Glucagon-Like Peptide-1 Increases Beta Cell Regeneration by Promoting Alpha- to Beta-Cell Transdifferentiation

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) can increase pancreatic β-cells, and α-cells could be a source for new β-cell generation. We investigated whether GLP-1 increases β-cells through α-cell transdifferentiation. New β-cells originating from non-β cells were significantly increased in recombinant adenovirus expressing GLP-1 (rAd-GLP-1)-treated RIP-CreER;R26-YFP mice. Proliferating α-cells were increased in islets of rAd-GLP-1-treated mice and αTC1-9 cells treated with exendin-4, a GLP-1 receptor agonist. Insulin$^+$glucagon$^+$ cells were significantly increased by rAd-GLP-1 or exendin-4 treatment *in vivo* and *in vitro*. Lineage-tracing to label the glucagon-producing α-cells showed a higher proportion of regenerated β-cells from α-cells in rAd-GLP-1-treated Glucagon-rtTA;Tet-O-Cre;R26-YFP mice than rAd-βgal-treated mice. In addition, exendin-4 increased the expression and secretion of fibroblast growth factor (FGF)21 in αTC1-9 cells and β-cell-ablated islets. FGF21 treatment of β-cell-ablated islets increased the expression of PDX-1 and neurogenin-3 and significantly increased insulin$^+$glucagon$^+$ cells. Generation of insulin$^+$glucagon$^+$ cells by exendin-4 was significantly reduced in islets transfected with FGF21 small interfering RNA or islets of FGF21 knockout mice. Generation of insulin$^+$ cells by rAd-GLP-1-treatment was significantly reduced in FGF21 knockout mice compared with wild-type mice. We suggest that GLP-1 has an important role in α-cell transdifferentiation to generate new β-cells, which might be mediated, in part, by FGF21 induction.

Keywords α-cell · β-cell · pancreas · islet · glucagon-like peptide-1 · FGF21 · insulin · glucagon · PDX-1 · Ngn3 · transdifferentiation

Introduction

Diabetes is caused by defective control of blood glucose levels resulting from an absolute or relative deficiency of functional pancreatic β-cells. Type 1 diabetes is characterized by absolute deficiency of insulin, due to autoimmune-mediated destruction of pancreatic β-cells, whereas type 2 diabetes is characterized by relative deficiency of insulin, due to insufficient insulin secretion to compensate for insulin resistance. Thus, strategies to manage diabetes by restoring functional β-cells are under investigation.

Glucagon-like peptide-1 (GLP-1), which is secreted from intestinal L-cells in response to nutrient ingestion, is known to have important physiological roles: it potentiates glucose-stimulated insulin secretion, induces insulin gene transcription and insulin biosynthesis, enhances β-cell proliferation and inhibits β-cell apoptosis (1; 2). In addition, GLP-1 has effects on the regeneration, differentiation, and neogenesis of pancreatic β-cells (3-6). In 70% pancreatectomized mice, the β-cell mass was significantly lower in GLP-1 receptor-knockout (KO) mice compared with wild-type mice, suggesting a potential role of GLP-1 in regulation of the β-cell mass (7).

In the pancreatic islets, α-cells primarily produce the hormone glucagon. However, recent observations indicate that α-cells can also produce GLP-1 (8-10). Another important role of α-cells is their ability to transdifferentiate into β-cells under conditions of extreme damage to β-cells (11). As well, increased physiological demand for insulin can result in increased α-cell proliferation (12). These results suggest that α-cells can be a source of newly generated insulin-producing cells and that GLP-1 may act as a stimulus through autocrine signaling (13).

Fibroblast growth factor (FGF)21 is a circulating protein that is highly expressed in the liver, and FGF21 protein expression is also detected in both α- and β-cells in the pancreas (14). Constant infusion of FGF21 for 8 weeks in *db/db* mice results in a higher number of islets per

pancreatic section and a higher number of insulin-positive cells per islet without β-cell proliferation compared with control mice (15). As well, glucagon receptor-KO mice display α-cell hyperplasia, and there is a correlation with the increase of plasma FGF21 in these mice (14). In addition, a GLP-1 analogue increases FGF21 expression and FGF21 activity in insulin resistant mice (16). Therefore, we hypothesized that GLP-1 might increase FGF21 production and FGF21 might play a role in the generation of new β-cells from α-cells by GLP-1. In the present study, we sought to determine the effects of GLP-1 on α-cells to generate new β-cells and to examine the mechanisms involved.

Research design and methods

Animals. RIP-CreER mice (a gift of Dr. D.Melton), Glucagon-rtTA mice (a gift of Dr. Pedro L Herrera), Tet-O-Cre mice (The Jackson Laboratory, Bar Harbor, ME, USA), ROSA26 (R26)-yellow fluorescent protein (YFP) mice (The Jackson Laboratory) and FGF21 KO mice (provided by Eli Lilly and Company) were used. We generated two types of mice: RIP-CreER;R26-YFP mice for tracing the β-cell lineage and Glucagon-rtTA;Tet-O-Cre;R26-YFP mice for tracing the α-cell lineage. These mice were maintained at the facility at Gachon University under a 12 h light:12 h dark photoperiod. Animals were fed *ad libitum* on a standard rodent diet. All animal experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Lee Gil Ya Cancer and Diabetes Institute, Gachon University.

Production of recombinant adenovirus producing GLP-1(7-37). Recombinant adenovirus (rAd) producing GLP-1 (rAd-GLP-1) or rAd producing β-galactosidase (rAd- βgal), as a control, were produced as previously described (17). The recombinant adenoviruses were produced and

amplified in a human embryonic kidney cell line (HEK-293). After purification of virus by CsCl-gradient ultracentrifugation, viral titer was determined by 50% tissue culture infectious dose.

4-Hydroxytamoxifen, doxycycline, streptozotocin, rAd-GLP-1 and BrdU treatment. Four week-old male RIP-CreER;R26-YFP mice were injected with 4-hydroxytamoxifen (Sigma, St. Louis, USA, 1 mg/mouse) daily for 5 days every other week, which was repeated 4 times. Doxycycline (1.5 mg/ml, sigma) was added to drinking water of four week-old male Glucagon-rtTA;Tet-O-Cre;R26-YFP mice for 2 weeks. After doxycycline removal, mice were kept for 14 days without treatment before streptozotocin (STZ) administration. β-cell destruction was achieved in RIP-CreER;R26-YFP mice, Glucagon-rtTA;Tet-O-Cre;R26-YFP mice and FGF21 KO mice by i.p. injection of STZ (Sigma, 150 mg/kg), a β-cell specific toxin. The mice were monitored for the development of hyperglycemia using a glucometer. STZ-induced diabetic mice (blood glucose levels > 300 mg/dl for 3 consecutive days) were injected via the tail vein with rAd-GLP-1 or rAd-βgal (3×10^9 plaque-forming units (PFU)). After viral injection, RIP-CreER;R26-YFP mice were injected with 5-bromodeoxyurine (BrdU; Sigma, 100 mg/kg) every day for 4 weeks.

Glucose tolerance tests. Mice were not fed for 4 h and a glucose solution (2 g/kg body weight) was injected intraperitoneally. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after glucose injection.

Immunohistochemical analysis. RIP-CreER;R26-YFP mice, Glucagon-rtTA;Tet-O-Cre;R26-YFP mice, and FGF21 KO mice were sacrificed at 4 weeks after rAd-GLP-1 or rAd-βgal treatment. Pancreata were removed, fixed in 10% formalin, and embedded in paraffin. More than

500 serial sections (4 μm thick) were prepared from each pancreas and every 25th section was used for immunohistochemical analysis. The tissue sections were boiled (100 °C for 10 min, 10 mM sodium citrate, pH 6.0) for antigen retrieval, and blocked with blocking solution (DAKO, Carpinteria, CA). The sections were then incubated with primary antibody solution: guinea-pig anti-insulin (DAKO, 1:100), rabbit anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100), rabbit anti-glucagon (DAKO, 1:100), mouse anti-glucagon (Sigma, 1:100), rabbit anti-green fluorescent protein (GFP), which cross-reacts with YFP (Abcam, Cambridge, UK, 1:50, Invitrogen, Vancouver, BC 1:100), or mouse anti-BrdU (DAKO, 1:50). Fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea-pig IgG (Santa Cruz Biotechnology, 1:200), Texas Red (TR)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology,1:200) or Alexa-Fluor-633-conjugated goat anti-guinea-pig IgG (Thermo Fisher Scientific, Rockford, IL, 1:200) were used as secondary antibodies. Fluorescence was imaged using a laser scanning confocal fluorescent microscope (LSM 700, Carl Zeiss MicroImaging, Jena, Germany) and colocalization was analyzed by ZEN 2009 Analysis Program.

Islet isolation and immunocytochemistry. Pancreatic islets were isolated from 4-6-week-old male FGF21 KO or C57BL/6 mice as described previously (18). Intact islets were dissociated at 37 °C in Accutase (Millipore, Bilerica, MA, USA), treated with STZ (1 mM) for 15 h, washed, and then cultured with exendin-4 (Sigma, 10 nM) or FGF21 (Sigma, 50 nM) in RPMI media. The islet cells were fixed in 4% paraformaldehyde, permeabilized in permeabilization buffer (Thermo Fisher Scientific), blocked in blocking solution (Thermo Fisher Scientific) and then incubated with mouse anti-glucagon (Sigma, 1:100), guinea-pig anti-insulin (DAKO, 1:100) or rabbit anti-pancreatic and duodenal homeobox (PDX)-1 (Cell Signaling Technology, Beverly MA, 1:25) antibodies. FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:200),

TR-conjugated goat anti-guinea-pig IgG (Santa Cruz Biotechnology, 1:200), or TR-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, 1:200) were used as secondary antibodies. Fluorescence was imaged using a laser scanning confocal fluorescent microscope (LSM 700).

αTC1-9 cell culture conditions. The αTC1 clone 9 (αTC1-9) is a pancreatic α-cell line that produces glucagon, but not preproinsulin mRNA (19; 20). Cells obtained from American Type Culture Collection (ATCC, CRL-2350) and were grown in Dulbecco's Modified Eagle Medium (DMEM) with 16.7 mM glucose supplemented with 10% heat-inactivated dialyzed fetal bovine serum, 15 mM HEPES, 0.1 mM nonessential amino acids and 0.02% BSA under an atmosphere of 95% humidified air-5% CO_2 at 37 °C.

Real-time quantitative PCR (RT-qPCR). PCR was carried out in a 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, CA). The specific PCR primers are given in supplementary Table 1. The relative copy number was calculated using the threshold crossing point (Ct) as calculated by the 7900HT fast real time PCR software combined with the delta delta Ct calculations.

Proliferation assays. αTC1-9 cells were seeded in 24-well plates at a density of 1×10^5 cells 1000 uL^{-1} /well and cultured with or without exendin-4 (10 nM) for 7 days (media were changed and exendin-4 was added daily). The cells were pulsed with [^{3}H]-thymidine (1 μCi/well). Eight hours after [^{3}H]-thymidine addition, the cells were analyzed for [^{3}H]-thymidine incorporation using a scintillation β-counter, 1450 LSC & Luminescence Counter MicroBeta TriLux (Perkin Elmer).

Western blot. Whole lysates of cells were prepared as described previously (21). Western blots were performed with rabbit-anti-cyclin D2 (Cell Signaling Technology), mouse-anti-β actin (Santa Cruz Biotechnology), goat-anti-FGF21 (R&D system, Minneapolis, MN), rabbit-anti-peroxisome proliferator-activated receptor (PPAR)-α (Santa Cruz Biotechnology), rabbit-anti-PDX-1 (Millipore), rabbit-anti-PDX-1 (Abcam), goat-anti-neurogenin (Ngn)3 (Santa Cruz Biotechnology), rabbit-anti-glucagon (Santa Cruz Biotechnology), and mouse-anti-β-tubulin (Santa Cruz Biotechnology).

