Original Article

MOLECULAR METABOLISM

Opposing effects of prostaglandin E_2 receptors EP3 and EP4 on mouse and human β-cell survival and proliferation

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ABSTRACT

Objective: Hyperglycemia and systemic inflammation, hallmarks of Type 2 Diabetes (T2D), can induce the production of the inflammatory signaling molecule Prostaglandin E_2 (PGE_2) in islets. The effects of PGE_2 are mediated by its four receptors, E-Prostanoid Receptors 1-4 (EP1-4). EP3 and EP4 play opposing roles in many cell types due to signaling through different G proteins, G_i and G_S, respectively. We previously found that EP3 and EP4 expression are reciprocally regulated by activation of the FoxM1 transcription factor, which promotes β-cell proliferation and survival. Our goal was to determine if EP3 and EP4 regulate β-cell proliferation and survival and, if so, to elucidate the downstream signaling mechanisms.

Methods: β-cell proliferation was assessed in mouse and human islets *ex vivo* treated with selective agonists and antagonists for EP3 (sulprostone and DG-041, respectively) and EP4 (CAY10598 and L-161,982, respectively). β-cell survival was measured in mouse and human islets treated with the EP3- and EP4-selective ligands in conjunction with a cytokine cocktail to induce cell death. Changes in gene expression and protein phosphorylation were analyzed in response to modulation of EP3 and EP4 activity in mouse islets.

Results: Blockade of EP3 enhanced β-cell proliferation in young, but not old, mouse islets in part through phospholipase C (PLC)-γ1 activity. Blocking EP3 also increased human β-cell proliferation. EP4 modulation had no effect on *ex vivo* proliferation alone. However, blockade of EP3 in combination with activation of EP4 enhanced human, but not mouse, β-cell proliferation. In both mouse and human islets, EP3 blockade or EP4 activation enhanced β-cell survival in the presence of cytokines. EP4 acts in a protein kinase A (PKA)-dependent manner to increase mouse β-cell survival. In addition, the positive effects of FoxM1 activation on β-cell survival are inhibited by EP3 and dependent on EP4 signaling.

Conclusions: Our results identify EP3 and EP4 as novel regulators of β-cell proliferation and survival in mouse and human islets *ex vivo*.

Keywords Pancreatic β-cell; Prostaglandin E_2; Proliferation; Cell death

1. INTRODUCTION

Type 2 diabetes (T2D) results from failure of the insulin-producing β-cells to compensate for increased metabolic demands, such as in the context of obesity, resulting in increased β-cell stress, impaired β-cell function, and decreased functional β-cell mass [1]. Many G protein-coupled receptors (GPCRs) expressed in islets play roles in β-cell function and/or regulation of β-cell mass [2]. As such, GPCRs, which represent 50–60% of all drug targets [3], are attractive candidates for T2D therapies. One GPCR currently used as a T2D drug target is the glucagon-like peptide 1 (GLP-1) receptor (GLP-1R). GLP-1R couples to stimulatory G proteins (G_S) and exogenous GLP-1 treatment increases rodent glucose-stimulated insulin secretion (GSIS), β-cell proliferation, and β-cell survival [4]. Drugs targeting the GLP-1R, such as the GLP-1 agonist exendin-4, have been used for over a decade in T2D treatment [5]. While these therapies have been successful in many cases, GLP-1 therapies are not effective in all individuals with T2D [6]. Increased activity of GPCRs that couple to inhibitory G proteins (G_i) in some individuals may provide an explanation for these variable responses to GLP-1-based treatments.

Islets express nearly 300 GPCRs, including G_S-, G_i-, and G_q-coupled receptors [7]. G_S-coupled receptors increase intracellular cAMP levels

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Abbreviations: GPCR, G protein-coupled receptor; PGE_2, prostaglandin E_2; EP1-4, E-Prostanoid Receptors 1-4; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PL, placental lactogen; PT, pertussis toxin; PKA, protein kinase A; COX-2, cyclooxygenase-2

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by activating adenylyl cyclase (AC) whereas G_i-coupled receptors inhibit AC, decreasing cAMP concentrations. G_q-coupled signaling activates phospholipase C (PLC)-β to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), resulting in increased intracellular Ca^{2+}. Islet G_S- and G_q-coupled GPCRs, such as GLP-1R and GPR40, respectively, increase GSIS, whereas G_i-coupled GPCRs, such as the somatostatin (SST) receptor, decrease GSIS (reviewed in [2]). In addition to GSIS, islet GPCRs have also been implicated in regulating dynamics of β-cell mass expansion, such as β-cell proliferation and/or survival. For example, activation of the G_S-coupled fatty acid receptor GPR119 stimulates β-cell proliferation [8]. On the other hand, signaling via G_i proteins negatively regulates β-cell proliferation and mass expansion *in vivo* [9]. Further, activation of the G_i-coupled SST receptor inhibits *ex vivo* glucose-induced β-cell proliferation in mouse and human islets [10]. Thus, G_i-GPCRs and G_S-GPCRs can have opposing effects on β-cell function and β-cell proliferation.

The receptors for the endogenous lipid signaling molecule prostaglandin E_2 (PGE_2), called E-Prostanoid Receptor 1-4 (EP1-4), are among the GPCRs expressed in rodent and human islets [11–16]. RNA-sequencing (RNA-seq) revealed that EP3 and EP4 are both expressed in whole islet populations and sorted mouse β-cells [14] and in sorted human α- and β-cells [12]; *Ptger2* (encoding EP2) was not detected by RNA-seq in mouse islets [14]. EP3 is also expressed in the mouse-derived αTC1 α-cell line [13]. EP1-4 can be distinguished by their signaling properties: EP1 couples to G_q; EP2 and EP4 couple to G_S; and EP3 primarily couples to G_i proteins [16], including the pertussin toxin (PTx) insensitive inhibitory protein $G\alpha_Z$ [17]. 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 [13,16,18–24]; however, these inhibitory effects have not been consistently observed [25–29].

