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FTY720 Normalizes Hyperglycemia by Stimulating β-Cell *in Vivo* Regeneration in *db*/*db* Mice through Regulation of Cyclin D3 and p57^{KIP2}*[S]

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Background: Preserving functional β-cell mass is essential for preventing and curing type 2 diabetes (T2D).
Results: Administration of FTY720 to *db*/*db* mice led to sustained normalization of hyperglycemia by stimulating *in vivo* β-cell regeneration through PI3K-dependent regulation of cyclin D3 and p57^{KIP2}.
Conclusion: FTY720 is capable of promoting *in vivo* β-cell regeneration in obesity diabetes.
Significance: Sphingosine 1-phosphate signaling pathway potentially is a therapeutic target for treatment of T2D.

Loss of insulin-producing β-cell mass is a hallmark of type 2 diabetes in humans and diabetic *db*/*db* mice. Pancreatic β-cells can modulate their mass in response to a variety of physiological and pathophysiological cues. There are currently few effective therapeutic approaches targeting β-cell regeneration although some anti-diabetic drugs may positively affect β-cell mass. Here we show that oral administration of FTY720, a sphingosine 1-phosphate (S1P) receptor modulator, to *db*/*db* mice normalizes fasting blood glucose by increasing β-cell mass and blood insulin levels without affecting insulin sensitivity. Fasting blood glucose remained normal in the mice even after the drug was withdrawn after 23 weeks of treatment. The islet area in the pancreases of the FTY720-treated *db*/*db* mice was more than 2-fold larger than that of the untreated mice after 6 weeks of treatment. Furthermore, BrdU incorporation assays and Ki67 staining demonstrated cell proliferation in the islets and pancreatic duct areas. Finally, islets from the treated mice exhibited a significant decrease in the level of cyclin-dependent kinase inhibitor p57^{KIP2} and an increase in the level of cyclin D3 as compared with those of untreated mice, which could be reversed by the inhibition of phosphatidylinositol 3-kinase (PI3K). Our findings reveal a novel network that controls β-cell regeneration in the obesity-diabetes setting by regulating cyclin D3 and p57^{KIP2} expression through the S1P signaling pathway. Therapeutic strategies targeting this network may promote *in vivo* regeneration of β-cells in patients and prevent and/or cure type 2 diabetes.

Type 2 diabetes is one of the most prevalent human metabolic diseases. It is characterized by insulin resistance and the reduction of functional pancreatic β-cell mass (1). Although there is an initial compensatory increase of β-cell mass in response to insulin resistance, diabetes occurs when the functional β-cell mass fails to expand sufficiently (2, 3). Finding ways to preserve or increase the mass of functional β-cells in diabetic patients is therefore a key step in controlling or curing type 2 diabetes in humans (4, 5).

Pancreatic β-cells are plastic cells that modulate their mass in response to a variety of physiological (pregnancy) (6) and pathophysiological (obesity or insulin resistance) states (3). New β-cells may arise from the proliferation of pre-existing β-cells (7) or pancreatic progenitor cells (5, 8, 9), and the transdifferentiation of pancreatic non-β-cells to β-cells under certain conditions (10–13). Recent islet transplantation in diabetes patients suggest that diabetes may be cured by replenishing β-cell mass (14). Importantly, it has been shown that β-cell volume in obese humans without diabetes is 50% higher than that in normal lean subjects (2, 15) and increases in islet mass occur during pregnancy in humans (16, 17), suggesting that human islets are capable of expanding their mass in response to metabolic demands, although much lower compared with mice (15). Our goal, therefore, is to develop a pharmacological agent that can stimulate an increase in β-cell mass *in vivo* (4, 5).

Various nutrients and peptide hormones have been implicated as regulators of β-cell mass (18, 19). However, we are particularly interested in a group of membrane-derived bioactive lysophospholipids that have growth factor and hormone-like biological activities (20). Lysophospholipids, including lysophosphatidic acid and sphingosine 1-phosphate (S1P),[2] regulate diverse biological processes including embryogenesis, vascular development, neurogenesis, uterine development, oocyte survival, immune cell trafficking, and inflammatory reactions through their receptors, a novel class of G protein-coupled receptors (GPCRs) (20, 21). Intriguingly, lysophospholipid levels are significantly increased during human pregnancy (22) and in obese mice (23). We have screened several lysophosphatidic acid and S1P analogs in a β-cell line and *db*/*db* mice,

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[2] The abbreviations used are: S1P, sphingosine 1-phosphate; GPCR, G protein-coupled receptor; FTY720, (2-amino-2-[4-octylphenyl]ethyl)-1,3-propanediol; BrdU, 5-bromo-2′deoxyuridine; PDX-1, pancreatic and duodenal homeobox 1.

which exhibit severe depletion of insulin-producing β-cells (24), and identified that intraperitoneal injection of FTY720, a structural analog of sphingosine, can normalize hyperglycemia in *db*/*db* mice.

FTY720 (Fingolimod), a derivative of ISP-1 (myriocin), a fungal metabolite of the Chinese herb *Iscaria sinclarii* as well as a structural analog of sphingosine, is a potent immunosuppressant that was approved as a new treatment for multiple sclerosis (25, 26). FTY720 becomes active *in vivo* following phosphorylation by sphingosine kinase 2 (SphK2) to form FTY720(S)-phosphate (FTY720-P), which binds to four of the five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ but not $S1P_2$) and prevents the release of lymphocytes from lymphoid tissue (27, 28). Here we report that oral administration of the FTY720 to *db*/*db* mice leads to normalization of hyperglycemia by stimulating β-cell *in vivo* regeneration through the PI3K-dependent regulation of cyclin D3 and $p57^{KIP2}$.

EXPERIMENTAL PROCEDURES

Materials—FTY720 (2-amino-2-[4-octylphenyl]ethyl)-1,3-propanediol, hydrochloride, $C_{19}H_{33}NO_2$·HC, 343.9), FTY720-P, S1P, SEW2871 (5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole), CAY10444 (2-undecyl-thiazolidine-4-carboxylic acid), W123 (3-(2-(3-hexylphenylamino)-2-oxoethylamino)propanoic acid), and FTY720(R)-phosphate were purchased from Cayman Chemical (Ann Arbor, MI).

Animals and Procedures—Five-week-old female *db*/*db* mice (BKS.Cg-*m*+/+*Leprdb*) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed under controlled light (12 h light/12 h dark) and temperature conditions, and had free access to food (normal rodent chow) and water. All procedures were conducted in accordance with the guidelines on Animal Care and were approved by the Institutional Animal Care and Use Committee (IACUC) of Mount Sinai School of Medicine.

After 1 week, the fasting glucose levels of the 6-week-old mice were measured. Mice with normal glucose levels (<126 mg/dl) were randomly divided into control and FTY720 treatment groups. Mice in the FTY720-treated group were fed 10 mg/kg of FTY720 daily by feeding tube and their food intake and body weight were measured twice a week. Fasting glucose levels were measured at the end of each week. At 12 weeks of age (when the mice had been treated for 6 weeks), all mice were subjected to metabolic analysis. After metabolic analysis, the pancreases were removed from the mice at 13 and 16 weeks of age for immunohistochemical analysis, quantitation of islet area, or islet isolation.

Intraperitoneal Glucose Tolerance Test or Insulin Tolerance Test—For the glucose tolerance test, the mice were fasted for 16 h and intraperitoneally injected with 10% glucose (1 mg/g of body weight). Glucose levels were then measured after 0, 30, 60, 90, and 120 min by a Glucometer Elite (Bayer Corp., Elkhart, IN) (29). For the insulin tolerance test, the mice were fasted for 6 h and injected intraperitoneally with human regular insulin (0.55 units/kg) (Sigma). Tail blood samples were collected at 0, 30, 40, 60, 90, and 120 min for glucose measurement (30, 31).

Determination of Serum Insulin Levels—After overnight fasting, blood samples (50 μl) were collected in a heparinized microhematocrit tube for the determination of insulin concentration using a Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL) (30).

