Pharmacological blockade of the EP3 prostaglandin E2 receptor in the setting of type 2 diabetes enhances β-cell proliferation and identity and relieves oxidative damage

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ABSTRACT

Objective: Type 2 diabetes is characterized by hyperglycemia and inflammation. Prostaglandin E2, which signals through four G protein-coupled receptors (EP1-4), is a mediator of inflammation and is upregulated in diabetes. We have shown previously that EP3 receptor blockade promotes β-cell proliferation and survival in isolated mouse and human islets ex vivo. Here, we analyzed whether systemic EP3 blockade could enhance β-cell mass and identity in the setting of type 2 diabetes using mice with a spontaneous mutation in the leptin receptor (Leprdb).

Methods: Four- or six-week-old, db/+ and db/db male mice were treated with an EP3 antagonist daily for two weeks. Pancreata were analyzed for α-cell and β-cell proliferation and β-cell mass. Islets were isolated for transcriptomic analysis. Selected gene expression changes were validated by immunolabeling of the pancreatic tissue sections.

Results: EP3 blockade increased β-cell mass in db/db mice through enhanced β-cell proliferation. Importantly, there were no effects on α-cell proliferation. EP3 blockade reversed the changes in islet gene expression associated with the db/db phenotype and restored the islet architecture. Expression of the GLP-1 receptor was slightly increased by EP3 antagonist treatment in db/db mice. In addition, the transcription factor nuclear factor E2-related factor 2 (Nrf2) and downstream targets were increased in islets from db/db mice in response to treatment with an EP3 antagonist. The markers of oxidative stress were decreased.

Conclusions: The current study suggests that EP3 blockade promotes β-cell mass expansion in db/db mice. The beneficial effects of EP3 blockade may be mediated through Nrf2, which has recently emerged as a key mediator in the protection against cellular oxidative damage.

Keywords Type 2 diabetes; Mouse model; Prostaglandin E2; Beta cell proliferation; Nrf2

1. INTRODUCTION

Type 2 diabetes (T2D) results from the failure of insulin-secreting β cells to compensate for elevated metabolic demand, thereby resulting in diminished expansion of functional β-cell mass [1,2]. G-protein-coupled receptors (GPCRs) expressed in islets play a role in β-cell function and/or regulation of β-cell mass [3]. One GPCR currently targeted as a T2D therapy is the glucagon-like peptide 1 (GLP-1) receptor (GLP-1R), which couples with stimulatory G proteins (Gs). In mice, exogenous GLP-1 treatment increases glucose-stimulated insulin secretion (GSIS), β-cell proliferation, and β-cell survival [4]. Although GLP-1-based therapies have been successful in many cases, they are not effective in all individuals with T2D [5]. The increased activity of GPCRs that couple with inhibitory G proteins (Gi) in some individuals may provide an explanation for these variable responses to GLP-1-based treatments. The chronic hyperglycemia and systemic inflammation observed in T2D are associated with increased levels of circulating prostaglandin

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E₂ (PGE₂; 6, 7), which has been implicated in β-cell dysfunction and loss of β-cell identity [7–15]. Downstream effects of PGE₂ signaling are carried out by the E-prostanoid (EP) receptors, EP1-4. The expression of EP3, a Gα-coupled receptor, is increased in islets from humans with T2D and mouse models of T2D [7,16]. Both EP3 and EP4, a Gα-coupled receptor, have emerged as potential therapeutic targets for modulating β-cell mass dynamics and function. Recently, our lab demonstrated that pharmacological EP3 blockade promotes β-cell proliferation and survival in ex vivo mouse and human islets [16]; activation of EP4 enhanced β-cell survival in both mouse and human islets but stimulated β-cell proliferation only in human islets. Our group has shown that the downstream effectors that block EP3 and/or activate EP4 in β cells include PLCγ-1 and/or CAMK/PKA pathways, respectively [7,16]. Although the modulation of EP3 and EP4 activity has been shown to be beneficial in the context of mouse models of diabetes, the mechanisms for these effects were not analyzed. In particular, whether in vivo PGE₂ receptor modulation in the setting of T2D alters β-cell mass dynamics or preserves β-cell identity has not been determined.

