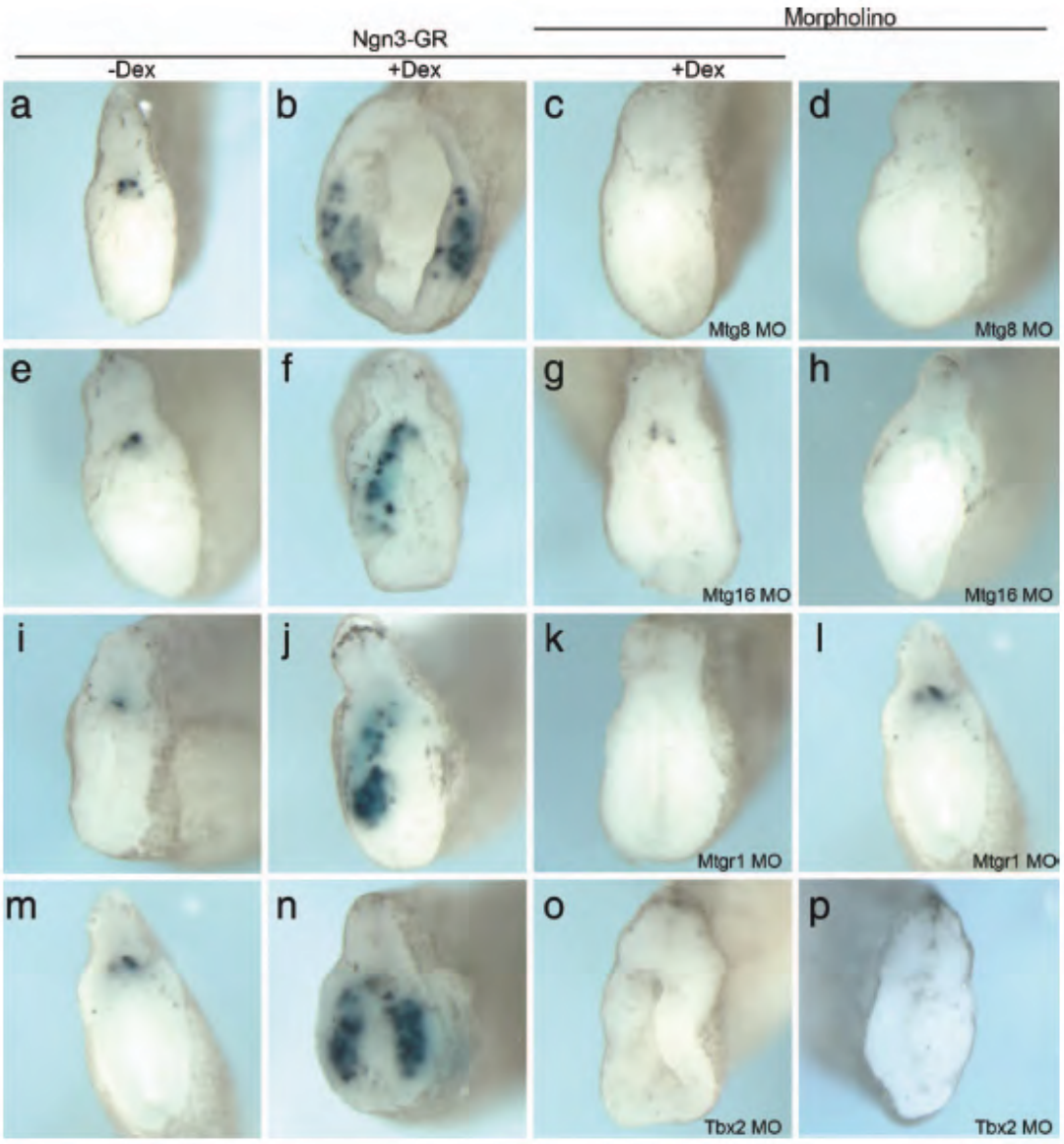

Figure 14. Tbx2, mtg8, mtg16, and mtgr1 function is required for ectopic and endogenous β-cell development at Stage 32. Tbx2 or Mtg morpholinos (40 ng each) were injected alone or with Ngn3-GR mRNA (1,800 pg), activated with Dex for 4 h at Stage 12 and the embryos grown to Stage 32 and analyzed for insulin expression. Coinjection with Ngn3-GR : (c) Mtg8 MO (n=20/27). (g), Mtg16 MO (n=16/20). (k) Mtgr1 MO (n=28/28). (o) Tbx2 MO (n=27/27) . Injection of each morpholino alone : (d) Mtg8 MO (n=40/77). (h) Mtg16 MO (n=27/52). (l) Mtgr1 (n=46/59). (p) Tbx2 MO (n=20/37).

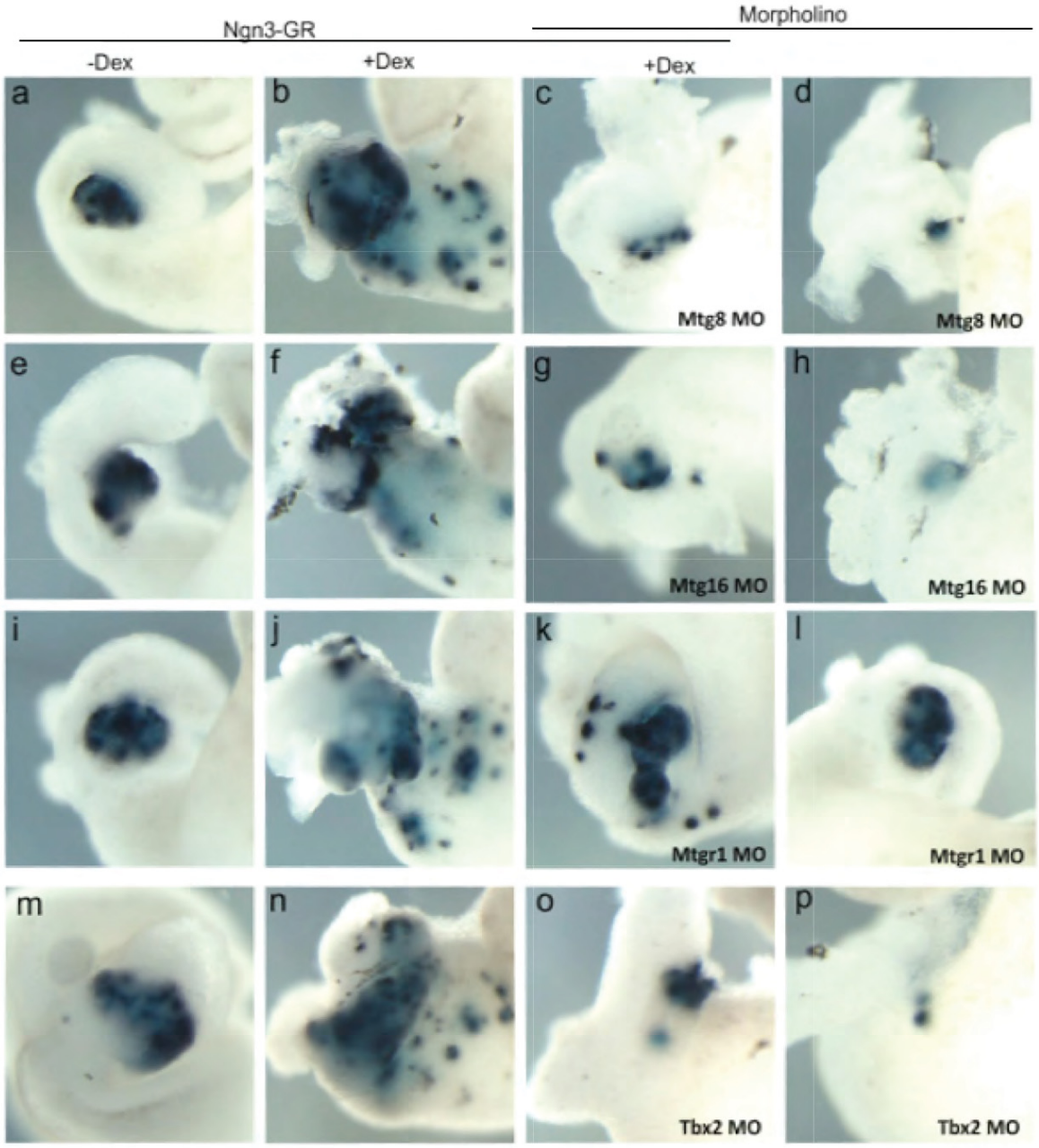

Figure 15. Tbx2, mtg8, mtg16 and mtgr1 function is needed for ectopic and endogenous β-cell development at stage 44. Tbx2 or Mtg morpholinos (40 ng each) were injected alone or with Ngn3-GR mRNA (1800 pg), activated with Dex for 4 h at Stage 12 and the embryos grown to Stage 44 and analyzed for insulin expression. Coinjection with Ngn3-GR: (c) Mtg8 MO (n=35/38). (g), Mtg16 MO (n=18/20). (k) Mtgr1 MO (n=16/21). (o) Tbx2 MO (n=43/43) . Injection of each morpholino alone: (d) Mtg8 MO (n=10/18). (h) Mtg16 MO (n=5/5). (l) Mtgr1 (n=16/20). (p) Tbx2 MO (n=18/18).

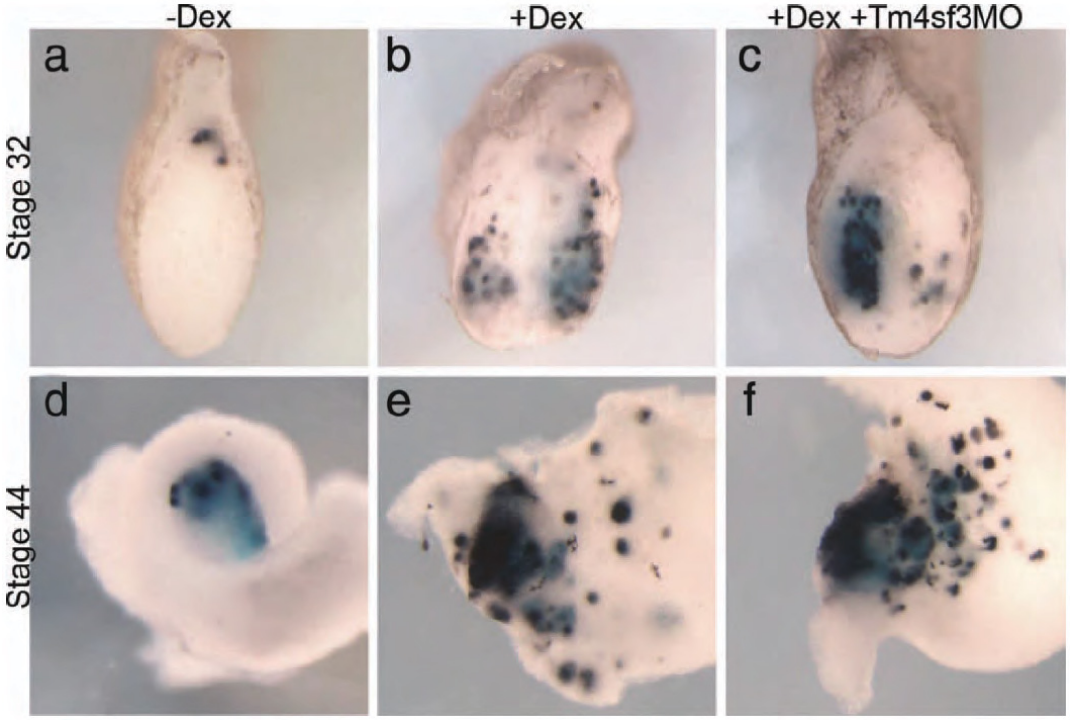

Figure 16. Knockdown of Tm4sf3 does not affect Ngn3 promotion of ectopic insulin expression. Ngn3-GR was injected alone or in combination with Tm4sf3 morpholino (25 ng). Insulin expression at Stage 32 in (a) control tadpoles; (b) +dex treated tadpoles (n=42/51), and (c) in tadpoles injected with Tm4sf3 morpholino (n=40/61). (d–f) Insulin expression at Stage 44 (for e, n=22/22; for f, n=40/51).

DISCUSSION

Our study demonstrates that transient overexpression of Ngn3 alone shortly after gastrulation can direct differentiation of *Xenopus* endoderm towards the β and δ-cell lineage and suggests that Ngn3 control of endocrine lineage specification is dependent on cell competence. By activating Ngn3 for a few hours at different times, we were able to pinpoint specific temporal windows for the ectopic generation of β and δ-cells specifically over α-cells. The observation that 4-hour activation of Ngn3-GR at Stage 12 promoted only insulin and somatostatin expression, while 4-hour activation at Stage 15 (or continuous activation from Stage 15 to stage 44) promoted expression of insulin, somatostatin, and glucagon argues against the hypothesis that prolonged Ngn3 activity is required for the ectopic formation of α, β and δ-cells. In other words, a transient burst of Ngn3 expression in a discrete developmental window, rather than simply obtaining a threshold of Ngn3 activity, can induce ectopic endocrine cells. In addition, we show for the first time that Ngn3 overexpression is capable of promoting ectopic formation of β-cells in the stomach and duodenum. However, even though stomach and duodenum markers were also decreased, we cannot rule out the possibility that these cells were generated from pancreatic progenitors that migrated throughout the endoderm. Taken together, our results suggest that the spatio-temporal context is crucial for directed and ectopic differentiation of β and δ-cells by Ngn3.

Our results agree with a previous study that also identified competency windows for Ngn3 function in mice by expressing Ngn3 at different timepoints during embryonic development in an Ngn3-knockout background (Johansson et al, 2007). However, in that article,

temporal windows were much longer as what we evaluated in our study (days rather than hours as we show here), and they expressed Ngn3 mirroring its endogenous temporal expression profile to recapitulate formation of endocrine cells. We show that transient Ngn3 activity in the early endoderm can be used to directly promote ectopic development specifically of β and δ-cells over α-cells, and activate insulin expression at an earlier time point than endogenous expression.

Our study has a few important caveats, we did not determine whether insulin and somatostatin were expressed in separate cells or co-expressed in the same cell. Even though there was an increase in Sur1, it is possible that the ectopic β–cells were not fully differentiated and could also be expressing somatostatin. We were also not able to ascertain if the ectopic β-cells generated were functional (i.e. could secrete insulin in response to glucose), as we did not isolate *Xenopus* embryonic tissue to make primary cell cultures. Since physiological or metabolic tests have not been extensively developed for the *Xenopus* frog we did not confirm if the ectopic β-cells created would have an impact on whole body glucose homeostasis.

We believe that the developmental state of the naïve endodermal cells in which Ngn3-GR is activated is comparable to embryonic or induced pluripotent stem cells that have been differentiated into definitive endoderm. Therefore, the identity and function of novel genes identified from our study could be applied to direct differentiation of stem cells into β–cells. We would predict that it could promote conversion of liver, stomach or intestinal progenitors specifically into the β and δ-cell fate and possibly decrease the number of α–cells in the resulting population of differentiated endocrine cells. Contamination from other endocrine or pancreatic cell lineages is one of the main problems facing *de novo* production of β–cells either *in vitro* or *in vivo* (Zhou et al 2008, Kroon et al, 2008). Even though we observed an increase in δ–cells, we believe eliminating the ectopic promotion of α-cells is an important improvement. Future

research can focus on identifying the factors or conditions that could promote the β–cell lineage over the δ–cell lineage following activation of Ngn3 in an effort to obtain a final homogeneous population of functional β-cells.

The ability to tightly control Ngn3 activity allowed us to isolate early downstream targets of Ngn3 and provided a specific list of genes that could function in the determination of the β-cell lineage. In particular, we have for the first time implicated Tbx2, Mtg8 and Mtg16 in normal pancreas development. We showed for the first time that Mtgr1 and Mtg8 are expressed in the pancreas during embryonic development and at the same time point as Ngn3. Although Mtg8 was found to be essential for development of enteroendocrine cells (Calabi et al, 2001), whether it is involved in pancreas development has not been reported. Previous studies have defined a relationship between Mtgs, Ngn1 and Ngn2 proteins in Xenopus ectoderm and in the chick developing neuronal system whereby Mtg proteins are downstream of Ngn proteins, and act as negative feedback regulators of Ngn function (Aaker et al, 2009, 2010; Cao et al, 2002).

In relation to pancreas development, this suggests that the pathway to β-cells might require a short activation by Ngn3 and subsequent inhibition by Mtg proteins. If so, then this could explain why previous studies promoted only α-cell development upon continuous Ngn3 overexpression. Alternatively, it is possible that the interaction between Ngn3 and Mtg proteins in β-cell development is not simply to block Ngn3 function, but to promote repression of genes required for other endocrine lineages. In agreement with this, a recent study showed that β-cell identity is maintained by DNA methylation-mediated repression of Arx by histone modifying enzymes (Dhawan et al, 2011). Since the Mtg proteins interact with histone modifying enzymes and transcriptional corepressors to orchestrate protein–protein interactions that cause epigenetic changes in gene regulation and silencing (Rossetti et al, 2004), it is possible that Ngn3 both

activates transcription of Mtg genes and interacts with the Mtg proteins to form complexes that repress key genes like Arx to promote a β-cell fate over other endocrine cell fates.

Tbx2 had not been previously implicated in β-cell development at the time we completed our study. Shortly after our work was published, it was shown that Tbx2 expression is dynamically regulated throughout mouse embryonic β–cell development (Begum and Papaioannou, 2011). It is expressed in β–cells as well as duct and other pancreatic endocrine cells during fetal development and in the adult pancreas. The Tbx genes are generally known for their role in mesoderm and ectoderm development, and previous studies demonstrate that Tbx3, which is thought to act redundantly with Tbx2, acts as a transcriptional repressor and plays a role in liver progenitor proliferation and cell-fate determination (Ludtke et al, 2009; Suzuki et al, 2008). Given the proposed model of a common pancreas and liver progenitor, the involvement of Tbx2 in early pancreas development would not be unexpected. Similar to Tbx3 in liver development, Tbx2 could work downstream of Ngn3 acting to inhibit expression and/or activity of Arx and promoting proliferation of β-cell progenitors. It will be interesting to see whether any key pancreatic genes are silenced following overexpression of Tbx2 and Mtg genes.

We found remarkably little overlap of our microarray gene list when compared to microarray data generated in previous studies using Ngn3 as a marker of endocrine progenitors or Ngn3-null mice (Gasa et al, 2004, 2008; Petri et al, 2006; Serafimidis et al, 2008; Treff et al, 2006; White et al, 2008). Of the 85 genes listed in Table 1, we found only 8 in common with other lists: Insm1, NeuroD4, Irf6, Znf38, Hes5, Stmn3, Hey1, and Mxi1. It is at present unclear why there was so little overlap. However, there were two main aspects of our study that were different from previous studies. First, we targeted naive endoderm *in vivo* at an early stage, while previous studies targeted pancreatic tissue *in vivo* after specification, pancreatic cells *in vitro* or

in ES cells *in vitro* that had been programmed down the endoderm lineage using culture conditions that do not fully recreate embryonic development. Second, we performed our analysis at a much earlier stage. We isolated and compared endoderm tissue 4 hours after Ngn3 activation, whereas the earliest time point previously examined was 12 hours. Because of these differences, we chose to begin our analysis on gene targets not previously identified in other screens. Our results with Mtg8, Mtg16, MtgR1, and Tbx2 are the first to demonstrate a role for these genes in pancreas development.

The pressing question is to confirm if the phenomenon observed in *Xenopus* could be reproduced during mammalian pancreatic development. The tamoxifen-inducible Cre-lox system could be adapted to cause transient overexpression of Ngn3 under the promoters of different pancreatic genes designed to probe various timepoints during embryonic development. Likewise, transgenic mice using an Ngn3-Cre line can be used to knock-out and overexpress Tbx2 and the Mtg proteins and see if they are involved in β–cell development and if they alone can promote ectopic β–cell fate. Additionally, it would be interesting to analyze if Tbx2 and the Mtg proteins promote *in vitro* stem cell differentiation into β–cells. It would also be important to determine if Tbx2 and the Mtg genes are direct transcriptional targets of Ngn3 and if the Mtg proteins directly interact with Ngn3. This information would help elucidate the regulatory mechanisms of these genes and determine whether it could be of therapeutic benefit to manipulate them.

In summary, transient overexpression of Ngn3 in *Xenopus* endoderm was sufficient to promote ectopic and early development of β-cells. We identified novel genes downstream of Ngn3 that are expressed during *Xenopus* embryonic pancreas development and are involved in endoderm development. We propose these genes might be novel players in the early specification of β–cell fate and may be useful targets to aid in the promotion of *de novo* β-cells.

CHAPTER II PREAMBLE

The development of an effective and efficient method to create *de novo* β-cells for cell replacement therapy is an important diabetes research goal. However, it is unknown how these transplanted β–cells will react in a diabetic environment in humans or if they will remain functional for prolonged periods of time under conditions of metabolic stress. If we understood the mechanisms by which β–cell dysfunction arises in diabetes, we could perform complete phenotypic characterization of cells destined for transplantation to ensure they do not posses inherent defects affecting insulin secretion and possibly engineer these cells to resist damage caused by the diabetic environment. Additionally, understanding the causes and pathways of β–cell dysfunction and death could allow for the development of β–cell specific drugs to reverse these processes. Towards this end, of particular interest are genes that show dysregulated expression in organs affected by diabetes or that have polymorphisms with a significant association with diabetes. Despite great advancements in the past decades, the mechanisms of β–cell dysfunction affecting the majority of type II diabetics remain elusive. This is in part because the catalytic and regulatory mechanisms of fuel-stimulated insulin secretion are still being assembled.

The next chapter consists of a study investigating the role of two structurally-related proteins previously linked to diabetes in β–cell physiology and insulin secretion. One of these proteins is decreased in islets of type II diabetics and has two single nucleotide polymorphisms significantly associated with increased risk of diabetes, as well as other metabolic diseases.

CHAPTER II

Inhibition of Pgc-1α and Pgc-1β function in mouse β–cells decreases insulin secretion without affecting mitochondrial respiration and mass

INTRODUCTION

Role of Mitochondria in glucose stimulated insulin secretion and associated β-cell dysfunction.

Mitochondria are central for efficient and optimal glucose stimulated insulin secretion (GSIS). The canonical and most effective activator of GSIS involves glucose oxidation in the mitochondria that increases intracellular ATP levels and directly stimulates insulin exocytosis (Reviewed in Prentki et al, 2013). Most of this pathway has been elucidated from work performed in rodents. GSIS initiates when glucose passively diffuses into the β–cell through the Glucose transporter 2 (Glut2); which is also expressed in the liver, intestine and kidney (Thorens et al, 1988). Glut2 has a low affinity, but high capacity, for glucose, allowing for uptake when glucose levels are high in the blood and insulin secretion is required (Jhonson et al, 1990). Compared to other related glucose transporters, Glut2 is most responsive to high glucose and is not expressed in α-cells of the islet (Tal et al, 1992). Glut2 is necessary for GSIS and cannot be functionally substituted by other glucose transporters. Genetic inactivation of Glut2 in every cell of the mouse abolishes GSIS, causes hyperglycemia, and death by three weeks of age (Guillam et al, 1997).

Following glucose entry into the β-cell, glucokinase phosphorylates glucose to promote entry into glycolysis. Glucokinase is a hexokinase with low affinity for glucose (only sensing concentrations above 4 mM) and is highly expressed in islets compared to neurons and liver (Maglasson et al, 1983; Matschinsky et al, 1993; Schuit et al, 2001). Under normal conditions, glycolysis is favored by the relative low levels of glucose-6-phosphatase (G6P) expression and

activity in the β-cell compared to other tissues (reviewed in van Schaftingen and Gerin, 2002). Indeed, overexpression of G6P in a mouse β–cell line decreases GSIS in a dose-dependent manner (Iizuka et al, 2000). Almost all of the pyruvate resulting from glycolysis is shuttled into the Krebs cycle (Schuit et al, 1997), tightly coupling glycolysis to mitochondrial oxidation. In fact, high glucose concentrations increase the ratio between glucose oxidation and glycolysis rates (Schuit et al, 1997); which is not observed in most cell types where glycolysis is preferred under high glucose.