FGF21 analysis. Mouse islets were isolated and STZ (1 mM) was added for 15 h to destroy the β-cells. The STZ-treated islets were given new media and treated with exendin-4 (10 nM added every 24 h) for 72 h. FGF21 secretion was analyzed by enzyme-linked immunosorbent assay (ELISA) (R&D System), with values normalized to protein.

Intracellular cAMP measurement. αTC1-9 cells (1×10^5) were seeded in 6-well plates in DMEM medium with 16.7 mM glucose. After 24 h, the medium was changed to that with 25 mM glucose, and exendin-4 (0, 5, 10, or 20 nM) was added for 2 h. The medium was removed and 1 ml of 0.1 M HCL was added for 20 min for lysis. The lysates were centrifuged at 5,000 rpm for 5 min to pellet the cellular debris. The supernatant was assayed for cAMP using ELISA (Enzo Life Sciences, Farmingdale, NY, USA).

Transfection with siRNA. FGF21 small interfering RNAs (siRNAs) were purchased from Bioneer (Daejeon, Korea). The target sequences were 5'-CUGAUGGAAUGGAUGAGAU-3' and 5'-AUCUCAUCCAUUCCAUCAG-3'. Intact islets from C57BL/6 mice were dissociated at 37°C in Accutase (Millipore). The single islet cells were seeded in 24-well plates at a density of

2×10^5 cells 250 uL^{-1} /well and cultured with RPMI media for 12 h. FGF21 siRNAs were transfected with RNAi Max reagent (Invitrogen) according to the manufacture's protocol.

Statistical analysis. Data are presented as means ± SE. Statistical significance of the difference between two groups or among multiple groups was analyzed by unpaired Student's *t*-test or ANOVA followed by Fisher's protected least significant difference test, respectively. $P<0.05$ was accepted as significant.

Results

New β-cells originating from non-β-cells are increased in rAd-GLP-1-treated mice. To investigate whether GLP-1-induced new β-cells come from remaining β-cells or non-β-cells, we used RIP-CreER;R26-YFP transgenic mice, bearing the transgenes RIP-CreER (inducible tagger) and R26-YFP as a reporter, to label pre-existing β-cells (11). Administration of 4-hydroxytamoxifen induced the expression of the reporter protein, YFP, in β-cells, and almost all of the β-cells expressed YFP (Sup. Fig. 1). STZ-induced diabetic RIP-CreER;R26-YFP male mice were injected via the tail vein with rAd-GLP-1 or rAd-βgal, and then blood glucose levels were monitored for 4 weeks. Blood glucose levels were significantly decreased, in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice and became normoglycemic (Sup. Fig. 2A). Intraperitoneal glucose tolerance tests in normoglycemic RIP-CreER;R26-YFP mice at 2 weeks after rAd-GLP-1 treatment showed that blood glucose levels in rAd-GLP-1-treated mice were properly controlled (Sup. Fig. 2B).

To determine whether β-cells are increased in diabetic rAd-GLP-1-treated mice, pancreatic sections were analyzed by immunostaining. The insulin-positive cell population, including $YFP^{+}insulin^{+}$ (new β-cells from surviving β-cells) and $YFP^{-}insulin^{+}$ (new β-cells from non-β cells), was significantly increased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 1A-C). $YFP^{-}insulin^{+}$ cells were 49.38 ± 3.95% of the insulin-positive cells in rAd-GLP-1-treated mice, and 23.25 ± 2.79% in rAd-βgal-treated mice. Thus, the proportion of new β-cells originating from non-β-cells ($YFP^{-}insulin^{+}$) was significantly higher in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 1D).

Proliferation of α-cells is increased in rAd-GLP-1-injected mice and exendin-4-treated α-cells. To explore the origin of new β-cells originating from non-β cells, we first examined the alteration of cells in the islets of rAd-GLP-1-treated RIP-CreER;R26-YFP mice after β-cell ablation by STZ injection. To monitor the proliferating cells, the mitotic marker, BrdU, was injected daily (i.p.) for 4 weeks. BrdU-positive cells in the islets increased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 2 A,B). Both $BrdU^+glucagon^+$ cells (Fig. 2C) and $BrdU^+insulin^+$ (Fig. 2D) cells were significantly increased in the islets of rAd-GLP-1-treated mice compared with rAd-βgal-treated mice, with $BrdU^+glucagon^+$ cells showing the greater increase.

To investigate whether GLP-1 receptor signaling directly increases proliferation in α-cells, we examined the effect of exendin-4 on proliferation of αTC1-9 cells by [^{3}H]-thymidine incorporation assay. Exendin-4 significantly increased proliferation of αTC1-9 cells dose dependently (Fig. 2E). Cyclins are important proteins that control the proliferation of cells; thus, we analyzed the expression of cyclins. The expression of cyclin D2 mRNA and protein was significantly increased (Fig. 2F,J), however the expression of cyclin A2, cyclin E, and cyclin D3 was not changed in exendin-4-treated αTC1-9 cells (Fig. 2G-I). Similarly BrdU-positive α-cells were increased in exendin-4-treated αTC1-9 cells when the cells were cultured in the presence of BrdU (Fig. 2K).

Bihormonal ($insulin^+glucagon^+$) cells are increased in rAd-GLP-1-injected mice and exendin-4-treated mouse islets. Recent studies show plasticity between pancreatic α- and β-cells. New β-cells can be produced from α-cells via a bihormonal $insulin^+glucagon^+$ transitional state in animals almost devoid of β-cells (11). Immunostaining of pancreatic sections from rAd-GLP-1- or rAd-βgal-treated RIP-CreER;R26-YFP mice with anti-glucagon and anti-insulin

antibodies revealed that insulin$^+$glucagon$^+$ double stained bihormonal cells were increased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 3A, B).

To investigate whether GLP-1 receptor signaling directly increases insulin$^+$glucagon$^+$ bihormonal cells, we isolated islet cells from C57BL/6 mice, treated them with STZ to destroy β-cells, and then treated them with exendin-4. In isolated islets, β-cells were specifically destroyed by STZ treatment whereas α-cells were preserved. In addition, the pattern of α-cell distribution in islets was similar to that observed in islets of STZ-treated mice (Supplementary figure 3). STZ-induced β-cell destruction was confirmed by staining with anti-insulin antibody. Insulin$^+$ cells were almost completely destroyed with only 3% of STZ-treated islet cells being insulin-positive (Fig. 3C). The STZ-treated islet cells were cultured with exendin-4 for 2 days and stained with anti-glucagon and anti-insulin antibodies (Fig. 3D). Insulin$^+$glucagon$^+$ bihormonal cells were significantly increased in STZ-exendin-4-treated islet cells compared with STZ only-treated islet cells (Fig. 3E). To investigate the change of the expression of β- or α-cell-related genes, we measured the expression of insulin, glucagon, PDX-1, and aristaless related homeobox (Arx) mRNA and protein expression of glucagon and PDX-1 in STZ-exendin-4-treated islet cells. The expression of glucagon and Arx mRNA and glucagon protein was significantly increased at 3 days after exendin-4 treatment in STZ-exendin-4-treated islet cells compared with STZ-treated islet cells. In addition, the expression of insulin and PDX-1 mRNA and PDX-1 protein was significantly increased at 7 days after exendin-4 treatment in STZ-exendin-4-treated islet cells compared with STZ-treated islet cells (Fig. 3 F-O).

GLP-1 increases β cell regeneration by promoting α- to β-cell transdifferentiation. To investigate the possibility of transdifferentiation of α-cells to β-cells by GLP-1, we used a genetic lineage tracing system. Glucagon-rtTA;Tet-O-Cre;R26-YFP mice have a doxycycline-inducible

glucagon-driven reverse tet transactivator (Gcg-rtTA) to direct Cre recombinase expression from a Tet-O-Cre transgene in glucagon$^+$ α-cells. Cre activates YFP transgene expression from the Rosa26 locus. Thus, doxycycline stimulates Cre recombinase expression specifically in glucagon$^+$ α-cells. Almost all of the glucagon$^+$ α-cells were labeled with YFP in pancreatic sections of Glucagon-rtTA;Tet-O-Cre;R26-YFP mice exposed to doxycycline for 2 weeks (Fig. 4A). After a washout period of 2 weeks after doxycycline treatment, diabetes was induced by STZ injection. The diabetic mice (blood glucose levels > 300 mg/dl for 3 consecutive days) were injected via the tail vein with rAd-GLP-1 or rAd-βgal. Blood glucose levels were significantly decreased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Sup. Fig. 4). At 4 weeks after virus injection, pancreatic sections were analyzed by immunostaining. The insulin-positive cell population, including YFP$^+$insulin$^+$ (new β-cells from α-cell transdifferentiation) and YFP$^-$insulin$^+$ (new β-cells from non-α cells), was significantly increased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 4B,C,F). New β-cells from α-cell transdifferentiation, YFP$^+$insulin$^+$ co-expressing cells, were 32% of insulin-positive cells observed in rAd-GLP-1 injected mice and 22% in rAd-βgal injected mice (Fig. 4G). In addition, YFP$^+$PDX-1$^+$ cells were significantly increased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 4D,E,H). These results suggest that GLP-1 increases new β-cell generation by promoting α- to β-cell transdifferentiation.

GLP-1 induces α-cell transdifferentiation into β-cells via FGF21. A recent report demonstrated that GLP-1 induces FGF21 production in the liver and adipose tissue (16). As well, pancreatic α-cells express FGF21 and hyperplastic α-cells highly express FGF21 (14). In addition, long-term treatment with FGF21 increases the number of insulin-positive cells per islet (15). To investigate whether FGF21 is truly involved in the increase of β-cells by GLP-1, STZ-

treated diabetic wild-type and FGF21 KO mice were injected with rAd-GLP-1, and then blood glucose levels were monitored. Blood glucose levels were decreased in both wild-type and FGF21 KO mice, but the glucose levels in FGF21 KO mice were significantly higher compared with wild-type mice (Fig. 5A). Intraperitoneal glucose tolerance tests at 4 weeks after rAd-GLP-1 treatment showed that blood glucose levels of FGF21 KO mice were significantly higher at 60, 90, and 120 minutes following glucose injection compared with wild-type mice (Fig. 5B). We then examined pancreatic sections by immunostaining. After rAd-GLP-1 treatment, the insulin$^+$ cell population was significantly lower and the glucagon$^+$ cell population was significantly higher in FGF21 KO mice compared with wild-type mice (Fig. 5C,D). To investigate the alteration of the expression of β- and α-cell-related genes, we measured the expression of insulin, glucagon, PDX-1, and Arx mRNA in STZ-treated dispersed islets from FGF21 KO or wild-type mice after exendin-4 treatment. The expression of all these mRNAs was significantly reduced in islets from FGF21 KO mice compared with wild-type mice (Fig. 5 E-H). These results suggest that increase of β-cells by GLP-1 is, in part, mediated by FGF21.

To investigate whether FGF21 directly increases insulin$^+$glucagon$^+$ bihormonal cells, we treated isolated islet cells from C57BL/6 mice with STZ to destroy β-cells, and then treated them with FGF21. FGF21 treatment of β-cell-ablated islets significantly increased the number of insulin$^+$glucagon$^+$ bihormonal cells (Fig. 6A,B). To investigate whether exendin-4-induced α-cell transdifferentiation into β-cells is mediated by FGF21, we inhibited the expression of FGF21 by siRNA in β-cell ablated islets and treated them with exendin-4. Knockdown of FGF21 significantly decreased the number of insulin$^+$glucagon$^+$ bihormonal cells generated by exendin-4 treatment (Fig. 6C-E). In addition, we confirmed this result using islets from FGF21 KO mice. The generation of insulin$^+$glucagon$^+$ cells by exendin-4 was significantly reduced in STZ-treated islets from FGF21 KO mice compared with wild-type mice (Fig. 6F,G). These results suggest

that generation of insulin$^+$glucagon$^+$ bihormonal cells by exendin-4 is mediated by FGF21 production.

