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RESEARCH ARTICLE

Age-dependent human β cell proliferation induced by glucagon-like peptide 1 and calcineurin signaling

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Inadequate pancreatic β cell function underlies type 1 and type 2 diabetes mellitus. Strategies to expand functional cells have focused on discovering and controlling mechanisms that limit the proliferation of human β cells. Here, we developed an engraftment strategy to examine age-associated human islet cell replication competence and reveal mechanisms underlying age-dependent decline of β cell proliferation in human islets. We found that exendin-4 (Ex-4), an agonist of the glucagon-like peptide 1 receptor (GLP-1R), stimulates human β cell proliferation in juvenile but not adult islets. This age-dependent responsiveness does not reflect loss of GLP-1R signaling in adult islets, since Ex-4 treatment stimulated insulin secretion by both juvenile and adult human β cells. We show that the mitogenic effect of Ex-4 requires calcineurin/nuclear factor of activated T cells (NFAT) signaling. In juvenile islets, Ex-4 induced expression of calcineurin/ NFAT signaling components as well as target genes for proliferation-promoting factors, including NFATC1, FOXM1, and CCNA1. By contrast, expression of these factors in adult islet β cells was not affected by Ex-4 exposure. These studies reveal age-dependent signaling mechanisms regulating human β cell proliferation, and identify elements that could be adapted for therapeutic expansion of human β cells.

Introduction

In type 1 or type 2 diabetes mellitus, an absolute or relative deficiency of functional pancreatic β cells underlies disease pathogenesis. Thus, investigations are focused on identifying native signaling pathways governing human pancreatic β cell proliferation, with the goal of expanding functional β cell mass. Islet β cells proliferate in neonatal humans and rodents, but the proliferation rate declines thereafter, and reaches its nadir by puberty (1–5). During postnatal β cell growth, studies in rodents suggest that hallmark β cell functions such as glucose sensing and insulin secretion mature (6–10). The mechanisms controlling age-dependent β cell proliferation and maturation are being intensely investigated, since deciphering these could accelerate development of β cell expansion strategies (3, 4, 8, 10–13).

Recent studies have identified age-dependent intrinsic and extrinsic regulators in mice and humans that influence or limit islet β cell proliferation. For example, with advancing age, increased expression of *CDKN2A* in β cells, which encodes the cell cycle inhibitor p16[INK4a], limits β cell regeneration in mice and humans (3, 4, 11–13). Native extrinsic signals that regulate β cell proliferation include PDGF, prolactin (PRL), and glucagon-like peptide 1 (GLP-1). Recent studies have elucidated crucial signal transduction elements of these mitogens in β cells (4, 14). For example, work on mouse and human islets suggests that the mitogenic function of PDGF in β cells is age-dependent. While islet β cells from neonatal mice and human children express PDGF receptors (PDGFRs) and proliferate in response to PDGF-A, β cells from adult mice and humans lack PDGFR expression and are unresponsive to PDGF stimulation (4). Thus, attenuated receptor expression underlies one mechanism of age-dependent mitogenic restriction in β cells, underscored by the finding that expression of activated PDGFR protein in adult β cells led to β cell proliferation (4). PRL-stimulated β cell proliferation is also lacking in adult human islets and is accompanied by little or no PRL receptor expression in adult β cells (14). However, unlike the effects of PDGF signaling, ectopic expression of PRL receptor in adult β cells does not restore responsiveness to PRL (14), suggesting that restriction of β cell competence for PRL includes both attenuated receptor expression and reduced intracellular signal transduction. Thus, mechanisms limiting human β cell responses to PDGF and PRL appear distinct, although both involve age-dependent loss of cognate receptor expression.

GLP-1 has a well-established role in stimulating β cell insulin secretion (the incretin effect), in addition to inducing insulin biosynthesis, and regulating β cell apoptosis (15–17). GLP-1 and its analogs have been previously reported to induce mouse β cell pro-

liferation in an age-dependent manner (18). Prior studies investigating whether GLP-1 or exendin-4 (Ex-4) stimulates human β cell proliferation have yielded conflicting results (15, 17–22). Thus, it remains unclear whether GLP-1 can stimulate human β cell proliferation. GLP-1 stimulates β cell Ca²⁺ transients (23, 24) through the GLP-1 receptor (GLP-1R), and these are known to activate the calcium-dependent calcineurin/nuclear factor of activated T cells (NFAT) signaling pathway, a crucial regulator of β cell proliferation and function in neonatal and adult islets (25–28). However, the links between GLP-1R responses and downstream intrinsic regulators of human β cell proliferation like calcineurin/NFAT signaling have not yet been established.

To test the hypothesis that human β cell proliferative response to the GLP-1 analog Ex-4 is age-dependent, we used an in vivo transplantation strategy with human islets from juveniles and adults (3, 4, 10, 26). Here we report that Ex-4 stimulates β cell proliferation in transplanted juvenile, but not adult, human islets, and that this response requires intact calcineurin/NFAT signaling. Thus, these studies reveal age-dependent signaling pathways and mechanisms that stimulate human β cell proliferation.

Results

Age-dependent human islet cell proliferation profile after transplantation. To investigate the age-dependent proliferative potential of human islet cells in vivo, we transplanted juvenile (aged 0.5–9 years) or adult (20 years of age and older) human islets under the renal capsule of NOD.Cg-*Prkdc^scid^Il2rg^tm1Wjl^*/Sz (NSG) mice, an immunocompromised strain favorable for xenograft studies (ref. 29 and Figure 1A). Ki67 immunostaining of juvenile islet grafts 4 weeks after transplantation revealed a greater number of proliferating cells in juvenile insulin-positive β cells, glucagon-positive α cells, and somatostatin-positive δ cells than engrafted adult islets (Figure 1, B, C, F, G, J, and K). Compared with engrafted adult islets, juvenile islets had 8-fold more Ki67⁺ β cells (Figure 1, B–E, and Figure 1E, inset). We assessed phospho–histone H3 (pHH3), an independent marker of proliferation, and found that the number of pHH3⁺ β cells was greater in juvenile islets, confirming progression into G₂ or M phases (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI91761DS1). Thus, the greater proliferation of juvenile human β cells continued after transplantation, indicating that age-dependent β cell proliferation is maintained and independent of the native pancreatic environment.

