Regulation of β-Cell Mass Expansion by Prostaglandin E_2 Signaling

By

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INTRODUCTION

General Background

Significance

Diabetes is a major healthcare concern in the United States, affecting more than 9% of the population [Center for Disease Control, National Statistics Report 2014]. Among those with diabetes, 90-95% are diagnosed with Type 2 Diabetes (T2D). In the face of increased metabolic demand, such as obesity-related insulin resistance, insulin output from the insulin-producing β-cells of the pancreatic islets normally increases and functional β-cell mass expands to compensate for the metabolic stress [1]. However, this β-cell plasticity is lost in the setting of T2D in humans and rodents [2]. Pancreatic β-cell failure, in combination with peripheral insulin resistance, ultimately results in T2D. The incidence of T2D increases with age, with 26% of individuals over the age of 65 affected by T2D [Center for Disease Control, National Statistics Report 2014]. The increased prevalence of T2D with age is multifaceted. In the β-cell, a combination of increased expression of cell cycle inhibitors and decreased capability to respond to proliferative cues with age likely contributes to the increase in disease incidence [3]. Understanding the signaling mechanisms that drive β-cell proliferation and the islet changes that occur with age will have important implications on therapeutics intended to enhance functional β-cell mass in patients with T2D.

Inflammation, Obesity, and T2D

Obesity-associated T2D is characterized by hyperglycemia and chronic low-grade inflammation. The idea that inflammation is involved in the etiology of diabetes dates back to the late 1800s when Ebstein noted that the anti-inflammatory drug sodium salicylate, a cyclooxygenase (COX) inhibitor, reduced the amount of glucose present in urine samples from patients with diabetes [4]. Historically, non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX-2 activity, such as aspirin and sodium salicylate, were used to treat diabetes [5]. Obesity-associated inflammation is defined by an increase in circulating cytokines, such as interleukin-1 (IL-1) β and tumor necrosis factor (TNF) α [6, 7], and elevated inflammatory mediators, including COX-2-derived prostaglandin (PG) E_2 (PGE_2) [7]. Seminal work from Hotamisligil and colleagues [8] illustrated that TNFα production and secretion is increased in the adipose tissue of obese rodents and this induction is correlated with severe obesity and insulin resistance. Following this work, several other groups identified additional inflammatory factors, such as leptin, IL-6, monocyte chemoattractant protein-1, and others, that are produced in the adipose tissue and lead to the onset of insulin resistance [9, 10]. These cytokines and inflammatory mediators can activate downstream signaling pathways that may contribute to the development of insulin resistance and T2D.

Pro-inflammatory cytokines, such as IL-1β, can activate c-Jun N-terminal kinase (JNK) and IκB kinase β (IKKβ)/nuclear factor-κB (NF-κB) signaling pathways, which have been linked to the development of insulin resistance [10]. IL-1β binding to its receptor results in activation of IKKβ, which then leads to phosphorylation and degradation of the inhibitory IκB protein, allowing for activation and nuclear

translocation of NF-κB. NF-κB promotes transcription of its target genes, including several cytokines and pro-inflammatory mediators, such as TNFα, IL-6, inducible nitric oxide synthase (iNOS), and COX-2 [7, 9]. Thus, NF-κB signaling leads to the development of insulin resistance through the transcriptional activation of cytokines and pro-inflammatory mediators [10]. Interestingly, the ability of NSAIDs to reverse hyperglycemia involves inhibition of IKKβ (NF-κB activator) [11], revealing a link between the COX pathway and insulin resistance. In addition to activation of the NF-κB pathway, IL-1β can activate JNK signaling. JNK causes insulin resistance by directly phosphorylating insulin receptor substrate (IRS)-1 on serine residues, inhibiting the insulin signaling pathway [10]. Other stimuli that can activate JNK or NF-κB pathways during metabolic stress include TNFα, IL-1, advanced glycation end products (AGE), and Toll by binding to their respective receptors (TNFR, IL-1R, RAGE, and TLR), reactive oxygen species (ROS) stress, and endoplasmic reticulum (ER) stress (Figure 1-1) [9].

In addition to the impact that inflammation has on the development of insulin resistance, cytokines and inflammatory mediators can also lead to changes in the pancreatic islets, including impaired β-cell function and enhanced β-cell death. In *ex vivo* assays using isolated rodent and human islets, the pro-inflammatory cytokines TNFα, IL-1β, and interferon (IFN)-γ work synergistically to reduce islet cell viability and impair glucose-stimulated insulin secretion (GSIS) [12-14]. The effects of these cytokines on β-cell death and dysfunction are mediated, in part, by activation of iNOS and subsequent generation of nitric oxide (NO) [12]. Short-term cytokine treatment (24-48 hours) results in NO-dependent β-cell DNA damage and necrosis [12, 15-17]

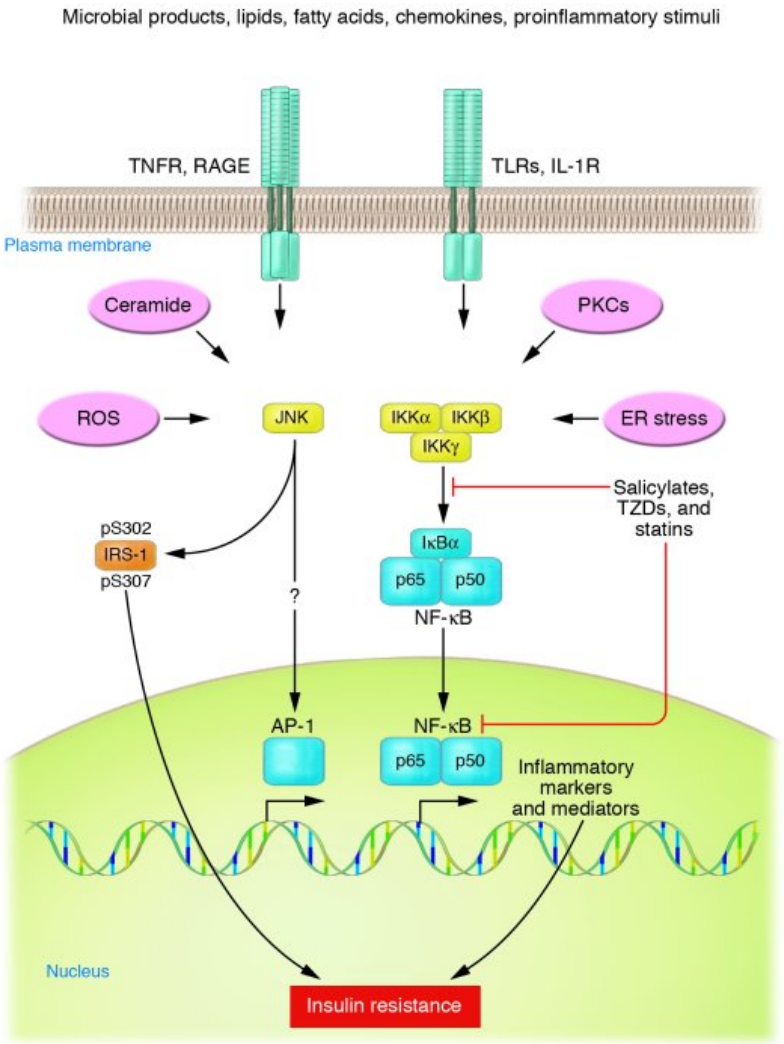

Figure 1-1. Potential cellular mechanisms for activating inflammatory signaling. Obesity and high-fat diet activate IKKβ/NF-κB and JNK pathways in adipocytes, hepatocytes, and associated macrophages. Stimuli known to activate these pathways during metabolic dysregulation include ligands for the TNF-α, IL-1, Toll, or AGE receptors (TNFR, IL-1R, TLR, or RAGE, respectively), intracellular stresses including ROS and ER stress, ceramide, and various PKC isoforms. Obesity-induced IKKβ activation leads to NF-κB translocation and the increased expression of numerous markers and potential mediators of inflammation that can cause insulin resistance. Obesity-induced JNK activation promotes the phosphorylation of IRS-1 at serine sites that negatively regulate normal signaling through the insulin receptor/IRS-1 axis. Examples include serine-302 (pS302) and serine-307 (pS307). By contrast, there is currently no evidence for obesity-induced effects on transcription factors such as AP-1 that are regulated by JNK. IKKβ and/or NF-κB are inhibited and repressed by the actions of salicylates, TZDs, and statins. Figure reprinted with permission from "Inflammation and Insulin Resistance." Shoelson, S.E., Lee, J., and Goldfine, A.B. *J. Clin. Invest.* 116:1793-1801, Copyright © 2006. DOI 10.1172/JCI29069.

whereas longer treatment (7-9 days) leads to NO-independent β-cell apoptosis [18, 19]. The ability of cytokines to induce β-cell dysfunction occurs during both short- and long-term treatments [15, 20, 21]. These mechanisms may account for β-cell death and dysfunction observed in both Type 1 Diabetes (T1D) and T2D. In T1D, there are high levels of TNFα, IL-1β, and IFN-γ, which are released by macrophages and other immune cells that infiltrate the pancreas [22]. In T2D, increased adipose tissue can enhance circulating levels of TNFα, as discussed above. In addition, hyperglycemia and elevated free fatty acids (FFAs) lead to elevated release of IL-1β by macrophages at the islets [23].

Glucose-stimulated insulin secretion

Proper regulation of insulin secretion from β-cells is critical for maintaining euglycemia. Insulin secretion is stimulated by elevated glucose levels and follows a biphasic secretion pattern: the initial peak in GSIS occurs minutes after stimulation as a result of the triggering pathway, and is followed by a lower, sustained level of secretion as a product of the amplifying pathway [24]. Glucose is the primary stimulus for insulin secretion, although metabolism of fatty acids and amino acids can also induce insulin secretion. The triggering pathway is initiated when glucose enters the β-cell through the glucose transporter GLUT2 (mouse β-cells) or GLUT1 (human β-cells) [25] where it is rapidly phosphorylated by glucokinase to generate glucose-6-phosphate (G-6-P). G-6-P is then metabolized via glycolysis and enters the mitochondrial tricarboxylic acid (TCA) cycle, which increases the cellular ATP/ADP ratio, leading to closure of the ATP-sensitive K_{ATP} channels and subsequent membrane depolarization.

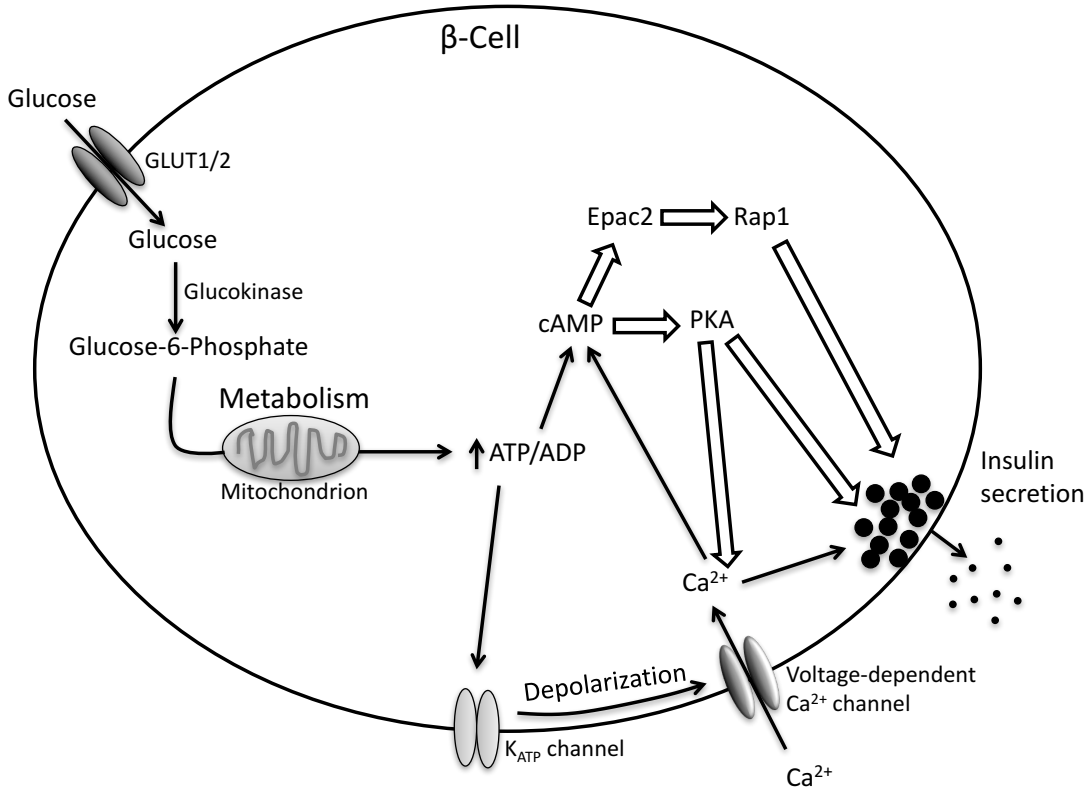

Figure 1-2. Glucose-stimulated insulin secretion pathway. Glucose enters the β-cell via GLUT2 (mouse) or GLUT1 (human) where it is phosphorylated to glucose-6-phosphate (G-6-P) by glucokinase. G-6-P is metabolized in the mitochondria, generating ATP. An increase in the ATP/ADP ratio leads to closure of K_{ATP} channels, membrane depolarization, and subsequent opening of voltage-dependent Ca^{2+} channels. Increased cytosolic Ca^{2+} levels triggers insulin granule release. ATP can also lead to formation of cAMP. cAMP signals via protein kinase A (PKA) or exchange factor directly activated by cAMP (Epac) 2 and can amplify insulin secretion. Hollow block arrows represent components also regulated by PGs that may be involved in altering insulin release. Figure reprinted with permission from "Regulation of pancreatic β-cell function and mass dynamics by prostaglandin signaling." Carboneau, B.A., Breyer, R.M, and Gannon, M. *J. Cell Commun. Signal.* 11[26]: 105-116. Copyright © 2017. DOI 10.1007/s12079-017-0377-7.

Depolarization causes voltage-dependent Ca^{2+} channels to open, resulting in an influx of Ca^{2+} and stimulation of insulin granule exocytosis (Figure 1-2). The amplifying pathway potentiates the effects of the triggering pathway and integrates different metabolic cues, such as free fatty acids, together with endocrine and neuronal signals to adjust insulin secretion [24].

The two phases of insulin secretion occur in a pulsatile manner. Under low glucose conditions (<3 mM), insulin secretion is very low. During stimulatory conditions, insulin secretion increases rapidly and peaks during the first phase and is sustained during the second phase due to secretory pulses of insulin [27]. Pulsatile secretion is driven by oscillations in insulin release due to oscillations in membrane potential, intracellular Ca^{2+}, and cAMP [28, 29]. This requires precise coordination and synchronization between cells within the islet. Gap junctions allow neighboring β-cells to become electrically coupled, which is essential for pulsatile insulin secretion. Mice lacking connexin 36, the pore-forming unit of the islet gap junction, do not have synchronous Ca^{2+} oscillations or insulin secretion, are glucose intolerant, and have a reduction in first phase secretion [30, 31]. Dissociated β-cells exhibit abnormal insulin secretion profiles compared to intact islets [32], further demonstrating the importance of cell-cell contacts in the process of insulin secretion. Additional cell-cell contacts required for normal GSIS include EphA-ephrin-A juxtacrine signaling and neuronal cell-adhesion molecule (NCAM) [33]. Pulsatile insulin release is important as it is more effective in lowering blood glucose than a continuous release of insulin [27]. This pulsatile nature of secretion along with the peak of insulin release during the first phase are lost in the

setting of T2D [34]. Pulsatile intravenous insulin therapy has been postulated to be an improved therapy in diabetes treatment [35].

β-Cell mass dynamics

Information in the following section was gained from studies in rodent models. Pancreatic β-cell mass is determined by the number and size of β-cells. Proper maintenance and expansion of β-cell mass is required in order to maintain euglycemia in the face of increased metabolic demands, including during pregnancy and obesity. Failure to maintain functional β-cell mass can result in the onset of T2D. Evidence from rodent models demonstrates that β-cell mass is a dynamic process that can be regulated by several different mechanisms. β-Cell mass can be increased by proliferation (increased cell number), hypertrophy (increased cell size), or neogenesis (generation of new β-cells from progenitors) and decreased by cell death (apoptosis/necrosis) or dedifferentiation (Figure 1-3) [36]. β-Cell mass expands from embryogenesis to early adulthood as the organism increases in size [37]. Neogenesis occurs during embryogenesis and early postnatal life and contributes to the initial β-cell mass of the organism. However, neogenesis is not thought to occur under normal circumstances beyond the early postnatal period [37]. Instead, adult β-cell mass expands as a result of increased β-cell proliferation [38]. β-cell proliferation is high (approximately 10%) during late embryogenesis [39] but declines postnatally until it reaches a basal level (around 1%), which further declines with age [40, 41]. In addition to this decline in replication with age, the ability of β-cells to respond to proliferative cues deteriorates with age. In young mice (2 months of age), pregnancy, the β-cell toxin

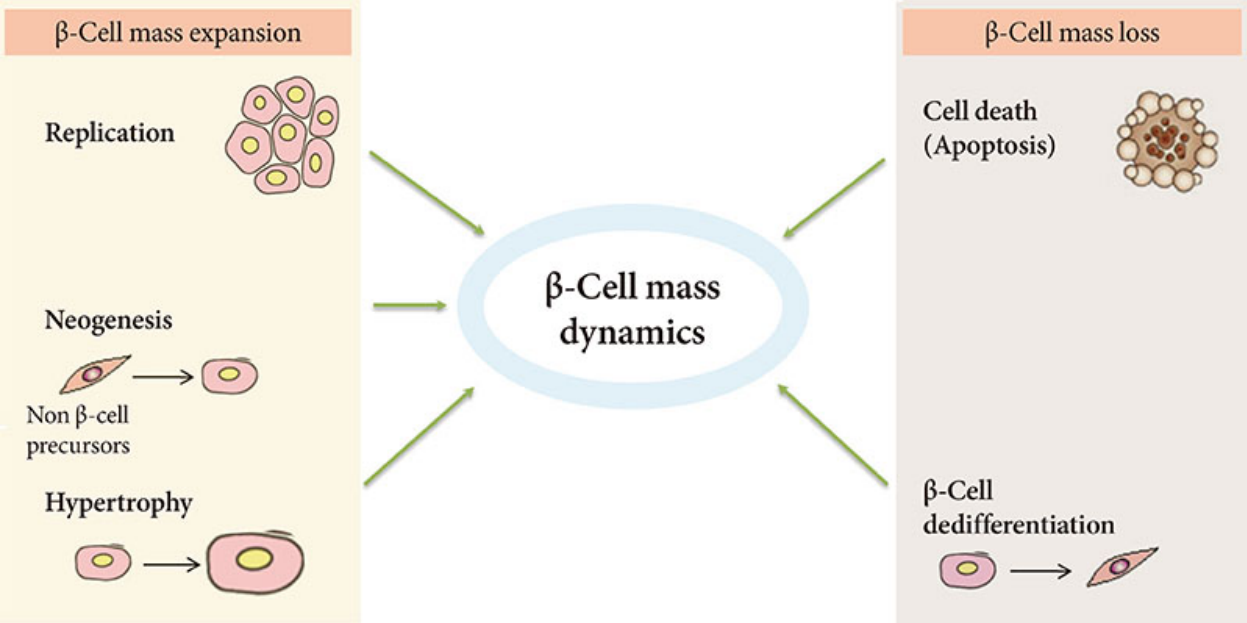

Figure 1-3. Regulation of pancreatic β-cell mass dynamics. β-Cell mass can be expanded (left side) by replication (increased cell number), neogenesis (generation of new β-cells from progenitors), or hypertrophy (increased cell size), and decreased (right side) by cell death (apoptosis/necrosis) or dedifferentiation. Figure reprinted with permission from "Therapeutic Approahces for Preserving or Restoring Pancreatic β-Cell Function and Mass." Jung, K.Y., Kim, K.M, and Lim. S. *Diabetes Metabl. J.* 38(6): 426-436. Copyright © 2014 Korean Diabetes Association. DOI 10.4093/dmj.2014.38.6.426.

streptozotocin (STZ), high fat diet (HFD), glucagon-like peptide-1 receptor (GLP-1R) activation by exendin-4 treatment, and partial pancreatectomy all stimulate a robust increase β-cell proliferation [40, 42]. However, these same stimuli fail to increase proliferation in aged mice: at 8 months of age only partial pancreatectomy can induce replication, and by 14 months of age none of these are able to increase proliferation over basal levels [40]. Since the incidence of T2D increases with age, understanding the signaling mechanisms that drive β-cell proliferation and the changes that occur with age will have important implications on therapeutics intended to enhance functional β-cell mass.

Although β-cell proliferation is low in the adult stage, there are settings that can increase β-cell proliferation in order to expand β-cell mass to compensate for the increased insulin demand and thus maintain euglycemia. Rodent studies have clearly demonstrated that β-cell mass expands in response to HFD-induced obesity or genetic obesity, as observed in the leptin-deficient *ob/ob* strain, due to an increase in β-cell proliferation [43-46]. In wildtype C57Bl/6J mice, the peak of HFD-induced β-cell proliferation occurs after only 3 days on the diet, whereas β-cell mass expansion does not begin until 3 weeks on HFD and increases significantly after 9 weeks [44]. The response to HFD may vary between genetic strains, nutrient composition of the diet, and the timing of diet induction [47]. Nonetheless, it is clear that increased weight gain and subsequent insulin resistance lead to increased β-cell proliferation as a way to expand β-cell mass. In addition, glucose, which is elevated in obesity, is a robust stimulus for β-cell proliferation. When glucose concentrations are elevated, many signaling molecules that regulate cell growth, such as mammalian target of rapamycin

complex 1 (mTORC1), are activated [48]. Thus, hyperglycemia and obesity induce β-cell replication in order to compensate for the increased glucose load.

β-Cell mass expansion is also observed during another state of high metabolic demand: pregnancy. During pregnancy, maternal β-cell mass is increased by β-cell replication and hypertrophy, which peak midway through gestation and return to normal levels soon after birth [49]. Circulating levels of placental lactogens (PLs) are increased during pregnancy and are the primary stimulus of pregnancy-induced β-cell proliferation [37]. PL binds to the prolactin receptor (PRLR) on β-cells and induces signaling through the Janus kinase (JAK) 2 – signal transducer and activation of transcription (STAT) 5 pathway, ultimately increasing expression of many cell cycle activators, such as cyclin D2 [50]. Several additional growth factors and signaling molecules are involved in the increased β-cell proliferation observed during pregnancy [49, 50].

The changes in β-cell proliferation described above are associated with increased expression and activity of many cell cycle regulators. Cell cycle progression depends on the activity of cyclin/cyclin-dependent kinase (Cdk) complexes. Elegant work using many different mouse models has led to the discovery of many key cell cycle regulators involved in β-cell proliferation. Factors involved in the G_1/S checkpoint are at the heart of driving β-cell proliferation (Figure 1-4) [50]. Two G_1/S cell cycle activators, cyclin D2 and Cdk4, are critical regulators of mouse β-cell proliferation. Genetic deletion of either cyclin D2 or Cdk4 results in reduced β-cell mass and impaired β-cell proliferation, ultimately resulting in glucose intolerance and diabetes [51, 52]. Cell cycle inhibitor proteins (Cips, Kips, and INKs) play an equally important role as cell cycle activators in regulating β-cell proliferation. Deletion of the inhibitor $p27^{Kip1}$ increases

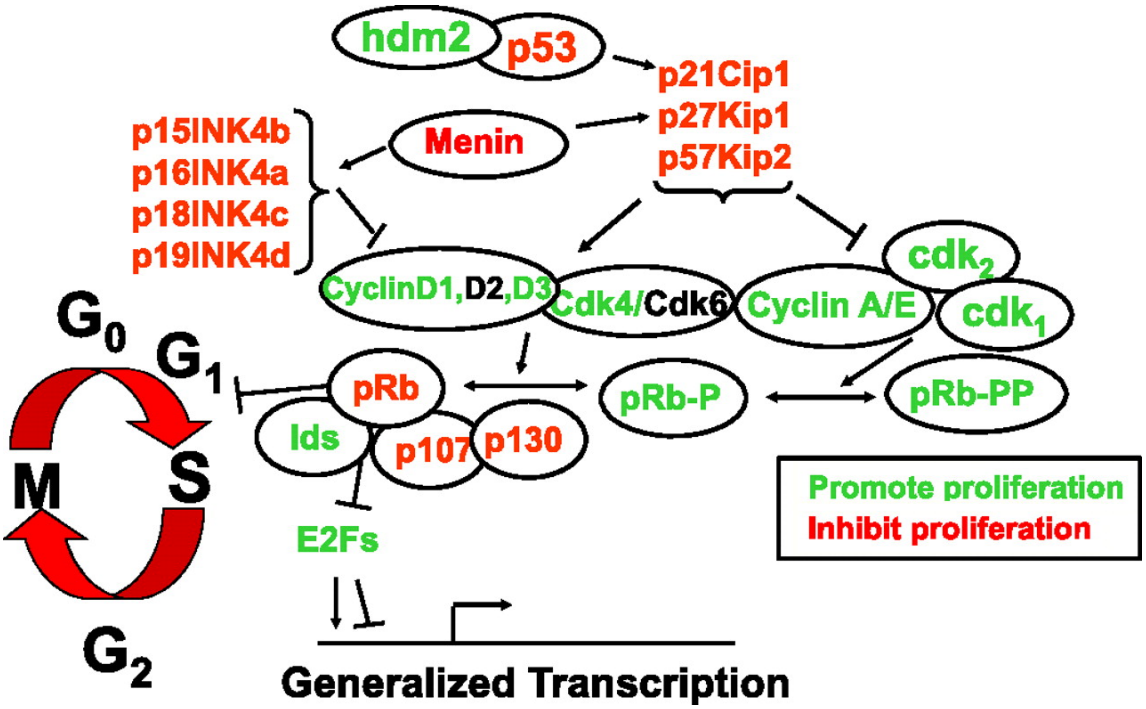

Figure 1-4. The rodent and human β-cell G_1/S molecule road map. These are the ultimate targets of upstream mitogenic signaling molecules. Those that activate cell-cycle progression (cyclins and cyclin-dependent kinases) are shown in green, and those that inhibit cell-cycle progression (the pocket proteins, INK4s, and the CIP/KIP families of cell-cyclin inhibitors) are shown in red. The two wiring diagrams in rodent and human β-cells appear to be similar, with two exceptions (cyclin D2 and cdk6), indicated in black: *1)* human islets have little cyclin D2, whereas this is abundant and critical in rodent β-cells; and *2)* human islets contain cdk6, a cdk that is absent in rodent β-cells. Figure reprinted with permission from "Human β-Cell Proliferation and Intracellular Signaling: Driving in the Dark Without a Road Map." Kulkarni, R.N., Mizrachi, E., Ocana, A.G., and Stewart, A.F. *Diabetes.* 61: 2205-2210. Copyright © 2012. DOI 10.2337/db12-0018.

β-cell proliferation and increases β-cell mass, thus alleviating hyperglycemia in models of T2D [53]. The expression of the cell cycle inhibitor $p16^{Ink4a}$ increases with age in β-cells [54], providing a potential explanation for the decline in β-cell proliferation with age.

Several transcription factors have emerged as critical regulators of cell cycle factors, including Forkhead box M1 (FoxM1). FoxM1 regulates the expression of several key cell cycle proteins, including cyclin B1, Cdk phosphatases Cdc25A and Cdc25B, and S-phase kinase-associated protein 2 (Skp2) and Cdk subunit 1 (Cks1), which together form the Skp1-Cullin1-F-box protein (SCF) ubiquitin ligase complex that is responsible for proteasomal degradation of $p27^{Kip1}$ and $p21^{Cip1}$ [37]. FoxM1 also indirectly regulates the transcription of many other genes important in cell cycle progression. For example, FoxM1 activates *c-Myc* and *Bmi1*, which in turn negatively regulate *Cdkn2a* ($p16^{Ink4a}$) expression [55, 56]. Work from our laboratory has revealed that FoxM1 is required for β-cell proliferation during postnatal growth, pregnancy, and partial pancreatectomy [57-59]. In addition, our group has demonstrated that the expression of *Foxm1* declines with age [60], providing an additional explanation for the decline in β-cell proliferation with age. Induction of an activated form of FoxM1 specifically in β-cells, in a model called β-FoxM1*, counteracts the age-related impairment in β-cell proliferation [60]. In addition, activation of FoxM1 results in protection against STZ- and cytokine-induced β-cell death [61], demonstrating that factors that enhance β-cell proliferation can also play important roles in regulating β-cell survival.

β-Cell death is an important mechanism in maintenance of β-cell mass and enhanced cell death can result in impaired β-cell mass. During embryogenesis, the

levels of β-cell apoptosis are very low. Apoptosis remains low in the adult stage, in which β-cell mass is fairly stable and unchanged [62]. There is an increase in apoptosis following pregnancy in rodents, thus returning β-cell mass to pregestational levels [63]; however, it is unclear if this occurs in human β-cells following pregnancy [49]. β-Cell apoptosis is increased following chronic high glucose exposure, a concept known as glucotoxicity. Glucotoxicity results in elevated inflammatory cytokine production and increased oxidative stress, which leads to β-cell death [64]. Glucotoxic-induced β-cell death ultimately leads to loss of β-cell mass and is thought to be a contributing factor to T2D progression [65].

The data discussed above primarily come from studies in rodent models. Studies on human β-cell mass dynamics are more difficult to perform. Human autopsy studies reveal that non-diabetic obese individuals have higher β-cell mass than lean non-diabetic subjects [66, 67] while β-cell mass is reduced in humans with T2D in both lean and obese settings [67]. These data suggest that β-cell compensation occurs during obesity in humans and loss of β-cell mass contributes to disease onset, as has been observed in rodent models. However, lack of reliable technology to longitudinally measure β-cell mass in living humans makes it difficult to assess the true dynamics of β-cell mass in humans. It is possible that some individuals are born with higher β-cell mass than others, thus protecting them against metabolic stressors later in life. It is also plausible that there are individuals who fail to adapt and properly expand β-cell mass in response to metabolic stress and therefore develop T2D. Finally, a third scenario is that humans can increase β-cell mass in response to obesity, yet certain individuals have

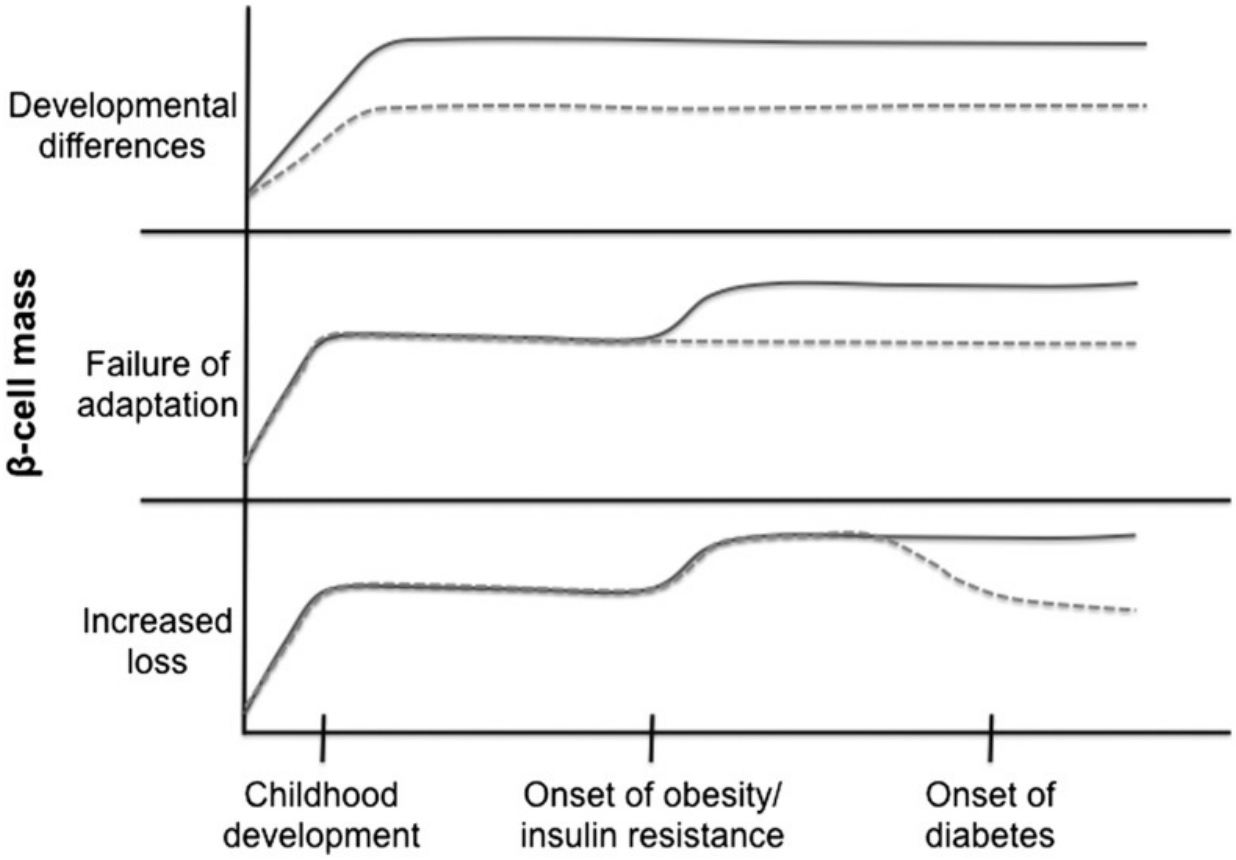

Figure 1-5. Three possible timelines resulting in diminished β-cell mass in T2D at the time of autopsy. In the top panel, a failure to achieve adequate β-cell mass during development or early childhood expansion results in decreased β-cell mass that persists throughout life, increasing susceptibility to T2D. In the middle panel, a failure to expand β-cell mass in adult life in response to obesity and insulin resistance results in failure to produce adequate insulin and the development of T2D. In the bottom panel, compensatory expansion occurs, but then there is increased loss of β-cells that results in lower β-cell mass when measured at autopsy. The dotted lines represent the timeline in an individual with increased susceptibility to diabetes. Figure reprinted with permission from "Pancreatic β-Cell Proliferation in Obesity." Linnemann, A.K., Baan, M., and Davis, D.B. *Adv. Nutr.* 5: 278-288. Copyright © 2014. DOI 10.3945/an.113.005488.

increased β-cell death, leading to T2D onset (Figure 1-5) [47]. Indeed, autopsy studies reveal that β-cell death is increased in humans with T2D [67], which may contribute to the failure to maintain proper β-cell mass.

In addition to limitations in studying human β-cell mass expansion, many factors that stimulate rodent β-cell proliferation do not induce human β-cell replication. The GLP-1R agonist exendin-4 results in a robust increase in mouse β-cell proliferation [68], yet the effect on human β-cells is controversial [69]. In addition, growth factors, such as hepatocyte growth factor (HGF), or lactogens, including PL, stimulate rodent, but not human, β-cell proliferation [50]. Despite being rather recalcitrant to proliferative stimuli, there are factors, such as cell cycle proteins and small molecules, that have been shown to induce human β-cell replication. Short hairpin (sh) RNA-mediated suppression of the cell cycle inhibitor $p57^{Kip2}$ induced more than a 3-fold increase in human β-cell proliferation. Interestingly, loss of $p57^{Kip2}$ increased replication in human islets from older donors, indicating that older, quiescent β-cells can still re-enter the cell cycle under the right conditions [70]. The small molecule harmine has also been shown to promote human β-cell replication. Harmine inhibits dual-specificity tyrosine-regulated kinase-1a (DYRK1A), an endogenous inhibitor of the cell cycle regulator nuclear factor of activated T cells (NFAT), in order to allow proliferation to progress [71]. However, harmine and its derivatives also stimulate α-cell proliferation. Therefore, identification of factors or small molecules that can induce human β-cell, but not α-cell, proliferation will have important therapeutic advantages over the currently available compounds.

Pancreas-specific Cre models

The Cre-*loxP* system of genetic recombination has been a powerful tool in the discovery of factors that regulate β-cell development, replication, and mass expansion. The P1 bacteriophage-derived Cre recombinase (Cre) protein excises DNA regions flanked by two *loxP* sequences (34 nucleotide-long DNA sequences). There are numerous mouse models currently available and widely used in which pancreas- and endocrine cell-type-specific promoters have been used to drive expression of Cre, allowing for cell- or tissue-specific recombination of DNA segments of interest (reviewed in [72]). This system has been refined to incorporate temporal control of Cre activity using Cre^{ERT2} (referred to herein as Cre^{ER}) for inducible recombination. Cre^{ER} is a fusion protein consisting of Cre and a mutated, tamoxifen (TM)-responsive estrogen receptor (ERT2) that is insensitive to endogenous 17β-estradiol [73, 74]. In the absence of TM, Cre^{ER} is sequestered in the cytoplasm; but upon TM binding, activated Cre^{ER} enters the nucleus and mediates recombination at *loxP* sites.