NADH feeds electrons into the electron transport chain (ETC) to raise levels of ATP in the β–cell. The importance of the ETC and mitochondrial respiration for GSIS is illustrated by the hindered insulin secretion observed in mitochondrial diabetes, where severe mutations in genes from mitochondrial DNA severely decrease ATP levels (Kadowaki et al, 1994; Maaseen et al, 2004) and by chemical and genetic studies. Incubating isolated rat islets in low levels of oxygen or with amytal, an inhibitor of mitochondrial respiration targeting Complex I at the level of NADH dehydrogenase, dramatically decreases GSIS (Ohta et al, 1990). Nuclear-encoded genes compose most of the proteins ETC complexes, but 13 proteins integral for the formation of ETC complexes are encoded by mitochondrial DNA and its lone transcription factor, Mitochondrial transcription factor A (Tfam). Removing mitochondrial DNA from β–cell lines strongly impairs GSIS, but does not affect secretion stimulated by other secretagogues that directly activate insulin exocytosis and bypass ATP production (Soejima et al, 1996 ; Kennedy et al, 1998). As proof of concept of the importance of mitochondria in β–cell function *in vivo*, Tfam inactivation in mouse β–cells using Cre-recombinase driven by the rat insulin 1 promoter causes hyperglycemia at seven weeks of age, along with severe mitochondrial and β–cell dysfunction (Silva et al, 2000). Mitochondria lacking Tfam lose all internal structure and isolated Tfam-

knockout islets have impaired GSIS and mitochondrial membrane hyperpolarization. β–cell mass significantly decreases with age in these mice and correlates with the severity of hyperglycemia.

Metabolism of glucose in the mitochondria raises ATP levels in the cell and causes the closing of ATP-sensitive K^+ channels (reviewed in Miki et al, 1999). This depolarizes the cell membrane, opening Ca^{2+} channels and increasing the levels of cytoplasmic Ca^{2+}, which in turn promotes the fusion of insulin granules to the plasma membrane and causes release of insulin into the bloodstream (reviewed in Wang and Thurmond, 2009). In the past decade, many regulatory and amplifying mechanisms of insulin secretion have been identified that are independent or complementary to mitochondrial function. These include ROS signaling, anaplerotic pathways and lipid metabolism occurring outside of the mitochondria (reviewed in Prentki et al, 2013). Nonetheless, mitochondria are intimately linked to these novel mechanisms by supplying or taking up intermediate metabolites and playing an important role in defining the metabolic state of the β–cell.

Mitochondrial dysfunction in β-cells has been observed in type II diabetics and mouse models of TIID (Anello et al, 2005; Lu et al, 2009). It is characterized by acute loss of internal mitochondrial structure and swelling, as well as, a significant decrease in mitochondrial membrane potential and ATP production capacity (Anello et al, 2005; Lu et al, 2009). Yet, it is not clear if this is a consequence of β-cell dysfunction or one of its primary causes.

To address this, recent research has targeted other proteins critical for mitochondrial function. Inactivation of Prohibitin, an integral structural protein of the inner mitochondrial membrane, in β-cells from fetal development causes progressive hyperglycemia and β–cell dysfunction starting at 4 weeks of age (Supale et al, 2013). Prohibitin knockout mice show significantly decreased GSIS and ATP production; yet internal mitochondrial structure is not

affected even though the mitochondrial web is fragmented (Supale et al, 2013). These changes culminate in significantly decreased β–cell mass caused by an increase in apoptosis (Supale et al, 2013). Targeting Optic atrophy 1 (Opa1), a key regulator of mitochondrial fusion dynamics, in β-cells from fetal development results in fragmented mitochondria with significant loss of internal structure (Zhang et al, 2011). At 8 week of age, β–cell Opa1 knockout mice are glucose intolerant, but do not become hyperglycemic; even though their islets showed decreased ATP production, GSIS, mitochondrial respiration and β–cell mass. While these models have provided essential information about the importance of mitochondrial function to β-cell biology, these proteins have fundamental roles in mitochondrial structure and function, often leading to severe phenotypes at an early age and there are currently no identified associations between genetic variation in the Tfam, Prohibitin or Opa1 genes and TIID. Thus, interest has increased in targeting molecules that both modulate mitochondrial function and have proven genetic or functional associations with diabetes in humans.

Pgc-1 genes are master regulators of mitochondrial biology in response to stress.

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Pgc-1α) is a transcriptional coactivator that regulates the transcriptional activity of a variety of transcription factors and is part of small family of proteins including Pgc-1α, Pgc-1β and the distantly related Pgc-1 related coactivator (PRC). The Pgc-1 proteins do not bind DNA, but instead orchestrate the formation of protein complexes that link transcription factors with basal transcriptional machinery (reviewed in Lin, 2009). The three members of the Pgc-1 family are transcribed by three separate genes that are located in different chromosomes in both mice and humans. They are mainly characterized by N-terminal transactivation and C-terminal RNA binding domains

which are highly conserved between the three proteins (Reviewed in Lin et al, 2005). Compared to PRC, Pgc-1α and Pgc-1β proteins are more closely related to each other as they contain domains in their central region not present in PRC, including a transcriptional repressor domain (Lin et al , 2005).

Pgc-1α was identified from a yeast two-hybrid screen used to pull out proteins that interact with the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), a regulator of fatty acid storage in brown fat cells (Puigserver et al, 1998). Pgc-1α expression is activated upon cold exposure in brown fat cells and binds PPARγ and Estrogen related receptor alpha (ERRα), strongly increasing the transcription of target genes. In pre-adipocytes, Pgc-1α increases the expression of mitochondrial genes including Uncoupling protein 1 (Ucp1), Cytochrome c oxidase subunit II (CoxII), CoxIV and ATP Synthase (Puigserver et al, 1998). Pgc-1-α is strongly expressed in both mouse and human tissues that have high metabolic demand including adipose tissue (white and brown), liver, heart, skeletal muscle and kidney (Puigserver et al, 1998; Wu et al, 1999; Esterbauer et al, 1999).

Extensive characterization in various *in vitro* systems demonstrate that Pgc-1α controls mitochondrial function in numerous cell types in which it is expressed. It achieves this by regulating transcriptional programs affecting mitochondrial physiology, including mitochondrial respiration, fatty acid oxidation (FAO) and reactive oxygen species (ROS) detoxification (reviewed in Finck and Kelly,2006; Scarpulla, 2008; Scarpulla, 2011). Pgc-1α function is cell-type specific, for example it activates expression of Ucp1 in pre-adipocytes, but not in muscle cells (Wu et al, 1999). Pgc-1α interacts with the nuclear mitochondrial transcription factor Nuclear respiration factor-1 (Nrf-1) to regulate expression of multiple genes involved in oxidative phosphorylation (OXPHOS) including Cytochrome C (CytC) (Wu et al, 1999). It also

increases the expression of other mitochondrial transcription factors including Nrf-2 and Tfam, the transcription factor responsible for transcribing mitochondrial DNA (Wu et al, 1999). In muscle cells, these effects on gene expression translate into increased mitochondrial respiration, mitochondrial mass and mitochondrial volume density (Wu et al, 1999; St-Pierre et al, 2003). In pre-adipocytes and liver cells, Pgc-1α interacts with the transcription factor PPARα, a master regulator of FAO, to increase expression of its targets including Medium and long chain acyl-coenzyme a dehydrogenases (MCAD and LCAD) and Carnitine palmitoyltransferase 1 (CPT1, Vega et al, 2000).

Pgc-1α upregulates genes involved in the detoxification of ROS, including Glutathione peroxidase (Gpx1) and Manganese Superoxide dismutase 2 (SOD2, St-Pierre et al, 2003). Further investigation showed that Pgc-1α is activated by elevated levels of ROS and increases expression of other ROS detoxification genes including SOD1 and catalase; thus having a protective role against ROS (St-Pierre et al, 2006). In osteosarcoma cells, Pgc-1α regulates the expression of Mitofusin 2 (Mtfn2), a gene involved in mitochondrial fusion dynamics (Cartoni et al, 2006). Taken together, Pgc-1α controls the expression of multiple gene pathways involved in mitochondrial metabolism and thus, is characterized as a master regulator of mitochondrial function.

Pgc-1α levels are significantly higher in muscle cells of Type I fibers, which have a higher mitochondrial mass compared to muscle cells of Type II fibers. An important role for Pgc-1α in mitochondrial biology was confirmed *in vivo* when Pgc-1α was overexpressed in all skeletal and cardiac muscle cells (Lin et al, 2002). Pgc-1α overexpression in muscle using the muscle creatine kinase (MCK) promoter, causes Type II muscle fibers to acquire the physiological characteristics of Type I fibers (Lin et al, 2002). It was later shown that this change

in phenotype is mainly driven by an increase in mitochondrial density and genes involved in OXPHOS (Choi et al, 2008). Hence, increased Pgc-1α expression impacts mitochondrial gene expression and is sufficient to modify mitochondrial biology.

Pgc-1β was identified from sequence homology to the N-terminal protein domains of Pgc-1α (Lin et al, 2002b). It has a similar expression pattern to Pgc-1α, being expressed in liver, muscle, kidney and adipose tissue (Lin et al, 2002b; Meirhaeghe et al, 2003). However, it is not always regulated in the same way as Pgc-1α. In liver, fasting induces expression of both Pgc-1β and Pgc-1α, while in brown fat cells, unlike Pgc-1α, Pgc-1β transcription is not affected by cold exposure, but rather factors that initiate differentiation (Lin et al, 2002b). It also interacts with many of the same transcription factors as Pgc-1α, including Nrf-1, PPARα and ERRα (Lin et al, 2002b; Kressler et al, 2002); but does not interact with others such as Hepatic nuclear factor 4 and Forkhead box protein O1 (Hnf4 and Foxo1, Lin et al, 2003). In liver cells, Pgc-1β increases the expression of OXPHOS and FAO genes but, unlike Pgc-1α, it does not increase expression of genes involved in gluconeogenesis (Lin et al, 2003). Pgc-1β increases mitochondrial respiration and density in muscle cells, possibly to a higher extent than Pgc-1α (St-Pierre et al, 2003; Meirhaeghe et al, 2003). Like Pgc1-α, Pgc-1β can modify mitochondria in muscle cells *in vivo*, but provides different physiological outcomes compared to over-expressed Pgc-1α. Overexpression of Pgc-1β in muscle under the MCK promoter increases mitochondrial mass and upregulates OXPHOS and FAO genes, but directs cells to acquire the characteristics of muscle cells of Type IIX fibers (Arany et al, 2007). These cells have 'fast twitch' biophysical properties compared with the muscle cells of Type I fibers produced by Pgc-1α.

PRC was also identified based on sequence homology to Pgc-1α, but it is ubiquitously expressed and activated by different cellular cues compared to Pgc-1α (reviewed in Andersson

and Scarpulla, 2001). It is not activated by cold exposure in brown fat cells, but instead is activated by serum stimulation in mouse quiescent embryonic fibroblasts (Andersson and Scarpulla, 2001). It interacts with Nrf-1, Nrf-2 and ERRα and activates expression preferentially of OXPHOS genes (Andersson and Scarpulla, 2001; Vercauteren et al, 2008). Activation by serum suggests a role of PRC in regulating mitochondrial function in proliferating cells in response to growth factors. This was later demonstrated in other cell systems and is thought to be a main function of PRC (reviewed in Scarpulla, 2008). The effects of PRC *in vivo* overexpression have not been reported to date and the whole body knockout of PRC causes preimplantation embryonic lethality (He et al, 2012).

In summary, Pgc-1 proteins modulate mitochondrial function allowing cells to adapt to various types of metabolic stress. They share a common group of interacting transcription factors, including Nrf-1 and ERRα, and transcriptional targets, but tend to be activated by different cues in a cell-type-specific manner. Pgc-1α and -1β are mainly activated in highly oxidative tissues by environmental stresses like exercise, fasting and cold, while PRC is more ubiquitous and seems to be activated by other cellular processes such as proliferation. Additionally, in many instances Pgc-1s can interact with distinct transcriptional partners, which dictate their individual functional differences.

Dysregulated Pgc-1 expression and single nucleotide polymorphisms are associated with metabolic disease and increased risk of diabetes.

Pgc-1α and β expression is often found dysregulated in highly oxidative tissues associated with diabetes. In muscles of type II diabetics, expression of Pgc-1α significantly decreases and is associated with a collective down-regulation of known Pgc-1 gene targets,

mainly key proteins involved in the OXPHOS pathway (Mootha et al, 2003). In another study, reduced Pgc-1α and Pgc-1β in diabetic muscle was confirmed in a separate group of diabetic patients and related family members with no disease, correlating again with reduced OXPHOS and FAO genes (Patti et al, 2003). Pgc-1α expression is also significantly decreased in liver of insulin resistant individuals (Westerbacka et al, 2007) and islets of type II diabetics have increased methylation of the Pgc-1α promoter and strongly decreased Pgc-1α mRNA levels by more than 90% (Ling et al, 2008). In fact, Pgc-1 mRNA levels in islets correlate positively with GSIS (Ling et al, 2008).

Polymorphisms in the human Pgc-1α coding sequence have also shown association with type II diabetes risk. A scan of seven common Pgc-1α coding sequence variants identified an association between a Glycine482Serine substitution and diabetes in a Danish population (Ek et al, 2001). Since then, this polymorphism has been associated with diabetes or its metabolic predictors in many different populations. In Pima Indians from Arizona it is not associated with diabetes, but in glucose-tolerant individuals, the Glycine allele is associated with decreased insulin secretion and fatty acid oxidation (Muller et al, 2003). Significant association with diabetes is also found in Japanese, North Indian, Western and Eastern European populations (Andrulionyte et al, 2006; Bhat et al, 2007; Hara et al, 2002; Kunej et al, 2004). In the North Indian population, another polymorphism in the coding sequence of Pgc-1, Thr394Thr, was also associated with diabetes (Bhat et al. 2007). In caucasians, complex haplotypes encompassing both the promoter and coding sequence of Pgc-1α are associated with increased risk for diabetes (Oberklofer et al, 2004). Several single nucleotide polymorphisms in the promoter of Pgc-1α are also associated with early onset diabetes in a Korean population (Kim et al, 2005). More specific in β–cells, islets from carriers of the 482Serine allele have decreased Pgc-1α mRNA expression

by more than 80% and insulin secretion is diminished by more than 30% (Ling et al, 2008).

Genetic variation in Pgc-1β has yet to be associated with diabetes, but there are considerably fewer targeted association studies on this gene locus. Nonetheless, there is one report associating a single nucleotide polymorphism in the coding region of Pgc-1β with obesity in a Danish population (Andersen et al, 2005).

Inactivation of Pgc-1α and Pgc-1β in peripheral tissues recapitulates features of diabetes.

Systematic inactivation of Pgc-1α and β in peripheral tissues has begun to unravel their roles in diabetes. Mice with skeletal muscle-specific Pgc-1α inactivation, using myogenin promoter driven expression of Cre-recombinase in floxed Pgc-1α mice, are slightly glucose intolerant, but show no difference in insulin sensitivity under normal chow diet (Handschin et al, 2007). Muscle cells lacking Pgc-1α decrease transcription of OXPHOS and ROS detoxification genes and Cox and Succinate dehydrogenase activity (Handschin et al, 2007). Skeletal muscle-specific Pgc-1β knockout mice, generated using the MCK promoter driven Cre-recombinase, show a modest, yet significant, decrease in OXPHOS, FAO and ROS detoxification genes; including SOD2, CoxIV and Cpt1b (Zechner et al, 2010). Pgc-1β knockout muscle cells have slightly decreased expression of Mitofusin1 (Mtn1), a gene involved in mitochondrial fusion, and their mitochondria have significantly decreased respiration. However, muscle Pgc-1β knockout mice show no difference in glucose tolerance or insulin sensitivity under a normal chow diet (Zechner et al, 2010). The differences in mitochondrial gene expression and respiration are more pronounced when both Pgc-1α and β are inactivated in skeletal muscle using the myogenin promoter (Rowe et al, 2013). Every gene analyzed encoding proteins of the ETC is significantly down-regulated, corresponding to decreased activity of every ETC complex. Interestingly, this

effect on ETC function does not impact muscle morphology, fiber type composition or muscle function under basal conditions (Rowe et al, 2013). Even more surprisingly, muscle cells from double knockout mice show no difference in mitochondrial mass, density, or morphology, even though their mitochondrial respiration is strongly impaired (Rowe et al, 2013).

In adipose tissue, inactivation of Pgc-1α in brown adipose tissue (BAT), inguinal white adipose tissue (IWAT) and epididymal white adipose tissue (EWAT, Kleiner et al , 2012) using Cre-recombinase expression by the adiponectin promoter has a strong effect on insulin resistance. Pgc-1α knockout BAT does not alter gene expression of known Pgc-1α targets, except for Fatty acid binding protein 3 (Fabp3), which is significantly decreased. In Pgc-1α knockout EWAT, only genes involved in thermogenesis and FAO are down-regulated, while in Pgc-1α knockout IWAT thermogenesis, FAO, OXPHOS and citric acid cycle genes are decreased. ROS detoxification genes were unaltered in any of the adipose tissues. Under chow diet, there were no gross morphological abnormalities in any of the adipose tissues and no difference in glucose or insulin tolerance (Kleiner et al, 2012).

Unlike the extensive characterization of Pgc-1 function in the muscle and adipose tissue, little is known about their function in β–cells and their impact on mitochondrial function.

Pgc-1α and Pgc-1β research in β-cells.

Given the role of mitochondria in GSIS and the decreased expression of Pgc-1α in islets of type II diabetics, the Pgc-1 genes are important candidates for regulation of β–cell function. Yet, little is known about the function of Pgc-1α and Pgc-1β in β-cells and most work has focused on Pgc-1α using *in vitro* systems. In rodent islets, transcription of Pgc-1α is induced by glucagon-like peptide-1 and forskolin (Hussein et al, 2006; Zhang et al, 2005), which are known

potentiators of GSIS. However, Pgc-1α expression is also induced by cold, glucocorticoids, obesity, chronic glucolipotoxic conditions, known inhibitors of GSIS and inducers of β-cell death (De Souza et al, 2003; Hussein et al, 2006; Zhang et al, 2005; Kim et al, 2009; Oberklofer et al, 2009; Valtat et al, 2013). Given that many stimuli thought to be detrimental to islet health and function also increase Pgc-1α expression in islets, it has been hypothesized that increased Pgc-1α expression is detrimental to β–cell function.

In support of this hypothesis, islets from rat models of diabetes and hyperglycemia have increased expression of Pgc-1α at the mRNA and protein level (Yoon et al, 2003). Over-expression of Pgc-1α in isolated rat islets using adenovirus also decreases insulin secretion and ATP production (Yoon et al, 2003). This reduction in GSIS is sufficient to impair normalization of glycemia after islets expressing Pgc-1α are implanted into mice rendered hyperglycemic by injection of streptozotocin (STZ) (Yoon et al, 2003). Overexpression of Pgc-1α decreases transcription of genes involved in glucose metabolism and β–cell function, including Glucokinase, Glut2, Pdx1 and Hnf4α (Yoon et al, 2003). In addition to the effects on transcription, there is an unexpected increase in G6P. Glucokinase overexpression could partially rescue the impaired GSIS caused by Pgc-1α overexpression, presumably by increasing the flow of glucose into glycolysis (Yoon et al, 2003).

Pgc-1α or β knockdown in isolated islets or β-cell lines improves β-cell function. However, Pgc-1α knockdown was only analyzed under conditions of cellular stress. In rat isolated islets, lowering Pgc-1α levels using antisense oligonucleotides or adenovirus expressing small interfering RNA (siRNA) rescues GSIS impaired by chronic exposure to cold or glucolipotoxicity (De souza et al, 2003; Kim et al, 2009). Knockdown of Pgc-1α *in vivo* in partially pancreatectomized rats (by injection of adenoviruses expressing siRNA into the celiac

artery, targeting the pancreas, liver and duodenum) increases serum insulin levels, protects against hyperglycemia and improves glucose intolerance (Kim et al, 2009). This was suggested to be due to Pgc-1α directly inhibiting transcription of NeuroD. In INS1 cells, a rat cancer β–cell line, Pgc-1α is recruited to the NeuroD promoter and Pgc-1α over-expression decreases its activity (Kim et al, 2009). Along these lines, exposing rat islets to chronically high glucose levels can in some instances decreases expression of Pgc-1α (Zhang et al, 2005).

Further supporting a negative role for Pgc-1 genes in β-cell function, transgenic over-expression of Pgc-1α driven by the rat insulin 1 promoter in mouse β-cells impairs GSIS *in vivo* (Valvat et al, 2013). Mice over-expressing Pgc-1α in β–cells from the fetal stage onwards are glucose intolerant and have decreased insulin secretion at six months of age, accompanied by a significant decrease in β–cell mass and density (Valvat et al, 2013). Islets isolated from these mice have decreased expression of insulin, glucokinase, Glut2 and Sur1 (a component of the ATP-sensitive K^+ channel), as well as Pdx1, MafA and Nkx2.2 (Valvat et al, 2013). In rat islets, Pgc-1α is recruited to the promoter of the Pdx1 gene, possibly mediating transcriptional repression at this locus (Valvat et al, 2013).

Surprisingly, glucose intolerance and decreased insulin secretion *in vivo* are only observed when Pgc-1α is over-expressed during fetal β-cell development. Overexpression of Pgc-1α in β-cells of 4-month-old mice has no impact on glucose tolerance or insulin secretion *in vivo* when mice are analyzed at 8 months of age; yet, these islets with high Pgc-1α still exhibit decreased transcription of insulin, glucokinase, Glut2, Pdx1, MafA and Nkx2.2 (Valvat et al, 2013). Furthermore, overexpression of Pgc-1α in β-cells *only* during fetal development (Embryonic day 0 to Postnatal day 1) causes glucose intolerance and impaired insulin secretion *in vivo,* without any effect on the expression of these genes. Thus, the precise timing of Pgc-1α

overexpression is critical to determining the biological and physiological consequences in β-cells. This is might be due to the importance of mitochondrial function and dynamics in rapidly dividing and differentiating tissue. However, it is not clear if the β–cell dysfunction observed is a secondary effect of cytoxicity caused by disproportionally high levels of Pgc-1α in the β-cell or a direct detrimental effect of Pgc-1α on β-cell physiology.

In general, despite some disparity in the results and different experimental conditions, taken as a whole, these early studies suggest that Pgc-1α activity is deleterious for β-cell function. Yet, when human islets are analyzed, this is not the case. A ~50% knockdown of Pgc-1α in human islets (by transfecting plasmids that express siRNA) is sufficient to cause a ~50% decrease in glucose-stimulated insulin secretion (Ling et al, 2008). The knockdown also causes a mild, yet significant, reduction in insulin expression, yet no change in glucagon expression. Furthermore, in contrast to Yoon et al., 2003, Pgc-1α mRNA levels were decreased in islets isolated from genetic rat models of diabetes or rats treated with STZ (Ling et al, 2008). Taken together with the fact that islets isolated from diabetic patients have decreased expression of Pgc-1α and corresponding decreased insulin secretion (Ling et al, 2008), loss of Pgc-1α expression may in fact be detrimental to β-cell function. Thus, it is still not clear what is the function of Pgc-1α function in β-cells.

Finally, only one study has addressed the function of Pgc1-β in β–cells. In INS1 cells, Pgc-1β overexpression increases expression of Ucp-2, which uncouples mitochondrial respiration from ATP production by draining protons from the intermembrane space into the mitochondrial matrix (Oberklofer et al, 2009). Consistent with this, knockdown of Pgc-1β in INS1 cells, by transfecting plasmids expressing siRNA, increases GSIS (Oberklofer et al, 2009) in this cultured β-cell line.

In summary, it remains unclear whether targeting Pgc-1 expression may be a viable option to improve β-cell function. Interestingly, given the important role of Pgc-1 genes in mitochondrial biology, very little has been done to characterize whether the Pgc-1s are important modulators of these organelles in β-cells. Furthermore, although controversial, some evidence from previous work suggests that altering Pgc-1 expression may impact beta-cell function independent of effects on mitochondria through yet unidentified molecular pathways. Thus, we set out to determine the importance of Pgc-1α and β to adult β–cell biology and whether they regulate mitochondrial function to impact insulin secretion.