The current study explores whether in vivo pharmacological antagonism of EP3 enhances β-cell proliferation and mass in the setting of T2D using the db/db mouse model, which contains a spontaneous mutation in the leptin receptor, resulting in hyperphagia, obesity, and hyperglycemia. Given our previous studies using isolated mouse and human islets ex vivo, we predicted that in vivo EP3 blockade would enhance β-cell proliferation and survival, which would ultimately result in β-cell mass expansion. We found that systemic blockade of EP3 enhanced β-cell proliferation and mass in db/db mice, with no effect on α-cell proliferation. Whole transcriptome analysis at the onset of diabetes revealed distinct gene expression changes in db/db mice compared with controls. EP3 blockade reversed the db/db islet phenotype, including changes in the islet architecture and the expression of some β-cell identity genes. GLP-1R protein, absent in db/db islets, was partially restored by treatment with the EP3 antagonist. EP3 blockade also increased the expression of the transcription factor nuclear factor E2-related factor 2 (Nrf2) and some of its downstream targets in islets from db/db mice. As Nrf2 has recently emerged as a key mediator in the protection against cellular oxidative damage [17–19], our results provide strong support for PGE₂ signaling modulating β-cell redox state and subsequently β-cell identity and function.

2. MATERIALS AND METHODS

2.1. Animal models

Leprdb/db (db/db) C57BL/6J (Jax #000697) mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and LeprΔb/db (db/db) mice were bred in-house. Mice were housed in a controlled-temperature environment with a 12-hr light cycle and ad libitum access to food (11% kcal from fat; 5% sucrose, Purina, St. Louis, MO) and water except when otherwise noted. Four- or six-week-old male mice were injected daily for two weeks with either 20 mg/kg DG-041 or vehicle-matched vehicle (phosphate buffered saline (PBS) + 10% dimethyl sulfoxide (DMSO)) subcutaneously. Body weights were assessed daily to ensure that the mice did not lose more than 20% body weight. Mice were euthanized at six or eight weeks of age. All mice were maintained on a C57BL/6J background and handled in accordance with the Guide for the Care and Use of Laboratory Animals (NIH). Mice were housed in the Vanderbilt University Medical Center (Nashville, TN) animal care facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

2.2. Mouse pancreas immunolabeling

Pancreata were dissected, weighed, and fixed for four hours in 4% paraformaldehyde and subsequently dehydrated in an ethanol series before paraffin embedding. Sections were rehydrated and subjected to sodium-citrate-induced antigen retrieval. The primary antibodies used were: guinea pig anti-insulin (1:400; Dako #A0564, Carpinteria, CA), mouse anti-glucagon (1:500; EMD Millipore #ABN238, Bellerica, MA), rabbit anti-Ki67 (1:500; Abcam #ab15580, Cambridge, MA), rabbit anti-GLP-1R (1:500; Abcam #ab218532, validated on Glp1−/− tissue in [20]), or mouse anti-GLP-1R (1:30, DSHB mAb #7F38, validated on Glp1−/− tissue in [21]). Apoptosis was detected using an ApoAlert terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit according to the manufacturer’s instructions (Clontech, Mountain View, CA). The primary antibodies were detected with the appropriate species-specific secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA); Cy2-conjugated anti-guinea pig IgG (1:400; #SP 706-225-148), Cy3-conjugated anti-rabbit IgG (1:400; #Rb 711-165-152), and Cy5-conjugated anti-mouse IgG (1:400; #715-175-151). Nuclei were visualized with 40,60-diamidino-2-phénylindole (DAPI, 1 µg/ml for 2 min; Molecular Probes D1306, Eugene, OR). Images were acquired with a ScanScope FL slide scanner (Aperio Technologies, Vista, CA) and quantified using MetaMorph 6.1 (Molecular Devices, Sunnyvale, CA). Positive staining was determined by double-blinded, manual quantification and cross-checked using macros generated with the CytoNuclearFL algorithm in eSlide Manager (Aperio Technologies, Vista, CA). A minimum of 3,000 β cells were counted for β-cell proliferation, death, and ziptβ ratio. Data are represented as Ki67−, TUNEL−, or glucagon-positive cells relative to the entire insulin+ and/or glucagon+ population. The primary antibodies used for Nrfl2 validation and 8-hydroxy-2-deoxyguanosine (8OHdG) immunolabeling included: guinea pig insulin (1:1000; Dako #A0564, Carpinteria, CA), mouse glucagon (1:1000, Abcam #ab10988 with Nrfl2 or 1:700, Abcam #ab63623 with 8OHdG), and either rabbit Nrfl2 (Cayman Chemicals #10214 at 1:250, Ann Arbor, MI) or mouse 8OHdG (Abcam #ab62623 at 1:100). The primary antibodies were detected with the appropriate species-specific secondary antibodies: Alexa488-conjugated anti-mouse IgG (Invitrogen #A11029), Alexa594-conjugated anti-rabbit IgG (Invitrogen #A11037), or Alexa647-conjugated anti-guinea pig IgG (Invitrogen #A21450). Nuclei were stained with DAPI. Cells were imaged with a Zeiss Axioplan 2 microscope or an Olympus DP74 microscope with cellSens Standard software version 2. For the quantification of nuclear Nrfl2 and 8OHdG, 250–800 insulin-positive cells per mouse were counted, and the percentage of the cells was calculated with nuclear labeling for Nrfl2 or 8OHdG.