We also noted a higher percentage of Ki67⁺ α cells (Figure 1, F–I, and Figure 1I, inset) and Ki67⁺ δ cells (Figure 1, J–M, and Figure 1M, inset) in transplanted juvenile islets. To our knowledge, age-dependent proliferation of these islet cell subsets in humans has not been previously reported. In β and α cells, greater Ki67 labeling was noted in donors up to 3 years of age (Figure 1, D and H), while in δ cells, we observed a lower proliferation rate by that age (Figure 1L). Within the limits of our donor sampling size, these data suggest that the tempo of declining proliferation with age is faster in δ cells than in β or α cells.

Ex-4 signaling promotes proliferation in juvenile β cells. GLP-1 and GLP-1R agonists, like Ex-4, stimulate insulin secretion (the incretin effect, reviewed in refs. 15, 17, 30) and are used in the treatment of type 2 diabetes. However, prior studies (15, 17–19, 21,

22) are in conflict about the ability of GLP-1R agonists to stimulate human islet cell proliferation, possibly due to different in vitro treatments and assays used. Maintenance of age-dependent islet cell proliferation in our transplant system permitted in vivo studies of candidate mitogens such as GLP-1/Ex-4 in a physiological context. We hypothesized that human islet β cells have an age-dependent capacity to respond to Ex-4, and used our transplant system to investigate this possibility.

First, we examined whether expression of GLP-1R in human β cells was different with age. *GLP1R* mRNA levels were similar in juvenile and adult human islets (Figure 2A), suggesting that human β cell GLP-1R expression is not age-dependent. Moreover, in FACS-isolated islet cell subsets, *GLP1R* mRNA was principally expressed in β cells and was very low, or not detectable, in α cells (Supplemental Figure 2, A–C), consistent with prior findings (10, 31–35).

To investigate human islet responses to Ex-4, we infused Ex-4 in mice transplanted with human islets from juvenile or adult donors (Figure 2B); this infusion established pharmacologically relevant serum Ex-4 levels (Supplemental Figure 2D and ref. 36). As expected, blood glucose levels were reduced by Ex-4 within 24 hours and remained lower during the subsequent treatment period (Figure 2C and Supplemental Figure 2E). Circulating human insulin levels in NSG mice bearing human islets (Figure 2, D and E) were greater in mice infused with Ex-4. Moreover, this incretin effect was observed in mice with juvenile or adult islets. Thus, Ex-4 stimulation of insulin secretion in vivo by human islets is age-independent, suggesting that downstream signaling that regulates GLP-1–stimulated insulin secretion is similar in human juvenile and adult islets.

To investigate proliferative responses to Ex-4, we assessed β cell proliferation in transplanted juvenile and adult islets. Ex-4 stimulated β cell replication, marked by Ki67, in juvenile donor samples up to age 9 (Figure 2, F, G, and I), but not in islets from older donors (20 years and above; Figure 2, H and I). Unlike what was observed in β cells, we did not detect changes of α cell or δ cell proliferation in response to Ex-4 in juvenile or adult islets (Supplemental Figure 3, A–C). The lack of Ex-4 mitogenic effect on α cells is likely explained by our finding that human α cells did not express *GLP1R* mRNA (Supplemental Figure 2, A–C). Thus, these studies revealed age-dependent mitogenic effects of Ex-4 on β cells, while the insulin secretion effect was age-independent. This suggests that divergent downstream pathways regulate these two distinct effects of GLP-1R signaling in human β cells.

Ex-4 stimulates calcineurin/NFAT signaling in juvenile human islets. To understand the mechanisms underlying Ex-4 mitogenic effects in juvenile human islet grafts, we compared the transcriptomes of Ex-4–treated and control PBS-treated juvenile human islet grafts and found that 1,440 genes were differentially expressed (Figure 3A). Gene sets associated with signaling pathways governing cell proliferation and vesicle secretion were among those mostly upregulated in Ex-4–infused islet grafts. Gene sets comprising the NFAT pathway were at the top of this list (Figure 3B).