Immunohistochemical Analysis—Pancreases were removed from the *db*/*db* mice, fixed overnight in 4% formaldehyde solution, and embedded in paraffin. Paraffin sections (10 μm thick) were rehydrated, and antigen retrieval in 10 mM sodium citrate solution was performed using a microwave, followed by blocking endogenous peroxidase in 3% H_2O_2 solution. The following primary antibodies were used: guinea pig anti-swine insulin (1:300; DAKO Corp., Carpinteria, CA), rabbit anti-glucagon (1:200; Thermo Fisher Scientific, Fremont, CA), mouse anti-cyclin D3 (1:40; Vector Laboratory, Burlingame, CA), mouse anti-BrdU (1:10; BD Biosciences), rabbit anti-Ki67 (1:100; Abcam, Cambridge, MA), and rabbit anti-$p57^{KIP2}$ (1:100; Abcam, Cambridge, MA). Goat anti-mouse/rabbit IgG (Vector Laboratory) and goat anti-guinea pig-mouse/rabbit IgG conjugated with the Alexa Fluor® dyes (Alexa Fluor® 488 and Alexa Fluor® 594; Invitrogen) were used for the secondary antibodies. All images were captured by a Zeiss Axioplan 2 microscope (32, 33).

Assessment of Islet Areas—To assess islet area in pancreas after 6 weeks of treatment with FTY720, six consecutive paraffin sections (10 μm) from a pancreas (10 pancreases for the control and 9 pancreases for the FTY720-treated group) were used. All islet images on a whole section were taken by a Axioplan 2 microscope at ×10 magnification, each islet area was measured by the Java-based image-processing program ImageJ (National Institutes of Health, Bethesda, MD), and the sum of all islet areas from a section was considered to be the islet area per pancreas.

Islet Isolation and Cell Culture—Islets were isolated from pancreases removed from the 13-week-old untreated and FTY720-treated *db*/*db* mice by Liberase (Roche Diagnostics) digestion, followed by discontinuous Ficoll gradient separation and manual stereomicroscopic selection to exclude contaminating tissues (30). Isolated islets (or INS-1 cells) were cultured in the medium (composed of RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% sodium pyruvate, 50 μM β-mercaptoethanol, 100 units/ml of penicillin, and 100 μg/ml of streptomycin). For treatment of INS-1 cells, the cells were plated in RPMI medium overnight and changed to Hanks' balanced salt solution containing 2 mM glucose and 0.5% FBS overnight. Then the cells were treated with various conditions in the same medium for 24 h.

Measurement of β-Cell Regeneration in Vivo—After overnight fasting, the mice were given 1 mg/ml of 5-bromo-2′-deoxyuridine (BrdU) in PBS intraperitoneally. Mice were sacrificed 24 h after BrdU injection and pancreases were removed and fixed with a 4% formaldehyde solution. The pancreases were then embedded in paraffin and sliced into 10-μm sections. Tissue sections were stained with the anti-BrdU monoclonal antibody provided in BrdU *in Situ* Detection Kit (BD Biosciences).

RT-PCR Analysis—Total RNA was extracted from islets isolated from treated and untreated mice using the RNeasy Mini Kit. The targeting cDNA sequences were obtained online (www.ncbi.nlm.nih.gov) and PCR primers were designed by PrimerQuest™ (IDT SciTools, Coralville, IA). Primers used in the conventional RT-PCR were listed in Table 1. RT-PCR was

TABLE 1
Sequences of primers and probes used for conventional and real-time RT-PCRs

Gene	Sequences
Primers for conventional RT-PCRs (mouse)	
GAPDH	F: 5′-AACTTTGGCATTGTGGAAGGGCTC
	R: 5′-ACCCTGTTGCTGTAGCCGTATTCA
Cyclin D1	F: 5′-TGCTGCAAATGGAACTGCTTCTGG
	R: 5′-TACCATGGAGGGTGGGTTGGAAAT
Cyclin D2	F: 5′-AGGCCCAAGTTGAATGAGTCTGGA
	R: 5′-ATCAGGTACCCAAGTTGCCCAAGA
Cyclin D3	F: 5′-TCCCACAGATGTCACAGCCATTCA
	R: 5′-AAGCTGGTTGAGTGGGAAGGAAGA
$p21^{CIP1}$	F: 5′-AATCCTGGTGATGTCCGACCTGTT
	R: 5′-AGACCAATCTGCGCTTGGAGTGAT
$p27^{KIP1}$	F: 5′-TGGCTCTGCTCCATTTGACTGTCT
	R: 5′-ATGATTGACCGGGCCGAAGAGATT
$p57^{KIP2}$	F: 5′-TGTACCATGTGCAAGGAGTACGCT
	R: 5′-TTGGTATGGGCAGTACAGGAACCA
$S1P_1$	F: 5′-TTCTCTTCTGCACCACCGTCTTCA
	R: 5′-AGCAGGCAATGAAGACACTCAGGA
$S1P_3$	F: 5′-TGGCTTCTCCATTTCCTCCTGTGT
	R: 5′-ACGGATCCACTGGGCATACATCAA
$S1P_4$	F: 5′-ATCTAGTGCTTGCCTCCCATCCAA
	R: 5′-GGCCTGGGTTTCCTGTTGTTTGAA
$S1P_5$	F: 5′-TTTCCAATAGCCGCTCTCCTGTGT
	R: 5′-AGCTTGCCGGTGTAGTTGTAGTGA
PI3Kr1	F: 5′-CAGCAACCGAAACAAAGCGGAGAA
	R: 5′-TGGCTTGAAGGTGAGGAACTGGAT
PI3Kr2	F: 5′-ATCAAAGTCTTCCACCGGGATGGT
	R: 5′-TGAGGTCAGGTTTGAGGCTGTTCA
PI3Kr3	F: 5′-TGCTTTGGAAAGAAGCTGGCTTGG
	R: 5′-AGGGCAAACAGGGTGATGAAGAGA
Akt1	F: 5′-ACGGGCACATCAAGATAACGGACT
	R: 5′-AAGGTGGGCTCAGCTTCTTCTCAT
Akt2	F: 5′-TGGACTGCTGAAGAAGGACCCAAA
	R: 5′-TTTCTGTACCACGTCCTGCCAGTT
Primers and probes for quantitative real-time RT-PCRs (mouse)	
GAPDH	F: 5′-TCAACAGCAACTCCCACTCTTCCA
	R: 5′-ACCCTGTTGCTGTAGCCGTATTCA
	Probe-/56-FAM/GGCTGGCAT/Zen/TGCTCTCAATGACAACT/3IABKFQ
Cyclin D3	F: 5′-TCT TCC TTC CCA CTC AAC CAG CTT
	R: 5′-TTT GGC AAC TGA GAA GGT TGG AGC
	Probe-/56-FAM/TCCTGGGCC/Zen/ATGATGGTCAGAGAAAT/3IABKFQ
$p57^{KIP2}$	F: 5′-ATG TAG CAG GAA CCG GAG ATG GTT
	R: 5′-TTT ACA CCT TGG GAC CAG CGT ACT
	Probe-/56-FAM/TGAGAACAC/Zen/TCTGTACCATGTGCAAGG/3IABKFQ
PI3Kr1	F: 5′-AAT AGG TTA CAG TGC GGG CCG TAT
	R: 5′-CAG TTT CCT TGG CTT TGC TCG GTT
	Probe-/56-FAM/CCAAGACAC/Zen/CATTACAAAGAAAGCCGGAC/3IABkFQ
PI3Kr2	F: 5′-TGC ATC CAG CAA GAT CCA AGG AGA
	R: 5′-ACC ATC CCG GTG GAA GAC TTT GAT
	Probe-/56-FAM/TCAGGAAAG/Zen/GCGGGAACAACAAGTTG/3IABkFQ
PI3Kr3	F: 5′-TTC AGA CAT TGC TGT GCG GTT GTG
	R: 5′-GCA AGT CTG CCA ACC ATT CCA AGT
	Probe-/56-FAM/GGGAAGGTA/Zen/AGGTGAGAACATTGTTGGG/3IABkFQ
PDX-1	F: 5′-ACT TAA CCT AGG CGT CGC ACA AGA
	R: 5′-GGC ATC AGA AGC AGC CTC AAA GTT
	Probe-/56-FAM/AATTCTTGA/Zen/GGGCACGAGAGCCAGTT/3IABkFQ
Bcl-2	F: 5′-AAT TGT AAT TCA TCT GCC GCC GCC
	R: 5′-ACA CTC CGG CTT CAC TGA GAA TGT
	Probe-/56-FAM/TGCGGTGCT/Zen/CTTGAGATCTCTGGTT/3IABkFQ
Bcl-xL	F: 5′-TTG CTG GTC GCC GGA GAT AGA TTT
	R: 5′-TGG GTC TGC TCT GTG TTT AGC GAT
	Probe-/56-FAM/ACTTATCTT/Zen/GGCTTTGGATCCTGGAAGAGA/3IABkFQ
Primers and probes for quantitative real-time RT-PCRs (rat)	
GAPDH	F: 5′-TGA TGC TGG TGC TGA GTA TGT CGT
	R: 5′-TCT CGT GGT TCA CAC CCA TCA CAA
	Probe-/56-FAM/AGTCTACTG/Zen/GCGTCTTCACCACCAT/3IABkFQ
Cyclin D3	F: 5′-TCA CTG CAT TTG GAT CTG GGT CCT
	R: 5′-AGT TTA CGG GCG CTT GCT CTT CTA
	Probe-/56-FAM/AG CCA ACC T/Zen/A GAT GGC TGC TGT GTA A/3IABkFQ
$p57^{KIP2}$	F: 5′-TCG AAT TCG CCC TTA GCG ATG GAA
	R: 5′-GCA CAT CCT GCT GGA AGT TGA AGT
	Probe-/56-FAM/TC CAG CGA C/Zen/A CCT TCC CAG TGA TA/3IABkFQ
PDX-1	F: 5′-AGT TGG GTA TAC CAG CGA GAT GCT
	R: 5′-TTG TCC TCA GTT GGG AGC CTG ATT
	Probe-/56-FAM/TC TGG GAC T/Zen/C TTT CCT GGG ACC AAT T/3IABkFQ