RATIONALE AND HYPOTHESIS

Mitochondria are a central player in achieving efficient GSIS and associated mitochondrial dysfunction is often observed in β–cells of diabetic rodents and humans. The Pgc-1 genes are master regulators of mitochondrial function and they have been shown to modify mitochondria in response to stress in various tissues and cell types. Coding polymorphisms and decreased mRNA levels of human Pgc-1α are associated with TIID and decreased GSIS. Despite evidence linking altered Pgc-1 expression to β-cell dysfunction, little data is available that addresses the functional role of Pgc-1 genes in β-cells. By genetically targeting both Pgc-1α and β, we will significantly decrease Pgc-1 function in β-cells and hypothesize this will cause significant mitochondrial dysfunction, decreased insulin secretion, and worsened whole body glucose homeostasis.

RESEARCH DESIGN AND METHODS

Generation of mT/mG:MIP-CreERT and β–cell-specific Pgc-1α/β knockout mice. Male heterozygote MIP-CreERT mice (Tamarina et al, 2014) were crossed with female homozygous mT/mG mice (Muzumdar et al, 2007) to generate mT/mG:MIP-CreERT mice. Previously described mice carrying *Pgc-1α* (Estall et al, 2009) and *Pgc-1β* floxed alleles (Lai et al 2008) interbred to generate $Pgc\text{-}1\alpha^{fl/fl}/\beta^{fl/fl}$ on a mixed C57BL/6N:C57BL/6J:129 background were subsequently bred with MIP-CreERT mice on a C57BL/6J background. Homozygous $Pgc\text{-}1\alpha^{fl/fl}/\beta^{fl/fl}$ (denoted WT-FL, littermate controls) and β-cell specific $Pgc\text{-}1\alpha^{-/-}/\beta^{-/-}$ knock-out (denoted β-KO) mice were then generated. To obtain MIP-CreERT control groups (CreERT transgene alone), $Pgc\text{-}1\alpha^{fl/fl}/\beta^{fl/fl}$,MIP-CreERT males were bred once with C57Bl/6J and interbred 2-3 generations to eliminate floxed alleles. Only male mice were used for all experiments. All test mice (unless specifically indicated) were gavaged at six weeks of age for ten days with 100 g/kg of tamoxifen (Sigma) suspended in 0.05% methylcellulose/distilled water. A minimum 5-week wash-out period followed tamoxifen dosing was provided prior to experimentation. Mice were maintained on a 12-hour dark/light cycle and given free access to water and standard laboratory chow (Teklad diets 2018). Mice were maintained and sacrificed according to approved protocols from the Clinical Research Institute of Montreal (IRCM).

Histological analysis of tissues in mT/mG reporter mice. At 13 weeks of age, mice were perfused via cardiac puncture with 10% formalin. Peripheral organs were frozen in OCT using isopentane. Brains were fixed overnight in 10% formalin, followed by 10% Sucrose/PBS for 24

hours prior to being frozen in OCT using isopentane. Tissues were sectioned (7-10 μm) using a Leica cryostat. Sections were visualized by fluorescence microscopy and images were captured for each wavelength (GFP, RFP) and merged using Velocity software. All brain sectioning and microscopy presented in Figure 3 were performed by Dr. Lionel Budry as part of collaboration with the laboratory of Dr. Thierry Alquier.

Isolation and culture of primary mouse islets. Mice were bled by cardiac puncture under anesthesia (ketamine/xylazine) and ~3 ml of 0.4 U/ml Liberase TL (Roche) in Hank's Balanced Salt Solution (HBSS) buffer with Ca2+ and Mg2+ were perfused into the pancreas through the bile duct. The pancreas was excised, placed in a 15 ml falcon tube and digested in 3 ml of 0.4U/ml Liberase TL in HBSS with Ca2+ and Mg2+ for 30 min at 37°C. Twelve ml of HBSS buffer without Ca2+ and Mg2+ supplemented with 0.1% BSA and 20 mM HEPES ph 7.4 (HBSS/BSA/HEPES) were added to each pancreas, transferred to a 50 ml falcon tube and gently shook 40 times. The homogenate was pelleted for 3 minutes at 4°C 500 g and washed three times with 40 ml of HBSS/BSA/HEPES. After the third wash, the supernatant was decanted and the walls of the tube were cleaned with autoclaved gauze. The pellet was resuspended with 9 ml of hystopaque 1077 (Sigma) and 9 ml of RPMI 1640 without glucose was gently overlaid prior to centrifugation 400 g (no break) for 30 min at 4°C. The supernanant was transferred into a new 50 ml falcon tube and volume was completed to 40 ml using HBSS without Ca2+ and Mg2+ and then centrifuged at 500 g (4°C) for 3 min. Islets were resuspended, hand picked and cultured overnight in 11 mM glucose RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. On average ~300 islets per mouse were obtained.

Gene expression analysis of whole islets, INS1 cells and peripheral tissues. The day following isolation, mouse islets were transferred into new 11 mM glucose RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. The next day, islets were transferred into fresh 11 mM glucose RPMI for a few hours, then batches of 120 islets were snap frozen in liquid nitrogen and RNA was isolated using the RNeasy Mini Kit (Qiagen) following manufacturers instructions. Peripheral tissues were snap frozen in liquid nitrogen immediately after mice were sacrificed and RNA was isolated using TRIZOL reagent (Invitrogen). RNA from INS1 cells cultures was isolated using the TRIZOL reagent (Invitrogen). cDNA was synthesized using the high capacity cDNA reverse transcription kit (Life technologies) following manufacturers instructions. Gene expression was analyzed using SYBR green reagent (Life Technologies) and the ViiA^{TM}7 real time PCR system (ABI) and relative expression was calculated by normalizing with hypoxanthine-guanine phosphoribosyltransferase (Hprt) levels. Human islets were obtained from the Human Islet Transplant Laboratory at McGill University and experimentation was carried out following guidelines approved by the Human Ethic Committee at the IRCM. Pgc-1 gene expression analysis of the brain presented in Figure 5C was performed by Dr. Lionel Budry as part of collaboration with the laboratory of Dr. Thierry Alquier.

Immunoblotting. Overnight-cultured islets (batches of 150 islets) were washed once with PBS, snap frozen in liquid nitrogen and lysed in RIPA buffer supplemented with protease inhibitor cocktail (EMD Millipore). Proteins were separated with SDS-PAGE, transferred to nitrocellulose membranes and blotted with primary mouse anti-PGC-1α (1:1000, Calbiochem 4Cl.3) and HRP-conjugated secondary antibodies were used to visualize PGC-1α protein by enhanced

chemilumiscence (BioRad Clarity Western ECL).

Metabolic tests and insulin measurements. Phenotypic analysis was carried out in mice at 13-17 weeks of age, fed a standard chow. Oral glucose tolerance tests were performed by gavaging 1.5 g/kg glucose (into the stomach) of 16 hr-fasted mice. Glucose or serum insulin levels were measured at indicated time points from the tail vein using a standard glucometer (FreeStyle Lite, Abbot Diabetes Care) and the mouse ultrasensitive insulin ELISA (Alpco), respectively. Insulin tolerance tests were performed by injecting human insulin (Eli Lilly, 1 U/kg) intraperitoneally in 4 hr-fasted mice and glucose measured from the tail vein at the indicated time points. Fasted and refed glucose and insulin levels were measured after a 16 hr-fast and following 2 hours of chow diet re-feeding.

Analysis of *in vivo* insulin secretion under hyperglycemic clamp. Hyperglycemic clamp was performed as previously described (Alquier et al, 2009). Briefly, 20-week-old β-KO male mice and WT-FL littermate controls under chow diet were fasted for four hours and infused with a 20% dextrose solution to clamp blood glucose levels at ~20 mM for 60 min. Throughout the procedure blood glucose levels were monitored and infusion rates adjusted to keep the mice at ~20 mM blood glucose. After 60 min, followed by an injection arginine (1 mmol/kg) to asses maximal insulin secretion. Blood was sampled at indicated time points for insulin measurements and plasma C-peptide levels were measured at 60 minutes. Insulin clearance was calculated as the ratio of C-peptide:insulin.

Mitochondrial mass and ATP measurements in dispersed islets. The day following isolation,

islets were digested for 3 minutes in 0.25% trypsin at 37°C and dispersed in 10% FBS by pipetting up and down 40 times. For mitochondrial mass, dispersed islet cells were incubated in 11 mM glucose RPMI 1640 supplemented with 10% FBS and Penicillin/Streptomycin (media) and 100 nM mitotracker green (Cell signaling) for 15 minutes at 37°C. Cells were washed with media and cultured under similar conditions with 100 nM mitotracker orange (Cell signaling). Cells were then washed once with media, resuspended in 1% FBS/PBS and analyzed by FACS (BD LSRFortessa^{TM}). For ATP, dispersed islet cells were resuspended in media and cultured overnight in poly-l-lysine coated 96-well opaque plates at a density of 7 x 10^4 cells/well. The next day, cells were incubated twice in Krebs-Ringer bicarbonate HEPES (KRBH) buffer supplemented with 2.8 mM glucose and 0.01 mM fatty-acid free BSA (Fisher) at 37°C for 20 minutes. Cells were then incubated in KRBH supplemented with 0.05 mM fatty-acid free BSA and 2.8 mM glucose, 20 mM glucose or 20 mM glucose plus 0.2 mM palmitate at 37°C for 15 min. ATP levels were quantified using the CellTiter Glo reagent (Promega).

Static isolated islet glucose stimulated insulin secretion. The day following isolation, batches of ten islets of similar size (measured using an eyepiece micrometer) were hand picked and placed in islet incubation chambers (Mecanique Universelle Hugo Bergeron) submerged in KRBH buffer (no glucose) supplemented with 0.01 mM fatty-acid free BSA (Fisher). Islets were then starved of glucose two times for 20 minutes at 37°C in KRBH buffer supplemented with 0.01 mM fatty-acid free BSA and 2.8 mM glucose. Islets were then sequentially cultured for 1 hr at 37°C in KRBH buffer supplemented with 0.05 mM fatty-acid-free BSA and 2.8 mM glucose, 16 mM glucose and 16 mM glucose plus 0.2 mM palmitate. Samples of media at the end of each treatment were collected for insulin measurements. Islets were lysed overnight in

1.5%/70% HCl/EtOH for the determination of islet insulin content. Insulin concentrations were measured using the mouse ultrasensitive insulin ELISA (Alpco).

INS1 culture and adenovirus infection. Parental INS1 cells (Asfari et al, 1992; Hohmeier et al, 2002; density of 1.50 x 10^8 cells per well divided by well area) were cultured in regular six-well culture plates overnight in 11 mM glucose RPMI 1640 supplemented with 10% heat inactivated FBS, penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 50 mM 2-ME (RPMI plus supplements). Cells were infected with adenoviruses (serotype 5) expressing short-hairpin RNAs targeting Pgc-1α (Sh-Pgc-1α,5'-GGTGGATTGAAGTGGTGTAGA-3', Koo et al, 2004) and/or Pgc-1β (Sh-Pgc-1β, 5'-GATATCCTCTGTGATGTTA-3'; Lin et al, 2005) for 8 hours, washed and cultured for 48 hours. Cells were then passaged into Seahorse XF^{e}24 cell culture plates (for mitochondrial respiration analysis, density of 3x10^4 cells/well) or regular six-well culture plates (for RNA extraction) and cultured for an additional 24 hours prior to experimentation.

Islet area, insulin and glucagon immunohistochemistry and mitochondrial morphology. For histological analysis, 4% paraformaldehyde/PBS fixed and paraffin-embedded pancreata were sectioned into 5 μm sections. To measure islet area, 10-12 sections distributed throughout the pancreas (separated by at least 200 μm) were stained with Haematoxylin and Eosin (H&E) for each mouse and average islet area calculated using Matlab script. The area of each islet in a pancreatic section was calculated and all islet areas from each mouse were pooled and grouped according to their size. β- and α–cells were stained using guinea pig anti-insulin (1:100, Dako) and mouse anti-glucagon (1:50, Sigma), horseradish peroxidase-conjugated secondary

antibodies. For ultrastructural analysis, whole islets were left in fixative (3% in glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)) overnight at 4°C. After extensive washing in 0.1 M sodium cacodylate buffer, the tissues were sequentially treated with osmium tetroxide, tannic acid and uranyl acetate, then dehydrated and embedded in Epon. Thin sections (60 nm) were counterstained with methanolic uranyl acetate and lead citrate and viewed using a Tecnai 12 transmission electron microscope at 120 kV. Mitochondrial density was calculated using ImageJ64 by dividing the number of mitochondria over cell area. All micrographs were taken with all nuclei having comparable area. Electron micrographs were captured by Lea Ringuette as part of collaboration with the laboratory of Dr. Elaine Davis.

Oxygen consumption rate analysis. To measure oxygen consumption the XF^{e}24 analyzer (Seahorse Bioscience) was used. Cells and islets were cultured in XF media (DMEM media supplemented with 4 mM glucose, 30 mM NaCl, 1 mM sodium pyruvate and 2 mM glutamine; adjusted pH to 7.4) for an hour in 37°C incubator without CO_2 prior to analysis. Oxygen consumption in INS1 cells was measured by sequentially incubating in 1 μM oligomycin (Calbiochem), 1 μM FCCP (Sigma) and 100 nM rotenone (Sigma). Islets were cultured in XF media supplemented with 1% FBS and sequentially incubated in 11 mM glucose plus 0.2 mM palmitate, 5 μM oligomycin, 1 μM FCCP and 5 μM rotenone.

Statistical analysis. All statistical tests were performed using Graphpad Prism software. Statistical analysis was performed using an unpaired two-tailed student's t-test or, where specified, using 2-way ANOVA.

RESULTS

Pgc-1s regulate mitochondrial gene expression and respiration in INS1 β-cells.

Given the importance of Pgc-1 genes to mitochondrial function in cells, we asked if lowering Pgc-1α and/or Pgc-1β expression in β-cells would significantly impact mitochondrial biology. We also sought to determine whether Pgc-1 family members play an additive, redundant, or synergistic role in β-cells. To do this, we first performed knockdown studies in INS1 cells with adenoviruses expressing sh-Pgc-1α and/or sh-Pgc-1β and measured mitochondrial gene expression and respiration. A ~50% decrease of Pgc-1α mRNA caused a significant decrease in expression of mitochondrial genes, including electron transport chain components, mtDNA transcription, and ROS detoxification (Figure 1A). In contrast, reduction of Pgc-1β expression decreased specific key regulators involved in ROS detoxification, including Sod1, Sod2 and Ucp2, while only decreasing Atp5b of the ETC components measured. Of various transcription factors involved in β–cell function, we noted a Pgc-1α-specific regulation of Pdx-1 expression and a Pgc-1β-specific regulation of NeuroD expression, suggesting that Pgc-1 activity is not completely over-lapping between the different family members in β-cells. Increased expression of endogenous Pgc-1α was observed following Pgc-1β knockdown (and vice versa), suggesting a possible compensatory up-regulation of other family members in the event one member is down-regulated (Figure 1A). Effects on gene expression translated into a significant decrease in oxygen consumption rate when mitochondrial respiration was assayed (Figure 1B). Knockdown of Pgc-1α and/or Pgc-1β caused significant differences in oxygen consumption rate when INS1 cells were treated with the ATP synthase inhibitor oligomycin or the respiratory chain uncoupler FCCP. When either sh-Pgc1α or sh-Pgc1β were used the

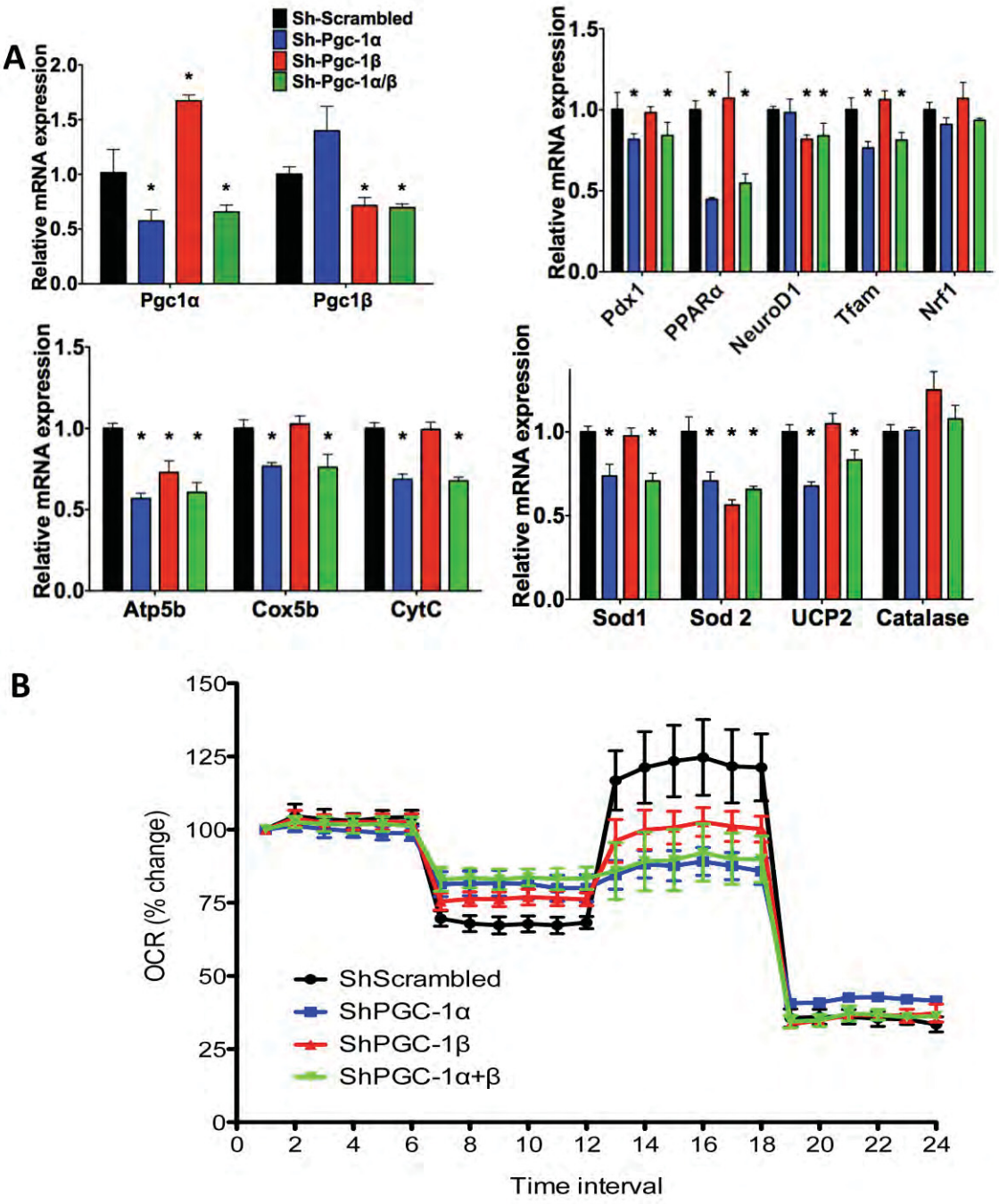

Figure 1. Pgc-1α and/or Pgc-1β knockdown in INS1 affects mitochondrial gene expression and respiration. INS1 cells were infected with adenoviruses expressing sh-Pgc-1α and/or sh-Pgc-1β and analyzed after 72 hours in culture. A-B: Gene expression and oxygen consumption rate (OCR, pmoles/min) analysis (n=3-4). Mean OCR data are presented from five different experiments. * P-value <0.05 compared to Sh-Scrambled, where indicated. All data expressed as means ± SD.

changes in oxygen consumption rate were not as pronounced compared to sh-Scrambled. Thus, there was a decrease in both coupled and uncoupled respiration. Taken together, these results indicate that Pgc-1α and Pgc-1β are important modulators of mitochondrial gene expression and respiration in INS1 β-cells. The data also suggest that in β–cells, Pgc-1α and Pgc-1β may compensate for each other in some aspects, in addition to regulating transcription of unique gene sets.

Given that down-regulation of Pgc-1 expression in a cultured rat β–cell model negatively affected mitochondrial function *in vitro*, we sought to address the impact of reducing Pgc-1 function in β–cells *in vivo* and determine whether it would affect whole body glucose homeostasis. To avoid any possible compensation between Pgc-1α and Pgc-1β (observed in previous in vivo studies [Lai et al, 2008; Sczelecki et al, 2014] and our unpublished work) and to fully assess the impact of Pgc-1 inhibition on mitochondrial function, we created β-cell-specific Pgc-1α and Pgc-1β double knockout mice.

MIP-CreERT line had no Cre-mediated recombination outside of the pancreatic islet.

Overexpression of Pgc-1α in β–cells during fetal development causes β–cell dysfunction characterized by decreased insulin secretion and β–cell mass (Valtat et al, 2013)**.** To overcome any developmental effects that dysregulation of Pgc-1 gene expression might cause during pancreatic development and assess the impact of Pgc-1 genes in adult β–cell biology, we used the MIP-CreERT mouse line (Tamarina et al, 2014) to create inducible, β-cell-specific Pgc-1α/β double knockouts. The MIP-CreERT mouse uses 8.5 kb of the mouse insulin 1 promoter (MIP) to drive expression of tamoxifen inducible Cre-recombinase specifically in β–cells.

Transgenic lines produced to express Cre-recombinase in specific tissues may exhibit leaky or ectopic transcription in other organs (Cui et al, 2004). The MIP-CreERT mouse has not been extensively characterized in terms of ectopic transgene expression; therefore we first performed experiments to detect any potential transcriptional or functional Cre-recombinase leakiness. We crossed the MIP-CreERT mouse with a global double fluorescent reporter line, the mT/mG mouse (Muzumdar et al, 2007). This line expresses a LoxP-Red fluorescent protein-Stop-LoxP-Green fluorescent protein (LoxP-RFP-Stop-LoxP-GFP) transgene under the control of the β-actin promoter, causing all cells in the mouse to be RFP+ under basal conditions. However, any cells with an active Cre-recombinase enzyme are permanently marked with GFP and RFP protein expression is halted.

First, we investigated if the MIP-CreERT exhibited Cre-mediated recombination in the absence of tamoxifen in the islet. We compared islets of double positive mT/mG:MIP-CreERT mice that had been gavaged with tamoxifen for ten days or received no tamoxifen. In the presence of tamoxifen, the majority of cells in the islets were positive for GFP, suggesting efficient excision and recombination of the RFP transgene (Figure 2A), while islets from double positive mT/mG:MIP-CreERT mice that did not receive tamoxifen had only very few GFP+ cells in the islets (Figure 2B). We also compared these images to pancreas sections from mT/mG mice to determine whether these green cells were due to mT/mG spontaneous recombination and detected no GFP positive cells in the islets (Figure 2C). This suggests that the MIP-CreERT transgene has only a very small amount of leaky Cre-recombinase-mediated recombination in the absence of tamoxifen and the MIP-CreERT mouse is effective at targeting the majority of β-cells in a drug-inducible manner.