A majority of the available β-cell type-specific Cre/Cre^{ER} models utilize the rodent *Insulin* promotor to drive expression of Cre in the β-cells. Two of the most commonly used models use the rat *Insulin2* promotor to drive Cre expression, called RIP-$Cre^{Magnuson}$ and RIP-$Cre^{Hererra}$ [75, 76]. Here, Cre is expressed in more than 80% of β-cells, yet the RIP-$Cre^{Magnuson}$ line displays leaky expression in the brain [77]. Several years ago, our group and others reported that some “pancreas-specific” Cre transgenes were also expressed in key brain regions involved in food intake and energy expenditure, complicating their use in metabolic studies, including the RIP-$Cre^{Magnuson}$ model [77].

The generation of the MIP-Cre^{ER} transgene, in which brain recombination did not occur, was a critical development in the field [78]. In this model, the mouse *Insulin1* promotor (MIP) drives expression of inducible Cre^{ER} in β-cells. However, several recent studies, including our own, have uncovered several unexpected phenotypes of this mouse line, which will be discussed in more detail in Chapter III.

Recently, two new β-cell-specific Cre models were generated, called Ins1^{Cre} and Ins1^{CreER}. In these models, the Cre cDNA has been inserted into the initiation codon of the mouse *Insulin1* gene. Importantly, Cre recombination has not been demonstrated in the brain nor have any unexpected phenotypes been uncovered to date [79]. The utility of the MIP-Cre^{ER} and Ins1^{Cre} models to generate EP3 and EP4 β-cell-specific knockouts will be discussed further in Chapter III.

In addition to β-cell-specific Cre transgenes, several Cre models exist to allow for specific recombination within the other endocrine cell types, including α, δ, ε, and pancreatic polypeptide (PP) cells. α-Cell-specific models have been generated making use of the rat glucagon (Gcg) promotor, resulting in two different lines called Gcg-Cre^{Herr} and Gcg-Cre^{Slib} [72]. The rat PP promotor has been used to successfully generate a PP transgenic strain, but this line induces recombination in both PP and β-cells [80]. Finally, the somatostatin (*Sst*) and ghrelin (*Grhl*) genes have been used to generate δ- and ε-cell-specific Cre transgenic models, respectively [72].

Current therapies to treat T2D

The primary goal of current treatments for T2D is to reduce blood glucose levels, as assessed by glycated hemoglobin (hemoglobin A_{1C}; HbA_{1C}) [81]. This can be achieved through several different mechanisms; including, improving β-cell function (insulin secretion) using secretagogues, enhancing the action of insulin in target tissues using insulin sensitizers, or by exogenous insulin administration [36]. Several different classes of drugs that lower blood glucose are currently used in the treatment of T2D, including biguanides, sulfonylureas, thiazolidinediones (TZDs), meglitinides (glinides), α-Glucosidase inhibitors, bile acid sequestrants, sodium-glucose co-transporter 2 (SGLT2) inhibitors, dipeptidyl peptidase (DPP)-4 inhibitors, GLP-1R agonists, dopamine-2 receptor agonists, amylin mimetics, and insulins (Figure 1-6) [81]. Several of the more commonly used drugs will be discussed in detail below.

The biguanide metformin is the first-line treatment for T2D due to its superior safety record, low cost, weight neutrality, and potential cardiovascular benefits [81]. Metformin acts to reduce blood sugar levels by increasing hepatic insulin sensitivity (reducing glucose output) and enhancing glucose uptake in peripheral tissues, particularly skeletal muscle [82]. The effects of metformin on hepatic gluconeogenesis occur through several different mechanisms: 1) activation of liver kinase B1 (LKB-1) and downstream transcriptional repression of gluconeogenesis enzymes [83]; 2) inhibition of complex I of the mitochondrial respiratory chain which decreases ATP production [82]; 3) activation of the AMP-activated protein kinase (AMPK) and downstream inhibition of cAMP response element binding protein (CREB)-regulated transcription coactivator 2 (CRTC2), leading to decreased expression of gluconeogenic genes [82]; and 4) AMPK-

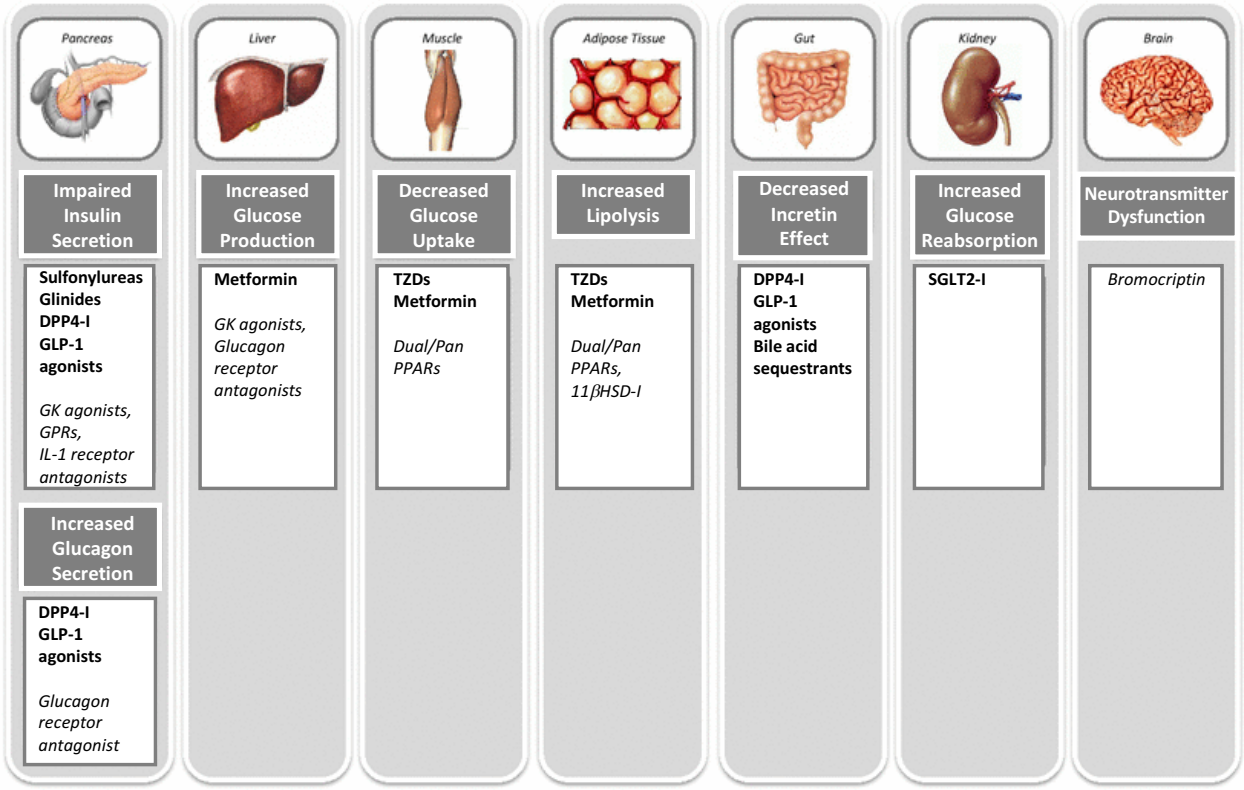

Figure 1-6. Current treatments for T2D. Treatment of T2D is becoming more targeted on the specific mechanisms contributing to hyperglycemia, disease progression, and development of complications. This figure summarizes the available (bold text) and potential future (italicized text) pharmacological agents based on the respective target tissues/organs. DPP-4I: dipeptidyl peptidase-4 inhibitors; GK: glucokinase; GLP: glucagon-like peptide; IL: interleukin; PPAR: peroxisome proliferator-activated receptor; SGLT2-I: sodium-glucose co-transporter-2 inhibitors; TZD: thiazolidinediones. Figure reprinted with permission from "Early Combination Therapy with Oral Glucose-Lowering Agents in Type 2 Diabetes." Bianchi, C., Daniele, G., Dardano, A., Miccoli, R., and Del Prato, S. *Drugs*. 77:247-264. Copyright © 2017. DOI 10.1007/s40265-017-0694-4.

independent mechanisms [82]. Metformin has been shown to improve glycemia in pre-diabetic patients [81]. The Diabetes Prevention Program (DPP) trial found that metformin reduced the incidence of T2D by 31% [84] and the diabetes incidence 10 years after the conclusion of study was reduced by 18% compared to placebo [85].

There are individuals who do not respond to metformin or cannot tolerate the drug, therefore several other second-line treatments are available [81]. Sulfonylureas are another commonly prescribed class of T2D drugs and are often used in combination with metformin to reduce HbA_{1C} levels [83]. The main action of sulfonylureas, such as glibenclamide, is to raise plasma insulin levels by stimulating β-cell insulin secretion [86]. Sulfonylureas bind to the sulfonylurea receptor (SUR1), which is part of the ATP-sensitive K_{ATP} channel. Binding of sulfonylureas to SUR1 blocks K_{ATP} channels, resulting in membrane depolarization and insulin secretion [87]. The combination of metformin + sulfonylureas has been shown to be more effective at controlling glycemia than either treatment alone due to their different mechanisms of action [88]. However, sulfonylureas have been associated with hypoglycemic events and weight gain [89].

TZDs, such as pioglitazone, enhance insulin action on the liver and improve insulin sensitivity in peripheral tissues, including adipose tissue and skeletal muscle, thus decreasing hyperglycemia and improving metabolic control in individuals with T2D [88, 90]. TZDs are agonists of the peroxisome proliferator-activated receptor-γ (PPARγ), which regulates genes involved in glucose and lipid metabolism [90]. Additional advantages of TZDs include drug durability, decreased triglyceride levels, and no hypoglycemic events. However, TZDs have been shown to cause weight gain and peripheral edema, increase the incidence of heart failure, and cause bone fractures in

women [81]. While caution should be used when prescribing TZDs, they may be an appropriate alternative therapy when metformin is not tolerated [88].

A newer class of glucose-lowering drugs is SGLT2 inhibitors, which targets the sodium-glucose co-transporter 2 (SGLT2) in the kidney. SGLT2 inhibitors are approved for monotherapy but are often used in combination with metformin and/or other drugs [81]. SGLT2 inhibitors act to inhibit SGLT2 in the renal proximal tubules, which results in decreased glucose reabsorption and increased urinary glucose excretion, thus decreasing blood sugar levels [81, 88]. SGLT-2 inhibitors have been shown to confer modest weight loss and decrease blood pressure [81]; however, they can stimulate hepatic glucose production and elicit a glucagon response [88]. The combination of SGLT2 inhibitors and metformin has been shown to reduce HbA_{1C} levels more effectively than either treatment alone [88].

Incretin-based therapies targeting the GLP-1R, such as exenatide, have been used clinically for over a decade. GLP-1R is a G_S-coupled G-protein coupled receptor (GPCR). Interestingly, drugs targeting GPCRs represent 50-60% of all drugs currently on the market [91], yet GLP-1R is the only GPCR-based T2D therapy to date. GLP-1 potentiates GSIS through cAMP signaling mechanisms, including protein kinase A (PKA) and exchange protein activated by cAMP (Epac). In patients with T2D, GLP-1R agonists have been shown to increase GSIS, decrease glucagon secretion, slow gastric emptying, and increase satiety [81]. Additional advantages of GLP-1-based therapies include no hypoglycemic events, modest weight loss, and a decrease in some cardiovascular risk events. However, these therapies do not work effectively in all

individuals with T2D [92] and there are several safety concerns surrounding these drugs, particularly concerning pancreatitis and gastrointestinal distress [81, 93].

Endogenous GLP-1 is rapidly degraded by the enzyme DPP-4, which results in a very short half-life for GLP-1. DPP-4 inhibitors block the activity of endogenous DPP-4, thereby increasing the half-life of active GLP-1 [36, 81]. In patients with T2D, DPP-4 inhibitors, such as sitagliptin, are generally well-tolerated, increase GSIS and decrease glucagon secretion, similar to GLP-1R agonists. The effects of DPP-4 inhibitors on cardiovascular events are controversial, though studies have observed an increase in hospitalizations due to heart failure. In addition, DPP-4 inhibitors are associated with an increased risk of pancreatitis, like GLP-1R agonists [81].

Islet G-proteins and GPCRs

GPCRs are membrane-spanning receptors that consist of seven transmembrane α-helices, three hydrophilic intracellular loops, three hydrophilic extracellular loops, an extracellular amino (N)-terminal end, and an intracellular carboxy (C)-terminal end. GPCRs signal through heterotrimeric G-proteins and β-arrestins. G-proteins are composed of α, β, and γ subunits. In the absence of a ligand, GDPα is associated with the β and γ subunits in an inactive state. Upon ligand binding to the extracellular N-terminal domain of the GPCR, the receptor undergoes conformational change, which catalyzes the exchange of GTP for GDP in the α subunit, thus releasing the active GTPα subunit from the β and γ subunits, allowing downstream signaling pathways to be activated (Figure 1-7) [69, 94]. The α and $\beta\gamma$ subunits are independently able to activate distinct downstream signaling pathways following ligand binding. There are four major

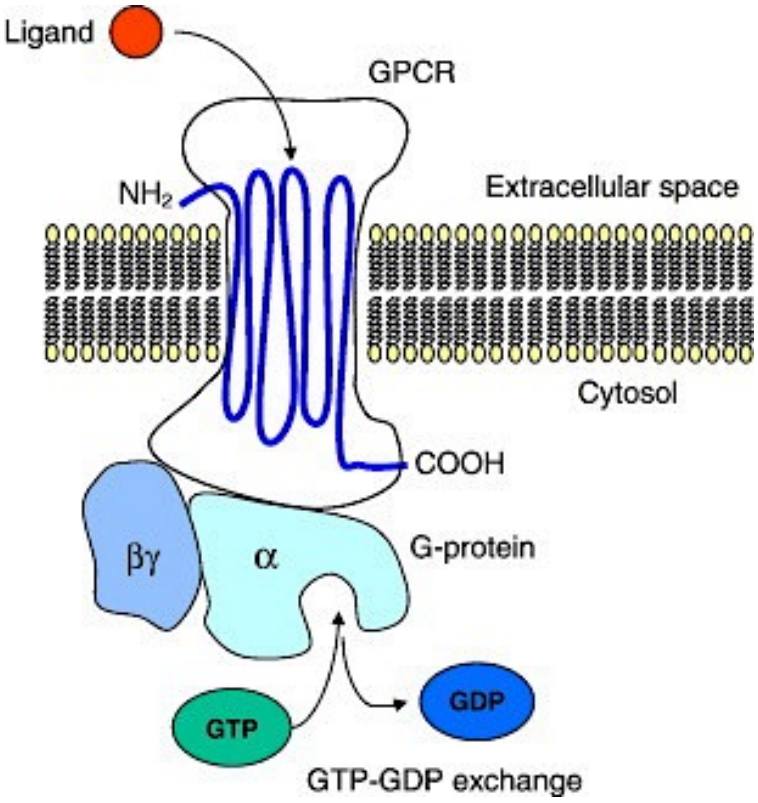

Figure 1-7. Schematic representation of a generic GPCR protein, with the 7-transmembrane-spanning domains, coupled to an intracellular G-protein. The G-protein consists of three subunits, α, β, and γ. Upon GPCR activation, GDP is exchanged against GTP, which dissociates the G-protein complex into two units, the α and $\beta\gamma$ subunits. These subunits in turn activate or inhibit downstream enzymes. Figure reprinted with permission from "G-protein-coupled receptors and islet function – Implications for treatment of type 2 diabetes." Winzell, M.S., and Ahrén, B. *Pharmacology & Therapeutics.* 116: 437-448. Copyright © 2007. DOI 10.1016/j.pharmthera.2007.08.002.

classes of G-proteins: G_S, G_i, G_q, and $G_{12/13}$ [95]. The G_S family increases intracellular cAMP levels by activating adenylyl cyclase (AC), which activates PKA and Epac. In contrast, the G_i family, which is made up of G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2}, and G_Z [93], inhibits AC, thereby decreasing intracellular cAMP concentrations. The G_q family activates phospholipase C (PLC)-β to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), resulting in increased intracellular Ca^{2+} and activation of protein kinase C (PKC), respectively. The $G_{12/13}$ family activates the small GTPase Rho, which is involved in actin cytoskeletal remodeling. In addition to activating G-protein signaling mechanisms, GPCRs can also signal via β-arrestins. β-Arrestins are involved in termination of GPCR signaling by uncoupling GPCRs from G-proteins, but also form scaffolds to activate alternative signaling pathways, such as extracellular signal-related kinase (ERK) 1/2 and JNK [69].

Pancreatic islets express over 300 GPCRs, including those coupled to G_S, G_i, and G_q proteins [96]. Islet GPCRs and G-proteins have been implicated in regulating β-cell function (insulin secretion) and/or β-cell mass. Islet GPCRs have primarily been studied for their ability to modulate insulin secretion. Many G_S-coupled GPCRs, such as GLP-1R and the lipid activated GPR119 receptor, and G_q-coupled GPCRs, including the acetylcholine M3 receptor, stimulate GSIS whereas G_i-coupled GPCRs, including the somatostatin (SST) and α_2-adrenergic receptors, inhibit GSIS (Figure 1-8) [94]. Several islet GPCRs have also been shown to regulate β-cell mass dynamics, including β-cell proliferation and survival. The G_S-coupled GLP-1R has been implicated in stimulating mouse β-cell proliferation and decreasing apoptosis, however the effect of GLP-1R on these mechanisms in human islets is less clear [69]. In contrast to G_S-GPCRs,

activation of G_i-coupled receptors negatively regulates β-cell proliferation. For example, the SST receptor inhibits *ex vivo* β-cell proliferation in both mouse and human islets [97].

The G-proteins found in islets have also been implicated in regulating β-cell function and β-cell mass dynamics. Interestingly, pertussis toxin (PTx), which blocks the action of inhibitory G-proteins excluding G_Z by ADP-ribosylation on a critical cysteine residue [98], was originally called islet-activating protein (IAP) due to its ability to reverse α-adrenergic inhibition of cAMP and to enhance insulin secretion from islets [99]. Blocking G_i signaling in the β-cell using an inducible mouse model that expresses PTx in islets resulted in improved glucose homeostasis, enhanced plasma insulin during a glucose challenge, increased β-cell area, and an increased percentage of cells in the G_2/M phase of the cell cycle [100]. Furthermore, mice with a global deletion of another inhibitory G-protein, G_Z, displayed improved glucose homeostasis due to an increase in plasma insulin levels, enhanced GSIS, increased islet cAMP production, increased β-cell proliferation and mass, and protection against STZ-induced hyperglycemia due to increased proliferation and decreased β-cell death [101-103]. In contrast, β-cell-specific deletion of G_S resulted in impaired glucose homeostasis, severe hyperglycemia and hypoinsulinemia, reduced islet insulin content, decreased islet area, decreased β-cell proliferation, and increased β-cell death [104]. Taken together, these data demonstrate that the inhibitory G-proteins and their receptors suppress insulin secretion, β-cell mass expansion, β-cell proliferation, and β-cell survival; whereas G_S-GPCRs enhance GSIS, β-cell proliferation, and β-cell survival, thus increasing functional β-cell mass.

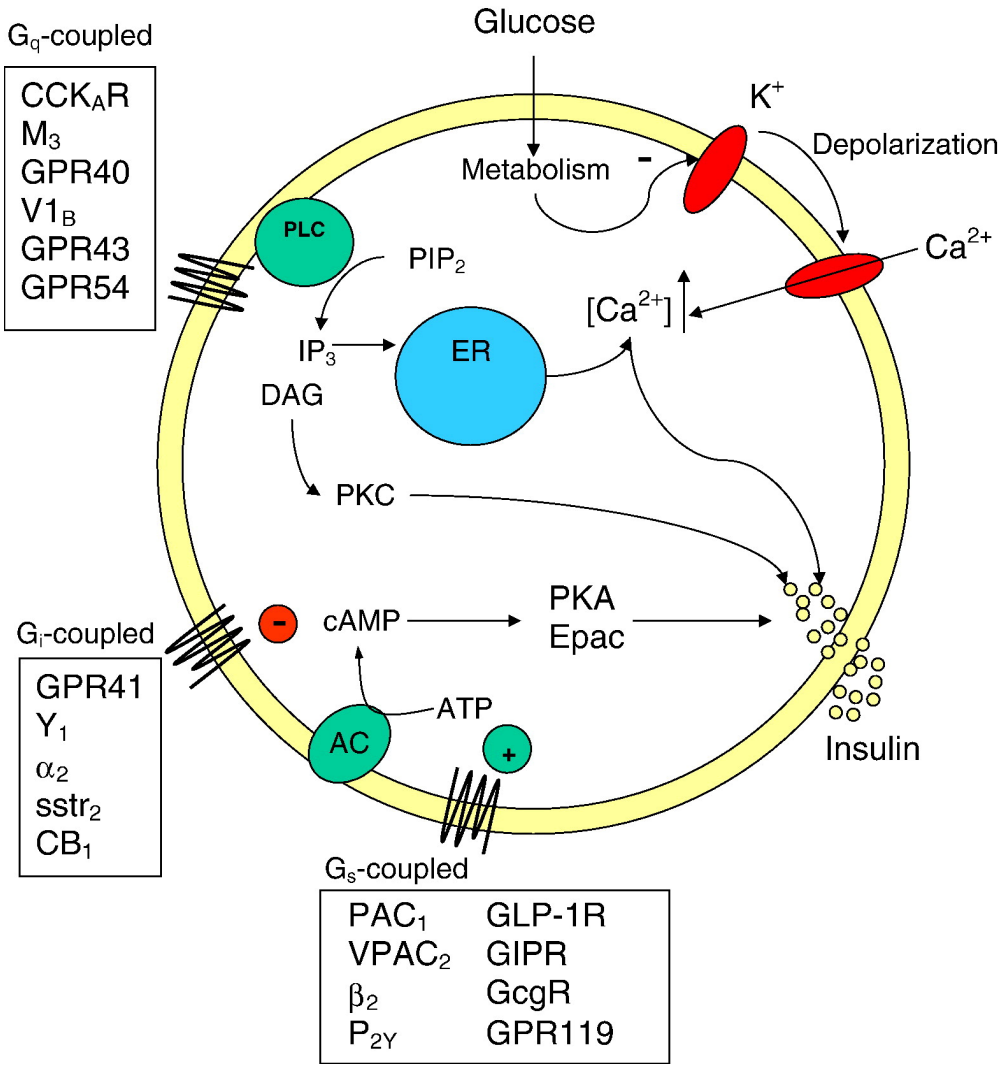

Figure 1-8. Schematic representation of selected islet GPCRs and their main secretory signaling pathways in β-cells. The G_S family increases intracellular cAMP levels by activating adenylyl cyclase (AC), which activates protein kinase A (PKA) and exchange protein activated by cAMP (Epac); whereas the G_i family inhibits AC, thereby decreasing intracellular cAMP concentrations. The G_q family activates phospholipase C (PLC)-β to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), resulting in increased intracellular Ca^{2+} and activation of protein kinase C (PKC), respectively. Examples of islet GPCRs that couple to the different families are indicated in the boxes. Figure reprinted with permission from "G-protein-coupled receptors and islet function – Implications for treatment of type 2 diabetes." Winzell, M.S., and Ahrén, B. *Pharmacology & Therapeutics.* 116: 437-448. Copyright © 2007. DOI 10.1016/j.pharmthera.2007.08.002.

Prostaglandins

Prostaglandin production

Eicosanoids, biologically active metabolites of the membrane lipid arachidonic acid (AA), play important roles in the pathogenesis of insulin resistance and T2D [105]. AA is metabolized into eicosanoids by three major pathways, which include the activity of COX, lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes. The COX-derived eicosanoids, called prostaglandins (PGs), are important lipid signaling molecules that mediate an array of physiological functions, including inflammation, and positively or negatively regulate insulin secretion from pancreatic β-cells [106, 107]. PGs are produced in a multi-step process (Figure 1-9). The first step involves release of AA from plasma membrane phospholipids by the action of phospholipase A_2 (PLA_2), which recognizes and hydrolyzes sn-2 acyl bonds of phospholipids, releasing AA. This is the rate-limiting step in PG synthesis [108]. The next steps in PG synthesis require the activity of the constitutively active COX-1 or the inducible COX-2. It has been demonstrated that COX-2 is predominantly expressed in islets [109, 110]. AA is first oxidized by COX-1 or -2 to generate PGG_2, and then undergoes reduction by COX-1 or -2 to form the unstable metabolite PGH_2. The final step in PG synthesis involves the activity of PG synthases on PGH_2 to form the five main PG family members: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and TXA_2 (thromboxane). These signaling molecules exert their action on the cell by signaling through their respective GPCRs, called DP1 and DP2 (also known as CRTH2), EP1-4, FP, IP, and TP [106].

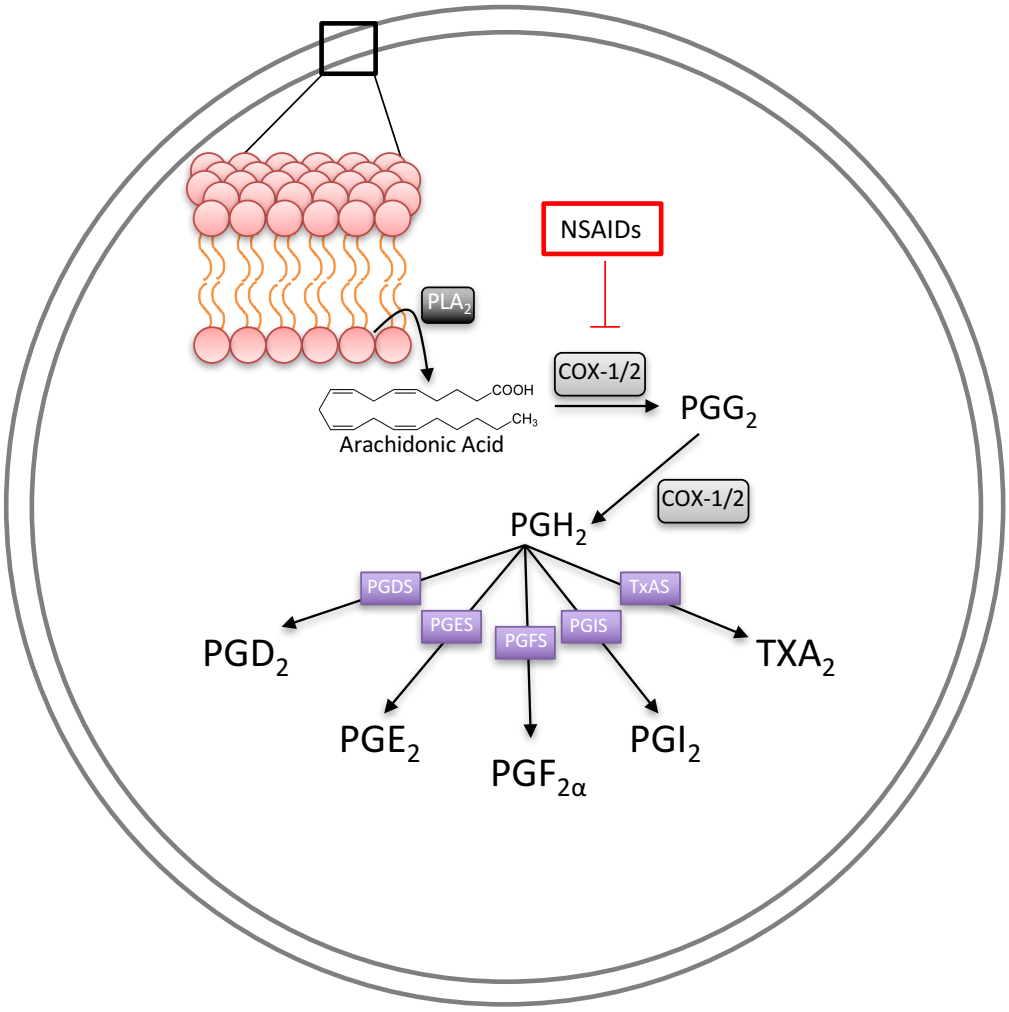

Figure 1-9. Prostaglandin synthesis. Arachidonic acid is released from plasma membrane phospholipids by the action of phospholipase A_2 (PLA_2) and is then metabolized by cyclooxygenase (COX)-1 or -2 to the unstable metabolite PGH_2, which serves as the precursor for the five main prostaglandin (PG) members. Activity of specific synthases for each individual PG (PGDS, PGES, PGFS, PGIS or TxAS) leads to generation of the five principle PGs: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and Thromboxane (TX) A_2. Non-steroidal anti-inflammatory drugs (NSAIDs) block the activity of COX-1 and -2. Figure modified and reprinted with permission from "Regulation of pancreatic β-cell function and mass dynamics by prostaglandin signaling." Carboneau, B.A., Breyer, R.M, and Gannon, M. *J. Cell Commun. Signal.* 11[26]: 105-116. Copyright © 2017. DOI 10.1007/s12079-017-0377-7.

Prostaglandins and PG receptors in the islet

Studies using radiolabeled AA demonstrated that PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 are all produced in rat islets [111-113]. Subsequent work by Vennemann and colleagues confirmed that these PG products are synthesized in mouse pancreata and demonstrated that production increases following treatment with STZ, a β-cell toxin [114]. This group also revealed that PGE_2 is the main PG produced in mouse pancreatic tissue [114]. In addition to being induced by STZ, PGE_2 and PGI_2 production are increased in mouse and human islets by high glucose culture conditions [115-117]. PGs have very short half-lives and thus act locally in an autocrine or juxtacrine manner to signal through their respective receptors [106, 118].

The receptors for each of the PGs are expressed in immortalized β-cell lines, rodent islets, and human islets. PGD_2 binds and signals through two different receptors called DP1 and DP2 (also known as Prostaglandin D_2 Receptor 2 (PTGDR2), chemoattractant receptor-homologous expressed on Th2 lymphocytes receptor (CRTH2), CD294, or GPR44) [106, 119]. DP1 and DP2 are expressed in islets and DP2 has endocrine-specific expression in human tissues [119-121]. The DP1 receptor couples to the stimulatory G_S-protein leading to increases in intracellular cAMP, whereas the DP2 receptor couples to inhibitory G-proteins and decreases cAMP [106]. PGE_2 signals through four receptors called EP1-4. EP1 couples to G_q, resulting in increases in intracellular Ca^{2+} levels. EP2 and EP4 couple to G_S whereas EP3 primarily couples to the G_i family, including G_i- and G_Z-proteins [93, 106]. PGE_2 binds with highest and equal affinity to EP3 and EP4 and lower affinity to EP1 and EP2 [122]. The PGE_2 synthase genes (*Ptges1-3*) as well as the receptor genes (*Ptger1-4*) are all

expressed in both mouse and human islets [110, 114, 120, 123, 124]. However, recent RNA-sequencing (RNA-seq) failed to detect the expression of *Ptger2* (EP2) in mouse islets [124]; thus further research is needed to clarify these discrepancies. $PGF_{2\alpha}$ binds to the FP receptor, which signals through the G_q-protein resulting in a rise in intracellular Ca^{2+} levels [106]. RNA-seq revealed that the FP receptor gene (*PTGFR*) is expressed in human islets [120]. PGI_2, also known as prostacyclin, signals through the IP receptor which primarily couples to G_S [106]. Expression of the PGI_2 synthase PGIS (*Ptgis*) and IP has been detected in β-cell lines, rat islets, and human islets [120, 125]. The TXA_2 receptor TP (*TBXA2R*) and synthase (*TBXAS1*) are expressed in human islets [120], yet there is no known role of TXA_2 in either β-cell function or mass dynamics.

Prostaglandins and diabetes

In 1876, Ebstein noted that the NSAID sodium salicylate, which inhibits COX activity, reduced the amount of glucose present in urine samples from patients with diabetes [4, 107]. As discussed previously, NSAIDs were historically used to treat diabetes [5]. In 1974, nearly 100 years after Ebstein's observations, Burr and Sharp demonstrated that PGE_1 inhibited GSIS by perifusion assay in rat islets [126], thus providing a potential explanation for Ebstein's early observations. Increased levels of mRNA and proteins associated with PG production have also been associated with T2D. The expression of *Ptgs2* (COX-2) can be increased by several different means: 1) by IL-1β treatment in the RIN 832/13 β-cell line [127], and rodent and human islets [109, 128, 129]; 2) in islets from the T2D *db/db* mouse model [116]; and 3) by hyperglycemia in rodent and human islets [115, 116]. Similarly, PGE_2 production is induced by IL-1β

and hyperglycemia in β-cells [110, 116, 129, 130] and is increased in T2D mouse and human islets [123]. These data unveil an interesting link between obesity, T2D, and PG signaling. Intriguingly, two single nucleotide polymorphisms (SNPs) in the *PTGS2* (COX-2) gene have been associated with T2D risk in Pima Indians [131], suggesting that COX-2 may play a role in the onset of T2D.

Prostaglandins and β-cell function

Based on the primary signaling mechanisms of the PG receptors (cAMP, Ca^{2+}), and the known mechanisms regulating GSIS (Figure 1-2), one might predict that PGs would function in regulation of insulin secretion. There are no reports to date implicating a role for either PGD_2 or $PGF_{2\alpha}$ in GSIS. One group found that PGD_2 administration promoted glucagon secretion, but had no impact on insulin secretion [132]. Therefore, only the roles of PGI_2, PGE_2, and their respective receptors in regulating GSIS will be discussed in this section.

PGI_2 and β-cell function

Early studies in the late 1970s and 1980s suggested that PGI_2 plays little to no role in altering GSIS. In perfused rat pancreata, PGI_2 did not affect insulin or glucagon secretion [132]. Similarly, in healthy human males, a two-hour PGI_2 infusion, at a dose sufficient to cause changes in platelets and vasculature, did not alter GSIS or glucose disposal during the course of the study [133]. A longer PGI_2 infusion of 48 hours in

patients with T2D that were being treated for vascular disease resulted in hyperglycemia but did not impact plasma insulin levels in response to glucose [134]. However, in non-diabetic participants, the results were variable with PGI_2 infusion decreasing, increasing, or not affecting GSIS [134]. To further investigate the hyperglycemic effect observed in patients with T2D, Sieradzki and colleagues performed GSIS studies in isolated rat islets using low, medium, and high concentrations of PGI_2 [135]. The low concentration of PGI_2 (2.7 nM) had no effect on GSIS in low or high glucose. The mid-range dose of PGI_2 (53.8 nM) initially stimulated but then decreased GSIS in high glucose conditions. The highest dose of PGI_2 (267 nM) resulted in inhibition of GSIS. These results suggest that there is a dose-dependent effect of PGI_2 on insulin release in isolated islets. Alternatively, it is possible that PGI_2 has off-target effects when used at high concentrations.