A prior study reported that signaling through the calcineurin/NFAT pathway is critical for juvenile β cell proliferation (26). For example, this pathway stimulates expression of multiple β cell transcription factors and cell cycle activators, including NFATC1, FOXM1, cyclin-dependent kinases (CDKs), and the A-type cyclins
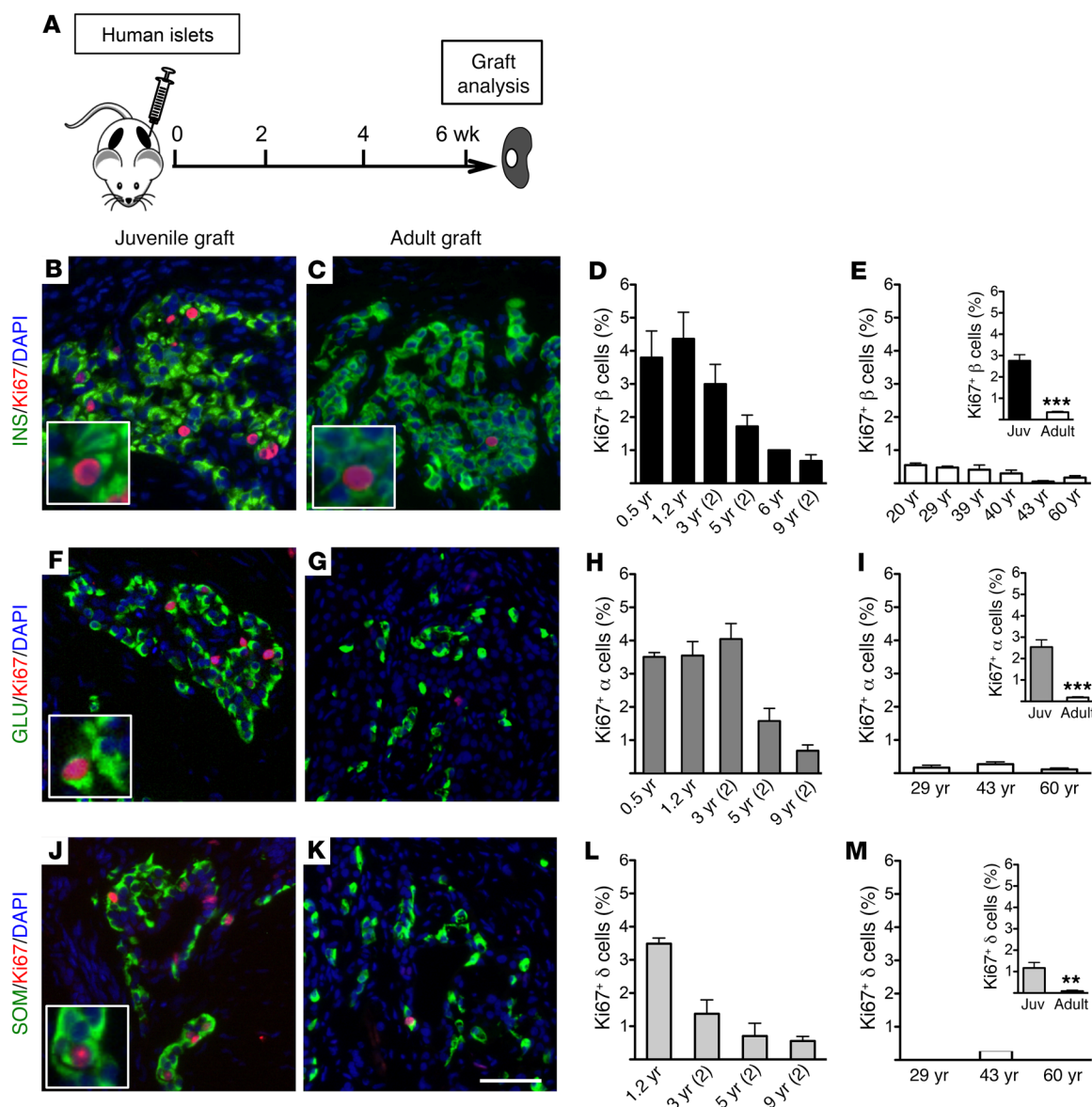
Figure 1. Endocrine cell proliferation is greater in transplanted human juvenile islets than in adult islets. (**A**) Schematic of experimental design. Grafts were removed for analysis 6 weeks after transplantation. (**B**, **C**, **F**, **G**, **J**, and **K**) Images of juvenile (left panels) and adult (right panels) grafts labeled with insulin (INS, **B** and **C**), glucagon (GLU, **F** and **G**), or somatostatin (SOM, **J** and **K**) in green; Ki67 in red; DAPI in blue. Insets show proliferating Ki67$^+$ cells. Scale bar: 50 μm (bar in **K** applies to all other images in this figure). (**D**, **E**, **H**, **I**, **L**, and **M**) Quantification of percentage Ki67$^+$ β (**D** and **E**), α (**H** and **I**), and δ (**L** and **M**) cells of transplanted grafts from individual juvenile (Juv) and adult donors (n = 2–5 grafts per donor; age shown on x axis). The average number of β, α, and δ cells counted in each donor sample was approximately 6,000, 3,000, and 2,000, respectively. Insets are average percentage proliferating cells in each age group (β cells: data from **D** and **E**; α cells: data from **H** and **I**; δ cells: data from **L** and **M**). Error bars represent SEM. **$P < 0.01$; ***$P < 0.001$. An unpaired 2-tailed Student's t test was used for statistical analysis. See also Supplemental Figure 1.

(26, 27, 37, 38). To unravel signaling links between Ex-4/GLP-1R and calcineurin/NFAT pathways in human islets, we first measured human islet mRNAs encoding all NFAT members and found that *NFATC2* and *NFATC3* were most abundant (Supplemental Figure 4, A and B). In human islets, levels of mRNAs encoding NFATC1, NFATC2, and NFATC4, but not NFATC3, declined with advancing age (Figure 3C), similar to a prior observation that levels of NFATC transcription factors are lower in older mouse islets (26). We also found that important cell cycle regulators (*CCNA1*, *CCNA2*, *CDK1*, and *CDK4*) and key transcription factors such as *FOXM1* are expressed more highly in juvenile human islets (Sup-

plemental Figure 4, C–F). By contrast, mRNA levels of *CDKN2A* and *CDKN2B*, which respectively encode the β cell cycle inhibitors p16^{INK4a} and p15^{INK4b}, were greater in adult human islets (Supplemental Figure 4C and refs. 4, 10, 11, 13).