2.3. β-cell mass quantification

Sections were cut serially at 5 μm and placed on slides pre-treated with Sta-On (Leica Biosystems, Lincolnshire, IL). Six to seven slides per animal (separated by at least 250 μm and sampling the entire pancreas) were immunolabeled for insulin, visualized via the DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA), and counterstained with eosin. Quantification of β-cell mass was calculated as described previously [22]. In brief, one pancreatic section per slide was scanned using a ScanScope CS scanner, and the images were processed identically with the ImageScope software (Aperio Technologies, Vista, CA). β-cell mass was measured by determining the ratio of insulin + area to the total pancreatic area of...
all the scanned sections per animal multiplied by the wet weight of the pancreas.

2.4. Islet isolation
Following euthanasia, the pancreata were perfused with 0.5 mg/mL type IV collagenase dissolved in Hanks Balanced Salt Solution (HBSS) containing Ca²⁺ and Mg²⁺, as described previously [23]. After digestion at 37°C, lysates were washed and resuspended in RPMI 1640 containing 5.6 mM glucose and 10% fetal bovine serum. The islets were handpicked and prepared for RNA isolation or static incubation assays.

2.5. Static incubation assays
Handpicked islets were allowed to recover in RPMI without FBS overnight, as described previously [23]. The islets were washed with Krebs solution containing the following (mM): 2.8 glucose, 102 NaCl, 5 KCl, 1.2 MgCl₂, 2.7 CaCl₂, 20 HEPES, 5 NaHCO₃, and 10 mg/mL BSA (pH 7.4). They were incubated at 37°C for 45 min and roughly 10 islet equivalents were transferred into the wells of a 12-well plate containing 1 mL pre-warmed KRB solution. GSIS was measured by calculating the total percentage of insulin secreted within a 45-minute treatment duration. Technical replicates were conducted for each experiment. Insulin ELISA kits (ALPCO, Salem, NH) were used to measure the insulin secretion, as per the manufacturer’s instructions.

2.6. Intraperitoneal glucose tolerance tests (IP-GTT)
For IP-GTT, the mice were fasted overnight. Glucose (2 mg/kg) was injected intraperitoneally. Blood glucose was measured via the tail vein (2 μL) at 0, 15, 30, 60, 90, and 120 min following injections using an Accu-check glucometer and glucose strips (Roche, Indianapolis, IN). Statistical analyses were performed on the area under the curve (AUC) to the baseline.

2.7. RNA isolation and sequencing
Pancreatic islets from vehicle- or DG-041-treated db/db or db/db mice were isolated and prepared for RNA extraction in 1 ml Trizol reagent (ThermoFisher, Waltham, MA). RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. The RNA concentration and integrity were assessed using a ND-1000 spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent, Santa Clara, CA) at the Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core. Libraries of 150 bp sequences using Cutadapt v2.5 [24] and aligned to the GENCODE vM24 gene annotations were provided to STAR to improve the accuracy of mapping. Quality control on both raw reads and adapter-trimmed reads was performed using FastQC v0.11.8 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). FeatureCounts v1.6.4 [26] was used to count the number of mapped reads to each gene. Significantly differentially expressed genes with an FDR-adjusted p-value < 0.05 and absolute fold change > 2.0 were detected by DESeq2 (v1.24.0) [27]. Heatmap3 [28] was used for cluster analysis and visualization. Gene ontology and KEGG pathway overrepresentation analyses were performed on differentially expressed genes using the WebGestaltR package [29]. One of the vehicle-treated db/db samples was statistically determined to be an outlier and was thus eliminated from further analysis.