More recently, PGI_2 has been shown to play a positive role in stimulating insulin release in β-cell lines. Interestingly, high glucose increases the expression of *Ptgis* and PGIS in rat islets suggesting that PGI_2 has a role in GSIS [117]. Overexpression of PGIS in the rat INS-1E β-cell line enhances GSIS during high glucose stimulation, but does not have an effect under substimulatory glucose conditions. The IP agonist iloprost also increased GSIS in INS-1E cells while the PGI_2 antagonist CAY10441 decreased insulin release in PGIS-overexpressing cells [117]. The mechanism underlying the PGIS-potentiated GSIS was not due to signaling via the PKA pathway but rather through the Epac2 pathway (Figures 1-2 and 1-10) [117]. Epac2 converts inactive GDP-Rap1 to active GTP-Rap1, which initiates downstream signaling, and potentiates GSIS

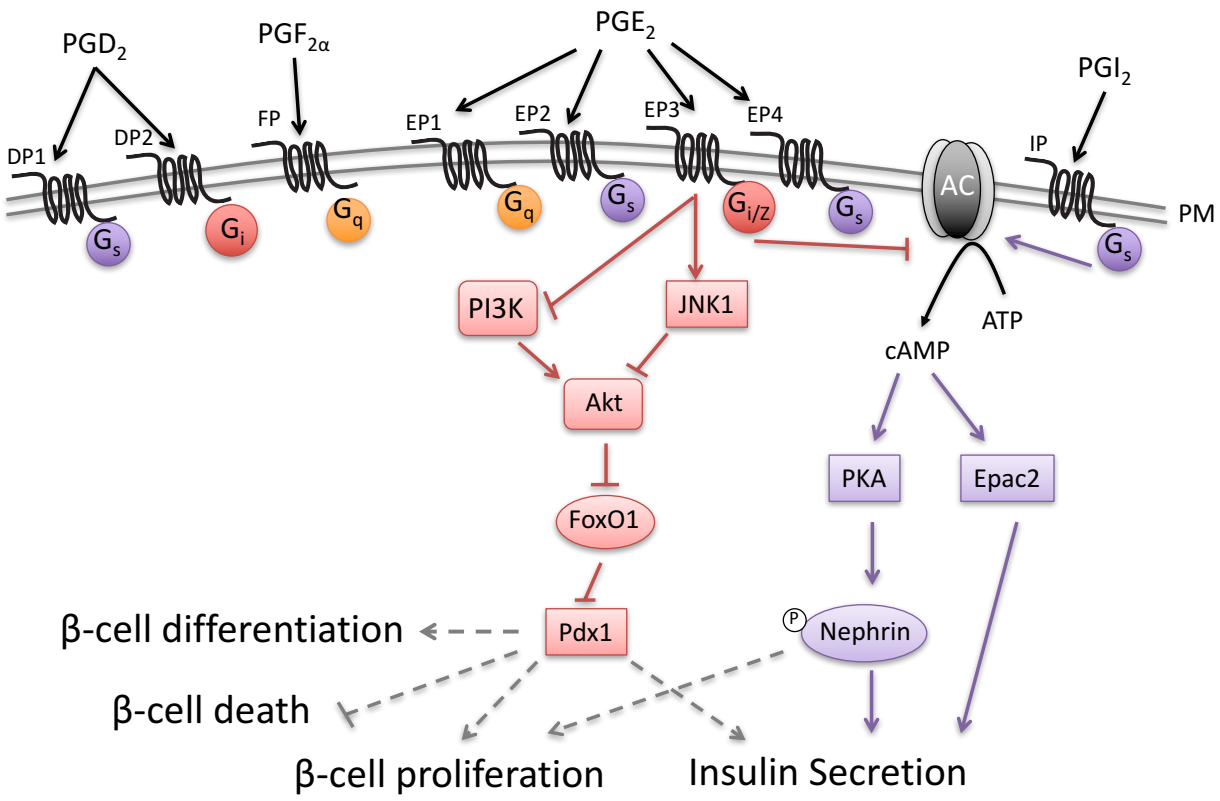

Figure 1-10. Summary of PG receptor signaling. A schematic of some of the proposed signaling mechanisms of PG receptors in the β-cell. EP2 and EP4 improve GSIS *in vivo*, but no mechanism has been determined. EP3 couples to G_i proteins, including G_Z, which inhibit AC and reduce islet cAMP levels. However, it is unclear if this is the mechanism responsible for alterations in GSIS and β-cell mass dynamics. In cell lines and rodent islets, EP3 signaling can either inhibit phosphatidylinositol 3-kinase (PI3K) or activate JNK, which leads to dephosphorylation and inhibition of Akt. Akt normally phosphorylates and inhibits Forkhead box O1 (FoxO1), so upon EP3 signaling, FoxO1 is dephosphorylated and undergoes nuclear translocation. Pancreatic and duodenal homeobox-1 (Pdx1) is important in regulating GSIS, β-cell differentiation, and β-cell mass dynamics, such as proliferation and cell death. FoxO1 and Pdx1 are mutually exclusive in the nucleus, thus providing a potential mechanism for EP3-induced decreases in GSIS and β-cell proliferation. PGI_2 via IP signaling results in G_S-mediated activation of AC and increased islet cAMP levels. cAMP activates protein kinase A (PKA) or exchange protein activated by cAMP (Epac) 2. In β-cell lines, IP signaling increases GSIS by either PKA activation and phosphorylation of Nephrin or by Epac2 signaling. The IP-induced downstream targets of Epac2 that increase GSIS have not been demonstrated. Red symbols and lines indicate PGE_2-EP3 signaling pathways. Purple arrows and symbols indicate PGI_2-IP signaling pathways. Dashed gray symbols represent potential but not confirmed events. Figure modified and reprinted with permission from "Regulation of pancreatic β-cell function and mass dynamics by prostaglandin signaling." Carboneau, B.A., Breyer, R.M, and Gannon, M. *J. Cell Commun. Signal.* 11[26]: 105-116. Copyright © 2017. DOI 10.1007/s12079-017-0377-7.

in a cAMP-dependent manner [136, 137]. Similarly, another IP agonist MRE-269 augmented GSIS in the MIN6 β-cell line [138]. The mechanism underlying this increase in GSIS involves PKA-dependent phosphorylation of nephrin (Figure 1-10) [138], distinct from what was observed in INS-1E cells. Nephrin is a transmembrane member of the immunoglobulin protein superfamily and has been shown to promote GSIS and induce β-cell survival signaling pathways [139, 140]. The authors of the latter study did not measure Epac2, thus it is unclear if this pathway also contributes to the observed enhanced GSIS in MIN6 cells. These two studies differ in many aspects, including: 1) PGIS overexpression versus IP agonism; 2) incubation time with different PKA inhibitors (24 hours versus 1.5 hours); and, 3) stimulatory glucose conditions (30 mM versus 11 mM glucose). These variations in experimental design may contribute to changes in signaling pathways that are altered in response to PGI_2 signaling. Despite these differences, these data demonstrate that PGI_2 can enhance insulin secretion in β-cell lines, potentially via multiple signaling mechanisms.

In agreement with the effects of PGI_2/IP signaling observed in β-cell lines, treatment with the IP agonist selexipag reduced the hyperglycemic effect of STZ injection in C57Bl/6 male mice [138]. The decreased hyperglycemia in selexipag-treated mice was due to an increase in plasma insulin levels, as observed during an intraperitoneal glucose tolerance test (IP-GTT), and maintenance of β-cell mass [138]. The signaling pathways contributing to enhanced GSIS have yet to be determined. Intriguingly, seleixpag treatment in the absence of STZ had no effect on glucose tolerance or plasma insulin during an IP-GTT [138]. Overall, the current literature on

PGI_2/IP suggests that PGI_2 signaling can alter GSIS, although there is a dose- and context-dependent role for PGI_2 in promoting insulin secretion.

PGE_2 and β-cell function

In comparison to the other PGs, the role of PGE_2 in β-cell function has been studied in greatest detail. This could stem from early reports demonstrating that the AA metabolite responsible for decreased insulin secretion was PGE_2 [107]. However, several groups have called into question the solely inhibitory effect of PGE_2 on insulin secretion.

There are numerous lines of evidence in support of an inhibitory role of PGE_2 on β-cell function in β-cell lines, isolated islets, and *in vivo*. *In vitro* studies have demonstrated that PGE_2 treatment decreases GSIS in several different β-cell lines, including the HIT-T15, βHC13, and INS-1 (832/3) lines [123, 141-143]. Early studies in the HIT cell line demonstrated that the action of PGE_2 to inhibit GSIS is mediated by a PTx-sensitive mechanism resulting in decreased cAMP levels [141, 142]. Since EP3 is the only PGE_2 receptor that couples to G_i-proteins, these data suggest that EP3 is the receptor responsible for this negative regulation of GSIS. PGE_2 also facilitates the inhibitory effect of IL-1β on GSIS *in vitro* [110], providing further evidence in support of an inhibitory role of PGE_2 on insulin secretion. Two structurally different COX-2 inhibitors were able to reverse the decreased GSIS in response to IL-1β treatment in HIT-T15 and βHC13 lines in part by decreasing PGE_2 production. When exogenous PGE_2 was added back to these cells, GSIS was once again decreased. The mechanism

of action was not determined but the authors predicted that PGE_2 signaling through the EP3 receptor is responsible for the observed decrease in GSIS [110].

Many of the *in vitro* data described above have been mirrored in *in vivo* settings and in isolated islets. Intravenous infusion of PGE_2 decreased circulating insulin levels and GSIS *in vivo* in early studies using animal models and humans [144-146]. As discussed previously, Burr and Sharp demonstrated that PGE_1 inhibited both first and second phases of GSIS in isolated rat islets in 1974 [126]. PGE_2 differs from PGE_1 in terms of side chain unsaturation: PGE_1 contains one double bond whereas PGE_2 has two double bonds [147]. Both PGE_1 and PGE_2 can act as agonists for the EP receptors [148]. The effects of PGE on insulin secretion were confirmed in later studies in which isolated rat islets [143, 149-152] or mouse islets [128, 143] were incubated in the presence of PGE_2 and demonstrated again that PGE_2 treatment decreases GSIS. In rat islets, treatment with the COX-2 inhibitor sodium salicylate decreased PGE_2 production and augmented GSIS [151]. However, in contrast to what was observed in cell lines, inhibition of GSIS by PGE_2 was not reversed upon PTx treatment [149]. This may be explained by PGE_2 signaling through the PTx-insensitive inhibitory G-protein, G_Z, discussed in more detail below. PGE_2 also mediates the negative effect of IL-1β on GSIS in isolated rat islets [150], as was described in β-cell lines above. Here, treatment of isolated islets with sodium salicylate blocked the IL-1β-induced decrease in GSIS [150].

PGE_2 also affects β-cell function *in vivo* in mice. Increased production of PGE_2 *in vivo* results in hyperglycemia and impaired glucose homeostasis in a transgenic mouse model [153]. When COX-2 and the microsomal PGE_2 synthase-1 (mPGES-1) were

overexpressed in mouse β-cells as a way to induce PGE_2 production, homozygous mice developed chronic hyperglycemia beginning at six weeks of age [153]. Heterozygous mice were euglycemic but displayed impaired glucose homeostasis due to a decrease in plasma insulin levels during an IP-GTT [153]. Thus, in many different experimental paradigms, PGE_2 decreases GSIS.

The mechanism for PGE_2-induced inhibition of insulin secretion downstream of G_i/G_Z-coupling has yet to be definitively determined but there is evidence for involvement of FoxO1. In rodent islets and the β-cell line HIT-TI5, decreased GSIS in response to PGE_2 occurred via activation of the FoxO1 pathway by either activation of JNK1 or inhibition of phosphatidylinositol 3-kinase (PI3K) [143, 152]. Here, PGE_2 activates JNK1 or inhibits PI3K leading to decreased phosphorylation of Akt and FoxO1. Akt normally phosphorylates FoxO1, retaining it in the cytoplasm [154]. In islets, PGE_2 inactivates Akt [143, 152] thereby decreasing the inhibitory action of Akt on FoxO1. Hypophosphorylated FoxO1 translocates to the nucleus of β-cells where it participates in nuclear exclusion of the critical β-cell transcription factor Pancreatic and Duodenal Homeobox 1 (Pdx1) [143, 155]. As Pdx1 regulates genes that promote GSIS [156, 157], this may explain the inhibitory role of PGE_2 on insulin secretion (Figure 1-10).

While there is a great deal of data indicating that PGE_2 inhibits GSIS, its role in this process is still controversial. An early study using rat islets found that PGE_2 did not affect GSIS using a wide range of doses [158]. In addition, several groups have reported that exogenous PGE_2 has no effect on GSIS in isolated rodent [129, 159] and human islets [129, 160], as measured by several different techniques. Intriguingly, PGE_2 treatment stimulated insulin release from human islets during substimulatory glucose

conditions [160]. Finally, in contrast to the studies described above [110], several groups have failed to demonstrate that COX-2 inhibition can rescue the inhibitory effects of IL-1β on GSIS in rat [129, 149, 158] or human islets [129], suggesting that PGE_2 is not required for the negative effects of IL-1β on insulin release.

The reasons for the inconsistencies described for the role of PGE_2 in regulating β-cell function are not readily apparent. Clearly it is not due to differences in experimental protocols for measuring insulin secretion, including static incubation and perifusion assays, as each have been used in both sides of the argument. However, initial perifusion assays demonstrated that the inhibitory effect of PGE_2 on GSIS could only be observed during the first phase of insulin secretion and not in response to additional secretagogues [144]. Thus, static incubation studies may miss an effect of PGE_2 since first phase secretion cannot be assessed by this methodology. Concentration of PGE_2 does not account for the observed differences as similar doses of exogenous PGE_2 (mainly 1 μM and 10 μM) have been used in all of the studies. One possible explanation for these discrepancies could be due to differences in experimental tissue studied. While β-cell lines have been used to demonstrate that PGE_2 impairs GSIS, none of the studies failing to observe an effect of PGE_2 used cell lines. However, there are examples of PGE_2 either having no effect or a negative effect on β-cell function in isolated islets. One group has suggested that variations in the culture media used prior to GSIS assays could explain some of these inconsistencies [129]. In a few of the reports demonstrating a negative role for PGE_2 in GSIS, islets were cultured in RPMI 1640 containing 11 mM glucose following islet isolation [110, 150]. In contrast, when islets were cultured in CMRL-1066 medium containing 5 mM glucose following

isolation, no effect of PGE_2 on GSIS was observed [129]. Indeed, as already discussed, high glucose conditions induce production of PGE_2 and thus could be affecting the results of exogenous PGE_2 on insulin secretion. While it is difficult to draw concrete conclusions from all of these data, similar to PGI_2 discussed above, it is likely that there are context-dependent roles for PGE_2 in GSIS, including in β-cell lines [110, 123, 141-143, 152], fetal rodent islets [149, 151], T2D mouse islets [123], and isolated islets cultured in high glucose [110, 143, 150, 152].

Another possible explanation for the discrepancies in PGE_2 effects on insulin secretion is that the different experimental conditions alter PGE_2 signaling through its different receptors. There are four EP receptors, all of which are expressed in islets, as described earlier. Most of the literature suggests PGE_2 signaling via the EP3 receptor is responsible for decreased GSIS. Based on its inhibitory G-protein signaling properties, one would predict that EP3 decreases GSIS whereas signaling via EP1-G_q or EP2/EP4-G_S would increase GSIS.

There is very little known in regards to the action of EP1, EP2, and EP4 on insulin secretion. The EP1 antagonist, AH-6809, does not affect GSIS alone nor does it alter the action of IL-1β on GSIS [150]. Further, the effect of STZ on glycemia in mice with a global deletion of EP1 does not differ from control mice [114]. These data suggest that EP1 does not affect GSIS.

EP2 and EP4 have been shown to indirectly promote insulin secretion. EP2-null mice treated with STZ and the EP4 antagonist ONO-AE3-208 have worsened STZ-induced hyperglycemia due to decreased plasma insulin [114]. Interestingly, EP2-null mice treated with STZ and the EP4 agonist ONO-AE1-329 showed an improvement in

glycemia. Further, control STZ-injected mice treated concurrently with ONO-AE1-259-01 (EP2 agonist) and ONO-AE1-329 (EP4 agonist) had even further protection against STZ-induced hyperglycemia compared to the EP2-null + EP4 agonist treated mice [114]. However, there were no direct measurements of GSIS in this study. In the T2D *db/db* mouse model, the EP4 agonist ONO-AE1-329 improved glucose homeostasis and insulin sensitivity as measured by IP-GTT and an insulin tolerance test (ITT); although, plasma insulin levels were not determined [161]. Although the mechanism is unknown, these data suggest that EP2 and EP4 promote insulin secretion *in vivo*.

In general, the literature supports an inhibitory role of EP3 in GSIS. EP3 signals through inhibitory G_i-proteins, including G_Z, all of which decrease cAMP production [103, 162]. In rat islets, treatment with the EP3 agonists misoprostol or sulprostone decreased GSIS during a static incubation assay [150]. This decrease in GSIS was reversed when islets were pre-treated with PTx before addition of the EP3 agonists [150], demonstrating that EP3 can signal through G_i-proteins in rat islets. In islets from C57Bl/6 *ob/ob* mice, the EP3 agonist sulprostone also decreased GSIS in a static incubation [103]. However, PTx treatment, which inactivates all G_i-proteins except G_Z, did not relieve the observed inhibition of sulprostone on GSIS [103]. This suggests that, at least in the context of *ob/ob* mice, G_Z is the primary G-protein coupled to EP3. G_Z itself negatively regulates GSIS in the INS1 832/13 β-cell line [162], *in vivo* in mice [102], and in isolated islets [103]. These conflicting data using PTx suggest that EP3 likely couples to multiple inhibitory G-proteins in islets, perhaps depending on the context. The diversity of the C-terminal cytoplasmic tail of EP3 results in alterations in G-protein coupling, receptor desensitization, and differences in constitutive versus

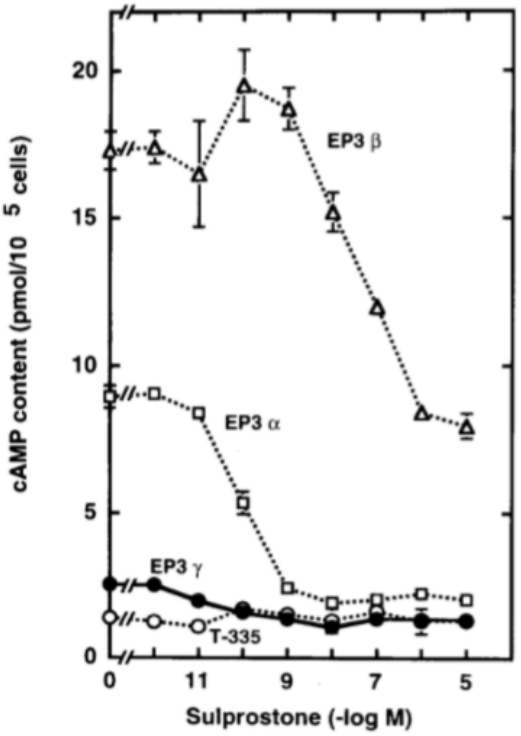

Figure 1-11. EP3 receptor variant activity. CHO cells expression EP3α (open square), EP3β (open triangle), EP3γ (closed circle), or a truncated EP3 lacking the C-terminal tail called T-335 (open circle) were incubated at 37°C for 10 min with 10 μM forskolin in the presence or absence of the indicated concentrations of sulprostone, then cAMP contents were determined. The results shown are the means ± S.E.M. for triplicate determinations. Figure reprinted with permission from "Prostagladin E receptor EP3γ isoform, with most fully constitutive Gi activity and agonist-dependent Gs activity." Negishi, M., Hasegawa, H., and Ichikawa, A. *FEBS*. 386: 165-168. Copyright © 1996. DOI 10.1016/0014-5793(96)00354-7.

ligand-dependent activity [106]. There are three EP3 receptor variants in mouse (EP3α, EP3β, and EP3γ) generated by alterative splicing of the C-terminal tail and at least eight EP3 variants have been identified in humans [148]. In mice, EP3γ displays the highest constitutive activity with very little ligand-dependent activity whereas EP3α shows modest constitutive activity and EP3β has no ligand-independent activity (Figure 1-11) [163].

Additional studies using EP3 agonists and antagonists have supported an inhibitory role for EP3 in GSIS. Islets from a T2D mouse model, $BTBR^{ob/ob}$, have increased GSIS when treated with the EP3 antagonist L-798,106, yet show decreased GSIS after stimulation with PGE_1 [123]. In human islets, the EP3 antagonist L-798,106 does not affect GSIS in islets from non-diabetic donors, yet improves insulin secretion in islets from donors with T2D [123]. Interestingly, *Ptger3* gene expression is upregulated in islets from $BTBR^{ob/ob}$ mice, obese humans, and humans with T2D [123]. Thus, an increase in EP3 expression may contribute to the impaired β-cell function in these settings. A recent study has demonstrated that PGE_2-EP3 signaling plays a role in the negative effects of *E. coli* infection on insulin secretion in INS-1E cells [164]. Treatment with the EP3 antagonist L-798,106 restored GSIS in response to long-term *E. coli* infection whereas the EP3 agonist sulprostone did not [164]. The EP3 antagonist L-798,106 also improves GSIS in MIN6 cells and isolated mouse islets [165]. Here, the authors demonstrated that the Group X secretory phospholipase, phospholipase A_2 (GX $sPLA_2$), which hydrolyzes phosphatidylcholine from the plasma membrane to produce PGE_2 [165], was involved in suppression of GSIS via PGE_2-EP3 signaling. Thus,

several lines of evidence indicate that the EP3 receptor serves to mediate the negative effect of PGE_2 on GSIS.

Surprisingly, it has been reported that pharmacological blockade of EP3 using the antagonist DG-041 does not alter GSIS in islets from wild-type mice fed a chow diet or in non-diabetic human islets [166]. While these data differ from those described above, it suggests that EP3 only affects GSIS in specific contexts. Pharmacological inhibition of EP3 only restored GSIS in the setting of T2D both in mouse and human islets [123]. Thus, the effects of inhibiting EP3 may only be observed during situations in which β-cell dysfunction is already present. Additionally, the Breyer group showed that global loss of EP3 ($EP3^{-/-}$) in mice did not affect GSIS as assessed by a perifusion assay [166]. Islets from $EP3^{-/-}$ mice fed a chow diet or HFD for 21 weeks had insulin secretion profiles that were indistinguishable from control mice [166]. A caveat to this study is that $EP3^{-/-}$ mice on HFD gained more weight than control HFD animals and displayed hyperglycemia, hyperinsulinemia, and insulin resistance, consistent with what has been previously reported [167]. Consequently, being able to parse apart the peripheral effects of EP3 from its role in β-cell function awaits conditional gene inactivation. It is possible that EP3 plays a role only in *in vivo* GSIS under specific circumstances, such as during T2D, as has been observed in isolated islets.

Prostaglandins and β-cell mass dynamics

In comparison to the literature on PGs and β-cell function, less is known about their role in regulating β-cell mass. Again, a majority of the available data focuses on PGE_2 and its receptors. There are no reports on PGD_2 and β-cell mass dynamics to date. Further, there is very little evidence to suggest a role for $PGF_{2\alpha}$ in this process. In one study performed in rat islets, $PGF_{2\alpha}$ had no effect on DNA synthesis [149]. In this section, the available information on PGI_2, PGE_2, and their receptors with regard to their roles in regulating β-cell mass will be discussed.

PGI_2 and regulation of β-cell mass

The PGI_2 analog beraprost sodium improves islet viability during islet isolation in a canine model [168], suggesting that PGI_2 may regulate β-cell mass by enhancing β-cell survival. Indeed, in multiple β-cell lines, PGI_2 and its receptor IP play a positive role in protecting against cell death [117, 125]. Overexpression of PGIS in the RINm5F β-cell line improved cell viability following cytokine treatment [125]. The protective effect of PGIS overexpression corresponded with prevention of caspase-3, -12, and -9 activation (apoptosis), decreased *Chop* expression (a marker of ER stress), decreased activation of the transcription factor NFκB, and blockade of the NO pathway. Interestingly, PGIS overexpression also prevented the cytokine-mediated reduction in cell proliferation [125]. These data suggest that PGI_2 may protect against cytokine toxicity by not only decreasing cell death but also by maintaining normal proliferation levels. In the INS-1E β-cell line, the IP antagonist CAY10441 decreases cell viability and cell proliferation in

both control and PGIS-overexpressing cells [117]. CAY10441 also induces caspase-3 activation in control cells while the IP agonist iloprost does not. Surprisingly, iloprost did not alter cell viability or proliferation itself. Overexpression of PGIS also increased cell proliferation but only during high glucose conditions [117]. Thus, high levels of PGI_2 and signaling via the IP receptor play cytoprotective roles in β-cell lines.

Activation of the IP receptor also plays a protective role *in vivo*. As already discussed, the IP agonist selexipag augmented GSIS in response to STZ treatment. This can be in part attributed to a preservation of β-cell mass in response to STZ [138]. However, in the absence of STZ, two-week administration of selexipag did not affect β-cell mass [138]. The *in vivo* mechanism for preservation of β-cell mass in response to STZ is unknown; however, evidence from β-cell lines suggest that it may be by protecting against cell death and maintaining proliferation.

PGE_2 and regulation of β-cell mass

PGE_2 alters several mechanisms responsible for regulating β-cell mass, including replication and cell death. However, as discussed in the following section, PGE_2 treatment can have different outcomes likely due to signaling through its different receptors, which couple to different downstream second messenger pathways.

In fetal rat islets, treatment with PGE_2 results in decreased DNA synthesis [149], suggesting that PGE_2 negatively regulates β-cell proliferation. In support of this notion, when PGE_2 production is increased *in vivo* using a transgenic mouse model of mPGES-1 and COX-2 overexpression, there is an observed decrease in β-cell ratio per islet and a corresponding decrease in total cell proliferation [153]. However, in these studies the

immunolabeling for Brd-U incorporation (cell cycle marker) was not counterlabeled for insulin, therefore one cannot comment specifically on the change in β-cell proliferation. Interestingly, there was an increase in α-cell ratio per islet in this mouse model [153]; yet, it is unknown how PGE_2 alters α-cell number.

The available literature suggests that a PGE_2-induced decrease in β-cell proliferation is the result of signaling via the EP3 receptor. Indeed, our group found that $EP3^{-/-}$ mice fed a HFD for 16 weeks have significantly increased β-cell proliferation compared to control mice on HFD; however, β-cell mass is not significantly different between genotypes (Figure 1-12) [166]. Intriguingly, chow-fed $EP3^{-/-}$ mice do not have altered β-cell proliferation compared to control mice [166], suggesting that EP3 activity only inhibits proliferation during specific circumstances, such as HFD feeding. Further support of an inhibitory role of EP3 on β-cell proliferation comes from one of its known downstream signaling proteins, specifically G_Z. Global loss of the G_Z in mice results in increased β-cell proliferation in chow- and HFD-fed animals and a corresponding increase in β-cell mass in HFD-fed mice [103].

The PGE_2-EP3 signaling pathways downstream of G-protein coupling responsible for mediating changes in β-cell proliferation and mass expansion during HFD-induced obesity are currently unknown. Based on the existing literature and knowledge of β-cell differentiation and proliferation, I hypothesize that a model similar to that described for GSIS is involved in mediating the effects of PGE_2-EP3 on β-cell proliferation and mass (Figure 1-10). I predict that increased PGE_2 production and

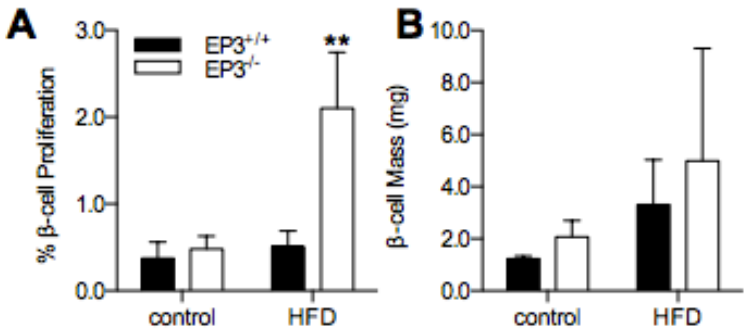

Figure 1-12. $EP3^{-/-}$ islets have increased β-cell proliferation after 16 weeks of high fat diet feeding. **A**) β-Cell proliferation (n=2 $EP3^{+/+}$ control, 2 $EP3^{-/-}$ control, 5 $EP3^{+/+}$ HFD, and 5 $EP3^{-/-}$ HFD) and **B**) β-cell mass (n=2 $EP3^{+/+}$ control, 2 $EP3^{-/-}$ control, 5 $EP3^{+/+}$ HFD, and 5 $EP3^{-/-}$ HFD) were measured in male $EP3^{+/+}$ and $EP3^{-/-}$ mice fed either HFD or control diet. Bonferroni multiple comparisons tests were performed comparing all means, significance compared to $EP3^{-/-}$ is indicated in the figures. Values are expressed as mean ± StdDev. Figure reprinted with permission from "The PGE_2 EP3 Receptor Regulates Diet-Induced Adiposity in Male Mice." Ceddia, R.P., Lee, D., Maulis, M.F., Carboneau, B.A., Threadgill, D.W., Poffenberger, G., Milne, G., Boyd, K.L., Powers, A.C., McGuinness, O.P., Gannon, M., and Breyer, R.M. *Endocrinology*. 157(1): 220-232, Copyright © 2016. DOI 10.1210/en.2015-1693.

Ptger3 (EP3) expression by hyperglycemia leads to activation of JNK and deactivation of Akt, resulting in hypophosphorylation and nuclear translocation of FoxO1, and therefore nuclear exclusion of Pdx1. Since Pdx1 is essential for maintenance of the β-cell differentiated state and is critical for embryonic and postnatal β-cell mass expansion [156, 169], this may provide an explanation for impaired β-cell proliferation in response to EP3 signaling. There are currently no reports revealing a role for EP1, EP2, or EP4 in β-cell proliferation. I predict that EP2 and/or EP4 signaling would enhance β-cell proliferation through G_S mechanisms to inhibit FoxO1 activity.

PGE_2 has also been implicated in regulating β-cell survival, another mechanism that can be involved in altering β-cell mass. Cytokines, such as IL-1β, which are known to induce β-cell death [12], induce the expression of *Ptgs2* (COX-2) and increase production of PGE_2 in isolated rodent and human islets with a corresponding decrease in cell viability [129]. PGE_2 treatment decreased cell viability in the β-cell line HIT-T15, yet did not alter apoptosis or cell cycle progression [152]. In contrast, exogenous PGE_2 decreased the level of apoptosis and caspase-3 activity in the MIN-6 β-cell line [170]. Interestingly, combined PGE_2 and IL-1β treatment in the β-cell line INS-1 (832/13) decreased *Ptger3* (EP3) and increased *Ptger4* (EP4) expression [127]. Based on their signaling properties and evidence from other tissue types, these data suggest that signaling via EP3 promotes cell death whereas EP4 protects against cell death. In support of this notion, RNA-seq of islets from a mouse model developed in our laboratory in which FoxM1 is activated in β-cells (β-Foxm1*) that displays enhanced β-cell proliferation and survival revealed that *Ptger3* expression was reduced while *Ptger4* was increased [61]. G_Z has also recently been shown to be involved in β-cell death. G_Z-

null mice treated with STZ show decreased β-cell death in addition to increased β-cell proliferation compared to control mice [101]. In response to another stimulus of cell death, IL-1β, islets from G_Z-null animals show no induction of ER stress or pro-apoptotic genes as was observed in control islets [101]. Taken together, these data demonstrate a role for G_Z signaling, and likely EP3, in β-cell proliferation and β-cell death. While the mechanism for PGE_2 in β-cell death has not been definitively established, I hypothesize that PGE_2 signaling via EP4 plays a protective role against β-cell death by G_S-mediated maintenance of Pdx1 whereas EP3 enhances β-cell death by FoxO1 activation and nuclear exclusion of Pdx1 (Figure 1-10).

Sections of this chapter were previously published under the title “Regulation of pancreatic β-cell function and mass dynamics by prostaglandin signaling” in the *Journal of Cell Communication and Signaling* (2017) 11(2): 105-116 and was modified and reused with permission.

Thesis overview

Ptger3 expression (encoding EP3) is increased in islets from humans and mice with T2D [123]. Mice null for one of the inhibitory G-proteins that couples to EP3 in the islet, G_Z, display increased β-cell proliferation and mass under both chow and HFD conditions [103]. In addition, G_Z-null mice are protected against STZ-induced

hyperglycemia due to a combination of increased β-cell proliferation and decreased β-cell death [101]. These data suggest that enhanced EP3 activity signaling via G_Z contributes to defects in β-cell compensatory mechanisms, leading to onset of T2D. However, a direct effect of EP3 in these processes remains unknown, since G_Z can couple to additional GPCRs. In collaboration with the Breyer laboratory, we found no differences in basal proliferation levels between control and $EP3^{-/-}$ mice fed a standard chow diet. Short-term HFD feeding (4 weeks) did not alter glucose homeostasis or β-cell proliferation in $EP3^{-/-}$ mice compared to controls. However, after prolonged HFD (16 weeks), which induces insulin resistance, $EP3^{-/-}$ mice had significantly increased β-cell proliferation compared to controls [166]. These data suggest that EP3 signaling impairs β-cell proliferation, but only under specific circumstances such as HFD-induced insulin resistance.

The role of EP4 in β-cell mass dynamics has not been studied to date. EP4 has been shown to promote proliferation and protect against cell death in other cell types, such as the gut epithelium [171] and myoblasts [172], suggesting that it may play a similar role in β-cells. EP4 activity in the islet has been demonstrated: EP4 agonist treatment improves hyperglycemia in the *db/db* mouse model of T2D [161]. Further, EP4 agonist treatment in EP2-null mice protects against STZ-induced hyperglycemia and death in mice [114], yet the mechanism for this protection is unknown. Intriguingly, RNA-seq analysis found that *Ptger4* (EP4) was up-regulated while *Ptger3* (EP3) was down-regulated in islets from β-FoxM1* mice, a model of enhanced β-cell proliferation and survival [61], suggesting that EP3 and EP4 play opposing roles in β-cell mass dynamics.

As T2D continues to be a major healthcare concern, identifying novel factors that can enhance functional β-cell mass, through regulation of β-cell proliferation and/or survival, could prove beneficial in generation of new therapies for T2D. The goals of the studies presented in this thesis were to determine the roles of EP3 and EP4 in regulating mouse and human β-cell proliferation and survival. We hypothesized that EP3 and EP4 play opposing roles in these processes: specifically, that EP3 inhibits β-cell proliferation and promotes β-cell death, while EP4 activation would enhance β-cell replication and promote β-cell survival, but that these effects are evident only when additional stimuli are present. Chapter II describes the methods used to test this hypothesis. Chapter III presents results from the characterization of frequently used Cre mouse models that I analyzed for their utility in *in vivo* studies of EP3 and EP4. Chapter IV presents experimental results probing the roles of EP3 and EP4 in isolated mouse and human islets using pharmacological tools. Chapter V discusses conclusions, implications, and future directions for the studies described in this dissertation.

The following publications resulted from the research presented in this thesis:

"Unexpected effects of the MIP-Cre^{ER} transgene and tamoxifen on β-cell growth in C57Bl6/J male mice." Carboneau, B.A., et al. 2016. *Physiological Reports*. 4(18): p.ii: e12863.

“Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation.” Carboneau, B.A., et al. 2017. *Molecular Metabolism.* 6: 548-559.

CHAPTER II

MATERIALS AND METHODS

Animals

$EP3^{flox}$ [173], $EP4^{flox}$ [174], MIP-Cre^{ER} [78], $Ins1^{Cre}$ [79], and β-FoxM1* [61] mice were generated as previously described. β-FoxM1* mice were of a mixed genetic background (C57Bl6J/DBA2J) whereas the rest of the mice discussed were of a pure C57Bl6/J background.

Mice carrying the MIP-Cre^{ER} allele were heterozygous for the Cre driver allele. MIP-Cre^{ER} mice received three subcutaneous injections of vehicle corn oil (C8267; Sigma) or 5 mg tamoxifen dissolved in corn oil (TM; T5648; Sigma) over a 5-day period beginning at 6 weeks of age. Injections were sealed with Vetbond tissue adhesive to prevent leakage. A one-week washout period following the final injection was provided prior to additional analyses.

To induce FoxM1 activation in β-cells, β-FoxM1* mice were given free access to water containing 2% doxycycline (Dox; Sigma-Aldrich) supplemented with Splenda, to avoid taste aversion, in a light-safe water bottle for 2 weeks prior to islet isolation. Drinking water was replaced three times per week.

Mice were housed in a 12-hour light/dark cycle and given *ad libitum* access to food (Lab Diet 5LJ5; Purina) and water, except where indicated. All experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University.

PCR and Genotyping

Genotyping was performed by PCR on DNA isolated from ear punches using the primers listed in Table 2-1. For studies using $Ins1^{Cre}$ mice, mice homozygous or heterozygous for the Cre allele were distinguished by genotyping for the *Insulin1* gene using the primers (mIns1 Forward and mIns1 Reverse) listed in Table 2-1.

HFD studies

Beginning at 8 weeks of age, male mice were placed on a high fat diet (60% kcal from fat; F3282; BioServ) for 3 days or 1 week. Food was pushed down into the wire rack every other day to ensure accessibility to the mice.

Intraperitoneal glucose tolerance tests (IP-GTTs)

IP-GTTs were performed on MIP-Cre^{ER} mice fed a chow or HFD and WT mice injected with TM fed a chow or HFD. Mice were fasted for 16 hours and fasting blood glucose (time = 0) was measured from the tail vein using an Accuchek glucometer and glucose strips. Animals then received an IP injection of filter-sterilized glucose in 1X phosphate buffered saline (PBS; 2 mg dextrose/g body weight) and blood glucose was measured from the tail vein at 15, 30, 60, 90, and 120 minutes following IP injection.

Tissue dissection and preparation for histology

For studies utilizing mouse pancreata, mice were sacrificed and pancreata were dissected and fixed for 4 hours in 4% paraformaldehyde (PFA) at room temperature

Table 2-1. Genotyping primers.

Primer Name	Mouse Strain	Sequence
EP3 flox Forward	$EP3^{flox}$	ACGTAACCCAAAGGCTGATG
EP3 flox Reverse	$EP3^{flox}$	CTCAGCTGCGGGATAGCAG
EP4 fl Forward	$EP4^{flox}$	TCTGTGAAGCGAGTCCTTAGGCT
EP4 fl Reverse	$EP4^{flox}$	GTTAGATGGGGGGAGGGGACAACT
Cre Forward	MIP-Cre^{ER}	TGCCACGACCAAGTGACAGC
Cre Reverse	MIP-Cre^{ER}	CCAGGTTACGGATATAGTTCATG
Ins1-Cre2 Forward	$Ins1^{Cre}$	GCTGGAAGATGGCGATTAGC
Ins1-Cre2 Reverse	$Ins1^{Cre}$	GGAAGCAGAATTCCAGATACTTG
mIns1 Forward	$Ins1^{Cre}$	GGACCCACAAGTGGAACAAC
mIns1 Reverse	$Ins1^{Cre}$	GCAGGAAGCAGAATTCCAGA
CMV Promoter Forward	β-FoxM1*	GGAGGTCTATATAAGCAGAGCTCG
DNRD Reverse 1	β-FoxM1*	CTCTCAGTGCTGTTGATGGCA
RIP-rtTA Forward	β-FoxM1*	GCGTGTGGGGCATTTTACTTTAG
RIP-rtTA Reverse	β-FoxM1*	CAT GTC CAG ATC GAA ATC GTC

(RT) on the orbital shaker, dehydrated in an ascending ethanol series, cleared in xylenes, and embedded in paraffin. Pancreata were serially sectioned at 5 μM.