After Ex-4 treatment, we observed an increase in mRNAs encoding NFATC1, FOXM1, CCNA1, and CDK1 in juvenile human islet grafts (Figure 3, E–G) but not in adult islet grafts (Supplemental Figure 5A), suggesting that calcineurin/NFAT signaling was stimulated by Ex-4 only in juvenile human islets. We also found increased mRNAs encoding other possible targets of calcineurin/NFAT signaling (Figure 3, E–G) thought to promote cell proliferation, includ-
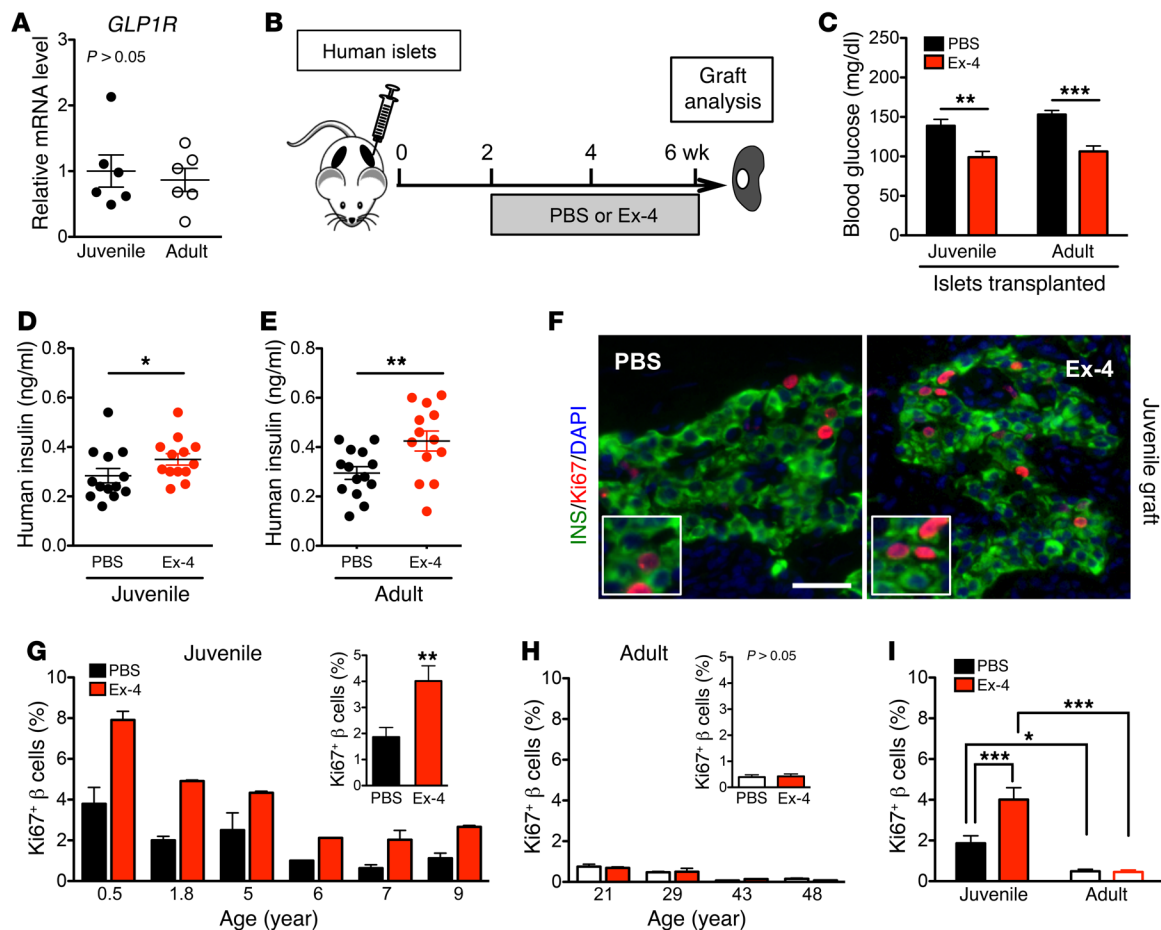
Figure 2. Ex-4 promotes β cell proliferation only in juvenile islets. (**A**) Both juvenile and adult islets express a similar level of *GLP1R* mRNA measured by qPCR (juvenile: *n* = 6, 0.5–9 years old; adult: *n* = 6, 20–60 years old). (**B**) Experimental design. After a 2-week islet engraftment period, PBS or Ex-4 was delivered by osmotic pump. The grafts were removed and analyses were performed after 4 weeks of treatment. (**C**–**E**) Mouse random glucose (**C**) and human insulin (**D** and **E**) in mice with transplanted juvenile (**D**, *n* = 3 donors) or adult (**E**, *n* = 3 donors) human islets 48 hours after the implantation of pumps with PBS or Ex-4 (*n* = 13–14 samples). (**F**) Representative images of juvenile grafts labeled with insulin (green), Ki67 (red), and DAPI (blue). Insets show proliferating Ki67+ cells. Scale bar: 35 μm. (**G** and **H**) Percentage of β cell proliferation in grafts from individual juvenile and adult donors (*n* = 4–8 grafts per donor). Insets are average percentage in each age group. (**I**) Statistical analysis of data sets in **G** and **H**. Error bars represent SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Unpaired 2-tailed Student's *t* test or 1-way ANOVA followed by Newman-Keuls multiple-comparisons test (**I**) was used for statistical analysis. See also Supplemental Figures 2 and 3.

ing NFATC3, NFATC4, CDK1 (39–41), and EGR3 (42). However, mRNA levels of *NFATC2*, *CDKN2A*, *CDKN2B*, or *EZH2*, a regulator of *CDKN2A* expression and proliferation in β cells (3, 4), were not detectably altered in juvenile islets after Ex-4 infusion. Together these data suggest that Ex-4 stimulates calcineurin/NFAT signaling and thus enhances expression of key transcriptional and cell cycle regulators that promote human juvenile β cell proliferation. These studies also reveal that the expression of other calcineurin/NFAT factors (e.g., NFATC2) and intrinsic inhibitors of β cell proliferation (e.g., CDKN2A) was not changed by Ex-4 exposure.

Ex-4 mitogenic effect on human β cells prevented by the calcineurin inhibitor FK506. To test whether the mitogenic effects of Ex-4 on juvenile human islets are mediated by calcineurin/NFAT signaling activation, we infused Ex-4 in mice engrafted with juvenile human islets and simultaneously infused FK506, a potent specific calcineurin inhibitor (Figure 3D). In mice coinfused with Ex-4 and saline, we observed increased nuclear localization of β cell NFATC4, and increased proliferation of transplanted human