3. RESULTS

3.1. EP3 antagonist treatment promotes β-cell mass expansion in pre-diabetic db/db mice
Mice homozygous for a spontaneous mutation in the leptin receptor (db/db) show elevations in blood glucose between four to eight weeks of age. To determine whether EP3 blockade in vivo has beneficial effects on β-cell mass in the setting of T2D, we treated db/db mice with the EP3 antagonist DG-041. As a guideline, we referred to the study by Guo et al. (2013), which identified a critical time window of increased β-cell proliferation and β-cell compensation between four and six weeks of age in db/db mice [30]. After this time point, β-cell proliferation declines and β-cell mass decreases, leading to overt diabetes. We thus utilized two different injection protocols: four to six weeks of age or six to eight weeks of age to determine whether modulation of EP3 receptor activity could enhance or extend β-cell compensation. DG-041 (20 mg/kg) or vehicle was subcutaneously administered daily for two weeks beginning at four or six weeks of age to db/db and db/db male mice; this dose was chosen based on published data demonstrating effective blockade of EP3 with minimal off-target effects [31]. Pancreata were collected at six or eight weeks of age and immunolabeled for insulin to quantify β-cell mass. EP3 blockade resulted in a significant, more than two-fold increase in β-cell mass only in db/db mice treated with DG-041 from six to eight weeks of age (vehicle: 1.11 mg ± 0.16; DG-041: 2.62 mg ± 0.35; p < 0.05; Figure 1). Notably, DG-041 treatment had no significant effect on β-cell mass in db/db mice at either time point. To understand the mechanism underlying β-cell mass expansion in response to EP3 antagonist, we analyzed β-cell proliferation and survival. Although we did not observe an effect on β-cell proliferation in db/db mice treated with DG-041 from six to eight weeks of age, an increase in β-cell proliferation was observed in mice treated from four to six weeks (as indicated by Ki67 labeling) compared with vehicle-treated animals (Figure 2). Systemic EP3 blockade had no significant effect on β-cell replication in db/db mice at either time point. Importantly, in agreement with our ex vivo studies [16], EP3 blockade did not increase z-cell proliferation in any genotype at either time point (data not shown). Despite the beneficial effects of EP3 blockade on β-cell survival in human and mouse islets, we did not observe a significant difference in TUNEL labeling across any treatment group within the respective cohorts after two weeks of DG-041 treatment at either time point (Supplemental Figure 1).

3.2. In vivo EP3 blockade does not alter insulin secretion in isolated islets from db/db mice
A previous study from our group demonstrated that ex vivo treatment of islets with the polyunsaturated fatty acid, eicosapentaenoic acid, enhanced β-cell function concomitant with reductions in Ptg2/EP3 expression [6]. However, our group has also reported that islets isolated from eight-week-old global EP3 KO mice do not display differences in GSIS when compared with their control counterparts [32]. In addition, EP3 KO mice fed a HFD do not show altered GSIS when compared with Chow-fed mice at approximately 30 weeks of age [32].
In the current study, insulin secretion in static incubation assays from islets isolated from db/+ or db/db mice was not significantly altered by in vivo EP3 blockade when examined at either low or high glucose concentrations (Supplemental Figure 2). The current DG-041 treatment protocol had no effect on insulin sensitivity (data not shown) or glucose tolerance in db/+ or db/db mice (Supplemental Figure 3). Since the global inactivation of Ptg3 resulted in increased hepatic lipid content and increased fat mass, we examined this in the DG-041-treated mice. Two weeks of EP3 antagonist treatment did not increase the hepatic lipid content over the db/db genotype alone (data not shown). However,
we did observe a slight, but significant increase in epididymal fat pad mass only in the DG-041-treated db/db mice compared with vehicle-treated mice (Supplemental Figure 4).