Immunolabeling

For histology, paraffin-embedded tissue sections were deparaffinized in xylenes and rehydrated in a descending ethanol series to distilled water. Indirect protein localization was obtained by incubation of tissue sections with primary antibodies. See Table 2-2 for details on immunolabeling. All primary antibodies were incubated overnight in a humid chamber at 4°C. For immunohistochemistry, detection of primary antibodies was achieved using a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) followed by visualization using a DAB peroxidase Substrate Kit (Vector Laboratories). For immunofluorescence, detection of primary antibodies was achieved by labeling with species-specific secondary antibodies conjugated to either Cy2 or Cy3 fluorophores (Jackson ImmunoResearch Laboratories) diluted 1:400 in 0.2% Triton-X + 5% normal donkey serum (NDS) in PBS. Nuclei were visualized with 1 μg/mL 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes). Fluorescent and bright field images were captured using the Aperio ScanScope FL or CS slide scanner (Aperio Technologies, Inc.), respectively.

β-cell mass

10-12 slides spaced 250 μM apart (1-2% of the total pancreas) per animal were immunolabeled for insulin (Table 2-2), as described above, followed by a peroxidase-conjugated donkey anti-guinea pig secondary antibody (1:400). The primary insulin

antibody was visualized using a DAB Peroxidase Substrate Kit and the tissue was counterstained with eosin. Images were acquired using a ScanScope CS slide scanner and were processed using ImageScope Software, as previously described [175]. β-Cell mass was calculated by measuring the ratio of insulin-positive area to total pancreas area of all sections scanned per animal and multiplying by the pancreatic wet weight.

β-cell proliferation

Five slides spaced at least 250 μM apart per animal were selected and immunolabeled for insulin and Ki67, as described above (see Table 2-2). One pancreatic section from each slide was imaged using a ScanScope FL slide scanner. A minimum of 4,000 cells were manually counted using Metamorph 6.1 software (Molecular Devices). The percentage of proliferating β-cells was determined by dividing the total number of Ki67-insulin double-positive cells by the total number of insulin-positive cells.

β-cell size and number

Five slides spaced at least 250 μM per animal were immunolabeled for insulin (Table 2-2). One pancreatic section from each slide was imaged using a ScanScope FL slide scanner. The total β-cell area for each islet was determined by circling the insulin-positive area using Metamorph 6.1 software. In addition, the number of β-cells in each circled islet was manually counted. Average β-cell size was determined by dividing the insulin-positive area by the total number of β-cell nuclei. A minimum of 100 islets per

Table 2-2. Primary antibodies used for immunohistochemistry.

Antibody	**Source**	**Dilution**	**Antigen Retrieval**
Guinea-pig anti-Insulin	DAKO	1:500	None
Rabbit anti-Ki67	AbCam	1:500	1X Sodium Citrate (pH 6.0), boil 14’ on high
Rabbit anti-Cre	Novagen	1:2000	1X TEG buffer (pH 9.0), 1’ fast boil (100% power), 7.5’ slow boil (10% power)

sample was assessed. β-cell number was measured as the total number of β-cells counted per 100 islets.

Islet size

Five slides spaced at least 250 μM apart per animal were immunolabeled for insulin as described above. One pancreatic section from each slide was imaged using a ScanScope FL slide scanner. Insulin-positive clusters were counted and binned by size (<8 or ≥8).

Islet isolation

Mouse islets were isolated from adult male mice at different ages by collagenase digestion through the main pancreatic duct and hand-picking from the exocrine tissue by the Vanderbilt Islet Procurement and Analysis Core [176]. Islets were either cultured overnight in mouse proliferation or survival media (Table 2-3) or processed for RNA or protein extraction, as discussed below.

A subset of human islets (n=21 donors) were isolated by the Alberta Diabetes Institute IsletCore [177] at the University of Alberta. The remainder of human islets were isolated by islet isolation centers participating in the Integrated Islet Distributed Program (IIDP; https://iidp.coh.org/). Upon arrival, human islets were immediately hand-picked into and cultured in human proliferation or survival media (Table 2-3) on ice, allowed to come to room temperature for 15 minutes, and cultured overnight in a 37°C, 5% CO_2 incubator prior to further analyses. Donor characteristics can be found in Table 2-4.

Table 2-3. Islet culture media.

Name	**Media**	**Serum**
Mouse proliferation media	RPMI 1640, 11 mM glucose	Horse Serum
Mouse survival media	RPMI 1640, 5.6 mM glucose	Fetal Bovine Serum (FBS)
Human proliferation media	DMEM 11885, 1 g/L D-glucose	Horse Serum
Human survival media	DMEM 11885, 1 g/L D-glucose	FBS

Table 2-4. Human donor characteristics. Abbreviations: ADI, Alberta Diabetes Institute; SC-ICRC, Southern California Islet Cell Resources Center; AVG, average.

Donor ID	Isolation Center	Age	Gender	BMI	HbA1c	T2D?	Assay
HI 1864	ADI	52	M	23.7	5.4	N	Gene Expression - Untreated
HI 1854	ADI	54	F	24.2	5.7	N	Gene Expression - Untreated
R116	ADI	45	M	27.7	N/A	N	Gene Expression - Untreated
R123	ADI	30	F	21.3	N/A	N	Gene Expression - Untreated
R137	ADI	56	F	36.7	5.5	Y – 2.5 years	Gene Expression – Untreated; 1 Day Treatment
R093	ADI	75	M	26.4	6.3	Y – 20 years	Gene Expression – Untreated
R131	ADI	52	M	26	6.8	Y – unknown duration	Gene Expression – Untreated; 1 Day Treatment
R125	ADI	70	M	29.4	7.7	Y – 20 years	Gene Expression – Untreated
R114	ADI	65	M	33.1	6	N	Gene Expression – Untreated
R138	ADI	71	F	37	5.9	N	Gene Expression – Untreated
R113	ADI	62	M	30.6	5.7	N	Gene Expression – Untreated
R120	ADI	60	M	31.9	5.8	N	Gene Expression – Untreated
R136	ADI	60	F	36.4	6.2	N	Gene Expression – Untreated
R071	ADI	28	M	28	6.6	N	Gene Expression – Untreated
R133	ADI	53	M	34.1	5.6	N	Gene Expression – 1 Day Treatment
R134	ADI	23	M	21.9	5.9	N	Gene Expression – 1 Day Treatment
R135	ADI	62	F	28.3	6.1	N	Gene Expression – 1 Day Treatment
R136	ADI	60	F	36.4	6.2	N	Gene Expression – 1 Day Treatment
R138	ADI	71	F	37	5.9	N	Gene Expression – 1 Day Treatment

R139	ADI	50	M	24.8	5.9	N	Gene Expression – 4 Day Treatment
H958	SC-ICRC	24	M	24.6	5	N	Gene Expression – 4 Day Treatment
H1443	Miami	32	M	27.8	5.7	N	β-Cell Proliferation
H1542	Pennsylvania	35	F	23.6	4.6	N	Gene Expression – 4 Day Treatment
H1550	Illinois	50	M	30.2	N/A	N	Gene Expression – 4 Day Treatment
H1567	Miami	49	M	27.9	5.6	N	β-Cell Proliferation
H1588	Wisconsin	54	F	30.1	5.8	N	Gene Expression – 4 Day Treatment; β-Cell Proliferation
H1608	Sharp-Lacey	33	M	22.7	5.1	N	β-Cell Proliferation
H1631	SC-ICRC	40	F	23	5.7	N	Gene Expression – 4 Day Treatment; β-Cell Proliferation
H1659	Pennsylvania	17	M	25.6	5	N	Gene Expression – 4 Day Treatment; β-Cell Proliferation
H1667	Sharp-Lacey	58	M	39.3	8.9	Y – 6-10 years	Gene Expression – 1 Day Treatment
H1690	SC-ICRC	52	F	39.9	7.4	Y – 6-10 years	Gene Expression – 1 Day Treatment
H1692	SC-ICRC	63	F	27.4	4.6	N	β-Cell Survival
H1714	Miami	60	F	29.3	N/A	N	β-Cell Survival
H1718	Wisconsin	37	M	27.6	5.6	N	β-Cell Survival
H1728	Wisconsin	55	F	25.4	5.2	N	β-Cell Survival
H1735	Sharp-Lacey	52	M	29.8	5.4	N	β-Cell Survival
H1738	Pennsylvania	28	M	24.6	5.1	N	β-Cell Survival
H1743	Sharp-Lacey	49	M	26.3	5.1	N	α-Cell Proliferation
H1748	Sharp-Lacey	39	M	23.7	4.5	N	α-Cell Proliferation
H1762	SC-ICRC	46	M	21.7	5.8	N	α-Cell Proliferation
H1767	SC-ICRC	33	M	29.8	5.2	N	α-Cell Proliferation
H1794	SC-ICRC	60	M	31.3	5.7	N	β-Cell Proliferation
H1812	Sharp-Lacey	37	F	19.7	4.6	N	β-Cell Proliferation
H1813	SC-ICRC	44	M	31.2	5.2	N	β-Cell Proliferation
H1826	SC-ICRC	38	M	26.4	5.2	N	β-Cell Proliferation; β-Cell Survival
H1827	Sharp-Lacey	29	M	26.7	5.5	N	β-Cell Proliferation; β-Cell Survival
H1847	Sharp-Lacey	57	F	25.9	5.3	N	β-Cell Survival

AVG		49	65% M	28.4	5.6		

RNA extraction

For untreated islets, islets were isolated from human donors or male mice aged 2, 4, 8, or 12 months, washed three times in cold 1X PBS, immediately placed in 1 mL TRIzol reagent (Life Technologies), lysed by vortexing, and stored at -80°C. A minimum of 100 human islets were used for each RNA extraction. RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol and eluted in 30 μL RNase-free dH_2O. RNA concentration and integrity were assessed using a ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent) at Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core.

For treated islets, 120 mouse islets were cultured in mouse proliferation media for 2 or 4 days in the presence of 0.1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and one of the following compounds: vehicle (PBS), DG-041, or CAY10598. See Table 2-5 for compound information and concentrations used. Another set of mouse islets was cultured in mouse survival media supplemented with cytokines (cytokine concentrations discussed below) for 2 days in the presence of vehicle, DG-041, or CAY10598. For treated human islets, 120 human islets were cultured in human proliferation media with 0.1 mM EGTA plus one of the following: vehicle, DG-041, or CAY10598 for 1 or 4 days. At the end of the treatment period, RNA isolation was performed as described above for untreated islets.

Table 2-5. Compounds used in *ex vivo* assays.

Compound	**Concentration**	**Source**
PBS	N/A	Gibco
Human Recombinant Placental Lactogen (PL)	0.5 μg/mL	Harbor-UCLA Research and Education Institute
Sulprostone	30 nM	Cayman Chemical
DG-041	30 nM	Vanderbilt Institute of Chemical Biology Synthesis Core
CAY10598	10 nM	Cayman Chemical
L-161,982	100 nM	Cayman Chemical
U-73122	1 μM	Sigma-Aldrich
Rapamycin	30 nM	Calbiochem
Rp-cAMPS	100 μM	Sigma-Aldrich
Forskolin	10 nM – 10 μM	Sigma-Aldrich

Quantitative Real-Time PCR (qRT-PCR)

100 ng or 175 ng cDNA was prepared from mouse and human islets, respectively, using the SuperScript III First Strand Synthesis System (ThermoFisher Scientific). Real-time reactions were performed in technical triplicate with iQ SYBR Green supermix (Bio-Rad) according to the manufacturer's instructions on a CFX Real-Time PCR Detection system (Bio-Rad) in the Vanderbilt Molecular and Cellular Biology Resource Core. Primers used to assess gene expression are listed in Table 2-6. Data are represented as $2^{-\Delta\Delta Ct}$ or ΔCt, as indicated.

Mouse *ex vivo* β-cell proliferation

Ex vivo proliferation was performed as previously described [178, 179]. Islets were pooled from multiple mice and 40 islets per replicate from young (8-12 weeks; n=3-8) or aged (1 year; n=3) mice were aliquoted into a well of a 96-well plate in 200 μL mouse proliferation media and allowed to recover from the isolation process overnight in a 37°C, 5% CO_2 incubator. The following day, 100 μL of media was removed from each well and replaced with 100 μL of fresh mouse proliferation media supplemented with 2X the final desired concentrations of EGTA (final concentration 0.1 mM) along with one or more compounds (Table 2-5) and incubated for 48 hours. After 48 hours, 150 μL of media was removed from each well and replaced with 150 μL of fresh mouse proliferation media supplemented with 0.1 mM EGTA and the same compounds as the previous treatment day and incubated for an additional 48 hours. All compounds were diluted in PBS to obtain desired concentrations.

Table 2-6. qRT-PCR primers.

Primer Name	Forward Primer	Reverse Primer
Hprt (internal control)	AGTCAACGGGGGACATAAAA	TGCATTGTTTTACCAGTGTCAA
Cre	TACCGGAGATCATGCAAGCTG	TGCCCCTGTTTCACTATCCAG
Ptger1	CGGCATTAGTGTGCAATACGCTCA	TGAAGTGATGGATGAGGCAGACGA
Ptger2	GAGACGGACCACCTCATTCTC	GGAGGTCCCACTTTTCCTTT
Total *Ptger3*	GCT ATC CCG CAG CTG AG	CGC AGT CGT CGG TAG TAC
Ptger3α	GCTTCAGCTCCACCTCCTT	CATCATCTTTCCAGCTGGTCACT
Ptger3β	GGAAGTTCTGCCAGATGATGAA	ATTCTCAGACCCAGGGAAACA
Ptger3γ	AGTTCTGCCAGGTAGCAAACG	GCCTGCCCTTTCTGTCCAT
Ptger4	CAGGAATTTGCTTCCAGGTTCGCA	AACCTCATCCACCAACAGGACACT
Ptgs2	TAGCAGATGACTGCCCAACTC	GGGTCAGGGATGAACTCTCTC
Ptges1	ATGCGCTGAAACGTGGAG	GTCCCAGGAATGAGTACACGA
Ptges2	AAGTACTGGCTCATGCTGGAC	GTTGGGAGAGATGAGATGCAC
Ptges3	GGGTGGTGATGAGGATGTAGA	TGACAACAGCCCTTACTCCAG
Gnaz	GTGGAGCTCAGTGGCTATGAC	CCAGTTGTTGTTGCAGATGG
Gnai1	GGGAGTACCAGCTGAACGATT	TCTGAGGACATCCTGCTGAGT
Gnai2	GCTCACGAGAATACCAGCTCA	CATCCTGCTGTGTAGGGATGT
Gnai3	CCCACTTCACCTTCAAGGAAC	GCTGTCACTCCCTCAAAACAG
Foxm1	CAC TTG GAT TGA GGA CCA CTT	GTC GTT TCT GCT GTG ATT CC
Ki67	AGCTTCTGTGCTGACCCTGATG	TGCAGAAAGGCCCTTGGCATAC
Ccnd1	CTGACACCAATCTCCTCAACGAC	GCGGCCAGGTTCCACTTGAGC
Ccnd2	CACCGACAACTCTGTGAAGC	TCCACTTCAGCTTACCCAACA
Ccna2	CTTGGCTGCACCAACAGTAA	CAAACTCAGTTCTCCCAAAAACA
Ccnb1	TCTTGACAACGGTGAATGGA	TCTTAGCCAGGTGCTGCATA
Cdkn1a	TCCACAGCGATATCCAGACA	GGACATCACCAGGATTGGAC
Cdkn1b	GAG CAG TGT CCA GGG ATG AG	TCT GTT CTG TTG GCC CTT TT
Cdkn2a	CCACTCCAAGAGAGGGTTTTC	ATCTGCACCGTAGTTGAGCAG
Bcl-xl	CCTTGGATCCAGGAGAACG	CAGGAACCAGCGGTTGAA
Birc5/survivin	TGATTTGGCCCAGTGTTTTT	CAGGGGAGTGCTTTCTATGC
Caspase 3	GACTGATGAGGAGATGGCTTG	CTTCCTGTTAACGCGAGTGAG
Caspase 8	TAGACTGCAACCGAGAGGAGA	CAGGCTCAAGTCATCTTCCAG
Caspase 9	CCTAGTGAGCGAGCTGCAAGT	TCCTGCCTGCTGAATATCCT

Bad	GAAGGGCTGGAGGACTTATCA	ATACTCTGGGCTGCTGGTCTC
Bax	CAAGAAGCTGAGCGAGTGTCT	GCAAAGTAGAAGAGGGCAACC
iNOS	GAGTTCACCCAGTTGTGCATC	CTCTGGATCTTGACCATCAGC
TBP (internal control)	GAACCACGGCACTGATTTTC	GCTGGAAAACCCAACTTCTG
PTGER1	AGCTTGTCGGTATCATGGTGGTGT	GATGTACACCCAAGGGTCCAGGAT
PTGER2	ACCCTTGGGTCTTTGCCATCCTTA	AGGTCAGCCTGTTTACTGGCATCT
PTGER3	TCACCTTTTCCTGCAACCTG	ACGCACATGATCCCCATAAG
PTGER4	TGGTGCGAGTATTCGTCAACCAGT	CAATGCGGCAGAAGAGGCATTTGA
FOXM1	GGAGGAAATGCCACACTTAGCG	TAGGACTTCTTGGGTCTTGGGGTG
KI67	CCCTGATGAGAGTGAGGGAAT	AGAGGCGTATTAGGAGGCAAG
CCND1	CCCTCGGTGTCCTACTTCAA	ACTTCTGTTCCTCGCAGACCT
CCND2	GGACATCCAACCCTACATGC	CGCACTTCTGTTCCTCACAG
CCND3	ACTGGATGCTGGAGGTATGTG	AAGACAGGTAGCGATCCAGGT
CCNA2	CATGGACCTTCACCAGACCTA	GGGTTGAGGAGAGAAACACCA
CCNB1	GTTATGCAGCACCTGGCTAAG	CATGCTTCGATGTGGCATAC
CDKN1A	GGAGACTCTCAGGGTCGAAAA	GATTAGGGCTTCCTCTTGGAG
CDKN1B	TGACTTGCATGAAGAGAAGCA	GCTGTCTCTGAAAGGGACATTAC
CDKN2A	GCACCAGAGGCAGTAACCAT	TTCTCAGAGCCTCTCTGGTTC

Following the treatment period, 150 μL of media was removed from each well and islets were gently washed with 150 μL of PBS. PBS was removed from each well and saved in a labeled 1.5 mL centrifuge tube. Islets were then dispersed using 200 μL of a 0.025% trypsin, 2 mM EDTA solution (Vanderbilt Molecular Biology Core), incubated at RT for approximately 5 minutes, and collected into the 1.5 mL centrifuge tubes containing PBS. Islets were further dissociated by vortexing for approximately 30 seconds. 200 μL of mouse proliferation media was added to each 1.5 mL centrifuge tube to stop the action of trypsin. Cells were pelleted by centrifugation for 5 minutes at 4,000 rpm and 4°C. Once pelleted, the supernatant was discarded and cells were resupended in 200 μL mouse proliferation media. The resuspended cell suspensions were transferred to an EZ cytofunnel (Thermo Scientific) fitted with a charged microscope slide (Leica) and centrifuged for 3 minutes at 800 rpm using a cytospin centrifuge (Dr. Powers' Laboratory). Slides were removed from the cytofunnels, a box was drawn around the cell spot with a hydrophobic pen, and slides were placed in a humid chamber.

For the immunolabeling process, cytospun cells were fixed on the slides in 200 μL 4% PFA for 10 minutes RT in a humid chamber. Cells were then washed three times in 1X PBS. Cells were permeablized in 200 μL 0.2% Triton-X100 in 1X PBS for 10 minutes RT in a humid chamber. Cells were washed three times in 1X PBS followed by incubation in blocking buffer (5% NDS in 1X PBS) for 30 minutes to 1 hour RT in a humid chamber. Blocking buffer was removed and cells were incubated in 200 μL primary antibodies diluted in blocking buffer for 1 hour RT in a humid chamber. Primary antibodies used include guinea pig anti-insulin (1:400) and rabbit anti-Ki67 (1:400). After

primary antibody incubation, cells were washed three times in 1X PBS and then incubated in secondary antibodies diluted in blocking buffer for 1 hour RT in a humid chamber. Secondary antibodies used include Cy2-conjugated anti-guinea pig (1:300) and Cy3-congugated anti-rabbit (1:300). After 1 hour, secondary antibodies were removed and cells were incubated with 200 μL DAPI (1 μg/mL diluted in H_20) for 2 minutes RT in a humid chamber. DAPI was removed and cells were washed with 1X PBS, mounted with aquamount, and coverslipped.

Images were obtained using a ScanScope FL slide. β-Cell proliferation was determined by quantifying the number of insulin-Ki67 dual-positive cells using a macro generated with the CytoNuclearFL algorithm in eSlide Manager (Aperio Technologies, Inc.). Data are represented as fold change in proliferation compared to vehicle-treated islets (which were set at "1") due to inherent variability between pooled islet samples.

Human *ex vivo* α- and β-cell proliferation

For human α- and β-cell proliferation, 40 islets were aliquoted per well into a well of a 96-well plate in 200 μL human proliferation media and allowed to recover from the picking process overnight in a 37°C, 5% CO_2 incubator. Islets were treated in the same manner and with the same concentrations of compounds described above for mouse β-cell proliferation and in Table 2-5. For the immunolabeling process, islets from two wells were pooled together for each replicate (80 islets total) following the dissociation by trypsin, prior to cytospin centrifugation.

To determine human β-cell proliferation, human islets were immunolabeled for insulin, Ki67, and DAPI as described for mouse *ex vivo* β-cell proliferation. Images were

obtained as described for mouse *ex vivo* β-cell proliferation. β-Cell proliferation was determined by quantifying the number of insulin-Ki67 dual-positive cells using a macro generated with the CytoNuclearFL algorithm in eSlide Manager. Each donor was assayed in duplicate and the values shown are the average of the duplicates. Data are represented as fold change compared to normalized vehicle-treated islets due to issues of inherent variability between donors.

To measure human α-cell proliferation, islets were immunolabeled with the primary antibodies mouse anti-glucagon (1:400) and rabbit anti-Ki67 (1:400); the secondary antibodies Cy2-conjugated anti-mouse (1:300) and Cy3-conjugated anti-rabbit (1:400); and nuclei were visualized with DAPI. α-Cell proliferation was determined by quantifying the number of glucagon-Ki67 dual-positive cells using a macro generated with the CytoNuclearFL algorithm in eSlide Manager. Data are represented as fold change in α-cell proliferation over normalized vehicle-treated islets due to issues of inherent variability between donors.

Ex vivo β-cell survival

Mouse β-cell survival was assayed in WT (n=3-5) and β-FoxM1* (n=3-4) islets from 8-10-week-old male mice as previously described [61]. To induce FoxM1 expression, 8-week-old male β-FoxM1* mice were given free access to water containing 2% Dox supplemented with Splenda for two weeks prior islet isolation [61]. After the islet isolation, islets from multiple mice were pooled together and 40 islets were aliquoted per well in a 96-well dish and were cultured in 200 μL mouse survival media supplemented with 1 μg/mL Dox in a 37°C, 5% CO_2 incubator overnight. The next day,

175 μL of media was removed and replaced with mouse survival media supplemented with a cytokine cocktail containing mouse tumor necrosis factor (TNF)-α (20 ng/mL; Sigma-Aldrich), mouse interleukin (IL)-1β (5 ng/mL; Sigma-Aldrich), and mouse interferon (IFN)-γ (10 ng/mL; Sigma-Aldrich) and islets were incubated for 48 hours. At the time of cytokine treatment, 0.1 mM EGTA and compounds (Table 2-5) were added to the mouse survival media.

After 48 hours of incubation, two wells of islets were pooled together (80 islets total per replicate), dissociated using trypsin-EDTA, cytospun onto charged slides, fixed in 4% PFA, and permeablizied with 0.2% Triton-X 100 as described for mouse *ex vivo* β-cell proliferation. To assess β-cell death, a terminal deoxynucletidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the ApoAlert DNA fragmentation assay kit (CLONTECH Laboratories, Inc.) according to the manufacturer's instructions. Following the TUNEL assay, cells were co-immunolabeled with guinea pig anti-insulin (1:400) and Cy3-conjugated anti-guinea pig (1:300). Nuclei were visualized with DAPI. Slides were mounted with aquamount and coverslipped.

Images were obtained using a ScanScope FL slide scanner. β-Cell death was determined by manually counting the number of insulin-TUNEL dual-positive cells using MetaMorph 6.1 software. Data are represented as fold change compared to vehicle + cytokine-treated islets (which were set at "1") due to inherent variability between islet preparations.

For human β-cell death, 40 islets per well were cultured in a 96-well dish in 200 μL human survival media overnight before being treated with the EP ligands (Table 2-5) in human survival media supplemented with 0.1 mM EGTA and a cytokine cocktail

consisting of human TNFα (20 ng/mL; R&D Systems), human IL-1β (5 ng/mL; R&D Systems), and human IFN-γ (10 ng/mL; R&D Systems). Data were obtained, quantified, and represented as described for mouse β-cell survival.

Phospho-protein microarray

Islets were isolated from 8-10-week-old WT male mice as described above. On the day of the isolation, 40 islets were aliquoted into wells of a 96-well plate in 200 μL mouse proliferation media and allowed to recover from the isolation process overnight in a 37°C, 5% CO_2 incubator. The following day, 100 μL of media was removed and replaced with fresh mouse proliferation media containing 0.1 mM EGTA and 2X the desired compounds from Table 2-5. Islets were incubated for 24 hours in the compounds. Islets from three mice were pooled together for each replicate. A total of two replicates per treatment were used in the microarray analysis.

After incubation, islets were collected into a 1.5 mL centrifuge tube and washed three times in cold 1X PBS. Following the last wash, PBS was removed without disturbing the islet pellet, the islet pellet was flash frozen on dry ice, and stored at -80°C until the samples were prepared for the Kinex™ KAM-900P Antibody Microarray Kit (Kinexus Bioinformatics Corporation). To prepare protein lysates, 50 μL of Kinexus Cell Lysis Buffer (supplied in kit) was added to each frozen islet pellet. Samples were vortexed for 1 minute each and stored on ice. Samples were then sonicated using a microprobe sonicator (Virtis Virsonic) 4 times for 10 seconds each with 10-second intervals on ice. Next, lysates were centrifuged for 30 minutes at 4°C at the maximum speed. The resulting supernatant was transferred to a new 1.5 mL centrifuge tube and

stored on ice or at -80°C. Protein concentration was determined by Bradford assay using the Protein Assay Dye Reagent Concentrate (Bio-Rad) according to the manufacturer's instructions. In order to obtain sufficient quantities of protein for the microarrays, samples were concentrated for 10-15 minutes using a speedvac (Savant).

Protein labeling and purification with chemical cleavage was performed using the maximum μg of protein possible. Equal amounts of protein for each experimental group were loaded on the microarrays and were as follows: 19.3 μg for vehicle vs. CAY10598 replicates 1 and 2; 18.74 μg for PL vs. PL + DG-041 replicate 1 and 20.1 μg for replicate 2. Protein labeling, protein purification with chemical cleavage, and microarray incubation were performed according to the manufacturer. Microarrays were stored at 4°C in the dark until they were shipped to Kinexus Bioinformatics Corporation for microarray scanning and analysis.

KiNetscape maps were generated by Kinexus Bioinformatics Corporation from the leads identified in the Kinex^{TM} KAM-900P Antibody Microarray analyses. To qualify as a lead, the compounds had to produce percent changes from control (%CFC) values that were at least 45% higher or lower with fluorescent signals that were at least 1,000 counts. The sum of the % errors in the mean of the averaged duplicates from the two separate experiments had to be less than 85% of the calculated %CFC values. The kinase-substrate relationships that were identified with these leads were retrieved from the KinaseNET website (www.kinasenet.ca) and used for pathway mapping with the Cytoscape 3.4.0 program (The Cytoscape Consortium).

Western blotting

Islets were isolated from 8-12-week-old male mice and 40 islets were aliquoted in 200 μL mouse proliferation media in a 96-well dish and allowed to recover overnight. The following day, islets were treated with 0.1 mM EGTA and one the compounds listed in Table 2-5 for 24 hours. After 24 hours, islets were washed three times in cold 1X PBS. The islet pellet was flash frozen on dry ice and stored at -80°C. To extract protein, the frozen islet pellets were immediately lysed in 50 μL cold RIPA buffer (Sigma-Aldrich) containing 1X PhosStop Phosphatase Inhibitor Cocktail (Roche) and 1X Complete Protease Inhibitor Cocktail (Roche). Islets were lysed on ice using a mortar and pestle gun, allowed to rest on ice for 30 minutes, and centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was transferred to a fresh 1.5 mL centrifuge tube and protein concentration was determined using the Bio-rad DC Protein Assay according to the manufacturer's instructions.

Equal amounts of protein per sample were electrophoresed on a 4-12% Bis-Tris gel under denaturing conditions and transferred onto a nitrocellulose membrane using the NuPAGE Western blotting system (Invitrogen). Membranes were blocked in 5% nonfat milk in 1X TBST for one hour and probed with the following primary antibodies in 5% bovine serum albumin (BSA) in 1X TBST overnight at 4°C: rabbit anti-phospho-PKA Substrate (1:1000, Cell Signaling), rabbit anti-Akt Substrate (1:1000, Cell Signaling), and rabbit anti-β-tubulin (1:1000, Cell Signaling). Membranes were incubated in peroxidase-conjugated goat anti-rabbit secondary antibody (1:20,000, Sigma-Aldrich) in 5% nonfat milk in 1X TBST for one hour RT. Proteins were detected using an ECL Plus

detection system (Perkin Elmer) using Kodak X-Omat Blue film. Protein levels were quantified using ImageJ software.

Determining islet cAMP

Isolated mouse (8-12-weeks-old) or human islets were allowed to recover overnight in a 35 mm petri dish in mouse or human proliferation media, respectively. The following day, islets were transferred to a new 35 mm petri dish containing 1X PBS to wash off the proliferation media. To measure changes in cAMP, islets were first incubated in low glucose (1 mM for human islets or 2.8 mM for mouse islets) Krebs-Ringer Buffer (KRB) solution (see below for details) and then transferred to high glucose (16.7 mM for mouse and human islets) KRB solution. The KRB solution consisted of 115 mM NaCL, 5 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 0.1% w/v BSA. The solution was made fresh for each day and allowed to warm up in a 37°C, 5% CO_2 incubator for 30 minutes before adjusting the pH to 7.4 using NaOH.

For incubation in KRB solutions, islets were washed in 2 mL low glucose KRB solution in a 35 mm petri dish. Following, islets were transferred into a new 35 mm petri dish and pre-incubated in 2 mL low glucose KRB solution for 1 hour in a 37°C, 5% CO_2 incubator. Following the pre-incubation period, approximately 20 mouse islets or 50 human islets were aliquoted into an 1.5 mL centrifuge tube for each treatment condition. 500 μL low glucose KRB solution was added to each 1.5 mL centrifuge tube containing islets. Islets were incubated for 1 hour with tube caps open in a 37°C, 5% CO_2 incubator. The islets were then pelleted by centrifugation at 1,000 rpm for 1 minute RT

and the supernatant was discarded. Islets were then stimulated in 500 μL high glucose KRB solution plus different compounds (Table 2-5) for 10 and 30 minutes with tube caps open in a 37°C, 5% CO_2 incubator. After the incubation, islets were pelleted by centrifugation at 1,000 rpm for 1 minute. The supernatant was discarded, the islet pellet was flash frozen in liquid nitrogen, and then stored at -80 °C.

Islet cAMP levels were measured using the Cyclic AMP XP Assay Kit (Cell Signaling). Cell lysates were prepared by vortexing samples for 30 seconds every 2 minutes for a total of 10 minutes, stored on ice, in 100 μL 1X cell lysis buffer (supplied in kit) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The Cyclic AMP XP Assay was performed according to the manufacturer's instructions.

Measuring islet PGE_2 levels

Media was collected during the β-cell proliferation and β-cell survival assays from mouse and human islets in order to measure the amount of PGE_2 secreted by islets. During the mouse and human β-cell proliferation or β-cell survival assays, media was removed from the 96-wells, as described for the treatments above, and collected into an 1.5 mL centrifuge tube. For the β-cell proliferation assay, media was collected on day 0 (after overnight recovery), day 2, and day 4 from vehicle-treated and PL-treated islets. For the β-cell survival assay, media was collected on day 0 (following overnight recovery) and after 48 hours of treatment from vehicle-treated with or without cytokines. Media from at least 4 different technical replicates (wells) were pooled together into one tube. 1.5 mL centrifuge tubes containing media were first placed on dry ice and then stored at -80°C. The amount of PGE_2 present in the media was quantified by the

Vanderbilt Eicosanoid Core Laboratory by liquid chromatography-mass spectrometry (LC-MS).

Statistics

Results are expressed as mean ± SEM. Statistical significance was calculated in GraphPad Prism (GraphPad Software, Inc.) using a one-way ANOVA and Bonferroni *post hoc* analysis, two-way ANOVA and Tukey *post hoc* analysis, Student's *t* test, or linear regression, as appropriate. *p* values of ≤0.05 were considered significant.

CHAPTER III

CHARACTERIZATION OF FREQUENTLY USED CRE MOUSE MODELS

Introduction

The *in vivo* roles of EP3 and EP4 in regulating β-cell mass dynamics have not been studied to date. In collaboration with the Breyer lab, we have shown that $EP3^{-/-}$ mice have increased β-cell proliferation compared to WT mice only after 16 weeks on HFD, in which insulin resistance is present [166]. A caveat to this study is that $EP3^{-/-}$ mice gain more weight than WT mice on HFD [166, 173]; thus, it is unclear whether the increase in β-cell proliferation after 16 weeks on HFD is due solely to loss of EP3 within the β-cell or if it is a consequence of obesity associated with loss of EP3 in other tissues, such as the hypothalamus. Therefore, in order to address the specific role for EP3 in β-cell proliferation, a β-cell-specific knockout mouse model is required.

In order to test the hypothesis that EP3 inhibits whereas EP4 enhances β-cell proliferation and mass expansion during HFD-induced obesity, I proposed to generate β-cell-specific knockout mouse models of the EP3 and EP4 receptors. As discussed in Chapter I, several "pancreas-specific" Cre transgenes were found to be expressed in key brain regions that affect food intake and metabolism [77]. Due to the lack of Cre expression in the brain, the mouse *Insulin1* promotor (MIP)-Cre^{ER} transgenic line was therefore chosen to generate EP3 and EP4 β-cell-specific knockout models [78].

During the process of generating EP3 and EP4 β-cell-specific knockout mice using MIP-Cre^{ER}, it was reported that some pancreas-specific Cre and Cre^{ER} models have unexpected phenotypes, in the absence of any *loxP*-containing loci, due to issues with the design of the Cre transgene [180-182]. These phenotypes include changes in glucose tolerance, glucose transporter *Glut2* expression, and β-cell mass due to the presence of a human growth hormone (hGH) minigene in the transgene construct [180, 181]. The hGH minigene was incorporated in the design of several transgenes, including MIP-Cre^{ER}, in order to enhance mRNA stability and expression levels [78, 183, 184]. Islets isolated from mice expressing Cre transgenes that contain hGH were subsequently found many years later to express *hGH* mRNA and protein [180, 181]. Further characterization of MIP-Cre^{ER} mice revealed that the presence of hGH alters pancreatic insulin content and β-cell mass in the presence of TM, as well as protects against hyperglycemia induced by combined high fat-high sugar diet and streptozotocin (STZ) treatment independent of TM [181]. Similar observations were also made in a different strain of mice that contains hGH in the transgenic construct: the Pdx1-Cre^{late} mouse [180]. Additionally, it was reported that TM, in the absence of Cre^{ER}, impairs compensatory β-cell proliferation in the settings of partial duct ligation (PDL), pregnancy, and development [182]. Thus, the utility of these commonly used tools, as well as the validity of findings related to β-cell proliferation, function, and mass, have recently been called into question.

As we embarked on obesity-induced β-cell mass expansion studies using EP3 and EP4 β-cell-specific knockouts, we wanted to determine if β-cell proliferation was altered by either the MIP-Cre^{ER} transgene or TM during HFD in the absence of any

additional genetic manipulation. To date, there are no reports on the impact of MIP-Cre^{ER} or TM on HFD-induced β-cell proliferation. Thus, β-cell proliferation and mass were evaluated in 8-week-old MIP-Cre^{ER} mice in the absence of TM as well as in WT mice injected with vehicle corn oil or TM and fed either chow diet or HFD. Results presented in this chapter demonstrate that the MIP-Cre^{ER} transgene by itself leads to increased β-cell mass via hypertrophy whereas TM treatment impairs HFD-induced β-cell proliferation in the absence of any genetic manipulation.

Upon completion of the characterization of MIP-Cre^{ER} mice and WT mice treated with TM, a new β-cell-specific Cre model was introduced to the field, called Ins1^{Cre}. Importantly, this model does not contain the hGH minigene and was reported not to be expressed in the brain [79]. Therefore, *Cre* mRNA, Cre protein expression, and EP4 gene recombination in Ins1^{Cre} mice with or without the floxed *Ptger4* gene were evaluated to assess the practicality of utilizing this recently developed model to generate EP3 and EP4 β-cell-specific knockout mice. Unfortunately, in our laboratory (and others at Vanderbilt), this model was found to have little-to-no *Cre* gene expression, very low levels of Cre protein, and was not sufficient to reduce *Ptger4* expression in EP4$^{flox/flox}$;Ins1^{Cre} mice. Therefore, an alternative β-cell-specific Cre model is required to address the *in vivo* roles of EP3 and EP4 in β-cell mass dynamics during obesity in the future.