performed according to the manufacturer's instructions. Band intensity was quantified by the Java-based image-processing program ImageJ.

Quantitative real-time RT-PCR was performed using an EXPRESS One-Step SuperScript quantitative RT-PCR Kit (from Invitrogen) according to the manufacturer's instructions

of *7900HT Real-Time* PCR Systems (Applied Biosystems, Foster City, CA). The mRNA levels were calculated by comparative C_T methods ($X_{\text{Test}}/X_{\text{GAPDH}} = 2^{\Delta\Delta CT}$) with GAPDH as the endogenous reference gene. Primers and probes used in quantitative RT-PCR were designed by PrimerQuest (IDT SciTools, Coralville, IA) as listed in Table 1.

Western Blot Analysis—Islets isolated from 8-week-old C57/BL6 mice were treated with 0.1 or 0.2 μM FTY720 in the absence of FBS. After 24 or 48 h, islets were collected and lysed in RIPA lysis buffer. Aliquots containing 60 μg of protein were separated on 10% SDS-PAGE (34). After electrophoresis, proteins were blotted onto nitrocellulose membranes and probed with rabbit anti-$p57^{KIP2}$ polyclonal antibody or mouse anti-cyclin D3 monoclonal antibody, followed by incubation with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase. Proteins were detected by enhanced chemiluminescence.

Statistical Analysis—Data were expressed as mean ± S.E. Significant differences among groups were evaluated by one-way analysis of variance and Turkey's multiple comparisons test or by unpaired two-tailed Student's *t* test by using PRISM. Significance levels are described in individual figure legends.

RESULTS

Oral Administration of FTY720 to db/db Mice Normalizes Hyperglycemia without Affecting Insulin Sensitivity—We have prescreened several lysophosphatidic acid and S1P analogs in islet β-cells (supplemental Fig. S1). We found that intraperitoneal injection of FTY720 led to relatively normal fasting glucose levels in *db*/*db* mice (supplemental Fig. S2). To rigorously determine the effects of FTY720 *in vivo*, pre-diabetic (age of 6 weeks, fasting glucose <126 mg/dl) and diabetic (age of 8–9 weeks, fasting glucose = 430 mg/dl) female *db*/*db* mice were fed daily with FTY720 (+FTY720) and monitored by weekly fasting blood glucose measurements. Although fasting glucose levels increased significantly in the untreated group (−FTY720) by the age of 8 weeks and continued to increase over time (to about 500 mg/dl by the age of 12 weeks), levels in the FTY720-treated pre-diabetic *db*/*db* mice remained normal (~126 mg/dl) and diabetic *db*/*db* mice also became normal after 6 weeks treatment with FTY720 (Fig. 1*A*). Importantly, this control of fasting glucose levels over time occurred despite the fact that FTY720 administration was changed from daily to weekly and ceased completely at the age of 29 weeks (Fig. 1*A*). In addition, the weight gain, a common side effect of insulin therapy, was significantly higher in the FTY720-treated group than in the untreated group (Fig. 1*B*).

After 6 weeks treatment, the fasting glucose levels in all mice were normalized (Fig. 1*C*) and the fasting serum insulin levels were significantly elevated (Fig. 1*D*). Consistent with these findings, we found that glucose tolerance improved significantly in the FTY720-treated *db*/*db* mice (Fig. 1*E*) as compared with untreated mice but that insulin sensitivity was unaffected (Fig. 1*F*). Specifically, initial fasting glucose levels in the untreated group were about 500 mg/dl but declined rapidly in the first hour after insulin administration; glucose levels in the treated and untreated groups were similar after 70 min. These results indicated that administration of FTY720 to the *db*/*db* mice can normalize fasting blood glucose leading to prevention and reversal of diabetes without affecting insulin sensitivity.

FTY720 Treatment Increases β-Cell Mass in db/db Mice—Because FTY720 treatment increased fasting insulin levels in the *db*/*db* mice and did not affect the glucose-stimulated insulin secretion *ex vivo* (supplemental Fig. S3), we compared islet morphology, size, and insulin content in pancreases from the two groups of animals. Both H&E staining (Fig. 2*A*) and immunofluorescent staining for insulin (Fig. 2*B*) indicated that the untreated *db*/*db* mice experience a deterioration in islet morphology and a reduction in β-cell mass over time. In contrast, the FTY720-treated group exhibited normal islet morphology and large islet size (Fig. 2, *A* and *B*). Stereological quantification demonstrated that the insulin-positive cell area in these mice were significantly larger than those in the untreated group (Fig. 2*C*). Interestingly, it was recently reported that FTY720 does not impair human islets function and affect glucose-stimulated insulin secretion after 48 h treatment (35). Taken together, we conclude that the increase in insulin levels in the FTY720-treated *db*/*db* mice results from the increased β-cell mass in *db*/*db* mice.

FTY720 Treatment Increases β-Cell Survival in db/db Mice—To determine the mechanism behind the increase in β-cell mass observed in the treated mice, first we examined β-cell survival because the S1P signal was previously reported to prevent cytokine-induced apoptosis in cultured islets (36). Although we detected few apoptotic β-cells in the pancreases of the untreated group, most likely due to the rapid clearance of apoptotic cells by phagocytes or adjacent cells *in vivo* (37), we found that levels of anti-apoptotic gene products, Bcl-2 (Fig. 3*A*) and Bcl-xL (Fig. 3*B*), were significantly increased in the islets from the FTY720-treated mice. Along with other report (38), these findings suggest that islet cells in these mice may have an increased survival ability through up-regulation of Bcl-2 and Bcl-xL.

FTY720 Treatment Increases β-Cell Proliferation in db/db Mice—Increases in islet mass occur during pregnancy in humans and animals (16, 17). Here we compared the degree of cell proliferation in islets from the two groups of animals by BrdU incorporation. In contrast to the untreated mice (Fig. 4*A*), BrdU-positive cells were easily observed in the islets of the FTY720-treated mice (Fig. 4, *B*–*D*).

We further examined β-cell proliferation in islets by staining of Ki67, a cellular marker for proliferation (39). Consistent with the results of BrdU incorporation, we did not observed any Ki67-positive cells within and outside islets in the untreated *db*/*db* mice. In contrast, Ki67 staining showed the proliferated cells in the islets, similar to BrdU incorporation in the islets of the FTY720-treated mice (Fig. 5). These data suggest that FTY720 induces β-cell proliferation *in vivo*.

