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Article

Dopamine D2 Receptor-Mediated Regulation of Pancreatic β Cell Mass

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SUMMARY

Understanding the molecular mechanisms that regulate β cell mass and proliferation is important for the treatment of diabetes. Here, we identified domperidone (DPD), a dopamine D2 receptor (DRD2) antagonist that enhances β cell mass. Over time, islet β cell loss occurs in dissociation cultures, and this was inhibited by DPD. DPD increased proliferation and decreased apoptosis of β cells through increasing intracellular cAMP. DPD prevented β cell dedifferentiation, which together highly contributed to the increased β cell mass. DRD2 knockdown phenocopied the effects of domperidone and increased the number of β cells. *Drd2* overexpression sensitized the dopamine responsiveness of β cells and increased apoptosis. Further analysis revealed that the adenosine agonist 5′-N-ethylcarboxamidoadenosine, a previously identified promoter of β cell proliferation, acted with DPD to increase the number of β cells. In humans, dopamine also modulates β cell mass through DRD2 and exerts an inhibitory effect on adenosine signaling.

INTRODUCTION

Diabetes is a progressive disorder characterized by elevated blood glucose levels, which causes organ complications. Both type 1 and late-stage type 2 diabetes lead to depletion of pancreatic islet β cells. Islet transplantation is a promising therapy to improve glycemic control. However, organ shortages and the loss of islet cells shortly after transplantation are major obstacles. Advances have been made in the generation of mature pancreatic β cells from pluripotent stem cells to overcome these problems. However, a thorough understanding of β cell mass regulation is required.

β Cells have been reported to have the potential to proliferate after experimental ablation, such as chemical treatment or partial pancreatectomy (Bonner-Weir et al., 2010) (Cano et al., 2008; Nir et al., 2007; Xu et al., 2008). Although controversial, β cells have also been reported to be generated from other cell sources, such as pancreatic ducts (Finegood et al., 1999; Sharma et al., 1999; Xu et al., 2008) and endogenous progenitor or *trans*-differentiated α, δ, or acinar cells, under specific conditions that lead to a nearly complete loss of β cells (Baeyens et al., 2005; Collombat et al., 2009; De Breuck et al., 2003, 2006; Xu et al., 2008). During regeneration, simple self-replication of β cells has been shown to be the primary source of new β cells (Dor et al., 2004; Kataoka et al., 2013). However, the mechanisms that regulate β cell proliferation are not well understood. Glucose has been identified as a signal that controls β cell proliferation through glucokinase acti-

vation and subsequent downregulation of cyclin D2 expression (Porat et al., 2011; Salpeter et al., 2011). In addition, several molecules, such as adenosine kinase inhibitors and glucagon-like peptide-1 (GLP-1), have been shown to promote β cell proliferation (Andersson et al., 2012; Bernal-Mizrachi et al., 2014). Moreover, dedifferentiation has also been shown to be a mechanism that triggers β cell loss (Talchai et al., 2012). Many studies have been aimed at identifying molecules that enhance β cell proliferation, which could restore proper β cell function and ultimately cure type 1 and type 2 diabetes. Recently, in a screening of a pharmacologically bioactive small-molecular compound library, we identified molecules that enhance the differentiation of mouse embryonic stem cells (ESCs) into pancreatic β cells. We extended our study and established a primary islet culture system for screening small chemical compounds that enhance β cell proliferation. Here, we identified domperidone (DPD), a dopamine D2 receptor (DRD2) antagonist, as a potent enhancer of β cell proliferation and investigated the underlying molecular mechanism.

RESULTS

A Screening System for Chemicals that Promote β Cell Proliferation

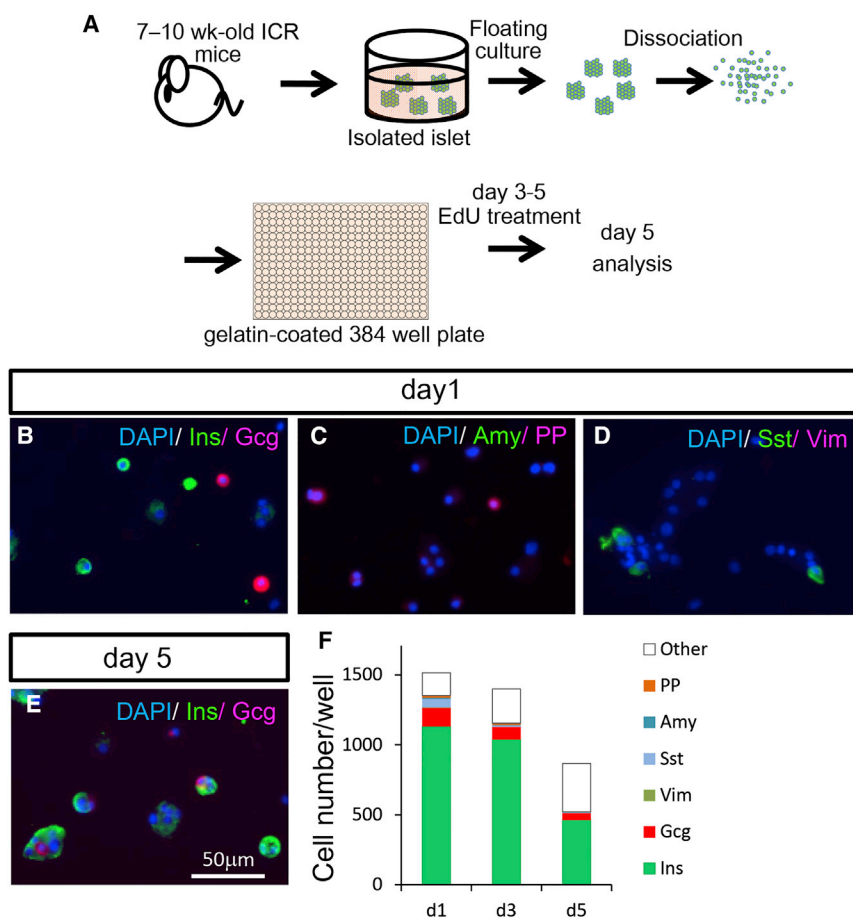To screen for chemicals that promote β cell proliferation, we established an islet dissociation culture system, in

Figure 1. A Screening System for Chemicals that Promote β Cell Proliferation

A high-throughput screening system was established to identify chemicals that promote β cell proliferation.

(A) Schematic drawing of the screening system. Dissociated islet cells were plated in a gelatin-coated 384-well plate at 2,000 cells/plate and cultured for 5 days, treated with EdU or test chemicals on days 3–5, and assayed on day 5.

(B–E) Immunocytochemistry analysis of the islet culture on day 1 (B, C, D) or day 5 (E).

(F) Total number of cells per well, showing the number of endocrine cells (PP$^+$, Sst$^+$, Gcg$^+$, Ins$^+$), exocrine cells (Amy$^+$), and mesenchymal cells (Vim$^+$) in the culture on day 1 (d1), d3, and d5 (mean ± SD, three independent experiments, each with four replicates).

which islets were dissociated into single cells and plated onto gelatin-coated 384-well plates.

In our islet dissociation culture, endocrine cell types were obtained at a proportion that reflected normal islet composition, i.e., approximately 80% insulin-positive (Ins$^+$) cells, 20% glucagon (Gcg)$^+$ cells, 5% somatostatin (Sst)$^+$ cells, and 1% pancreatic polypeptide (PP)$^+$ cells (Figures 1B–1F, day 1). Other cell types, such as vimentin (Vim)$^+$ mesenchyme cells, amylase (Amy)$^+$ exocrine cells, and others, were also observed (Figure 1F). In the culture, the cells appeared to gradually become apoptotic, and the number of islet cells decreased with time.

To determine the assay conditions, we first tested the period of 5-ethynyl-2′-deoxyuridine (EdU) exposure of the dissociated islets. EdU incorporation in Ins$^+$ cells increased for up to 72 hr of exposure to EdU (Figure S1A). EdU incorporation in Ins$^+$ cells was observed at a substantial level on days 1–3 or days 3–5, and significantly decreased on days 5–7 after islet dissociation (Figure S1B). Therefore in our initial screen, the 48-hr EdU exposure was adopted and chemicals were added to the cultures beginning on days 3–5, and the numbers of total cells

and Ins$^+$ cells were counted and EdU positivity in Ins$^+$ cells determined on day 5.

Domperidone, a DRD2 Antagonist, Promotes β Cell Proliferation

We tested a library of 1,120 biologically active compounds arrayed as single compounds in DMSO. Candidate compounds that increased the numbers of total cells and Ins$^+$ cells were selected as primary hits. Of our hit compounds, a DRD2 antagonist, DPD, showed the strongest effect.