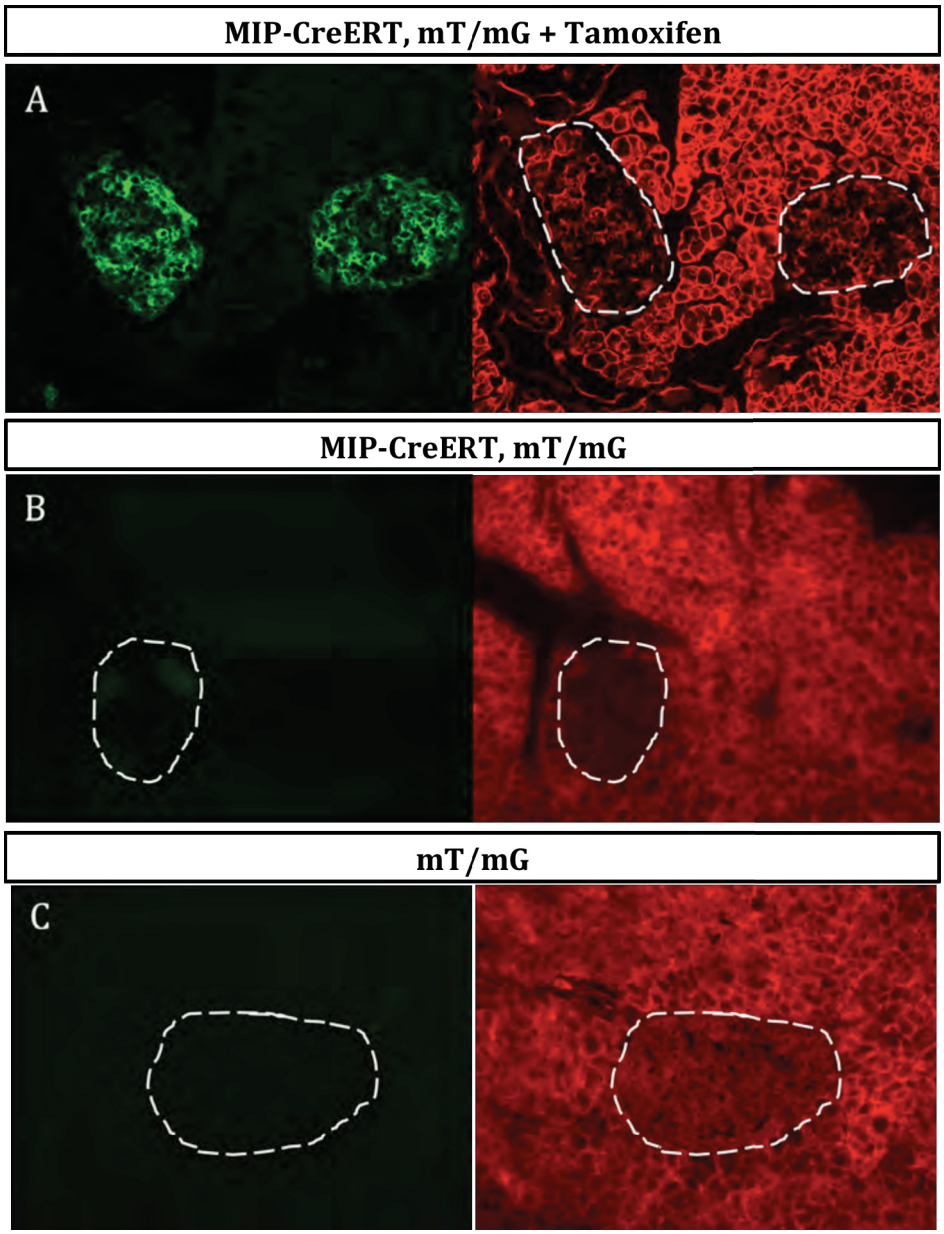

Figure 2. Assessment of tamoxifen-induced and tamoxifen-independent recombination in pancreatic islets of mT/mG:MIP-CreERT mice. A: MIP-CreERT, mT/mG male and female mice were gavaged with 100 g/kg of tamoxifen everyday for ten days at six weeks of age and pancreata were extracted for analysis at 13 weeks (representative n=6). B,C: Pancreata from MIP-CreERT,mT/mG or mT/mG male and female mice were extracted for analysis at 13 weeks (representative n=3).

Strong and widespread misexpression of Cre-recombinase throughout various brain regions, including the hypothalamus and brain stem, has been reported when promoters of β-cell specific genes are used, such as the rat insulin promoter (RIP)-Cre or pancreatic and duodenal homeobox 1 (Pdx1)-Cre lines (Cui et al, 2004; Wicksteed et al, 2010). We assessed for ectopic Cre-recombinase mediated loxP excision in the brains of mT/mG:MIP-CreERT, mice five weeks after tamoxifen gavage. Whole brain coronal and sagittal sections (Figures 3A-B) were negative for GFP expression and no single GFP+ cells were detected in areas known to be involved in glucose homeostasis; namely the hypothalamic arcuate nucleus, ventromedial hypothalamus, dorsomedial hypothalamus, dorsal motor nucleus of vagus nerve, nucleus of the solitary and parabrachial nucleus (Figures 3C-G.). To confirm targeted Cre-recombinase activity, islets in the corresponding pancreas of each mouse were shown to have a strong GFP signal (Figure 3H). Next we analyzed peripheral tissues for the presence of any GFP+ cells. As in the brain, we detected no GFP+ cells in the liver, spleen, muscle, kidney or testes (Figure 4). We concluded that MIP-CreERT line used to generate our β-KO line had no significant Cre-recombinase activity outside of the islet in multiple peripheral tissues. This gave us confidence to use the MIP-CreERT line to create a β-cell specific knock-out of the Pgc-1 proteins that would have little to no confounding effects due to reducing Pgc-1 expression in other non-β-cell populations (Kleiner et al, 2012; Rowe et al, 2013).

β-KO mice had significantly decreased Pgc-1α and Pgc-1β levels but no changes in islet architecture or area.

Islets of male β-KO mice showed an ~80% reduction in both Pgc-1α and Pgc-1β gene expression at 13 weeks of age (Figure 5A). As Pgc1-α protein levels are highly regulated

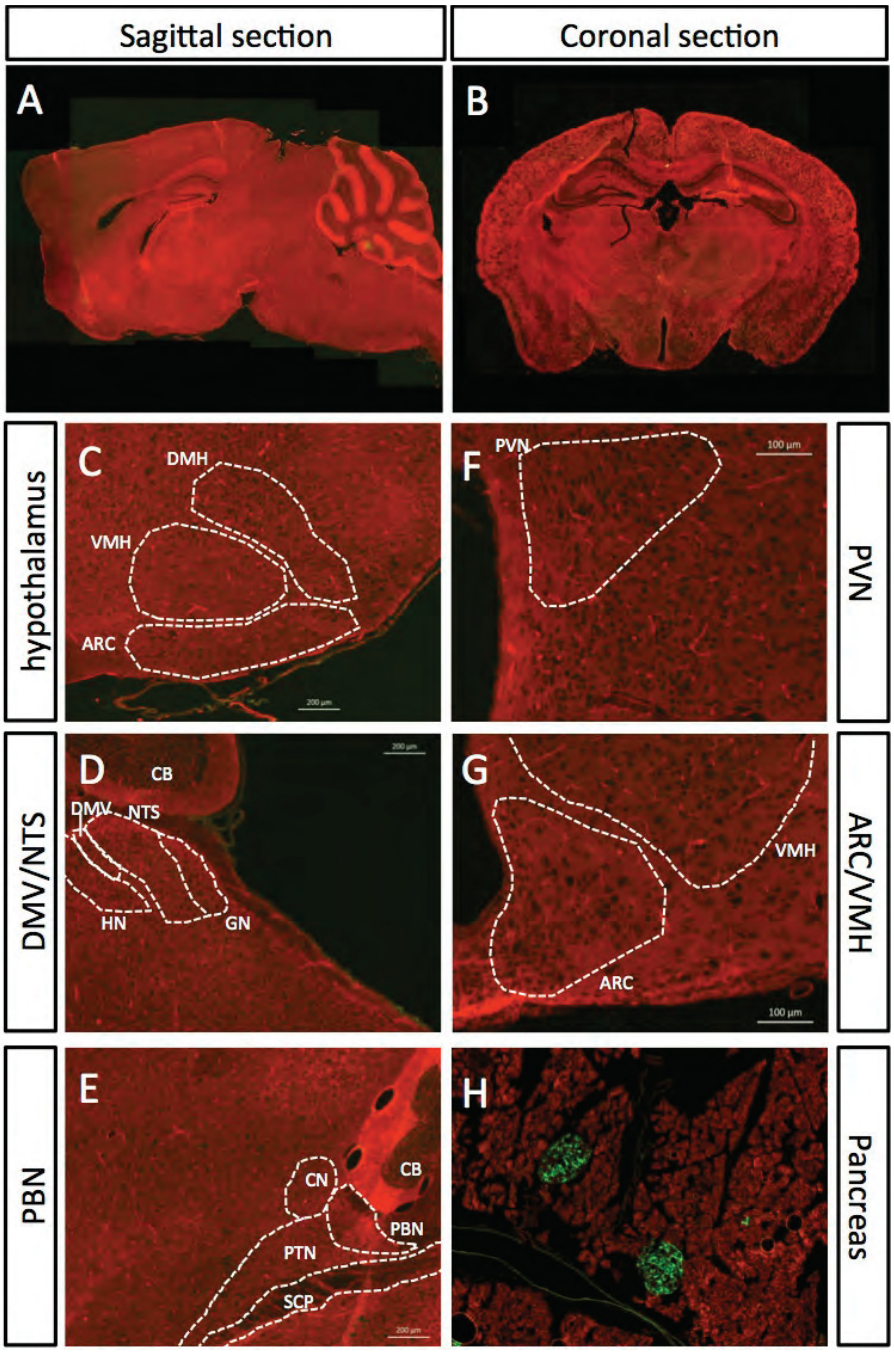

Figure 3. Assessment of tamoxifen-induced recombination in central nervous system of MIP-CreERT,mT/mG mice. Leakiness of the MIP-CreERT transgene was assessed in MIP-CreERT, mT/mG mice that were gavaged with 100 g/kg of tamoxifen everyday for ten days at six weeks of age. Both sagittal (A,C, D, E) and coronal (B, F, G) 20 µm brain cryosections at 13 weeks are shown here. (representative of n=2). Higher magnification images of the hypothalamus were taken on coronal sections at the level of the paraventricular nucleus (PVN) and more ventrally at the level of the arcuate nucleus (ARC) (representative of n=3). Pancreatic islets (H) are shown here as a positive control. Other structures shown : gracile nucleus (GN), hypoglossal nucleus (HN), cerebellum (CB), cuneiform nucleus (CN), pedunculopontine tegmental nucleus (PTN), and superior cerebellar peduncle (SCP).

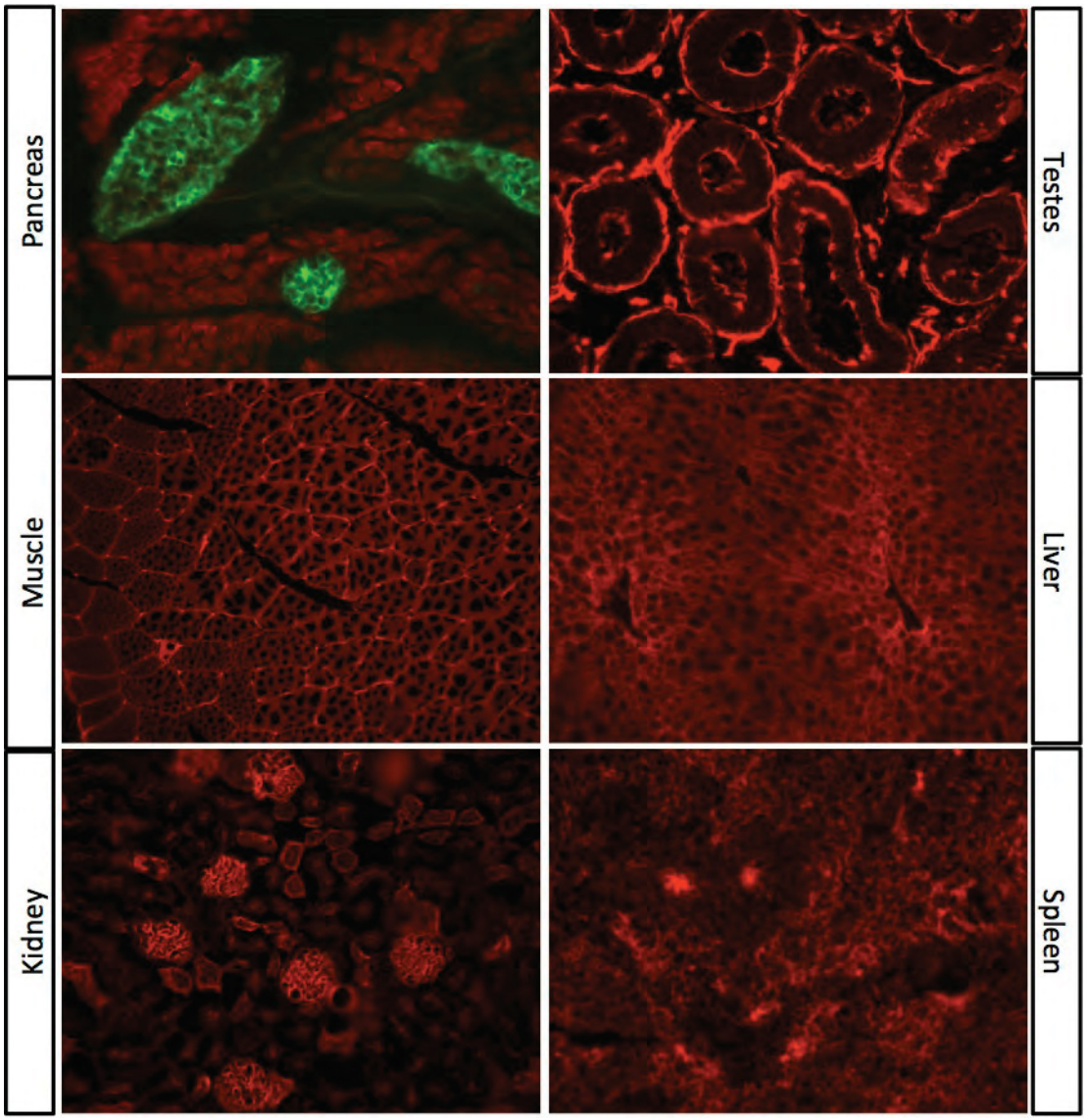

Figure 4. Assessment of tamoxifen-induced recombination in peripheral tissues of MIP-CreERT, mT/mG mice. MIP-CreERT, mT/mG male mice were gavaged with 100 g/kg everyday for ten days at six weeks of age and peripheral tissues were extracted for analysis at 13 weeks (representative of n=2). All pictures were taken at the same magnification (20X) except for the spleen which was taken at 40X.

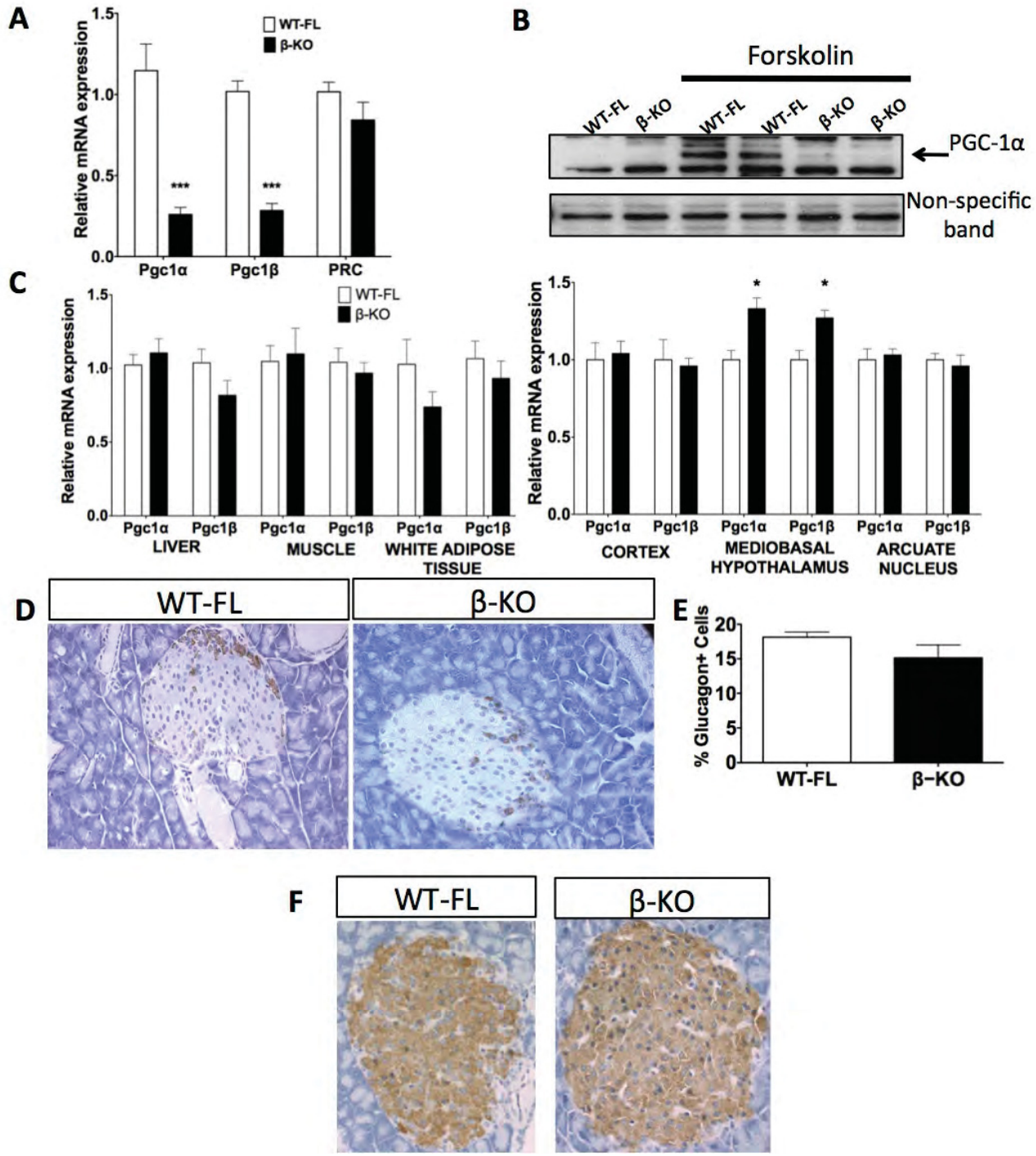

Figure 5. β-KO mice had no difference in Pgc-1α/β mRNA levels in peripheral tissues or brain and no difference in islet architecture. A: qPCR of isolated islets (n=10). B: Immunoblot of Pgc1α in isolated islets following four hrs of culture in 10 μM forskolin. C: qPCR analysis in peripheral tissues and brain (n=10). D-F: Representative glucagon staining images and corresponding proportion of glucagon+ cells per islet for all islets in one pancreatic section per pancreas (n=6). F: Representative insulin staining images. All data expressed as means ± SEM. * P-value<0.05.

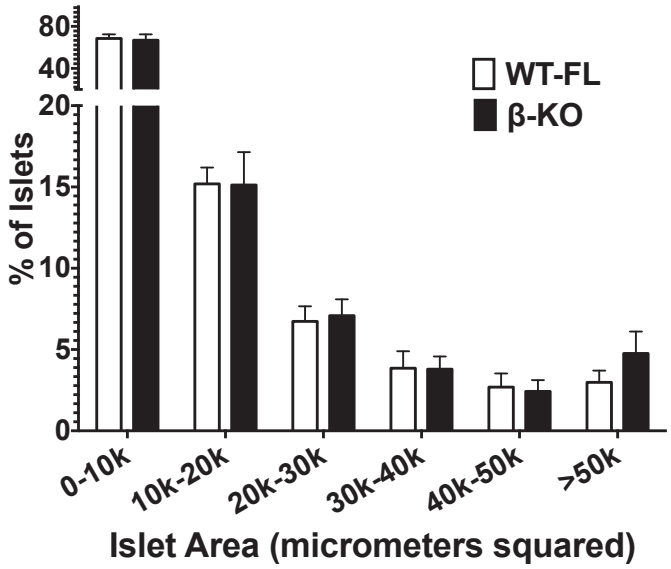

Figure 6: β-KO mice under chow diet show no difference in islet area. Islet area quantification using 12 H&E stained sections per pancreas from 5-month-old β-KO mice (n=5-6) sacrificed after hyperglycemic clamp. All data expressed as means ± SEM.

under basal conditions it is not possible to detect Pgc-1α protein levels with western blot analysis (Figure 5B), hence we treated β-KO islets with forskolin to confirm a significant decrease of Pgc-1α at the protein level (Figure 5B). No significant decrease in Pg1α/β gene expression was observed in the brain, liver, muscle or white adipose tissue from β-KO mice compared to WT-FL controls (Figure 5C). However, there was a significant increase in expression for both Pgc-1α and Pgc-1β in the mediobasal hypothalamus. In summary, along with results from the previous section, β-KO mice had a significant decrease of Pgc-1α/β expression in the islet without detectable excision of floxed genes or decrease in Pgc-1α/β expression in the brain regions or peripheral tissues analyzed.

We next asked if the significant loss of Pgc-1 expression in the islet caused any changes in islet composition or architecture. We performed histological analysis on pancreata of 4-month-old β-KO and found no difference in insulin staining or islet area, yet a trend towards

decreased proportion of glucagon-positive cells that did not reach statistical significance (Figures 5D-F and Figure 6).

β-KO mice had decreased insulin secretion *in vivo* and *in vitro* but no changes in glucose homeostasis and insulin sensitivity.

To confirm our hypothesis, we determined whether β–KO mice have impaired glucose homeostasis or insulin secretion. At 13 weeks of age, β-KO mice showed no difference in glucose or insulin tolerance (Figures 7A-B). Also, no difference in fasted and refed glucose or insulin levels were observed (Figure 7C). However, β-KO mice had significantly dysregulated *in vivo* insulin secretion kinetics and reduced insulin secretion five minutes following an oral glucose challenge (Figure 7D-E). Despite the insulin responses having no significant difference in area under the curve, 2-way analysis of variance showed a significant difference in the shape of the insulin curve in β–KO mice (ANOVA p-value for interaction = 0.031); the insulin secretion curve was consistently blunted presenting a prolonged plateau instead of a first phase secretion peak. This result was highly reproducible throughout four different experiments with sample sizes of 10-12 mice per genotype. To ensure the phenotype was not due to expression of the MIP-CreERT transgene alone, corresponding control experiments with MIP-CreERT positive mice and littermate, wild-type controls (Cre-recombinase-alone control groups) were performed in parallel. MIP-CreERT mice showed no difference in glucose tolerance or insulin secretion *in vivo* following an oral glucose tolerance test (Figures 8 A-B).