FTY720 Treatment Increases β-Cell Neogenesis in db/db Mice—New β-cells may arise from the proliferation of pancreatic progenitor cells located in the ductal lining (8, 9). We also examined BrdU incorporation in the pancreatic duct area. We were not able to detect any BrdU staining in the islets and ductal line of the untreated mice (Fig. 6*A*). As shown in Fig. 6, *B*–*D*, BrdU-positive cells were easily observed in the pancreatic duct lines of the FTY720-treated mice. Furthermore, the insulin-

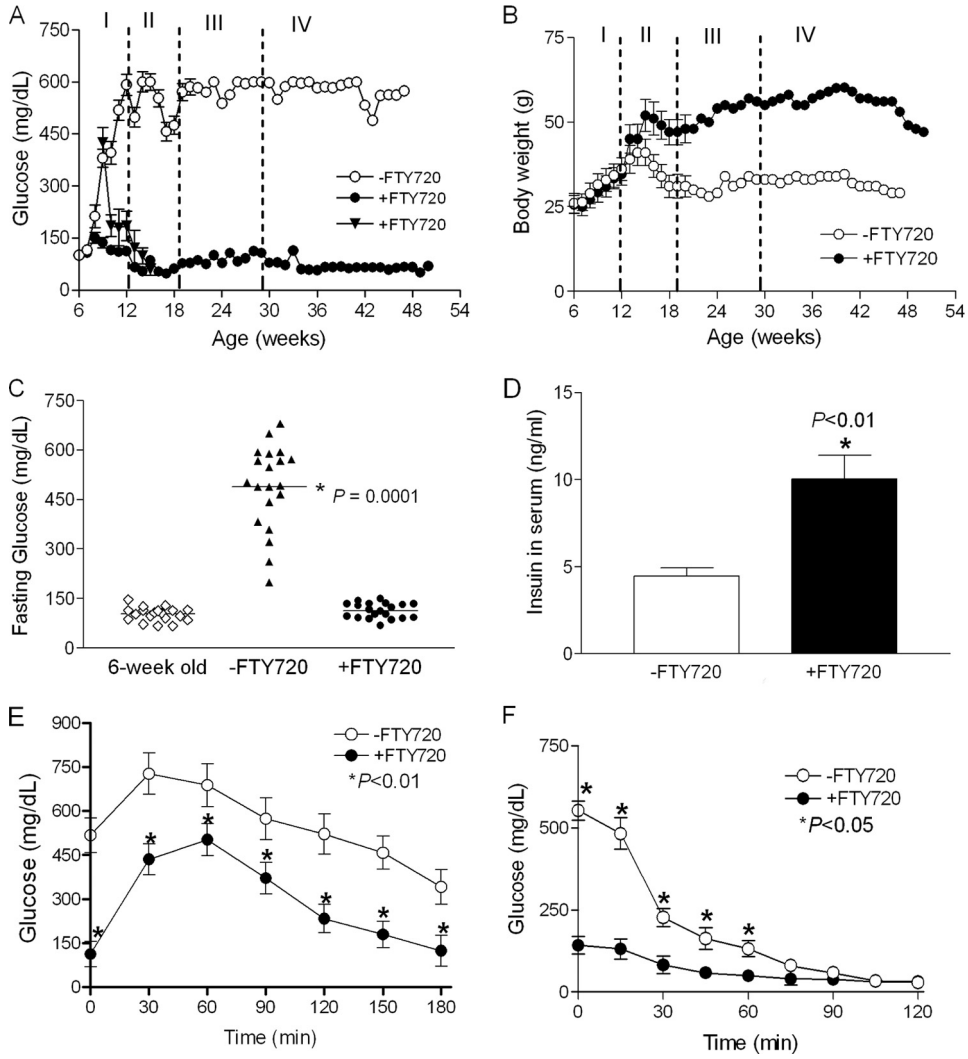

FIGURE 1. **Metabolic features of the FTY720-treated *db*/*db* mice.** *A,* fasting glucose levels. ○, FTY720-untreated group; ●, pre-diabetic *db*/*db* mice (FG < 126 mg/dl) at 6 weeks old treated with 10 mg/kg of FTY720; and ▲, diabetic *db*/*db* mice (FG ≅ 430 mg/dl) at 8–9 weeks old treated with 10 mg/kg of FTY720 (n = 4). *I*, fasting glucose of mice at the age of 6 to 12 weeks at a daily dosage of 10 mg/kg of FTY720 (n = 20 for the control and FTY720 treated pre-diabetic mice, respectively); *II*, fasting glucose levels of mice at the age of 12 to 20 weeks at the daily dosage of 10 mg/kg of FTY720 (n = 6 for the control and FTY720 treated mice, respectively); *III*, fasting glucose levels of mice at the age of 20 to 29 weeks at the weekly dosage of 10 mg/kg of FTY720 (n = 2 for control and FTY720-treated mice, respectively); *IV*, glucose levels from the mouse without FTY720 treatment after the age of 29 weeks (n = 2 for control and FTY720-treated mice, respectively). *B,* body weights. The mice were treated as in *A* and the body weights were measured weekly. *C*, the distribution of fasting glucose levels after 6 weeks treatment (*, $p < 0.0001$, one way analysis of variance test). *D*, fasting serum insulin levels of *db*/*db* mice with or without FTY720 treatment (*, $p < 0.01$). *E*, glucose tolerance test (1 g/kg 10% glucose) (*, $p < 0.01$) and *F*, insulin tolerance test (0.55 units/kg) (*, $p < 0.05$) were performed after 6 weeks treatment.

positive cells or small islets were also identified in the pancreatic duct area of the treated mice (Fig. 6, *E* and *F*). Our data suggested that FTY720 induces β-cell regeneration *in vivo* at least in the obese-diabetes setting.

FTY720 Treatment Down-regulates p57^{KIP2} and Up-regulates Cyclin D3 in db/db Islets—Cell proliferation is controlled by cyclin-dependent kinases, which are regulated positively by cyclins and negatively by inhibitors (CKIs). There are two families of CKIs: INK4 and CIP/KIP. Because p16^{INK4a} is involved in the age-dependent decline in islet regenerative potential (40), we first examined p16^{INK4a} levels in islets isolated from untreated and FTY720-treated *db*/*db* mice. We were unable to detect the expression of p16^{INK4a} at 13 weeks of age, consistent with previous reports that the gene is repressed in young mice (41). Next we examined the CIP/KIP family, including p21^{CIP1}, p27^{KIP1}, and p57^{KIP2} (42). Of these, p57^{KIP2} was the most highly expressed in *db*/*db* islets from untreated mice (Fig. 7*A*); conversely, its expression was significantly down-regulated in the islets from the FTY720-treated *db*/*db* mice. In contrast, the expression of p21^{CIP1} and p27^{KIP1} did not vary significantly between the two groups. Interestingly, most of the p57^{KIP2}-expressing cells are, like β-cells, terminally differentiated (42). Therefore, our results suggest that down-regulating p57^{KIP2} may allow these cells to re-enter the cell cycle from the differentiated state and begin proliferating.

Furthermore, we examined the expression of the cyclin D family members cyclin D1, cyclin D2, and cyclin D3. Of these, cyclin D3 was expressed at the lowest levels in islets from untreated *db*/*db* mice (Fig. 7*B*). Although the expression of cyclin D1 and D2 was elevated in islets from the FTY720-