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. Mice null for EP3 ($EP3^{-/-}$) show no difference in β-cell proliferation when fed a chow diet yet display increased β-cell proliferation after 16 weeks of high fat diet (HFD) feeding [28]. $EP3^{-/-}$ mice gain more weight than control mice on HFD [28,30]; thus, it is unclear whether the increase in β-cell proliferation is due solely to loss of EP3 in islets or is a consequence of a combination of loss of EP3 and obesity. A β-cell-specific role for EP3 in proliferation has yet to be shown. In agreement with the $EP3^{-/-}$ data, global loss of $G\alpha_Z$, which couples to EP3 in the islet [17], results in increased β-cell proliferation during chow and HFD conditions [31]. In addition, $G\alpha_Z$-null mice are protected against streptozotocin (STZ)-induced hyperglycemia due to decreased β-cell death and increased proliferation [32]. These data suggest that EP3 normally inhibits β-cell proliferation and promotes β-cell death, yet the direct effect of EP3 in these processes remains unknown, since $G\alpha_Z$ 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 [11]. In other cell types, such as mouse gut epithelial cells and primary myoblasts, EP4 agonist treatment increases cell proliferation and decreases cell death [33,34].

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 active form of the critical cell cycle-regulating transcription factor FoxM1 is induced in β cells (β-FoxM1*) [35]. 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 [36] has no effect on basal levels of β-cell proliferation but enhances the proliferative response to placental lactogen (PL) in young wild type (WT) mouse islets but not in islets from old 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.

2. MATERIALS AND METHODS

2.1. Animals

Wild type (WT) male C57BL/6J mice were purchased from Jackson Laboratory, and β-FoxM1* mice were generated as previously described [35]. β-FoxM1* mice were maintained on a mixed C57BL6J/DBA2J background. Only male mice were used for *ex vivo* assays. Analyses were performed on islets from 8 to 10-week- or one-year-old mice, as indicated in each figure legend. Mice were housed in a 12-hour light/dark cycle and given *ad libitum* access to food (Lab Diet 5LJ5, Purina) and water. The Vanderbilt University Institutional Animal Care and Use Committee approved all mouse studies.

2.2. Mouse and human islet isolations and culture media

Mouse islets were isolated from male mice by the Vanderbilt Islet Procurement and Analysis Core. Islets were handpicked and cultured overnight in either mouse proliferation media (RPMI 1640 (ThermoFisher Scientific), penicillin/streptomycin (1% v/v), 11 mM glucose, and 10% horse serum (ThermoFisher Scientific)) or mouse survival media (RPMI 1640, penicillin/streptomycin (1% v/v), 5.6 mM glucose, and 10% fetal bovine serum (FBS; ThermoFisher Scientific)).

A subset of human islets ($n = 20$ donors) was obtained from the Alberta Diabetes Institute IsletCore [37] at the University of Alberta. The remainder of donor islets ($n = 24$ donors) was obtained from islet isolation centers participating in the Integrated Islet Distribution Program (http://iidp.coh.org/). Upon arrival, human islets were handpicked and cultured in either human proliferation media (low-glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, 110 mg/L sodium pyruvate (ThermoFisher Scientific), penicillin/streptomycin (1% v/v), and 10% horse serum) or human survival media (low-glucose (1 g/L) DMEM with L-glutamine, 110 mg/L sodium pyruvate, penicillin/streptomycin (1% v/v), and 10% FBS). Each set of donor islets was tested in duplicate, and the results shown are the average of the duplicates for each individual donor. Donor characteristics for each experiment can be found in Supplemental Table 1.

2.3. Ex vivo mouse and human proliferation

Ex vivo mouse β-cell proliferation was performed as previously described [38,39]. Briefly, 40 mouse islets per replicate were cultured

for four days in mouse proliferation media in the presence of 0.1 mM EGTA, which mildly loosens cell—cell contacts to enhance nutrient and compound accessibility to the core of the islet [38] and one or more of the following compounds: vehicle (phosphate buffered saline (PBS)), 0.5 μg/mL recombinant human placental lactogen (PL; Harbor-UCLA Research and Education Institute), 30 nM sulprostone (Cayman Chemical), 30 nM DG-041 (Vanderbilt Institute of Chemical Biology Synthesis Core [40]), 10 nM CAY10598 (Cayman Chemical), 100 nM L-161,982 (Cayman Chemical), 1 μM U-73122 (Sigma), or 30 nM rapamycin (Calbiochem). All compounds were diluted in PBS to obtain desired concentrations. Dispersed cells were immunolabeled with guinea pig anti-insulin (1:400; Dako), rabbit anti-Ki67 (1:400; AbCam), Cy2-conjugated anti-guinea pig IgG (1:300; Jackson ImmunoResearch Laboratories), and Cy3-conjugated anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories). Nuclei were visualized with 4′,6′-diamidino-2-phenylindole (DAPI, 1 μg/mL; Molecular Probes). Images were obtained using a ScanScope FL slide scanner (Aperio Technologies, Inc.). 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.