To better characterize the *in vivo* insulin secretion defect observed in β-KO mice using a more sensitive and informative measurement, we performed a hyperglycemic clamp on 20-week-old β-KO mice. Unlike with the OGTT where glucose levels in the blood are more variable depending on how glucose is absorbed (i.e. in the intestine), the hyperglycemic clamp ensures all

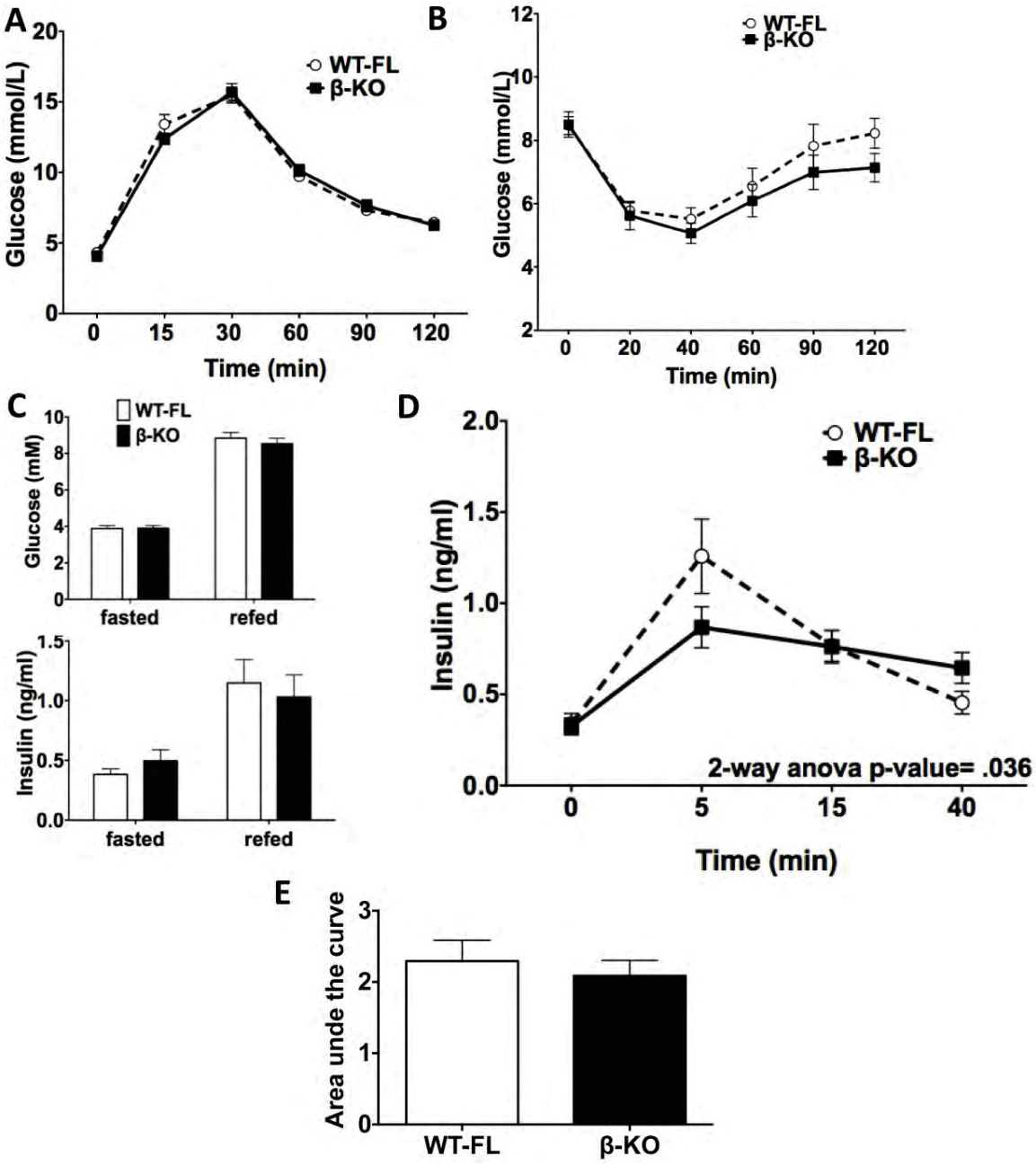

Figure 7. β-KO mice had no difference in glucose or insulin tolerance but GSIS *in vivo*. A: Glucose levels following an oral glucose tolerance test (1.5 g/kg) in 16 hr-fasted mice. B: Insulin tolerance test (1 IU/kg) following a 4 hr fast. C: Fasted and refed glucose and insulin levels of mice following 16 hr fast and 2 hrs refeeding. D-E: Insulin levels following an oral glucose tolerance test (1.5 g/kg) in 16 hr-fasted mice and corresponding area under the curve quantification. All experiments were done with 13-17 week old mice (n=12-13). Data are expressed ± as means SEM.

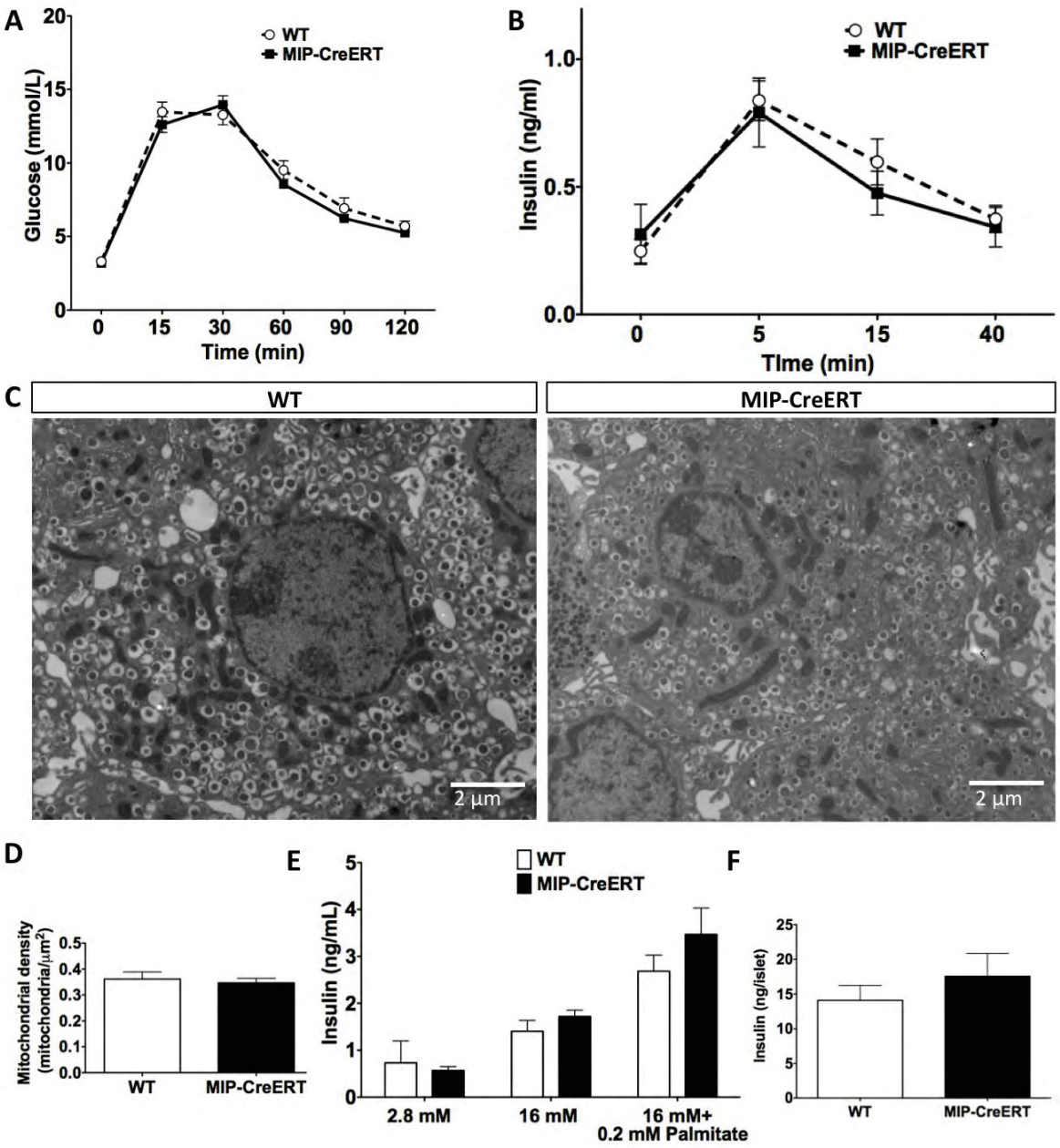

Figure 8. MIP-CreERT mice show no significant difference in *in vivo* insulin secretion, glucose tolerance, *in vitro* insulin secretion, mitochondrial morphology, mitochondrial mass or mitochondrial density. A-B: Glucose and insulin levels following an oral glucose tolerance test (1.5 g/kg) in 16 hr-fasted mice. C-D: Ultrastructural analysis of mitochondrial morphology in β–cells of isolated whole islets and corresponding quantification of mitochondrial density. E-F: In vitro glucose/palmitate stimulated insulin secretion of isolated islets and corresponding insulin content (n=4). All experiments were done using mice and isolated islets at 13-17 weeks of age. All data expressed as means ± SEM.

mice maintain a range of 15-20 mM blood glucose concentration, preventing lowering of blood glucose by physiological means. This technique can also provide an indirect estimation of insulin sensitivity, as mice that are more insulin sensitive require a higher glucose infusion rate to be clamped. Additionally because the mice are kept at high glucose for a prolonged period of time, their β–cells are under acute metabolic stress providing a significant physiological challenge that could uncover additional effects on insulin secretion. We observed no difference in basal insulin levels between WT-FL and β-KO mice, but there was a significant decrease in insulin secretion for β-KOs throughout the 60-minute clamp (Figure 9A). The general insulin secretion machinery was not affected, as insulin secretion in response to arginine was the same in β-KO mice compared to controls (Figure 9B). C-peptide levels were also significantly decreased, but the insulin clearance rate was not affected (Figures 9C-D). Differences in insulin levels were likely driven by defects in insulin secretion, as the glucose infusion rate and MI index (glucose infusion rate/serum insulin concentration) showed no statistical difference (Figures 9E-F); pointing to no significant difference in insulin sensitivity, in accordance with results from the insulin tolerance test. Taken together, these results suggest that β-KO mice had no difference in glucose or insulin tolerance, despite decreased *in vivo* insulin secretion.

To determine whether the decreased insulin values observed *in vivo* were the result of a β-cell autonomous defect in GSIS, we performed static 1-hour incubations in stimulatory conditions of 16 mM glucose and 16 mM glucose plus 0.2 mM palmitate. β-KO islets generally showed no statistical difference in basal secretion at 2.8 mM glucose, but showed a significant blunting of insulin secretion when stimulated with high glucose or glucose plus palmitate (Figure 10A), while insulin content was not significantly different (Figure 10B). Corresponding MIP-CreERT control experiments confirmed that the differences in insulin secretion observed in the

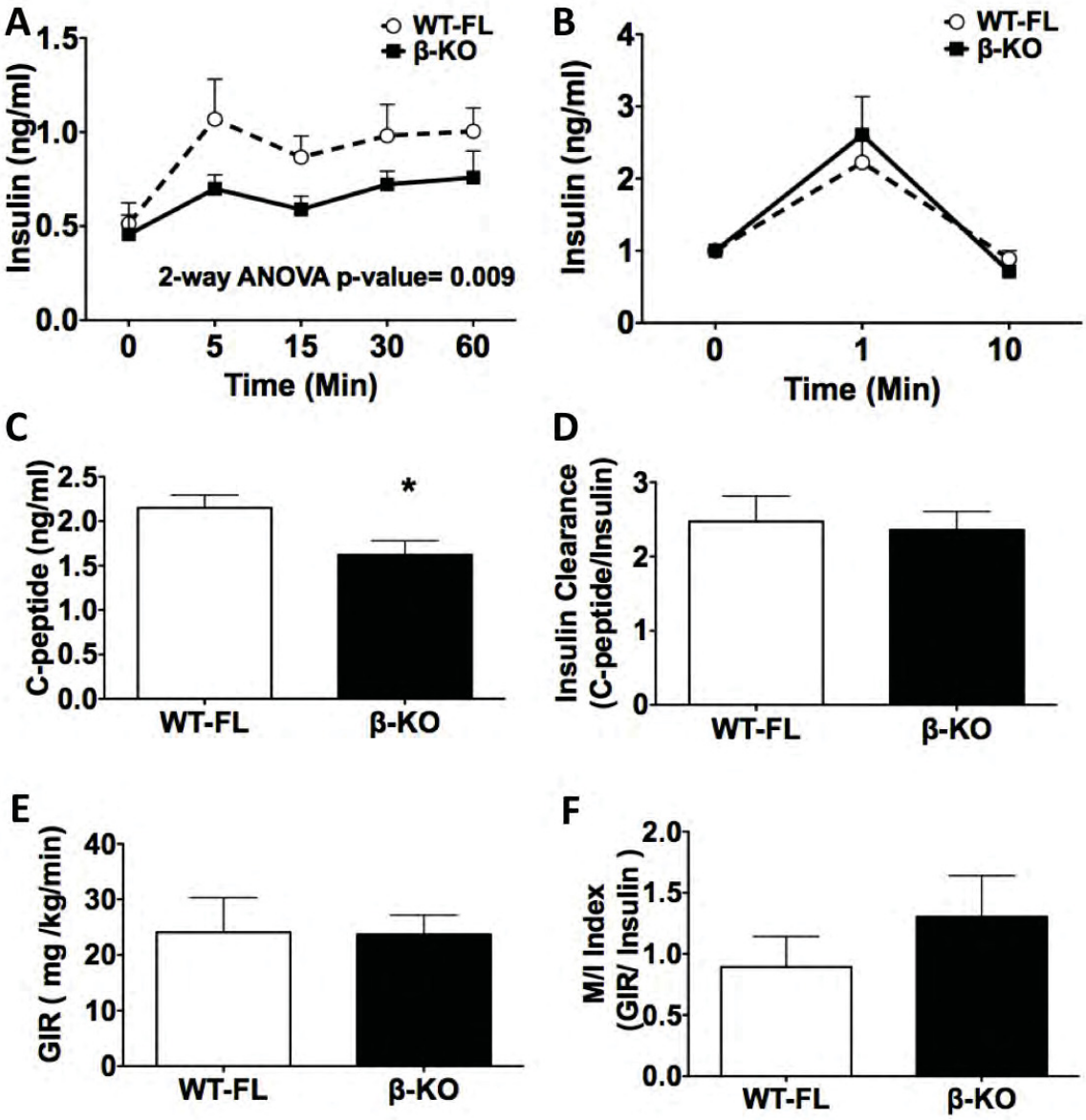

Figure 9. Hyperglycemic clamp of β-KO mice shows a decrease in insulin secretion, but unchanged MI index and insulin clearance. A: Insulin levels throughout 60 minutes of hyperglycemic clamp. B: Insulin levels after arginine bolus following 60 minutes of hyperglycemic clamp. C: C-peptide levels at 60 min time point of. D: Insulin clearance at 60 min time point, calculated as the ratio of C-peptide levels over insulin levels. E-F: Glucose infusion rate (GIR) and MI index (GIR/Insulin) throughout the hyperglycemic clamp. Experiments were done using 20 week old mice (n=8-9). * P-value<0.05. Data are expressed as mean ± SEM.

β-KO mice were not due to presence of the MIP-CreERT transgene (Figures 8D-E).

These data suggested that Pgc-1s are important for the regulation of nutrient-stimulated insulin secretion in β-cells; thus, we asked if Pgc-1 gene expression is acutely regulated by conditions that promote insulin secretion. As previously shown (Hussain et al, 2006), forskolin strongly increased expression of Pgc-1α in mouse islets. In addition, forskolin decreased expression of Pgc-1β, while PRC showed no difference in mRNA expression (Figure 10C). In human islets, forskolin also increased expression of Pgc-1α, caused no change in Pgc-1β mRNA and modestly increased PRC expression (Figure 10C). When both mouse and human islets were stimulated for one hour with glucose and /or palmitate, Pgc-1α showed a mild, but significant increase in expression with all conditions (Figure 10D). Taken together with previous data demonstrating that reduced Pgc-1 expression blunts insulin secretion in response to glucose and fatty acids, this data suggests that acute up-regulation of Pgc-1α level in islets may be a mechanism by which β-cells regulates insulin secretory capacity in response to nutritional cues.

β-KO islets showed little change in mitochondrial biology, but dysregulated expression of genes involved in lipid metabolism.

To begin investigating the mechanism of defective insulin secretion, we isolated islets from β-KO mice and analyzed their gene expression profiles and mitochondrial function. Although we assayed expression of multiple known Pgc-1-responsive genes involved in ETC subunit expression, only Atp5b and ERRα were significantly down-regulated (Figure 11A). Genes involved in mitochondrial fission were significantly dysregulated, including decreased Drp1 and increased Fis1 expression. Glut2 was also significantly decreased, while NeuroD, Pdx1 and Sur1 mRNAs were unaffected by loss of Pgc-1 expression. Next, we analyzed mitochondrial

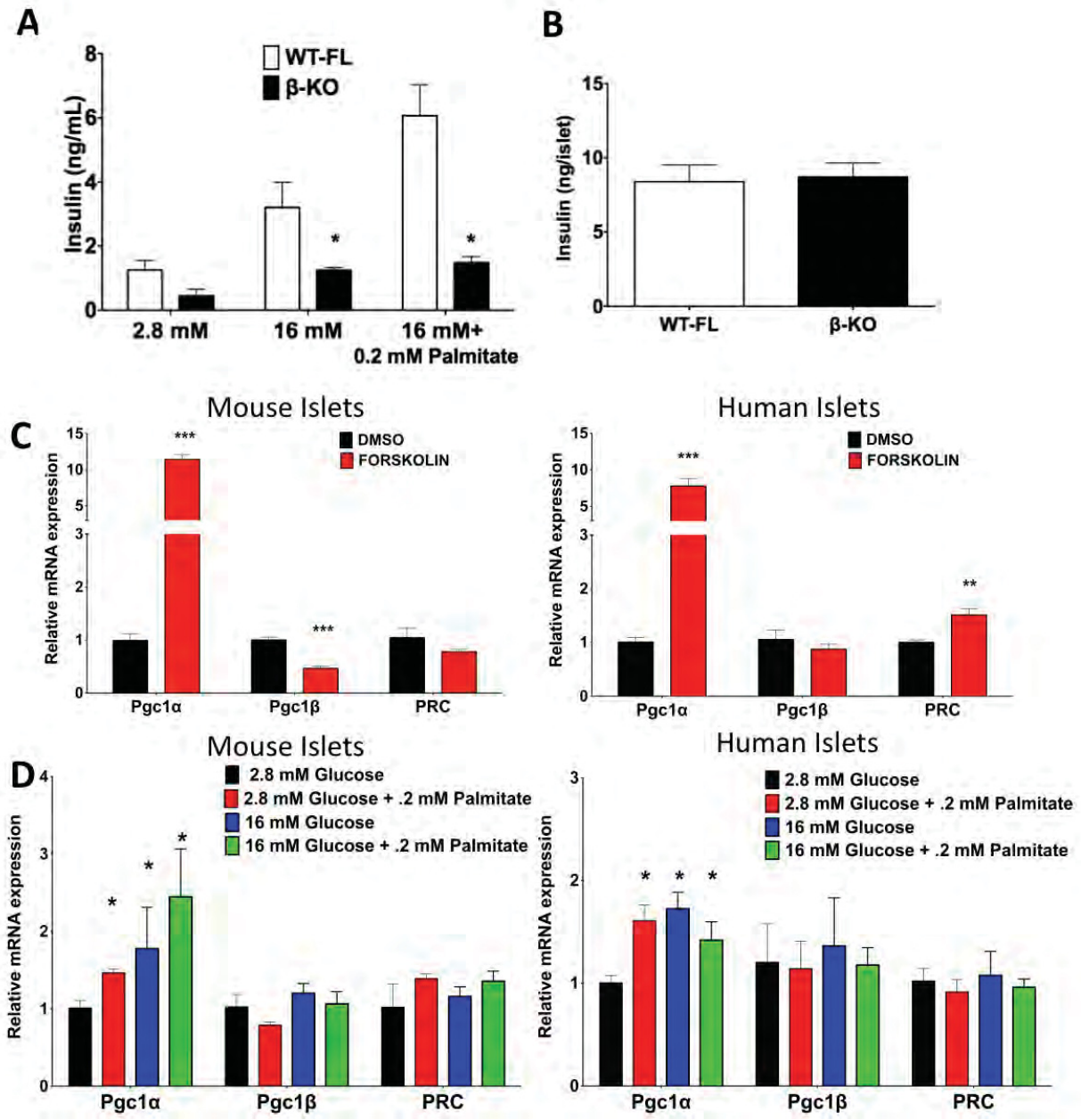

Figure 10. β-KO isolated islets have decreased *in vitro* insulin secretion, but the same insulin content. A-B: *In vitro* glucose/palmitate stimulated insulin secretion of isolated β-KO islets and corresponding insulin content (n=4). C: qPCR of isolated C57/BL6N mouse islets or human islets stimulated with 10 μM forskolin for two hours (n=4). D: qPCR of isolated C57/BL6N mouse islets or human islets stimulated for one hour with glucose and/or palmitate at the indicated concentrations (n=4). All experiments were done using isolated islets from 13-17 week old mice. Data are expressed as mean ± SEM.

mass and morphology by electron microscopy and mitotracker staining. There was a significant decrease in mitochondrial density in islets from β-KO mice (Figure 11B-C), but no difference in gross morphology (Figure 11D). Despite the change in density, there was no significant difference in ATP levels in dispersed β-KO islet cells following stimulation with 20 mM glucose (Figure 11E). Likewise, there was no difference in coupled or uncoupled mitochondrial respiration when whole islets were stimulated with 11 mM glucose and cultured in the presence of oligomycin, FCCP and rotenone (Fig 11F-G).

Because we observed blunted palmitate-potentiated GSIS in β-KO islets, we also analyzed expression of genes controlling fatty-acid oxidation, lipolysis and lipogenesis. Extensive research has been performed showing that lipid metabolism plays a crucial role in β–cell function and GSIS regulation (reviewed in Nolan et al, 2006). There was a significant decrease in the transcription factor PPARα and genes involved in fatty acid transport and catabolism, such as Fabp3 and Hormone sensitive lipase (Hsl, Figure 12). In contrast, there was a significant increase in two genes involved in lipogenesis, Diglyceride acyltransferase 1 (Dgat1) and Acyl-coA synthetase long-chain family member 4 (Acsl4, Figure 12). Taken together, these data suggest that, unexpectedly, overall Pgc-1 genes are not needed for mitochondrial function in β-cells, yet are important for regulation of efficient lipid metabolism, possibly contributing to the blunted nutrient stimulated insulin secretion observed in β-KO islets.

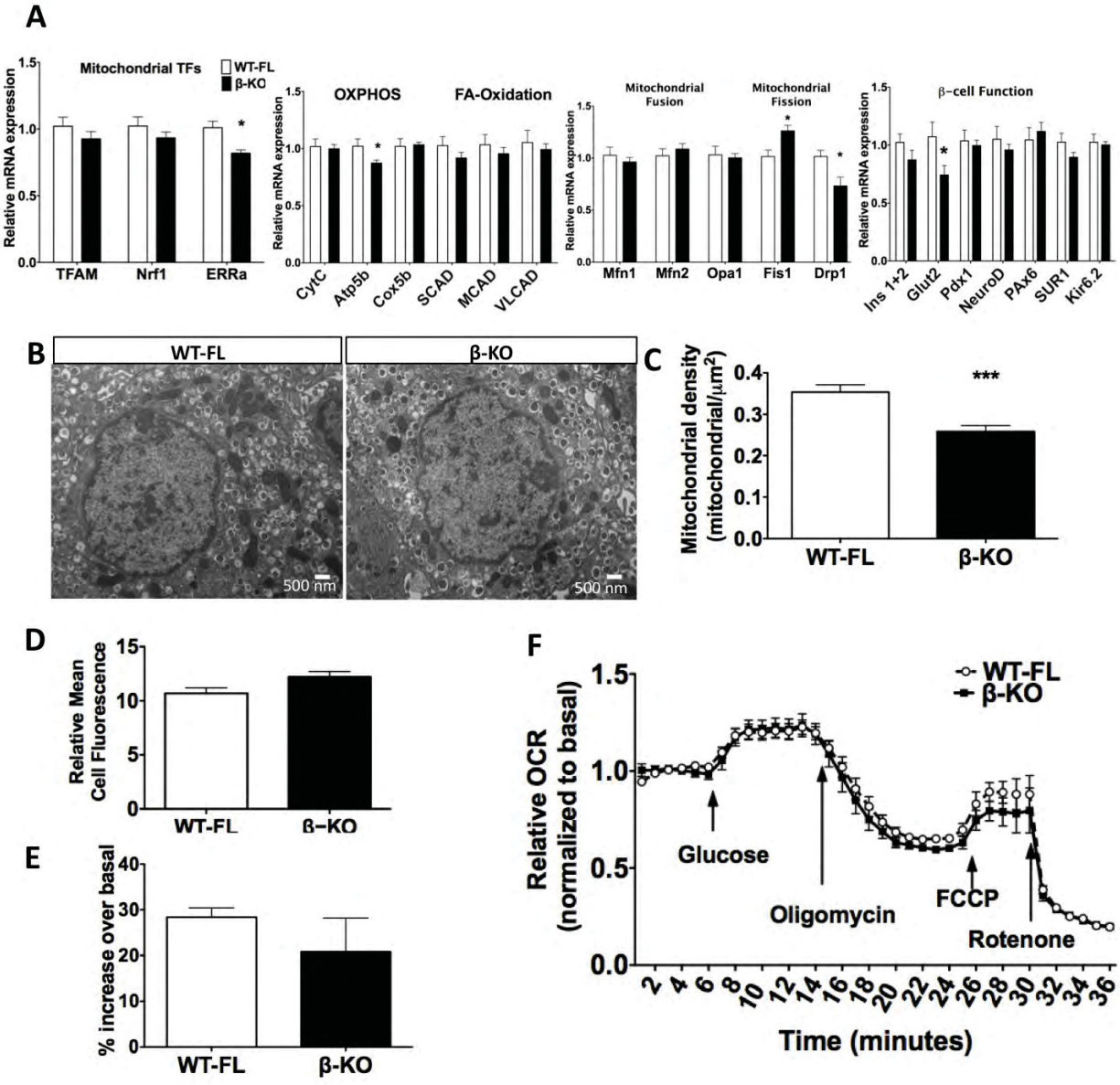

Figure 11. β-KO isolated islets have mild effect on mitochondrial gene expression, morphology but no effect on mitochondrial respiration or ATP levels following glucose stimulation. A. Gene expression analysis of whole islets (n=10). B-C. Ultrastructural analysis of mitochondrial morphology in β–cells of whole islets and corresponding quantification of mitochondrial density (n=50 cells). D. Mitochondrial mass quantification using mitotracker green or orange and FACS analysis of dispersed islet cells (n=4). E: ATP fold increase over basal in dispersed islet cells following stimulation with 20 mM glucose (n=5). F-G. Oxygen consumption rate (pmoles/min) of whole islets after sequential addition of glucose, oligomycin, FCCP and rotenone (n=8). All experiments were done using isolated islets from 13-17 week old mice. * P-value<0.05. Data are expressed mean ± SEM.