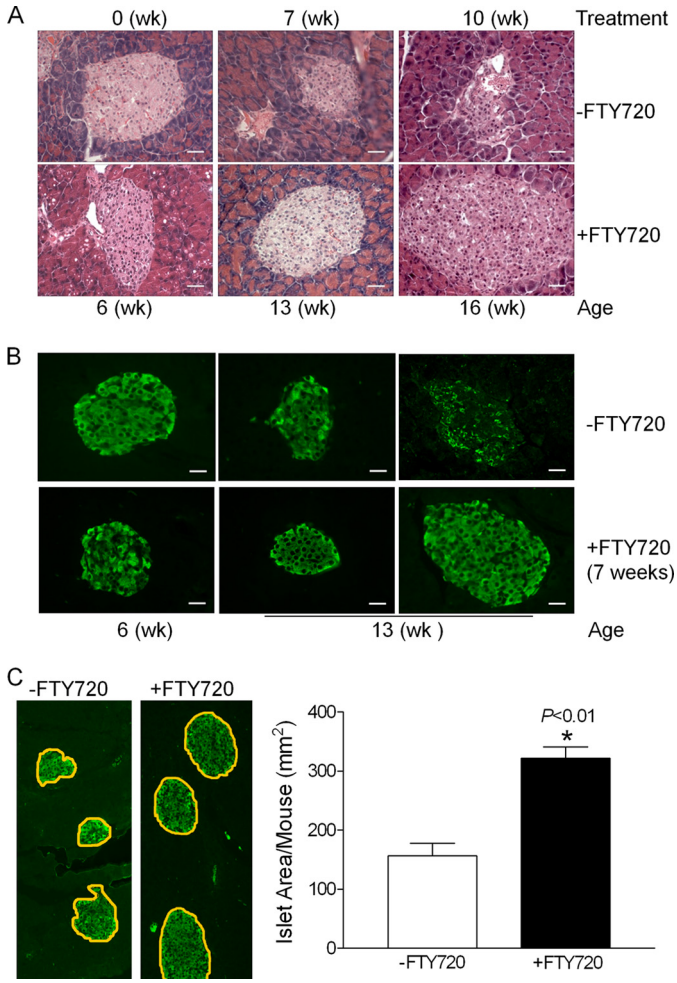

FIGURE 2. **Effect of FTY720 on β-cell mass of *db*/*db* mice.** *A*, H&E staining of pancreas from the untreated and FTY720-treated mice after 6 and 9 weeks treatment (*scale bar*, 50 μm). *B*, insulin immunostaining (green fluorescence) of pancreas after 6 weeks FTY720 treatment (*scale bar*, 50 μm). *C*, stereological quantification of the insulin positive areas in the control and FTY720-treated *db*/*db* mice. Six consecutive paraffin sections (10 μm) from each pancreas (10 pancreases from the untreated and 9 from the FTY720-treatment *db*/*db* mice) were used for islet area measurements. *, $p < 0.01$.

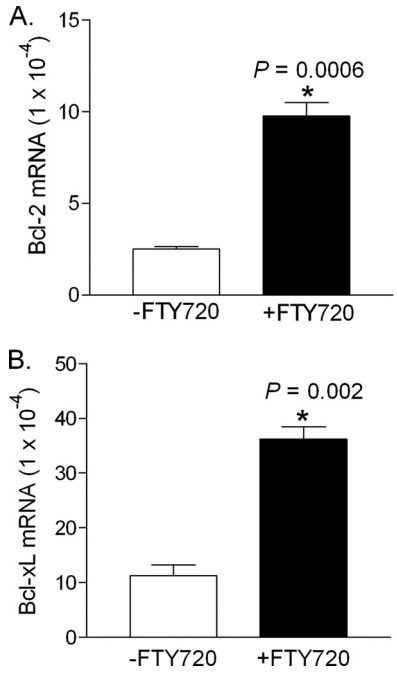

FIGURE 3. **Effect of FTY720 on the expression of Bcl-2 and Bcl-xL in islets.** Total RNAs were extracted from the islets isolated from the control and FTY720-treated mice at the age of 13 weeks and Bcl-2 (*A*) and Bcl-xL mRNA (*B*) in the *db*/*db* islets were quantitated by real time RT-PCT. *, $p < 0.01$.

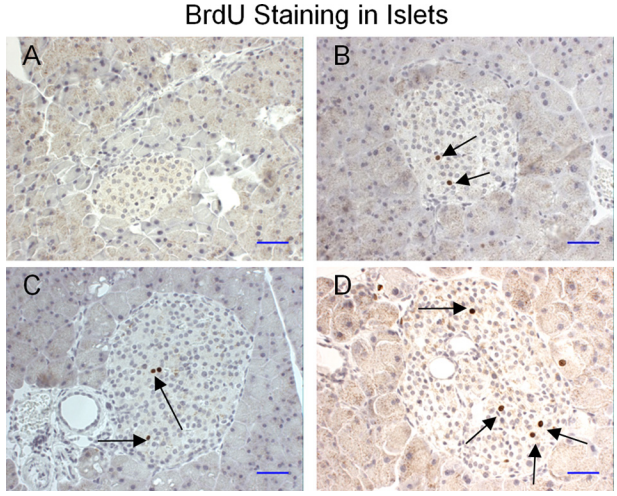

FIGURE 4. **BrdU staining in pancreatic islets of the *db*/*db* pancreas.** *A*, BrdU staining of islets in control *db*/*db* mice. *B–D*, BrdU staining of islets in FTY720-treated *db*/*db* mice. BrdU-positive cells (*brown*) were indicated by *black arrows* (*scale bar*, 100 μm).

treated *db*/*db* mice when compared with the untreated mice, the expression of cyclin D3 increased the most. Immunohistochemical analysis showed a dramatic increase in cyclin D3 expression in several islet cells (Fig. 7*C*) similar to that of BrdU incorporation (Fig. 4) and Ki67 staining (Fig. 5).

Finally, treatment of freshly isolated wild type islets with FTY720 or FTY720-P caused a dramatic down-regulation in $p57^{KIP2}$ expression and a corresponding up-regulation in cyclin D3 expression in both proteins (Fig. 8*A*) and mRNAs (Fig. 8, *B* and *C*). These *in vivo* and *ex vivo* findings reveal an important role for $p57^{KIP2}$ and cyclin D3 in regulation of the *in vivo* β-cell regeneration by FTY720.

FTY720 Treatment Up-regulates S1P Receptors in db/db Islets—It is well characterized that FTY720 is phosphorylated *in vivo* to form FTY720-P that binds $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ (25). We determined the expression of these receptors in the islets isolated from the untreated and FTY720-treated *db*/*db* mice. As shown in Fig. 9*A*, $S1P_1$ and $S1P_3$ were predominantly expressed in the *db*/*db* islets, whereas $S1P_4$ and $S1P_5$ were hardly detected. However, after FTY720 treatment of *db*/*db* mice, $S1P_1$, $S1P_3$, and $S1P_4$ but not $S1P_5$ were significantly elevated in the islets. Consistent with the findings *in vivo*, treatment of freshly isolated wild type islets with FTY720 caused a significant increase of $S1P_1$, $S1P_3$, and $S1P_4$ but not $S1P_5$ (Fig. 9*B*).

We further evaluated the role of S1P receptors in regulation of cyclin D3 and $p57^{KIP2}$ in INS-1 cells. Consistent with the findings *in vivo* and *ex vivo*, FTY720 or FTY720-P treatment significantly increased cyclin D3 expression in INS-1 cells, which was reversed significantly by CAY10444 (also known as BML-241), a selective antagonist of $S1P_3$ (43), or by W123, a selective antagonist of $S1P_1$ (44) (Fig. 9*C*). Furthermore,

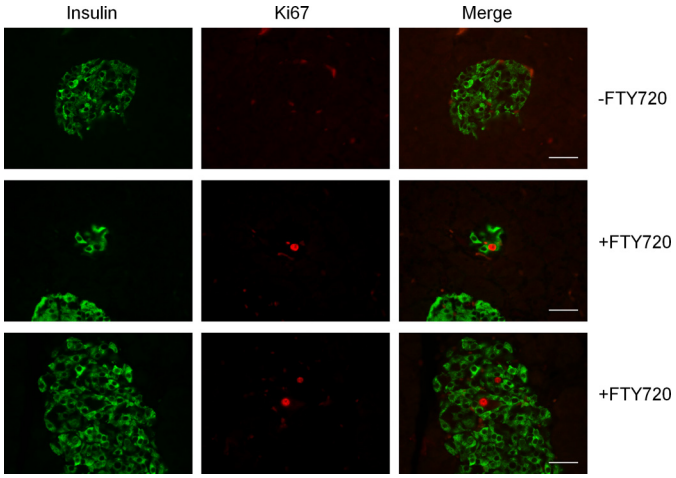

FIGURE 5. **Ki67 staining in pancreatic islets of the *db*/*db* pancreas.** The pancreatic sections were double-stained for insulin (*green*) and Ki67 (*red*). The Ki67-stained nucleus of the cell was identified within small and large islets of the FTY720-treated *db*/*db* mice (*scale bar*, 50 μm).