DPD is known to be a specific antagonist of DRD2, which is a G-protein-coupled receptor (GPCR). DRD2 is known to mediate Gα$_i$ signaling, which inhibits adenylate cyclase and decreases cyclic AMP (cAMP) upon dopamine binding. Addition of DPD increased the number of EdU$^+$ proliferative Ins$^+$ β cells (Figures 2A–2B′) in the culture on day 5. DPD specifically increased the proportion of EdU$^+$ cells among the Ins$^+$ cells (Figure 2C), resulting in a specific increase in Ins$^+$ cells but not other cell types (Figure 2D). Time-dependent application of DPD revealed that early application of DPD from day 1 or day 3 increased β cell
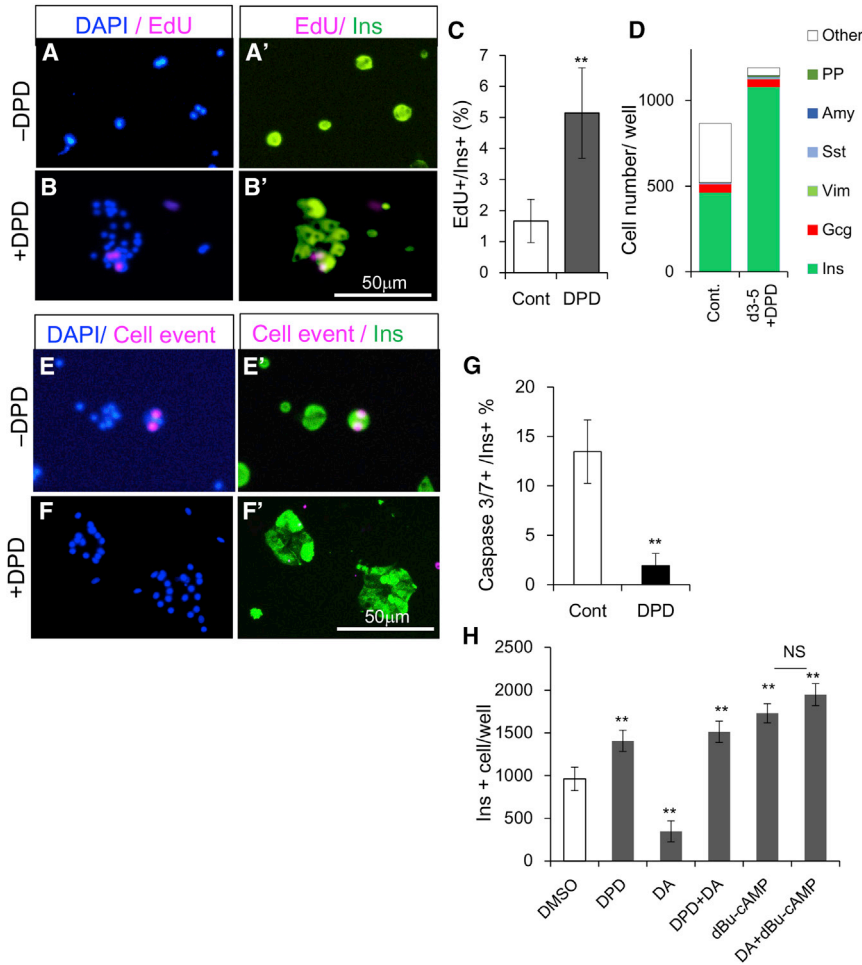
Figure 2. Domperidone Enhances β Cell Proliferation by Antagonizing the Dopamine Apoptotic Signal in Islets

Treatment with dopamine increased EdU incorporation and decreased cell event positivity in β cells, analyzed by immunocytochemistry.

(A–B′) Representative images are shown. EdU incorporation increased in insulin-positive (Ins+) cells treated with domperidone (DPD).

(C) The proportion of EdU-positive proliferating cells within the Ins+ cell population increased following DPD treatment (12 independent experiments, each with three to four replicates).

(D) Total cell numbers per well, showing the number of endocrine cells (PP+, Sst+, Gcg+, Ins+), exocrine cells (Amy+), and mesenchymal cells (Vim+) in the culture on day 5 without (Cont) or with DPD. The data shown in Cont are identical to those in Figure 1F d5 (three independent experiments, each with four replicates).

(E–F′) The Ins+ cells in the islet cells become cell event-positive apoptotic cells on day 5. The appearance of apoptosis was inhibited by DPD treatment on days 3–5.

(G) Percentage of apoptotic cells among the Ins+ cells on day 5 (six independent experiments, each with three to four replicates).

(H) The number of Ins+ cells per well after 4 days of chemical treatment (days 3–7) (four independent experiments, each with three to four replicates). Data represent mean ± SD. **p < 0.01 compared with control (open bars); NS, not significant. DA, dopamine. Scale bar, 50 μm.

number effectively, but late application (on day 5) produced minimal effects (Figure S2).

Over time, the islet cells in our culture assays formed clusters, stopped proliferating, and became caspase-3/7-positive (cell event-positive) apoptotic cells, which led to a decrease in cell number. We then assessed whether DPD could prevent β cell apoptosis. As expected, in the presence of DPD, apoptosis was significantly reduced (Figures 2E–2G). Taken together, our results showed that DPD increased cell proliferation and decreased apoptosis of Ins+ cells by antagonizing the action of dopamine through DRD2.

To determine whether dopamine acts as an upstream signaling molecule that modulates β cell proliferation and cell death, we tested the effects of dopamine and cAMP on proliferation and apoptosis. The results showed that dopamine decreased the number of Ins+ cells, which was reversed by the addition of DPD. In contrast, the addition of a cell-permeable cAMP analog, dibutyryl cAMP (dBu-cAMP) increased the number of Ins+ cells; however, this was not affected by the addition of dopamine, indicating that cAMP is likely downstream of dopamine. These results suggested that dopamine functions to negatively regulate β cell proliferation and promote cell death by decreasing cAMP levels through DRD2 (Figure 2H).

DPD Treatment Protects β Cells from Dedifferentiation and Increases β Cell Mass

In our islet dissociation culture, we observed an increase in the proportion of "other" cells that were not stained with the antibodies tested. DPD treatment resulted in an increase in Ins+ cells and simultaneous decrease in "other" cells (Figure 2D). The results thus urged us to hypothesize that β cell dedifferentiation occurs, so that "other" cells
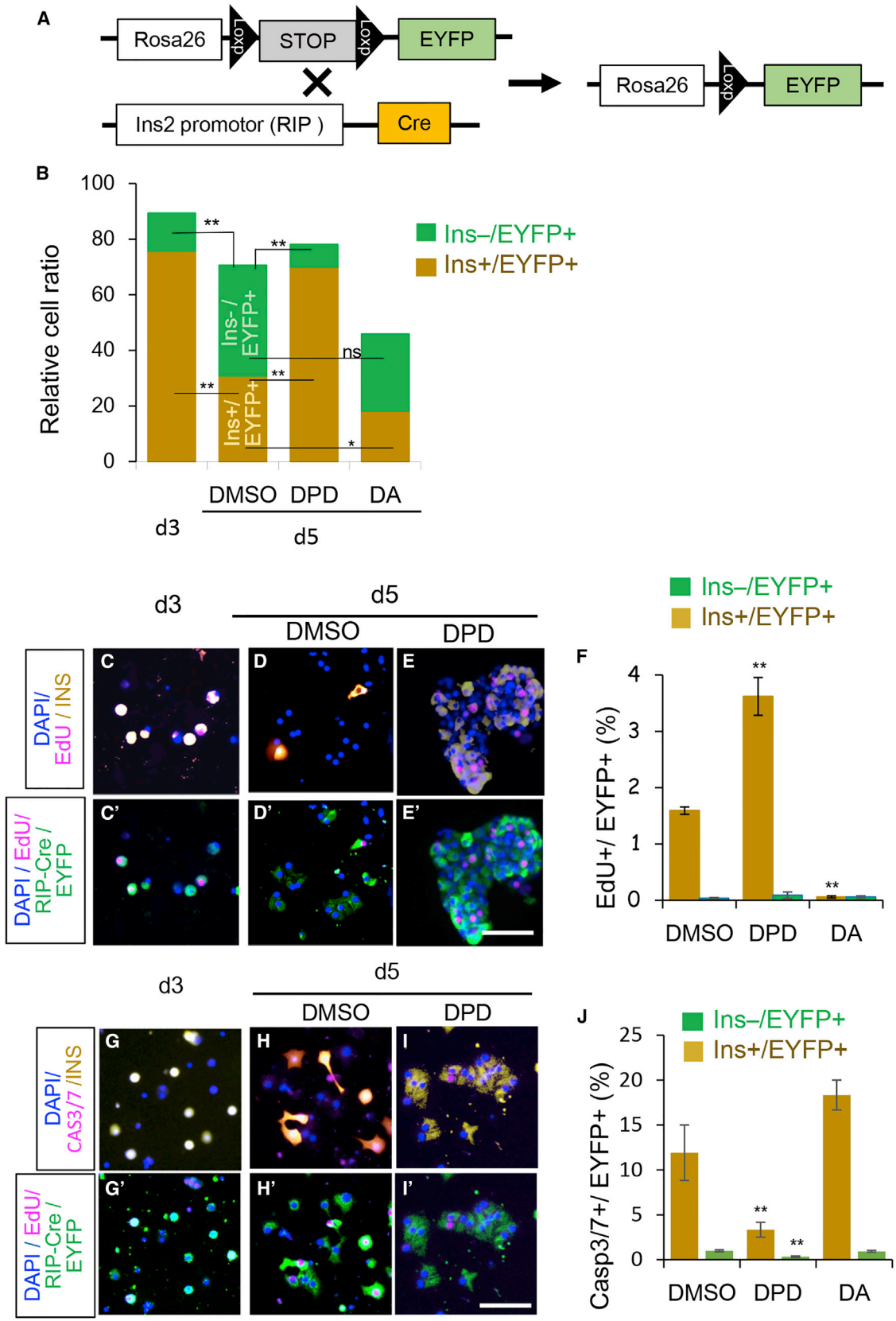
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that represent dedifferentiated β cells increase, and that DPD might protect β cells from dedifferentiation. To test this possibility, we used islets harvested from mice bearing both *Ins2-Cre* (*RIP*-Cre) and *Rosa26-loxP-Stop*-loxP-EYFP transgenes, in which β cells that once expressed insulin would be labeled permanently with EYFP (Figure 3A). If β cell dedifferentiation occurs during dissociation culture, the EYFP-labeled Ins$^+$ β cells would turn into Ins-negative (Ins$^-$) cells. As a result, we found that dedifferentiation of β cells actually occurred. On day 3 after dissociation, many EYFP$^+$/Ins$^+$ cells were observed. By contrast, on day 5, the ratio of EYFP$^+$/Ins$^+$ cells decreased and EYFP$^+$/Ins$^-$ cells increased. DPD addition rescued the reduction of EYFP$^+$/Ins$^+$ cells and prevented the increase of Ins$^-$/EYFP dedifferentiated cells, whereas dopamine addition accelerated the reduction of EYFP$^+$/Ins$^+$ cells (Figure 3B).