FGF21 induces PDX-1 and Ngn3 production in α-cells. To investigate whether FGF21 induces the expression of β-cell transcription factors in α-cells, we isolated mouse islets from C57BL/6 mice, ablated the β-cells with STZ, treated the islets with FGF21, and then analyzed mRNA expression of β-cell transcription factors in α-cells. We found that PDX-1 mRNA expression was significantly increased, but the expression of glucagon mRNA was significantly decreased in β-cell-ablated islets treated with FGF21. However, the expression of Arx, MafA, MafB, and insulin mRNA was not changed by FGF21 treatment (Fig. 7A-F). The protein level of PDX-1 and Ngn3, an important transcription factor in endocrine pancreas development and transdifferentiation of α- to β- cells (22), was also significantly increased in FGF21 treated β-cell ablated islets compared with cells without FGF21 treatment (Fig. 7G-J). To investigate whether the expression of PDX-1 is observed in glucagon$^+$ α-cells after FGF21 treatment, we stained β-cell-ablated islet cells with anti-glucagon and anti-PDX-1 antibodies. Immunocytochemical analysis showed that co-expression of glucagon and PDX-1 in α–cells was clearly observed after FGF21 treatment. The intensity of PDX-1 staining in glucagon-producing α-cells was significantly increased in FGF21-treated cells compared with cells without FGF21 treatment as measured by a laser scanning confocal fluorescent microscope (Fig. 7K,L).

Exendin-4 treatment induces FGF21 production in α-cells via a cAMP-dependent pathway

It was reported that pancreatic α-cells express FGF21 (14). We found that α-cells are increased by GLP-1 in this study, and FGF21 is involved in GLP-1-induced α-cell transdifferentiation into β-cells. Therefore, we wanted to know whether α-cells produce FGF21 after treatment with GLP-

1. In αTC1-9 cells, the expression of FGF21 mRNA was increased at 12 h after exendin-4 treatment (Fig. 8A), and FGF21 protein was increased at 24 h after exendin-4 treatment (Fig. 8C), both dose dependently. FGF21 is regulated by PPAR-α (23). Thus, we investigated the expression of PPAR-α in exendin-4 treated αTC1-9 cells. The expression of PPAR-α mRNA was up-regulated at 6 h and the protein level of PPAR-α was increased at 24 h after exendin-4 treatment (Fig. 8B, C). To investigate whether FGF21 expression is regulated by GLP-1 in α-cells, we used islets in which β-cells had been ablated by STZ treatment. The expression of FGF21 mRNA (Fig. 8D) and FGF21 protein secretion (Fig. 8E) were also significantly increased in STZ-exendin-4-treated islets compared with STZ-treated islets. In addition, we investigated whether the expression of FGF21 is increased *in vivo* in rAd-GLP-1-injected mice. At 1 week after virus injection of diabetic Glucagon-rtTA;Tet-O-Cre;R26-YFP mice, pancreatic sections were analyzed for FGF21 expression. FGF21+ cells were significantly increased in islets of rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Sup. Fig. 5).

The major pathway for GLP-1 receptor signaling is via a cAMP-dependent pathway (24). Thus, we determined whether the cAMP pathway is involved in FGF21 production by exendin-4 in αTC1-9 cells. The production of cAMP was increased at 2 h after exendin-4 treatment in αTC1-9 cells (Fig. 8F). The expression of FGF21 mRNA was significantly increased by exendin-4 treatment, but it was inhibited by KH7, which is a selective inhibitor of soluble adenylyl cyclase (Fig. 8G). These results suggest that GLP-1 increases FGF21 expression through cAMP in α-cells. Next we determined the expression of FGF receptors after exendin-4 treatment. The mRNA expression of FGFR1, FGFR4, and co-receptor β-klotho was significantly increased in exendin-4-treated αTC1-9 cells (Fig. 8H-L). These results suggest that GLP-1 increases both FGF21 and FGF receptor expression in α-cells.

Discussion

Diabetes is caused by absolute or relative deficiency of functional pancreatic β-cells, resulting in defective control of blood glucose. Thus, strategies to increase the β-cell mass, for example, the proliferation of remaining β-cells, differentiation of progenitors of β-cells, and transdifferentiation of non-β-cells into β-cells, have been investigated for management of diabetes.

GLP-1 is known to have potential effects in regulation of the β-cell mass through regeneration, differentiation, and neogenesis of pancreatic β-cells (25-27). However, GLP-1 is rapidly inactivated by the enzyme dipeptidyl peptidase IV (28). We previously constructed a recombinant adenovirus containing cytomegalovirus promoter and albumin leader sequence, followed by GLP-1 cDNA (rAd-GLP-1) to facilitate GLP-1 secretion in the circulation (29). In mice treated with rAd-GLP-1, serum levels of GLP-1 are dramatically increased and remain significantly higher for at least 4 weeks compared with rAd-βgal-treated mice (17), indicating that a substantial amount of circulating GLP-1 could effectively act throughout the whole body including pancreas. In this study, hyperglycemia was rapidly reversed in less than 2 days, which might be due to the large amount of GLP-1 produced after rAd-GLP-1 administration. Reduced blood glucose levels were maintained for 28 days when the experiment was terminated. These effects can be due to the increase of newly generated β-cells.

In the present study, we analyzed the contribution of pre-existing β-cells to become new β-cells using the tamoxifen-dependent Cre/loxP system - transgenic mice bearing the transgenes RIP-CreERT (inducible tagger) and R26-YFP as a reporter. After β-cell destruction by STZ, we found that both existing β-cells and non-β-cells contributed to the generation of new β-cells in response to GLP-1 produced by rAd-GLP-1. Interestingly, we found that a larger proportion of

insulin$^+$ cells originated from non-β-cells than from pre-existing β-cells in rAd-GLP-1-treated mice compared with rAd-βgal-treated control mice.

Besides the production of glucagon, α-cells in the pancreatic islets can be a source of new β-cells (11). α-Cell hyperplasia occurs in response to hyperglycemia resulting from injury of β-cells as well as in pancreatic islets of diabetic animals and human diabetic patients (30-32), suggesting that the new α-cells might serve as a source for β-cell regeneration. In our study, we found increased proliferation of α-cells in the pancreas of rAd-GLP-1-treated mice compared with rAd-βgal-treated mice after ablation of β-cells by STZ. Similarly, we found that treatment of αTC1-9 cells, which produce glucagon but not preproinsulin mRNA (20; 33), with exendin-4, a GLP-1 receptor agonist, increased the proliferation of α-cells.

An increased α-cell mass might be expected to result in an increase of glucagon production, which promotes hepatic glucose output and deterioration of metabolic control in the diabetic condition. Although α-cells were increased after rAd-GLP-1 treatment in STZ-induced diabetic mice, serum glucagon levels were not significantly different from rAd-βgal treated mice (Sup. Fig. 6). As well, normal blood glucose levels were maintained in rAd-GLP-1-treated STZ-induced diabetic mice for 4 weeks, suggesting that the proliferation of α-cells caused by GLP-1 did not adversely affect metabolic control of glucose. Similarly, continuous administration of GLP-1 for 6 weeks in type 2 diabetic patients did not change plasma glucagon concentrations and decreased heamoglobin A1c (34). In our study, we found that the pancreatic α-cells are a target for GLP-1 action. GLP-1 promotes α-cell proliferation *in vitro* and *in vivo*. However, the pancreatic α-cell proportion was similar between STZ-rAd-βgal-treated mice and STZ-rAd-GLP-1 treated mice, even though the proliferation of α-cells was increased in STZ-rAd-GLP-1-treated mice. Furthermore, the pancreatic β-cell proportion was increased in rAd-GLP-1-treated mice (Sup. Fig. 7). These results suggest that α-cells may transdifferentiate into β-cells in rAd-GLP-1-

treated mice. In addition, rAd-GLP-1 treatment *in vivo* or exendin-4 treatment *in vitro* increased insulin$^+$glucagon$^+$ bihormonal cells after β-cell ablation. In conditions of extreme loss of β-cells, bihormonal cells are frequently observed, and they are not from original β-cells but are generated from pre-existing α-cells that start to produce insulin and transdifferentiate into β-cells (11). In our study, bihormonal cells were actually quite rare *in vivo* (Fig. 3A-B) compared with the *in vitro* experiment (Fig. 3D-E). This difference may be due to observational time point. *In vivo*, we measured bihormonal cells after 4 weeks of rAd-βgal or rAd-GLP-1 injection. At that time, many bihormonal cells might be already transdifferentiated to new β-cells. However, *in vitro*, we measured bihormonal cells after 2 days of exendin-4 treatment, perhaps before complete transdifferentiation occurred. Although α- to β-cell transdifferntiation is known to occur in the case of extreme β-cell loss, other mechanisms such as generation of new β-cells from progenitor cells, or the trans-differentiation of PP- or δ-cells to new β-cells, could also explain the larger proportion of insulin$^+$ cells originated from non-β-cells than from pre-existing β-cells in rAd-GLP-1-treated mice compared with rAd-βgal-treated control mice. Lineage tracing studies using Glucagon rtTA;Tet-O-Cre;R26-YFP mice showed that new β-cells originating from α-cells were significantly increased in rAd-GLP-1 injected mice compared with rAd-βgal injected mice. These results indicate that pre-existing α-cells transdifferentiated into insulin producing β-cells by GLP-1 treatment. Thus, α-cell expansion and transdifferentiation by GLP-1 may contribute to β-cell compensation. However, the mechanism for the transdifferentiation of α- to β-cells remains enigmatic. In addition, rAd-GLP-1 treatment induced proliferation in non-α-/non-β-cells compared to rAd-βgal treatment (Fig. 2 B-D). rAd-GLP-1 treatment might increase proliferation of PP-, ε− or δ-cells, which also might transdifferentiate to new β-cells. However, the percentage of non-α/non-β cells in the islets did not increased by exendin-4 (Sup. Fig. 8). The identity of non-α/non-β cells as a source of transdifferentiated cells needs further study.

GLP-1 is produced in pancreatic α-cells by activation of PC1/3 under demands for β-cell regeneration such as pregnancy, *ob/ob,* or *db/db* conditions and in prediabetic NOD mice (35). In addition, more GLP-1 is released from freshly isolated islets of hyperglycemic animals than from normoglycemic animals; healthy islets secrete more GLP-1 following culture with high glucose (36). In our study, we found that exendin-4 treatment increased PC1/3 expression and GLP-1 production in αTC1-9 cells and β-cell ablated islets (Sup. Fig. 9). Locally produced GLP-1 in the pancreatic islets contributes to maintaining β-cell function (9), and GLP-1 restores leucine-induced α-cell dysfunction (37). Thus, local production of GLP-1 from pancreatic α-cells on demand may be beneficial to protect and to regenerate β-cells.