For *ex vivo* human β-cell and α-cell proliferation, 80 islets from each donor were cultured for four days in human proliferation media containing 0.1 mM EGTA and one of the following compounds: vehicle (PBS), 0.5 μg/mL recombinant human PL, 30 nM DG-041, or 10 nM CAY10598. To determine β-cell proliferation, human islets were immunolabeled for insulin, Ki67, and DAPI as described above. To measure α-cell proliferation, islets were immunolabeled for mouse anti-glucagon (1:400; Millipore), rabbit anti-Ki67 (1:400), Cy2-conjugated anti-mouse IgG (1:300; Jackson ImmunoResearch Laboratories), Cy3-conjugated anti-rabbit IgG (1:400), and DAPI. β- and α-cell proliferation was determined by quantifying the number of insulin-Ki67 or glucagon-Ki67 dual-positive cells, respectively, as described for mouse β-cell proliferation. Data are represented as fold change in proliferation over vehicle treated islets.

2.4. Ex vivo mouse and human β-cell survival

β-cell survival was assayed in WT and β-FoxM1* mouse islets similarly to our previously published work [35]. To induce FoxM1 expression, β-FoxM1* mice were given free access to water containing 2% doxycycline (Dox; Sigma—Aldrich) supplemented with Splenda for two weeks prior to islet isolation; drinking water was replaced 3 times per week [35]. After islet isolation, 80 WT or β-FoxM1* islets were cultured overnight in mouse survival media supplemented with 1 μg/mL Dox. The next day, islets were treated 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) for 48 h. At the time of cytokine treatment, 0.1 mM EGTA and one or more of the following compounds: 1 nM sulprostone, 30 nM DG-041, 10 nM CAY10598, 100 nM L-161,982, or 100 μM Rp-cAMPS (Sigma) were supplemented in the mouse survival media. After 48 h, islets were dissociated and cytospun onto charged slides. β-cell death was assessed using the ApoAlert DNA fragmentation kit (Clontech) according to the manufacturer. Dispersed cells were co-immunolabeled with guinea pig anti-insulin (1:400) and Cy3-conjugated anti-guinea pig (1:300) and nuclei were visualized with DAPI (1 μg/mL). Images were acquired as described in Section 2.3. TUNEL-insulin double-positive cells were manually counted using MetaMorph 6.1 software (Molecular Devices) to determine the percentage of cell death. Data are represented as fold change compared to vehicle + cytokine treated islets.

For human β-cell survival, 80 islets per group were cultured in human survival media overnight before being treated with the EP ligands as described for mouse islets. Cytokines used for human islets include 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 quantified and represented as described for mouse islets.

2.5. Islet gene expression analysis

For untreated islets, islets were isolated from human donors or two-, four-, eight-, or 12-month old WT male mice, washed three times in cold 1X PBS and immediately prepared for RNA isolation using the TRIzol reagent (Life Technologies), and stored at −80 °C. RNA was isolated using the RNeasy Mini Kit (Qiagen). A total of 100 ng or 175 ng cDNA was prepared from mouse and human islets, respectively, using the SuperScript III First Strand Synthesis System (ThermoFisher Scientific). Quantitative RT-PCR (qRT-PCR) was performed as described previously [1]. Primer sequences are available upon request. Data are normalized to *Hprt* or *TBP* for mouse and human, respectively. Mouse islet data are represented as $2^{-\Delta\Delta Ct}$ compared to the two-month-old group. Human islet data are shown as ΔCt relative to *TBP*.

For treated islets, 120 mouse islets were cultured in mouse proliferation media for two or four days in the presence of 0.1 mM EGTA and one of the following compounds: vehicle (PBS), 30 nM DG-041, or 10 nM CAY10598. Another group of mouse islets was cultured in mouse survival media supplemented with cytokines (as described in Section 2.4) for two days in the presence of vehicle (PBS), 30 nM DG-041, or 10 nM CAY10598. 120 human islets were cultured with the same compounds described for mouse islets in human proliferation media for one or four days. At the end of the treatment period, RNA isolation, cDNA preparation, and qRT-PCR were performed as described above. Data are represented as $2^{-\Delta\Delta Ct}$ compared to vehicle treated islets.

2.6. Phospho-protein microarray

Islets were isolated from 8 to 10-week-old WT male mice, allowed to recover overnight, and treated with vehicle (PBS), 10 nM CAY10598, 0.5 μg/mL PL, or 0.5 μg/mL PL + 30 nM DG-041 for 24 h in mouse proliferation media. Islets from three mice were pooled together for two replicates from each treatment. After 24 h, islets were collected and washed three times in cold 1X PBS. The islet pellet was then flash frozen and stored at −80 °C until samples were prepared for the Kinex™ KAM-900P Antibody Microarray Kit (Kinexus Bioinformatics Corporation). Protein lysate preparation, protein labeling, protein purification with chemical cleavage, and microarray incubation were performed according to the manufacturer. Protein concentration was determined by Bradford assay using the Protein Assay Dye Reagent Concentrate (Bio-Rad). Equal amounts of protein for each replicate 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 PL vs. PL + DG-041 replicate 2. Microarrays were scanned and analyzed by Kinexus Bioinformatics Corporation.

KiNetscape maps were generated from the leads identified in the Kinex™ 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 1000 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).

2.7. Statistics

All results are expressed as mean ± SEM. Statistical significance was calculated in GraphPad Prism Version 6.0d (GraphPad Software, Inc.) using a one-way ANOVA and Bonferroni *post hoc* analysis, Student's *t* test, or linear regression, as appropriate. *p* values of ≤ 0.05 were considered significant.

3. RESULTS

3.1. Placental lactogen treatment decreases *Ptger3γ* expression

PL is a pregnancy hormone known to stimulate rodent β-cell proliferation [41]. 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 [41]. 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 1). Although we observed a variable increase in *Ptgs2* (COX-2) expression in each islet preparation in response to PL, it did not reach statistical significance (Figure 1; Supplemental Figure 1). Intriguingly, PL treatment specifically decreased expression of *Ptger3γ* (Figure 1).