3.3. Transcriptomic analysis reveals changes in gene expression in db/db mice at diabetes onset

To begin to understand the mechanisms whereby EP3 blockade enhances β-cell mass dynamics, we performed transcriptomic analysis on whole islets isolated from eight-week-old db/þ or db/db mice treated with or without DG-041 for two weeks. We observed modest variability in gene expression between the biological replicates of each genotype (Figure 3A). Comparisons between the data sets revealed distinct patterns of differentially expressed (DE) genes, in particular between islets from db/þ and db/db mice regardless of the EP3 antagonist treatment, with fewer DE genes between untreated and treated mice within a given genotype. (Supplemental Figure 5A).

We identified 2,986 DE genes between the vehicle-treated db/þ and db/db groups, including 42 key islet genes that were altered in db/db islets compared with db/þ islets (Figure 3B). For example, several transcription factors involved in β-cell identity and maturity (Mafa, Nkx2.2, Nkx6.1, Pdx1) were downregulated. Other genes involved in β-cell function that were downregulated in islets from db/db include Ins1, Gck, Slc2a2, and Ucn3, while glucagon (Gcg) and Cck were upregulated. Several key mediators of the insulin secretion pathway (Kcnj11, Vamp2, Slc30a8, G6pc2) were also downregulated in islets obtained from db/db mice (Figure 3B), mirroring a previous proteomic analysis of db/db islets at 12 weeks of age [33]. The expression of Glp1r was also downregulated in db/db islets compared with db/þ islets (Figure 3B). Other upregulated genes included transcription factors associated with immature endocrine cells (Oc1, Matb) (Figure 3B) and several β-cell disallowed genes, including Idha, Aldob, and Aldh1a3. Ptger3, the gene encoding EP3, was also upregulated in db/db mice compared with db/þ mice (Figure 3B), as was Ptger4, which encodes the EP4 receptor.

3.4. EP3 antagonist treatment restores aspects of gene expression and islet architecture

Treatment with the EP3 antagonist DG-041 resulted in changes in the expression of key islet genes in islets from both db/þ mice (Figure 3C) and db/db mice (Figure 3D). Gene ontology analysis revealed that many DE genes were involved in signaling and communication, extracellular matrix, and protein binding (Supplemental Figure 5B). Only 17 genes were significantly DE in the islets from DG-041-treated db/db mice compared with vehicle-treated db/db mice (Supplemental Figure 5D). Of these 17 DE genes, most were upregulated including oxidoreductases involved in xenobiotic metabolism (Cyp2e1, Cyp3a13), while genes involved in extracellular matrix accumulation were downregulated (e.g. Col10a1). This supports previous studies implicating the role of EP3 in the regulation of extracellular matrix protein accumulation [34].
In islets from db/db mice treated with DG-041, there was also an upregulation of genes correlated with β-cell identity (Mafa, Nkx6.1), β-cell function (Ins1, Gck), and insulin secretion (Figure 3D). Treatment with the EP3 antagonist reversed many of the gene expression changes observed in db/db islets (Figure 3D). Of note, the Mki67 expression was decreased in six-to-eight-week-old db/db mice treated with DG-041, which supports the immunolabeling data showing no increase in Ki67 labeling at this time point (Figure 2C).

Comparison between vehicle- and DG-041-treated db/db mouse islets revealed an overlap with several key cell adhesion genes that were upregulated in db/db mice compared with db/+ mice (Madcam1, Tgfbi), whereas others were downregulated (Hapln4, Tenm4) (Figure 4A), suggesting differences in cell–cell and cell–extracellular matrix interactions in db/db islets. Other DE genes encoding for proteins involved in cell–cell interactions and cell–matrix interactions such as collagens, integrins, and Eph receptors that were upregulated in db/db islets compared with db/+ islets were not affected significantly by treatment with the EP3 antagonist (Figure 4B).