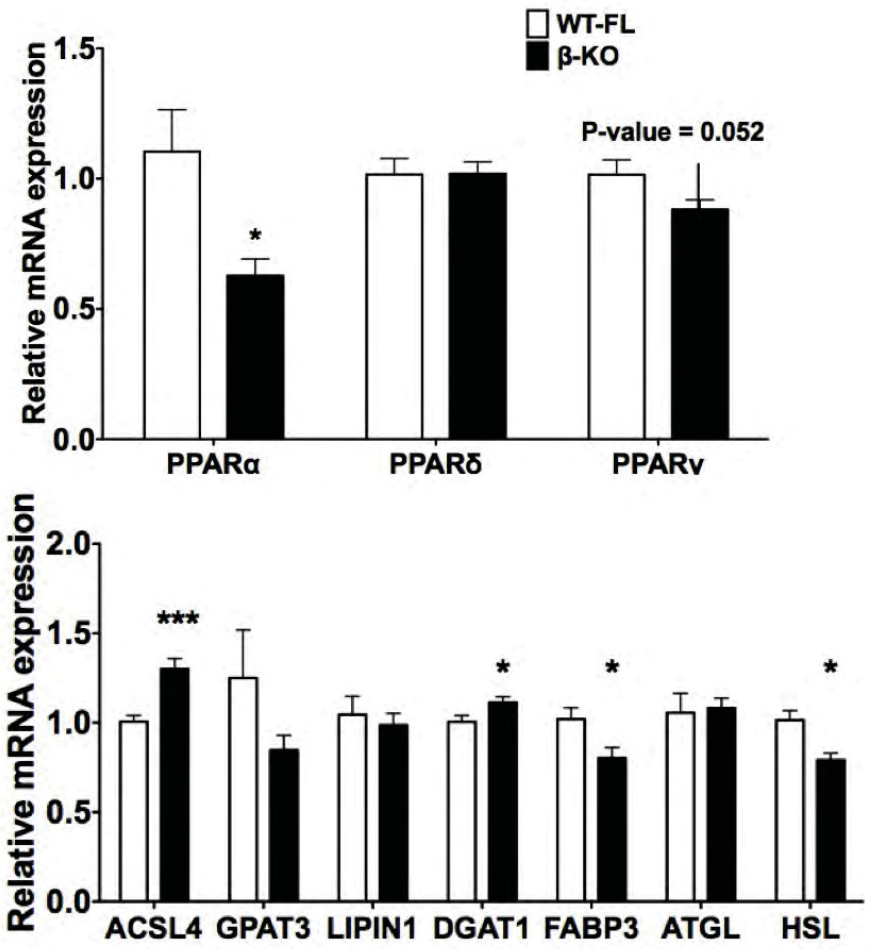

Figure 12 . β-KO isolated islets have dysregulated expression of genes involved in lipid metabolism. qPCR of isolated islet from β-KO 13-17 week old mices (n=10). Data are expressed as mean ± SEM.

DISCUSSION

Although decreased Pgc-1 mRNA expression in β-cells is associated with the development of TIID, little is known about the physiological role of these co-activators in β-cells. Our study is the first to address the *in vivo* consequences of Pgc-1α and Pgc-1β inactivation in β–cells using transgenic mice. We generated Pgc-1α and β double knockouts based on compensatory increases in related family member mRNA expression observed in our INS1 cells (Figure 1) and in work done by others and us in other cells/tissues. Increased mRNA expression of Pgc-1α when Pgc-1β expression is reduced was observed in heart cells of Pgc-1β whole body knockout mice, and vice versa, increased Pgc-1β mRNA levels are observed in the Pgc-1α muscle-specific knockout (Lai et al, 2008; Sczelecki et al, 2014). It has been suggested that the majority set of genes controlled by Pgc-1α and Pgc-1β overlap, but there is also a group of genes that respond to only one or the other. Our results in INS1 cells suggest that in β–cells, NeuroD and catalase were only sensitive to Pgc-1β knockdown, while the majority of genes analyzed like Ucp2 and CytC were sensitive only to Pgc-1a knockdown, and the double knock-down did not have an additive effect for most genes. This suggests that Pgc-1α and Pgc-1β may have very distinct and separate roles in β-cell function and/or Pgc-1α plays a more predominant role is regulating these genes. To avoid any compensation between Pgc-1α and β and to fully assess the consequence of Pgc-1 inactivation in β–cells, we chose to first analyze β-cell-specific Pgc-1α/β double knockouts produced using the recently generated MIP-CreERT transgenic mouse line.

Characterization of the MIP-CreERT mouse using the mT/mG reporter mouse showed no Cre-mediated recombination outside of the pancreatic islet in the peripheral tissues analyzed.

Our results in the brain agree with previous analysis of the MIP-CreERT mouse performed using whole mount X-gal staining and a LacZ reporter (Wicksteed et al, 2013). However, in that study, individual sections of the brain were not analyzed at high resolution and the resolution of whole mount X-gal staining is poor when looking at small populations of cells. Thus, this method would not detect recombination in a few key neurons that might impact glucose homeostasis. By using the mT/mG reporter, we are able to have single cell resolution with a clear yes/no answer, since cells were either RFP+ or GFP+. We showed no single neurons with Cre-mediated recombination in areas of the brain previously associated with the regulation of peripheral glucose homeostasis (Figure 2). We also saw no decrease in Pgc1α or -1β expression in the brain or multiple other peripheral tissues in our floxed mice carrying the MIP-CreERT transgene. Therefore, the phenotype we observed in β-KO mice is likely not confounded by knockout of Pgc-1α or -1β in other tissues. Unexpectedly, there was a significant increase in expression of both Pgc-1α and Pgc-1β in the mediobasal hypothalamus of β–KO mice. Since we ruled out the possibility of ectopic CreERT expression in this region of the CNS, increased Pgc-1 expression in the mediobasal hypothalamus may be an indirect effect of reduced pancreatic Pgc-1 expression. However, we have yet to measure CNS expression of the Pgc-1s in control MIP-CreERT mice, so it remains possible that this increase in Pgc-1 could be the result of a non-specific effect of the MIP-CreERT transgene.

Our hypothesis that inhibition of Pgc-1α and β function in β–cells would cause impaired glucose homeostasis was incorrect. Despite blunted GSIS, β–KO mice showed no difference in glucose tolerance or insulin sensitivity. We also expected that inhibition of Pgc-1α and β function in β–cells would cause mitochondrial dysfunction. Surprisingly, this did not occur to any significant degree other than a decrease in mitochondrial density. To our knowledge, this is

the only instance in which *in vivo* knockout of both Pgc-1 genes did not significantly affect expression of OXPHOS genes or decrease mitochondrial respiration. While knockout of only Pgc-1α has, in some instances, been shown to have little to no effect on OXPHOS gene expression (Kleiner et al, 2012), this was believed to be due to compensatory activity of the other family members, as the double knock-out of both Pgc-1α and Pgc-1β has dramatically reduced mitochondrial gene expression and function in adipose, muscle, and liver cells (Uldry et al, 2006; Rowe et al, 2013; Zechner et al, 2010; our unpublished results in liver). On the other hand, our observed dysregulation of genes involved in mitochondrial dynamics (Figure 10A) was shown previously in the skeletal muscle Pgc-1α/β double knockouts (Zechner, 2010; Rowe et al, 2013). In those studies, Drp1 and Mtfn1/2 showed a decrease mRNA abundance, while in β-KO islets it was Drp1 expression that was decreased, and Mfn1/2 expression was unaltered.

Mitochondrial mass, morphology and respiration was unchanged in β-KO isolated islets. Interestingly, despite an abundance of data in overexpression models suggesting the Pgc-1s as master regulators of mitochondrial biogenesis and function, when assessed, cells from almost all previous Pgc-1α or Pgc-1β knockout studies also show no change in mitochondrial mass, size or morphology. Only cardiac muscle cells of whole body Pgc-1α/β double knockouts had abnormal mitochondrial morphology (Lai et al, 2008). However, mitochondrial respiration was consistently reduced and this was associated with a down-regulation of electron transport chain assembly in the skeletal muscle-specific Pgc-1α/β double knockouts, despite no change in mitochondrial content, morphology, area or internal structure (Rowe et al, 2013). Conversely, decreased mitochondrial density in β–KO β-cells was the only significant change in mitochondrial physiology in the β–KO islets, which was also demonstrated in previous Pgc-1α/β double knockout models (Uldry et al, 2006; Lai et al, 2008; Zechner et al, 2010).

Assessment of mitochondrial density is performed by analyzing mitochondrial number or area in electron micrographs that represent sections across certain regions of the cell. Given Pgc-1 regulation of genes involved in mitochondrial dynamics, it is possible that the Pgc-1s regulate the distribution and arrangement of the mitochondrial web. Thus, while total mitochondrial mass remains unchanged, altered arrangement of mitochondria within the cell may cause density changes in certain regions. Few studies have been performed has been performed that investigate the regulation of mitochondrial dynamics and web formation by Pgc-1 proteins (Liesa et al, 2008; Martin et al, 2014), even though it is known that Pgc-1α can directly increase expression of Mfn, in general it is not known how they can regulate mitochondrial fusion and fission. In β–cells, changes in the dynamics of the mitochondrial web are closely related to metabolic fluxes, exposure to glucose or fatty acids and can affect insulin secretion (Molina et al, 2009; Supale et al, 2013). Nonetheless, it still not clear in what way rearrangement of the mitochondrial web may affect insulin secretion. It would be interesting to determine whether loss of Pgc-1 expression controls β-cell mitochondrial dynamics by staining β-KO β-cells with mitochondrial probes that measure both total and functional mitochondria and analyze the dynamics of the mitochondrial web under basal and nutrient stimulated conditions.

The lack of stronger differences in mitochondrial gene expression or mitochondrial biology in our β-KO mice could be due to multiple reasons. First, results from our INS1 knockdown experiments suggest that acute knockdown of Pgc-1α/β is capable of decreasing β–cell mitochondrial gene expression and respiration, yet compensatory mechanisms might be occurring in the weeks following Pgc-1 knockdown in the mice eliminating these effects. If indeed the increase in expression of Pgc-1α/β in the mediobasal hypothalamus is caused by the β-KO, these might also be part of a compensatory phenotype to ameliorate the effect of Pgc1-α/β

KO in the β-cells. For example, it is known that neurons can control β–cell function through acetylcholine signaling (Bereiter et al, 1981; Gautam et al, 2006). Second, Pgc-1s are stress response genes and we performed much of our characterization under basal conditions. In muscle and adipose tissue Pgc-1α knockout mice (Handschin et al, 2007; Kleiner et al, 2012), significant metabolic differences are only observed when mice are challenged with exercise or with a high fat diet. It is possible that subjecting β–KO mice to different types of metabolic stress would cause more significant differences in glucose homeostasis, gene expression and mitochondrial respiration. For example culturing the β–KO islets in glucolipotoxic conditions for 72 hours or putting β–KO mouse on a high-fat or high-glucose diet, may reveal additional physiological consequences of reduced Pgc-1 expression.

Another possibility is that Pgc-1α/β function in adult β-cells is not crucial and PRC is the predominant family member responsible for maintaining mitochondrial function in mature β-cells. We found significant expression of PRC mRNA in both mouse and human β-cells and further studies to investigate the role of PRC in β-cell function could answer this question. Lastly, we are the first to create an inducible Pgc-1 knockout, and all other *in vivo* Pgc-1 knockout models were generated using Cre-recombinase driven by promoters activated early in fetal development. In light of the findings from the inducible Pgc-1α over-expressing mouse line, it is possible that inhibition of Pgc-1 function during fetal development or for more prolonged periods of time is necessary to cause a more pronounced phenotype. Indeed, aging Pgc-1α muscle knockout mice for two years on a chow diet uncovered glucose intolerance and insulin resistance that was not present in young mice (Sczelecki et al, 2014). Also, it has been shown that the MIP-CreERT mice have CreERT-recombinase expression in 89 $\pm$ 8 % of β-cells (Wicksteed et al, 2010), thus residual Pgc-1 activity in β–cells that do not express CreERT may

be sufficient to mask the effects of Pgc-1 knockout. Finally, our test mice were on a mixed background, which could have masked some of the consequences of losing Pgc-1 function in β-cells. Strong effects of genetic background on metabolic phenotypes from knockout mice have been observed before, for example with the Ucp1 knockout mouse (Hoffman et al, 2013). Backcrossing the test mice onto a pure C57B/J background could uncover more significant differences or other phenotypes.

Previous overexpression studies in β–cells suggested that Pgc-1α expression might be detrimental for β-cells (Yoon et al, 2003; Valtat 2013). Overexpression of Pgc-1α in adult rat islets using adenoviruses or transgenically in mouse β-cells during fetal development decreased GSIS. Pgc-1α mRNA and protein levels are usually low under basal conditions, but are strongly increased with stress or nutritional cues (Puigserver et al, 1998; Lin et al, 2002b; Meirhaeghe et al, 2003; Estall et al, 2009; Kleiner et al, 2012). Pgc-1α protein is short lived as it contains protein motifs that target it for quick degradation by the proteasome (Sano et al, 2007). Indeed, in isolated islets we could not detect Pgc-1α protein by Western blot without stimulation with forskolin (Figure 5B). Constitutive overexpression of Pgc-1α in β-cells under conditions where it is highly regulated and usually quickly degraded might confer a cytotoxic effect that affects physiology independent of their function in β–cell biology. We show that Pgc-1 expression is increased in β-cells after just one-hour stimulation with 16 mM glucose or 0.2 mM palmitate and is required for efficient GSIS, suggesting that Pgc-1α is involved in regulating the acute response to nutrients and insulin secretion. Previous reports show that Pgc-1α expression is also increased 2-fold when mouse islets are exposed to chronic glucolipotoxic conditions (Zhang et al, 2005) and knock-down of Pgc-1α was protective in this context (Kim et al, 2009) implicating Pgc-1α activity in mediating the negative effects of this β-cell stress. However, chronic exposure to high

glucose and free fatty acids may result in prolonged and inappropriate activation or expression of Pgc-1α, which could explain the negative effects seen under this stressful environment. It is possible the Pgc-1 proteins, in particular Pgc-1α, are involved initially in the mechanism of fuel stimulated insulin secretion, but sustained increase in their levels caused by hyperglycemia or hyperlipidemia eventually affects β–cell function.

Our hypothesis was correct in that β-KO mice have impaired insulin secretion both *in vivo* and *in vitro*. This is in accordance with data in human islets where Pgc1-α was knocked down using siRNA (Ling et al, 2008). Taken together with our results showing increased Pgc-1α gene expression following glucose or palmitate stimulation in human islets (Figure 11 D-E), this suggests that the β-KO mouse is a good model to analyze the mechanism of impaired insulin secretion observed in humans islets with decreased Pgc-1α. In isolated β–KO islets, the decrease in palmitate-potentiated insulin secretion and expression of PPARα, Fabp3, Hsl suggests defects in lipid and fatty-acid metabolism. The adipose tissue-specific Pgc1-α knockout also showed differences in gene expression involved in lipid metabolism when mice were put on a high-fat diet, specifically a decrease in Fabp3 (Kleiner et al, 2012). The general consensus in the field is that lipid and fatty acid metabolism are necessary for fuel stimulated insulin secretion in rodents (Prentki et al, 2013). Inactivation of Desnutrin in β-cells, the enzyme catalyzing the first step in triglyceride catabolism, as well as depletion of β-cell triglyceride by overexpressing leptin in the liver caused a substantial decrease in insulin secretion (Koyama et al, 1997; Tang et al, 2013). In β-KO islets, downregulation of Hsl could cause the accumulation of diacyl-glycerol (DAG) to affect insulin secretion (Haemmerle et al, 2002; Badin et al, 2011). Increased levels of DAG could then cause the observed increases in Acsl4 and Dgat1 in an effort to compensate for the lack of free fatty acids being generated by triglyceride catabolism. To test for this, we could

quantify triglycerides, diacyl and monoacylglycerols and free fatty acids both under basal conditions and after stimulation with glucose and palmitate.

In conclusion, inhibition of Pgc1-α and β in β–cells decreased glucose stimulated insulin secretion both *in vitro* and *in vivo*, but did not impact glucose tolerance in mice. Surprisingly, apart from the decreased mitochondrial density at the level of the nucleus, the mechanism for impaired insulin secretion does not appear to involve significant mitochondrial dysfunction, but appears to involve dysregulated lipid metabolism that might occur outside of the mitochondria. Our results suggest that Pgc1-α and β function is dispensable for mitochondrial function in adult β-cells under basal conditions, however any genetic variation that affects Pgc-1 function in β-cells could cause a decrease in GSIS without affecting blood glucose levels. It is possible that in humans, variation in Pgc-1 genes decrease GSIS without affecting blood glucose levels and this could contribute to the pre-diabetic state . If under different kinds of metabolic stress, such as a high-glucose diet, β–KO mice present problems with glucose homeostasis, this could suggest that decreased or augmented Pgc-1 function in β–cells might be one of the early determinants that contribute to progression into TIID.

CHAPTER III PREAMBLE

In the past decade it has become apparent that transgenes inserted in the mouse genome to express Cre-recombinase protein under the control of promoters can, on their own, have a strong impact on various aspects of mouse biology and physiology. In many cases, the mechanisms through which these effects arise are largely uncharacterized, yet given the widespread occurrence in multiple Cre-recombinase lines affecting different organs, interest has grown into understanding the causes for these unexpected effects and increasing care is taken when using newly generated Cre-recombinase lines.

The study presented in the following chapter originated from a remarkable phenotype observed in MIP-CreERT-only control groups when we challenged β-KO mice with acute metabolic stress and β–cell death by feeding them a high-fat diet and injecting STZ, which selectively kills β–cells. We have begun to explore the conditions and mechanisms by which this phenotype arises.

The results from this chapter will advance our understanding into how Cre-recombinase technology can impact the expected outcome of diabetes models, so that in the future this sort of artifacts may be avoided when new Cre-recombinase lines are produced. Additionally, because the observed phenotype arises in a mouse line that expresses Cre-recombinase in β-cells, it is possible that investigating the causes of such Cre-recombinase transgene-mediated effects would identify novel regulatory pathways of β–cell function and could lead into new therapeutic applications targeting β–cells in diabetes.

CHAPTER III

The MIP-CreERT mouse is protected against High-fat diet / Streptozotocin-induced hyperglycemia

INTRODUCTION

Problems associated with mouse transgenic lines expressing Cre-recombinase in β-cells

One of the main disadvantages of the Cre-lox technology is the negative side-effects that could arise from having the viral Cre-recombinase protein expressed in animal cells, specially when a transgene expressing Cre-recombinase has leaky expression in other tissues. There are several reports showing that Cre-recombinase transgenes can affect cell function or cause unexpected physiological phenotypes in the mouse. Expression of Cre-recombinase protein decreases activity of cAMP-dependent protein kinase (PKA) in mouse embryonic fibroblasts and causes oxidative stress in sertoli cells (Gangoda et al, 2012; Xiao et al, 2012). Mice with a transgene expressing Cre-recombinase under the nestin promoter to target the central nervous system have leaky expression of Cre-recombinase in other tissues (including the pancreas) and a metabolic phenotype characterized by a decrease in body length and weight (reviewed in Harno et al, 2013).

Numerous transgenic mouse lines expressing Cre-recombinase in β–cells have been created using different stretches of the rat insulin 2 (RIP-Cre) and mouse Pdx1 promoters (Pdx1-Cre; reviewed in Magnuson and Osipovich, 2013). There are many problems associated with these lines, including Cre-recombinase expression only in a fraction of β–cells or throughout many different regions of the brain, possibly because they were generated using the promoter of the insulin 2 gene (Magnuson and Osipovich, 2013; Wicksteed et al, 2010). In mice, insulin 2, but not insulin 1, is expressed in several regions of the brain including the hippocampus, cortex and cerebellum (Mehran et al, 2012). Alarmingly, one popular RIP-Cre line was used in 16

different studies targeting 15 different genes without corresponding experiments with RIP-Cre-only control groups before it was reported that the presence of the RIP-Cre transgene alone was sufficient to cause significant glucose intolerance and impaired insulin secretion on some genetic backgrounds (Lee et al, 2006). However, in general there is little information about the mechanisms that cause this phenotype in the RIP-Cre mouse. New β–cell-specific Cre lines using different promoters are being created in an effort to avoid these kinds of problems.

The tamoxifen-inducible Cre-lox system and the MIP-CreERT mouse

In order to temporally control tissue-specific gene inactivation, researchers created a fusion protein, the CreERT protein, using the sequence of Cre-recombinase fused to a mutated ligand binding domain of the human or mouse estrogen receptor (Feil et al, 1996; Danielian, 1998). This mutated estrogen receptor domain has strongly decreased binding to endogenous estrogens, but has high affinity for the synthetic steroid tamoxifen (Littlewood et al, 1995). This domain sequesters the Cre-recombinase protein in the cytoplasm and it is only after the binding of tamoxifen that the CreERT fusion protein translocates into the nucleus to allow Cre-recombinase-mediated recombination at loxP sites (Reinert et al, 2012). In β–cells, high tamoxifen doses maintain the CreERT protein in the nucleus for up to one month and recombination is observed several weeks after the last exposure to tamoxifen, albeit at a much lower efficiency (Reinert et al, 2012).

To overcome the ectopic Cre-recombinase expression in the brain observed with previous lines, a new mouse line was recently generated that drives the expression of CreERT under the mouse insulin 1 promoter (MIP) (Tamarina et al, 2014). The MIP-CreERT mouse contains a transgene consisting of an 8.3-kb fragment of the mouse insulin 1 promoter, cDNA of Cre-

recombinase fused to the ligand binding domain of the mouse estrogen receptor (CreERT) and a 2.1-kb fragment of the human growth hormone gene that does not contain any coding DNA, but provides a polyadenylation signal (Tamarina et al, 2014). Previous characterization of the MIP-CreERT mouse shows no differences in glucose tolerance, *in vivo* insulin secretion, islet architecture or calcium oscillations in response to glucose compared to wild-type, littermate controls (Kaihara et al, 2013; Tamarina et al, 2014). Additionally, no Cre-recombinase-mediated recombination in α–cells was reported (Kaihara et al, 2013). We demonstrate in the previous chapter that the MIP-CreERT mouse exhibits no Cre-recombinase-mediated recombination in liver, muscle, kidney, multiple regions of the brain, testis or spleen (Chapter 2, Figures 3 and 4). MIP-CreERT mice did not show differences in glucose tolerance, β-cell ultrastructure or *in vitro* or *in vivo* GSIS (Chapter 2, Figure 8). Given the lack of Cre-recombinase expression in the brain and ample expression of CreERT in β-cells, this line is being championed as the best mouse line available for inducible β–cell-specific recombination (Magnuson and Osipovich, 2013). However, it still remains important to evaluate whether the transgene alone augments the physiologically or cellular responses to different models of diabetes or hyperglycemia, such as high-fat diet and streptozotocin-induced β-cell death, as these are common protocols used in our field to investigate the mechanisms underlying the development of diabetes in mice.