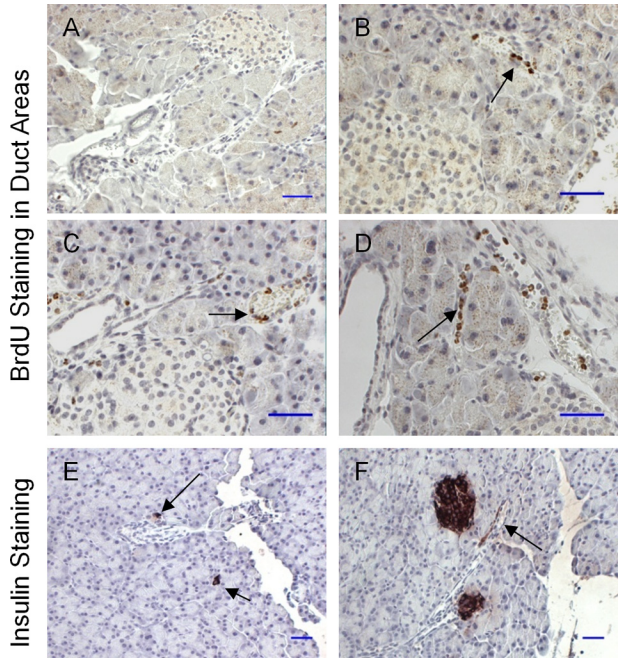

FIGURE 6. **BrdU staining in the pancreatic duct areas of the *db*/*db* pancreas.** *A*, BrdU staining of pancreas from control *db*/*db* mice. *B–D*, BrdU staining of pancreas from the FTY720-treated *db*/*db* mice. BrdU-positive cells (*brown*) in the duct lines are indicated by *black arrows* (*scale bar*, 100 μm). *E* and *F*, insulin immunostaining. *Black arrows* indicate the newly formed islets near the duct area (*arrow*) and insulin positive ductal line (*scale bar*, 100 μm).

FTY720 or FTY720-P treatment significantly decreased $p57^{KIP2}$ expression in INS-1 cells, which was reversed by CAY10444 or W123 (Fig. 9*D*). Interestingly, FTY720(R)-P, one of the stereoisomers of FTY720-P that is apparently not formed *in vivo* (45), did not affect cyclin D3 significantly but dramatically increased $p57^{KIP2}$ expression (Fig. 9, *C* and *D*). These results suggest that at least $S1P_1$ and $S1P_3$ are involved in the FTY720-stimulated β-cell regeneration *in vivo*.

FTY720 Treatment Activates PI3K Signaling Pathway in db/db Islets—The PI3K/Akt pathway is known to play an important role in S1P/GPCR-mediated cellular responses, including survival and proliferation (46, 47). We determined

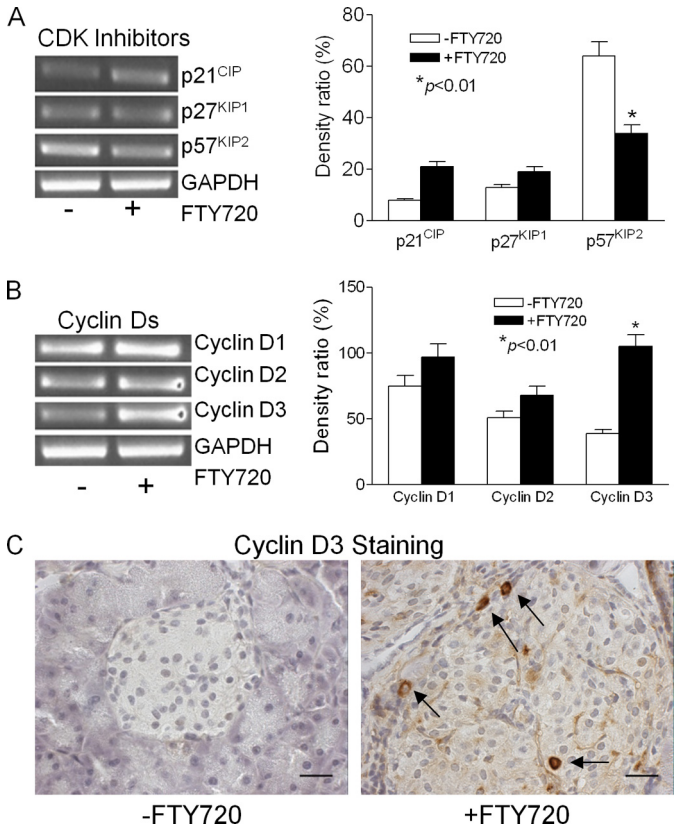

FIGURE 7. **Effect of FTY720 on the expression of cyclin Ds and cyclin-dependent kinase (*CDK*) inhibitors in *db*/*db* islets.** *A*, the mRNA levels of cyclin-dependent kinase inhibitors. *, $p < 0.01$. *B*, the mRNA levels of cyclin Ds. *, $p < 0.01$. Total RNAs were extracted from islets isolated from control and FTY720-treated mice at the age of 13 weeks, and the mRNA levels of interested targets were analyzed by RT-PCR. *C*, cyclin D3 immunostaining of pancreas after 6 weeks FTY720 treatment. The cells in the islet with cyclin D3-staining in their nucleus (*brown*) are indicated by *black arrows* (*scale bar*, 50 μm).

the expression of three major PI3K regulatory subunits, *PIK3r1* (encodes p85α, p55α, and p50α through differential promoter usage), *PIK3r2* (encodes p85β), and *PIK3r3* (encodes p55γ) (48), in the islets isolated from the untreated and FTY720-treated *db*/*db* mice. As shown in Fig. 10*A*, *PI3Kr1* was moderately expressed and increased by FTY720 *in vivo*. *PI3Kr2* and *PI3Kr3*, however, were undetectable in the islets from the untreated *db*/*db* mice and dramatically elevated in the islets from the FTY720-treated *db*/*db* mice, suggesting the involvement of PI3K. There was no difference in Akt1 and Akt2 between the treated and untreated groups because Akts are regulated by protein phosphorylation. It is known that expression of pancreatic and duodenal homeobox 1 (PDX-1), a transcription factor necessary for pancreatic development and β-cell maturation (49), is regulated by PI3K activation during pancreatic regeneration (50, 51). We found that the PDX-1 expression in the islets from the FTY720-treated *db*/*db* mice was 6-fold higher than that in the islets from the untreated mice (Fig. 10*B*), consistent with PI3K activation.

Furthermore, treatment of the wild type islets with FTY720 or FTY720-P dramatically increased the expression of *PI3Kr1* and *PI3Kr3* (Fig. 10, *C* and *E*) but not PI3Kr2, probably due to its high expression (about 100-fold higher than *PI3Kr1* and *PI3Kr3*) in the wild type islets (Fig. 10*D*). These results suggest

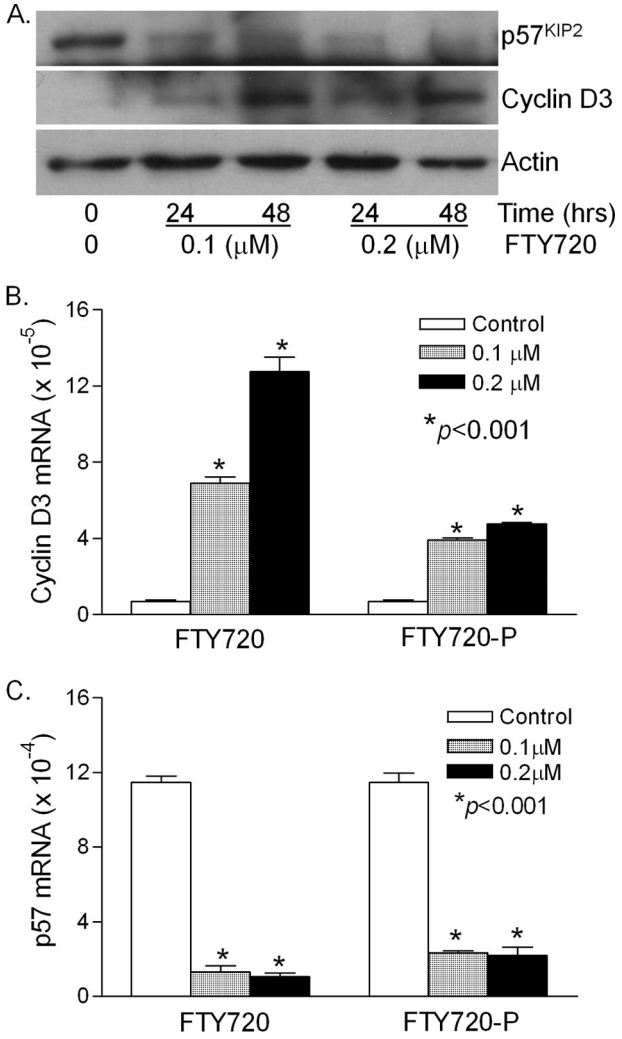

FIGURE 8. **Effect of FTY720 on p57^{KIP2} and cyclin D3 expression in the wild type islets.** *A*, Western blot analysis. Islets were isolated from 8-week-old wild type C57/BL6 mice and treated with 0.1 or 0.2 μM FTY720 in cell culture medium without FBS. After 24 and 48 h, protein was extracted and analyzed by Western blot analysis. *B*, quantitation of cyclin D3 mRNA and *C*, p57^{KIP2} mRNA levels. Isolated islets were treated with 0 (control), 0.1 or 0.2 μM FTY720 or FTY720-P for 48 h, and total RNAs were extracted and quantitative real-time RT-PCR was performed. *, $p < 0.001$, *versus* control.