A GLP-1 analogue was shown to increase FGF21 protein in liver and plasma (16). FGF21 protein expression is detected in both α- and β-cells in the pancreas (14) and there is a correlation between the increase of plasma GLP-1 and FGF21 in glucagon receptor knockout mice ((14). Constant infusion of FGF21 for 8 weeks in *db/db* mice normalized blood glucose levels and increased plasma insulin levels. In addition, FGF21-treated mice showed a higher number of insulin-positive cells per islet compared with control mice without β-cell proliferation (15). Therefore, we hypothesized that FGF21 production was induced by GLP-1 in α-cells and FGF21 might play a role in the generation of new β-cells by GLP-1. We found that exendin-4 increased the expression of FGF21 mRNA and protein in αTC1-9 cells, β-cell ablated islets, and rAd-GLP-1 injected mouse islets. We then investigated whether FGF21 plays a role in GLP-1-induced new β-cell generation. Exendin-4 treatment of β-cell ablated islets increased the insulin$^+$glucagon$^+$ bihormonal cell population, and inhibition of FGF21 by siRNA FGF21 reduced this exendin-4-induced bihormonal cell population. In vivo studies using FGF21 KO mice revealed that the insulin$^+$ cells in islets were significantly reduced compared with wild-type mice after treatment with rAd-GLP-1.

PDX-1 is an important transcription factor for both pancreatic development and the differentiation of progenitor cells into the β-cell phenotype (38). Forced PDX-1 expression induces α-to-β cell conversion (39), whereas adult β-cell-specific removal of PDX-1 results in the reverse – a rapid transdifferentiation to α-cells (40). Ngn3 is also a critical gene for pancreatic development of endocrine cells, and the combined expression of PDX-1 with Ngn3 improves the differentiation efficiency of embryonic stem cells into insulin-producing cells (41-43). As well, the ectopic expression of Ngn3 induces conversion of other cells into endocrine cells (44) and the expression of Ngn3 is necessary for the transdifferentiation of α-cells to β-cells (22). Therefore, we examined whether FGF21 can induce PDX-1 or Ngn3 expression in α-cells. We found that FGF21 treatment of β-cell-ablated islets increased both PDX-1 and Ngn3 expression and augmented PDX-1 intensity in glucagon-producing cells. We speculate that FGF21 may stimulate β-cell formation by inducing Ngn3 and PDX-1 expression. The percentage of α-cells in the islets of the rAd-GLP-1-injected FGF21 KO mice was higher than wild type mice. In addition, it was reported that constant infusion of FGF21 increased β-cell mass without β-cell proliferation in mice (15). Therefore FGF21 probably contributes to the increase of α-cell transdifferentiation into β-cells. Further studies are needed to determine the detailed mechanisms by which FGF21 induces the expression of these transcription factors. One possibility is that FGF21 might increase PDX-1 expression by increase of PPARγ, as it is known that FGF21 promotes PPAR-γ activity and expression (45), and PPAR-γ enhances the expression of PDX-1 and Nkx6.1 in INS-1 cells (46).

In summary, we found that GLP-1 contributes to new β-cell generation from α-cells. Circulating GLP-1 and GLP-1 produced from pro-α-cells could induce FGF21 and subsequently increase β-cell transcription factors, such as PDX-1 and Ngn-3. These results suggest that GLP-1 may act on α-cell transdifferentiation to β-cells via FGF21 induction.

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Duality of Interest. The authors declare that there is no duality of interest associated with this manuscript.

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Figure Legends

Figure 1. New β cells originating from non-β cells are increased in rAd-GLP-1-treated mice. RIP-CreER;R26-YFP mice were injected daily with hydroxytamoxifen (1 mg/mouse) for 5 days every other week, which was repeated 4 times, and diabetes was induced by STZ (150 mg/kg). Diabetic mice were treated with (A) rAd-βgal (n=4) or (B) rAd-GLP-1 (n=4). Four weeks after virus injection, pancreatic sections were prepared and double-stained with anti-GFP and anti-insulin antibodies. White arrows indicate double-positive cells ($YFP^+Insulin^+$) and red arrows indicate insulin-single-positive cells ($YFP^-Insulin^+$). (C) The insulin-positive cells expressed as a percentage of the number of islet cells. (D) $YFP^+Insulin^+$ or $YFP^-Insulin^+$ cells were counted and expressed as percentage of the $insulin^+$ cells found in islets of rAd-GLP-1 or rAd-βgal mice (20 or 14 sections, 65 or 26 islets, 541 or 195 $insulin^+$ cells per group). Data are means ±SE. * $P<0.05$ compared with rAd-βgal-treated mice.

Figure 2. Proliferation of α-cells is increased in rAd-GLP-1-treated mice and exendin-4-treated αTC1-9 cells. STZ-induced diabetic RIP-CreER;R26-YFP mice were treated with (A) rAd-βgal (n=4) or rAd-GLP-1 (n=4). Mice were injected daily with BrdU (100 mg/kg body weight, i.p.) for 4 weeks beginning on the first day after virus injection. Four weeks after virus injection, pancreatic sections were prepared and triple-stained with anti-glucagon (Gcg), anti-insulin (Ins), and anti-BrdU antibodies. (B) The total $BrdU^+$ cells. (C) $BrdU^+Glucagon^+$, and (D) $BrdU^+Insulin^+$ cells were measured and expressed as a percentage of the number of islet cells in rAd-GLP-1 or rAd-βgal mice (19 or 11 sections, 104 or 74 islets per group). αTC1-9 cells were cultured without (Un) or with exendin-4 (10 nM) for 7 days. The culture media was changed every 24 h. (E) [^{3}H]-thymidine incorporation was measured. The expression of (F) cyclin D2,

(G) cyclin A2, (H) cyclin E, (I) cyclin D3 mRNA, and (J) cyclin D2 protein was measured in exendin-4-treated αTC1-9 cells. (K) Exendin-4-treated αTC1-9 cells were double-stained with anti-BrdU and anti-glucagon antibodies. Data are means ± SE from three to four independent experiments and are expressed as a ratio of the control (F-I). * $P<0.05$, ** $P<0.005$ compared with rAd-βgal treated group or untreated cells.

Figure 3. Bihormonal cells (insulin$^+$glucagon$^+$ cells) are increased in rAd-GLP-1-injected mice and exendin-4-treated mouse islets. STZ-induced diabetic RIP-CreER;R26-YFP mice were treated with rAd-βgal (n=4) or rAd-GLP-1 (n=4). Mice were injected daily with BrdU (100 mg/kg body weight, i.p.) for 4 weeks beginning on the first day after virus injection. (A) Pancreatic sections were prepared and triple-stained with anti-glucagon (Gcg), anti-insulin (Ins) and anti-BrdU antibodies. (B) The bihormonal (insulin$^+$glucagon$^+$) cells were counted and expressed as a percentage of the number of islet cells. (C) Islets were isolated from C57BL/6 mice, treated with STZ (1 mM) for 15 h and then stained with anti-glucagon or anti-insulin antibodies. (D-O) Mouse islets were treated with STZ (1 mM) for 15 h and then with or without exendin-4 (10 nM, add per 24 h) for (D-E) 2 days, (F-J) 3 days, or (K-O) 7 days (400 islets per group). (D) Islets were double-stained with anti-glucagon and anti-insulin antibodies. (E) The bihormonal (insulin$^+$glucagon$^+$) cells were counted and expressed as percentage of the number of islet cells (n = 1468 STZ-treated cells, 1204 STZ-exendin-4-treated cells). (F-O) The expression of insulin, glucagon, Arx, and PDX-1 mRNA was analyzed by real-time quantitative PCR, with values normalized to cyclophilin expression. The expression of glucagon and PDX-1 protein was analyzed by western blot, with values normalized to β-tubulin expression. The fold change was calculated as ratio of the expression in STZ islets. Data are means ± SE from three to four independent experiments. * $P<0.05$ compared with STZ-treated islets.

Figure 4. GLP-1 increases α- to β-cell transdifferentiation. Four week-old male Glucagon-rtTA;Tet-O-Cre;R26-YFP mice were given doxycycline in water (1.5 mg/ml) for 2 weeks. (A) The pancreas stained with anti-GFP and anti-glucagon antibodies. (B-H) Two weeks after doxycycline was stopped, Glucagon-rtTA;Tet-O-Cre;R26-YFP mice were treated with STZ (150 mg/kg) and then monitored for the development of hyperglycemia (blood glucose levels > 300 mg/dl for 3 consecutive days). The diabetic mice were injected with rAd-βgal (n=4) or rAd-GLP-1 (n=7). (B, C, F, G) After 4 weeks, pancreatic sections were prepared and double-stained with anti-GFP and anti-insulin antibodies. The percentage of the insulin$^+$ cells in the islets and the percentage of insulin$^+$YFP$^+$ co-expressing cells as a proportion of insulin-positive cells was analyzed by ZEN 2009 Light Edition Analysis Programme. (31 or 23 sections, 335 or 184 islets in rAd-GLP-1 or rAd-βgal injected mice group) (D, E, H) At 1 week after rAd-βgal (n=4) or rAd-GLP-1 (n=4) injection, pancreatic sections were prepared and double-stained with anti-GFP and anti-PDX-1 antibodies. (H) The percentage of YFP$^+$PDX-1$^+$ co-expressing cells was expressed as a proportion of islet cells in rAd-GLP-1 or rAd-βgal injected mice (8 or 10 sections, 23 or 44 islets per group). Data are means ± SE. * $P<0.05$ compared with rAd-βgal treated mice.

Figure 5. FGF21 KO mice have reduced rAd-GLP-1-induced β cells. FGF21 KO and wild-type mice were injected with STZ (150 mg/kg). Diabetic mice were treated with rAd-βgal or rAd-GLP-1. (A) Blood glucose levels (BGL) were measured (n=5-9 per group) (black squares, FGF21 KO/STZ/rAd-βgal; white squares, wild-type/STZ/rAd-βgal; black circles, FGF21 KO/STZ/rAd-GLP-1; white circles, wild-type/STZ/rAd-GLP-1). (B) Four weeks later, glucose tolerance tests were performed (n=4-5 per group). (black squares, FGF21 KO/STZ/rAd-βgal; white squares, wild-type/STZ/rAd-βgal; black circles, FGF21 KO/STZ/rAd-GLP-1; white circles,

wild-type/STZ/rAd-GLP-1). (C) Pancreata were removed at 4 weeks after virus injection. Pancreatic sections were double-stained with anti-glucagon and anti-insulin antibodies. (D) Glucagon- or insulin-positive cells were counted and expressed as a percentage of the cell number found in islets of FGF21 KO/STZ/rAd-GLP-1 (red squares) or wild-type/STZ/rAd-GLP-1 (blue circles) mice (n=9 or 5 animals, 33 or 22 sections, 224 or 151 islets per group). Data are means ± SE. * $P < 0.05$, ** $P < 0.01$ compared with wild-type/STZ/rAd-GLP-1 treated mice. (E-H) Islet cells prepared from FGF21 KO (n=4) or wild-type (n=4) mice were treated with STZ (1 mM) for 15 h and then exendin-4 (10 nM) for 2 days (add per 24 h). The expression of (E) insulin, (F) glucagon, (G) PDX-1, and (H) Arx mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. Data are means ± SE from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with wild-type/STZ/exendin-4-treated islet cells.