We further assayed for EdU incorporation and apoptosis. Our results revealed that proliferation was observed in EYFP$^+$/Ins$^+$ β cells, but not in the dedifferentiated EYFP$^+$/Ins$^-$ cells (Figures 3C–3E′). EdU incorporation revealed that DPD promoted proliferation of the differentiated β cells (Ins$^+$/EYFP$^+$ cells), but not the dedifferentiated β cells (Ins$^-$/EYFP$^+$ cells). Dopamine decreased proliferation of the Ins$^+$/EYFP$^+$ β cells (Figure 3F). Caspase-3/7 staining revealed that apoptosis was detected mainly in the Ins$^+$/EYFP$^+$ β cells but not in the dedifferentiated Ins$^-$/EYFP$^+$ cells. DPD protected and dopamine increased apoptosis of the Ins$^+$/EYFP$^+$ β cells (Figures 3G–3I′, 3J). The dedifferentiated β cells showed low adherence properties and tended to detach from plates; therefore, their cell numbers might be underestimated (Figure 2D, "other" cells and Figure 3, green bars).

Taken together, our results revealed that β cell dedifferentiation is the major mechanism for β cell loss that occurs during islet dissociation culture, which is mediated through dopamine signaling. Inhibition of dopamine signal increased β cell number mainly through the maintenance of the differentiated state of β cells, although its effects on decreasing apoptosis and increasing proliferation of β cells also account for the increased β cell mass.

Loss-of-Function and Gain-of-Function Studies of the Role of Dopamine-DRD2 Signaling in Regulating Cell Number and Apoptosis in MIN6 Cells

To examine the function of dopamine signaling in detail we used MIN6 cells, a β cell line. The number of MIN6 cells increased in cultures treated with DPD or dBu-cAMP. By contrast, dopamine treatment decreased the number of MIN6 cells (Figure 4A). DPD treatment increased proliferation assayed by EdU incorporation (Figure 4B). These results in MIN6 cells phenocopied those observed in our primary islet culture. We then assayed intracellular cAMP levels after various chemical treatments. DPD increased cAMP levels whereas dopamine decreased cAMP levels. When DPD was added together with dopamine, the dopamine-mediated decrease in cAMP was reversed (Figure 4C). Taken together, our results strongly suggest that MIN6 proliferation is mediated by cAMP signaling and that dopamine activates DRD2, decreases intracellular cAMP levels, and reduces the number of MIN6 cells.

We then established a *Drd2*-knockdown (D2KD) MIN6 cell line by introducing a *D2shRNA-mRFP* construct, in which D2shRNA expression could be monitored as mRFP expression (Figure 4D). The D2KD MIN6 cell lines showed that *Drd2* expression was approximately 40% that of the wild-type MIN6 cells (Figure 4E). Cell number was significantly increased in D2KD MIN6 cells, to a level similar to that of the DPD-treated control vector-introduced non-silencing (NS) cells (Figure 4F). DPD treatment did not further increase the number of D2KD MIN6 cells. However, due to the partial knockdown of *Drd2* in D2KD MIN6 cells, dopamine treatment still inhibited cell proliferation, but to a lesser degree than that in the vector-transfected control MIN6 cells (Figure 4F, NS). The addition of dBu-cAMP to the D2KD MIN6 cells did not further increase cell numbers, suggesting that in D2KD MIN6 cells, cAMP mediates the increase in cell number. Taken together, the results show that in MIN6 cells, treatment with DPD increased cell numbers by antagonizing dopamine signaling through DRD2, and that dopamine negatively regulates cell proliferation by

Figure 3. DPD Protects Pancreatic β Cells from Dedifferentiation and Thus Enhances Proliferation and Decreases Apoptosis during In Vitro Dissociation Culture

The effects of DPD and dopamine (DA) in dedifferentiating β cells.

(A) R26-EYFP homozygote (+/+) and *RIP-cre* homozygote (+/+) mouse were crossed and *RIP-Cre*(+/−)/*R26-EYFP*(+/−) mice were generated.

(B) The dissociated islet cells were cultured, and applied with different chemicals on days 3–5. Dedifferentiated β (Ins$^-$/EYFP$^+$) cells were observed in control (DMSO)-, DPD-, or dopamine-containing culture conditions, assayed on day 5. DPD treatment reduced Ins$^-$/EYFP$^+$ dedifferentiated cells.

(C–F) DPD treatment (days 3–5) (C–E′) increased EdU (labeled on days 3–5) incorporation in Ins$^+$/EYFP$^+$ cells (golden bars), but not in dedifferentiated cells (Ins$^-$/EYFP$^+$, green bars).

(G–J) DPD treatment (days 3–5) (G–I′) reduced caspase-3/7$^+$ cells in Ins$^+$/EYFP$^+$ cells (golden bars).

Data represent mean ± SD. All data are obtained from three to four independent experiments, each with three to four replicates. **p < 0.01, *p < 0.05, Student's t test. Scale bar represents 50 μm.
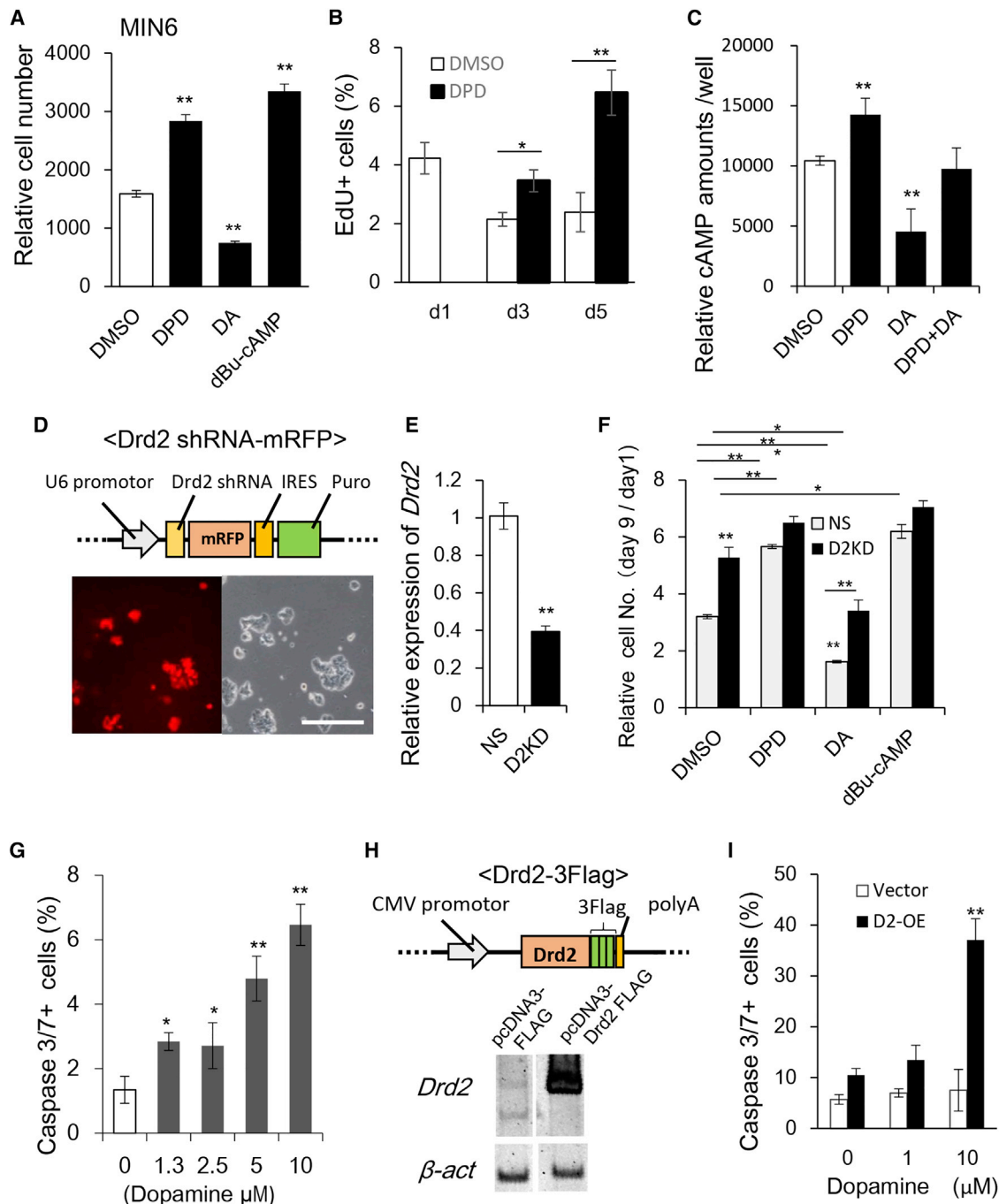
Figure 4. Loss-of-Function and Gain-of-Function Studies of the Dopamine D2 Receptor DRD2 in MIN6 Cells

The effects of DPD and dopamine (DA) in control (A, B, C, G) and *Drd2*-knockdown (D, E, F) or *Drd2*-overexpressing (H, I) MIN6 cells.