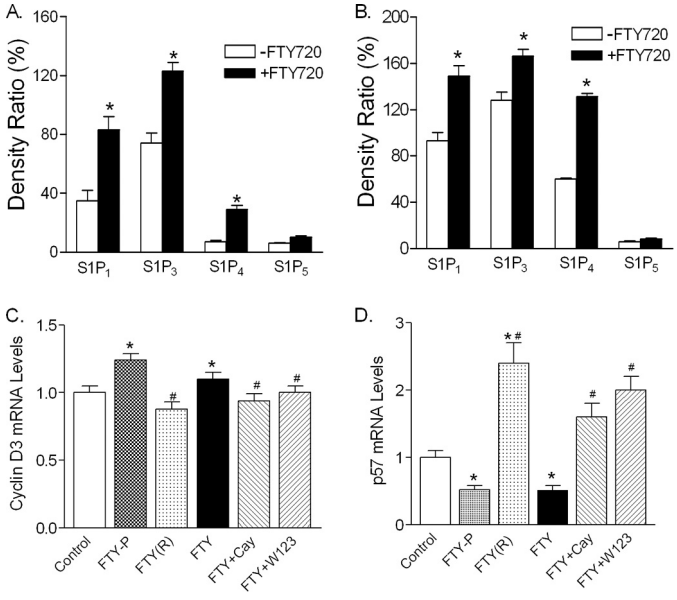

FIGURE 9. **Effect of FTY720 on the expression of S1P receptors in islet β-cells.** *A*, expression of S1P receptors *in vivo*. The total RNAs were extracted from islets isolated from the untreated and FTY720-treated *db*/*db* mice at the age of 13 weeks, and the mRNA levels of S1P receptors were analyzed by RT-PCR and the band intensities were quantified by ImageJ ($n = 5$), *, $p < 0.05$. *B*, expression of S1P receptors *ex vivo*. Isolated wild type islets were treated with or without 0.2 μM FTY720 for 24 h and total RNAs were extracted and analyzed by RT-PCR for S1P receptors and the band intensities were quantified by ImageJ, *, $p < 0.05$. *C*, quantitation of cyclin D3 and *D*, p57^{KIP2} mRNA levels. INS-1 cells were treated with FTY720-P (FTY-P, 0.1 μM), FTY720(R)-P (FTY(R), 10 nM), FTY720 (FTY, 0.1 μM), FTY720 + Cay10444 (Cay, 10 μM), and FTY720 + W123 (20 μM) for 24 h and total RNAs were extracted and quantitative real-time RT-PCR was performed. The levels relative to the control were plotted. *, $p < 0.05$, *versus* control; #, $p < 0.05$, *versus* FTY720 or FTY720-P-treated groups.

that S1P signaling may mediate PI3K function partly through regulating expression of the PI3K regulatory subunits.

To substantiate the role of PI3K in the FTY720-stimulated β-cell regeneration, INS-1 cells were treated with FTY720, FTY720-P, or S1P, respectively, in the presence or absence of wortmannin, a specific PI3K inhibitor (52). We found that wortmannin completely blocked the increase of PDX-1 by FTY720, FTY720-P, or S1P (Fig. 10*F*). Moreover, wortmannin essentially reversed the effects of FTY720, FTY720-P, and S1P on cyclin D3 (Fig. 10*G*) and p57^{KIP2} (Fig. 10*H*). Our data strongly suggest that FTY720-stimulated *in vivo* β-cell regeneration is PI3K-dependent.

DISCUSSION

In this study, we showed that orally administered FTY720 can normalize blood glucose levels of *db*/*db* mice by preserving β-cell mass *in vivo* through increasing in survival ability and regeneration of β-cells, which in turn contributes to the well controlled fasting blood glucose levels observed in FTY720-treated *db*/*db* mice. Moreover, we demonstrated that FTY720 treatment of *db*/*db* mice up-regulates the expression of PDX-1 and cyclin D3 and down-regulates p57^{KIP2} in the islets through S1P receptors in a PI3K-dependent manner.

FTY720 is a structural analog of sphingosine and becomes active *in vivo* following phosphorylation by sphingosine kinase 2 to form FTY720(S)-P, which binds to four of the five S1P receptors (25, 53). We found that expression of $S1P_1$, $S1P_3$, and $S1P_4$ was significantly increased in the islets from the FTY720-treated *db*/*db* mice and the effects of FTY720 are attenuated by S1P receptor antagonists, suggesting the involvement of S1P signaling in the *db*/*db* islets.

Intriguingly, it was recently reported that S1P signaling plays a key role in adiponectin-mediated survival of pancreatic β-cells, nutrient uptake, nutrient utilization, and mitochondrial proliferation (54, 55). Our findings suggest that FTY720 when administered to *db*/*db* mice acts on an intrinsic pathway that is physiologically important for β-cell survival and regeneration under metabolic stress.

Our findings identify a novel way to control β-cell regeneration by down-regulating p57^{KIP2} and up-regulating cyclin D3 expression through S1P/GPCR signaling. p57^{KIP2} is an imprinted gene that is conserved between rodents and humans and is required for normal development and differentiation (42). It has been reported that p57^{KIP2} is paternally imprinted and highly expressed in human pancreatic β-cells (56), and con-