Figure 6. Exendin-4 induces bihormonal cells (insulin$^+$glucagon$^+$) via an increase of FGF21. Mouse islets were isolated from C57BL/6 mice (n=10) and dissociated with Accutase (Millipore) treatment. Single islet cells (2 x 10^5 cells) were treated with STZ (1 mM) for 15 h and then FGF21 (50 nM) for 24 h. (A) Cells were double-stained with anti-glucagon and anti-insulin antibodies. (B) The bihormonal cells (insulin$^+$glucagon$^+$ cells) were counted and expressed as a percentage of the cell number. N = 816 for STZ and 879 for STZ/FGF21. Data are means ± SE from six independent experiments. * $P < 0.05$ compared with STZ treated islets. (C-E) Single islet cells (2 x 10^5 cells) were transfected with siRNA scramble (Scr) or siRNA FGF21. After 9 h, the islets were treated STZ (1 mM) for 15 h and then exendin-4 (10 nM) for 2 days (add per 24 h). (C) The inhibition of FGF21 mRNA was analyzed by RT-qPCR. (D) Islet cells were double-stained with anti-glucagon and anti-insulin antibodies. (E) The bihormonal cells (insulin$^+$glucagon$^+$ cells) were counted and expressed as a percentage of the cell number. N =

504 for scramble siRNA/STZ, 525 for scramble siRNA/STZ/exendin-4, and 441 for FGF21 siRNA/STZ/exendin-4. Data are means ± SE from three independent experiments. * $P < 0.05$ compared with scramble siRNA/STZ-treated islet cells. # $P<0.005$ compared with FGF21 siRNA/STZ/exendin-4-treated islet cells. (F, G) Islets were isolated from FGF21 KO (red) (n=10) or wild-type (blue) (n=10) mice and dissociated with Accutase (Millipore). Islet cells (2 x 10^5 cells) were treated with STZ (1 mM) for 15 h and then exendin-4 (10 nM) for 2 days (add per 24 h). (F) The islet cells were double-stained with anti-glucagon and anti-insulin antibodies. (G) The bihormonal cells ($insulin^+glucagon^+$ cells) were counted and expressed as a percentage of the cell number. N = 835 for wild/STZ, 586 for wild/STZ/exendin-4, 1204 for FGF21KO/STZ, and 1233 for FGF21KO/STZ/exendin-4. Data are means ± SE from three independent experiments. * $P < 0.05$ compared with wild/STZ-treated islet cells. # $P<0.05$ compared with FGF21KO/STZ/exendin-4-treated islet cells.

Figure 7. FGF21 induces PDX-1 and Ngn3 production in α-cells. Mouse islets were isolated from C57BL/6 mice (n=10) and treated with STZ (1 mM) for 15 h and then FGF21 (50 nM) for 24 h. (A-F) The expression of PDX-1, Arx, MafA, MafB, insulin, and glucagon mRNA was analyzed with RT-qPCR and normalized by cyclophilin expression. (G-J) Mouse islets (n=20) were isolated and treated with STZ (1 mM) for 15 h and then FGF21 (50 nM) for 48 h. PDX-1 and Ngn3 proteins were analyzed by western blot. (K) Islets were stained with anti-PDX1 and anti-glucagon antibodies. (L) The mean intensity was measured by confocal laser scanning microscope LSM 700 (Carl Zeiss) and analyzed. STZ or STZ/FGF21 treated islet numbers were 83 and 73. Data are means ± SE from three to four independent experiments and are expressed as a ratio of the control (STZ). * $P < 0.05$ compared with STZ-treated islets.

Figure 8. Exendin-4 treatment promotes FGF21 production in α-cell via a cAMP-dependent pathway. (A) αTC1-9 cells were treated without (Un) or with exendin-4 (5, 10, 20 nM) for 12 h. The expression of FGF21 mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (B) αTC1-9 cells were treated with exendin-4 for 6 h. The expression of PPAR-α mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (C) αTC1-9 cells were treated with exendin-4 for 24 h. FGF21 and PPAR-α protein was analyzed by western blot and β-actin was analyzed as a loading control. (D, E) Mouse islets (n=20) were isolated and treated with STZ (1 mM) for 15 h and then with or without exendin-4 (10 nM) for (D) 12 h or for (E) 72 h. (D) FGF21 mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (E) FGF21 protein was analyzed by ELISA with values normalized to protein. (F) αTC1-9 cells were treated with exendin-4 for 2 h. The cAMP concentration was measured by ELISA. (G) αTC1-9 cells were pretreated with KH7 (Sigma, 10 μM) for 30 min and then with or without exendin-4 (10 nM) for 12 h. The expression of FGF21 mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (H-L) αTC1-9 cells were treated with exendin-4 for 12 h. The expression of FGFR1, FGFR2, FGFR3, FGFR4, and β-Klotho was analyzed by RT-qPCR and normalized by cyclophilin expression. Data are means ± SE from three independent experiments and are expressed as a percentage or ratio of the control. * $P < 0.05$, ** $P < 0.01$, compared with untreated and # $P<0.05$ compared with exendin-4 treated.

Fig. 1

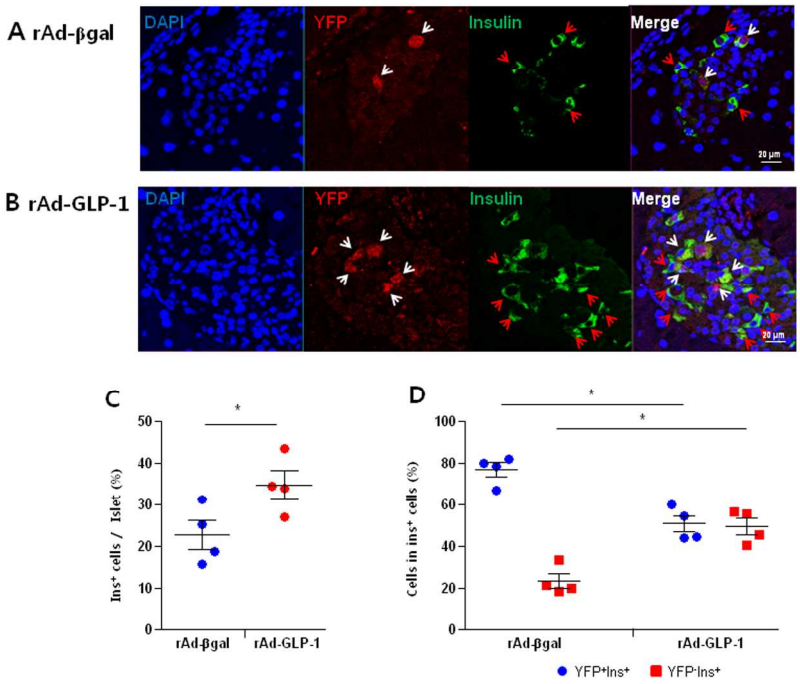

Figure 1. New β cells originating from non-β cells are increased in rAd-GLP-1-treated mice. RIP-CreER;R26-YFP mice were injected daily with hydroxytamoxifen (1 mg/mouse) for 5 days every other week, which was repeated 4 times, and diabetes was induced by STZ (150 mg/kg). Diabetic mice were treated with (A) rAd-βgal (n=4) or (B) rAd-GLP-1 (n=4). Four weeks after virus injection, pancreatic sections were prepared and double-stained with anti-GFP and anti-insulin antibodies. White arrows indicate double-positive cells (YFP+Insulin+) and red arrows indicate insulin-single-positive cells (YFP-Insulin+). (C) The insulin-positive cells expressed as a percentage of the number of islet cells. (D) YFP+Insulin+ or YFP-Insulin+ cells were counted and expressed as percentage of the insulin+ cells found in islets of rAd-GLP-1 or rAd-βgal mice (20 or 14 sections, 65 or 26 islets, 541 or 195 insulin+ cells per group). Data are means ±SE. * P<0.05 compared with rAd-βgal-treated mice.

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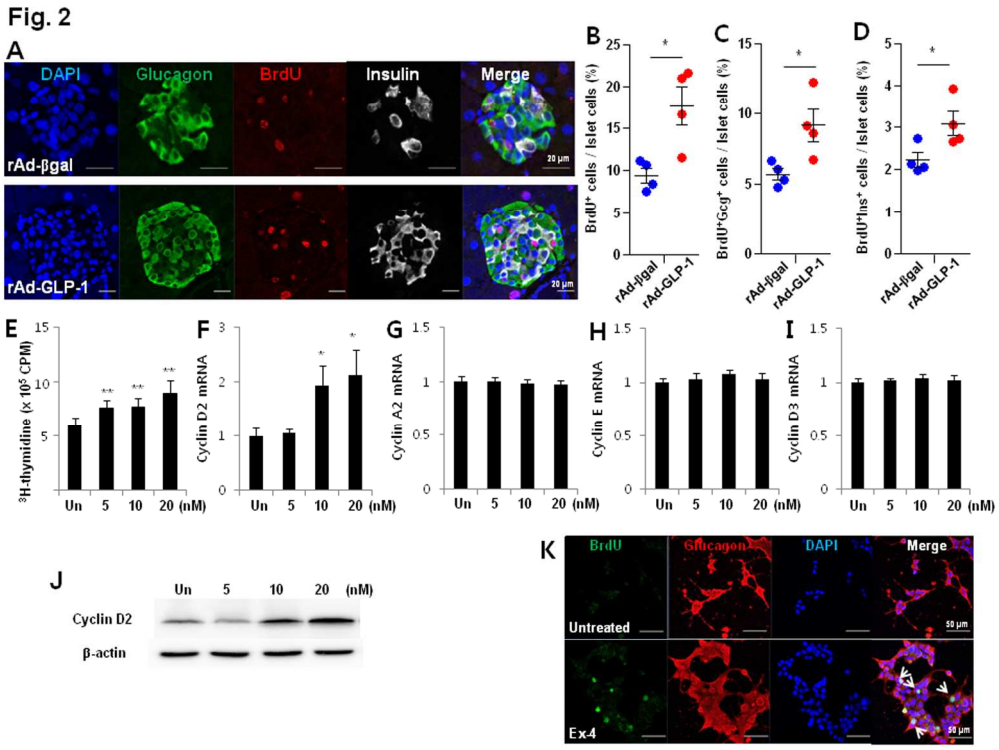

Figure 2. Proliferation of α-cells is increased in rAd-GLP-1-treated mice and exendin-4-treated αTC1-9 cells. STZ-induced diabetic RIP-CreER;R26-YFP mice were treated with (A) rAd-βgal (n=4) or rAd-GLP-1 (n=4). Mice were injected daily with BrdU (100 mg/kg body weight, i.p.) for 4 weeks beginning on the first day after virus injection. Four weeks after virus injection, pancreatic sections were prepared and triple-stained with anti-glucagon (Gcg), anti-insulin (Ins), and anti-BrdU antibodies. (B) The total BrdU+ cells. (C) BrdU+Glucagon+, and (D) BrdU+Insulin+ cells were measured and expressed as a percentage of the number of islet cells in rAd-GLP-1 or rAd-βgal mice (19 or 11 sections, 104 or 74 islets per group). αTC1-9 cells were cultured without (Un) or with exendin-4 (10 nM) for 7 days. The culture media was changed every 24 h. (E) [3H]-thymidine incorporation was measured. The expression of (F) cyclin D2, (G) cyclin A2, (H) cyclin E, (I) cyclin D3 mRNA, and (J) cyclin D2 protein was measured in exendin-4-treated αTC1-9 cells. (K) Exendin-4-treated αTC1-9 cells were double-stained with anti-BrdU and anti-glucagon antibodies. Data are means ± SE from three to four independent experiments and are expressed as a ratio of the control (F-I). * P<0.05, ** P<0.005 compared with rAd-βgal treated group or untreated cells.