(A) The number of MIN6 cells per well after 4 days of chemical treatment (days 3–7). Dopamine treatment decreased the number of cells, whereas DPD or cAMP treatment increased the number of MIN6 cells (five independent experiments, each with three to six replicates).

(B) DPD increased proliferation of MIN6 cells revealed by EdU incorporation (48 hr) assay (four independent experiments, each with four replicates).

(C) Relative cAMP levels after 6 hr of chemical treatment on day 3. DPD increased intracellular cAMP levels, whereas dopamine decreased intracellular cAMP levels, and DPD reversed the dopamine-mediated decrease.

(D–F) *Drd2* knockdown increased cAMP levels and phenocopied DPD effects. (D) Upper panel: a schematic of the lentiviral vector used for *Drd2* shRNA-*mRFP* expression. Lower panel: fluorescence and transmission images of the *Drd2*-knockdown MIN6 cells (D2KD). (E) D2KD

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decreasing cAMP levels through DRD2. In D2KD MIN6 cells, this negative regulation is shut down, mimicking the effects of DPD.

We then examined dopamine-dependent apoptosis in MIN6 cells. Dopamine dose-dependently induced apoptosis, and approximately 6.5% of MIN6 cells underwent apoptosis in the presence of 10 μM dopamine (Figure 4G). The expression of *Bcl2*, an anti-apoptotic gene, was downregulated upon dopamine treatment and upregulated upon DPD treatment. In contrast, the expression of *Bax*, a pro-apoptotic gene, was downregulated upon DPD treatment (Figure S3). These results suggest that dopamine treatment increased apoptosis, whereas DPD treatment inhibited apoptosis through regulating *Bcl2* and *Bax* expressions.

We next examined the effects of overexpressing *Drd2* in MIN6 cells. MIN6 cells transfected with *D2-3Flag* expressed much higher levels of *Drd2* than the control vector-transfected cells (Figure 4H). The effects of dopamine treatment were compared between the *Drd2*-overexpressing and control MIN6 cells. *Drd2*-overexpressing MIN6 cells showed a dopamine dose-dependent increase in apoptosis because of their increased sensitivity to dopamine. Up to 37% of cells became apoptotic following treatment with 10 μM dopamine. Compared with control cells, *Drd2*-overexpressing cells showed an approximately 5-fold increase in apoptosis (Figure 4I). These results suggest that dopamine is an apoptotic signal and that overexpression of *Drd2* increased sensitivity to the signal.

Dopamine Modulates β Cell Proliferation by Acting as an Inhibitory Signal for Adenosine

The adenosine signaling pathway has been reported to be a potent signal for β cell regeneration (Andersson et al., 2012). The adenosine agonist 5′-N-ethylcarboxamidoadenosine (NECA), which acts through the adenosine receptor A2a (ADORA2A), was reported to increase β cell proliferation. ADORA2A is a GPCR that is known to mediate $G\alpha_s$ signaling to activate adenylyl cyclase and increase intracellular cAMP. ADORA2A and DRD2 have been reported to be highly co-localized and to form heterodimers (Canals et al., 2003).

To gain insight into the relationship between adenosine signaling and dopamine function in terms of β cell prolif-eration, we examined the possible interaction between ADORA2A and DRD2. Duolink in situ proximity ligation assays revealed that DRD2 and ADORA2A are expressed and form a heterodimer in dissociated mouse pancreatic β cells (Figures 5A–A″). The interaction of DRD2 and ADORA2A was further confirmed by co-immunoprecipitation with antibodies against ADORA2A and DRD2 (Figure 5B). DRD2-ADORA2A heterodimer formation was enhanced by dopamine but suppressed by DPD or NECA addition (Figures 5C–5G). The results suggest an inhibitory effect of heterodimer formation against adenosine signaling.
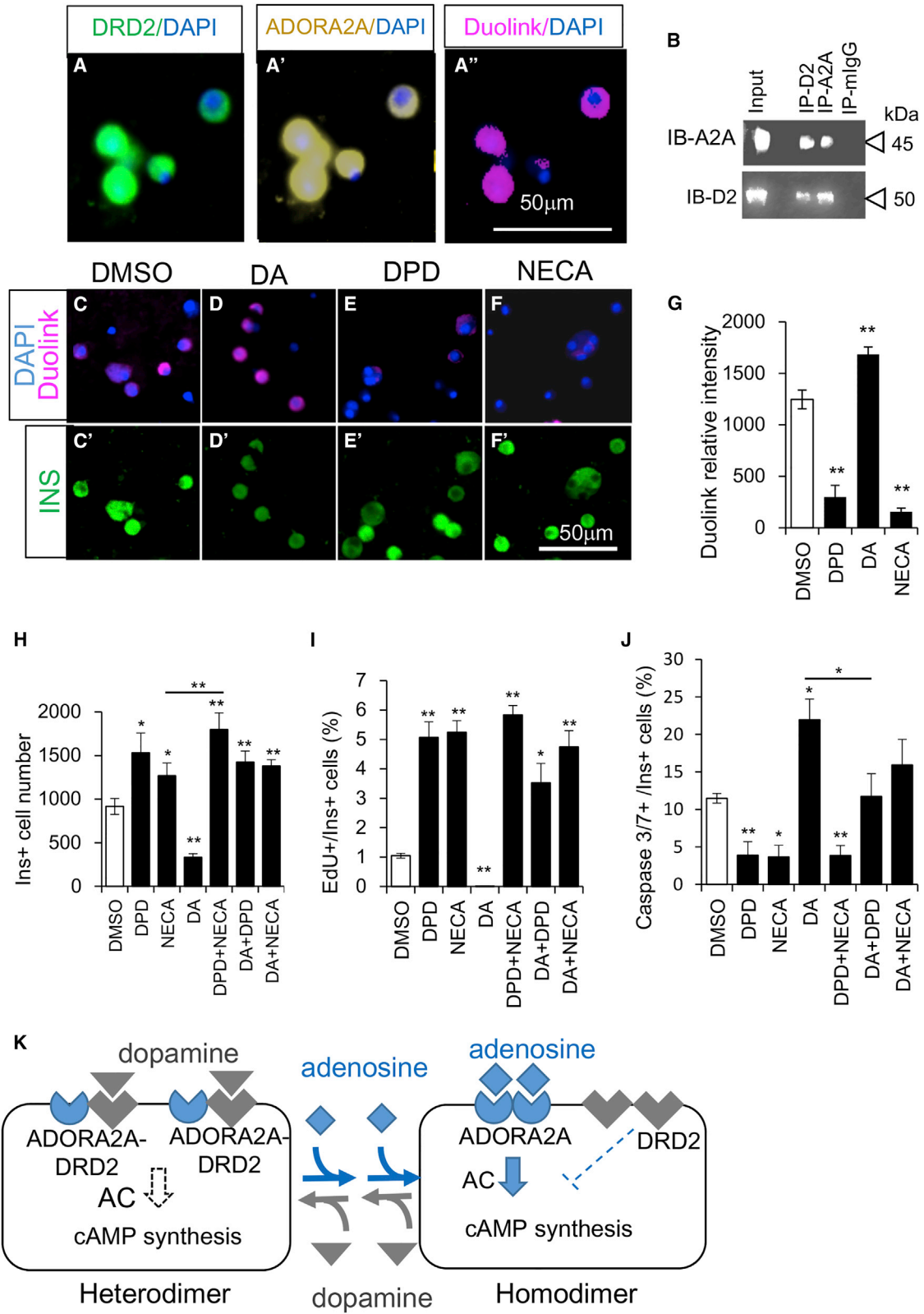
We then tested islet β cells using our primary culture system (Figures 5H–5J). NECA alone increased the number of β cells and reversed the negative effects of dopamine, similar to the dopamine-inhibitory effect of DPD. NECA was shown to increase EdU incorporation (proliferation) of β cells and decrease apoptosis. Similar to DPD, NECA effectively rescued the proliferation of dopamine-treated β cells. However, NECA seemed to be less effective than DPD for rescuing dopamine-triggered apoptosis (Figure 5J; p = 0.06).

We also tested the effect of NECA in MIN6 cells (Figure S4A). Treatment with NECA or DPD alone increased the number of MIN6 cells when present at ≥1.0 μM (D.S. et al., unpublished data). At lower concentrations, DPD and NECA work together to yield even more numbers of MIN6 cells. Under *Drd2* knocked-down background, NECA was more effective in increasing cell number compared with control wild-type MIN6 cells (Figure S4B). These results suggest that a basal dopamine signal might exert negative effects on ADORA2A -mediated β cell proliferation.