Results

Chow-fed MIP-Cre^{ER} mice have a tamoxifen-independent increase in β-cell mass due to hypertrophy

Chow-fed MIP-Cre^{ER} mice treated with TM have a strong trend toward increased β-cell mass (p=0.051) in the absence of any gene inactivation, likely due the presence of hGH in the transgenic construct [181]. This is similar to the findings of another commonly used model that also contains the hGH minigene, the Pdx1-Cre^{late} strain. Pdx1-Cre^{late} mice have significantly increased β-cell mass at 24 weeks of age [180]. However, neither group determined the mechanism for increased β-cell mass. Since a major focus of the EP3 and EP4 β-cell-specific knockout studies is regulation of adult β-cell proliferation, we wanted to determine whether this parameter could be affected by the hGH-containing MIP-Cre^{ER} transgene.

β-Cell mass was measured in 8-week-old untreated MIP-Cre^{ER} and WT male mice fed a chow diet. Compared to WT mice, MIP-Cre^{ER} mice had significantly increased β-cell mass (Figure 3-1A). β-Cell proliferation and β-cell size were assessed to determine if either of these compensatory mechanisms could explain the increase in β-cell mass in MIP-Cre^{ER} mice. While β-cell proliferation was similar between WT and MIP-Cre^{ER} mice (Figure3-1B), individual β-cell size was significantly increased in MIP-Cre^{ER} mice (Figure 3-1C). To determine whether the increase in β-cell mass in MIP-Cre^{ER} mice could be explained by an increase in proliferation earlier than 8 weeks of age, β-cell number was evaluated in each genotype. No differences in total β-cell number were observed between chow-fed MIP-Cre^{ER} mice and WT mice (Figure 3-1D),

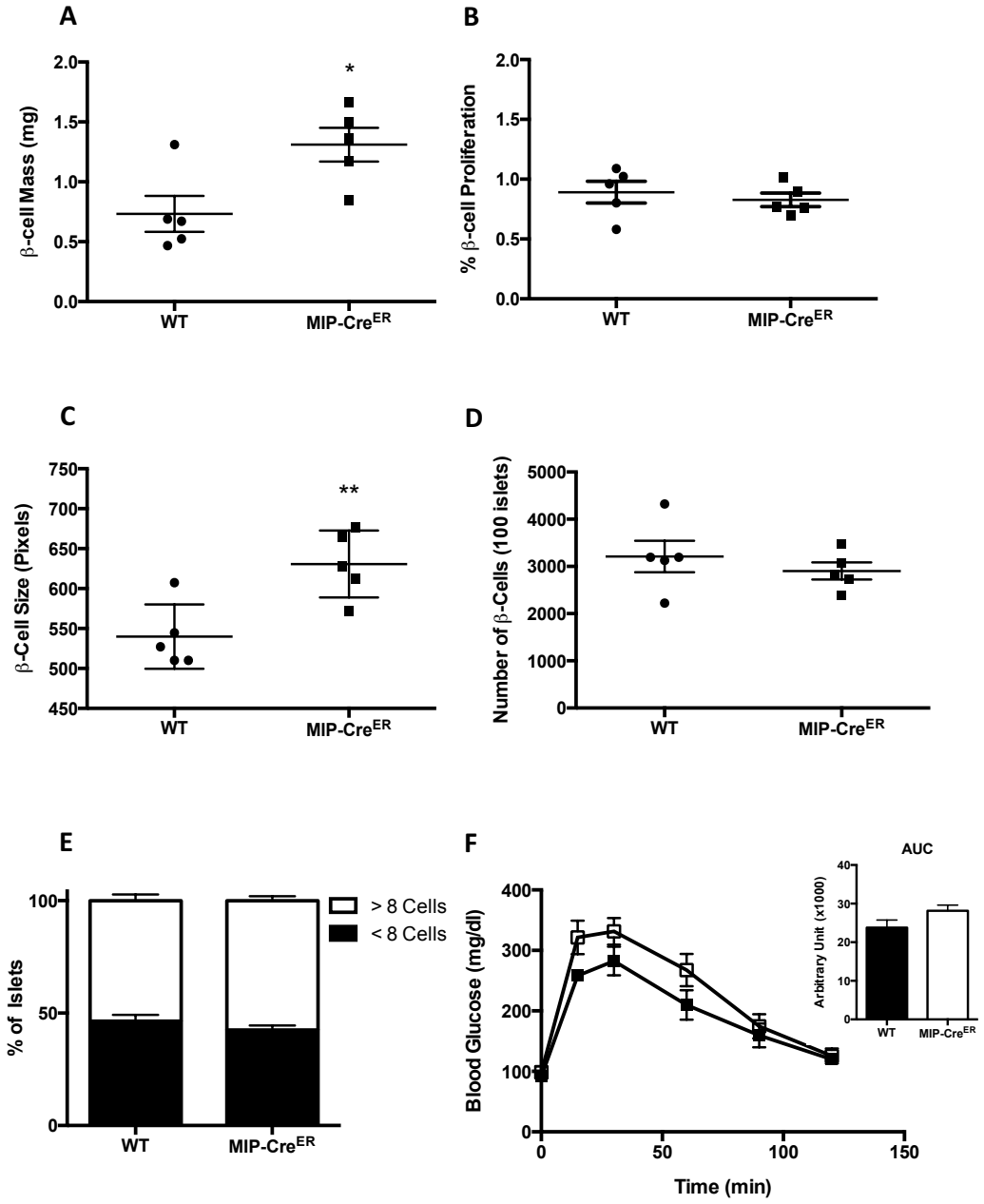

Figure 3-1. Chow-fed MIP-Cre^{ER} mice have enhanced β-cell mass due to increased β-cell size. **A**) β-cell mass, **B**) β-cell proliferation, **C**) β-cell size, **D**) β-cell number, **E**) percentage of small insulin+ clusters, and **F**) IP-GTT and Area Under the Curve (AUC) of IP-GTT for chow-fed 8-week-old WT (n=5) or MIP-Cre^{ER} (n=5) male mice. Black boxes represent WT mice; open boxes represent MIP-Cre^{ER} mice. Data are shown as mean ± SEM. *p*-values were calculated using Student's *t* test for A-E and two-way ANOVA followed by Tukey *post hoc* analysis for F. *p=0.02 in A; **p=0.0081 in C. Figure reprinted with permission from "Unexpected effects of the MIP-Cre^{ER} transgene and tamoxifen on β-cell growth in C57Bl6/J male mice." Carboneau, B.A., Le, T.D.V., Dunn, J.C., and Gannon, M. *Physiol. Rep.* 4(18): pii: e12863. Copyright © 2016. DOI 10.148/phy2.12863.

suggesting that the increase in β-cell mass in MIP-Cre^{ER} mice at 8 weeks of age is not due to an earlier increase in proliferation. Next, the possibility that islet neogenesis was responsible for the increase in β-cell mass in MIP-Cre^{ER} mice was explored. As a surrogate for neogenesis, the number of small insulin+ clusters (<8 cells) was quantified. No change in the number of small islet clusters was observed (Figure 3-1E), suggesting that neogenesis does not contribute to the increase in β-cell mass. However, in the absence of a lineage tracer, this cannot be completely ruled out. Thus, these data demonstrate that MIP-Cre^{ER} mice have increased β-cell mass due to individual cell hypertrophy and not due to changes in β-cell proliferation.

Whole-body glucose homeostasis was also assessed in MIP-Cre^{ER} mice since the production of Cre in β-cells has led to impaired glucose homeostasis in other models in the absence of TM [185]. Consistent with a recent report [181], chow-fed untreated MIP-Cre^{ER} mice showed no significant difference in glucose clearance as measured by IP-GTT (Figure 3-1F).

β-cell proliferation is affected similarly in HFD-fed WT and MIP-Cre^{ER} mice

When β-cell compensatory mechanisms are operating normally, β-cell mass is increased during obesity in response to increases in metabolic demand [42, 43, 67]. It is well documented that in rodents, this increase in β-cell mass during obesity occurs via increased β-cell proliferation [47]. Although MIP-Cre^{ER} mice on a chow diet did not show changes in β-cell proliferation compared to WT mice, we wanted to investigate if HFD-induced replication was altered due to the presence of the hGH minigene in this strain. Eight-week-old WT and MIP-Cre^{ER} mice were placed on a HFD for one week: a time

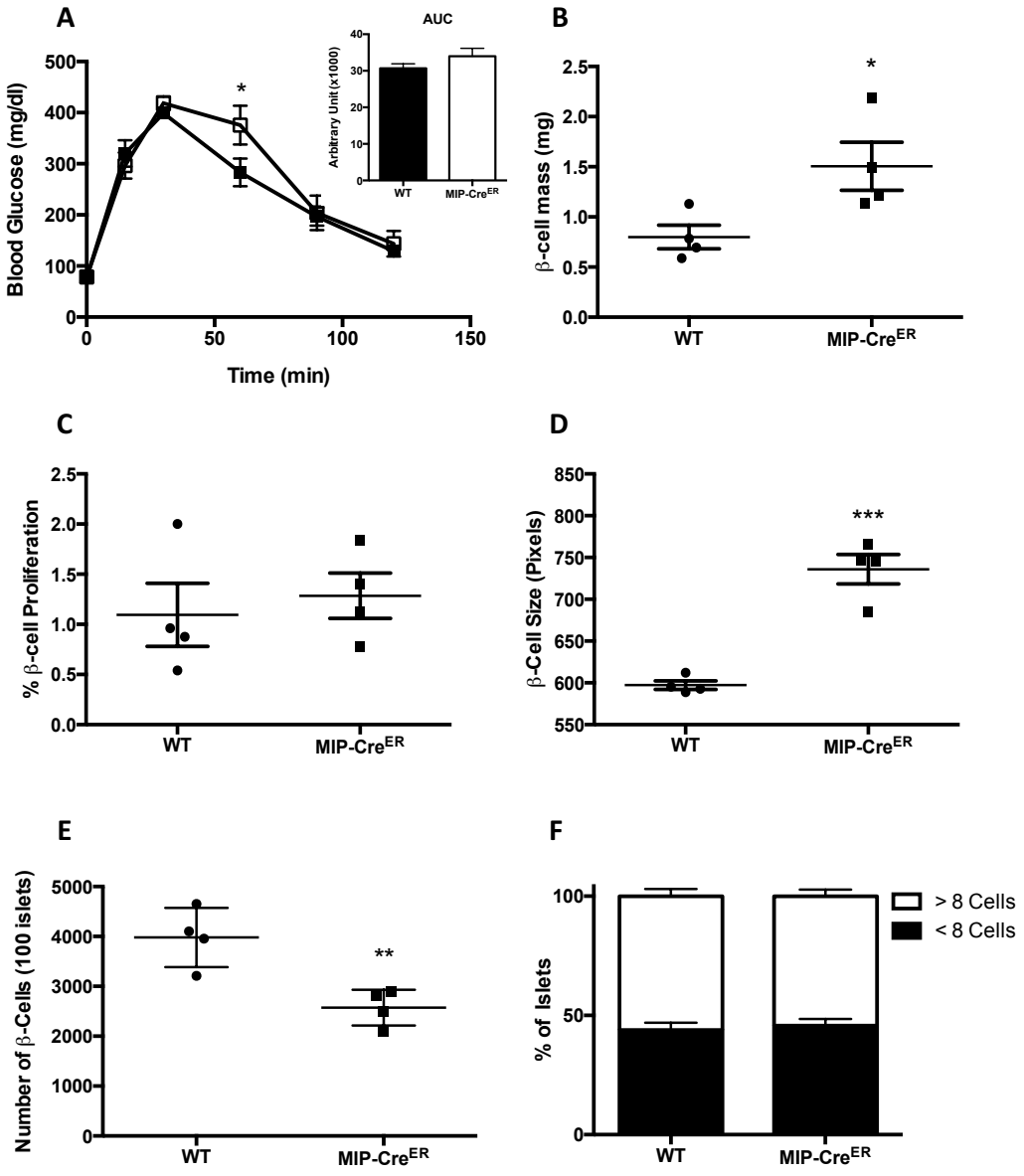

Figure 3-2. WT and MIP-Cre^{ER} mice show similar levels of β-cell proliferation in response to 1 week HFD feeding. **A**) IP-GTT and AUC (WT n=6, MIP-Cre^{ER} n=3), **B**) β-cell mass, **C**) β-cell proliferation, **D**) β-cell size, **E**) β-cell number, and **F**) percentage of small insulin+ clusters for 8-week-old WT or MIP-Cre^{ER} male mice placed on a HFD for 1 week. Black boxes represent WT mice; open boxes represent MIP-Cre^{ER} mice. Data are shown as means ± SEM. *p*-values were calculated using Student's *t* test for B-F and two-way ANOVA followed by Tukey *post hoc* analysis for A. *p<0.05 in A; *p=0.0385 in B; and ***p=0.0003 in D; **p=0.0067 in E. Figure reprinted with permission from "Unexpected effects of the MIP-Cre^{ER} transgene and tamoxifen on β-cell growth in C57Bl6/J male mice." Carboneau, B.A., Le, T.D.V., Dunn, J.C., and Gannon, M. *Physiol. Rep.* 4(18): pii: e12863. Copyright © 2016. DOI 10.148/phy2.12863.

point that corresponds to robust increases in proliferation [44, 45]. Whole-body glucose homeostasis was unchanged between WT and MIP-Cre^{ER} mice following HFD-feeding as measured by IP-GTT (Figure 3-2A). Further, HFD-fed MIP-Cre^{ER} animals have increased β-cell mass and β-cell size compared to WT (Figure 3-2B, D), similar to chow-fed mice. There was no difference in β-cell proliferation in response to one-week HFD feeding between WT and MIP-Cre^{ER} mice (Figure 3-2C). The increased β-cell mass can be explained again by hypertrophy, rather than proliferation or neogenesis, as there was a decrease in the number of β-cells in MIP-Cre^{ER} mice on HFD (Figure 3-2E), and no difference in the number of small insulin+ clusters (Figure 3-2F). Collectively, these data suggest that neither the presence of hGH nor the MIP-Cre^{ER} transgene itself affects β-cell proliferation during metabolic stress, but that one or both affects β-cell growth.

Tamoxifen inhibits HFD-induced β-cell proliferation

The above studies were performed in MIP-Cre^{ER} mice in which Cre^{ER} had not been activated by TM injection and was retained in the cytoplasm (Figure 3-3A**,** top panels). Upon TM injection, MIP-Cre^{ER} undergoes nuclear translocation (Figure 3-3A, bottom panels). Recently, TM was reported to impair β-cell proliferation during PDL, pregnancy, and development in the absence of any genetic manipulation [182]. However, the effects of TM on HFD-induced β-cell proliferation are unknown. As TM is required for activation of MIP-Cre^{ER}, we sought to determine if the presence of TM, in the absence of Cre, affects basal and/or HFD-induced β-cell proliferation.

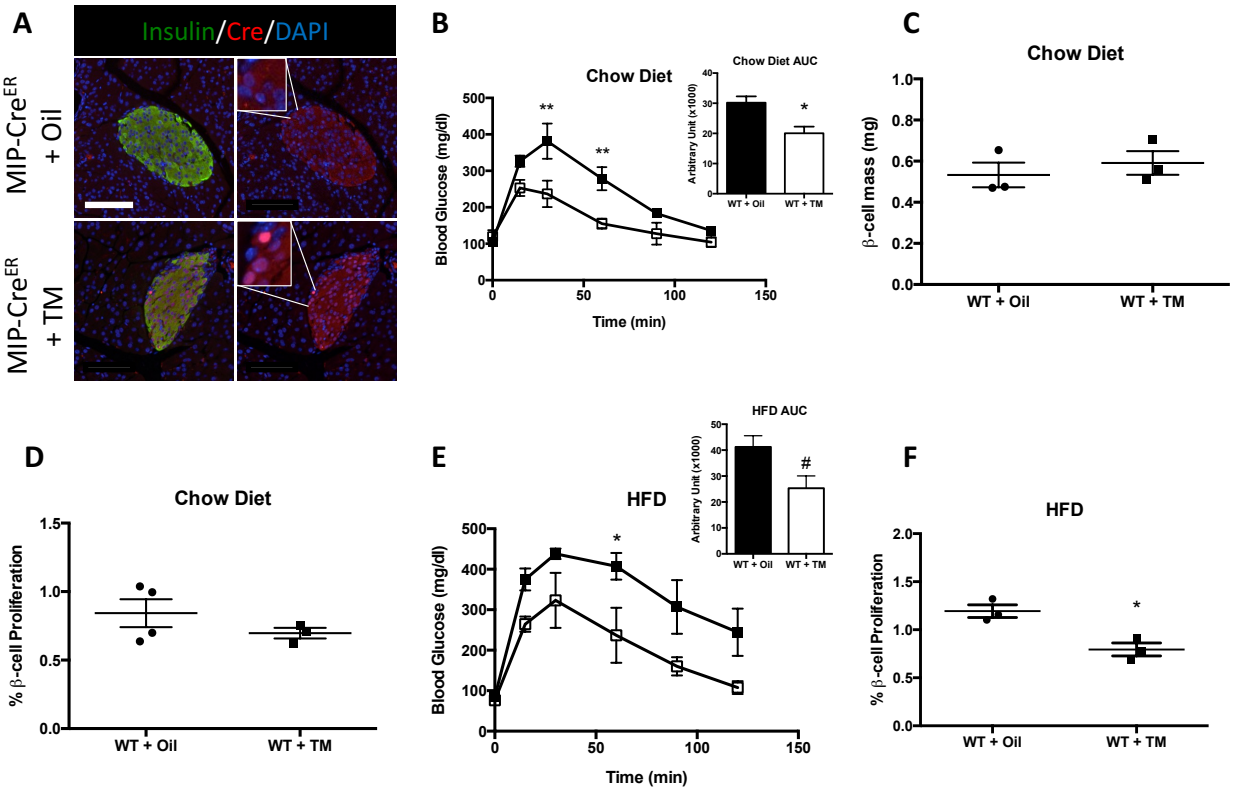

Figure 3-3. TM inhibits β-cell proliferation after 3 days HFD feeding in WT mice. **A**) Immunolabeling of Cre localization in MIP-Cre^{ER} mice injected with corn oil (top panels) or TM (bottom panels). In the absence of TM, Cre displays cytoplasmic localization (top panels). Upon TM treatment, Cre immunolabeling can be visualized in the nuclei, as marked by DAPI (bottom panels). The brightness was adjusted +20% and contrast -20%. Scale bars represent 100 µm. **B**) IP-GTT and AUC (WT + oil n=3, WT + TM n=3), **C**) β-cell mass, and **D**) β-cell proliferation for chow-fed WT mice injected with corn oil or TM at 6 weeks of age and analyzed at 8 weeks of age. **E)** IP-GTT and AUC (WT + oil n=3, WT + TM n=3) and **F**) β-cell proliferation for WT mice placed on HFD for 3 days following the washout period. Black boxes represent WT + Oil-treated mice; open boxes represent WT + TM mice. Data are shown as means ± SEM. *p*-values were calculated using Student's *t* test for C, D, and F and two-way ANOVA followed by Tukey *post hoc* analysis for B and E. *p=0.0291, **p<0.01 in B; #p=0.0696, *p<0.05 in E**;** and *p=0.0133 in F. Figure modified and reprinted with permission from "Unexpected effects of the MIP-Cre^{ER} transgene and tamoxifen on β-cell growth in C57Bl6/J male mice." Carboneau, B.A., Le, T.D.V., Dunn, J.C., and Gannon, M. *Physiol. Rep.* 4(18): pii: e12863. Copyright © 2016. DOI 10.148/phy2.12863.

First, glucose homeostasis and β-cell proliferation were measured in chow-fed mice. For these experiments, 6-week-old WT male mice were injected with corn oil (vehicle) or TM dissolved in corn oil (3x5 mg injections over 5 days). Following a one-week washout period, IP-GTTs were performed. Surprisingly, WT mice injected with TM have improved glucose homeostasis compared to oil-injected mice (Figure 3-3B). No significant effect of TM on β-cell mass or β-cell proliferation was observed (Figure 3-3C-D). These findings are similar to previously published data in non-pregnant female mice [182].

Next, the impact of TM on compensatory β-cell proliferation was investigated. To address this question, WT male mice were placed on a HFD for 3 days following the one-week washout period mentioned above. Our group has previously published that 3 days of HFD feeding is the peak in HFD-induced β-cell proliferation [44]. There was a trend for improved glucose homeostasis in WT mice injected with TM (Figure 3-3E). In contrast to chow-fed mice, β-cell proliferation was blunted in TM-treated mice compared to oil-injected mice following 3 days of HFD (Figure 3-3F). These data indicate that TM suppresses β-cell proliferation during HFD feeding, in addition to PDL, pregnancy, and development [182].

Cre is not expressed in Ins1^{Cre} mice and is not sufficient to reduce EP4 receptor gene expression

Based on the confounding factors associated with MIP-Cre^{ER} transgene and TM described above, it was concluded that this mouse strain was not the best model

system to study the dynamics of β-cell mass *in vivo*. The newly developed Ins1^{Cre} model was therefore an attractive candidate for these studies as it does not contain the hGH minigene and is not TM-dependent.

Cre mRNA expression was measured in isolated islets from Ins1^{Cre} male mice and was compared to mice containing only floxed alleles of EP4 (control) or to MIP-Cre^{ER} mice injected with TM as a positive control for *Cre* expression. Surprisingly, islets from Ins1^{Cre} mice do not express *Cre* whereas islets from MIP-Cre^{ER} + TM mice have an 80-fold increase in *Cre* expression compared to floxed-only control samples (Figure 3-4A). Immunofluorescence for Cre protein corroborated the gene expression analysis: very few insulin-positive cells were positive for Cre (Figure 3-4B), whereas 80% of β-cells from MIP-Cre^{ER} + TM mice were dually positive for insulin and Cre (Figure 3-4B, C). The ability of the Ins1^{Cre} allele to mediate recombination of floxed *Ptger4* (EP4) alleles was examined by qRT-PCR in islets isolated from control (EP4$^{flox/+}$ or EP4$^{flox/flox}$) and EP4$^{flox/flox}$;Ins1^{Cre} mice. The qRT-PCR primers used to detect *Ptger4* expression lie within the floxed region of the gene (Figure 3-4D). Thus, *Ptger4* should not be detected in islets isolated from EP4$^{flox/flox}$;Ins1^{Cre} mice. However, there were no differences in *Ptger4* expression between control and EP4$^{flox/flox}$;Ins1^{Cre} islets (Figure 3-4E). Taken together, these data reveal that Ins1^{Cre} is not abundantly expressed in β-cells and thereby is not sufficient to reduce *Ptger4*.

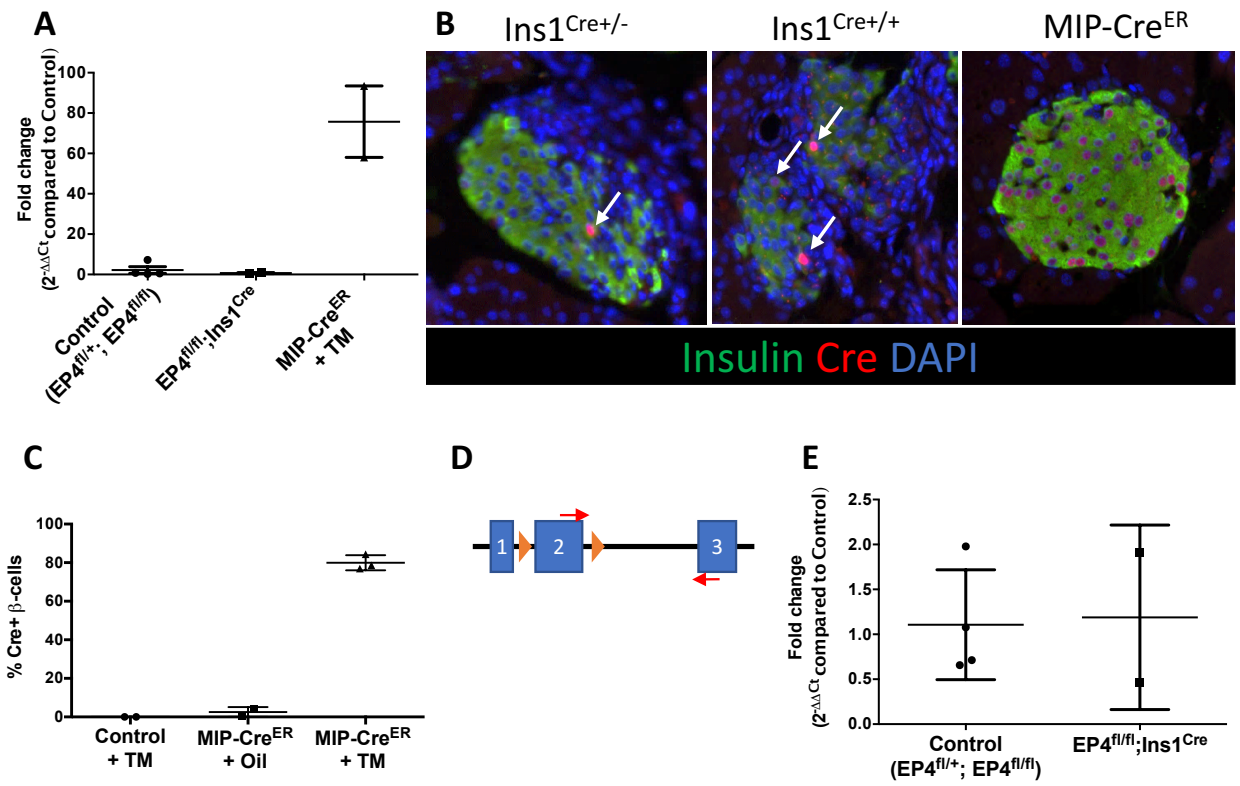

Figure 3-4. Characterization of Ins1^{Cre} mice. **A**) *Cre* mRNA expression in isolated islets from control (EP4$^{flox/+}$ or EP4$^{flox/flox}$), EP4$^{flox/flox}$;Ins1^{Cre}, and MIP-Cre^{ER} + TM-treated mice. **B**) Immunolabeling of Cre localization in Ins1$^{Cre+/-}$ (left panel), Ins1$^{Cre+/+}$ (middle panel), and MIP-Cre^{ER} mice injected with TM (right panel). White arrows point to Cre-positive cells in Ins1^{Cre} samples. The brightness was adjusted +20% and contrast -20%. **C**) Quantification of Cre-positive β-cells from control (no MIP-Cre^{ER}), MIP-Cre^{ER} + oil, and MIP-Cre^{ER} + TM mice. **D**) Schematic of primer design to detect recombination in the *Ptger4* allele. Blue boxes represent *Ptger4* exons, orange triangles represent *loxP* sites, and red arrows designate the position of qRT-PCR primers used in E. **E**) *Ptger4* gene expression in isolated islets from 8-week-old control (EP4$^{flox/+}$ or EP4$^{flox/flox}$) and EP4$^{flox/flox}$;Ins1^{Cre} mice.

Discussion

In this study, we confirm and expand upon recent findings characterizing commonly used mouse models in the islet field [181, 182]. Here, MIP-Cre^{ER} mice do not display impaired whole-body glucose homeostasis yet have increased β-cell mass in the absence of TM. This study demonstrates for the first time that the increase in β-cell mass can be explained by hypertrophy and not changes in replication. The results presented in this chapter further show that the presence of the MIP-Cre^{ER} transgene does not alter β-cell proliferation during HFD conditions. Additionally, we found that TM administration improves glucose homeostasis in chow-fed WT mice but impairs β-cell proliferation after HFD feeding. Surprisingly, islets from Ins1^{Cre} mice do not express high levels of Cre mRNA or protein, which impairs the ability of Cre to recombine floxed alleles. These findings reveal several new concerns associated with transgenic mouse models regularly used to study β-cell development, function, and mass dynamics.

Growth hormones (GH) can act on the β-cell by activating prolactin receptors (PRLRs) [186] and exert similar effects as prolactin (PRL) signaling [187]. During pregnancy, PRL and placental lactogen (PL) induce production of serotonin and increase β-cell proliferation, thus expanding β-cell mass to compensate for insulin resistance [49]. It has recently been reported that hGH expressed from Cre transgenes can contribute to pregnancy-like phenotypes [180, 181]. Specifically, MIP-Cre^{ER} mice have increased expression of the enzymes responsible for serotonin synthesis, *tryptophan hydroxylase 1* (*Tph1*) and *Tph2*, in addition to increased serotonin production [181]. These effects have been observed in different genetic backgrounds of

mice, including the C57Bl6/J, CD1, and BTBR strains [180, 181, 188]. Here, we show that, in addition to the previously reported phenotypes, MIP-Cre^{ER} mice have increased individual β-cell size resulting in significantly increased β-cell mass. hGH has been shown to augment β-cell proliferation both *in vivo* [188] and *ex vivo* in cultured islets [189]. However, in this study, the presence of the hGH in the MIP-Cre^{ER} transgene does not alter β-cell replication. These differences may be due to the genetic makeup of transgenes used in the different studies [78, 188]. Consistent with the findings herein, β-cell proliferation is not different between untreated MIP-Cre^{ER} and WT mice placed on a high fat-high sugar diet and treated with STZ [181].

In addition to the effects of hGH on β-cell dynamics, the consequences of TM have recently been brought to light. TM is a synthetic ER antagonist that binds more strongly to a mutated ER that is insensitive to circulating endogenous estrogens [190]. Estrogen and ER signaling are important in β-cell function, survival, and replication, yet the potential effect of TM on β-cell proliferation through effects on endogenous ER was previously unknown [182]. Inhibition of ER signaling via TM administration, in the absence of Cre, impairs the induction of β-cell proliferation following PDL, during pregnancy, and in development [182]. β-Cell proliferation is also impaired in response to PDL and during development when ER signaling is turned off by genetic deletion of the ERα receptor [182], demonstrating the importance of ER signaling in promoting β-cell proliferation. We have now shown that TM administration also blunts the proliferative capacity of β-cells in response to short-term HFD feeding.

To our surprise, the impaired proliferation in TM-injected mice was accompanied by improved glucose homeostasis. Steroid hormones and ER modulators, such as TM,

have been shown to alter food consumption and appetite [191]. Male mice treated with TM consume less food than control treated mice, resulting in less weight gain over a 28-day period [192]. Decreased food consumption in TM treated mice may explain the improved glucose homeostasis observed in this study. Higher doses of TM, similar to that used here, can have long-term toxic effects in mice [193]. It is unknown how lower doses of TM, a longer washout period, or route of administration (gavage, subcutaneous, or IP) may impact β-cell proliferation during HFD. Based on previous results [182], I speculate that lower doses of TM would also inhibit HFD-induced replication. TM administered subcutaneously is present in the system for up to 4 weeks post-injection [193]. Therefore, a washout period of longer than 4 weeks may help avoid the TM-induced impaired proliferation observed here and by Yuchi and colleagues; however, this has not been empirically tested.

The generation of the $Ins1^{Cre}$ model is an important development in the field for several reasons: 1) it does not contain the hGH minigene, 2) recombination has not been observed in the brain, 3) the presence of the $Ins1^{Cre}$ construct does not alter body weight or glucose homeostasis, and 4) lineage tracing demonstrates efficient recombination in β-cells (98%) [79]. However, $Ins1^{Cre}$ mice in our colony do not express *Cre* mRNA or Cre protein, and are not effective at reducing *Ptger4* expression in $EP4^{flox/flox}$;$Ins1^{Cre}$ mice. The reason for these discrepancies are unknown. One potential explanation could be due to differences in the gut microbiome between different animal facilities, which is causing gene silencing of some transgenes. Our lab and others have observed this phenomenon with other transgenes in the past.

In conclusion, these data unveil additional unexpected phenotypes of commonly used models for studies on β-cell mass expansion, underscoring the importance of proper controls for utilization and interpretation of data related to β-cell function, mass, and proliferation. In particular, inclusion of mice expressing only MIP-Cre^{ER} or Cre^{ER} mice treated with TM without further genetic manipulation will be necessary as control groups, particularly for studies related to β-cell dynamics.

CHAPTER IV

OPPOSING EFFECTS OF EP3 AND EP4 ON MOUSE AND HUMAN *EX VIVO* β-CELL SURVIVAL AND PROLIFERATION

Introduction

Exploration into the GPCR signaling pathways that enhance β-cell proliferation and survival may reveal novel pathways for the development of drugs to treat T2D. As discussed in Chapter I, GLP-1R-based therapies have proven to be beneficial in many patients with T2D, however they are not effective in all individuals [92]. Increased activity of GPCRs, such as EP3, that couple to inhibitory G-proteins in some individuals may provide an explanation for these variable responses to GLP-1R-based treatments.

The role of PGE_2 in GSIS has been widely studied and is primarily demonstrated as being inhibitory to GSIS in *in vitro, ex vivo,* and *in vivo* settings [107, 123, 141-143, 149, 150, 152, 153]; however, these inhibitory effects have not been consistently observed [128, 129, 158, 159, 166]. In contrast, less is known about the role of PGE_2 and the EP receptors in regulating β-cell proliferation and survival, which can affect β-cell mass dynamics. As previously discussed, $EP3^{-/-}$ mice have increased β-cell proliferation after prolonged HFD feeding [166]; yet the β-cell-specific effect of EP3 in this process remains undetermined as $EP3^{-/-}$ mice gain more weight than WT mice on HFD [166, 173]. Evidence from studies of an inhibitory G-protein that couples to EP3 in the islet, G_Z [162], further suggests that EP3 acts to inhibit β-cell proliferation and

increase β-cell death [101, 103]. However, the direct effect of EP3 in these processes remains unknown since G_Z may couple to multiple GPCRs. The role of EP4 in regulating β-cell proliferation or survival has yet to be determined. Pharmacological activation of EP4 in conjunction with genetic loss of EP2 protects against STZ-induced hyperglycemia and death in mice, yet the mechanism for this protection remains to be determined [114]. In other cell types, such as mouse gut epithelial cells and primary myoblasts, EP4 agonist treatment increases cell proliferation and decreases cell death [171, 172].

We previously reported that *Ptger3* (EP3) gene expression is reduced and *Ptger4* (EP4) is increased in islets from a transgenic mouse model of enhanced β-cell proliferation and survival in which an activated form of the critical cell cycle-regulating transcription factor FoxM1 is induced in β-cells (β-FoxM1*) [61]. Taken together, these data led us to hypothesize that EP3 and EP4 play opposing roles in β-cell proliferation and survival, such that EP3 signaling inhibits β-cell proliferation and promotes β-cell death, whereas EP4 enhances replication and protects against β-cell death. To test this hypothesis, mouse and human islets were exposed *ex vivo* to selective agonists and antagonists for EP3 and EP4 and the effects on β-cell proliferation and survival were analyzed. Here, we show that pharmacological blockade of EP3 with the antagonist DG-041 [194] has no effect on basal levels of β-cell proliferation, but enhances the proliferative response to placental lactogen (PL) in young WT mouse islets, but not in islets from aged WT mice. EP3 blockade also enhances human β-cell proliferation, even in the absence of PL, but has no effect on α-cell proliferation. Activation of EP4 alone does not affect mouse or human β-cell proliferation, but increases human β-cell

proliferation when EP3 signaling is simultaneously blocked. In mouse and human islets, EP4 activation increases β-cell survival following cytokine treatment, while the EP3 pathway impairs β-cell survival. In addition, the positive effects of FoxM1 activation on β-cell survival require the activation of EP4 and inhibition of EP3 activity. These data, combined with previously published work, suggest that inhibition of EP3 and/or activation of EP4 may improve outcomes in the settings of obesity and T2D by enhancing β-cell proliferation, survival, and GSIS.

Results

Placental lactogen treatment decreases *Ptger3γ* expression

PL is a pregnancy hormone known to stimulate rodent β-cell proliferation [195]. We thus wanted to determine whether PL treatment could alter the expression of EP receptors or enzymes involved in PGE_2 production as a mechanism to induce β-cell proliferation. WT mouse islets were treated with PL for four days, which corresponds to the time required to observe the maximum effect of PL [195]. qRT-PCR was used to analyze the expression of the four PGE_2 receptors EP1-4 (encoded by the genes *Ptger1-4*), including the three mouse EP3 splice variants (α, β, and γ), and the enzymes required for PGE_2 production (cyclooxygenase (COX)-2: *Ptgs2*; prostaglandin E synthases: *Ptges1-3*).