When compared with their db/+ counterparts at eight weeks of age, we found that vehicle-treated db/db mice displayed reduced β/α-cell ratios (Figure 4C). At this age, db/db mice also have a “mixed islet” phenotype, where the normal islet architecture is disrupted, with α cells infiltrating the islet core [35,36] (Figure 4D). This phenotype is likely the result of the migration of the existing α cells located on the mantle into the core of the islet, although islet cell transdifferentiation cannot be ruled out at this time [37,38]. Treatment with the EP3 antagonist significantly improved the β/α-cell ratio in db/db mice (Figure 4C) and even more dramatically restored the islet architecture (Figure 4D).

Glp1r mRNA was dramatically decreased in db/db islets compared with db/+ islets, but restoration of gene expression was variable with EP3 blockade. To determine whether there was more of an effect on GLP-1R protein expression, we examined GLP-1R protein levels using immunolabeling with two different antibodies previously validated on tissue from GLP-1R knockout mice (Figure 5 and Supplemental Figure 6). GLP-1R protein was undetectable in db/db islets from untreated mice. Treatment with the EP3 antagonist partially restored GLP-1R, increasing its expression on the surface of a sub-population of...
β-cells. EP3 blockade had no effect on GLP-1R protein in db/+ mice (Figure 5 and Supplemental Figure 6). In addition, we noticed a consistent increase in insulin protein immunolabeling with EP3 antagonist treatment in db/db mice (Figures 5e7).

3.5. EP3 blockade activates the Nrf2 antioxidant pathway

Nfe2l2, the gene encoding Nrf2, was upregulated in response to DG-041 treatment in db/db mice compared with db/+ mice (1.9-fold, adjusted p-value = 1.83E-07; Figure 6B). Nrf2 is a master transcriptional regulator of cellular antioxidant responses and plays an important role in promoting β-cell survival and proliferation [19,39,40]. Moreover, Nrf2 is activated by the prostaglandin signaling pathway [41–43], as well as the cAMP signaling pathway via GLP-1R [44]. Therefore, to assess whether Nrf2 induction might mediate the beneficial effects of DG-041, we performed immunolabeling of mouse pancreatic sections with insulin, glucagon, and Nrf2 antibodies. EP3 blockade increased the overall Nrf2 protein signal in db/+ and db/db mice, and significantly increased the nuclear localization of Nrf2 in db/db mice (Figure 6A–A'). Analysis of the RNA-Seq data showed a strong upregulation of a set of canonical Nrf2 target genes (Figure 6C), including those with antioxidant functions (Nqo1, Txn1, Gsr, Gclc, Gstm1), those involved in iron metabolism (Ftl1 and Fth1), and those that produce NADPH (Me1, G6pdx, Idh1) [17], while the expression of Keap1, a negative regulator of Nrf2 [20], was decreased. In addition, the levels of 8-hydroxydeoxyguanosine (8OHdG), a marker of DNA damage, were significantly decreased in both db/+ and db/db mice after DG-041 treatment (Figure 7A–B). Together, these data demonstrate that EP3 blockade activates the Nrf2 pathway, providing a possible explanation for the sparing of β-cell mass by DG-041.

4. DISCUSSION

The current study reveals that in vivo pharmacological blockade of the G-coupled PGE2 receptor EP3 improves the β-cell phenotype in a mouse model of T2D. The major findings are as follows: 1) increase in β-cell proliferation and mass in response to EP3 blockade, with no significant effect on β-cell survival, 2) restoration of islet morphology and improved expression of genes that enhance β-cell identity and function, and 3) activation of the Nrf2 antioxidant pathway.