islet β cells quantified by Ki67 labeling (Figure 4, A–C). In contrast, simultaneous infusion of FK506 with Ex-4 prevented increases of β cell NFATC4 nuclear localization and proliferation (Figure 4, A–C). In control mice receiving PBS plus saline or PBS plus FK506, we did not detect differences in human islet β cell NFATC4 nuclear localization or proliferation (Figure 4, A–C), indicating that basal proliferation in transplanted juvenile human β cells was insensitive to FK506. Furthermore, little apoptosis was observed in human islet grafts after either treatment (Figure 4D and Table 1). Collectively, these data suggest that calcineurin/NFAT signaling is required for Ex-4–stimulated human juvenile islet β cell proliferation. Supporting this view, we observed that increased expression of calcineurin/NFAT signaling targets induced by Ex-4 was reduced to basal levels in human islet grafts recovered from mice simultaneously infused with Ex-4 and FK506, including *NFATC1*, *NFATC3*, *NFATC4*, *FOXM1*, *CCNA1*, and *CDK1* (Figure 3, E–G). Together, these data demonstrate that calcineurin signaling in human juvenile islets is required for β cell proliferation.
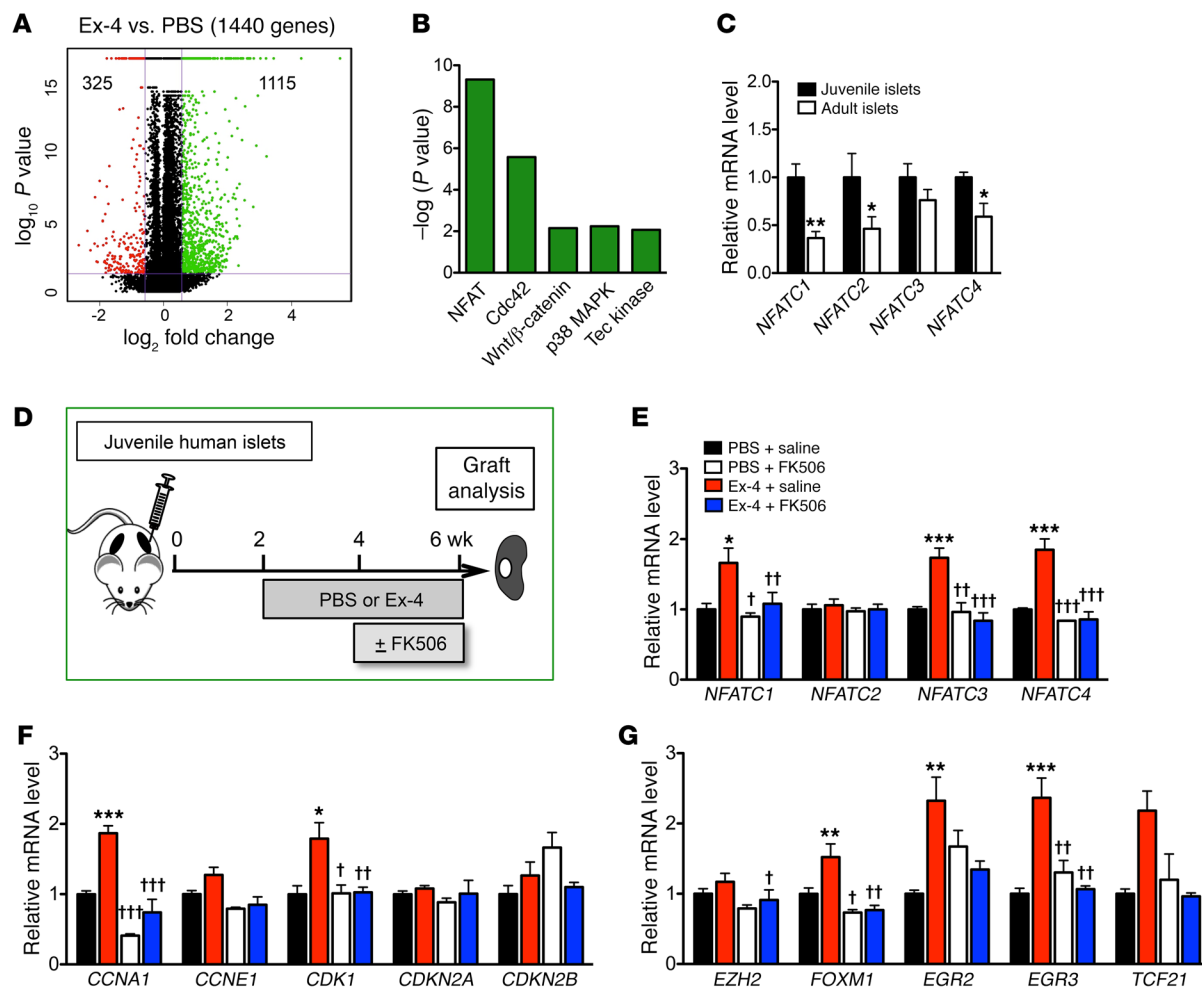
Figure 3. Gene expression in human islets and juvenile grafts after treatment with Ex-4 and/or FK506. (**A** and **B**) RNA-Seq analysis of PBS- or Ex-4–treated juvenile grafts ($n = 3$ grafts per treatment, 6-year-old donor). (**A**) Number of genes regulated by Ex-4 (≥1.5-fold) in juvenile grafts (Ex-4 vs. PBS); 1,115 genes are upregulated and 325 are downregulated by Ex-4 treatment. (**B**) Signaling pathways upregulated by Ex-4. (**C**) Genes of the NFATC family are expressed more highly in juvenile islets ($n = 5$ donors, age: 0.5, 1.2, 1.7, 3, and 4 years) than adult ($n = 5$ donors, age: 29, 43, 50, 53, and 60 years). The average expression level in juvenile islets is defined as 1.00 for each gene. (**D**) Schematic of transplantation and treatment with Ex-4 plus FK506. Two weeks after transplantation, a pump with PBS or Ex-4 was implanted, and a second pump with saline or FK506 was implanted in the last 2 weeks. Grafts were removed at 4 weeks. (**E–G**) Juvenile graft gene expression measured by qPCR. Ex-4 upregulates genes of the NFAT family (**E**), cell cycle regulators (**F**), and transcription factors (**G**), and the effects were diminished by FK506 ($n = 5$ graft samples per treatment from 0.2- and 6-year-old donors). The average expression level in PBS+saline–treated islets is defined as 1.00 for each gene. Error bars represent SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. PBS+saline; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ vs. Ex-4+saline; no significant differences between PBS+FK506 and Ex-4+FK506. Unpaired 2-tailed Student's t test or 1-way ANOVA followed by Newman-Keuls multiple-comparisons test (**E–G**) was used for analysis of statistical significance. See also Supplemental Figures 4 and 5.

Discussion

Understanding the basis of declining proliferation observed with advancing age in human pancreatic islet β cells (1–3, 5) would reveal potential strategies for therapeutic human β cell expansion in diabetes. Studies with rodent islets have suggested that GLP-1 stimulates cell proliferation, but most studies on human islets have relied on in vitro assays and reported variable effects of GLP-1 on proliferation (21, 22, 43). To test the hypothesis that Ex-4, a GLP-1 analog, stimulates human islet cell proliferation in an age-dependent fashion, we developed an in vivo engraftment system that maintained the proliferative competence of juvenile human islet cells and an infrastructure to systematically compare human juvenile and adult islets. We found that Ex-4 stimulates human β cell

proliferation in human juvenile, but not adult, islets. The unresponsiveness to Ex-4 in adult human β cells did not reflect absence of GLP-1R expression or signal transduction, since Ex-4 stimulated insulin secretion in both juvenile and adult human β cells. We show that this β cell proliferative response to Ex-4 requires calcineurin/NFAT signaling and also suggest that β cell mitogenic responses to Ex-4 may be limited in adult islets by calcineurin/NFAT–independent factors, including potent inhibitors of β cell proliferation like the cyclin inhibitor CDKN2A (p16[INK4A]). Thus, this work reveals an age-dependent pathway for β cell proliferation in human juvenile islets (Figure 5).