Taken together, our results suggest that dopamine-DRD2 signaling negatively regulates proliferation and increases apoptosis, through inhibition of adenosine-ADORA2A signaling that positively regulates proliferation and reduces apoptosis. DRD2 and ADORA2A form homo- or heterodimer. We hypothesize that binding of dopamine to DRD2 promotes DRD2-ADORA2A heterodimer formation, through which dopamine negatively regulates adenosine-ADORA2A signaling, and vice versa (Figure 5K). Integration of these two signals is important for the regulation of β cell

cells showed a partial decrease in *Drd2* expression (two independent experiments, each with three replicates). (F) Responses of D2KD cells to dopamine chemicals. DPD increased cell number for 9 days culture, but not in D2KD cells. The effect of dopamine decreased in knockdown cells (four independent experiments, each with four replicates).
(G–I) Dopamine treatment of wild-type (G) or *Drd2*-overexpressing (I) MIN6 cells on days 3–7. Dopamine dose-dependently increased the number of apoptotic cells (G) (three independent experiments, each with four replicates). Upper panel in (H) shows a schematic of the FLAG-tagged *Drd2*-expressing vector, and lower panel shows RT-PCR analysis of the exogenously overexpressed *Drd2* in MIN6 cells. MIN6 cells overexpressing *Drd2* (D2-OE) showed increased sensitivity to dopamine. Up to 37% of the cells became apoptotic after treatment with 10 μM dopamine (I) (three independent experiments, each with three replicates). **p < 0.01, *p < 0.05 compared with control DMSO-treated cells. Data represent mean ± SD. Student's t test. Scale bar, 100 μm.

A DRD2/DAPI   A' ADORA2A/DAPI   A" Duolink/DAPI   50μm

B

        Input IP-D2 IP-A2A IP-mIgG   kDa
IB-A2A   ◁ 45
IB-D2   ◁ 50

DMSO   DA   DPD   NECA

DAPI/Duolink: C   D   E   F
INS: C'   D'   E'   F'   50μm

G
Duolink relative intensity
DMSO, DPD**, DA**, NECA**

H
Ins+ cell number
DMSO, DPD*, NECA*, DA**, DPD+NECA**, DA+DPD**, DA+NECA**

I
EdU+/Ins+ cells (%)
DMSO, DPD**, NECA**, DA**, DPD+NECA**, DA+DPD*, DA+NECA**

J
Caspase 3/7+ /Ins+ cells (%)
DMSO, DPD**, NECA*, DA*, DPD+NECA**, DA+DPD, DA+NECA

K

dopamine   adenosine   adenosine

ADORA2A-DRD2   ADORA2A-DRD2   ADORA2A   DRD2
AC   AC
cAMP synthesis   cAMP synthesis

Heterodimer   dopamine   Homodimer

(legend continued on next page)

mass, and the differentiation versus dedifferentiation states of β cells.

DPD Is Also Effective in Human β Cells

We then tested the effects of DPD to examine whether a similar mechanism could be applied to human islet β cells. Human β cells from a single donor were dissociated and subjected to assays for EdU incorporation and apoptosis. With human islets, chemical treatments were done on days 1–3 and assayed on day 3. We found that treatment with DPD, dopamine + NECA, dBu-cAMP, or 5IT (iodotubercidin), an adenosine kinase inhibitor, increased the expression level of an anti-apoptotic protein *BCL2* (Figure 6A). Although NECA alone did not induce *BCL2* expression in the human β cells, it induced *Bcl2* in the mouse β cells (D.S., unpublished data). Expression of a pro-apoptotic protein, *BAX*, was increased with dopamine treatment (Figure 6B). DPD, NECA, DPD + NECA, dBu-cAMP, or 5IT increased total Ins+ cell numbers and EdU (24 hr) incorporation and reduced apoptosis, whereas dopamine decreased total Ins+ cell number and EdU incorporation and increased apoptosis (Figures 6C–6E). Our results suggest that a similar mechanism exists in human islet β cells and that dopamine acts as a negative regulator for β cell mass. Dopamine inhibits proliferation and triggers apoptosis, and inhibition of dopamine signal reduces apoptosis and increases β cell number.

DISCUSSION

Here, we utilized an adult islet dissociation culture system to identify chemicals that promote β cell proliferation. We revealed that in islet cell dissociation culture, β cell proliferation was triggered and at the same time β cell loss due to apoptosis and dedifferentiation of β cells was observed. Therefore, our islet cell dissociation culture system might represent a state seen in type 1 or type 2 diabetes, where disruption of cell-cell communication between islet cells promotes β cells to trigger failures in maintaining their identities and undergo apoptosis. Utilizing this assay system, we identified DPD, an antagonist of DRD2 signaling, as a chemical that specifically increased β cell mass by antagonizing dopamine signaling. Dedifferentiation of β cells seems to contribute to the major part of β cell loss in our present islet dissociation culture. Dopamine signaling, which is mediated by DRD2, decreases cAMP and triggers dedifferentiation and apoptosis of β cells. Antagonizing dopamine signaling by adding DPD protected β cells from dedifferentiation, apoptosis, and increased proliferation.

In MIN6 cells, *Drd2* knockdown increased cell number. Therefore, dopamine might function as an autocrine inhibitor produced from β cells themselves. Activation of dopamine signaling through DRD2 seems to be a default state for β cells to inhibit proliferation, so that removal of the dopamine signal might be necessary for re-entry into the cell cycle. Dopamine has been reported to modulate proliferation and apoptosis of Ins+ cells (Garcia Barrado et al., 2015; Garcia-Tornadu et al., 2010). These previous reports and our present results provide strong evidence for the importance of dopamine signaling in regulating β cell mass. MIN6 cells treated with DPD showed higher cAMP levels, suggesting that DPD enhanced proliferation by regulating cAMP. Importantly, we further revealed that DPD also increased Ins+ cell number and proliferation, and decreased apoptosis in human islet β cells. Therefore, the mechanism found in mice also functions in humans.

There are five different dopamine receptor subtypes, one of which, DRD2, is expressed in pancreatic β cells of

Figure 5. Synergistic Effects of NECA, an ADORA2A Agonist, and DPD on β Cell Proliferation and Cell Death through Interaction between DRD2 and ADORA2A

(A and B) DRD2 and ADORA2A are expressed and form heterodimer in dissociated mouse pancreatic β cells. (A–A″) Islets were dissociated and Duolink assay was performed immediately after dissociation. (B) Co-immunoprecipitates of the dissociated islet cells using anti-DRD2 (IP-D2), anti-ADORA2A (IP-A2A), or mouse IgG (mIgG) antibody were immunoblotted with anti-ADORA2A (IB-A2A) or anti-DRD2 Ab (IB-D2), respectively. The results indicated that DRD2 and ADORA2A formed a heterodimer.

(C–G) DRD2-ADORA2A dimerization states detected by Duolink assay were changed by dopamine (DA), DPD, or NECA, an ADORA2A agonist (C–F′). Dissociated islet cell cultures on day 3 were treated with chemicals for 6 hr and subjected to Duolink assay. (G) DRD2-ADORA2A heterodimer formation was suppressed by DPD or NECA, and enhanced by dopamine.

(H–J) The effect of NECA on β cell proliferation was examined using islet dissociation cultures. (H) The number of Ins+ cells after 4 days of chemical treatment (days 3–7) were counted and was increased by NECA treatment. The dopamine-mediated reduction in the number of β cells was reversed by treatment with NECA or DPD. (I) The number of EdU+ among the Ins+ cells after 4 days of chemical treatment (days 3–7) were counted. EdU incorporation (48 hr) in β cells was increased by NECA treatment. Decreased β cell proliferation was reversed by treatment with NECA or DPD. (J) β cell apoptosis was assessed after 2 days of chemical treatment (days 3–5). The number of apoptotic β cells was decreased by treatment with NECA or DPD. Dopamine-triggered apoptosis was rescued by DPD but not by NECA.