3.2. 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 [28,33,34], 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 [38]. After four days of treatment, PL increased β-cell proliferation 3–4-fold over vehicle-treated islets (Figure 2**A-B**). Basal levels of β-cell proliferation were not affected by treatment with any of the EP3 or EP4 ligands alone (Figure 2**A-B**). Based on the decreased *Ptger3γ* expression in response to PL (Figure 1), we tested whether EP3 activity acts to inhibit the proliferative response to PL by treating WT islets with a combination of PL and either sulprostone or DG-041. Activation of EP3 with sulprostone combined with PL failed to increase β-cell proliferation over that of vehicle-treated islets, in agreement with our prediction (Figure 2**A**). 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 2**A**). We also tested whether EP4 activation would enhance the proliferative response to PL. However, neither CAY10598 nor L-161,982 treatment in combination with PL significantly affected β-cell proliferation compared to PL alone (Figure 2**B**). In addition, combined blockade of EP3 and activation of EP4 did not increase mouse β-cell proliferation (Supplemental Figure 2). Thus, we conclude that EP3 function 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 [42]. 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 2**C**), 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 old islets (Figure 2**C**). 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.

3.3. Ptger 3 (EP3) gene expression is altered with age in mouse islets

Given the lack of response to DG-041 in aged β-cells, we wanted to determine if expression of PGE_2 receptors changed during aging. qRT-PCR was performed on islets isolated from WT mice aged two, four,

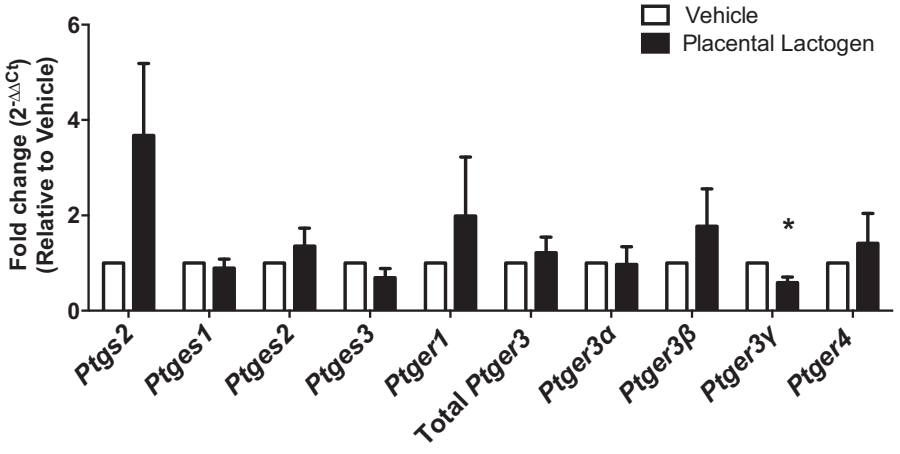


Figure 1: Placental lactogen decreases *Ptger3γ* in mouse islets. Wild type mouse islets (8–10 weeks old) were cultured for four days in the presence of vehicle (n = 4) or placental lactogen (n = 4). qRT-PCR was performed for PGE_2 synthesis genes (*Ptgs2*, *Ptges1-3*) and receptor genes (*Ptger1-4*). *p = 0.0147 vs vehicle. All data are represented as $2^{-\Delta\Delta Ct}$ relative to vehicle. Data were analyzed using a Student's *t test*.