PL treatment did not significantly alter the expression levels of the PGE_2 synthases *Ptges1-3* or the expression of *Ptger1, Ptger2,* total *Ptger3*, or *Ptger4* (Figure 4-1A). Although we observed a variable increase in *Ptgs2* (COX-2) expression in each

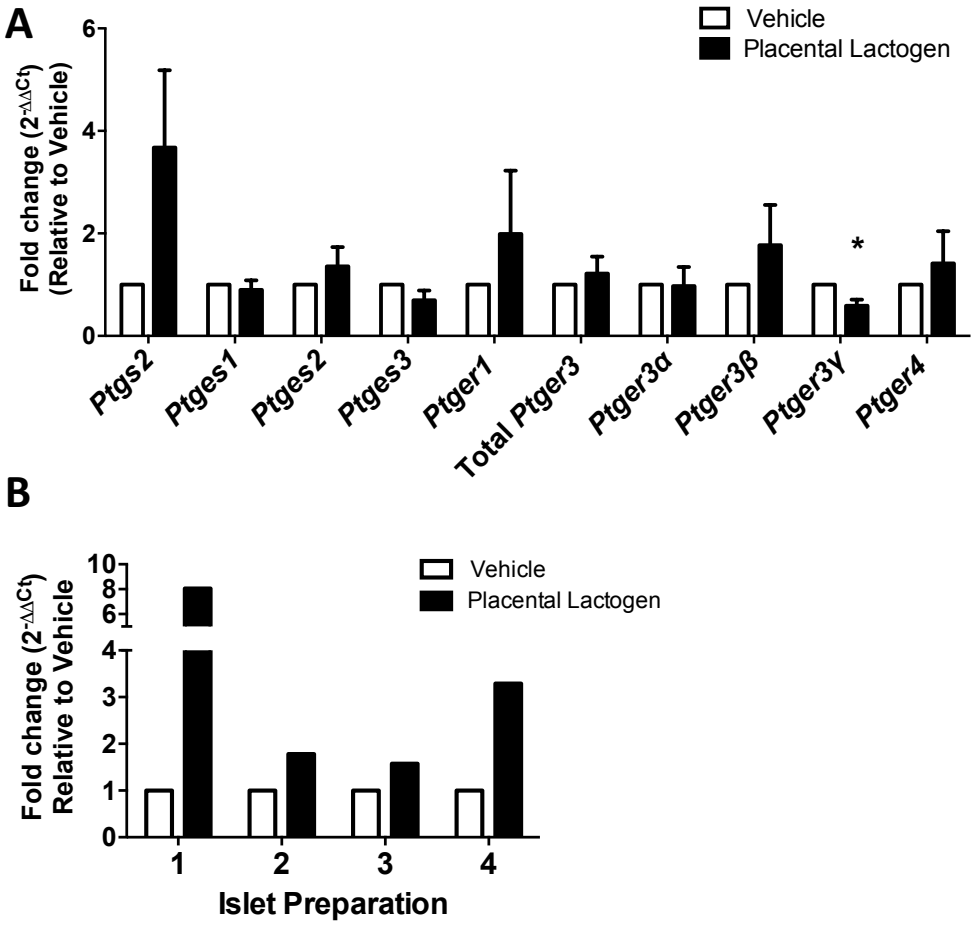

Figure 4-1. Placental lactogen decreases *Ptger3*γ in mouse islets. **A**) Wild type mouse islets (8-10-weeks-old) were cultured for four days in the presence of vehicle (n=4) or PL (n=4). qRT-PCR was performed for PGE_2 synthesis genes (*Ptgs2, Ptges1-3*) and receptor genes (*Ptger1-4*). *p=0.0147 vs vehicle. **B**) Individual mouse islet preparations are plotted on the X-Axis to show individual *Ptgs2* changes in response to PL treatment. All data are represented as $2^{-\Delta\Delta Ct}$ relative to vehicle. Data were analyzed using Student's *t test.* Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

islet preparation in response to PL, it did not reach statistical significance (Figure 4-1A-B). Intriguingly, PL treatment specifically decreased expression of *Ptger3γ* (Figure 4-1).

EP3 inhibits, whereas EP4 has no effect on, mouse β-cell proliferation *ex vivo*

Data from EP3$^{-/-}$ mice and other cell types suggest that EP3 plays an inhibitory role while EP4 plays a positive role in regulating replication [166, 171, 172], yet a specific role for EP3 and EP4 in β-cell proliferation has yet to be identified. To investigate this, β-cell proliferation was measured in young (8-10-weeks-old) WT mouse islets treated *ex vivo* with the EP3 agonist sulprostone, the EP3 antagonist DG-041, the EP4 agonist CAY10598, or the EP4 antagonist L-161,982 using a proliferation assay developed by our laboratory [178]. After four days of treatment, PL increased β-cell proliferation 3-4-fold over vehicle-treated islets (Figure 4-2A-B), in agreement with previously published results [59, 178, 195]. Basal levels of β-cell proliferation were not affected by treatment with any of the EP3 or EP4 ligands alone (Figure 4-2A-B). Based on the decreased *Ptger3γ* expression in response to PL (Figure 4-1A), the ability of EP3 activity to inhibit the proliferative response to PL was tested by treating WT islets with a combination of PL and either sulprostone or DG-041. Activation of EP3 with sulprostone in the presence of PL failed to increase β-cell proliferation over that of vehicle-treated islets, in agreement with our prediction (Figure 4-2A). Further, blockade of EP3 using DG-041 in combination with PL led to a significant increase in β-cell proliferation over that of DG-041 or PL alone (Figure 4-2A). Next, the possibility that activation or blockade of EP4 signaling could alter the proliferative response to PL was tested.

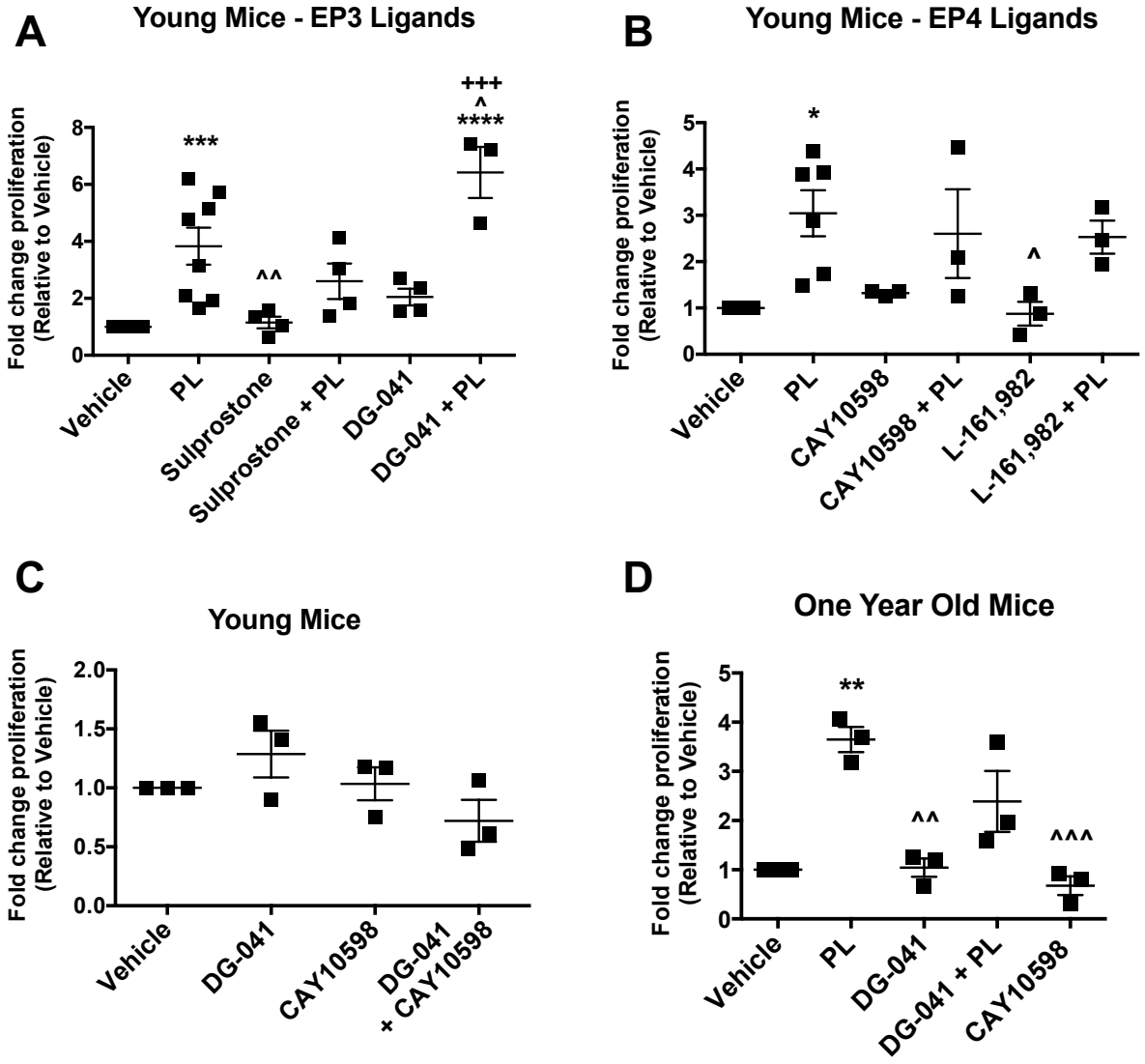

Figure 4-2. EP3 inhibits, while EP4 does not affect, mouse β-cell proliferation. **A**) Young mouse islets (8-10-weeks-of-age) were treated with vehicle, PL, sulprostone ± PL, or DG-041 ± PL for 4 days before being immunolabeled for insulin, Ki67, and DAPI. *versus vehicle; ^versus PL; +versus DG-041 alone. **B**) Young mouse islets were treated with vehicle, PL, CAY10598 ± PL, or L-161,982 ± PL as described in A. *versus vehicle; ^versus PL. **C**) Young mouse islets were treated with vehicle or DG-041 ± CAY10598 as described in A. **D**) Aged mouse islets (one-year-old) were treated with vehicle, PL, DG-041 ± PL, or CAY10598 as described in A. *versus vehicle; ^versus PL. All data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. One symbol represents $p<0.05$, two symbols indicate $p<0.01$, four symbols represent $p<0.0001$. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

However, neither CAY10598 nor L-161,982 treatment in combination with PL significantly affected β-cell proliferation compared to PL alone (Figure 4-2B). In addition, combined blockade of EP3 and activation of EP4 did not increase mouse β-cell proliferation (Figure 4-2C). Taken together, these data show that EP3 inhibits β-cell proliferation in the presence of PL in young mouse islets and that EP4 does not play a role in modulating mouse β-cell proliferation under the conditions tested.

The ability of β-cells to respond to proliferative cues, such as the GLP-1R agonist exendin-4, declines with age, beginning at 8 months of age in the mouse [40]. We therefore asked whether blockade of EP3 would stimulate β-cell proliferation in islets from older mice. In islets from one-year-old WT mice, PL treatment resulted in a 3.5-fold increase in β-cell proliferation (Figure 4-2D), as it did in young islets. Similar to what was observed in young islets, treatment with either DG-041 or CAY10598 alone had no effect on β-cell proliferation in aged islets (Figure 4-2D). In contrast to young islets, in older islets, DG-041 treatment (EP3 antagonist) in combination with PL did not result in a further enhancement of β-cell proliferation over that of PL alone.

Ptger3 (EP3) gene expression is altered with age in mouse islets

Given the lack of response to DG-041 in aged β-cells, the possibility that expression of PGE_2 receptors changes during aging was tested. qRT-PCR was performed on islets isolated from WT mice aged two, four, eight, or 12 months. The total level of *Ptger3* (EP3) was increased in islets at 12 months of age due to a specific increase in expression of *Ptger3*γ (Figure 4-3). No differences in gene expression of *Ptger3*α, *Ptger3*β, or the other EP receptors were identified during aging (Figure 4-3).

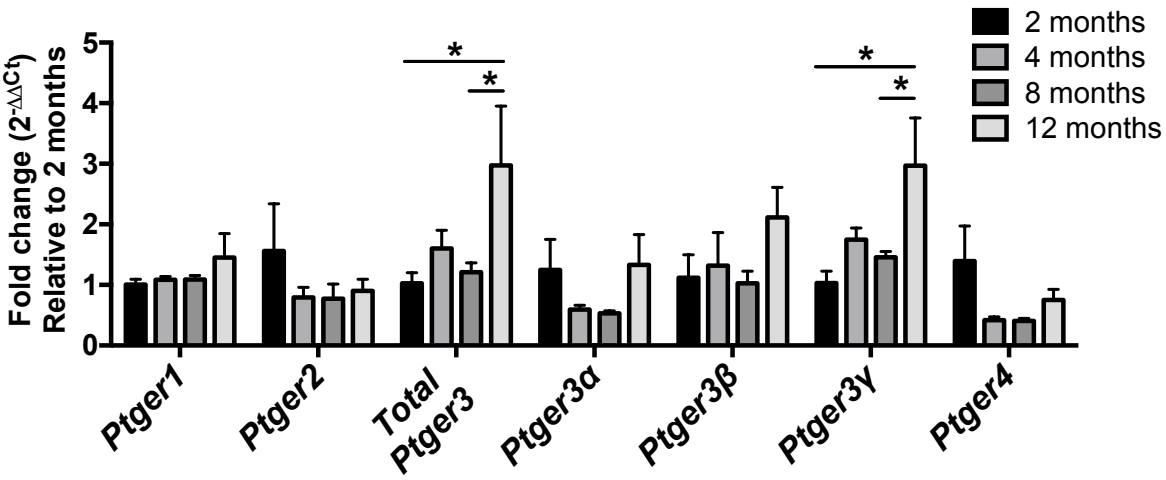

Figure 4-3. *Ptger3* (EP3) displays increased expression with age. RNA was isolated from mouse islets at 2 (n=3), 4 (n=4), 8 (n=4), or 12 (n=5) months of age. qRT-PCR was performed for PGE_2 receptor expression. Data are expressed as $2^{-\Delta\Delta Ct}$ relative to 2 months old. All data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. *p<0.05. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

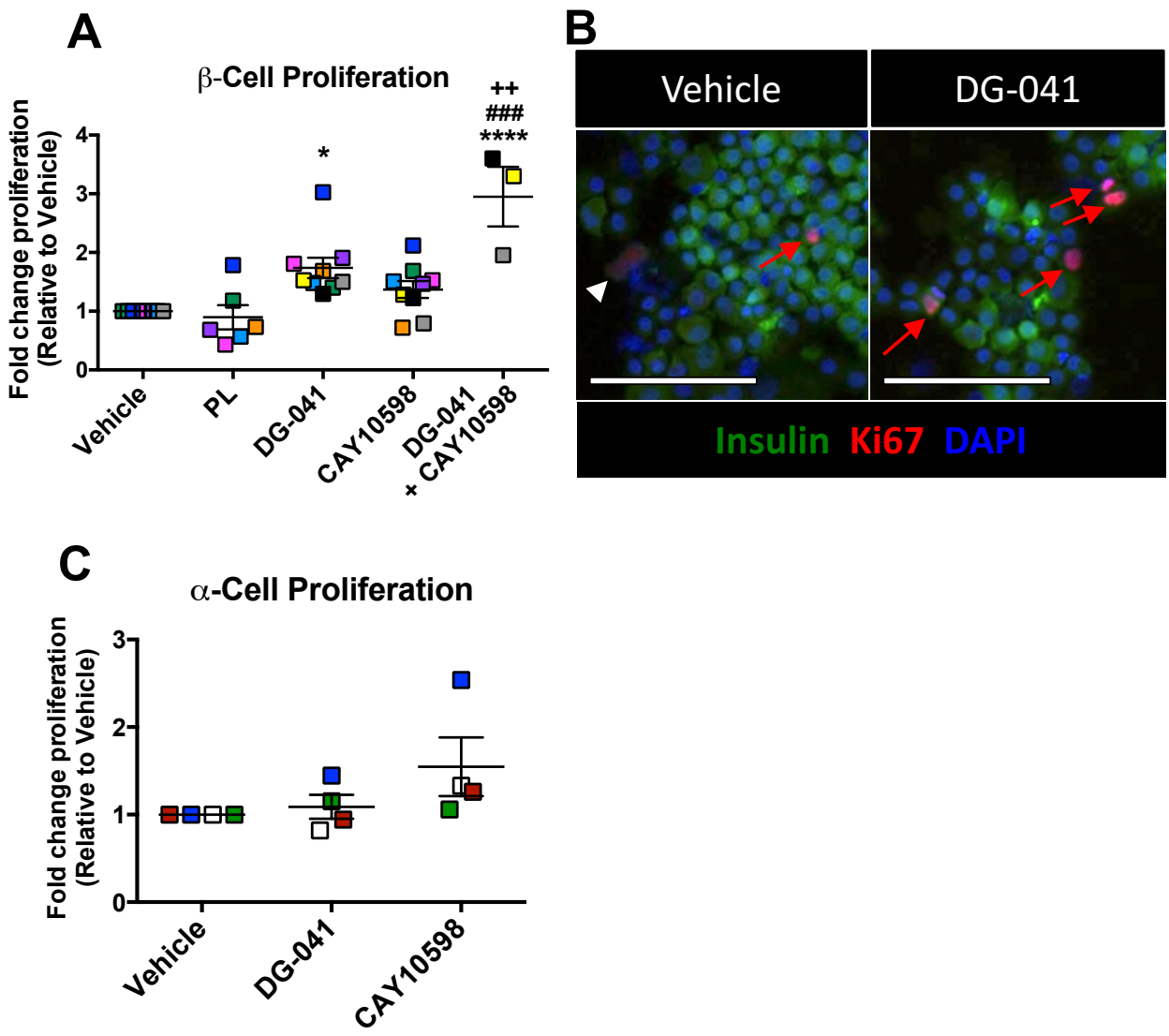

Figure 4-4. EP3 and EP4 have opposing effects on human β-cell proliferation. **A**) Human islets were treated with vehicle, PL, DG-041, or CAY10598 and immunolabeled for insulin (green), Ki67 (red), and DAPI (blue). *p=0.0177 vs vehicle; ****p<0.0001 vs vehicle; ++ p=0.0038 vs DG-041; ###p<0.001 vs CAY10598. **B**) Representative images of vehicle- and DG-041-treated human islets (donor H1588). Scale bars represent 100 μM. Red arrows point to proliferating β-cells; white arrowhead indicates non-specific immunolabeling. **C**) Human islets were treated with vehicle, PL, DG-041, or CAY10598 and immunolabeled for glucagon, Ki67, and DAPI (not shown). Each distinct human donor is represented by a different colored symbol. All data are represented as fold change in β- or α-cell proliferation compared to vehicle-treated islets. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

EP3 and EP4 have opposing effects on human β-cell proliferation *ex vivo*

It is critical to determine whether discoveries in mouse translate to human β-cells. Thus, we examined whether blocking EP3 or activating EP4 could increase human β-cell proliferation. In contrast to mouse islets, PL did not increase human β-cell proliferation (Figure 4-4A). This divergence in responsiveness to PL in human versus mouse islets may be explained by differences in prolactin receptor (*Prlr*) expression: *PRLR* is expressed in human β-cells, but is approximately 15-fold lower than the levels observed in mouse β-cells by RNA-seq [196]. Interestingly, blockade of EP3 with DG-041 increased human β-cell proliferation compared to vehicle-treated islets in the absence of any other stimulus (Figure 4-4A, B). Similar to mouse islets, activation of EP4 by CAY10598 did not increase human β-cell proliferation (Figure 4-4A). Strikingly, in contrast to mouse islets, combined blockade of EP3 and activation of EP4 led to a significant increase in human β-cell proliferation, over that of DG-041 or CAY10598 treatment alone (Figure 4-4A). Importantly, neither DG-041 nor CAY10598 treatment increased human α-cell proliferation (Figure 4-4C). Thus, EP3 signaling inhibits β-cell proliferation in both mouse and human islets, whereas EP4 signaling increases human β-cell proliferation only when EP3 signaling is blocked.

Increased *PTGER3* (EP3) expression is observed in islets from lean T2D individuals

A previous study found that *PTGER3* (EP3) expression was increased in human islets from obese and T2D donors [123]. PGE_2 production is increased in T2D [123] and T2D incidence increases with age [197], highlighting the potential significance of this

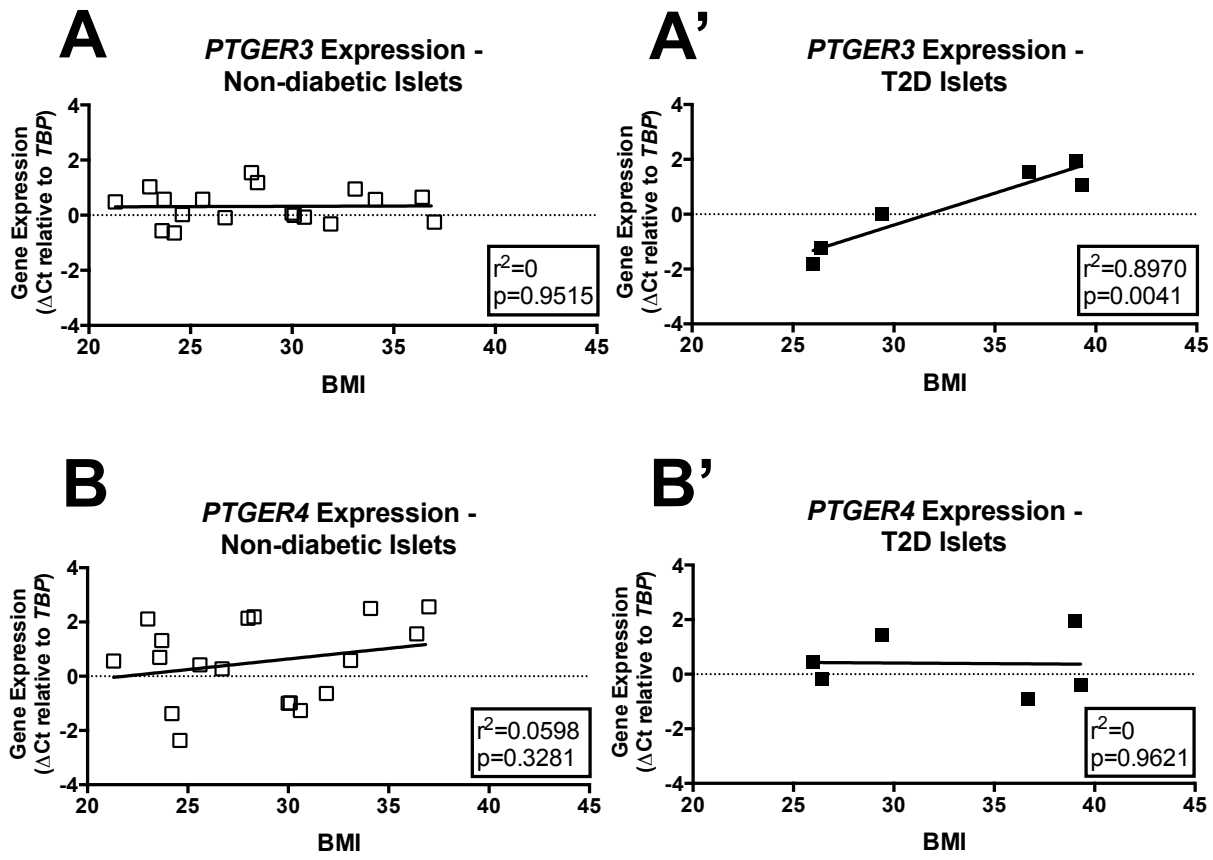

Figure 4-5. Increased *PTGER3* expression in T2D human islets is correlated with lower BMI. RNA was isolated from human islets and qRT-PCR was performed for EP3 **A**) and EP4 **B**) expression in non-diabetic (**A, B**) and T2D (**A', B'**) islets. Data are represented as ΔCt relative to *TBP* and were analyzed using a linear regression. Figure reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

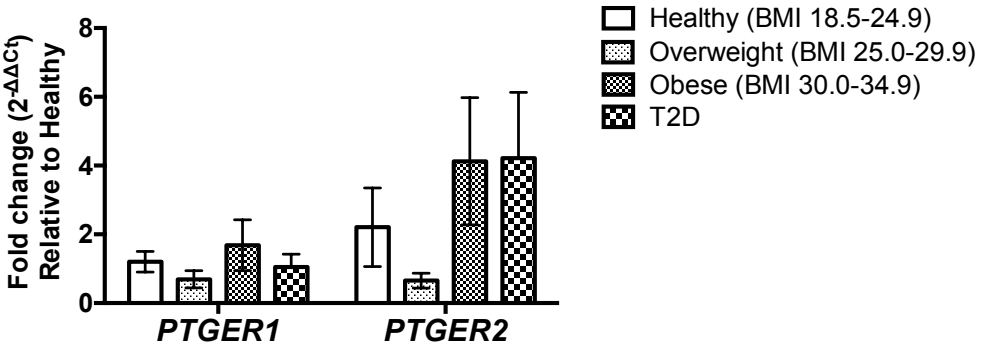

Figure 4-6. *PTGER1* and *PTGER2* expression in human islets is not correlated with BMI or T2D. RNA was isolated from human islets and qRT-PCR was performed for *PTGER1* (EP1) and *PTGER2* (EP2) expression. n=7 for healthy; n=3 for overweight; n=8 for obese; n=6 for T2D. Data are expressed as $2^{-\Delta\Delta Ct}$ relative to healthy. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Figure reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

pathway in T2D etiology. EP receptor expression was analyzed in human islets and classified by BMI and T2D status. In contrast to the previously published dataset [123], *PTGER3* expression was not increased in islets from this set of obese non-diabetic donors (Figure 4-5A). However, islets from T2D donors did show a significant correlation between increased *PTGER3* expression and lower BMI (r^2=0.8970, p=0.0041; Figure 4-5A'). These data are consistent with previous work demonstrating that *PTGER3* is increased in T2D donor islets (BMI 25.4 ± 2.1 kg/m^2) compared to non-diabetic donor islets (BMI 28.1 ± 7.3 kg/m^2) [123]. There were no significant differences in *PTGER4* expression in either non-diabetic donor islets (Figure 4-5B) or T2D donor islets (Figure 4-5B'). Additionally, no significant changes in *PTGER1* or *PTGER2* expression were detected (Figure 4-6).

EP3 induces, while EP4 protects against, β-cell death in mouse and human islets *ex vivo*

Ptger3 is decreased, whereas *Ptger4* is increased, in islets from β-FoxM1* mice, which display increases in both β-cell proliferation and β-cell survival [61]. In these mice, an activated form of the *Foxm1* cell cycle transcription factor was induced in β-cells in 8-week-old mice for two weeks. We hypothesized that EP3 and EP4 play opposing roles in β-cell survival in this model. G_Z-null mice have decreased β-cell death in response to STZ [101], suggesting that EP3 signaling negatively affects β-cell survival. Thus, to probe the roles of EP3 and EP4 in β-cell survival, EP3 signaling was blocked using DG-041 and EP4 was activated with CAY10598 in WT islets that were treated with a cytokine cocktail (TNFα, IFNγ, and IL-1β) known to induce β-cell death [61]. In the

presence of these cytokines, both DG-041 and CAY10598 treatments resulted in significant improvements in β-cell survival compared to the vehicle-treated WT islets (Figure 4-7A). The levels of β-cell survival in DG-041- and CAY10598-treated islets are comparable to those of vehicle-treated islets in the absence of cytokines (Figure 4-7A). Next, to determine if decreased EP3 activity and/or increased EP4 activity mediate the pro-survival effects of FoxM1, EP3 signaling was activated using sulprostone and EP4 was blocked with L-161,982 in β-FoxM1* islets treated with cytokines. β-FoxM1* islets treated with cytokines had significantly less β-cell death compared to vehicle-treated WT islets (Figure 4-7A), consistent with our previously published work [61]. In contrast, β-FoxM1* islets treated with sulprostone or L-161,982 had increased β-cell death compared to vehicle-treated β-FoxM1* islets and no longer exhibited the protective effect on β-cell survival induced by *Foxm1* over-expression (Figure 4-7A).

To investigate if EP3 and/or EP4 play roles in human β-cell survival, human islets were treated with cytokines and the EP ligands. Consistent with the mouse β-cell data, blockade of EP3 by DG-041 or activation of EP4 via CAY10598 decreased cytokine-induced human β-cell death compared to vehicle-treated islets (Figure 4-7B). Conversely, activation of EP3 via sulprostone led to a trend in increased β-cell death over cytokine treatment alone ($p=0.0713$, Figure 4-7B); blockade of EP4 signaling with L-161,982 did not alter cytokine-induced β-cell death (Figure 4-7B). Taken together, these data demonstrate antagonistic roles of EP3 and EP4 activity on mouse and human β-cell death, with EP3 decreasing β-cell survival and EP4 improving β-cell survival.

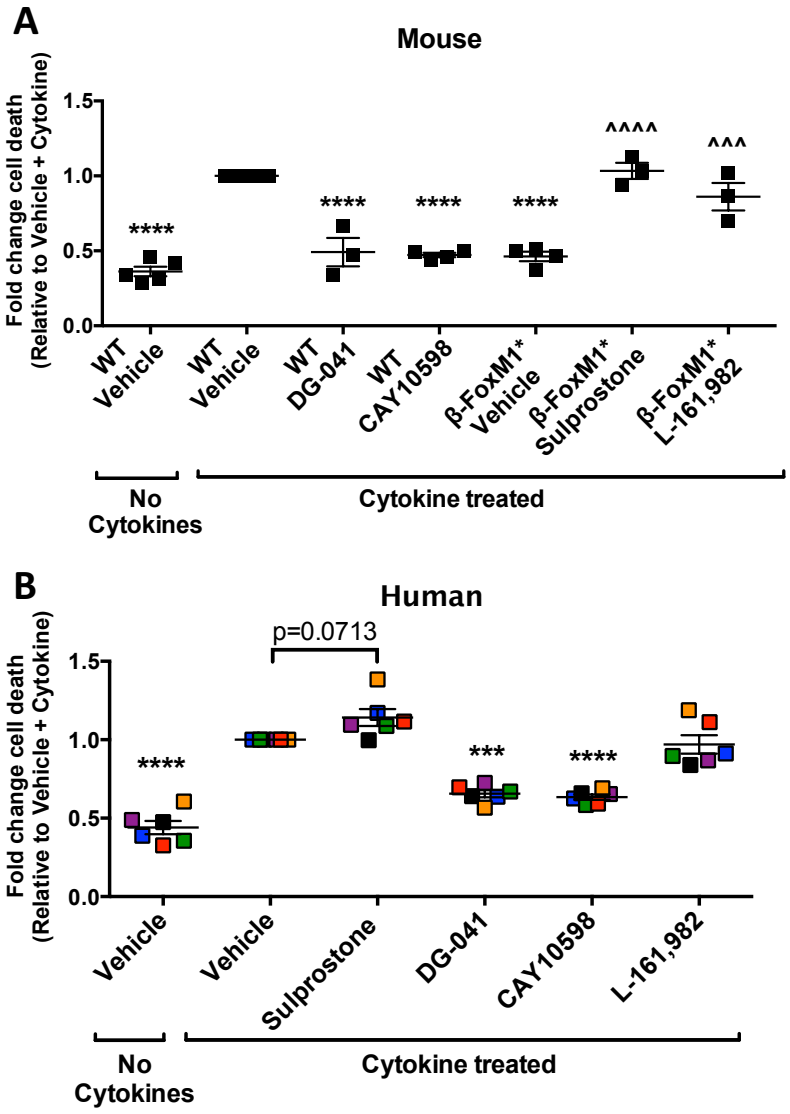

Figure 4-7. EP3 increases, while EP4 protects against, β-cell death in mouse and human islets. **A**) WT or β-FoxM1* mouse islets (8-10-weeks-old) or **B**) human islets were treated for 48 hours with a species-specific cytokine cocktail plus one of the following compounds: vehicle, sulprostone, DG-041, CAY10598, or L-161,982. Following treatment, islets were immunolabeled for insulin, TUNEL, and DAPI. Each distinct human donor is represented by a different colored symbol. **A**) *Significant versus WT Vehicle + Cytokines; ^significant vs β-FoxM1* Vehicle + Cytokines. **B**) *Significant vs Vehicle + Cytokines; #p=0.0713 vs Vehicle + Cytokines. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Three symbols indicate p<0.001, four symbols represent p<0.0001. Figure reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

Identification of downstream effectors of EP3 and EP4

It is well-established that PGE_2 inhibits GSIS through an EP3-inhibitory $G_{i/Z}$-protein coupled mechanism that results in decreased cAMP levels [103, 141, 150]. However, in this study, I was unable to detect any changes in cAMP when islets were treated with EP3 or EP4 ligands despite being able to measure an increase in cAMP in response to forskolin (data not shown; see Chapter II). In addition, hyperglycemic islet culture conditions, similar to those used in this study, are known to increase endogenous islet PGE_2 production [115, 116]. However, in my studies, the levels of endogenous PGE_2 produced during the *ex vivo* assays were below the detectable limit for analysis, and thus we cannot determine whether the culture conditions or various treatments had an effect on PGE_2 production (data not shown; see Chapter II).