(K) Schematic of crosstalk between dopamine-DRD2 and adenosine-ADORA2A through heterodimer formation. Data represent mean ± SD. All data are obtained from three independent experiments, each with three replicates. **p < 0.01, *p < 0.05 compared with control (open bars), Student's t test.
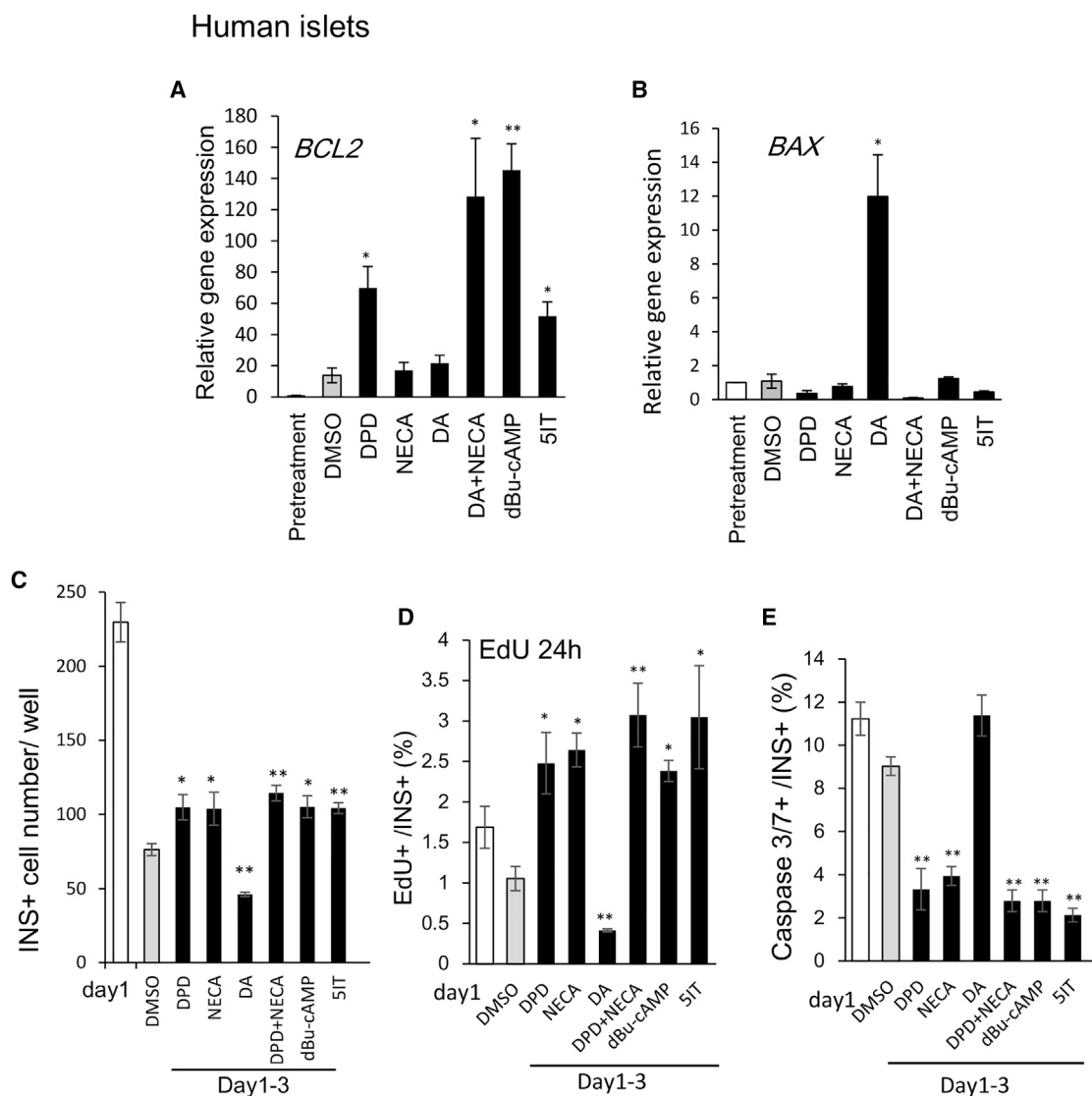
Figure 6. DPD and NECA Promoted Proliferation and Suppressed Cell Death in Human β Cells

(A and B) The effects of dopamine (DA) and adenosine signaling were tested in human primary culture by treating human islet cells with chemicals on days 1–3, and assayed on day 3. DPD, NECA, dBu-cAMP, and 5-iodotubericidin (5IT), an inhibitor of adenosine kinase, increased the expression level of *BCL2* (A), whereas dopamine increased the expression of *BAX* (B) in human islet cells.

(C) Total cell number per well, showing the number of Ins⁺ cells increased by adding DPD, NECA, DPD + NECA, cAMP, or 5IT.

(D) EdU incorporation assayed for 24 hr. DPD, NECA, cAMP, and 5IT increased EdU incorporation whereas dopamine decreased EdU incorporation in human β cells.

(E) Dopamine promoted apoptosis, whereas DPD, NECA, cAMP, and 5IT decreased apoptosis. Data represent mean ± SD. Data are from a single donor and each point represents three replicates. **$p < 0.01$, *$p < 0.05$ compared with control (open bars), Student's t test.

humans and rodents (Rubi et al., 2005; Ustione and Piston, 2012). In the pancreas, dopamine is reported to function through the DRD2 receptor as an inhibitory signal against insulin secretion. In β cells, dopamine is synthesized from circulating L-Dopa, which is produced by the gastrointestinal tract (Ustione et al., 2013). Knockdown of DRD2 in INS-1 cells (a β cell line) resulted in increased insulin secretion (Wu et al., 2008). However, global *Drd2*-knockout mice

showed impaired insulin secretion, which caused glucose intolerance (Garcia-Tornadu et al., 2010). Since dopamine is reported to regulate insulin secretion, we then tested whether insulin affects β cell proliferation or cell death in our culture, and found that insulin at high concentration (>100 nM) inhibited β cell proliferation regardless of DPD (Figure S5A). Insulin also promoted apoptosis at low concentrations, which was not rescued by DPD treatment

(Figure S5B). We interpret the seemingly contradictory previous reports and our results to mean that the insulin signal is toxic for β cells and that the inhibitory dopamine signal against insulin secretion might be important for protecting β cells from a high workload. The inhibitory effects of the dopamine signal on β cell proliferation might also function as a brake. Determining how the dopamine signal is regulated is important for understanding its action.

Recently, we found that vesicular monoamine transporter 2 (VMAT2) regulates monoamine (including dopamine) storage by acting as a negative regulator, such that high monoamine levels inhibit the progression from $Pdx1^+$ pancreatic progenitor cells to $Neurog3^+$ endocrine progenitor cells of ESC-derived pancreatic progenitor cells and during embryonic pancreatic development (Sakano et al., 2014). VMAT2, which regulates dopamine storage, and tyrosine hydroxylase, which synthesizes dopamine, are expressed in both the embryonic pancreas and the adult islets (Persson-Sjogren et al., 2002; D.S. et al., unpublished data). Our results suggest that the VMAT2-dopamine signal plays important roles both during pancreas development and in the maintenance of β cell mass.

It was previously reported that NECA promotes β cell regeneration in a zebrafish model and that adenosine kinase inhibitors increase primary β cell proliferation (Annes et al., 2012). We found that NECA functions by antagonizing dopamine signaling to increase β cell proliferation and decrease β cell apoptosis.

The detailed mechanism of the crosstalk between ADORA2A and DRD2 signaling in β cell proliferation is not yet known. Adenosine signaling is mediated by ADORA2A, which is a $G\alpha_s$-coupled receptor that increases cAMP. Dopamine signaling is mediated by a $G\alpha_i$-coupled receptor DRD2, which decreases cAMP. Thus, ADORA2A and DRD2 exert opposing effects on cAMP levels, and they have been reported to be highly co-localized and form heterodimers in the striatum (Fuxe et al., 2001) and neuronal cells (Kamiya et al., 2003) where the heterodimer antagonizes homodimer formation of ADORA2A/ADORA2A or DRD2/DRD2, to modulate dopaminergic activity (Ferre et al., 1991; Hall and Strange, 1999). L-Dopa treatment, which activates dopamine signaling, was reported to disrupt heteromer formation (Bonaventura et al., 2014; Pinna et al., 2014).

Crosstalk between adenosine and dopamine signaling through other mechanisms has also been observed. ADORA2A mediates the phosphorylation of dopamine- and cAMP-regulated phosphoprotein of M_r 32,000 (DARPP-32) at Thr34 (the cAMP-dependent protein kinase site) in striatopallidal neurons, and has been reported to oppose DRD2 signaling (Shuto et al., 2006; Yabuuchi et al., 2006). Similarly, dopamine signaling has been reported to exert opposing effects on GLP-1 through

phosphorylation of the serine/threonine kinase AKT, its downstream substrate GSK3b, and several other downstream signaling molecules (Ustione et al., 2013).

Our results, showing that activation of the adenosine signaling pathway by NECA together with DPD application in MIN6 cells and islet β cells further increased cell numbers compared with their single applications, thereby suggest that DRD2 and ADORA2A signal via different pathways. This notion is supported by the ability of NECA to overcome dopamine-dependent cell loss and the dopamine-dependent decrease in β cell proliferation. Our results suggest that dopamine inhibits adenosine signaling at least in part through ADORA2A-DRD2 heterodimer formation, so that ADORA2A receptor cannot activate downstream signaling events. However, it remains unknown how heterodimerization acts on cAMP levels and how it affects downstream signaling events.

In conclusion, the present study revealed the role of dopamine signaling in the control of β cell proliferation and apoptosis, and the maintenance of β cell differentiation; dopamine acts through DRD2 to decrease cAMP. Dopamine also functions through negatively regulating other signaling pathways such as the adenosine pathway. Our finding could be beneficial for the development of new targets for increasing β cell mass.

EXPERIMENTAL PROCEDURES

Animals

RIP-Cre (Postic et al., 1999) and *Rosa26-EYFP* (Srinivas et al., 2001) have been previously described. All animal procedures were performed in accordance with the guidelines for the care and use of animals of Kumamoto University and Tokyo Institute of Technology.

Chemicals

DPD and NECA were purchased from Tocris Bioscience. dBu-cAMP was purchased from BIOMOL International. Dopamine was obtained from LKT Laboratories. 5IT was purchased from Toronto Research Chemicals. Test compounds were dissolved in DMSO and were added to cells on day 3 and assayed on day 5, unless otherwise indicated. Final concentrations of the chemicals used were: 0.5 μM DPD, 0.25 μM NECA, 1.0 μM dBu-cAMP, 1.0 μM dopamine, and 1.0 μM 5IT unless otherwise indicated.

Islet Isolation and Screening Culture

Mouse islets from 10-week-old C57BL/6 mice (SLC) and RIP-Cre/R26-eYFP mice were isolated as described previously (Kikawa et al., 2014). Isolated islets were handpicked and dissociated into single cells by incubation with 0.05% trypsin-EDTA (Invitrogen) for 5 min at 37°C and 5% CO_2 and pipetting. The dissociated cells were plated in 96-well plates at a density of 2,000 cells per well in DMEM supplemented with 10% fetal bovine serum, 100 μM nonessential amid acids, 2 mM L-glutamine, 50 units/ml penicillin,

50 μg/ml streptomycin, and 100 μM 2-mercaptoethanol (Sumitomo Bakelite).