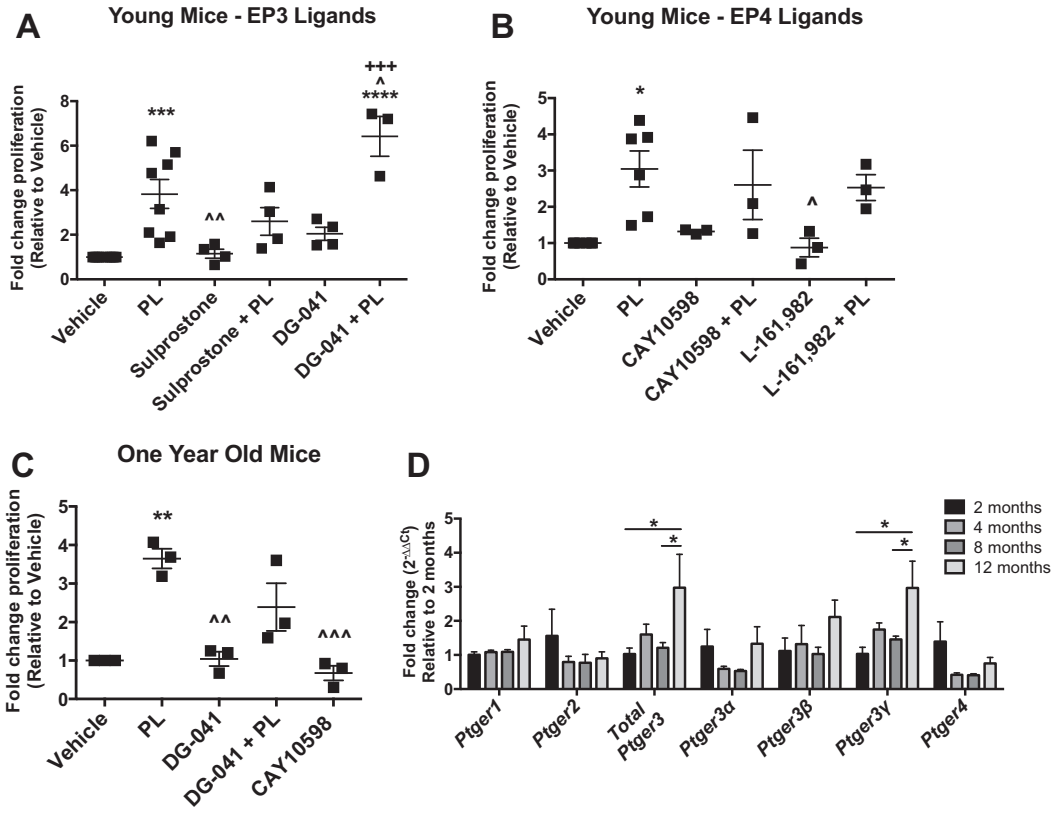


Figure 2: EP3 inhibits, while EP4 does not affect, mouse β-cell proliferation and displays increased expression with age. (**A**) Young mouse islets (8–10 weeks of age) were treated with vehicle, PL, sulprostone +/− PL, or DG-041 +/− PL for 4 days before being immunolabeled for insulin, Ki67, and DAPI. *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**) Aged mouse islets (1 year old) were treated with vehicle, PL, DG-041 +/− PL, or CAY10598 as described in A. *versus vehicle; ^versus PL. (**D**) 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. For A–C, one symbol represents $p < 0.05$, two symbols indicate $p < 0.01$, four symbols represent $p < 0.0001$. For D, *$p < 0.05$.

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 *Ptger3γ* (Figure 2**D**). We did not identify any differences in gene expression of *Ptger3α*, *Ptger3β*, or the other EP receptors during aging.

3.4. 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 3**A**). 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 [43]. 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 3**A**, **B**). Similar to mouse islets, activation of EP4 by CAY10598 did not increase human β-cell proliferation (Figure 3**A**). 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 3**A**). Importantly, neither DG-041 nor CAY10598 treatment increased human α-cell proliferation (Figure 3**C**). 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.

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

In a previous study, *PTGER3* (EP3) expression was found to be increased in human islets from obese and T2D donors [13]. PGE_2 production is increased in T2D [13], and T2D incidence increases with age [44], highlighting the potential significance of this pathway in T2D etiology. We analyzed EP receptor expression in human islets classified by BMI and T2D status. In contrast to the previously published dataset [13], *PTGER3* expression was not increased in islets from this set of obese non-diabetic donors (Figure 4**A**). 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**A'**). 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) [13]. There were no significant differences in *PTGER4* expression in either non-diabetic donor islets (Figure 4**B**) or T2D donor islets (Figure 4**B'**). Additionally, we did not detect any significant changes in *PTGER1* or *PTGER2* expression (Supplemental Figure 3).

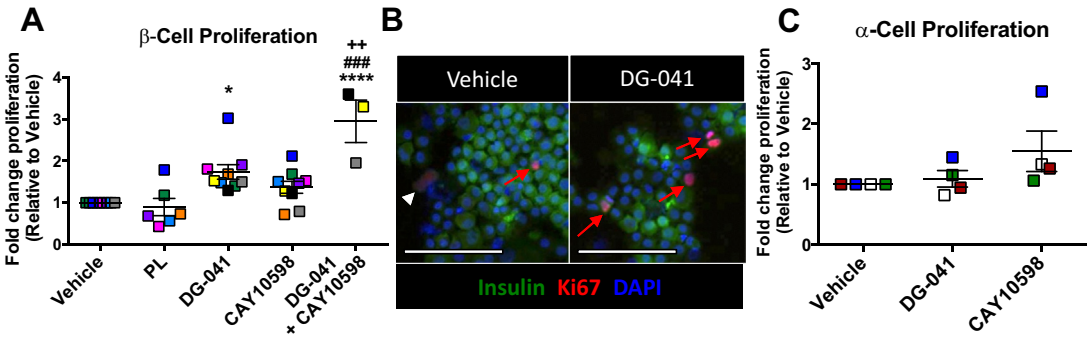


Figure 3: EP3 and EP4 have opposing effects on human β-cell proliferation. (**A**) Human islets were treated with vehicle, PL, DG-041, or CAY10598 and immunolabeled for insulin (green), Ki67 (red), and DAPI (blue). *p = 0.0177 vs vehicle; ****p < 0.0001 vs vehicle; $^{++}$p = 0.0038 vs DG-041; ###p < 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.