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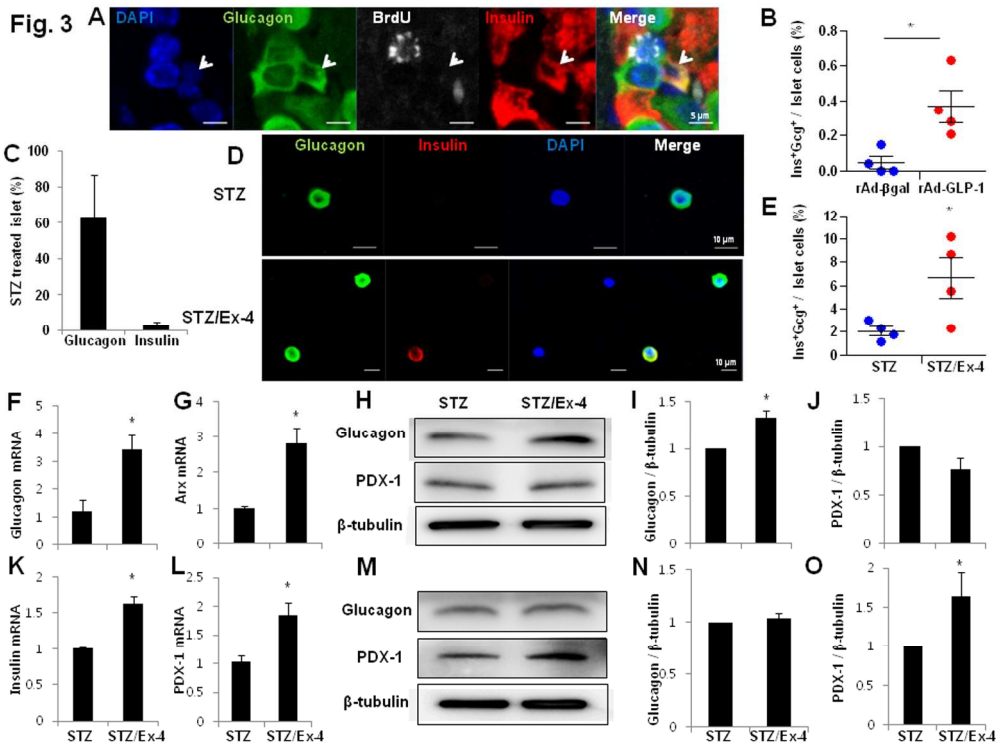

Figure 3. Bihormonal cells (insulin+glucagon+ cells) are increased in rAd-GLP-1-injected mice and exendin-4-treated mouse islets. STZ-induced diabetic RIP-CreER;R26-YFP mice were treated with rAd-βgal (n=4) or rAd-GLP-1 (n=4). Mice were injected daily with BrdU (100 mg/kg body weight, i.p.) for 4 weeks beginning on the first day after virus injection. (A) Pancreatic sections were prepared and triple-stained with anti-glucagon (Gcg), anti-insulin (Ins) and anti-BrdU antibodies. (B) The bihormonal (insulin+glucagon+) cells were counted and expressed as a percentage of the number of islet cells. (C) Islets were isolated from C57BL/6 mice, treated with STZ (1 mM) for 15 h and then stained with anti-glucagon or anti-insulin antibodies. (D-O) Mouse islets were treated with STZ (1 mM) for 15 h and then with or without exendin-4 (10 nM, add per 24 h) for (D-E) 2 days, (F-J) 3 days, or (K-O) 7 days (400 islets per group). (D) Islets were double-stained with anti-glucagon and anti-insulin antibodies. (E) The bihormonal (insulin+glucagon+) cells were counted and expressed as percentage of the number of islet cells (n = 1468 STZ-treated cells, 1204 STZ-exendin-4-treated cells). (F-O) The expression of insulin, glucagon, Arx, and PDX-1 mRNA was analyzed by real-time quantitative PCR, with values normalized to cyclophilin expression. The expression of glucagon and PDX-1 protein was analyzed by western blot, with values normalized to β-tubulin expression. The fold change was calculated as ratio of the expression in STZ islets. Data are means ± SE from three to four independent experiments. * P<0.05 compared with STZ-treated islets.

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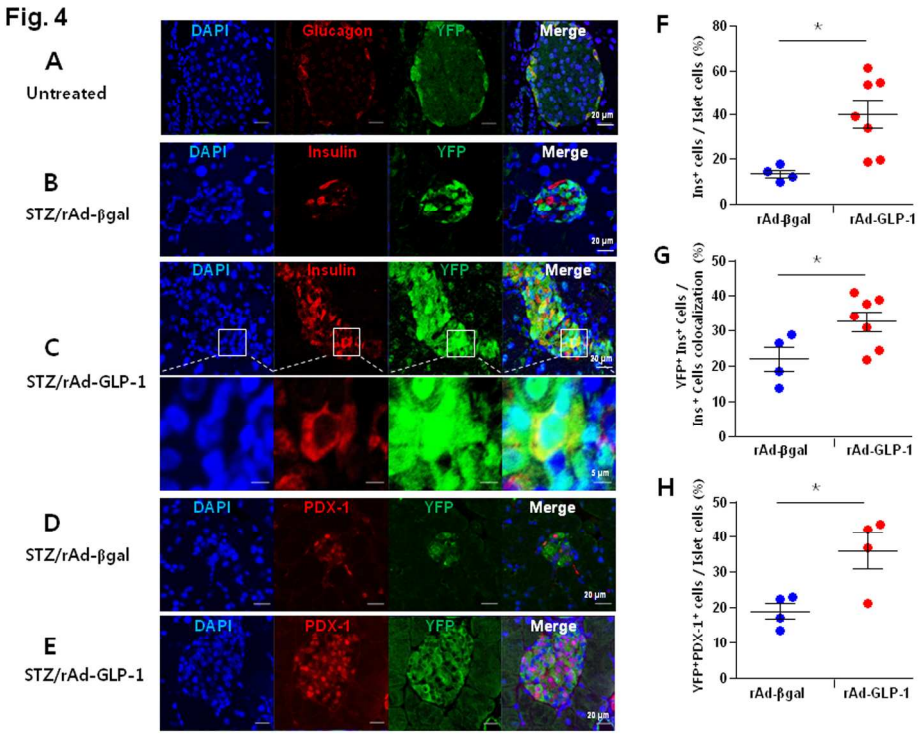

Figure 4. GLP-1 increases α- to β-cell transdifferentiation. Four week-old male Glucagon-rtTA;Tet-O-Cre;R26-YFP mice were given doxycycline in water (1.5 mg/ml) for 2 weeks. (A) The pancreas stained with anti-GFP and anti-glucagon antibodies. (B-H) Two weeks after doxycycline was stopped, Glucagon-rtTA;Tet-O-Cre;R26-YFP mice were treated with STZ (150 mg/kg) and then monitored for the development of hyperglycemia (blood glucose levels > 300 mg/dl for 3 consecutive days). The diabetic mice were injected with rAd-βgal (n=4) or rAd-GLP-1 (n=7). (B, C, F, G) After 4 weeks, pancreatic sections were prepared and double-stained with anti-GFP and anti-insulin antibodies. The percentage of the insulin+ cells in the islets and the percentage of insulin+YFP+ co-expressing cells as a proportion of insulin-positive cells was analyzed by ZEN 2009 Light Edition Analysis Programme. (31 or 23 sections, 335 or 184 islets in rAd-GLP-1 or rAd-βgal injected mice group) (D, E, H) At 1 week after rAd-βgal (n=4) or rAd-GLP-1 (n=4) injection, pancreatic sections were prepared and double-stained with anti-GFP and anti-PDX-1 antibodies. (H) The percentage of YFP+PDX-1+ co-expressing cells was expressed as a proportion of islet cells in rAd-GLP-1 or rAd-βgal injected mice (8 or 10 sections, 23 or 44 islets per group). Data are means ± SE. * P<0.05 compared with rAd-βgal treated mice.

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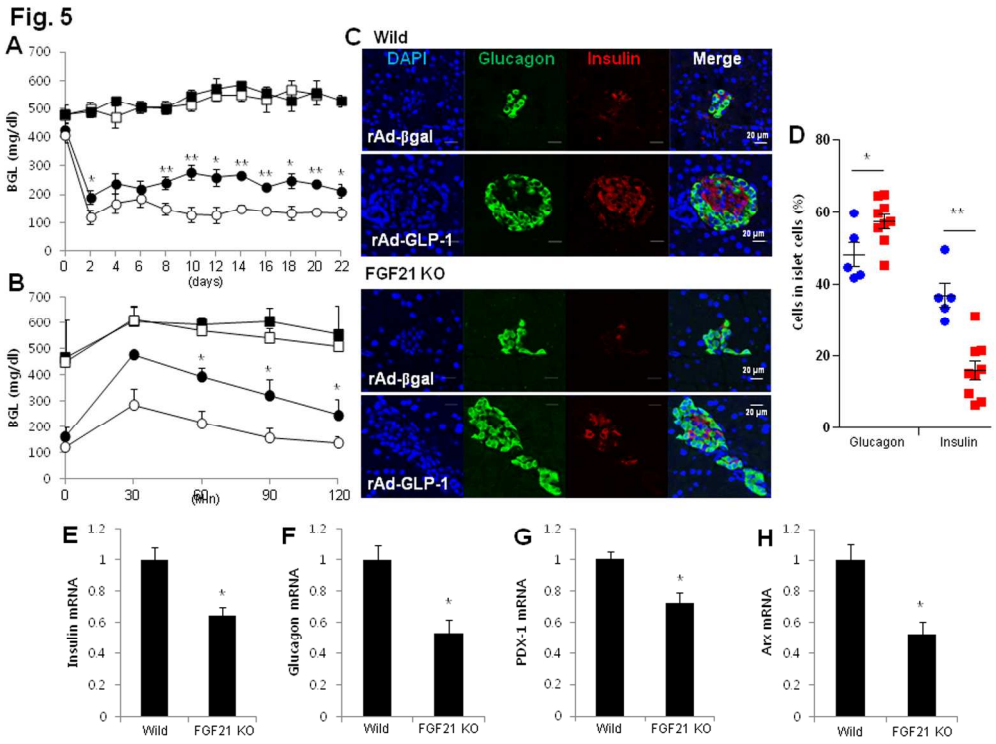

Figure 5. FGF21 KO mice have reduced rAd-GLP-1-induced β cells. FGF21 KO and wild-type mice were injected with STZ (150 mg/kg). Diabetic mice were treated with rAd-βgal or rAd-GLP-1. (A) Blood glucose levels (BGL) were measured (n=5-9 per group) (black squares, FGF21 KO/STZ/rAd-βgal; white squares, wild-type/STZ/rAd-βgal; black circles, FGF21 KO/STZ/rAd-GLP-1; white circles, wild-type/STZ/rAd-GLP-1). (B) Four weeks later, glucose tolerance tests were performed (n=4-5 per group). (black squares, FGF21 KO/STZ/rAd-βgal; white squares, wild-type/STZ/rAd-βgal; black circles, FGF21 KO/STZ/rAd-GLP-1; white circles, wild-type/STZ/rAd-GLP-1). (C) Pancreata were removed at 4 weeks after virus injection. Pancreatic sections were double-stained with anti-glucagon and anti-insulin antibodies. (D) Glucagon- or insulin-positive cells were counted and expressed as a percentage of the cell number found in islets of FGF21 KO/STZ/rAd-GLP-1 (red squares) or wild-type/STZ/rAd-GLP-1 (blue circles) mice (n=9 or 5 animals, 33 or 22 sections, 224 or 151 islets per group). Data are means ± SE. * $P < 0.05$, ** $P < 0.01$ compared with wild-type/STZ/rAd-GLP-1 treated mice. (E-H) Islet cells prepared from FGF21 KO (n=4) or wild-type (n=4) mice were treated with STZ (1 mM) for 15 h and then exendin-4 (10 nM) for 2 days (add per 24 h). The expression of (E) insulin, (F) glucagon, (G) PDX-1, and (H) Arx mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. Data are means ± SE from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with wild-type/STZ/exendin-4-treated islet cells.