In our previous ex vivo studies with isolated islets, β-cell proliferation in response to EP3 blockade required the presence of an additional proliferative cue (placental lactogen) in mouse islets but not human islets [16]. It is possible that in db/db mice, the chronic inflammatory state and/or β-cell oxidative stress [45,46] contribute to the response
to an EP3 antagonist, leading to increased β-cell proliferation. Inflammation-associated PGE2 production is increased in the setting of T2D [7] and correlates with reduced GSIS [47,48] — an effect that is relieved with EP3 blockade ex vivo [7]. Inhibition of PGE2 production itself has yielded conflicting effects on β-cell function [47], possibly because of the beneficial effects of PGE2 acting through the EP4 receptor, effects that would be lost by decreasing the PGE2 ligand [49]. Thus, EP3 antagonism could alleviate the negative effects of PGE2 on β-cells, thereby allowing PGE2 to signal through EP4 to enhance β-cell function [16,50]. Alternatively, hyperglycemia may act as the additional proliferative cue required for the EP3 antagonist to induce its proliferative effect, since the current studies were performed at a time point when db/db mice already show elevated blood glucose. It is interesting to note that while we observed an increase in proliferation in the 4–6 week age group, we did not see an increase in mass at this time point. This may reflect a delay between the induction of proliferation and a detectable expansion of mass, as reported previously [51]. In addition, the increase in mass despite a lack of proliferation seen in the 6–8 week treatment group may be explained by increased proliferation at an earlier time point in the treatment paradigm that decreases prior to tissue harvesting. Future studies using tissue harvesting at intermediate time points are needed to address these outstanding questions. Modulation of EP3 has been shown to affect β-cell death [16,52–54]. In our study, TUNEL labeling was not significantly altered between db/+ and db/db mice at either time point. However, there was a trend toward increased β-cell death in DG-041-treated db/db mice at six weeks of age and decreased β-cell death at eight weeks of age. This may also modestly contribute to the effects seen on β-cell mass. A previous analysis of islet gene expression in db/db mice performed at 13 weeks of age, during overt diabetes, revealed significant downregulation of many genes involved in the insulin secretion pathway [55]. Proteomic analysis was performed to characterize the alterations in the signaling pathways in islets from 13-week-old db/db mice, with a particular focus on the GSK3-Pdx1 axis [33]. We observed changes in the expression of a similar subset of genes in db/db islets at eight weeks of age compared with that of db/+ islets and also detected an upregulation of glucagon expression. Many of the gene expression changes were reversed following EP3 blockade. We also found that the expression of 123 genes was altered in db/+ islets upon exposure to the EP3 antagonist. To our surprise, DG-041 treatment led to different sets of DE genes in db/+ versus db/db mice, suggesting that the effect of EP3 blockade on gene expression differs in the setting of hyperglycemia present in db/db mice.

In several mouse models of diabetes, including db/db mice, the characteristic core and mantle architecture of the mouse islet is lost [56], which occurs concomitantly with lower β/α-cell ratios [30,35]. We found that treatment with the EP3 antagonist preserves normal islet architecture and partially restores the β/α-cell ratio. Our RNA-Seq analysis revealed that many genes encoding integrins, ephrin/EPH signaling, and collagens were upregulated in the setting of hyperglycemia but were not changed following EP3 inhibition. In contrast, a number of genes involved in cell adhesion were altered in the setting of

**Figure 6: EP3 blockade activates the Nrf2 antioxidant pathway.** (A) Paraffin-embedded sections from pancreata isolated from the indicated genotype and treatment groups were immunolabeled with antibodies directed against insulin (Ins; white), glucagon (Gcg; green), or Nrf2 (red). Nuclei were stained with DAPI (blue). Depicted are representative images of three individual mice in each group. (A) Quantification of nuclear Nrf2 in β-cells. One asterisk indicates *p* < 0.05. (B) Heatmap depicting alterations in the expression of Nrf2(Flk2) and selected Nrf2 target genes between vehicle-treated db/+ and db/db mice. (C) Heatmap depicting alterations in the expression of Nrf2(Flk2) and selected target genes between vehicle-treated and DG-041-treated db/db mice. Mice were treated from 6 to 8 weeks of age. Red indicates high expression; blue indicates low expression. (Scale bar, 20 μm)
EP3 blockade as well as during hyperglycemia. This suggests that EP3 inhibition either reverses the mixed islet phenotype after it has already been established or attenuates the migration of glucagon+ cells into the core of the islet.

The role of EP3 in modulating β-cell function in mouse and human islets has been postulated [6,7,23,48,49,53,57–59]. Eicosapentae-noic acid-enriched islets display enhanced β-cell function concomitant with reductions in EP3 expression [6]. In contrast, islets obtained from EP3 K0 mice do not display differences in GSIS [32]. In the current study, in vivo EP3 inhibition did not lead to increased insulin secretion in static incubation assays conducted using isolated islets, nor did it lead to improved glucose tolerance in db/db mice. Potential explanations for these discrepant findings include: 1) EP3 does not regulate β-cell function in db/db mice; 2) the effects of EP3 receptor modulation differ between strains/backgrounds of mice and the underlying metabolic derangements; 3) an inhibitory effect of the increase in Sst expression is observed in islets from DG-041-treated db/db mice (although the protein levels were not examined) as has been shown by others [60,61]; 4) longer treatment duration is needed to translate into beneficial effects on glucose homeostasis.