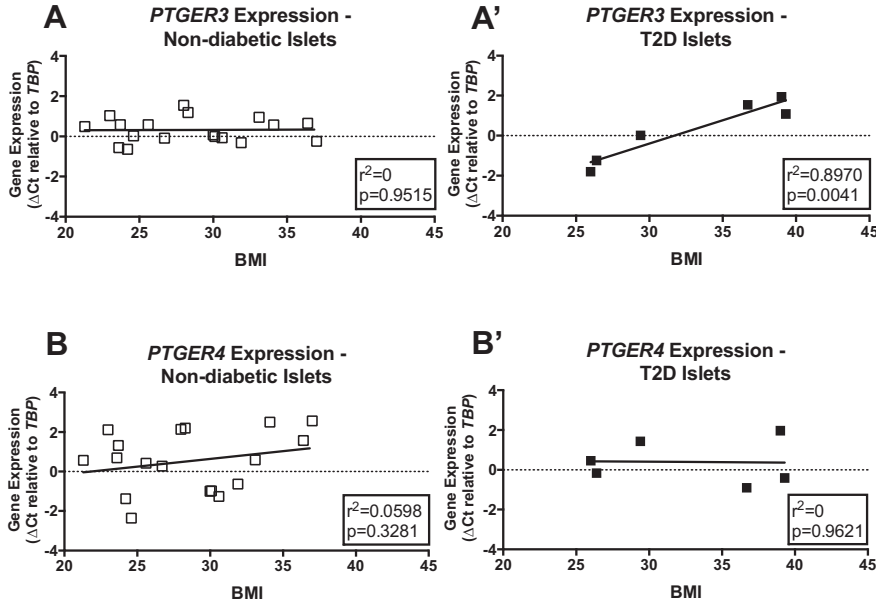


Figure 4: Increased *PTGER3* expression is correlated with lower BMI in T2D human islets. RNA was isolated from human islets and qRT-PCR was performed for EP3 (**A**) and EP4 (**B**) expression in non-diabetic (**A, B**) and T2D (**A', B'**) islets. Data are represented as ΔCt relative to *TBP* and were analyzed using a linear regression.

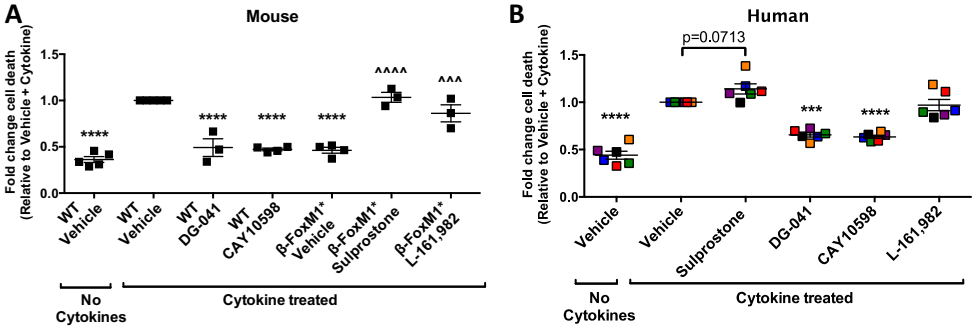
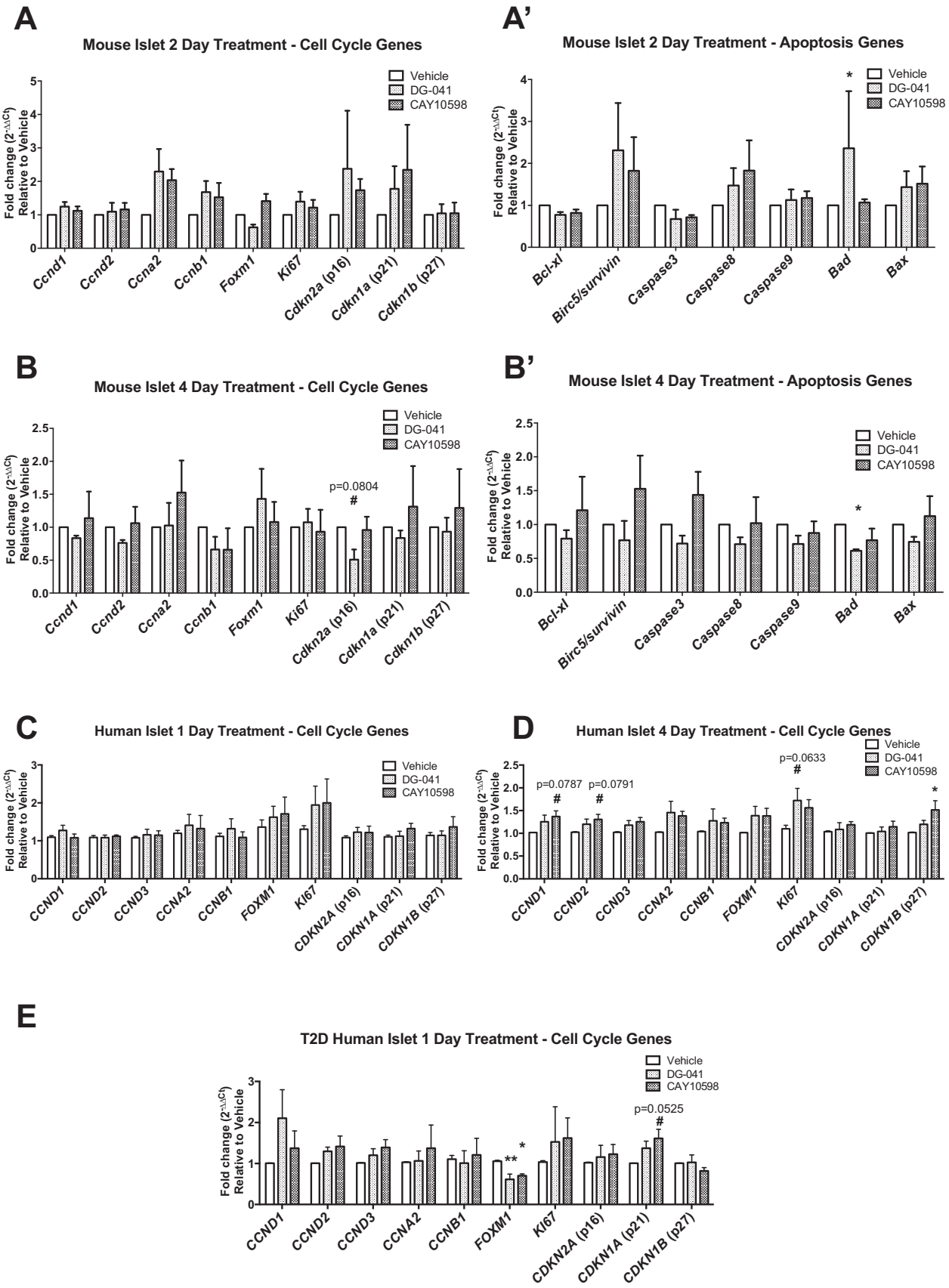


Figure 5: EP3 increases, while EP4 protects against, β-cell death in mouse and human islets. (**A**) Wild type (WT) or β-FoxM1* islets (8–10 weeks) or (**B**) human islets were treated for 48 h with a species-specific cytokine cocktail plus one of the following compounds: vehicle, sulprostone, DG-041, 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. 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.