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Fig. 6

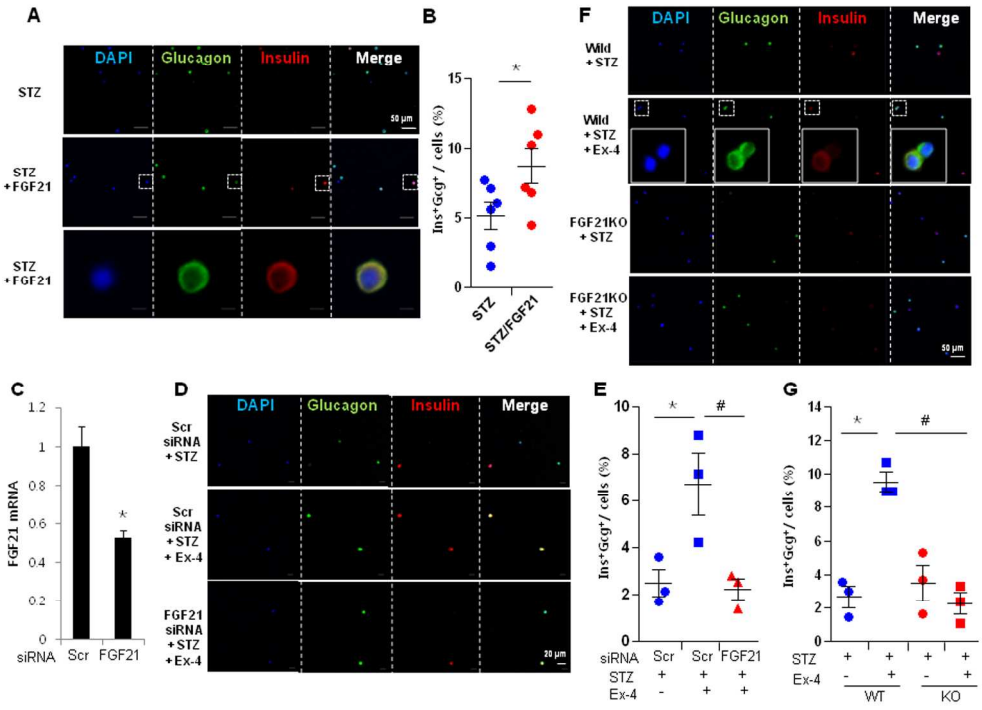

Figure 6. Exendin-4 induces bihormonal cells (insulin+glucagon+) via an increase of FGF21. Mouse islets were isolated from C57BL/6 mice (n=10) and dissociated with Accutase (Millipore) treatment. Single islet cells (2 x 105 cells) were treated with STZ (1 mM) for 15 h and then FGF21 (50 nM) for 24 h. (A) Cells were double-stained with anti-glucagon and anti-insulin antibodies. (B) The bihormonal cells (insulin+glucagon+ cells) were counted and expressed as a percentage of the cell number. N = 816 for STZ and 879 for STZ/FGF21. Data are means ± SE from six independent experiments. * P < 0.05 compared with STZ treated islets. (C-E) Single islet cells (2 x 105 cells) were transfected with siRNA scramble (Scr) or siRNA FGF21. After 9 h, the islets were treated STZ (1 mM) for 15 h and then exendin-4 (10 nM) for 2 days (add per 24 h). (C) The inhibition of FGF21 mRNA was analyzed by RT-qPCR. (D) Islet cells were double-stained with anti-glucagon and anti-insulin antibodies. (E) The bihormonal cells (insulin+glucagon+ cells) were counted and expressed as a percentage of the cell number. N = 504 for scramble siRNA/STZ, 525 for scramble siRNA/STZ/exendin-4, and 441 for FGF21 siRNA/STZ/exendin-4. Data are means ± SE from three independent experiments. * P < 0.05 compared with scramble siRNA/STZ-treated islet cells. # P<0.005 compared with FGF21 siRNA/STZ/exendin-4-treated islet cells. (F, G) Islets were isolated from FGF21 KO (red) (n=10) or wild-type (blue) (n=10) mice and dissociated with Accutase (Millipore). Islet cells (2 x 105 cells) were treated with STZ (1 mM) for 15 h and then exendin-4 (10 nM) for 2 days (add per 24 h). (F) The islet cells were double-stained with anti-glucagon and anti-insulin antibodies. (G) The bihormonal cells (insulin+glucagon+ cells) were counted and expressed as a percentage of the cell number. N = 835 for wild/STZ, 586 for wild/STZ/exendin-4, 1204 for FGF21KO/STZ, and 1233 for FGF21KO/STZ/exendin-4. Data are means ± SE from three independent experiments. * P < 0.05 compared with wild/STZ-treated islet cells. # P<0.05 compared with FGF21KO/STZ/exendin-4-treated islet cells.

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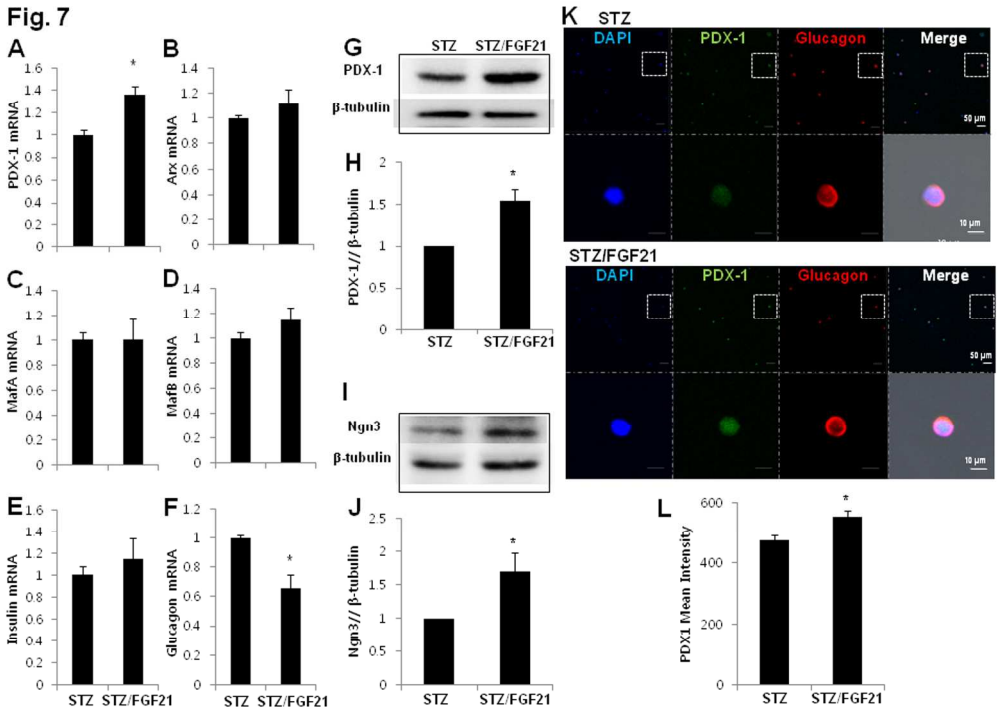

Figure 7. FGF21 induces PDX-1 and Ngn3 production in α-cells. Mouse islets were isolated from C57BL/6 mice (n=10) and treated with STZ (1 mM) for 15 h and then FGF21 (50 nM) for 24 h. (A-F) The expression of PDX-1, Arx, MafA, MafB, insulin, and glucagon mRNA was analyzed with RT-qPCR and normalized by cyclophilin expression. (G-J) Mouse islets (n=20) were isolated and treated with STZ (1 mM) for 15 h and then FGF21 (50 nM) for 48 h. PDX-1 and Ngn3 proteins were analyzed by western blot. (K) Islets were stained with anti-PDX1 and anti-glucagon antibodies. (L) The mean intensity was measured by confocal laser scanning microscope LSM 700 (Carl Zeiss) and analyzed. STZ or STZ/FGF21 treated islet numbers were 83 and 73. Data are means ± SE from three to four independent experiments and are expressed as a ratio of the control (STZ). * $P < 0.05$ compared with STZ-treated islets.

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Fig. 8

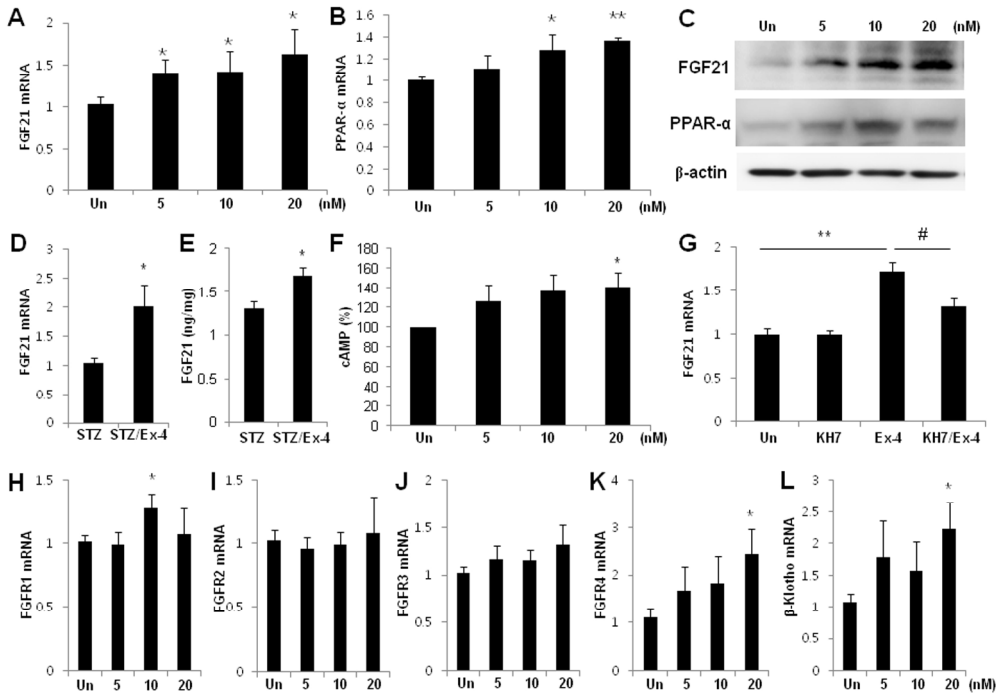

Figure 8. Exendin-4 treatment promotes FGF21 production in α-cell via a cAMP-dependent pathway. (A) αTC1-9 cells were treated without (Un) or with exendin-4 (5, 10, 20 nM) for 12 h. The expression of FGF21 mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (B) αTC1-9 cells were treated with exendin-4 for 6 h. The expression of PPAR-α mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (C) αTC1-9 cells were treated with exendin-4 for 24 h. FGF21 and PPAR-α protein was analyzed by western blot and β-actin was analyzed as a loading control. (D, E) Mouse islets (n=20) were isolated and treated with STZ (1 mM) for 15 h and then with or without exendin-4 (10 nM) for (D) 12 h or for (E) 72 h. (D) FGF21 mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (E) FGF21 protein was analyzed by ELISA with values normalized to protein. (F) αTC1-9 cells were treated with exendin-4 for 2 h. The cAMP concentration was measured by ELISA. (G) αTC1-9 cells were pretreated with KH7 (Sigma, 10 μM) for 30 min and then with or without exendin-4 (10 nM) for 12 h. The expression of FGF21 mRNA was analyzed by RT-qPCR and normalized by cyclophilin expression. (H-L) αTC1-9 cells were treated with exendin-4 for 12 h. The expression of FGFR1, FGFR2, FGFR3, FGFR4, and β-Klotho was analyzed by RT-qPCR and normalized by cyclophilin expression. Data are means ± SE from three independent experiments and are expressed as a percentage or ratio of the control. * $P < 0.05$, ** $P < 0.01$, compared with untreated and # $P<0.05$ compared with exendin-4 treated.