We used transplantation of human juvenile and adult islets into immunodeficient NSG mice (44, 45) to investigate mecha-
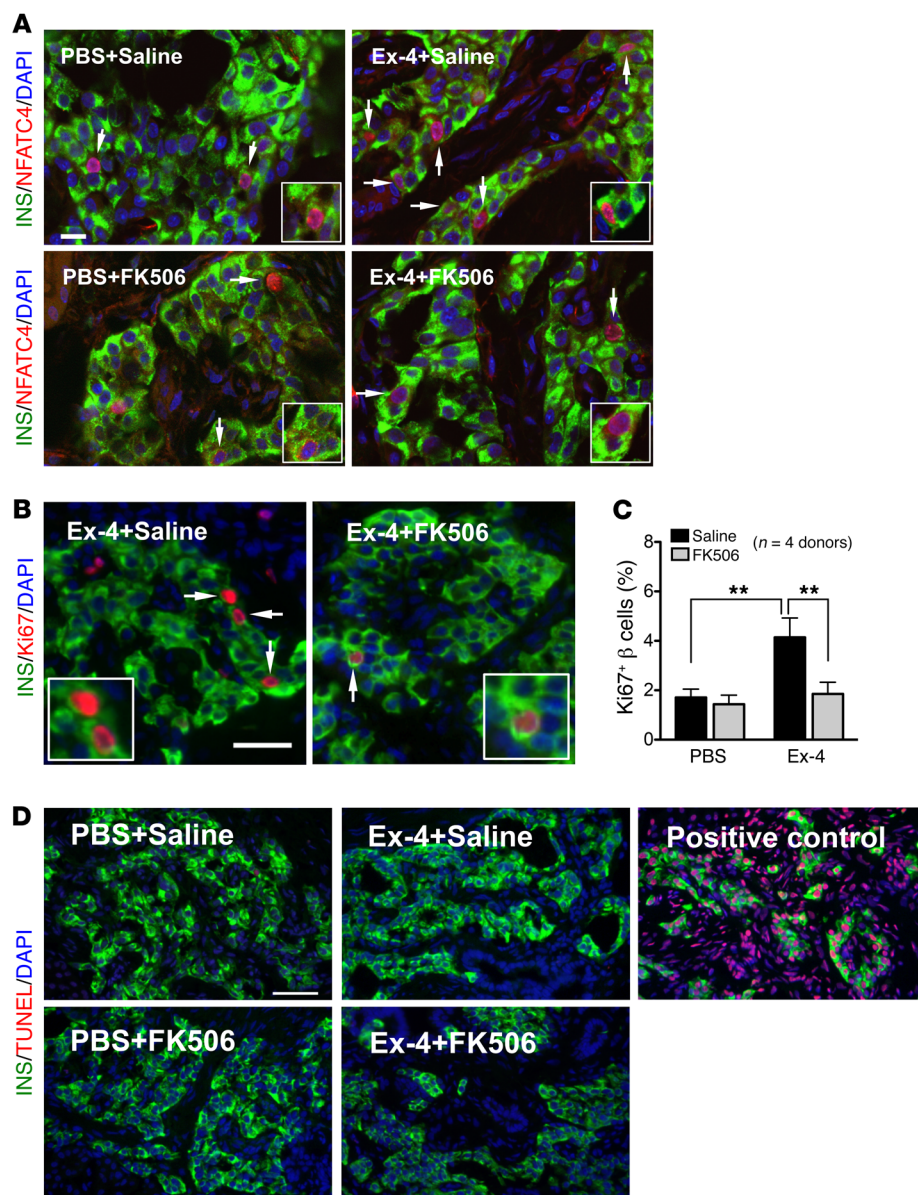
Figure 4. Calcineurin/NFAT signaling mediates the mitogenic effect of Ex-4 in juvenile β cells. (**A**) Ex-4 stimulated NFAT translocation. Insulin (green), NFATC4 (NFAT3) (red), DAPI (blue). Scale bar: 15 μm (applies to the other images in **A**). Arrows point to NFATC4^{+} β cells, and insets show NFATC4^{+} β cells. (**B**) Representative images of β cell proliferation in juvenile grafts. Insulin (green), Ki67 (red), DAPI (blue). Arrows point to proliferating Ki67^{+} cells (also showed in insets). Scale bar: 35 μm. (**C**) FK506 blocked the β cell proliferation stimulated by Ex-4 in juvenile grafts (*n* = 4 donors, 0.2, 1.8, 6, and 9 years old). See also Supplemental Figure 6. (**D**) Representative images of TUNEL assay including positive control. Insulin (green), TUNEL (red), DAPI (blue). Scale bar: 50 μm (applies to all images in **D**). See Table 1 for the quantification of TUNEL^{+} β cells in PBS+saline–treated, Ex-4+saline–treated, PBS+FK506–treated, and Ex-4+FK506–treated graft samples from 3 juvenile donors. Error bars represent SEM. ***P < 0.01. Unpaired 2-tailed Student's *t* test or 1-way ANOVA followed by Newman-Keuls multiple-comparisons test (**C**) was used for statistical analysis. See also Supplemental Figure 6.

nisms controlling human islet cell proliferation. Compared with retrospective studies of cadaveric pancreata or in vitro islet culture–based assays, this in vivo transplantation strategy afforded experimental flexibility, including relatively prolonged observation times, prospective exposure of islets to sequential or concurrent schedules of drugs, and serial measures of insulin secretion in response to Ex-4. In our transplantation model, the maintenance of greater levels of β cell proliferation after transplantation indicates that the proliferation resulted from intrinsic human islet cell signals, and was not dependent on the pancreatic environment or circulating human factors.