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3.6. 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 increased β-cell proliferation and β-cell survival [35]. In these mice, an activated form of the *Foxm1* cell cycle transcription factor was induced in β-cells at two months of age for two weeks. We hypothesized that EP3 and EP4 play opposing roles in β-cell survival in this model. $G\alpha_z$-null mice have decreased β-cell death in response to STZ [32], 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 consisting of TNFα, IFNγ, and IL-1β to induce β-cell death [35]. 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 5**A**). 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 5**A**). Next, to determine if EP3 and/or EP4 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 5**A**), consistent with our previously published work [35]. 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 5**A**).

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 5**B**). Conversely, activation of EP3 via sulprostone led to a trend in increased β-cell death over cytokine treatment alone ($p = 0.0713$, Figure 5**B**); blockade of EP4 signaling with L-161,982 did not alter cytokine-induced β-cell death (Figure 5**B**). 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.

3.7. Identification of downstream effectors of EP3 and EP4

It has been established that PGE_2 inhibits GSIS through an EP3-inhibitory G_i-protein coupled mechanism that results in decreased cAMP levels [16,23,31]. However, the EP3 and EP4 signaling mechanisms downstream of G-protein coupling that promote their effects on proliferation and survival 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 6**A**) or four days (Figure 6**B**) 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 6**A'**; Figure 6**B'**), with the exception of *Bad*, a pro-apoptotic gene, after two and four days of DG-041 treatment. We also analyzed gene expression 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 (Supplemental Figure 4**A-C**).

In human islets, one day of DG-041 or CAY10598 treatment did not alter the expression of cell cycle genes (Figure 6**C**). 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 3**A**), there is a trend ($p = 0.0633$) toward increased *KI67* expression (Figure 6**D**). 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 6**E**). These data suggest that islets from T2D individuals respond differently to DG-041 and CAY10598 than islets from humans without T2D.

Since there were not dramatic changes in gene expression in response to manipulation of EP3 and EP4 activity, we next asked if changes in protein activity, via phosphorylation, were contributing to the observed changes in β-cell proliferation and survival. Using antibody microarrays, 878 phosphorylation sites were probed in protein lysates from WT 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 (Supplementary Table 2 and Supplementary Figure 5). Interestingly, both of these phosphorylation events are stimulated by mitogens [45]. PLC-γ1 Y783 phosphorylation, which increases enzymatic activity [46], and phosphorylation of several proteins involved in cell cycle regulation were also increased in response to DG-041 (Supplementary Table 2 and Supplementary Figure 5). 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 7**A**). Treatment with the mTOR inhibitor rapamycin essentially eliminated β-cell proliferation in untreated or DG-041 + PL treated islets (Figure 7**A**), 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 7**A**). However, the effect of DG-041 on PL-induced proliferation was lost when islets were treated with U-73122 (Figure 7**A**). These observations suggest that inhibition of EP3 improves β-cell proliferation in part through activation of PLC-1γ.

Figure 6: Changes in cell cycle and apoptosis genes with 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. (**B** and **B'**) Wild type mouse islets (8–10 weeks old; $n = 4$) were cultured for four days as described in A and qRT-PCR was performed for cell cycle (**B**) and apoptosis (**B'**) genes. *$p < 0.05$ vs vehicle. (**C, D**) Human islets ($n = 5–7$) were cultured for one (**C**) or four (**D**) 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. (**E**) Human islets ($n = 4$) from donors with T2D were cultured for one day as described in C. *$p < 0.05$ 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.

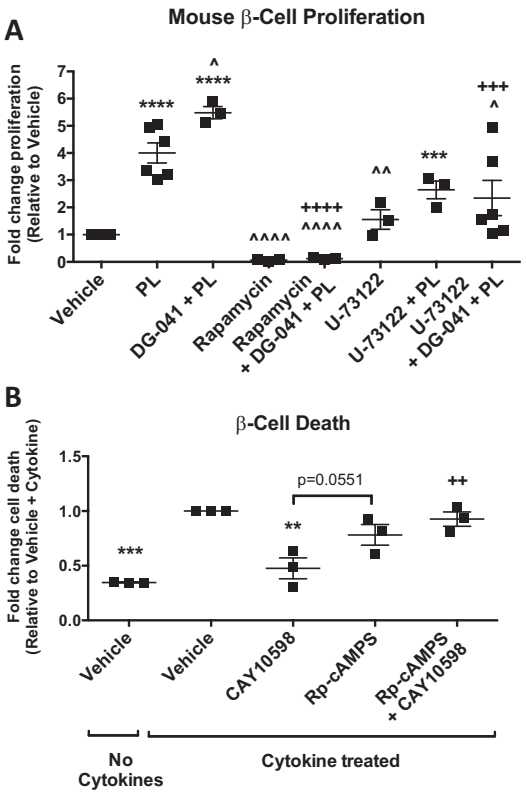

Figure 7: EP3 modulation of β-cell proliferation involves PLC-γ1 while EP4-induced protection against β-cell death involves PKA. (**A**) Young WT mouse islets (8–10 weeks old) were treated with vehicle, PL, DG-041 + PL, rapamycin (mTOR inhibitor) +/− DG-041 + PL, or U-73122 (PLC-γ1 inhibitor) +/− DG-041 + PL for 4 days before being immunolabeled for insulin, Ki67, and DAPI. *versus vehicle; ^versus PL; $^{+}$versus DG-041 + PL. (**B**) WT mouse islets (8–10 weeks old) were treated for 48 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. 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 $p < 0.0001$.