Of particular significance was the induction of gene transcription and nuclear localized protein of Nrf2 in response to EP3 blockade in islets from db/db mice. Nrf2 plays a critical role in counteracting oxidative stress. Under homeostatic conditions, Nrf2 is maintained at low basal levels with a short half-life of approximately 10–30 min; however, under oxidative stress, Nrf2 is stabilized and translocates to the nucleus, where it activates the transcription of target genes in cooperation with the members of the small Maf protein family [17]. Transcriptional targets of Nrf2 include many antioxidant and detoxification enzymes that are integral to the glutathione and thioredoxin antioxidant system, NADPH regeneration, reactive oxygen species (ROS) and xenobiotic detoxification, as well as heme and iron homeostasis [17,62]. Previous studies have shown that Nrf2 suppresses inflammation, improves insulin resistance, and protects β-cells from ROS-induced damage [40,63]. Oxidative stress is increased in diabetic patients as well as in pancreatic islets of db/db mice and is associated with impaired regulation of blood glucose [63]. Pancreatic β-cells are unique in that they contain low levels of antioxidant enzymes [64], suggesting that unresolved oxidative stress contributes to the development of diabetes mellitus. Indeed, the transgenic expression of glutathione peroxidase in β-cells of db/db mice reverses diabetes [65]. Transgenic expression of Nrf2 in β-cells leads to increased insulin expression and secretion in iNOS transgenic mice [39], and activation of Nrf2 via Keap1 deletion prevents the onset of diabetes in db/db mice [66]. These results strongly suggest that activation of Nrf2 under increased ROS conditions prevents β-cell dedifferentiation and dysfunction [67]. Thus, the restoration of insulin protein and decrease in 8OHdG observed in response to EP3 blockade could be due, in part, to Nrf2 induction. Overexpression of Nrf2 in human islets resulted in an increase in β-cells under both low- and high-glucose conditions, indicating that Nrf2 overexpression is sufficient for human β-cell proliferation [19]. Overall, current evidence suggests that the activation of Nrf2 protects pancreatic β-cells from varying kinds of damage and promotes β-cell proliferation. Thus, elucidating the functional connections between EP3 and Nrf2 are of great translational significance. The mechanisms whereby EP3 blockade leads to increased Nrf2 warrant further investigation.

The current investigation utilizes a model of systemic EP3 blockade. Since EP3 is expressed in α, β, and δ cells [7,68,69], the effects of an
EP3 antagonist on β-cell mass dynamics could be due to the signaling mechanisms elicited via EP3 receptor blockade on the other endocrine cells of the pancreas. In addition, the effects observed within the islets could be due to EP3 blockade on other tissues/organisms that crosstalk with pancreatic endocrine cells. Therefore, future investigations will include the use of β-cell-specific PtgE3 inactivation.

5. CONCLUSION

The current study provides evidence that EP3 blockade promotes β-cell mass expansion in a mouse model of T2D. The increase in the GLP-1 receptor expression observed in db/db mice treated with the EP3 antagonist supports the concept that blocking EP3 activity would provide added benefit in the setting of GLP-1 pathway agonist therapy, especially given the lack of efficacy of these treatments in some individuals [70,71].

AUTHOR CONTRIBUTIONS

S.R.A., M.E.K., D.K.S., R.M.B., and M.G. conceived the studies. K.J.B., S.R.A., A.A.S., S.B-A., M.A.R., E.M.O., and E.M.W. generated the figures. K.J.B., S.R.A., A.A.S., L.S.K., S.B-A., M.A.R., E.M.O., E.M.W., M.E.K., Q.S., D.K.S., R.M.B., and M.G. analyzed the data. S.R.A. and M.G. wrote the first draft of the manuscript. K.J.B., S.R.A., M.E.K., D.K.S., R.M.B., and M.G. edited and finalized the manuscript.

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