The EP3 and EP4 signaling mechanisms downstream of G-protein coupling that promote their effects on proliferation and survival are unknown and may differ from those that affect GSIS. We first asked whether EP3 or EP4 signaling alters gene expression as a way to induce changes in β-cell proliferation and survival. To test this, several genes involved in cell cycle control and apoptosis were analyzed in WT mouse islets treated with DG-041 (EP3 inhibition) or CAY10598 (EP4 activation) for two or four days, corresponding to the halfway and end points of the proliferation assay, respectively. There were no significant changes in the cell cycle genes that were assessed after two (Figure 4-8A) or four days (Figure 4-9A) of treatment, although four days of DG-041 treatment resulted in a trend ($p=0.0804$) toward decreased expression of the cell cycle inhibitor *Cdkn1a* (p21). Furthermore, there were no changes in expression levels of apoptosis genes at either time point (Figure 4-8A', 4-9A'), with the

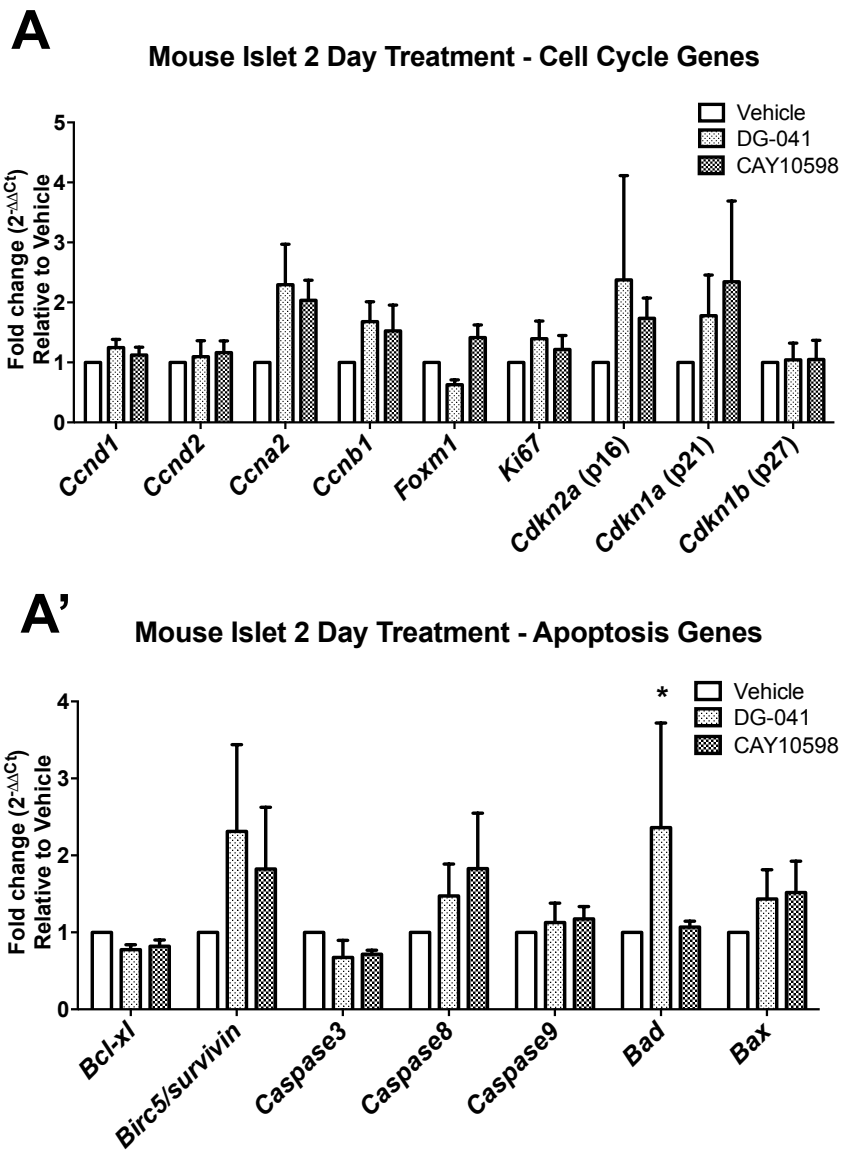

Figure 4-8. Assessment of mouse cell cycle and apoptosis gene expression after two days of inhibition of EP3 or activation of EP4. (**A** and **A'**) Wild type mouse islets (8-10 weeks old; n=4) were cultured for two days in the presence of vehicle, DG-041, or CAY10598. Following treatment, qRT-PCR was performed for cell cycle (**A**) and apoptosis (**A'**) genes. *p<0.05 vs vehicle. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

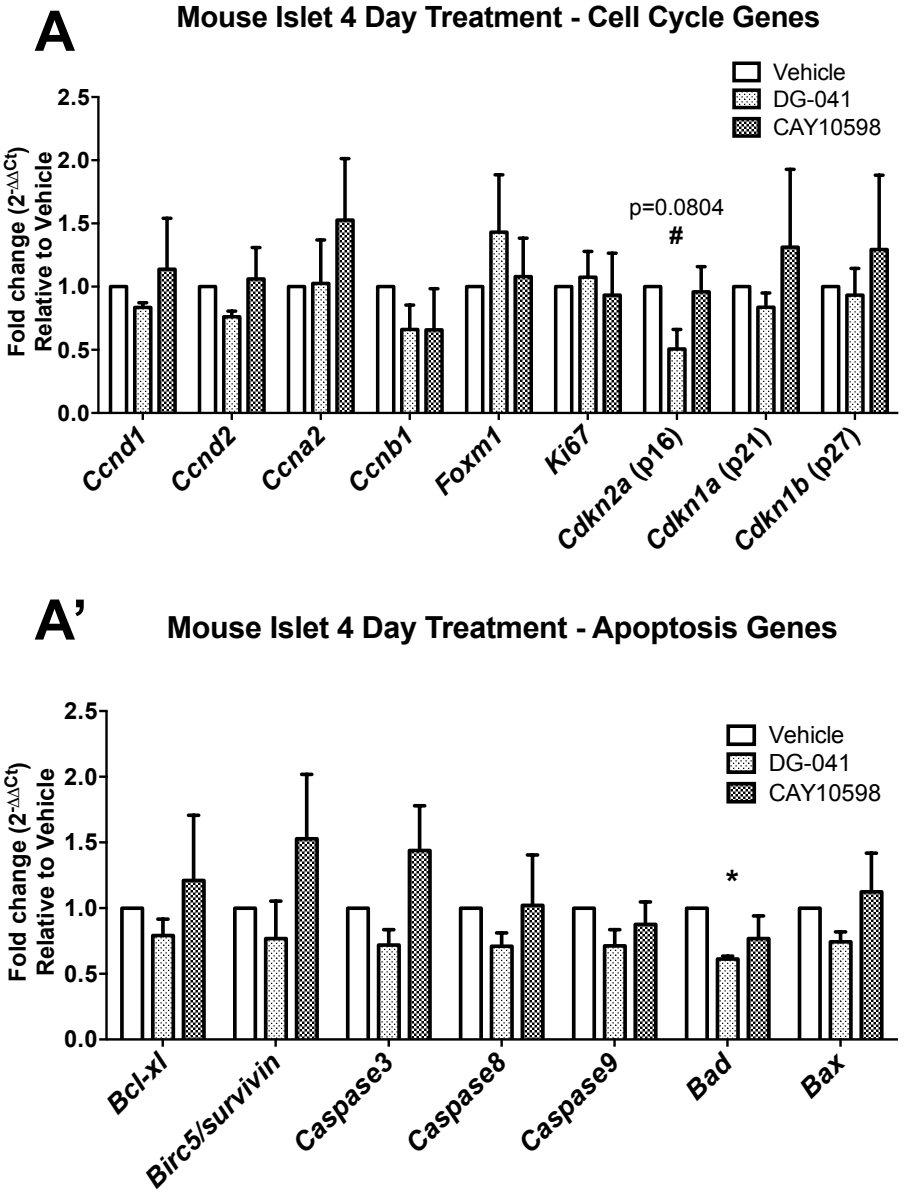

Figure 4-9. Assessment of mouse cell cycle and apoptosis gene expression after four days of inhibition of EP3 or activation of EP4. (**A** and **A'**) Wild type mouse islets (8-10 weeks old; n=4) were cultured for four days as described in Figure 4-8 and qRT-PCR was performed for cell cycle (**A**) and apoptosis (**A'**) genes. *p<0.05 vs vehicle. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

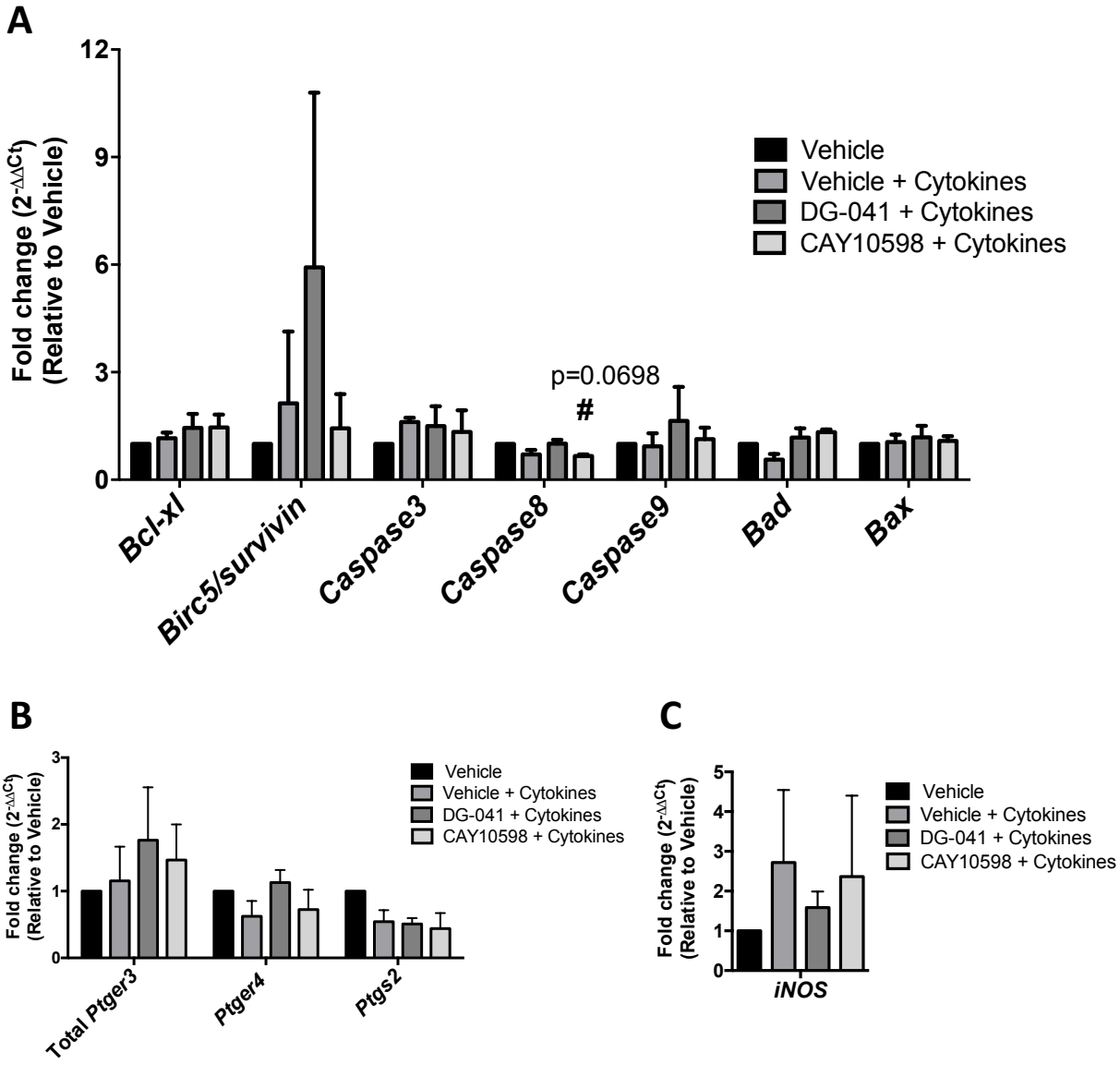

Figure 4-10. Effects of EP3 and EP4 on selected gene expression in response to cytokine treatment in mouse islets. **A**) Wild type mouse islets (8-10 weeks; n=3) were treated for 48 hours in the presence of cytokine cocktail and one of the following compounds: vehicle, DG-041, or CAY10598. Following treatment, qRT-PCR was performed for apoptosis genes, **B**) PGE_2 synthesis (*Ptgs2*) and receptor (*Ptger3, Ptger4*) genes, and **C**) *iNOS* gene expression. All data are represented as $2^{-\Delta\Delta Ct}$ relative to Vehicle + Cytokines. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Figure reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

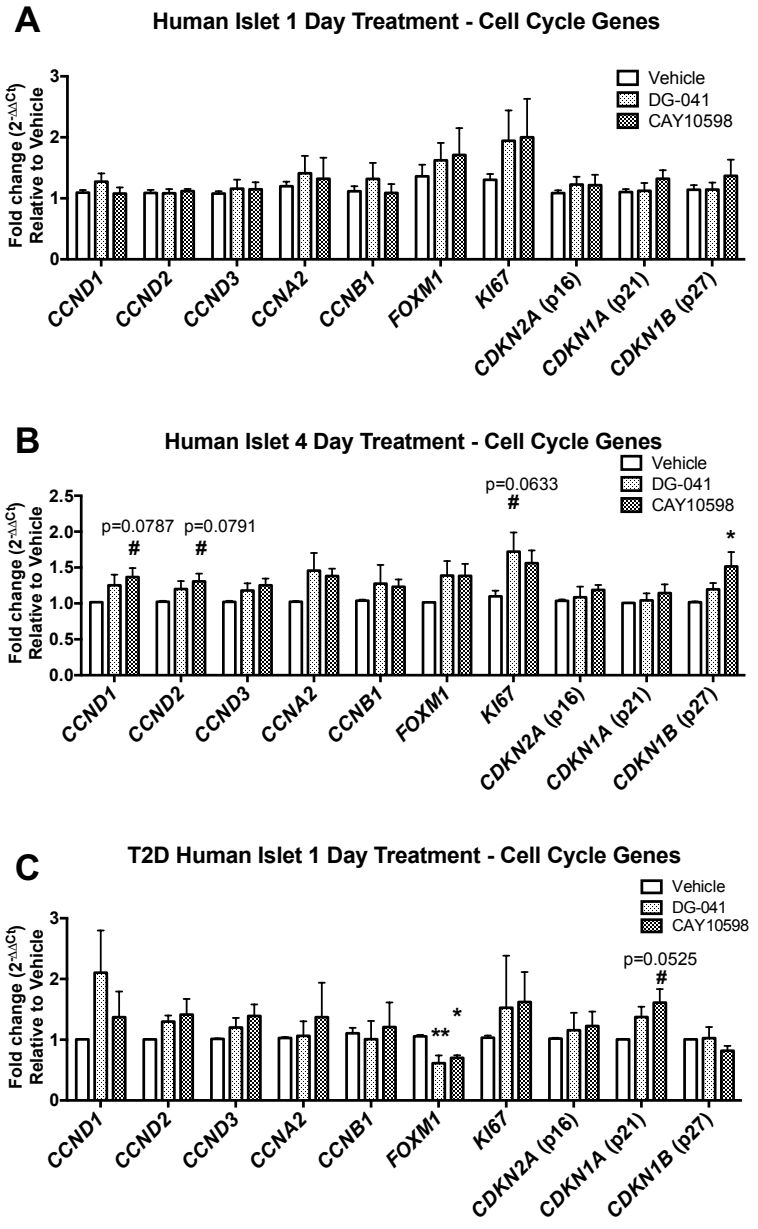

Figure 4-11. Assessment of human cell cycle gene expression following inhibition of EP3 or activation of EP4. Human islets (n=5-7) were cultured for one (**A**) or four (**B**) days in the presence of vehicle, DG-041, or CAY10598. Following treatment, qRT-PCR was performed for cell cycle genes. *p<0.05 vs vehicle. (**C**) Human islets (n=4) from donors with T2D were cultured for one day as described in A. *p<0.05 vs vehicle; **p<0.001 vs vehicle. All data are represented as $2^{-\Delta\Delta Ct}$ relative to vehicle. Data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

exception of *Bad,* a pro-apoptotic gene, after two and four days of DG-041 treatment. Gene expression was also analyzed in WT islets treated with cytokines and DG-041 or CAY10598. There were no significant changes in apoptosis genes, *Ptger3, Ptger4, Ptgs2*, or *iNOS* expression in response to cytokines with or without DG-041 or CAY10598 (Figure 4-10A-C).

In human islets, one day of DG-041 or CAY10598 treatment did not alter the expression of cell cycle genes (Figure 4-11A). After four days of DG-041 (EP3 antagonist) treatment, which corresponds to the time point at which human β-cell proliferation is increased in response to DG-041 (Figure 4-4A), there is a trend (p=0.0633) toward increased *KI67* expression (Figure 4-11B). Expression of cell cycle genes was also assessed in human islets from T2D donors treated with DG-041 and CAY10598 for one day. Both compounds resulted in a significant decrease in *FOXM1*; treatment with CAY10598 caused a trend (p=0.0525) in increased expression of *CDKN1a* (p21) (Figure 4-11C). These data suggest that islets from T2D individuals respond differently to DG-041 and CAY10598 than islets from humans without T2D. Due to the limited availability of T2D donor islets, β-cell proliferation has not been measured to date in T2D human islets treated with DG-041 or CAY10598.

Since there were no dramatic changes in gene expression in response to manipulation of EP3 and EP4 activity in either mouse or human islets, we next asked whether changes in protein activity, via phosphorylation, could contribute to the observed changes in β-cell proliferation and survival. Using antibody microarrays, 878 phosphorylation sites were probed in protein lysates from WT mouse islets treated with PL vs. DG-041 + PL or vehicle vs. CAY10598 for one day. The level of protein

phosphorylation in PL-treated islets was compared to that of DG-041 + PL to identify the specific effects of blocking EP3 signaling. DG-041 + PL treatment resulted in increased phosphorylation of proteins in the mammalian target of rapamycin (mTOR) pathway, including increased T252 and T412 phosphorylation of p70S6K (Table 4-1, Figure 4-12). Interestingly, both of these phosphorylation events are stimulated by mitogens [198]. PLC-γ1 Y783 phosphorylation, which increases enzymatic activity [199], and phosphorylation of several proteins involved in cell cycle regulation were also increased in response to DG-041 (Table 4-1, Figure 4-12). Based on these results, the mTOR and PLC-γ1 pathways were pursued as potential downstream targets of EP3. As shown above, both PL alone or DG-041 + PL treatment increased mouse β-cell proliferation (Figure 4-13A). Treatment with the mTOR inhibitor rapamycin essentially eliminated β-cell proliferation in untreated or DG-041 + PL treated islets (Figure 4-13A), and thus a specific effect of EP3 signaling could not be determined. In contrast to rapamycin, basal β-cell proliferation was not significantly altered compared to vehicle-treated islets in presence of the general PLC inhibitor U-73122 and PL was still capable of increasing β-cell proliferation in the presence of U-73122 (Figure 4-13A). However, the enhancing effect of DG-041 on PL-induced mouse β-cell proliferation was lost when islets were treated with U-73122 (Figure 4-13A). Additionally, treatment with U-73122 blocked the ability of DG-041 to increase human β-cell proliferation (Figure 4-13B). These observations suggest that inhibition of EP3 improves mouse and human β-cell proliferation in part through activation of PLC-1γ.

Table 4-1. Phospho-protein microarray data from WT mouse islets treated with PL or PL + DG-041 for 24 hours. Each replicate represents a pool of islets from three mice. PL served as the control group for analysis. %CFC (change from control) represents increases (orange) or decreases (blue) in phosphorylation compared to the control group (PL). A Z ratio of ± 1.2-1.5 was considered significant. Table modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

Protein Name	Phosphosite	%CFC in DG-041 + PL vs. PL Replicate 1	%CFC in DG-041 + PL vs. PL Replicate 2	Z-ratio Replicate 1	Z-ratio Replicate 2
BRCA1	S1497	137	77	1.19	1.04
p53	S6 and S33	125	218	1.16	1.90
Cyclin E1	T395	53	138	0.68	1.49
COT	Pan antibody	-70	119	-1.22	1.28
p70 S6K	T252 and T412	135	111	1.18	1.17
CHK2	Pan antibody and T383	-54	85	-0.75	1.17
Caldesmon	S789	810	82	2.85	0.96
PTEN	Pan antibody and S380+T382+T383	92	-97	0.94	-6.54
CSK	Pan antibody and Y184	-66	231	-1.08	1.91
PKR1	Pan antibody and T446	-81	82	-1.82	1.10
CDKL5	Y171	62	73	0.73	1.04
PLCγ1	Y783	60	67	0.71	0.85
PKCθ	S676	1046	51	3.11	0.65
SRPK1	S222	-54	-49	-0.72	-1.28

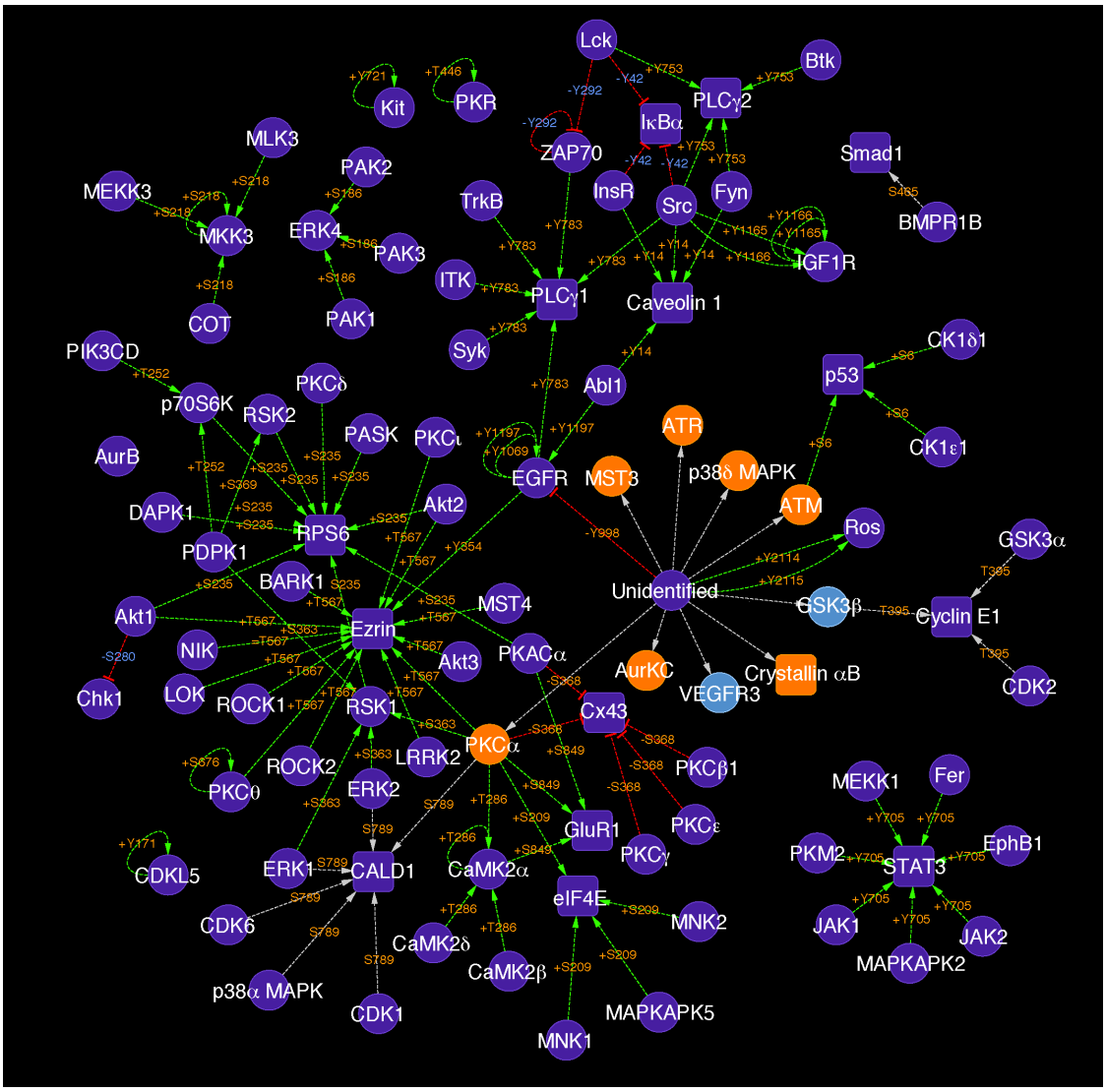

Figure 4-12. Phosphorylation network map comparing PL versus PL + DG-041 treatment. Kinases are represented as circles and non-kinases as rounded squares. Orange: %CFC was increased by 45% or greater; blue: %CFC was decreased by 45% or greater; purple: changes less than 45%. Known phosphorylation events of target substrates by protein kinases are shown with dashed arrows with the phospho-site amino acid type and position indicated and prefixed with a "-" for inhibiting or a "+" for activating substrates. When the phosphorylation of the substrate was increased by PL + DG-041 by 45% or more, this text is orange, and is blue if the phosphorylation was inhibited by 45% or greater. Effects less than 45% appear light grey. The dashed arrows are colored green for stimulatory phosphorylation, red for inhibitory phosphorylation, and grey if the effect of phosphorylation is unclear. Different proteins represented on the map are shown as unique icons only once. Figure reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

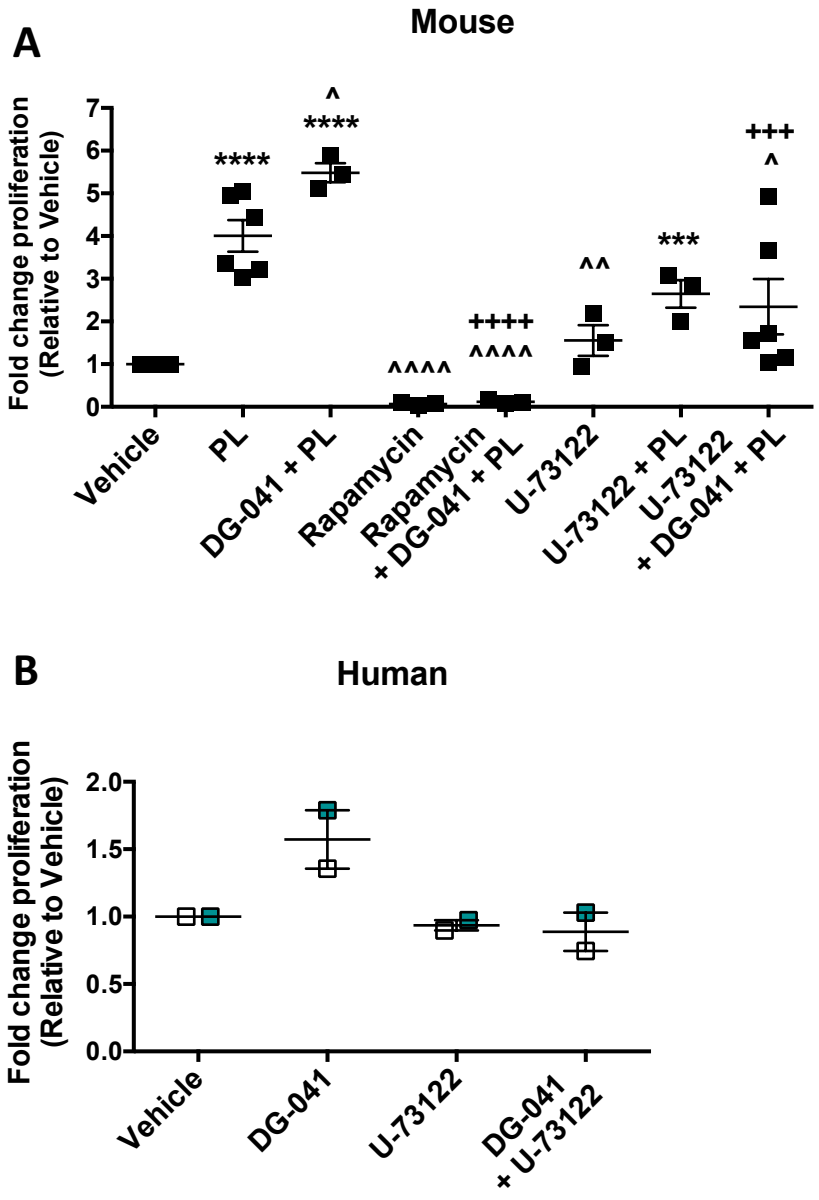

Figure 4-13. EP3 modulation of β-cell proliferation involves PLC-γ1. **A**) Young WT mouse islets (8-10 weeks old) were treated with vehicle, PL, DG-041 + PL, rapamycin (mTOR inhibitor) ± DG-041 + PL, or U-73122 (PLC inhibitor) ± DG-041 + PL for 4 days before being immunolabeled for insulin, Ki67, and DAPI. *versus vehicle; ^versus PL; +versus DG-041 + PL. **B**) Human islets were treated with vehicle, DG-041, or U-73122 (PLC inhibitor) ± DG-041 as described in A. All data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. One symbol represents $p<0.05$, two symbols indicate $p<0.01$, three symbols denote $p<0.001$, four symbols represent $p<0.0001$. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

When protein phosphorylation of CAY10598 (EP4 agonist)-treated islets was compared to vehicle treatment, S209 phosphorylation of eukaryotic initiation factor 4E (eIF4E) was increased and phosphorylation of protein kinase C (PKC) ε was altered, albeit variably between samples (Table 4-2**,** Figure 4-14). eIF4E and PKCε have been implicated in PKA signaling previously [200, 201]; thus, the role of PKA as a downstream target of EP4 was investigated as a mechanism for protection against cytokine-induced β-cell death. As shown above, CAY10598 (EP4 activation) treatment inhibited cytokine-mediated β-cell death (Figure 4-15A). Addition of the PKA-specific inhibitor Rp-cAMPS alone had no effect on cytokine-mediated β-cell death (Figure 4-15A). However, in the presence of Rp-cAMPS, activation of EP4 by CAY10598 was no longer able to decrease cytokine-induced β-cell death in mouse islets (Figure 4-15A). These results were mirrored in human islets treated with Rp-cAMPS ± CAY10598: CAY10598 failed to decrease cytokine-induced β-cell death in the presence of Rp-cAMPS (Figure 4-15B). Taken together, these data demonstrate that EP4 promotes mouse and human β-cell survival through activation and downstream signaling induced by PKA.

Table 4-2. Phospho-protein microarray data from WT mouse islets treated with vehicle or CAY10598 for 24 hours. Each replicate represents a pool of islets from three mice. Vehicle-treated islets served as the control group for analysis. %CFC (change from control) represents increases (orange) or decreases (blue) in phosphorylation compared to the control group (vehicle). A Z ratio of ± 1.2-1.5 was considered significant. DOI 10.1016/j.molmet.2017.04.002.

Protein Name	Phosphosite	%CFC in CAY10598 vs Vehicle Replicate 1	%CFC in CAY10598 vs Vehicle Replicate 2	Z-ratio Replicate 1	Z-ratio Replicate 2
eIF4E	S209	97	76	2.05	1.72
CDK1/2	Y15	46	45	1.14	1.10
PKCε	S729/Pan Antibody	49	-48	1.20	-1.96

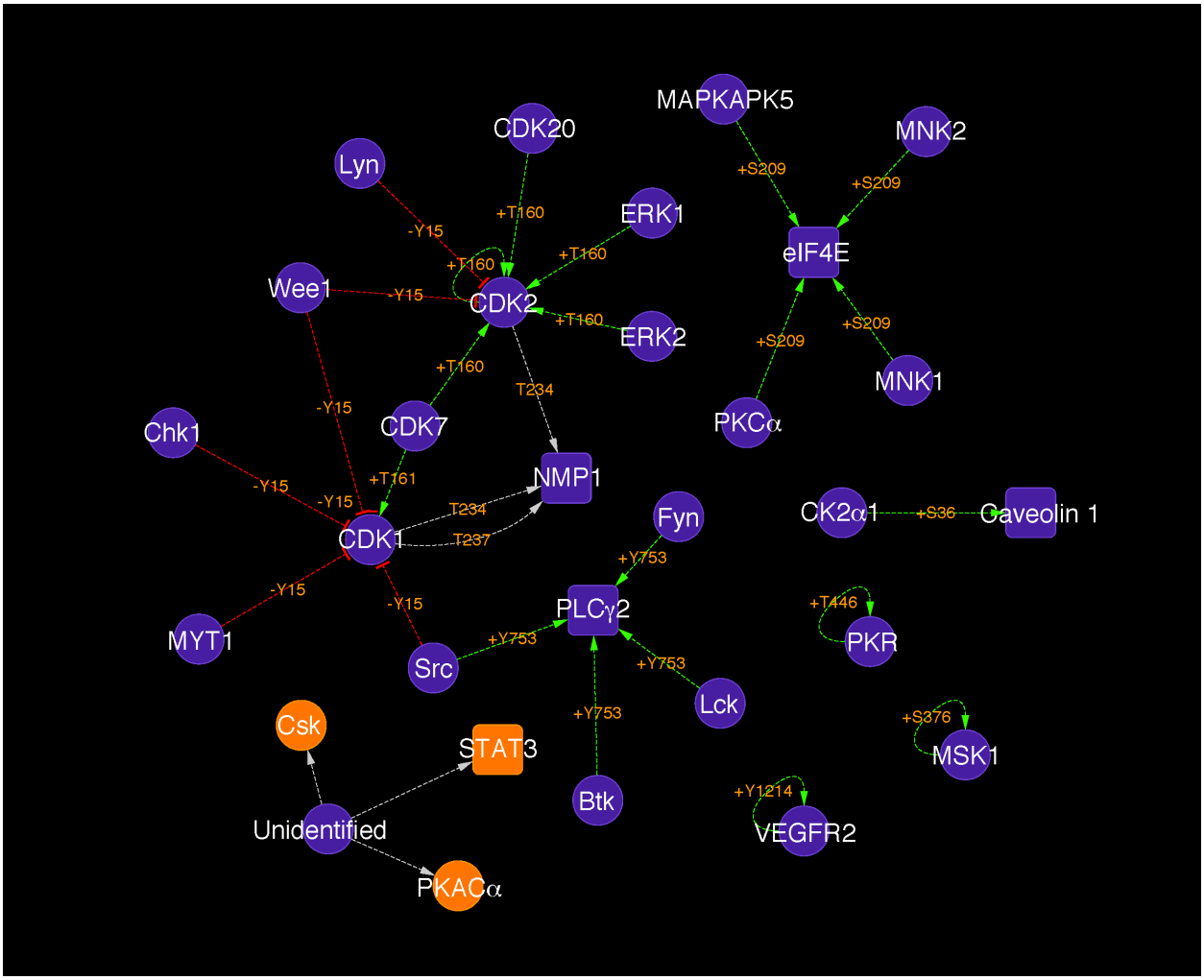

Figure 4-14. Phosphorylation network map comparing vehicle versus CAY10598 treatment. Kinases are represented as circles and non-kinases as rounded squares. Orange: %CFC was increased by 45% or greater; blue: %CFC was decreased by 45% or greater; purple: changes less than 45%. Known phosphorylation events of target substrates by protein kinases are shown with dashed arrows with the phospho-site amino acid type and position indicated and prefixed with a "-" for inhibiting or a "+" for activating substrates. When the phosphorylation of the substrate was increased by CAY10598 by 45% or more, this text is orange, and is blue if the phosphorylation was inhibited by 45% or greater. Effects less than 45% appear light grey. The dashed arrows are colored green for stimulatory phosphorylation, red for inhibitory phosphorylation, and grey if the effect of phosphorylation is unclear. Different proteins represented on the map are shown as unique icons only once. Figure reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

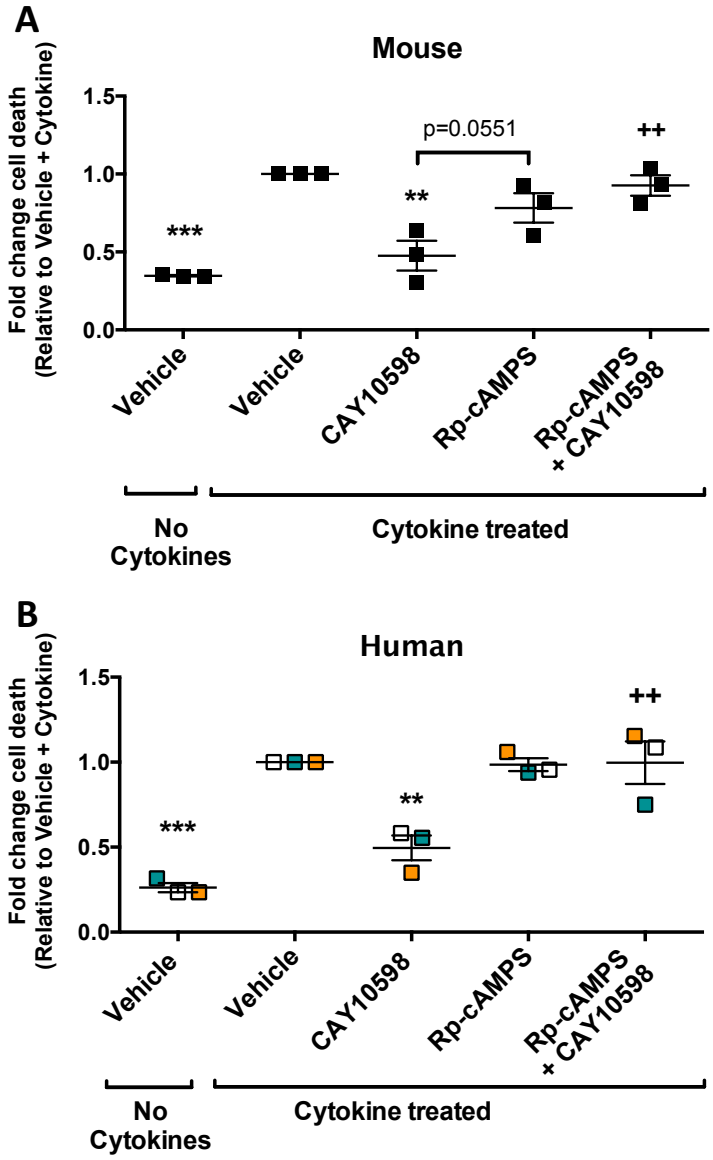

Figure 4-15. EP4-induced protection against β-cell death involves PKA. **A**) WT mouse islets (8-10 weeks old) were treated for 48 hours with a species-specific cytokine cocktail plus one or more of the following compounds: vehicle, CAY10598, or Rp-CAMPS (PKA inhibitor). Following treatment, islets were immunolabeled for insulin, TUNEL, and DAPI. *versus vehicle + cytokines; + versus CAY10598. **B**) Human islets were treated for 48 hours with a species-specific cytokine cocktail and the compounds described in A. Islets were immunolabeled as described in A. *versus vehicle + cytokines; + versus CAY10598. All data were analyzed using a One-way ANOVA with Bonferroni *post hoc* analysis. One symbol represents $p<0.05$, two symbols indicate $p<0.01$, three symbols denote $p<0.001$, four symbols represent $p<0.0001$. Figure modified and reprinted with permission from "Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation." *Molecular Metabolism* 6(6): 548-559. Copyright © 2017. DOI 10.1016/j.molmet.2017.04.002.

Discussion

The goal of the current study was to determine the roles of EP3 and EP4 in mouse and human β-cell proliferation and β-cell survival. We show that the proliferative response to the β-cell mitogen PL is enhanced when EP3 signaling is blocked by DG-041 in young mouse islets, but not in aged islets. Importantly, blockade of EP3 signaling increased human β-cell proliferation, in the absence of an additional proliferative stimulus, but had no effect on α-cell proliferation. Modulation of EP4 signaling alone did not lead to changes in mouse or human β-cell proliferation or human α-cell proliferation. Notably, activation of EP4 increased human, but not mouse, β-cell proliferation when EP3 signaling was simultaneously blocked. In addition, we demonstrated roles for both EP3 and EP4 in β-cell survival in mouse and human islets: EP3 signaling increases cytokine-induced β-cell death, while EP4 activation promotes β-cell survival. Since EP3 and EP4 are expressed in both α- and β-cells [120, 123, 124], it remains possible that the observed effects on β-cell proliferation and β-cell survival are due to EP3 or EP4 activity in non-β-cells of the islet, which then secondarily affect the β-cell. Resolution of this issue awaits cell-type-specific inactivation of these receptors in α- and β-cells or treatment of isolated cell populations with the pharmacological tools used here.

Our interest in EP3 and EP4 stemmed from work on the critical cell cycle transcription factor FoxM1. Transgenic over-expression of an activated form of FoxM1, called β-FoxM1*, uncovered a novel role for FoxM1 in promoting β-cell survival [61]. RNA-seq of β-FoxM1* islets identified EP3 and EP4 as potential downstream effectors of FoxM1. Expression of *Ptger3* (EP3) was down-regulated in response to FoxM1

induction, while expression of *Ptger4* (EP4) was increased [61]. Here, we demonstrate that the pro-survival effects of FoxM1 are mediated, in part, by regulation of EP3 and EP4. Reciprocal regulation of *Ptger3* and *Ptger4* by FoxM1 provides a mechanism to dampen β-cell death pathways and enhance pro-survival signaling pathways thereby protecting against β-cell death. These results support the notion that pathways influencing β-cell proliferation can also play important roles in β-cell survival.