EdU Incorporation

Cells were incubated in culture medium containing 20 μM EdU for 48 hr prior to harvest (days 3–5), unless otherwise indicated, then processed using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen), and stained with DAPI and anti-insulin antibodies.

Screening of Small Molecules and Quantitative Imaging Analysis

Small molecules from the bioactive, pharmacologically defined Prestwick Chemical Library were screened for pro-differentiation activity. Compounds were dissolved in DMSO and added at a 1:100 dilution on day 3. Cells were assayed by immunostaining with an anti-insulin antibody on day 5. Fluorescent images were quantified by counting the number of pixels, representing the number of positive cells, using an ImageXpress micro scanning system and MetaXpress cellular image analysis software (Molecular Devices). Data are reported as the relative fold changes normalized to the DMSO-only controls. A hit compound was defined as a compound causing a 2-fold or higher increase in the number of EdU-positive insulin-positive β cells. Candidate compounds were tested for dose dependency and reproducibility.

Apoptosis Assay

For the apoptosis assay, caspase-3/7 activity was measured using CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min. The following antibodies were used: guinea pig anti-insulin (A0564, Dako Denmark; 1:500), mouse anti-glucagon (G2654, Sigma-Aldrich; 1:500), rabbit anti-DRD2 receptor (251384, ABBIOTEC; 1:500), rabbit anti-GFP (598, Medical & Biological Laboratories; 1:1,000), Alexa 488 goat anti-rabbit immunoglobulin G (IgG) (A-11008 Life Technologies; 1:1,000), Alexa 568 goat anti-guinea pig IgG (A-11075, Life Technologies; 1:1,000), and Alexa 647 goat anti-mouse IgG (A-21235, Life Technologies; 1:1,000). Cells were counterstained with DAPI (Roche Diagnostics; 1:1,000). Mouse anti-ADORA2A, clone 7F6-G5-A2 (ab115250, Abcam; 1:500) was obtained from Millipore.

Real-Time PCR Analysis

RNA was extracted from cells using the RNeasy mini-kit (Qiagen) and treated with DNase I (Qiagen). cDNA was synthesized from 1 μg of total RNA using Revertra Ace qPCR RT Master Mix (Toyobo).

For the real-time PCR analysis, mRNA expression was quantified by amplification in an ABI 7500 thermal cycler (Applied Biosystems) with SYBR Green. Gene expression of target genes was normalized to that of β-actin. The PCR conditions were as follows: denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s, for up to 40 cycles. Each measurement was normalized to β-actin (mouse) or *GAPDH* (human) by subtracting the average β-actin or *GAPDH* expression level. Target mRNA levels were ex-

pressed as a.u. and were determined using the standard curve method. All primers used for real-time PCR are listed in Table S1.

Gene Silencing

For the *Drd2*-knockdown assays, cells were transfected with either Expression Arrest, non-silencing, control short hairpin RNA (shRNA) (Open Biosystems; #RHS4080) or *Drd2* shRNA (Open Biosystems; #TRCN0000025738). The lentiviral vectors were constructed as previously described (Golla and Seethala, 2002), and MIN6 cells were infected with viral supernatants. After a 24-hr incubation, the virus-containing medium was replaced with fresh ESC maintenance medium. After another 24 hr incubation, infected cells were selected by the addition of 1.5 μg ml^{-1} puromycin. The surviving cells were harvested, and clones were selected to establish knockdown and control cell lines.

Drd2 Overexpression

A cDNA fragment of mouse *Drd2* (nucleotides 95–1,429; NM_010077.2; amino acids 1–444) was inserted into the *Xho*I–*Bam*HI sites of the pCDNA3-cFlag vector (Addgene). MIN6 cells were plated at 4×10^4 cells per well in 96-well culture plates on the day of transfection (0.3 μg of plasmid DNA). DNA was mixed with Opti-MEM medium (Life Technologies) containing Hilymax (Dojin) at a ratio of 3:1 to total DNA and then added to the cells.

cAMP Content Measurement

cAMP levels were calculated using the cAMP-Glo Assay (Promega) following the manufacturer's protocol 36. In brief, MIN6 cells were grown in 96-well plates and lysed with the buffer provided in the kit. Intracellular cAMP levels were detected using the detection solution provided in the kit. Luminescence was measured using a GloMax 96 Microplate Luminometer (Promega). A standard curve was generated in parallel for each experiment using serial dilutions of the cAMP standard provided with the kit.

Co-immunoprecipitation and Western Blot Analysis

Co-immunoprecipitation was performed using rabbit anti-DRD2 receptor antibody (251384, ABBIOTEC) or mouse monoclonal [7FG-G5-A2] against ADORA2A (ab115250, Abcam). In brief, mouse islet cells were lysed with RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 0.1% SDS, 1.5 mM MgCl$_2$) and the cellular extracts were incubated for 16 hr with the antibody coupled to Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, #sc-2003) at 4°C under rotation. Immunoprecipitates were spun down and washed six times in RIPA buffer. Co-immunoprecipitates were dissolved with 2× Laemmli buffer and boiled for 5 min for SDS-PAGE.

SDS-PAGE was carried out using an 8% acrylamide gel. Western blot analysis of DRD2 or ADORA2A after co-immunoprecipitation was carried out on polyvinylidene fluoride membranes using primary antibody and Immuno-Aptamer rabbit IgG (Nippon Gene, 310-07,211) or mouse IgG (Nippon Gene, 310-07,201) dissolved in TBST-Mg (Tris-buffered saline containing 0.05% Tween 20 and 5 mM MgCl$_2$) containing 1% acetylated BSA (Nippon Gene, 316-07,291). After washing with TBST (TBS containing 0.05% Tween 20), the membrane was incubated with TBST containing streptavidin-horseradish peroxidase conjugate (1:10,000; Perkin Elmer, #NEL750001EA) antibody. Finally the membrane was reacted

with ECL Prime reagents (GE Healthcare) and scanned using a Fusion Solo (Vilber-Lourmat) imaging system.

Duolink In Situ Proximity Ligation Assay

Direct protein-protein interactions were investigated by means of a proximity ligation assay (PLA) (Soderberg et al., 2006) using Duolink In Situ Red Starter Kit Mouse/Rabbit (Sigma). Dispersed islet cells were washed with PBS and then either immediately fixed, or cultured for 3 days and treated with chemicals for 6 hr then fixed on the culture dish at room temperature for 30 min in 4% paraformaldehyde, for further use in Duolink PLA. Cells were further rinsed for 10 min with PBS containing 1% Triton X-100 (Sigma) and then washed for 10 min three times with 0.05% Tween 20 in PBS to allow permeabilization. Duolink II blocking solution was then added into wells, followed by incubation for 1 hr at room temperature. The blocking solution was then tapped off and cells were incubated for 1 hr at 37°C with primary antibodies. Primary antibodies used were: rabbit anti-DRD2 receptor antibody (1:500; ABBIOTEC, #251384) or mouse monoclonal (7FG-G5-A2) to ADORA2A (1:500; Abcam, #ab79714). Duolink anti-rabbit PLUS and anti-mouse MINUS secondary antibodies and red detection reagents were used and antibody incubation, ligation, amplification, and washing steps were performed according to the supplier's manual. Cells were then immunocytochemically stained with anti-insulin antibody and DAPI. The average intensity of PLA signals in insulin-positive cell area was calculated by MetaMorph software (Molecular Devices).

Human Islets

Non-diabetic human islets were obtained from Prodo Laboratories, and cultured in Prodo Islet Medium (Prodo Laboratories). Donor information is as follows. Age, 56; gender, female; ethnicity, African American; body mass index, 33.4; diabetes, no history; and hemoglobin A1c, 5.2%. Chemicals were added on days 1–3 after dissociation, considering the islets used were not freshly isolated (more than ~1 week after isolation).

Statistics

Data are expressed as the mean ± SD. Differences between groups were analyzed by Student's t test. A p value of less than 0.05 was considered statistically significant. Data were confirmed in three to four independent experiments for reproducibility, each experiment with three to six biological replicates, unless otherwise indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.05.015.

AUTHOR CONTRIBUTIONS

D.S., N.S., and S.K. conceived the project and designed the study; D.S., S.C., and M.K. performed most experiments; M.U. provided the chemical library; D.S., N.S., M.U., K.K., and S.K. contributed to data analysis and interpretation; D.S. and S.K. wrote the paper; all the authors approved the final version of the manuscript.

REFERENCES

Andersson, O., Adams, B.A., Yoo, D., Ellis, G.C., Gut, P., Anderson, R.M., German, M.S., and Stainier, D.Y. (2012). Adenosine signaling promotes regeneration of pancreatic beta cells in vivo. Cell Metab. *15*, 885–894.

Annes, J.P., Ryu, J.H., Lam, K., Carolan, P.J., Utz, K., Hollister-Lock, J., Arvanites, A.C., Rubin, L.L., Weir, G., and Melton, D.A. (2012). Adenosine kinase inhibition selectively promotes rodent and porcine islet beta-cell replication. Proc. Natl. Acad. Sci. USA *109*, 3915–3920.

Baeyens, L., De Breuck, S., Lardon, J., Mfopou, J.K., Rooman, I., and Bouwens, L. (2005). In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia *48*, 49–57.

Bernal-Mizrachi, E., Kulkarni, R.N., Scott, D.K., Mauvais-Jarvis, F., Stewart, A.F., and Garcia-Ocana, A. (2014). Human beta-cell proliferation and intracellular signaling part 2: still driving in the dark without a road map. Diabetes *63*, 819–831.