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 (Supplementary Table 3 and Supplementary Figure 6). eIF4E and PKCε have been implicated in PKA signaling previously [47,48]; 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 7**B**). Addition of the PKA-specific inhibitor Rp-cAMPS alone had no effect on cytokine-mediated β-cell death (Figure 7**B**). However, in the presence of Rp-cAMPS, activation of EP4 by CAY10598 was no longer able to decrease cytokine-induced β-cell death (Figure 7**B**). Taken together, these data demonstrate that EP4 promotes β-cell survival through activation and downstream signaling induced by PKA.

4. 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 old 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 [12–14], it remains possible that the observed effects on β-cell proliferation and β-cell survival are due to EP3 or EP4 signaling 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 [35]. 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 [35]. 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 mechanism [16], 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. We have identified novel downstream targets of EP3 and EP4 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 [46] and on sites in p70S6K, a downstream target of mTOR, known to be involved in mitogen stimulation and important for its activity [45]. 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 [49]). 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. PLC-γ1 has been implicated in promoting proliferation in other tissue types through ERK activation [50,51], but this is the first report, to our knowledge, revealing a role for PLC-γ1 in mouse 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 [19,20]. As Akt is known to regulate β-

cell proliferation through several targets including mTOR [52], 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 mouse β-cell proliferation.

CAY10598 treatment (EP4 activation) in mouse islets resulted in increased phosphorylation at the primary phosphosite in eIF4E [53] along with variable changes in phosphorylation of PKCε. PKA is activated by G_S-coupled mechanisms and has been shown to regulate eIF4E and PKCε in other tissues [47,48]. The PKA inhibitor Rp-cAMPS blocked the positive effect of CAY10598 (EP4 activation) on mouse 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 [54]. Therefore, we 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.

Identifying therapeutic targets that are capable of inhibiting β-cell loss in addition to increasing β-cell proliferation are of interest to the T2D field. This study highlights EP3 and EP4 as 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 could protect against T2D-associated β-cell loss as well as increase expansion of functional β-cell mass. EP3 and EP4 have antagonistic roles in many systems, such as in regulating blood pressure [55], and data from this report demonstrate that this phenomenon is also observed in islets. The endogenous ligand PGE_2 binds with equal affinity to EP3 and EP4 [56], so the balance of EP3 and EP4 receptor expression may be an important factor determining the final effect of PGE_2 signaling. In this study, PL treatment resulted in a variable, but not significant, increase in *Ptgs2* (COX-2) expression, yet did not change the expression of the prostaglandin synthases, *Ptges1-3*. In islets, levels of COX-2, and not the prostaglandin synthases, primarily regulate PGE_2 production [29], suggesting that PL treatment may increase PGE_2 production. We also identified situations in which *Ptger3* (EP3) gene expression is altered, which may impact the effects of PGE_2. In mouse islets, PL treatment decreases *Ptger3γ* expression and aging results in increased levels of *Ptger3γ* and overall *Ptger3*. In human islets, *PTGER3* expression is increased in islets from lean T2D patients. This observation is consistent with previous work demonstrating increased *PTGER3* expression in islets from T2D donors [13]. In contrast to EP3 expression, mouse *Ptger4* expression was unaffected by PL treatment and age and *PTGER4* was not altered by BMI or T2D status in human islets. Therefore, an increase in EP3 gene expression during aging and T2D may 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 EP3 expression, such as by PL, could result in enhanced PGE_2-EP4 signaling which would protect against β-cell death and preserve functional β-cell mass.

Our data suggest that the previously observed increase in *Ptger3* (EP3) mRNA in islets from mice and humans with T2D [13] is context-specific. In islets from individuals with T2D, there was an inverse correlation between *PTGER3* expression and BMI. 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. Indeed, EP3 activation decreases GSIS in mouse and human islets only in specific settings, such as insulin resistance and T2D [13,23]. Thus, 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 T2D where *PTGER3* is elevated. This idea is consistent with our observed 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.

EP3 and EP4 may also serve to 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. We propose that increased EP3 signaling in some T2D individuals may be counteracting the beneficial effects of GLP-1R agonists. In line with this notion, activation of EP3 antagonizes the effects of GLP-1R activation on cAMP production in mouse islets [13]. GLP-1 treatment fails to induce β-cell proliferation in aged mouse islets [42], similar to the failure of DG-041 (EP3 inhibition) to enhance PL-induced β-cell proliferation in one-year old mice shown here. Increased expression of the *Ptger3γ* variant may prevent DG-041 from blocking EP3 activity in aged mouse islets. The three mouse EP3 splice variants (α, β, and γ) result from alternative splicing of the C-terminal tail and differ in terms of constitutive agonist-independent activity and receptor desensitization [57,58]. 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 [58]. An increase in constitutively active *Ptger3γ* in old 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) 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 [54], 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. Thus, it may be beneficial when designing future therapeutics to activate GLP-1R while simultaneously activating EP4 and/or inhibiting EP3 to improve GSIS, increase β-cell proliferation, and protect against β-cell loss. Overall, our studies have identified novel roles of EP3 and EP4 as regulators of β-cell proliferation and survival in mouse and human islets.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.04.002.

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