While it is known that PGE_2-EP3 signaling impairs GSIS via a $G_{i/Z}$ mechanism [141], the factors downstream of G-protein coupling for EP3 and EP4 that impact β-cell proliferation and/or survival are not defined. Data reported herein demonstrate that regulation of phosphorylation status, rather than gene expression, is the primary mechanism responsible for the effects of EP3 and EP4 on mouse β-cell proliferation and β-cell death. In this work, novel downstream targets of EP3 and EP4 have been identified in mouse islets, including PLC-γ1, p70S6K, and PKA. DG-041 treatment (EP3 antagonist) resulted in increased phosphorylation on a site in PLC-γ1 that enhances enzymatic activity [199] and on sites in p70S6K, a downstream target of mTOR, known to be involved in mitogen stimulation and important for its activity [198]. In the presence of the PLC inhibitor U-73122, PL was still able to increase β-cell proliferation, yet the magnitude of PL's effect appears diminished in the presence of U-73122, although this difference is not statistically significant. There is one study suggesting that PLC-γ1 can mediate some effects of PL, but the specific role it plays in this pathway is unknown (reviewed in [202]). U-73122 prevented the effect of EP3 blockade (DG-041) on PL-induced β-cell proliferation in mouse islets, demonstrating that this pathway is normally inhibited by EP3 signaling as a mechanism to inhibit β-cell proliferation. Further, U-

73122 blocked the ability of DG-041 treatment to increase human β-cell proliferation. PLC-γ1 has been implicated in promoting proliferation in other tissue types through ERK activation [203, 204], but this is the first report, to our knowledge, revealing a role for PLC-γ1 in mouse and human β-cell proliferation. The ability of EP3 to regulate the mTOR pathway is less clear since treatment with rapamycin, an mTOR inhibitor, resulted in very low levels of β-cell proliferation and PL + DG-041 (EP3 antagonist) treatment was unable to overcome this inhibitory effect. Interestingly, PGE_2 inactivates Akt in islets, leading to impaired GSIS (see Figure 1-10) [143, 152]. As Akt is known to regulate β-cell proliferation through several targets including mTOR [50], our data suggest that PGE_2-EP3 signaling can result in decreased Akt and downstream mTOR activities, leading to impaired β-cell proliferation. Taken together, multiple signaling pathways are inhibited by EP3 signaling to reduce β-cell proliferation.

CAY10598 treatment (EP4 activation) in mouse islets resulted in increased phosphorylation at the primary phospho-site in eIF4E [205] along with variable changes in phosphorylation of PKCε. PKA is activated by G_S-coupled mechanisms and has been shown to regulate phosphorylation of eIF4E and PKCε in other tissues [200, 201]. The PKA inhibitor Rp-cAMPS blocked the positive effect of CAY10598 (EP4 activation) on mouse and human β-cell survival. PKA downstream signaling can increase transcription of cAMP response element binding protein (CREB) target genes, some of which are involved in pro-survival signaling [69]. Therefore, I propose that EP4 signals by activating PKA, leading to enhanced phosphorylation of downstream target proteins, including eIF4E and PKCε, in the mouse β-cell to promote cell survival.

EP3 and EP4 may modulate the response to G_S-coupled GLP-1R signaling. GLP-1R agonists, such as exendin-4, are not efficacious in all individuals with T2D. Increased EP3 signaling in some T2D individuals may be counteracting the beneficial effects of GLP-1R agonists. In agreement with this idea, activation of EP3 antagonizes the effects of GLP-1R activation on cAMP production in mouse islets [123]. Interestingly, GLP-1 treatment fails to induce β-cell proliferation in aged mouse islets [40], similar to the failure of DG-041 (EP3 inhibition) to enhance PL-induced β-cell proliferation in one-year-old mice shown here. qRT-PCR analysis revealed that expression of *Ptger3γ* was increased in islets from one-year-old mice. This is particularly interesting as the EP3γ splice variant displays high constitutive activity and very little ligand-dependent activity whereas EP3α shows modest constitutive activity and EP3β has no ligand-independent activity [163]. Increased expression of the *Ptger3γ* variant may prevent DG-041 from blocking EP3 activity in aged mouse islets. The role of EP3γ constitutive activity cannot be assessed with DG-041, as DG-041 only blocks ligand-dependent activity. An inverse agonist is required to inhibit constitutive receptor activity and there are currently no known inverse agonists for EP3 available. An increase in constitutively active *Ptger3γ* in older islets may result in tonic inhibition of β-cell proliferation in aged islets, providing an explanation for the lack of responsiveness to PL + DG-041 (EP3 antagonist) and GLP-1 agonist treatment. Our data combined with GLP-1 studies suggest that aged rodent islets are refractory to GPCR-based β-cell proliferation. In contrast, while the ability of GLP-1 to increase human β-cell proliferation is controversial [69], we show here that blockade of EP3 does stimulate human β-cell proliferation, even in islets from middle-aged individuals. In addition, concurrent

activation of EP4 and blockade of EP3 further increases human β-cell proliferation over that of EP3 blockade alone. There are at least eight splice variants in the human EP3 receptor compared to the three splice variants in mice [206]. Although the human EP3II/D/c appears to most closely resemble EP3γ [148], it is unknown how/if the expression level of the human EP3 splice variants changes during age or metabolic stress in islets. The expression of the different human EP3 variants has not been measured to date and awaits testing in our laboratory. It is possible that EP3 activity in human islets is counteracting the effects of GLP-1R agonist treatments, thus explaining the discrepancies between mouse and human β-cell proliferation in response to GLP-1R activation.

Intriguingly, *Ptger3γ* expression is reduced in response to PL treatment in mouse islets. This may serve as a mechanism for PL signaling to dampen the constitutive activity of EP3 in order to exert its beneficial effects on β-cell proliferation. In this study, it appears that EP3γ is the most highly regulated splice variant in mouse islets. As discussed, the splice variants alter the specificity of G-protein coupling, receptor desensitization, and the constitutive receptor activity [148, 207]. However, the mechanism(s) that regulate splicing of *Ptger3* remains to be determined.

Overall, this study has identified novel roles of EP3 and EP4 as regulators of β-cell proliferation and survival in mouse and human islets. Furthermore, this report highlights EP3 and EP4 as potential druggable targets for the treatment of T2D due to their roles in both β-cell survival and β-cell proliferation in human islets. Simultaneously inhibiting EP3 and activating EP4 may protect against T2D-associated β-cell loss and promote expansion of functional β-cell mass.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

PGE_2 is an important modulator of many cellular processes, including insulin secretion and systemic inflammation. The effects of PGE_2 are mediated by its four receptors, EP1-4. The EP3 and EP4 receptors often play opposing roles in different cell types due to signaling through different G-proteins. For example, PGE_2 can have either pro- or anti-inflammatory effects in different cell types depending on which receptor is activated [127]. EP3 is coupled to inhibitory G-proteins, leading to inhibition of AC and a decrease in intracellular cAMP. Conversely, EP4 is coupled to the stimulatory G-protein, which activates AC and increases cAMP levels. In the pancreatic islet, evidence from the literature and from our group suggests that EP3 and EP4 play opposing roles in regulating different processes in the β-cell, including GSIS, β-cell proliferation, and β-cell death. In islets isolated from β-FoxM1* mice, which display enhanced β-cell proliferation and β-cell survival, *Ptger3* (EP3) expression was down-regulated whereas *Ptger4* (EP4) expression was upregulated [61]. In collaboration with the Breyer lab, we demonstrated that mice with a global deletion of EP3 have enhanced β-cell proliferation only during a state of obesity-associated insulin resistance [166]. These data led us to hypothesize that EP3 and EP4 play opposing roles in regulating β-cell mass dynamics, such that EP3 inhibits β-cell proliferation and survival whereas EP4 enhances β-cell proliferation and survival, but that these effects are evident only when additional stimuli are present. Many islet investigators are searching for factors or receptors that can stimulate human

insulin secretion, enhance β-cell proliferation, and protect against β-cell death in the hopes of developing new T2D therapies. Thus, EP3 and EP4 are ideal receptors to investigate for these purposes.

The focus of this dissertation is the function of EP3 and EP4 signaling and their downstream effectors in adult mouse and human β-cell mass dynamics. The results presented in Chapter III discussed the complications of several β-cell-specific Cre models. Consequently, the *in vivo* requirements for EP3 and EP4 in β-cell proliferation and β-cell death were not assessed. The RIP-Cre^{Herrera} mouse [75] may be an adequate tool to generate EP3 and EP4 β-cell-specific knockout models as this mouse strain does not contain the hGH minigene and displays very little recombination in the hypothalamus [75, 77]. Alternatively, mice could be treated systemically with EP3 and EP4 ligands to test the effects of activating or blocking receptor signaling *in vivo*. Data from our group and others suggests that EP3 only affects GSIS and β-cell proliferation under specific circumstances, such as T2D or obesity-induced insulin resistance [123, 166]. Therefore, the effects of EP3 and EP4 ligand treatment will need to be studied in both non-diabetic mice and mice with T2D and/or insulin resistance. Pilot studies are beginning in the laboratory utilizing the T2D mouse model of leptin receptor deficiency, *db/db* mice. *Db/db* mice will be treated with the EP ligands used in Chapter IV as a first step towards understanding the *in vivo* effects of EP3 and EP4 in regulating β-cell proliferation and survival. Blood glucose is elevated beginning at four weeks of age and plateaus at 12 weeks in male *db/db* mice [208]. In addition, four weeks of age corresponds to a period of β-cell compensation in which 20% of β-cells are proliferating, yet only 1% of β-cells are proliferating at six weeks [209]. The goal of these studies is to

determine if blocking EP3 using DG-041 and/or activating EP4 with CAY10598 could protect against the decline in β-cell proliferation that occurs during this critical time window of four to six weeks of age and if this treatment could delay the progression of hyperglycemia in *db/db* mice. If blockade of EP3 or activation of EP4 enhances β-cell proliferation and/or delays the onset of hyperglycemia in *db/db* mice, the question will still remain: are the observed effects due to alterations in EP3/EP4 signaling in the β-cell itself or are they due to changes in receptor activity in other cell types that then secondarily affect the β-cell? Systemic treatment with DG-041 (EP3 antagonist) may be very similar to the studies in EP3 global knockout mice. $EP3^{-/-}$ become obese, have ectopic lipid storage in the liver, and are insulin resistant [166]. Thus, systemic treatment may mask any effect of EP3 activity in the β-cell due to blockade of EP3 signaling in other important tissues. The EP3 and EP4 receptors are also expressed in other endocrine cell types of the islet. RNA-seq on sorted islet cell populations has revealed that EP3 and EP4 are expressed in human and mouse α-cells [120, 124], yet the function of these receptors in α-cells remains unknown.

In the absence of β-cell-specific EP3 and EP4 knockout models, Chapter IV of this dissertation probed the roles of EP3 and EP4 in mouse and human β-cell proliferation and β-cell survival *ex vivo* using selective receptor agonists and antagonists. In mouse islets, blockade of EP3 enhanced PL-induced β-cell proliferation in young, but not aged, islets in part through PLC-γ1 activity. Blocking EP3 increased human β-cell proliferation, in the absence of any additional proliferative stimulus, also via PLC-γ1 activity. Modulation of EP4 had no effect on mouse or human *ex vivo* β-cell proliferation alone. However, blockade of EP3 in combination with activation of EP4

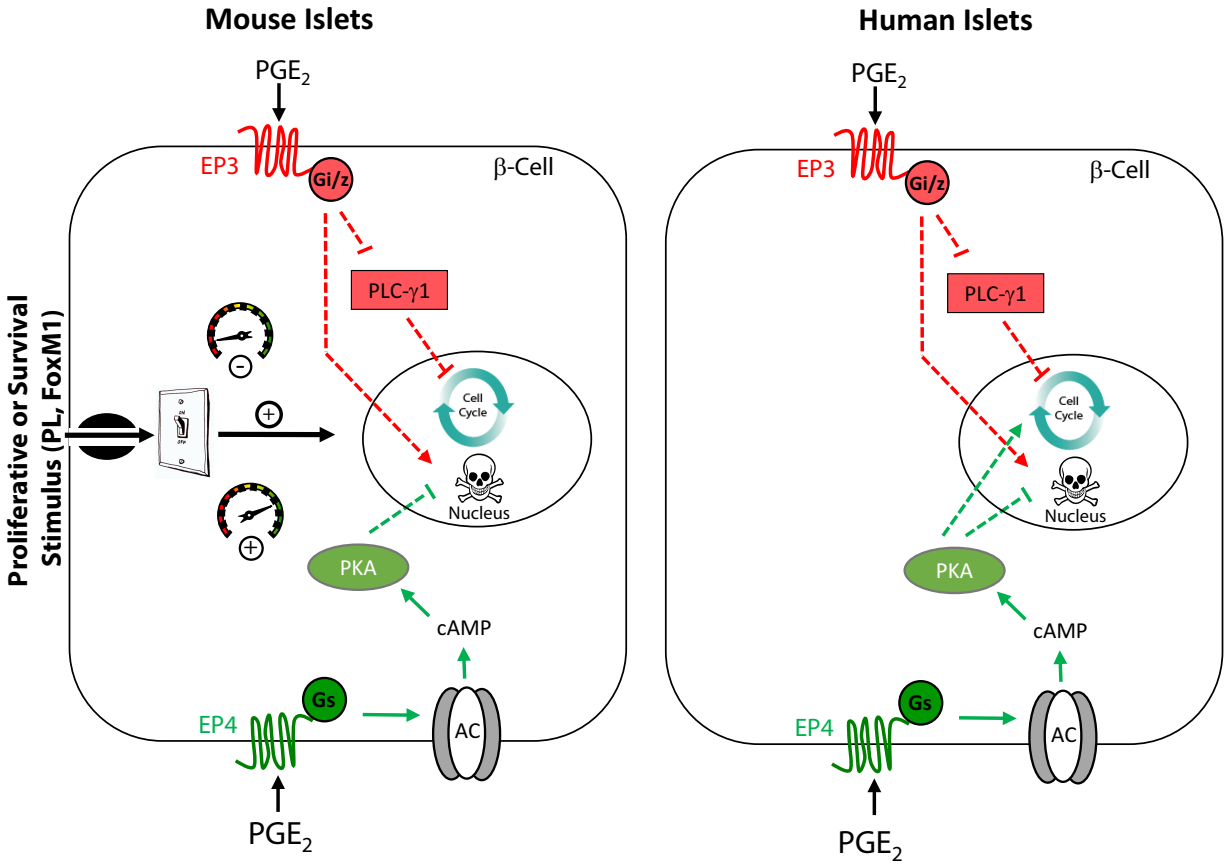

Figure 5-1. Model of EP3 and EP4 signaling in mouse and human β-cells. In mouse β-cells (left), EP3 increases β-cell death and inhibits β-cell proliferation through decreased phosphorylation and activity of PLC-γ1. In contrast, mouse EP4 signaling has no effect on basal or mitogen-stimulated β-cell proliferation, yet does improve β-cell survival through PKA signaling. In human β-cells (right), EP3 and EP4 have opposing effects on β-cell proliferation in the absence of any proliferative stimulus: inhibition of EP3 enhances β-cell proliferation through the activity of PLC-γ1, which is further increased when EP4 is simultaneously activated. Additionally, EP3 increases whereas EP4 protects against β-cell death via PKA signaling. The dotted lines represent pathways with unknown intermediates.

enhanced human, but not mouse, β-cell proliferation. In both mouse and human islets, blockade of EP3 or activation of EP4 enhanced β-cell survival in the presence of cytokines. The ability of EP4 to promote human and mouse β-cell survival is mediated by downstream PKA signaling. Interestingly, the pro-survival effects of FoxM1 are mediated, in part, by down-regulation of EP3 and up-regulation of EP4. These data demonstrate for the first time that EP3 and EP4 have antagonistic roles in the islet to regulate processes involved in the dynamics of β-cell mass (Figure 5-1).

The endogenous ligand PGE_2 binds with equal affinity to EP3 and EP4 [122], so a balance of EP3 and EP4 receptor expression may be an important factor in determining the final effect of PGE_2 signaling. Several novel situations in which *Ptger3* (EP3) and *Ptger4* (EP4) gene expression are altered have been identified in this work. In mouse islets, PL treatment decreases *Ptger3*γ expression and aging increases the level of overall *Ptger3*. This increased expression of *Ptger3*γ may increase the amount of constitutive EP3 signaling present, which DG-041 is unable to block, providing tonic inhibition of β-cell proliferation in aged mouse islets. This observation may also partially explain the lack of responsiveness to GLP-1R activation in aged mouse islets and the decline in β-cell proliferation that occurs with age [40]. In human islets, *PTGER3* expression is increased in islets from lean T2D patients, consistent with previous work [123]. Increased *PTGER3* may shift PGE_2 signaling towards EP3 in the diabetic state, thereby decreasing β-cell proliferation and enhancing β-cell death. This may contribute to the decline in β-cell mass that has been observed in human T2D autopsy pancreata [67]. It is currently unknown if specific human EP3 splice variants display altered expression during different metabolic states, such as obesity and T2D (discussed in

more detail below). In contrast to EP3 expression, mouse *Ptger4* expression is unaffected by PL treatment and is not significantly changed throughout aging, although there is a trend toward decreased expression beginning at 4 months of age (see Figure 4-3). A decrease in *Ptger4* with age may further drive PGE_2 to signal via EP3, which may also contribute to the age-induced decrease in mouse β-cell proliferation. Interestingly, in islets from the pro-survival β-FoxM1* model, *Ptger4* expression is increased whereas *Ptger3* is decreased while the expression of genes involved in PGE_2 production were unchanged [61]. It would be of interest to determine which of the EP3 splice variants are decreased in β-FoxM1* islets to see if there are any similarities to the changes in *Ptger3γ* expression observed in response to PL treatment. The reciprocal regulation of *Ptger3* and *Ptger4* expression by FoxM1 may serve as a means to enhance pro-survival signaling pathways in order to protect against β-cell death. In human islets, *PTGER4* expression was not altered by BMI or T2D status. Overall, the gene expression data suggest that an increase in EP3 gene expression during aging and T2D could lead to enhanced PGE_2 signaling via EP3, resulting in impaired β-cell proliferation and increased β-cell death. On the other hand, a decrease in *Ptger3* expression and/or increased *Ptger4* (for example, during pregnancy or FoxM1 activation) could result in enhanced PGE_2-EP4 signaling, which would protect against β-cell death and preserve functional β-cell mass. However, it should be noted that receptor expression does not always correlate with function.

This work has identified alterations in mouse EP3 splice variant expression, yet it is currently unclear how their expression is regulated. There are three alternatively spliced variants of EP3 in the mouse [207] and at least eight splice variants in human

tissues [206]. The mouse *Ptger3* promoter contains an NFκB binding site [105] and the human *PTGER3* gene contains an IFN-γ response element [206], suggesting that inflammatory cytokines can regulate EP3 expression. The EP3 splice variants are encoded by the same gene, so why would PL or aging lead to selective changes in mouse *Ptger3γ* expression? *Ptger3α* and *Ptger3β* result from alternative splicing whereas generation of *Ptger3γ* does not require splicing. Intriguingly, it has recently been recognized that splicing defects are associated with age-associated diseases. Further, microarray analysis has revealed that the expression of many splicing factors decreases with age [210]. A decrease in splicing factor expression with age may explain the increase in *Ptger3γ* observed with age in our study. Several questions remain for human EP3 splice variants: Are there changes in the expression of the human EP3 variants, and do they change with age, obesity, T2D, or other metabolic stressors? As discussed in Chapter IV, human EP3II/D/c appears to be the most similar to mouse EP3γ [148]. The expression of the human *PTGER3* splice variants was not measured in human islets in this study. Thus, future studies to identify specific changes in the human EP3 splice variants during different physiological settings, such as obesity and T2D, will be immensely informative. It will also be interesting to determine if any of the changes in the human receptor variants correspond to changes that were observed in mouse islets.

Data presented in this document suggest that the previously observed increase in *PTGER3* mRNA in islets from humans with T2D [123] is context-specific. Here, there is an inverse correlation between *PTGER3* expression and BMI in islets from individuals with T2D, with *PTGER3* expression decreasing with higher BMIs. Interestingly, there

are certain populations that develop T2D at lower BMIs than other populations, including those of Asian descent [211]. The expression level of islet *PTGER3* across different populations is unknown, but would be an interesting question to address. Enhanced *PTGER3* and EP3 signaling in lean, but not obese, individuals with T2D suggests that the etiology of β-cell dysfunction and ultimately T2D may differ in these two groups. It is possible that the genetic makeup of lean individuals who develop T2D results in higher levels of islet *PTGER3*, making them more susceptible to subtle stressors later in life, such as age-associated weight gain, ultimately leading to onset of T2D. Alternatively, subtle stressors could induce *PTGER3* expression and result in disease onset in more susceptible lean individuals. The technology does not currently exist to distinguish between these possibilities. The utility of EP3 and EP4 as therapeutic targets for the treatment of T2D might be applicable in only a particular subset of individuals, such as lean individuals with T2D where *PTGER3* is elevated. This idea is supported by the effects of EP3 inhibition and EP4 activation on gene expression from islets from obese T2D donors (average BMI 35.48 kg/m^2), which showed less *PTGER3* expression: DG-041 (EP3 inhibition) and CAY10598 (EP4 activation) decreased expression of *FOXM1*, a critical β-cell proliferation factor. Due to limited availability of T2D donor samples, β-cell proliferation was not assessed in islets from T2D donors. Future studies to explore whether β-cell proliferation is increased in human T2D β-cells in response to DG-041 ± CAY10598 are planned in the laboratory. Based on the gene expression discussed above, it will be important to measure β-cell proliferation in both lean and obese T2D donor islets to determine if there are in fact differences in the response to blockade of EP3 and activation of EP4, as was observed

in the qRT-PCR analysis. The information gained from these experiments will prove useful for the evaluation of these receptors as potential T2D therapeutics.

The work in this dissertation has uncovered differences in mouse versus human EP3 and EP4 signaling. In mouse islets, EP3 only inhibits β-cell proliferation when an additional proliferative stimulus is present. In addition, EP4 does not increase mouse β-cell proliferation, even when EP3 is simultaneously blocked. Conversely, blockade of EP3 is able to enhance human β-cell proliferation in the absence of any additional mitogen. Further, activation of EP4 increases human β-cell proliferation when EP3 is blocked. Presently, the reason(s) for these differences is unknown. As discussed above, there are at least eight splice variants in the human EP3 receptor and only three splice variants in mice. In both mice and humans, these variants differ in terms of receptor desensitization, constitutive activity, and second messenger signaling pathways [148]. In addition to differences in constitutive activity already discussed, mouse EP3γ can couple to G_S, in addition to $G_{i/Z}$, in an agonist-dependent manner, whereas EP3α and EP3β cannot [163]. Similar differences in downstream signaling have also been observed between the human EP3 splice variants. For example, human the EP3-II and EP3-III variants can stimulate ERK1/2 phosphorylation whereas EP3-Ia leads to minimal changes in ERK1/2 phosphorylation due to differences in G-protein coupling [212]. Perhaps human islets are expressing EP3 variants that have less constitutive activity than mouse EP3γ, the constitutively active mouse variant. Mouse EP3γ may be causing tonic inhibition of β-cell proliferation that is too strong for DG-041 treatment (EP3 antagonist) alone to overcome, and thus only has an effect when PL reduces *Ptger3γ* expression, whereas human islets may not be expressing high levels of a constitutively

active variant of EP3 and are thus more sensitive to DG-041 treatment. Alternatively, G-protein coupling and downstream signaling may differ between mouse and human islets, which is affecting the final output on β-cell proliferation. Future studies to explore the expression of human EP3 splice variants will help to address this possibility. Currently, there are no known EP3 inverse agonists; thus, the role that the constitutive EP3 receptor variant itself plays in regulating *ex vivo* β-cell proliferation cannot be tested.

There are many other variables that may explain the differences between mouse and human islets in response to the EP ligands, including differences in the glucose concentration of the culture media or endogenous islet cytokine production. The mouse *ex vivo* β-cell proliferation assay was performed in media containing 11 mM glucose whereas the media for the human β-cell proliferation assay contained 5.5 mM glucose. As previously discussed, hyperglycemic culture conditions induce PGE_2 production in mouse and human islets [115, 116]. Therefore, it is possible that there is more PGE_2 is present in the mouse β-cell proliferation assay compared to the human β-cell proliferation assay and this is responsible for the differential outcomes observed. This possibility could be addressed by blocking PGE_2 production with an NSAID and then adding back exogenous PGE_2. Unfortunately, the amount of PGE_2 produced during the proliferation assays was undetectable in these assays (discussed in more detail below). It is also plausible that there are different cytokines produced by resident inflammatory cells in mouse and human islets. Variation in cytokines may alter PGE_2 production or gene expression of the EP3 receptor since the mouse *Ptger3* promoter contains an NFκB binding site [105] and the human *PTGER3* gene contains an IFN-γ response

element [206]. Thus, subtle differences in cytokine production may alter the level of EP3 expression between mouse and human islets during the proliferation assays, which affects the response to DG-041 (EP3 antagonist). This outcome was not addressed as the expression of PGE_2 receptor genes and cytokine genes were not measured during the time course of the mouse and human β-cell proliferation assays.

It is possible that the discrepancies in EP3 and EP4 activity in mouse and human islets are due to differences in tissue processing, rather than inherent differences in the receptors themselves. Human islets are obtained from recently deceased donors and often have long cold ischemia times whereas mouse islets are isolated on the day of sacrifice and do not undergo cold ischemia periods. The Weir group elegantly demonstrated that Ki67 immunolabeling (proliferation) is dramatically decreased in mouse and pig β-cells from tissue that underwent warm and cold ischemia periods, similar to the processing of human pancreata, compared to freshly fixed tissue [213]. Other islet isolation variables that could influence β-cell proliferation include time of death, shipping duration, and islet shipping culture medium. It is plausible that any combination of these factors influence the response of human β-cells to the EP3 and EP4 ligands. It would be interesting to isolate and culture mouse islets in a similar manner and time course to the human islet isolation process and assess how mouse islets respond to DG-041 ± CAY10598 in this setting. These experiments would clarify if the differences in mouse versus human β-cell proliferation are due to differences in receptor activity itself or merely due to the tissue processing procedure and duration.

The EP3 and EP4 signaling pathways that regulate β-cell proliferation and survival are not well defined, thus phospho-protein antibody microarrays were utilized to

identify downstream targets of these receptors. From this analysis, PLC-γ1 and mTOR were of primary interest for EP3 signaling and PKA was implicated in EP4 signaling. Use of a PLC inhibitor revealed that PLC-γ1 is involved in inducing β-cell replication when EP3 blocked in both mouse and human islets. DG-041 (EP3 antagonist) treatment increased tyrosine 783 phosphorylation on PLC-γ1 in mouse islets, a phosphorylation event that has been shown to enhance the enzymatic activity [199]. PLC-γ1 is a member of the PLC family that hydrolyzes plasma membrane phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates release of intracellular Ca^{2+} while DAG activates PKC signaling [199]. G_q-proteins activate the PLC-β members of the PLC family, while PLC-γ members are activated by non-receptor tyrosine kinases and receptor tyrosine kinases, including platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) [214, 215]. The link between EP3, PLC-γ1, and β-cell proliferation remains to be determined. PLC-γ1 itself has been implicated in promoting proliferation in other tissue types through ERK1/2 activation [203, 204]. Intriguingly, ERK1/2 mediates PDGFR-induced β-cell proliferation [216]. It is possible, then, that blockade of EP3 releases inhibition of PDGFR and thus activation of PLC-γ1, allowing for activation of ERK1/2 and induction of β-cell proliferation. To probe this possibility, mouse and human islets can be cultured in the presence of a PDGFR inhibitor imatinib [217] or a selective ERK1/2 inhibitor FR180204 [218] in combination with DG-041 in the β-cell proliferation assay. For mouse islets, the addition of PL treatment will be necessary as DG-041 treatment alone does not increase mouse β-cell proliferation. Alternatively, IP_3-mediated release of Ca^{2+} may be the mechanism for enhanced β-cell proliferation in response to DG-041.

Rises in intracellular Ca^{2+} can activate the phosphatase calcineurin, which dephosphorylates the nuclear factor of activated T cells (NFAT) transcription factor, allowing NFAT to undergo nuclear translocation [216]. Once in the nucleus, NFAT activates transcription of many cell cycle activators, including cyclins, cyclin-dependent kinases, and FoxM1, thus activating β-cell replication [219]. According to this scenario, blockade of EP3 would lead to activation of PLC-γ1, generation of IP_3, a rise in intracellular Ca^{2+}, activation of NFAT, and induction of β-cell proliferation. To test this possibility, mouse and human islets can be cultured in the presence of a selective inhibitor of calcineurin-NFAT [220] and DG-041 (with PL for mouse islets) in the β-cell proliferation assay.

The phospho-protein microarray and subsequent inhibitor analyses revealed that mouse and human EP4 utilize the canonical G_S-coupled PKA signaling mechanism to promote β-cell survival. GLP-1R is another islet G_S-coupled GPCR that promotes β-cell proliferation and survival via activation of PKA [69]. It is interesting to consider that EP4 and GLP-1R may have many overlapping roles in the β-cell since they are both G_S-coupled receptors that can enhance β-cell replication and survival. The ability of GLP-1R to induce human β-cell proliferation is controversial with some groups showing no effect and others reporting a modest increase [69]. In our study, we only observed an increase in human β-cell proliferation in response to EP4 receptor activation when EP3 was also blocked. It is possible that EP3 signaling is blocking the activity of several G_S-GPCRs, such as GLP-1R, and not just EP4. Future studies that use the EP3 antagonist DG-041 in combination with a GLP-1R agonist in the human β-cell proliferation assay would address this question. If the ability of EP4 to induce human β-cell proliferation is

indeed a conserved mechanism used by many G_S-GPCRs, including GLP-1R, I would predict that combined blockade of EP3 and activation of GLP-1R would uncover a positive role for GLP-1R in human β-cell proliferation. In support of this idea, blockade of EP3 with simultaneous GLP-1R activation enhances intracellular cAMP levels and GSIS in diabetic mouse islets [123]. How these receptors interact in human islets, particularly in terms of β-cell proliferation and survival, remains to be tested.

The *ex vivo* studies presented in Chapter IV demonstrated that blockade of EP3 or activation of EP4 can promote human β-cell proliferation and protect against cytokine-induced β-cell death. However, the function of human EP3 and EP4 receptors in an *in vivo* setting remain to be determined. Future endeavors will investigate the response of human islets to modulation of EP3 and EP4 signaling in an *in vivo* setting using an islet transplant model [221]. To this end, human islets will be co-transplanted with poly(lactic-co-glycolic acid) (PLGA) microspheres loaded with DG-041 (EP3 antagonist) or CAY10598 (EP4 agonist) into immunodeficient NOD-SCID mice. PLGA microspheres are a safe, biodegradable, FDA approved tool for therapeutic drug delivery [222, 223]. Moreover, our group has successfully performed this assay using human islets and PLGA microspheres loaded with recombinant connective tissue growth factor (CTGF) [Dr. Raymond Pasek, unpublished data]. Using this methodology, we will be able to assess β-cell proliferation and survival of human islets co-transplanted with DG-041 ± CAY10598 microspheres. As previously discussed, work from the Kimple lab and our own studies with $EP3^{-/-}$ mice suggest that EP3 only has an inhibitory effect on GSIS and β-cell proliferation during certain *in vivo* conditions. It is possible, then, that there will not be any changes in human β-cell proliferation or survival when human islets

are co-transplanted with DG-041- or CAY10598-loaded microspheres into non-diabetic mice. However, in my studies using human islets, I saw an increase in human β-cell proliferation when EP3 was blocked in the absence of any secondary stressor/stimulus. If there are not significant increases in β-cell proliferation or survival in the transplant studies, mice could be injected with STZ to induce hyperglycemia and diabetes, as a secondary "hit".

All of the *ex vivo* experiments discussed above were performed in the absence of an NSAID (COX-2 inhibitor). The mouse β-cell proliferation assay was performed under hyperglycemic conditions (11 mM glucose), which has been shown to induce COX-2 expression and islet PGE_2 production [115, 116]. Islet PGE_2 production is also increased by cytokine treatment [110, 129]. Thus, it is possible and likely that PGE_2 is produced under these different experimental settings. As mentioned in Chapter IV, we were unable to measure PGE_2 production from mouse and human islets during both the β-cell proliferation and β-cell survival assays. The levels of PGE_2 were below the detectable limit for the assay. The number of islets required to accurately and reliably measure PGE_2 production during the *ex vivo* assays will need to be optimized in collaboration with the Vanderbilt Eicosanoid Core. Once PGE_2 is readily detectable in these assays, inhibiting its production using an NSAID, such as ibuprofen, will be very informative.

Evidence from the literature has primarily pointed to an inhibitory role for EP3 signaling on GSIS and β-cell proliferation during T2D and insulin resistance. It would be interesting to investigate if EP3 and EP4 regulate β-cell mass during other physiologically important periods, such as development and pregnancy. Currently, there

are no reports that have investigated the *in vivo* role of EP4 in β-cell proliferation and mass. Data from EP3$^{-/-}$ mice shows that EP3 does not affect β-cell proliferation during development, a period of robust β-cell proliferation. The levels of β-cell proliferation and mass in adult EP3$^{-/-}$ mice are indistinguishable from those of control mice [166], demonstrating that loss of EP3 during development did not impair the ability of β-cell mass to expand normally.

During pregnancy, several physiological changes occur in order to maintain euglycemia in the mouse; including increased GSIS, enhanced insulin production, and elevated β-cell mass via increased hypertrophy and proliferation [49]. The peak of pregnancy-induced β-cell proliferation occurs at gestational day (GD) 14.5, resulting in the maximal increase in mass at GD16.5 [224]. Many proteins have been demonstrated to be important factors during pregnancy to induce β-cell proliferation and maintain euglycemia, including FoxM1 and PL [59, 195]. Intriguingly, mouse islet *Ptger3* expression is significantly reduced at GD14.5 [225]. Previous work from our group and work presented in this thesis demonstrate that FoxM1 and PL negatively regulate *Ptger3* expression. Further, PL-induced *ex vivo* β-cell proliferation was enhanced when EP3 was blocked. These observations suggest that the decrease in *Ptger3* observed at GD14.5 may serve as an additional mechanism to enhance β-cell proliferation during pregnancy. It would be interesting to study β-cell proliferation during pregnancy in an EP3 β-cell-specific knockout mouse model. Alternatively, islets could be isolated from pregnant mice and treated with sulprostone (EP3 agonist) or DG-041 (EP3 antagonist) in the β-cell proliferation assay to probe the function of EP3 during pregnancy.

Identifying therapeutic targets that are capable of inhibiting β-cell loss in addition to increasing β-cell proliferation are of great interest to the T2D research field. The link between PGs and diabetes has been known since the late 1800s and NSAIDs were historically used to treat diabetes [4, 5]. The data in this thesis reveal that the PGE_2 EP3 and EP4 receptors have opposing actions on β-cell proliferation and survival in mouse and human islets, highlighting their potential as T2D drug targets. As previously discussed (see Chapter I), GLP-1R agonists, such as exendin-4, are very common treatments for T2D, but are not efficacious in all individuals with T2D. Based on their G-protein signaling mechanisms, it is possible that EP3 and EP4 modulate the response to islet GLP-1R signaling. I propose that increased EP3 signaling in some T2D individuals may be counteracting the beneficial effects of GLP-1R agonists. Thus, it may be beneficial when designing future therapeutics to activate GLP-1R while simultaneously inhibiting EP3 and/or activating EP4 to improve GSIS, increase β-cell proliferation, and protect against β-cell loss. Before these receptors can be pursued as T2D therapeutics, β-cell-specific targeting strategies must be developed. EP3 and EP4 are widely expressed and play important roles in many other physiological processes, including, but not limited to, inflammation, blood pressure, pain, and renal function [226]. EP3 especially has critical functions in the brain, as $EP3^{-/-}$ mice develop obesity and consume more food than WT mice [166, 173]. EP3 signaling in the hypothalamus is thought to be responsible for the described phenotypes. If β-cell-specific targeting is not possible, utilizing an EP3 antagonist that is at least peripherally restricted (i.e., cannot cross the blood brain barrier) may suffice, although there are still potential off-target effects of systemic blockade of EP3, such as increased adipose lipolysis and ectopic

lipid storage in the liver [166]. Additional off-target effects due to GLP-1R targeting, including pancreatitis and gastrointestinal distress, will likely still be present in any systemic drugs designed to target the EP receptors in combination with GLP-1R.

Despite these hurdles to overcome, EP3 and EP4 ligands have been used in human clinical trials, suggesting that they may prove useful as T2D therapeutics. The EP3 antagonist DG-041 was used in a European phase II trial to test its ability to treat peripheral arterial disease (PAD) [EU Clinical Trials Register]. However, the results of this study were not published, therefore the efficacy of DG-041 in treating PAD and the side effects caused by systemic DG-041 treatment are unknown. Both agonists and antagonists of EP4 have been used in human clinical trials. An EP4 agonist has been used in a small phase II study (7 patients total) to study the effects of EP4 activation in patients with mild to moderate ulcerative colitis (UC). Importantly, no adverse events were observed during the trial [227]. Another EP4 agonist, called Lubiprostone (Amitiza), is currently used to treat chronic idiopathic constipation, irritable bowel syndrome with constipation, and constipation caused by opioids in adults [228]. Lubiprostone is a derivative of PGE_1 and has been shown to stimulate secretion of intestinal fluid through the activation of chloride channel protein 2 chloride channels. Lubiprostone additionally targets and activates the EP4 receptor in many different model systems [229]. There have not been any adverse events of Lubiprostone reported in the clinical trials data [230]. EP4 antagonists have used in clinical trials to study several different outcomes, including headaches and rheumatoid arthritis [ClinicalTrials.gov].

Overall, the work presented in this thesis has furthered our knowledge of the EP3 and EP4 receptors and their influence on β-cell proliferation and β-cell survival in mouse and human islets. Specifically, these studies show that pharmacological blockade of EP3 enhances β-cell proliferation through activation of PLC-γ1 and prevents cytokine-induced β-cell death. Activation of EP4 enhances human β-cell proliferation, only when EP3 is simultaneously blocked, and promotes β-cell survival in a PKA-dependent manner in mouse and human islets treated with cytokines. These data support the notion that pathways that activate proliferation also play pivotal roles in activating survival pathways, which are of interest for future therapeutics.

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