Bonaventura, J., Rico, A.J., Moreno, E., Sierra, S., Sanchez, M., Luquin, N., Farre, D., Muller, C.E., Martinez-Pinilla, E., Cortes, A., et al. (2014). L-DOPA-treatment in primates disrupts the expression of A(2A) adenosine-CB(1) cannabinoid-D(2) dopamine receptor heteromers in the caudate nucleus. Neuropharmacology *79*, 90–100.

Bonner-Weir, S., Li, W.C., Ouziel-Yahalom, L., Guo, L., Weir, G.C., and Sharma, A. (2010). Beta-cell growth and regeneration: replication is only part of the story. Diabetes *59*, 2340–2348.

Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S.R., Neve, K., Fuxe, K., Agnati, L.F., Woods, A.S., et al. (2003). Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J. Biol. Chem. *278*, 46741–46749.

Cano, D.A., Rulifson, I.C., Heiser, P.W., Swigart, L.B., Pelengaris, S., German, M., Evan, G.I., Bluestone, J.A., and Hebrok, M. (2008).

Regulated beta-cell regeneration in the adult mouse pancreas. Diabetes *57*, 958–966.

Collombat, P., Xu, X., Ravassard, P., Sosa-Pineda, B., Dussaud, S., Billestrup, N., Madsen, O.D., Serup, P., Heimberg, H., and Mansouri, A. (2009). The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell *138*, 449–462.

De Breuck, S., Lardon, J., Rooman, I., and Bouwens, L. (2003). Netrin-1 expression in fetal and regenerating rat pancreas and its effect on the migration of human pancreatic duct and porcine islet precursor cells. Diabetologia *46*, 926–933.

De Breuck, S., Baeyens, L., and Bouwens, L. (2006). Expression and function of leukaemia inhibitory factor and its receptor in normal and regenerating rat pancreas. Diabetologia *49*, 108–116.

Dor, Y., Brown, J., Martinez, O.I., and Melton, D.A. (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature *429*, 41–46.

Ferre, S., von Euler, G., Johansson, B., Fredholm, B.B., and Fuxe, K. (1991). Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. Proc. Natl. Acad. Sci. USA *88*, 7238–7241.

Finegood, D.T., Weir, G.C., and Bonner-Weir, S. (1999). Prior streptozotocin treatment does not inhibit pancreas regeneration after 90% pancreatectomy in rats. Am. J. Physiol. *276*, E822–E827.

Fuxe, K., Stromberg, I., Popoli, P., Rimondini-Giorgini, R., Torvinen, M., Ogren, S.O., Franco, R., Agnati, L.F., and Ferre, S. (2001). Adenosine receptors and Parkinson's disease. Relevance of antagonistic adenosine and dopamine receptor interactions in the striatum. Adv. Neurol. *86*, 345–353.

Garcia-Tornadu, I., Ornstein, A.M., Chamson-Reig, A., Wheeler, M.B., Hill, D.J., Arany, E., Rubinstein, M., and Becu-Villalobos, D. (2010). Disruption of the dopamine d2 receptor impairs insulin secretion and causes glucose intolerance. Endocrinology *151*, 1441–1450.

Garcia Barrado, M.J., Iglesias Osma, M.C., Blanco, E.J., Carretero Hernandez, M., Sanchez Robledo, V., Catalano Iniesta, L., Carrero, S., and Carretero, J. (2015). Dopamine modulates insulin release and is involved in the survival of rat pancreatic beta cells. PLoS One *10*, e0123197.

Golla, R., and Seethala, R. (2002). A homogeneous enzyme fragment complementation cyclic AMP screen for GPCR agonists. J. Biomol. Screen. *7*, 515–525.

Hall, D.A., and Strange, P.G. (1999). Comparison of the ability of dopamine receptor agonists to inhibit forskolin-stimulated adenosine 3′5′-cyclic monophosphate (cAMP) accumulation via D2L (long isoform) and D3 receptors expressed in Chinese hamster ovary (CHO) cells. Biochem. Pharmacol. *58*, 285–289.

Kamiya, T., Saitoh, O., Yoshioka, K., and Nakata, H. (2003). Oligomerization of adenosine A2A and dopamine D2 receptors in living cells. Biochem. Biophys. Res. Commun. *306*, 544–549.

Kataoka, M., Kawamuro, Y., Shiraki, N., Miki, R., Sakano, D., Yoshida, T., Yasukawa, T., Kume, K., and Kume, S. (2013). Recovery from diabetes in neonatal mice after a low-dose streptozotocin treatment. Biochem. Biophys. Res. Commun. *430*, 1103–1108.

Kikawa, K., Sakano, D., Shiraki, N., Tsuyama, T., Kume, K., Endo, F., and Kume, S. (2014). Beneficial effect of insulin treatment on islet transplantation outcomes in Akita mice. PLoS One *9*, e95451.

Nir, T., Melton, D.A., and Dor, Y. (2007). Recovery from diabetes in mice by beta cell regeneration. J. Clin. Invest. *117*, 2553–2561.

Persson-Sjogren, S., Forsgren, S., and Taljedal, I.B. (2002). Tyrosine hydroxylase in mouse pancreatic islet cells, in situ and after syngeneic transplantation to kidney. Histol. Histopathol. *17*, 113–121.

Pinna, A., Bonaventura, J., Farre, D., Sanchez, M., Simola, N., Mallol, J., Lluis, C., Costa, G., Baqi, Y., Muller, C.E., et al. (2014). L-DOPA disrupts adenosine A(2A)-cannabinoid CB(1)-dopamine D(2) receptor heteromer cross-talk in the striatum of hemiparkinsonian rats: biochemical and behavioral studies. Exp. Neurol. *253*, 180–191.

Porat, S., Weinberg-Corem, N., Tornovsky-Babaey, S., Schyr-Ben-Haroush, R., Hija, A., Stolovich-Rain, M., Dadon, D., Granot, Z., Ben-Hur, V., White, P., et al. (2011). Control of pancreatic beta cell regeneration by glucose metabolism. Cell Metab. *13*, 440–449.

Postic, C., Shiota, M., Niswender, K.D., Jetton, T.L., Chen, Y., Moates, J.M., Shelton, K.D., Lindner, J., Cherrington, A.D., and Magnuson, M.A. (1999). Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J. Biol. Chem. *274*, 305–315.

Rubi, B., Ljubicic, S., Pournourmohammadi, S., Carobbio, S., Armanet, M., Bartley, C., and Maechler, P. (2005). Dopamine D2-like receptors are expressed in pancreatic beta cells and mediate inhibition of insulin secretion. J. Biol. Chem. *280*, 36824–36832.

Sakano, D., Shiraki, N., Kikawa, K., Yamazoe, T., Kataoka, M., Umeda, K., Araki, K., Mao, D., Matsumoto, S., Nakagata, N., et al. (2014). VMAT2 identified as a regulator of late-stage beta-cell differentiation. Nat. Chem. Biol. *10*, 141–148.

Salpeter, S.J., Klochendler, A., Weinberg-Corem, N., Porat, S., Granot, Z., Shapiro, A.M., Magnuson, M.A., Eden, A., Grimsby, J., Glaser, B., et al. (2011). Glucose regulates cyclin D2 expression in quiescent and replicating pancreatic beta-cells through glycolysis and calcium channels. Endocrinology *152*, 2589–2598.

Sharma, A., Zangen, D.H., Reitz, P., Taneja, M., Lissauer, M.E., Miller, C.P., Weir, G.C., Habener, J.F., and Bonner-Weir, S. (1999). The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. Diabetes *48*, 507–513.

Shuto, T., Kuroiwa, M., Hamamura, M., Yabuuchi, K., Shimazoe, T., Watanabe, S., Nishi, A., and Yamamoto, T. (2006). Reversal of methamphetamine-induced behavioral sensitization by repeated administration of a dopamine D1 receptor agonist. Neuropharmacology *50*, 991–997.

Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K., Leuchowius, K.J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L.G., et al. (2006). Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat. Methods *3*, 995–1000.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced

by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Talchai, C., Xuan, S., Lin, H.V., Sussel, L., and Accili, D. (2012). Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell *150*, 1223–1234.

Ustione, A., and Piston, D.W. (2012). Dopamine synthesis and D3 receptor activation in pancreatic beta-cells regulates insulin secretion and intracellular [Ca(2+)] oscillations. Mol. Endocrinol. *26*, 1928–1940.

Ustione, A., Piston, D.W., and Harris, P.E. (2013). Minireview: dopaminergic regulation of insulin secretion from the pancreatic islet. Mol. Endocrinol. *27*, 1198–1207.

Wu, W., Shang, J., Feng, Y., Thompson, C.M., Horwitz, S., Thompson, J.R., MacIntyre, E.D., Thornberry, N.A., Chapman, K., Zhou, Y.P., et al. (2008). Identification of glucose-dependent insulin secretion targets in pancreatic beta cells by combining defined-mechanism compound library screening and siRNA gene silencing. J. Biomol. Screen. *13*, 128–134.

Xu, X., D'Hoker, J., Stange, G., Bonne, S., De Leu, N., Xiao, X., Van de Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D., et al. (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell *132*, 197–207.

Yabuuchi, K., Kuroiwa, M., Shuto, T., Sotogaku, N., Snyder, G.L., Higashi, H., Tanaka, M., Greengard, P., and Nishi, A. (2006). Role of adenosine A1 receptors in the modulation of dopamine D1 and adenosine A2A receptor signaling in the neostriatum. Neuroscience *141*, 19–25.