Recent studies suggest that insulin secretion by human islets may mature in an age-dependent manner (10), similar to findings in rodents (6–9). Using our transplant-based system, however, we found that Ex-4 stimulated insulin secretion similarly in both juvenile and adult islet grafts, consistent with similar levels of GLP-1R expression in these islets. By contrast, Ex-4 stimulated β cell proliferation only in islets from juvenile donors. This suggests that

age-dependent factors in adult β cells may suppress proliferation or that adult β cells lack factors mediating the proliferative effect of Ex-4/GLP-1R signaling, without affecting the incretin effects of this signaling pathway (reviewed in ref. 46). These findings are also supported by studies here (Figure 2A) and elsewhere (10, 33) revealing that *GLP1R* expression did not detectably change with age in human islet β cells. An additional finding from our work is that human α cell and δ cell proliferation also appears to decline with age, corroborating a recent mass cytometry–based study (47). However, unlike in juvenile human β cells, Ex-4 did not stimulate proliferation of these other islet cell subsets. This is consistent with findings here and from prior studies that *GLP1R* expression in human islet cells is restricted to β cells (Supplemental Figure 2, A–C, and refs. 10, 31–35). The circulating Ex-4 level in our system (~4 ng/ml) was much higher than the basal plasma GLP-1 level in mice (48, 49) or healthy humans (50) but close to the therapeutic concentration target for GLP-1R agonists in clinical use (36). The duration of Ex-4 infusion in our study was shorter (28 days)

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Table 1. Quantification of TUNEL⁺ β cells

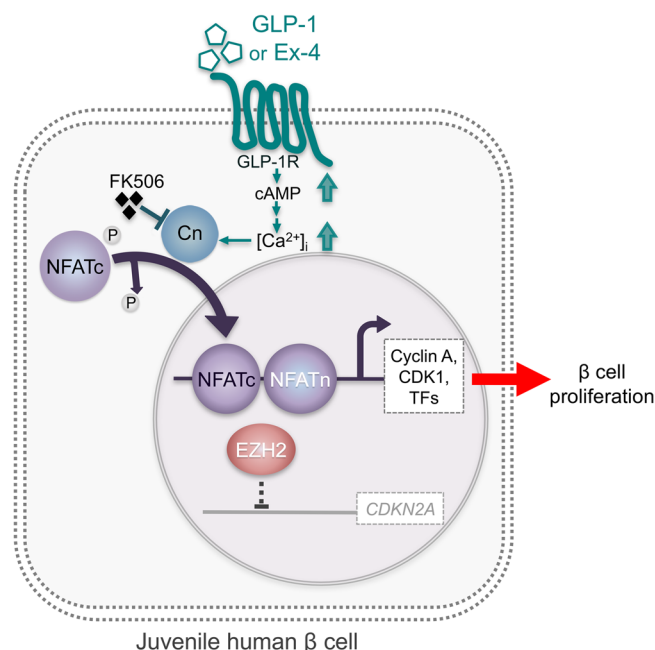| Donor age	1.8 yr		6 yr		9 yr	
	Total β cells	TUNEL⁺ β cells	Total β cells	TUNEL⁺ β cells	Total β cells	TUNEL⁺ β cells
PBS + saline	2,400	1	4,789	1	1,056	0
Ex-4 + saline	2,458	0	4,610	0	1,489	0
PBS + FK506	401	0	2,264	0	679	0
Ex-4 + FK506	986	0	4,980	0	819	0

in comparison with a recent study (16) in which continuous exposure (>250 days) of transplanted islets to the GLP-1R agonist liraglutide was found to impair β cell function. Our findings raise the possibility that relatively brief exposure to pharmacological levels of Ex-4 may be sufficient to stimulate human β cell proliferation, and should motivate further studies of Ex-4 dosing and schedule effects on β cell growth and function.

Another principal finding here is that Ex-4 effects on human β cell proliferation and gene expression require calcineurin/NFAT signaling. Calcineurin/NFAT signaling is a calcium-responsive pathway required for postnatal β cell expansion in mice and humans (26, 27, 51). Prior observations that Ex-4/GLP-1R signaling may provoke Ca²⁺ transients in β cells (19, 23, 24, 52) suggested a connection between GLP-1R and calcineurin/NFAT signaling pathways. Our findings here provide direct evidence for stimulation of calcineurin/NFAT signaling by Ex-4 in juvenile human islets. FK506 is a potent calcineurin inhibitor used in multiple clinical settings, and a role for calcineurin/NFAT in human β cell function has also been inferred from the striking observation that 10%–30% of patients requiring immunosuppression with FK506 develop diabetes mellitus (28). Here we showed that infusion of FK506 eliminated the increased juvenile β cell proliferation and gene expression alteration evoked by Ex-4. Together, these findings suggest that the calcineurin/NFAT signaling pathway is crucial for mediating the mitogenic effect of GLP-1R in human juvenile β cells. In addition to NFAT, calcineurin signaling regulates other factors, including CTRC2, RCAN1, and IRS2 (53–55). Our study did not detect an effect of Ex-4 or FK506 on the transcript levels of these factors (Supplemental Figure 5). However, we cannot exclude roles of these or additional factors in the Ex-4 effects we observed in human β cells.

Age-dependent decline in human β cell responsiveness to mitogens was found previously to reflect loss of mitogen receptor expression in β cells. For example, age-dependent reduction of receptors for PDGF and PRL in human β cells has been noted (4, 14). Similarly to PDGFR, GLP-1R signal transduction can stimulate Ca²⁺ transients to activate NFATC transcription factors (47). Thus, we speculate that PDGFR and GLP-1R may signal in parallel to regulate β cell proliferation, a possibility requiring further studies. In contrast to age-dependent loss of PDGFR expression and signaling competence observed previously (4), our current work indicates that loss of mitogenic responses by adult human β cells to Ex-4 is not the result of reduced GLP-1R expression (Figure 2A) or failure to activate GLP-1R signaling in cells (Figures 2, C–E). Instead, other age-dependent mechanisms constraining β cell proliferation appear to limit adult β cell responses to Ex-4. For example, expression of intrinsic β cell growth regulators like *CDKN2A*, *CDKN2B*, *NFATC2*, and *EZH2* (3, 4, 12, 56) was not detectably affected by Ex-4 exposure, suggesting a cell-autonomous basis for age-dependent mitogenic responses by human islets to Ex-4. Thus, findings here provide motivation for future investigations aiming to rejuvenate human adult β cell mitogenic responses to GLP-1 agonists.

In summary, use of a system for investigating age-dependent phenotypes in human pancreatic islets revealed that human β cell mitogenic responses to GLP-1 are age-dependent and require intact calcineurin/NFAT signal transduction. Identification of direct and indirect targets of calcineurin/NFAT activation in human islet cells could suggest steps for achieving therapeutic aims like functional β cell expansion.