Streptozotocin-induced hyperglycemia.

Streptozotocin (STZ) is a glucose analog initially isolated from the soil bacterium *streptomyces achromogenes* (Vavra et al, 1959). It is a glucosamine-nitrosourea compound that acts as a DNA alkylating agent and enters the cell through the Glut2 transporter (reviewed in Wilson and Leitner, 1990). This confers STZ with β–cell specificity within the islet, as no other

pancreatic endocrine cell expresses Glut2. Its chemical structure is composed of a nitrosourea moiety with a methyl group attached at one end and a glucose molecule at the other end (Wilson and Letiner, 1990). Once it enters the β-cell, it spontaneously decomposes into isocyanate and methyldiazohydroxide; the latter then being metabolized into alkylating agents that can destabilize DNA and protein structure by changing regional charge; which in turn activates cellular stress pathways such as the DNA damage response (Wilson and Leitner, 1990). It is believed that it is mainly through damage to DNA structure that STZ promotes β–cell death, but other mechanisms including increase in ROS or nitric oxide are also thought to contribute to β–cell toxicity (Wilson and Leitner, 1990; Lenzen, 2008).

The dose of STZ determines the β–cell death pathway that is activated. In mice, if administered in multiple low doses (40-50 mg/kg daily for five days), STZ will activate apoptosis (O'brien et al, 1999; Li et al, 2000; Tonne et al, 2013). In this case, apoptosis occurs in two phases, the first arising a few days after STZ injection as result of cellular toxicity and the second arising from immune cell infiltration into the islet and insulinitis (O'brien et al, 1999; Wilson and Leitner, 1990; Lenzen, 2008). Consequently, this treatment causes a significant decrease in islet area within the first three days after the last injection, followed by a slight recovery after 14 days and a decreasing plateau thereafter (Li et al, 2000). On the other hand, a single high STZ dose (usually between 100-200 mg/kg) causes a strong cytotoxic effect that activates necrotic β–cell death within 48 hours, without any recovery thereafter and with a dramatic decrease in islet area (Zhang et al, 2012). Concomitant to β–cell death is an increase in α–cell and δ–cell mass that peaks between one to three weeks after the last STZ injection, depending on the dose, treatment and mouse strain (Li et al, 2000; Zhang et al, 2012). Regardless of the treatment regime, the resulting hyperglycemia and β–cell death is largely the same. In both

cases, mice become hyperglycemic around 3-5 days after the last STZ injection and there is significant β-cell death within a week. While not an accurate model of the pathological β-cell death associated with either TID or TIID, STZ treatment is considered a model of hyperglycemia and glucotoxicity as a consequence of decreased β-cell mass and is often used in molecular studies assessing effects on β-cell apoptosis.

RATIONALE AND AIM

There are many problems associated with various mouse transgenic lines expressing Cre-recombinase in different tissues. Such problems have complicated analysis of mouse gene knockouts because in several previous studies the appropriate Cre-recombinase expressing control groups were not included. Thus, it is important to characterize the newly generated β-cell MIP-CreERT line under commonly used models of TIID like high-fat diet and STZ.

The aim of this study is to investigate whether the MIP-CreERT transgene may confound results generated when we challenge our β-cell specific Pgc1α/β KO mice with a high-fat-diet and STZ to assess β–cell death in a model of TIID.

RESEARCH DESIGN AND METHODS

Generation of MIP-CreERT and β–cell-specific Pgc-1α/β knockout mice. Previously described mice carrying *Pgc-1α* (Estall et al, 2009) and *Pgc-1β* floxed alleles (Lai et al, 2008b) interbred to generate $Pgc\text{-}1\alpha^{fl/fl}/\beta^{fl/fl}$ on a mixed C57BL/6N:C57BL/6J:129 background were subsequently bred with MIP-CreERT mice on a C57BL/6J background. Homozygous $Pgc\text{-}1\alpha^{fl/fl}/\beta^{fl/fl}$ (denoted WT-FL, littermate controls) and β-cell specific $Pgc\text{-}1\alpha^{-/-}/\beta^{-/-}$ knock-out (denoted β-KO) mice were then generated. To obtain MIP-CreERT control groups (CreERT transgene alone), $Pgc\text{-}1\alpha^{fl/fl}/\beta^{fl/fl}$,MIP-CreERT males were bred once with C57Bl/6J and interbred 2-3 generations to eliminate floxed alleles. Only male mice were used for all experiments. To obtain Pdx1-Cre mice, Pdx1-Cre (Hingorani et al, 2005) male mice were bred with C57Bl/6J females. All test mice (unless specifically indicated) were gavaged at six weeks of age for ten days with 100 g/kg of tamoxifen (Sigma) suspended in 0.05% methylcellulose/distilled water. A minimum 5-week wash-out period followed tamoxifen dosing was provided prior to experimentation. Mice were maintained on a 12-hour dark/light cycle and given free access to water and standard laboratory chow (Teklad diets 2018). Mice were maintained and sacrificed according to approved protocols from the Clinical Research Institute of Montreal (IRCM).

Induction of hyperglycemia with high-fat feeding and low dose STZ injections. To render the mice hyperglycemic, we used a combination of high-fat diet feeding and STZ administration that has been reported previously (Mu et al, 2006), with the following modifications. Test mice starting at eight weeks of age were fed a 45% high-fat diet (D12451 ResearchDiets) for three

weeks. They were then administered an intraperitoneal injection (i.p.) of 50 mg/kg streptozotocin (Bioshop) resuspended in 100 mM sodium citrate pH 4.5 following a four hour fast. Five days later, mice received a second intraperitoneal injection of 75 mg/kg STZ under the same conditions. Mice were kept on the high-fat diet until sacrifice. Non-fasting blood glucose was measured at the indicated time points from the tail vein using a standard glucometer (FreeStyle Lite, Abbot Diabetes Care). For MIP-CreERT mice not gavaged with tamoxifen, a third intraperitoneal injection of 100 mg/kg STZ was given three days after the 75 mg/kg STZ injection because the control wild type mice did not become hyperglycemic following the first 2 doses.

Metabolic tests and insulin measurements. Oral glucose tolerance tests were performed by gavaging glucose (1 g/kg) into 16 hr-fasted mice. Glucose or serum insulin levels at indicated time points from the tail vein were measured using a glucometer (FreeStyle Lite, Abbot Diabetes Care) and the mouse ultrasensitive insulin ELISA (Alpco), respectively. Fasted and re-fed glucose and insulin levels were measured after a 16 hr-fast and following 2 hours of chow diet re-feeding. These tests were performed 1-3 weeks after the second (75 mg/kg) STZ injection.

Islet area and cleaved caspase-3 staining. For histological analysis, pancreata were kept overnight in 4% paraformaldehyde/PBS, washed with PBS once and embedded in paraffin. To measure islet area, 10-12 5-μm sections throughout the pancreas separated by at least 200 μm were stained with Haematoxylin and Eosin (H&E) following standard procedures and the area of each islet was assessed using Matlab. Briefly, the area was calculated by circling all islets in each pancreatic section. The area values for all islets in each mouse were pooled and grouped

depending on their size to create a distribution. Islet density was calculated as the ratio of the number of islets in a pancreatic section divided by the area of the section. For caspase-3 staining, mouse anti-cleaved caspase-3 (1:150, Cell signaling) antibody was used with standard immunostaining procedures, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and diaminobenzidine to detect cleaved caspase-3 positive cells.

Statistical analysis. All statistical tests were performed using Graphpad Prism software. Statistical analysis was performed using an unpaired two-tailed student's t-test or, where specified, using 2-way ANOVA.

RESULTS

The MIP-CreERT mouse was protected against streptozotocin-induced hyperglycemia.

Pgc-1α has been associated with regulation of apoptosis in a cell-specific manner. In ovarian cancer cells, overexpression induces apoptosis, yet muscle cells with reduced Pgc-1α function are more susceptible to cell death (Zhang et al, 2007; Adihetty et al, 2009). We hypothesized that loss of PGC-1s in β-cells would render them more susceptible to cellular stress resulting in increased cell death. To determine whether loss of Pgc-1 expression in β-cells impacted cell survival, we fed β-KO mice and WT-FL littermate controls a high-fat diet (HFD) for three weeks followed by STZ. The WT-FL littermate controls became hyperglycemic three days after the second STZ injection as expected, while strikingly, the β-KO mice showed no significant increase in blood glucose levels (Figure 1A). To ensure that the protection from hyperglycemia was due to Pgc-1 reduction and not the simple presence of the MIP-CreERT transgene, we repeated the experiment in MIP-CreERT mice and WT littermate controls. Surprisingly, the same results were observed with MIP-CreERT+ mice compared to WT littermate controls (Figure 1B). Even three weeks after the second STZ injection, on average, MIP-CreERT mice did not have significantly increased random glycemia, while WT mice had blood glucoselevels above 18 mM. This difference in glycemia was not a result of a significant difference in weight as in both cases MIP-CreERT and β–KO mice showed no significant difference in weight compared to their respective controls (Figure 1C-D). We performed oral glucose tolerance tests (OGTT) one week after the 75 mg/kg STZ injection and all mice harboring a MIP-CreERT transgene (regardless of the presence of floxed genes) showed a significant improvement in

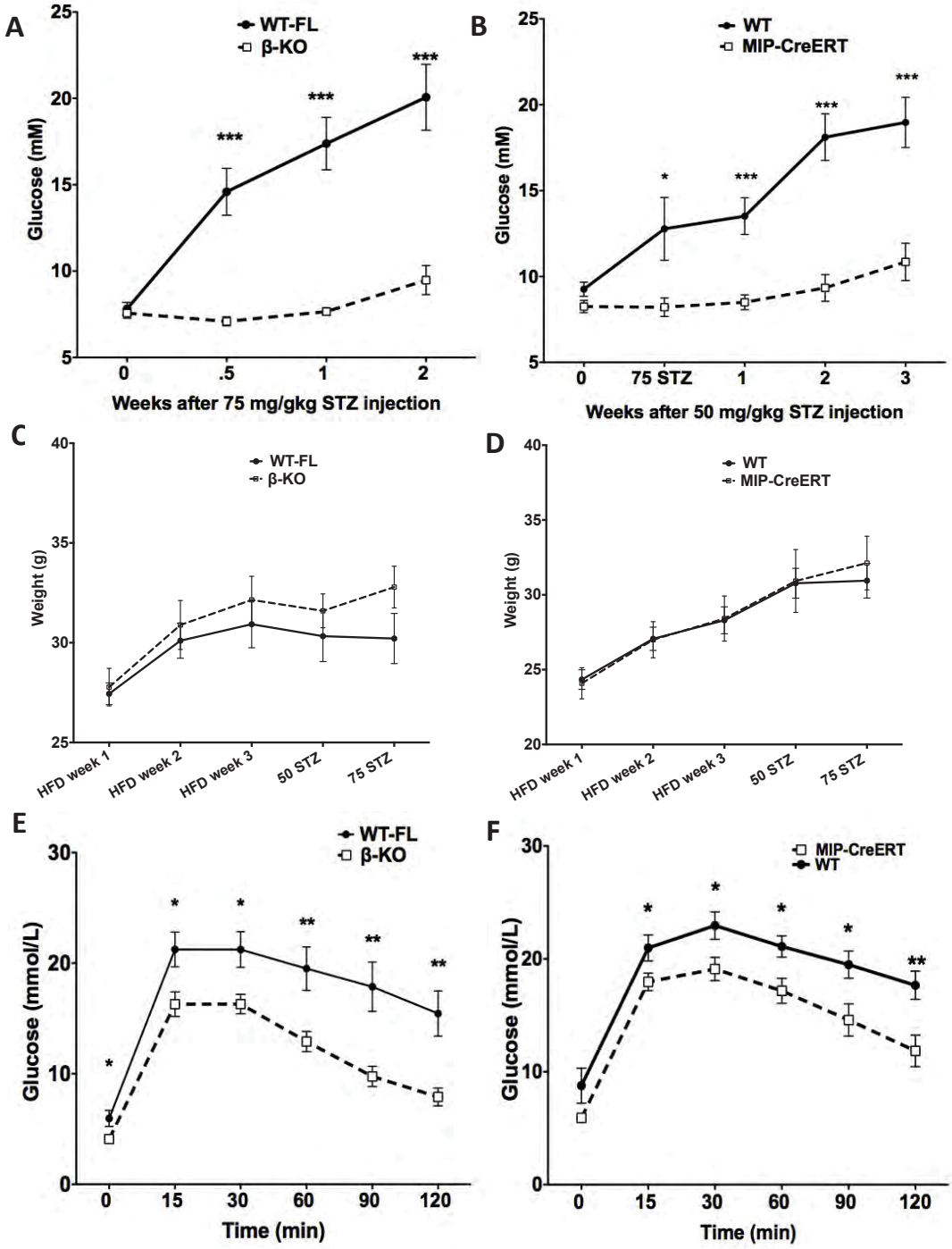

Figure 1. MIP-CreERT+ mice are protected against Streptozotocin-induced hyperglycemia. A-B: Non-fasting blood glucose of β-KO and MIP-CreERT male mice on a HFD and after two i.p. injections of 50 and 75 mg/kg streptozotocin. C-D: Weight gain after HFD feeding of corresponding mice in A-B. E-F: Oral glucose tolerance tests of corresponding mice in A-B one week after the second 75 mg/kg streptozotocin injection. Data is expressed as mean ± SEM (n=8-10 for all groups). Each panel is representative of at least two independent experiments. * P-value< 0.05, ** P-value<0.01.

glucose tolerance at all time points compared to their respective controls (Figure 1C-D). Thus, we concluded that the MIP-CreERT transgene (MIP-CreERT+) conferred protection against HFD/STZ-induced hyperglycemia.

MIP-CreERT+ mice treated with STZ had increased levels of insulin in the blood and a similar degree of cleaved caspase-3 in the islet.

To begin exploring the underlying mechanism of the protection against HFD/STZ-induced hyperglycemia in MIP-CreERT+ mice, we first asked if blood insulin levels were increased. We measured glucose and insulin levels in fasted and re-fed mice and performed an OGTT three weeks after the second STZ injection. MIP-CreERT+ mice showed no difference in fasting insulin levels, but a significant increase in blood insulin levels following re-feeding and at all time points following an oral glucose challenge (Figure 2). The largest difference was observed 5 minutes after gavage of glucose, where blood insulin levels in MIP-CreERT+ mice were, on average, 1.8 fold higher than littermate controls.

MIP-CreERT+ mice treated with STZ had similar islet area and a similar degree of cleaved caspase-3 in the islet

A simple explanation for the increased levels of insulin would be that β–cells from MIP-CreERT+ islets are protected against STZ-induced β–cell death. We next analyzed cleaved caspase-3 staining, islet density and islet area from MIP-CreERT+ mice three weeks after the last STZ injection. There was no significant difference in the proportion of islet cells positive for cleaved caspase-3 staining (Figure 3A-B) between groups.

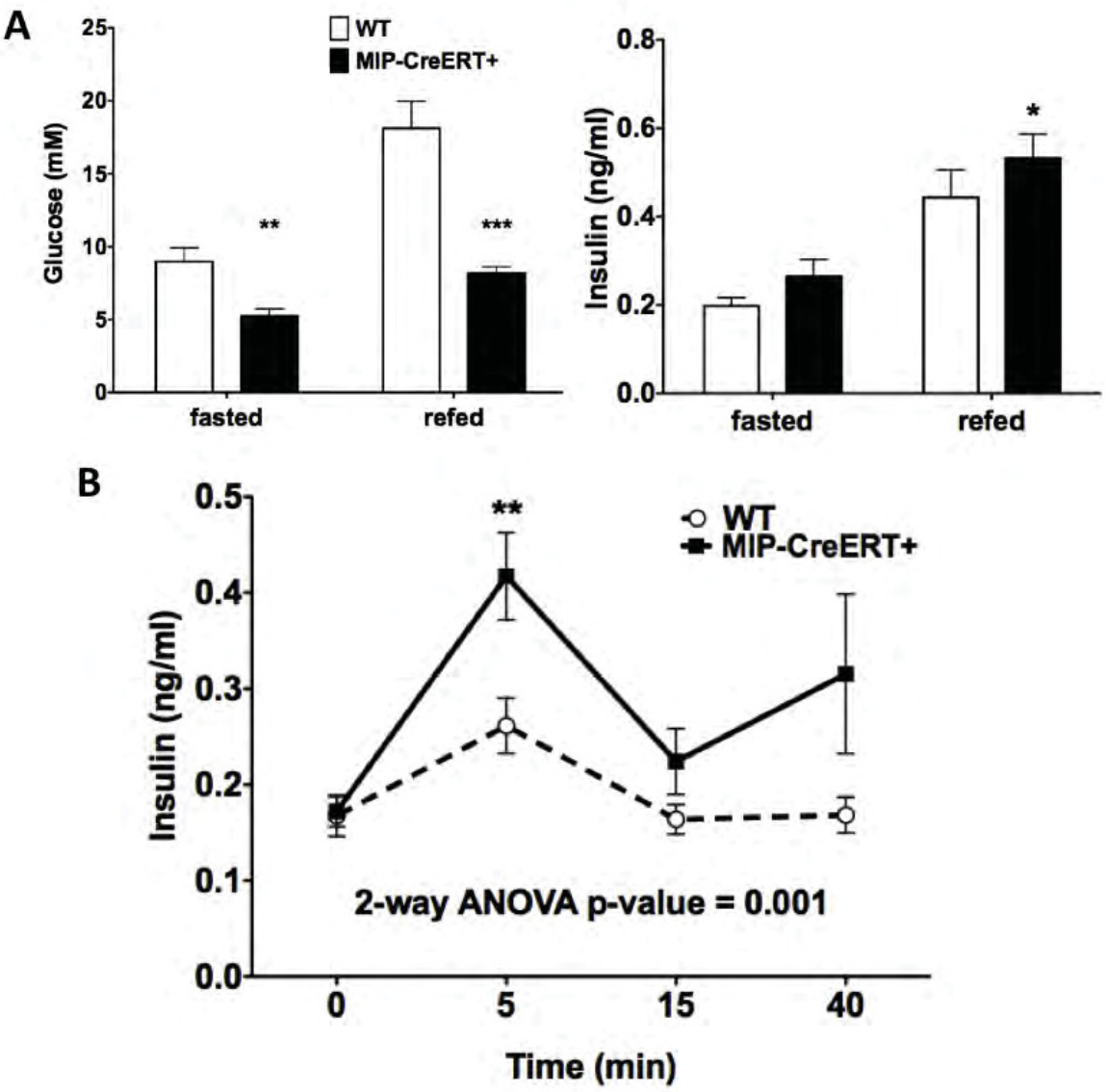

Figure 2. MIP-CreERT+ mice injected with STZ have increased blood insulin levels. A: Fasting and re-fed blood glucose and insulin levels of MIP-CreERT+ mice on HFD/STZ protocol. B: Insulin levels following an oral glucose tolerance test. All tests were done 1-3 weeks after the 75 mg/kg STZ injection. Data is expressed as mean $\pm$ SEM (n=8-10 for all groups). * P-value< 0.05, ** P-value<0.01.

There was a trend towards increased islet density (number of islets over area of pancreatic section) in MIP-CreERT+ mice that did not reach statistical significance (Figure 3C). There was no significant difference in the number of islets with an islet area below 20k μm^2 (Figure 3D); but there was a trend towards more islets with an area of 20-30k μm^2 and 30-50k μm^2 that did not reach statistical significance. On average, islets with an area of 20-50k μm^2 represents ~3% of all islets, which in most mice represent ~5 individual islets per 10 sections analyzed. Given only a slight trend toward increased islet area, no difference in cleaved caspase-3,and similar levels of fasting blood insulin levels, our data suggest that MIP-CreERT+ mice may have increased *in vivo* glucose-stimulated insulin secretion that is not caused by a protection against β–cell death following STZ treatment.

Protection against STZ is not a general consequence of the presence the Cre-recombinase enzyme in the β–cell.

Next, we asked if the protection against hyperglycemia was a general consequence of expressing Cre-recombinase protein in the β-cell. To do this, we used a different mouse line that expresses Cre-recombinase in mouse islets. Pdx1-Cre mice fed a HFD (which were not gavaged with tamoxifen) and given two injections of STZ showed no statistical difference in glycemia compared to control mice, with average blood glucose levels rising to over 20 mM two weeks after the second STZ injection (Figure 4).

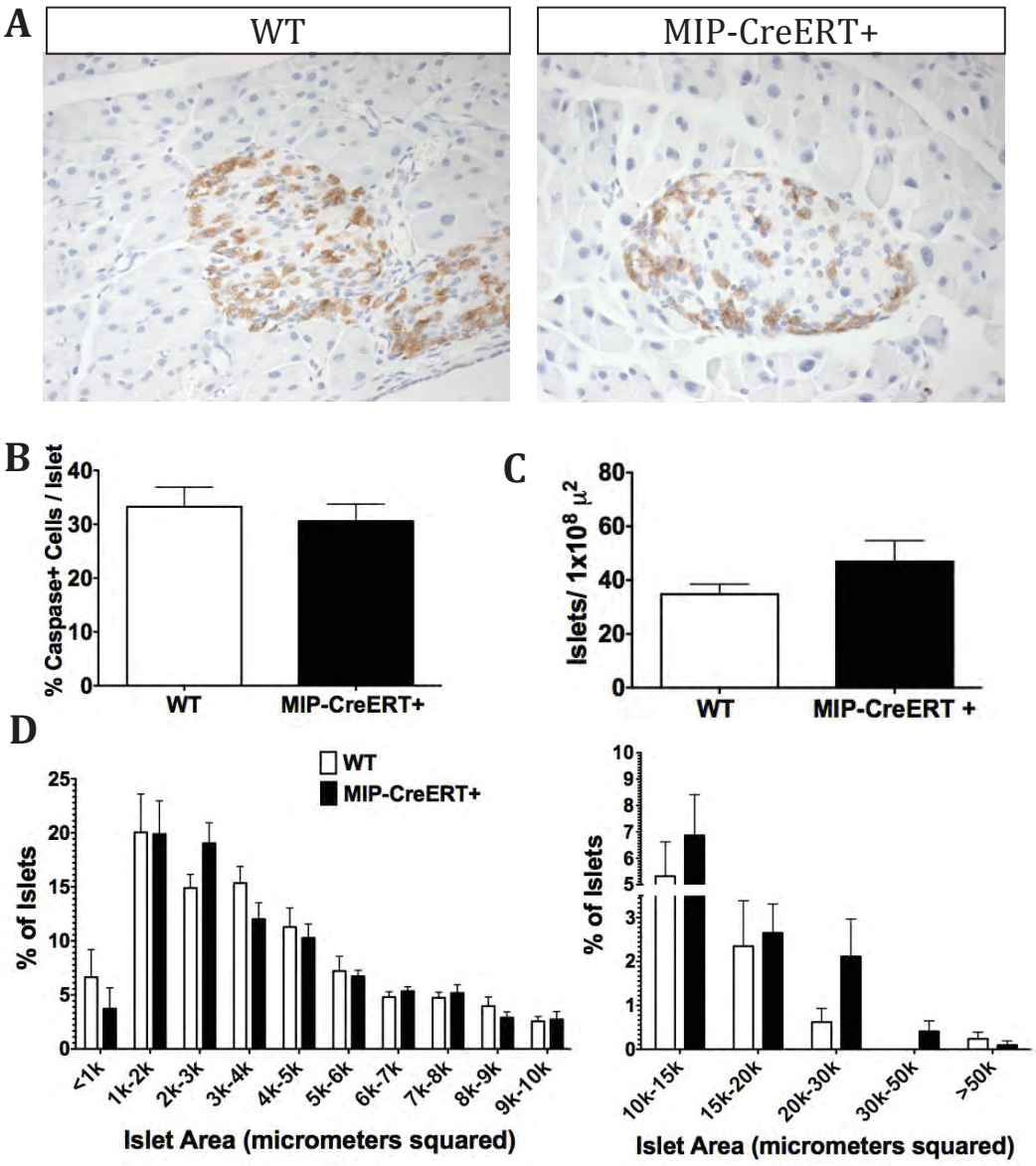

Figure 3. MIP-CreERT+ mice injected with STZ have the same proportion of Caspase+ cells and islet area. A-B: Representative cleaved caspase-3 staining of islets and their corresponding quantification. C: Islet density quantified from H&E stained sections. D: Islet area quantified from H&E stained sections. Pancreata were analyzed 3 weeks after the 75 mg/kg STZ injection. Data is expressed as mean ± SEM (n=5-6 for all panels).