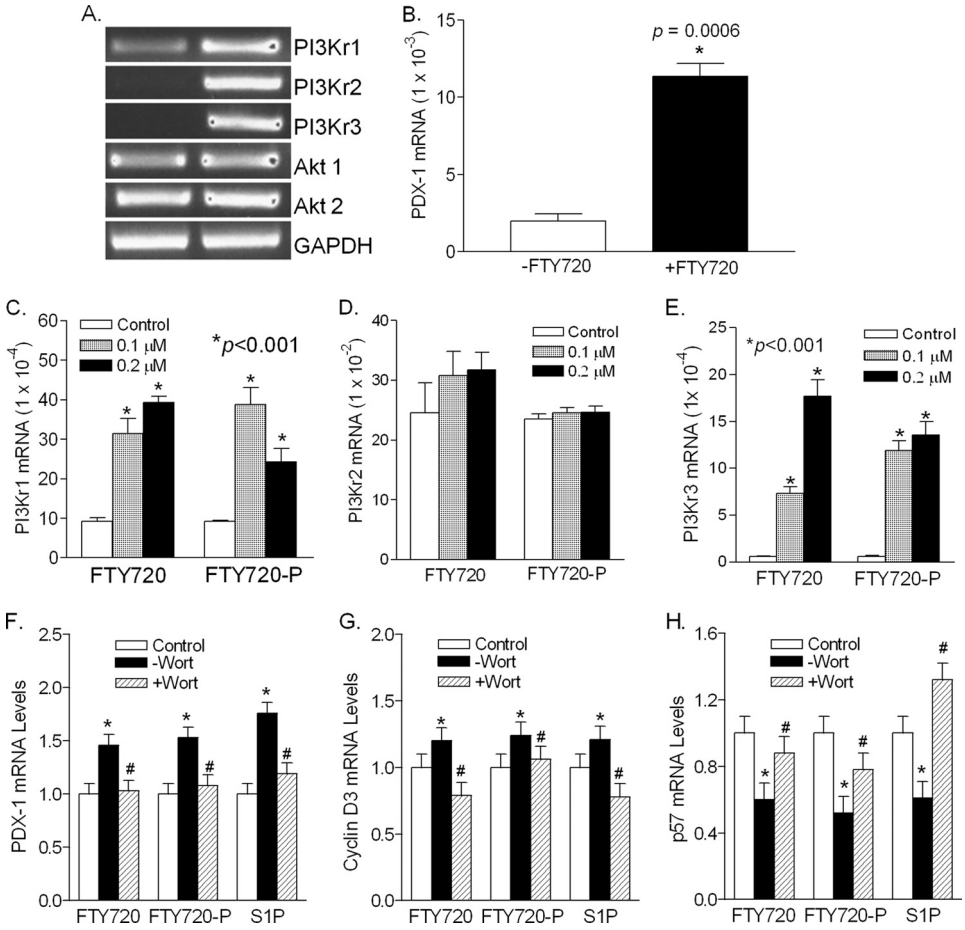

FIGURE 10. **Effect of FTY720 on the expression of PI3K regulatory subunits in the islet β-cells.** Expression of PI3K regulatory subunits (*A*) and PDX-1 (*B*) *in vivo*. The total RNAs were extracted from the islets isolated from the untreated (−FTY720) and FTY720-treated *db*/*db* mice at the age of 13 weeks, and the mRNA levels of PI3K regulatory subunits, PI3Kr1, PI3Kr2, and PI3Kr3, as well as Akt1 and Akt2, were amplified by RT-PCR and visualized by agarose gel electrophoresis. The PDX-1 mRNA levels were quantified by quantitative RT-PCR. *, *versus* −FTY720 group. Expression of PI3Kr1 (*C*), PI3Kr2 (*E*), and PI3Kr3 in wild type islets. Isolated islets were treated with 0 (control), 0.1 or 0.2 μM FTY720 or FTY720-P for 48 h and total RNAs were extracted, and quantitative real time RT-PCR was performed. *, $p < 0.001$, *versus* control. Effects of wortmannin on the expression of (*F*) PDX-1, (*G*) cyclin D3, and (*H*) $p57^{KIP2}$ in INS-1 cells. INS-1 cells were treated with FTY720 (FTY, 0.1 μM), FTY720-P (FTY-P, 0.1 μM), or S1P (0.1 μM) in the presence (+*Wort*) or absence (−*Wort*) of wortmannin (0.1 μM) for 24 h and total RNAs were extracted and quantitative real-time RT-PCR was performed. The levels relative to the control were plotted. *, $p < 0.05$, *versus* control, #, $p < 0.05$, *versus* the group without wortmannin.

trols both self-renewal and exit from the cell cycle of pancreatic progenitors during pancreatic development (57). $p57^{KIP2}$ expression, but not $p21^{CIP1}$ or $p27^{KIP1}$, is induced by transcription factor E47, which is involved in the transition from proliferation to cell cycle exit (58). Down-regulation of $p57^{KIP2}$ by FTY720 may reverse this transition *in vivo*. Interestingly, cyclin D3, which is required for normal expansion of immature T lymphocytes (59), is a target of $p57^{KIP2}$ inhibition (42). Our findings suggest that orchestrating $p57^{KIP2}$ and cyclin D3 expression may be critical to inducing β-cell regeneration *in vivo*.

S1P receptors are a group of GPCRs, and S1P/GPCR signaling has been reported to control cell proliferation and survival through activation of AMP-activated protein kinase or PI3K/Akt pathways (46, 60). We found that *PI3Kr2* and *PI3Kr3*, which are highly expressed in normal islets, were hardly detected in *db*/*db* islets and dramatically increased after FTY720 treatment, which is accompanied with a 6-fold increase of PDX-1. Inhibition of PI3K essentially reverses the effect of FTY720 on PDX-1, cyclin D3, and $p57^{KIP2}$, suggesting that PI3K is required for FTY720-stimulated β-cell regeneration *in vivo*.

Based on these findings and others (54, 55), we propose the mechanism for S1P signaling in β-cell regeneration and survival *in vivo* as elucidated in Fig. 11. In this model, S1P signaling is a core component of the adiponectin-mediated pleiotropic actions (54) and FTY720 acts on this core, which is physiologically important for β-cell regeneration and survival under metabolic stress.

Although FTY720 promotes β-cell regeneration, the risk of tumorigenesis is low. Concentrations of FTY720 identical to those used in this study (10 mg/kg) reportedly inhibit growth, migration, and invasion of pancreatic cancer cells (61). The compound has also been used in phase III clinical trials in patients with relapsing multiple sclerosis with a few reported cancer formation (26).

Is the FTY720-induced β-cell regeneration observed in *db*/*db* mice relevant to humans? β-Cell volume in obese humans with non-diabetes is 50% higher than that in normal lean subjects (2, 15) and increases in islet mass occur during pregnancy in humans (16, 17), suggesting that the mechanism in humans for β-cell mass expansion may be triggered by increased metabolic demand. Surprisingly, in contrast to the

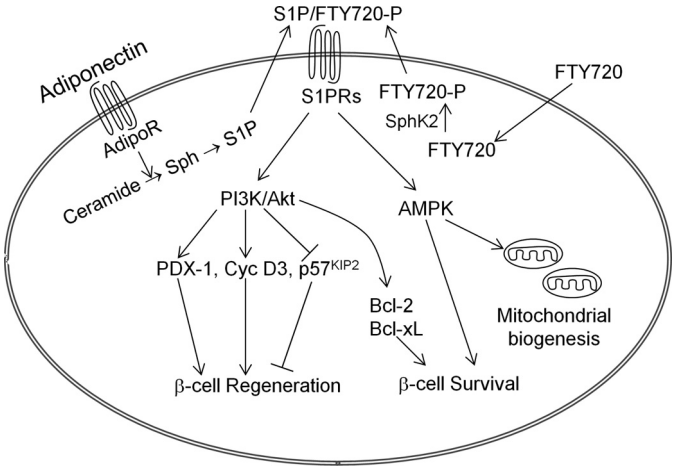

FIGURE 11. **Proposed mechanism for FTY720-stimulated β-cell regeneration *in vivo*.** S1P signaling is a core component of the adiponectin-mediated pleiotropic actions (54). Binding of adiponectin to its receptors enhances deacylation of ceramide to sphingosine, which can then be phosphorylated to form S1P (54). FTY720, a structural analog of sphingosine, is phosphorylated *in vivo* to form FTY720-P (25). Inside-out signaling of S1P/FTY720-P via S1P receptors activates the PI3K/Akt pathway, which leads to up-regulation of PDX-1 and cyclin D3 and down-regulation of $p57^{KIP2}$, and subsequently β-cell regeneration *in vivo*. FTY720 also promotes cell survival through activation of PI3K/Akt (38) and expression of Bcl-2 and Bcl-xL. In addition, S1P receptor signaling plays a key role in adiponectin-mediated AMP-activated protein kinase activation, which is important for mitochondrial biogenesis and β-cell survival (54). Thus, FTY720 acts on an intrinsic pathway that is physiologically important for β-cell regeneration and survival under metabolic stress. *Sph*, sphingosine; *S1PRs*, S1P receptors; *AdipoR*, adiponectin receptors.

adult mouse, in which β-cell progenitors can be activated by pancreatic injury (8), partial pancreatectomy in adult humans does not provoke β-cell regeneration (62). These findings suggest that metabolic demand, but not pancreatic injury, can trigger human β-cell regeneration. It has been reported that $p57^{KIP2}$ is highly expressed in human pancreatic β-cells (56) and controls both self-renewal and exit from the cell cycle of pancreatic progenitors during pancreatic development (57). High expression of $p57^{KIP2}$ may inhibit β-cell regeneration in humans, unless $p57^{KIP2}$ expression is reduced by some means and allows cells to re-enter the cell cycle (57).

In addition, human islets contain abundant cyclin D3, but a variable amount of cyclin D1 and little cyclin D2 (63). In contrast to mice in which cyclin D2 is required for β-cell expansion (64), overexpression of cyclin D3 and cdk6 effectively drives human β-cell replication (63), suggesting that increasing in cyclin D3, but not cyclin D1 and D2, is essential for human β-cell replication. It is worth noting that FTY720 does not have any toxic effects on human islets *in vitro* and *in vivo* after human islets were transplanted into the chemically induced diabetic immunodeficient mice (35). Therefore, it is possible that FTY720 that down-regulates $p57^{KIP2}$ and up-regulates cyclin D3 expression in *db*/*db* islets may also increase islet mass in humans with metabolic demand, such as obesity and type 2 diabetes patients. Further study in human islets will validate the potential role of FTY720 in stimulating β-cell proliferation in type 2 diabetes.

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