Figure 5. Model of GLP-1 mitogenic effect mediated by calcineurin/NFAT signaling in juvenile human cells. GLP-1 (or Ex-4) binds and activates GLP-1R, which increases intracellular calcium concentration (56), Ca²⁺-sensitive phosphatase, and calcineurin (Cn). Calcineurin dephosphorylates NFATc factors to expose their nuclear localization sequences, which triggers rapid entry into the nucleus. In the nucleus, NFATc proteins assemble on DNA with partner proteins (termed NFATn) to activate transcription of target genes, including cell cycle activators (e.g., cyclins, CDKs) and proliferation-promoting transcription factors (TFs) such as *FOXM1*, which are all produced at a low level in adult islets. GLP-1/calcineurin/NFAT signaling does not appear to regulate the expression of age-associated cell cycle inhibitors such as *CDKN2A* (normally very low level in juvenile islets or its repressor *EZH2* in juvenile human β cells, which may explain the absence of proliferative response to GLP-1 or Ex-4 in adult islets.

Methods

Human islet procurement, quality assessment, and transplantation. Deidentified human pancreatic islets were obtained from previously healthy, nondiabetic organ donors. Islets from 14 juvenile donors (ages 0.2, 0.5, 1.2, 1.7, 1.8, 3, 3, 4, 5, 5, 6, 7, 9, and 9 years) were procured through the National Disease Research Interchange (http://ndriresource.org) and the International Institute for the Advancement of Medicine (http://www.iiam.org). Juvenile islets were isolated at the Institute of Cellular Therapeutics of the Allegheny Health Network (Pittsburgh, Pennsylvania, USA) as previously described (3, 4, 26). Islets from 12 adult donors (ages 20, 21, 29, 30, 40, 43, 43, 48, 49, 53, 60, and 60 years, BMI ranges 24.1–29.6) were obtained from the Integrated Islet Distribution Program (http://iidp.coh.org). Islet function (i.e., glucose-induced insulin secretion) was assessed by islet perifusion assay on the day of arrival, as previously described (57, 58). Each human islet preparation was handpicked on the day of arrival for gene expression studies (57). Male NOD.Cg-*Prkdc^scid^Il2rg^tm1Wjl^*/Sz (NSG) mice (44, 45), aged 12–18 weeks, were used for transplantation. Human islets were transplanted under the kidney capsule as previously described (29, 59, 60).

Ex-4 and FK506 administration. Two weeks after human islet transplantation, Ex-4 (24 nmol/kg/d; California Peptide Research Inc.) or 1× PBS was delivered by micro-osmotic pumps (Alzet 1004) implanted in recipient NSG mice (Figure 2B). For FK506 (tacrolimus) experiments, a second pump (Alzet 1002) loaded with FK506 (0.25 mg/kg/d; Astellas Ireland Co.) or saline was implanted 2 weeks after the Ex-4 pump was placed (Figure 4A). Serum Ex-4 levels in recipient mice were measured using the Exendin-4 EIA kit (Phoenix Pharmaceuticals Inc.).

Immunohistochemistry of human islet grafts. Recovered human islet grafts were fixed in 4% paraformaldehyde in PBS and cryopreserved. Five-micrometer-thick frozen sections were cut and stained as previously described (60). Primary antibodies used were rabbit anti–human Ki67 (Abcam, ab15580), guinea pig anti–human insulin (DAKO, A0564), mouse anti-glucagon (Abcam, ab10988), goat anti-somatostatin (Santa Cruz Biotechnology, sc7819), and rabbit anti–histone H3 (phospho S10) (Abcam, ab5176). Images were obtained using an Olympus BX41 fluorescence microscope. Proliferating islet cells were quantified by counting of Ki67+ and hormone-positive cells as described previously (57, 60, 61). Apoptosis was assessed by TUNEL (Millipore, S7165) following the manufacturer's instructions.

RNA isolation, cDNA synthesis, and quantitative reverse transcriptase PCR. Total RNA was extracted from isolated human islets or recovered human islet grafts using an RNAqueous RNA isolation kit (Ambion). RNA quality control and quantity assessment (QC/QA) was performed using a Bioanalyzer instrument in the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core laboratory. Only samples with a 28S/18S ratio greater than 1.2 and an RNA integrity number greater than 8.1 were used for subsequent analysis. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using TaqMan assays (Supplemental Table 1) and reagents from Applied Biosystems as previously described (57). *ACTB* and *TFRC* were used as endogenous controls in islet gene measurement, while *SYPL1*, *SV2A*, and *CHDA* were used for control genes in graft gene assay. Relative changes in mRNA expression were calculated by the comparative ΔCt method using the Applied Biosystems StepOne Plus System.

RNA-Seq library preparation, sequencing, and data normalization. The PolyA-enriched RNA sequencing library was prepared as previously described (62). Paired-end sequencing (100 million 100-bp paired-end reads) was performed on an Illumina HiSeq2500 sequencer (Illumina Inc.). The quality checks on raw sequence data for each sample were performed using FastQC (Babraham Bioinformatics). Raw reads were mapped to the reference *hg19* using TopHat version 2.0. The alignment metrics of the mapped reads were estimated using SAMtools. Aligned reads were imported to the commercial data analysis platform AvadisNGS (Strand Scientifics). Samples were grouped and transcript abundance was quantified for this final read list using trimmed means of M-values (TMM) as the normalization method (63). RNA sequencing data have been deposited in the NCBI's Gene Expression Omnibus (GEO) with ID code GSE100660.

Statistics. Statistical significance of differences was determined by 2-tailed Student's *t* test or 1-way ANOVA followed by Newman-Keuls multiple-comparisons tests. A *P* value less than 0.05 was considered statistically significant. Values represent mean ± SEM. In RNA-Seq analysis, differential expression of genes was calculated on the basis of fold changes (using the default cutoff ≥ ±1.5) observed in comparisons between defined conditions, and the *P* value of the differentially expressed gene list was estimated by ANOVA using Benjamini-Hochberg corrections of 0.05 for FDR. Differentially expressed genes were used for the functional pathway analysis using Ingenuity Pathway Analysis (IPA; Qiagen Inc.).

Study approval. All human islet studies were approved by the Vanderbilt Institutional Review Board. All animal studies were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Author contributions

CD, YH, SKK, and ACP designed the experiments. YH, CD, SKK, and ACP wrote the original manuscript draft, and YH, CD, SEL, DLG, LDS, RB, SKK, and ACP reviewed and edited the manuscript. CD, AS, GP, NH, N Prasad, N Phillips, and RB performed the experiments and analyzed the data.

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