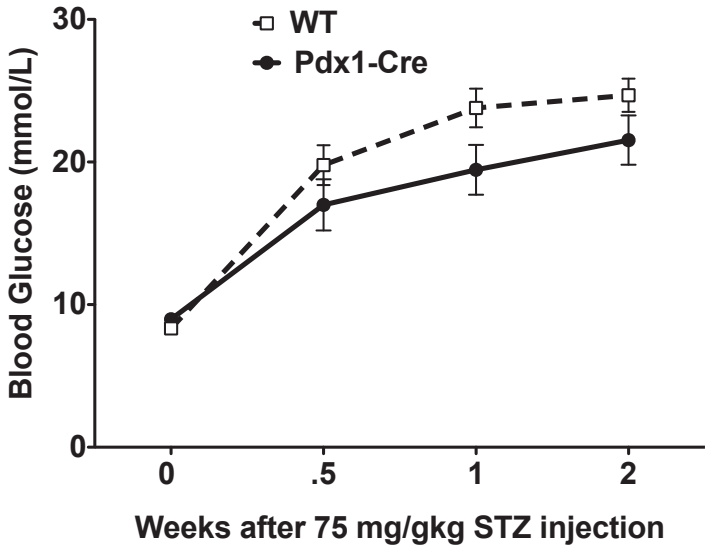

Figure 4. Pdx1-Cre mice are not protected against STZ-induced hyperglycemia. Non-fasting blood glucose of Pdx1-Cre mice on a HFD and after i.p. injections of 50 and 75 mg/kg STZ. Data is expressed as mean ± SEM (n=6-8).

Protection against STZ is not dependent on CreERT translocation into the nucleus or exposure to tamoxifen toxicity.

In the Pdx1-Cre mouse, the Cre-recombianse protein is localized to the nucleus. In the MIP-CreERT+ mice, the CreERT protein is sequestered in the cytoplasm until tamoxifen induces translocation into the nucleus (Reinert et al, 2012). Hence, we tested if the observed protection against HFD/STZ in MIP-CreERT+ was dependent on translocation of the CreERT protein. To test this, we subjected MIP-CreERT+ mice not exposed to tamoxifen to a HFD with STZ, except an additional injection was required to produce hyperglycemia in control mice (Figure 5). Even though Cre-recombinase remained cytoplasmic and theoretically inactive, MIP-CreERT+ mice still had significantly lower blood glucose compared to control littermates. Three to four weeks after the last STZ injection, wild type controls had average blood glucose levels of 20 mM, while

MIP-CreERT+ mice had 12.5 mM. These results demonstrate that protection against STZ in MIP-CreERT+ mice is not dependent on CreERT translocation into the nucleus. As an aside, this also shows that exposure to tamoxifen, which can be a β-cell toxin (Hashimoto et al, 1997), is not required to reveal the phenotype associated with the MIP-CreERT+ mice.

Protection against STZ is not caused by excessive glycosuria in MIP-CreERT+ mice

Lastly, we asked if protection against STZ was caused by increased clearance of glucose by excretion in the urine. As Glut2 is expressed in the kidney, STZ can cause significant renal damage at high doses (Thorens et al, 1988; Chow et al, 2004). It is possible that MIP-CreERT+ mice are less susceptible to STZ-induced kidney damage or have modified kidney function allowing them to excrete more glucose in the urine. To address this, we measured urine glucose levels in MIP-CreERT+ (not administered tamoxifen, same mice from Figure 5) 3.5 weeks after the last STZ injection when blood glucose was significantly different between groups. Arguing against a mechanism of increased glucose clearance via the kidney, MIP-CreERT+ mice showed a trend towards decreased glycosuria compared to controls that did not reach statistical significance (Figure 6).

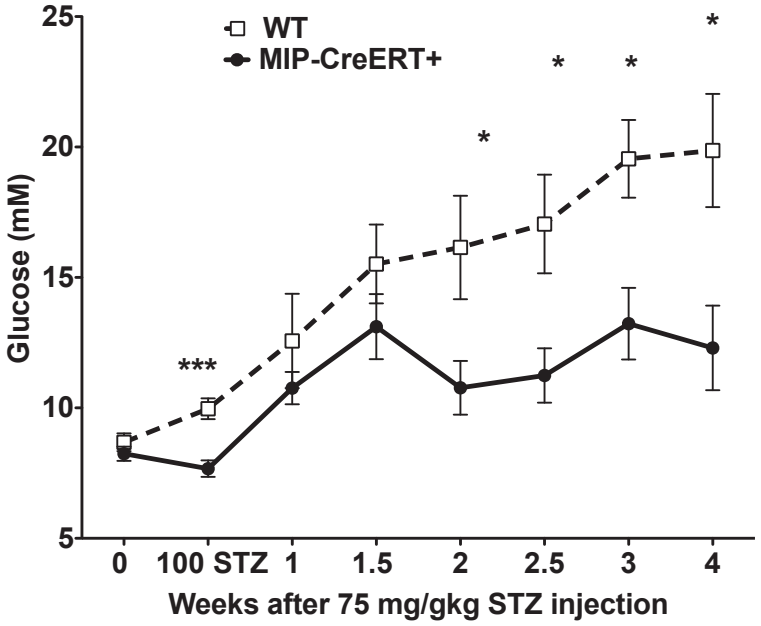

Figure 5. MIP-CreERT+ mice are protected against STZ independent of tamoxifen. Non-fasting blood glucose of MIP-CreERT+ mice on a HFD/STZ (no tamoxifen) and after i.p. injections of 50, 75 and 100 mg/kg STZ. Data is expressed as mean ± SEM (n=6-8). * P-value< 0.05, *** P-value<0.001.

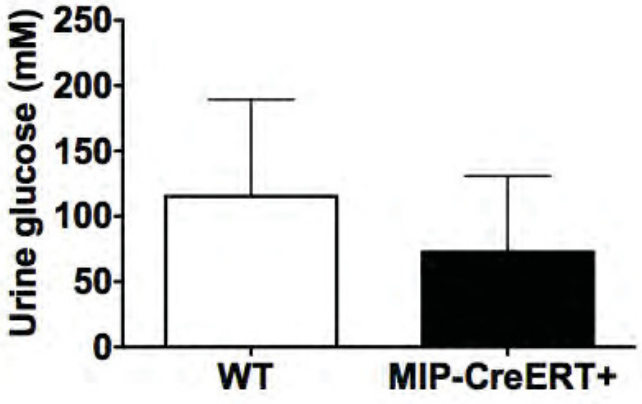

Figure 6. MIP-CreERT+ mice injected with STZ have the same levels of glycosuria. Urine glucose levels of mice on a HFD four weeks after the 75 mg/kg STZ injection. Data is expressed as mean ± SEM (n=6-8).

DISCUSSION

In this study we show that an islet-targeted CreERT transgene can protect mice against the effects of a β-cell specific toxin. In this case, the MIP-CreERT transgene caused a striking protection against HFD/STZ-induced hyperglycemia. Even four weeks following the last STZ injection, the majority of MIP-CreERT+ mice (in the absence or presence tamoxifen) did not increase their blood glucose levels over 12 mM, while all control mice had blood glucose of 20 mM or more. Some of our results are still preliminary and some experiments have yet to be repeated. As some of the MIP-CreERT+ mice in initial experiments also lacked expression of Pgc-1α/β (Figure 2), particularly experiments were blood insulin levels were increased; it is possible that the loss of Pgc-1α/β confounds effects of the MIP-CreERT transgene alone. We believe this is unlikely, as both β-KO and MIP-CreERT mice showed the same level of protection against STZ-induced hyperglycemia and glucose intolerance (Figure 1) and we show in Chapter 2 that loss of Pgc-1α/β actually results in the opposite effect (decreased insulin secretion) and has no effect on glycaemia. In the case of the MIP-CreERT+ experiment performed in the absence of tamoxifen (Figure 5), there was more variability between mice and half of the MIP-CreERT+ mice became hyperglycemic one week after the additional third STZ injection; in contrast with previous experiments in mice receiving tamoxifen, where none of the MIP-CreERT+ mice became hyperglycemic (Figure 1). This variability in blood glucose likely explains the variability in the urine glucose levels in MIP-CreERT+ mice and we only observed a mild, yet insignificant, decrease compared to control mice, despite significantly lower blood glycemia (Figure 6). Because of these reasons, these experiments will need to be repeated to

confirm our results and to rule out confounding variables that interfere with interpretation.

To our knowledge, this is the first time a transgene expressing Cre-recombinase in β–cells has been shown to protect against hyperglycemia. Previously available β–cell-specific Cre lines, such as some RIP-Cre strains, exhibited a negative impact on whole body glucose homeostasis and β–cell function in mice fed a standard chow diet (Lee et al, 2005; Magnuson and Osipovich, 2013), albeit these experiments were done under different metabolic conditions than we tested and the effects are likely mediated by different mechanisms. The causes and mechanisms of the affected glucose metabolism observed with previous Cre lines are unclear and limited work has been performed to clarify this. In those cases, it is possible that ectopic expression of Cre-recombinase in the brain might direct the negative effects on glucose tolerance (Wicksteed at al, 2010). However, ectopic Cre-recombinase expression in the brain is most likely not a cause of the STZ protection in the MIP-CreERT mouse as previous reports (Wicksteed at al, 2010), as well as our results presented in Chapter 2, show that there is no detectable Cre-mediated recombination in many regions of the brain.

Our data sheds light on several important aspects of this protective phenotype. MIP-CreERT+ mice have increased levels of insulin in the blood in response to STZ treatment that do not appear to be the consequence of protection against STZ-induced β–cell cell death. There are at least three possible causes for the protection against HFD/STZ-induced hyperglycemia. First, this phenotype could be the result of an inability of STZ to cause toxic effects in MIP-CreERT+ β–cells. For example, the CreERT protein in the cytoplasm could interact directly with STZ, preventing conversion to a toxic metabolite or causing the cell to actively secrete or inactive it. Second, the presence of CreERT protein in the cytoplasm of the β–cell (as opposed to the majority of Cre-recombinase mouse lines that express the protein in the nucleus) may somehow

modify β-cell physiology and improve insulin secretion capacity or insulin biosynthesis and processing. Third, the insertion of the MIP-CreERT transgene disrupts a gene locus that either directly improves β-cell physiology or modifies the glucose-regulatory function of other peripheral organs (i.e. enhanced glucose disposal).

To determine which one of these possibilities is driving the phenotype, key experiments need to be performed. First, although we see no difference in cleaved caspase-3 staining or islet area in MIP-CreERT+ mice, we need to definitively rule out protection against β–cell death as a possible mechanism. It is possible that the temporal dynamics of HFD/STZ-induced β–cell death in MIP-CreERT+ mice follow a strong peak of apoptosis a few days after the second STZ injection followed by a plateau, instead of a steady and sustained increase in control mice. Thus, it could be argued that by analyzing apoptosis three weeks after the last STZ injection, we missed the peak of HFD/STZ-induced β–cell death where significant differences could be observed. However, if this were the case, we would expect the MIP-CreERT mice to have a significantly higher islet area or density because of protection against the initial peak of STZ-induced β–cell death. There was only a slight increase in islet density and number of islets with an area larger than 30k μm^2, but this did not reach statistical significance. Additionally, because the number of islets with an area bigger than 30 thousand μm^2 was very small (in some cases only one or two islets per mouse, <3% of total islets), it is unlikely this small number would make such a large impact on blood glucose levels. To confirm that there is no difference in β–cell death *in vivo* in MIP-CreERT+ mice treated with STZ, we could stain pancreatic sections with a general marker of cell death such as Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). We could also isolate islets from MIP-CreERT+ mice, treat them with STZ *in vitro* and quantify apoptotic and necrotic markers using FACS. Confirming that there is

no difference in β–cell death in response to STZ would suggest that the MIP-CreERT transgene is not hindering the ability of STZ to act as a β-cell toxin.

If the MIP-CreERT transgene is not affecting the mechanism by which HFD/STZ kills β-cells, then it could be improving cell physiology of the remaining β-cell population in MIP-CreERT+ mice, rendering them more efficient and/or increasing secretory capacity to compensate for equivalent β-cell loss. Improved β–cell function could arise as a general effect of having CreERT protein in the β–cell or it could be a MIP-CreERT-specific effect. Our data in Pdx-1-Cre mice suggests that protection from STZ is not a general effect of having Cre-recombinase in the β–cell. Thus, to address the possibility that the effect is due to the presence of CreERT protein in the β-cell cytoplasm (specific to the tamoxifen-inducible system), we are repeating the experiment with another β–cell-specific CreERT line, either the Pdx1-CreERT or the RIP-CreERT (Dor et al, 2004; Zhang et al, 2005b). If these β-cell-specific CreERT mouse lines are not protected against HFD/STZ hyperglycemia, this would suggest that this is a MIP-CreERT-specific phenotype, possibly caused by disruption of the locus where the transgene was inserted.

To test whether MIP-CreERT+ mice have improved β–cell function in our experimental conditions, we could isolate islets from MIP-CreERT+ mice subjected to HFD/STZ and perform *in vitro* GSIS experiments to assess whether they are secreting more insulin. Following STZ treatment, β–cells that are able to escape cell death can have STZ-mediated methylation of proteins that affect β–cell function (Lenzen, 2008; Wilson and Leitner, 1990). Indeed, islets isolated from mice treated with STZ or islets treated with STZ in culture for short periods of time show a significant decrease in *in vitro* GSIS (Noh et al, 2013; Strandell et al, 1988; Rasschaert et al, 1992) that is not due to differences in β-cell mass. Thus, it could be possible that the MIP-

CreERT transgene, either by the direct action of the CreERT protein (a general CreERT effect) or by disrupting a gene locus, modifies β–cell biology to protect against protein methylation.

To determine where the MIP-CreERT transgene is inserted, we could sequence the entire genome of the MIP-CreERT mouse or use a combination of genome digestion, southern blot, PCR and fluorescent in situ hybridization techniques. This would show how many copies of the transgene were inserted in the genome and whether the transgene disrupts one or several gene loci. If important loci are disrupted, it could be responsible for improving the function of MIP-CreERT+ β-cells. Ideally, the identity of the gene locus (or loci) disrupted by the MIP-CreERT transgene could give initial clues as to the physiological mechanism and target tissue(s) underlying the phenotype.

If the locus of insertion is in fact mediating the phenotype of the MIP-CreERT+ mice, it could also be via disruption of glucose metabolism or homeostasis in organs apart from the β-cells. These effects could indirectly cause an increase in blood insulin concentrations or directly decrease blood glucose levels (i.e. improved insulin sensitivity or glucose excretion). For example, its possible that modified kidney function directs the MIP-CreERT+ mice to excrete large amounts of excess glucose in urine. However, if this were the case, we would expect significantly higher levels of glycosuria in MIP-CreERT+ mice compared to controls. Our results in Figure 6 do not support this hypothesis. To address whether non-β-cell-specific mechanisms are mediating the phenotype, we could analyze insulin sensitivity in MIP-CreERT+ mice following STZ injection and perform histological analysis of peripheral organs to look for differences in cell morphology, suggesting altered function or development.

Various genes expressed in β-cells and other peripheral tissues have been implicated in protective mechanisms against hyperglycemia caused by multiple low dose STZ injections.

However, in most cases, these proteins protected against β-cell apoptosis and preserved β-cell mass to normalized glucose levels, such as overexpression of CD39 in peripheral blood leukocytes (Chia et al, 2013), Menin reduction in β–cells (Yang et al, 2010) or whole body deletion of the NADPH oxidase Nox2 or the small heterodimer partner transcription factor (SHP) (Xiang et al, 2010; Noh et al, 2013). Interestingly, whole body deletion of the glucagon receptor or insulin hypersensitivity caused by inactivating phosphatase and tensin homolog (PTEN) in adipose tissue protects against STZ-induced hyperglycemia without inhibiting β–cell apoptosis (Lee et al, 2011; Kurlawalla-Martinez et al, 2005). Given that the MIP-CreERT+ mice do not appear to be protected against β–cell death, it will be exciting to investigate if they are protected from STZ by a novel mechanism that improves β–cell physiology or whole body glucose homeostasis.

In summary, MIP-CreERT+ mice were protected against HFD/STZ-induced hyperglycemia, independent of tamoxifen exposure, nuclear Cre-recombinase localization or protection against HFD/STZ-induced β–cell death. Furthermore, we show that the protection was not a general effect of expressing Cre-recombinase protein in the β–cell nucleus. These results will expand our knowledge about how Cre-recombinase technology impacts β–cell biology. Not only does this underline the importance of designing experiments to control for these effects (i.e. including all the necessary Cre-recombinase-alone controls when assessing the effects of gene deletion using the Cre/Lox system), but may also allow for better screening of lines that are generated in the future to avoid these kind of artifacts. For example, expression of all genes identified that can modify resistance or susceptibility to STZ could be screened in new Cre mouse lines generated or targeted transgenesis can be performed so that the locus on insertion is known. On the other hand, if the effect is a direct CreERT protein effect on the β-cell, the

CreERT protein sequence could be modified to control for this. Additionally, new mechanisms regulating the action of STZ could be identified that might lead to STZ being applied in other areas of β–cell research.

CONCLUSION

Diabetes is a complex multifactorial disorder for which one therapy will most likely not provide long lasting management of glycemia. A combination of different treatments is probably needed in order to help diabetics cope with a changing physiology as the disease progresses. A promising approach to treat ailing β–cells in diabetics could be through cell replacement with functional, *de novo* generated β–cells combined with therapies that target β–cell dysfunction and its causes.

Our results with Ngn3 in *Xenopus* demonstrate that ectopic development of β and δ-cells *in vivo* throughout the foregut can be achieved, without any significant increase in α-cell numbers. Our experiments provide information about novel gene targets, including the Mtgs and Tbx2, which may be involved in this process. Further research may ascertain whether this phenomenon also occurs in mammals and whether manipulation of Ngn3 function or expression of these novels genes could improve current protocols for *de novo* generation of β–cells.

However, Ngn3 manipulation has its problems. When overexpressed, we generated both β- and δ cells in vivo. Ideally, we would like to identify a protein (or set of proteins) that can reliably and efficiently promote the development of functional β-cells while minimizing the number of other endocrine cells. The Xenopus system provides a unique system to identify these key factors. In general, overexpression and knockdown screens in *Xenopus* (even using two different mRNAs or morpholino oligos at the same time in the same embryo) can be completed fairly quickly in a matter of weeks; hence, it would be interesting to increase the scale of this type of approach and couple it with *in vitro* differentiation of human ESCs or iPSCs. To this end, transgenic *Xenopus* lines expressing enhanced GFP under the insulin promoter can be utilized as a surrogate to directly visualize increases in insulin expression *in vivo,* instead of processing embryos and performing i*n situ* hybridization. In this way, large genetic screens manipulating

numerous genes at once could identify novel targets to promote ectopic β–cell development in *Xenopus.* These targets could be rapidly tested by virus-mediated overexpression or knockdown for their potential to direct *in vitro* differentiation of human stem cells into β–cells. Promising target genes could then be studied in detail in the mouse to understand their function in mammal embryonic β–cell development and identify signaling pathways and ligands that could activate them.

Our results stemming from reduction of Pgc-1 gene function in β-cells *in vivo* provided unexpected results, as mitochondrial function was largely unaffected, and instead pointed to a putative new Pgc-1 dependent pathway of insulin secretion through regulation of lipid metabolism. Additionally, as Pgc-1 genes are considered master regulators of mitochondrial biogenesis and function, our results suggest the existence of strong compensatory mechanisms of mitochondrial function in β–cells or to other proteins as the predominant regulators of mitochondria in adult β–cells. Nonetheless, inactivation of Pgc-1α and β in mouse β-cells under basal conditions caused a mild, yet significant decrease in insulin secretion. As TIID is a polygenic and chronic disease that usually arises with advanced age, β–cell dysfunction in diabetics is most likely the result of genetic variation affecting several genes that have a mild effect on insulin secretion under basal conditions but collectively have a bigger impact with different types of metabolic stress including a high fat or high glucose diet. Thus, given the human Pgc-1α gene has the Gly482Ser polymorphism that has been shown to be associated with TIID and decreased GSIS , our results suggest that the Pgc-1 genes might be contributing to β–cell dysfunction in TIID and could be important determinants for the progression into hyperglycemia.

Pairing the expertise and techniques of β–cell physiology with those of development and

differentiation could help tackle many of the current problems in the β–cell field. A complete physiological characterization of *de novo* generated β–cells is an essential step to ensure their insulin secretion capacity and survival is comparable to endogenous β–cells. *De novo* generated β–cells should be subjected to different assays to examine β–cell physiology, including analysis of mitochondrial biology (genomic and proteomic analysis as well as measuring ATP levels, oxygen consumption rates and calcium fluctuations), characterization of insulin secretion under different secretagogues, and analysis of apoptosis and insulin secretion under chronic glucolipotoxic conditions. Characterization of *de novo* generated β-cells might reveal that specific differentiation protocols produce β–cells with different physiological characteristics, and could point to particular aspects of β–cell biology that need to be focused on or improved during directed differentiation in order to produce functional β-cells.

Comprehensive knowledge of β–cell physiology and directed differentiation could also allow for the development of patient-specific cell engineering of β-cells *in vitro* for transplantation. In this context, *de novo* β-cells could be manipulated to be more responsive or resistant to a particular pathological physiology, which is likely unique for each diabetic patient. For example, *de novo* β-cells that have increased fatty acid-potentiated insulin secretion might pose a risk of exacerbating β-cell exhaustion or insulin resistance in obese patients and might be more beneficial for lean diabetics, and likewise, β-cells that are resistant to lipotoxic conditions would more suited to obese rather than lean diabetics.

Finally, there is little information about the metabolic state of the endodermal, pancreatic and endocrine progenitors that generate β–cells during embryonic development. It is possible that the progression through these different stages of differentiation is intimately linked to the genetic and protein profiles that define the metabolic characteristics of progenitors giving rise to

β–cells. It would be interesting to investigate if Ngn3, Pax4 or other master regulators of endocrine development control the expression of genes involved in mitochondrial function or fuel metabolism during β–cell embryonic development. For example, our microarray results in *Xenopus* endoderm following 4 hours of Ngn3-GR activation identified pyruvate dehydrogenase kinase (Chapter 1 Table 1) and preliminary *in situ* hybridization data confirmed this result (Chapter 1, Figure 12). It is possible that specific transitions through different cellular states of fuel metabolism is necessary to create the metabolic profile of β-cells and any deviations from this would be involved in the pathology of β–cell dysfunction observed in diabetes. Additionally, these metabolic states might need to be maintained in the culture conditions (like availability of certain fuel substrates or metabolites) to ensure proper differentiation. Thus, further expanding our knowledge of the metabolic transitions possibly taken by endocrine progenitors throughout β–cell embryonic development might also bring insight into key pathways needed to create a functional β–cell and that could be targeted for therapy in adult β–cells.

In conclusion, our findings provide an initial dissection of novel mechanisms of β–cell physiology and development. β–cell differentiation and metabolism might be more intimately linked than previously thought. Molecules such as Pdx1 and NeuroD are important for development but are also needed for adult β–cell function. Thus, master regulators of cellular metabolism might interact with crucial β–cell differentiation genes, for example Pgc-1α might bind and regulate transcriptional activity of Ngn3 or Pdx1, both during embryonic development and in adult β–cells. Integrating different avenues of physiological and developmental research will provide further insight into β–cell biology and will help tackle the obstacles towards an efficient therapy targeting β–cells in diabetics.

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