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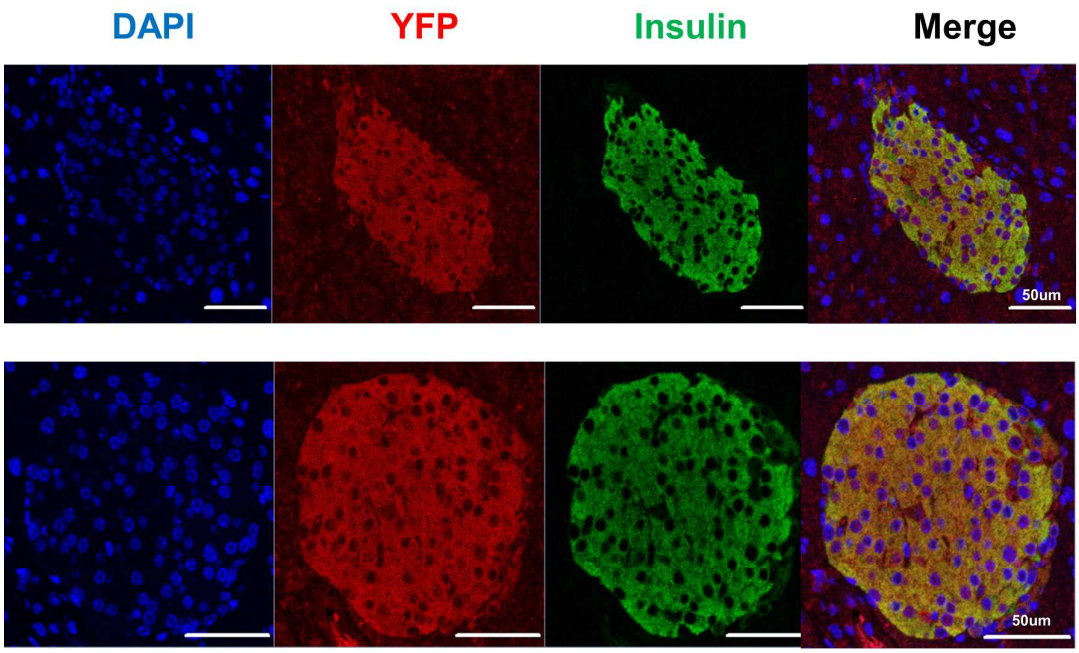
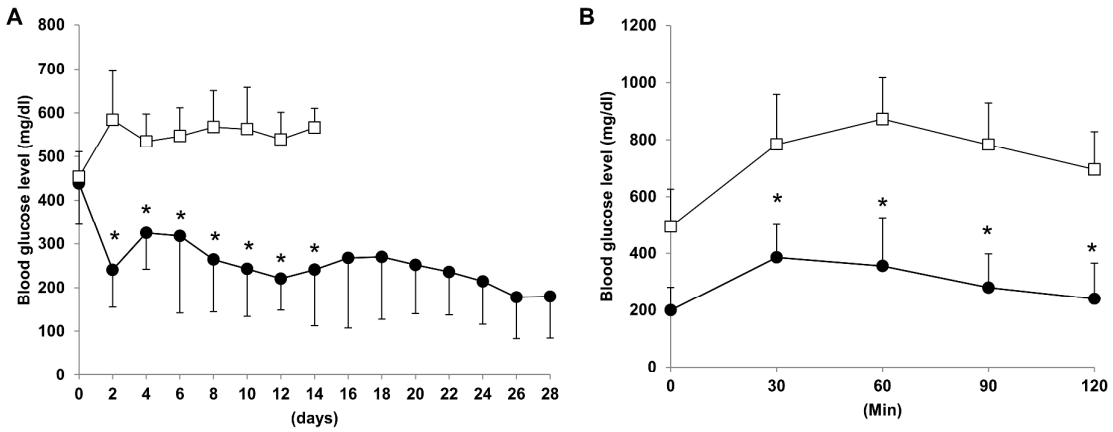
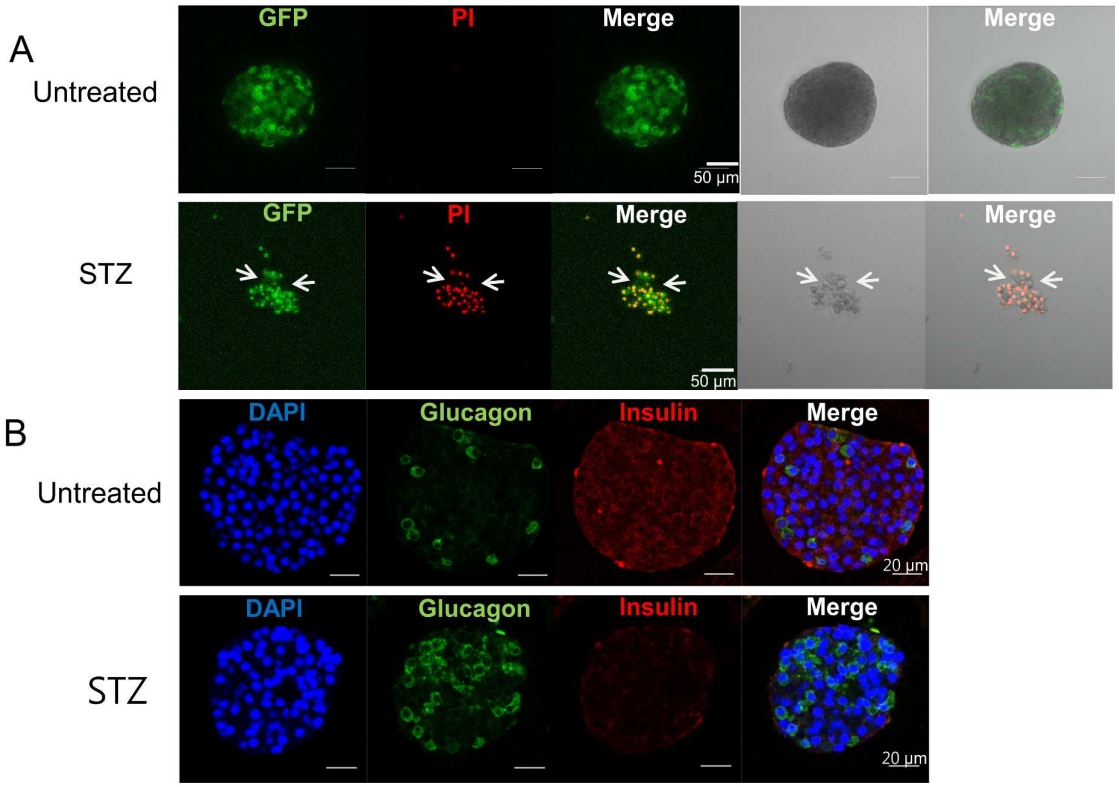


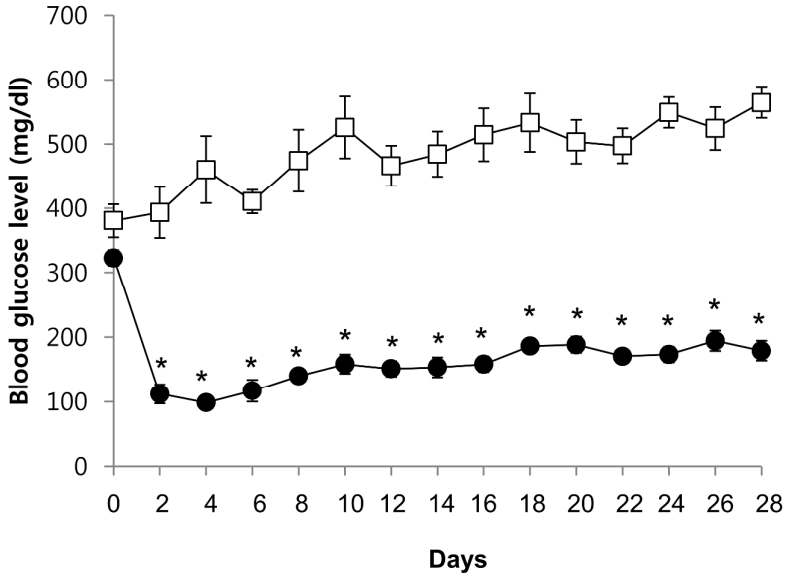
Supplemental Figure 1. **YFP expression in the pancreas of 4-hydroxytamoxifen-treated RIP-CreER;R26-YFP mice.** RIP-CreER;R26-YFP mice were injected daily with 4-hydroxytamoxifen (1 mg/mouse) for 5 days every other week, which was repeated 4 times. Pancreatic sections were prepared and stained with anti-GFP and anti-insulin antibodies.



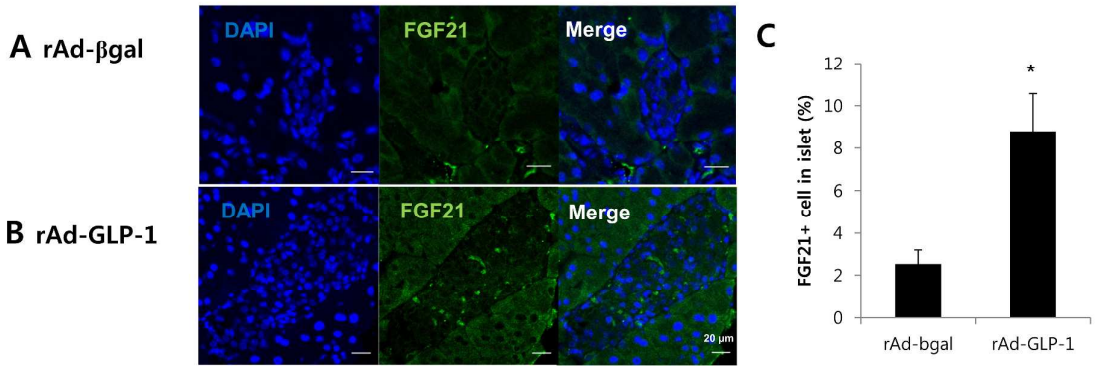
Supplemental Figure 2. **Remission of diabetes in rAd-GLP-1-injected RIP-CreER;R26-YFP mice.** (A) Blood glucose levels and (B) glucose tolerance test of rAd-GLP-1-injected (black circles, n = 6) or rAd-βgal-injected (white squares, n = 3) RIP-CreER;R26-YFP mice. Data are means ± SE. * $P<0.05$ compared with rAd-βgal-treated mice.



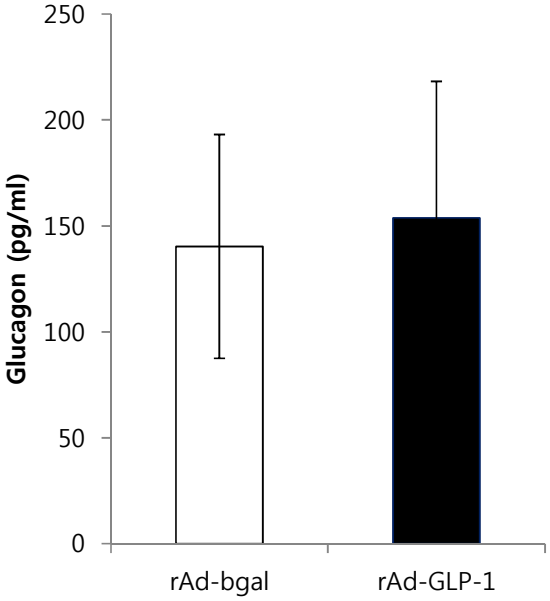
Supplemental Figure 3. **Changes of morphology in STZ-treated islets.** (A) Islets were isolated from Ins-2-luc/EGFP/TK mice (n=3) and treated with or without STZ (1 mM) for 15 h. The islets were then stained with propidium iodide (PI). White arrows indicate non-beta cell which are unstained by PI. (B) Islets (100 islets/group) isolated from C57BL/6 mice (n=3) were treated with STZ (1 mM) for 15 h and then maintained for 2 days in fresh media. The islets were fixed in 10% formaldehyde and embedded in paraffin. Sections were prepared and double-stained with anti-glucagon and anti-insulin antibodies (10-20 islets per group, three independent experiments).



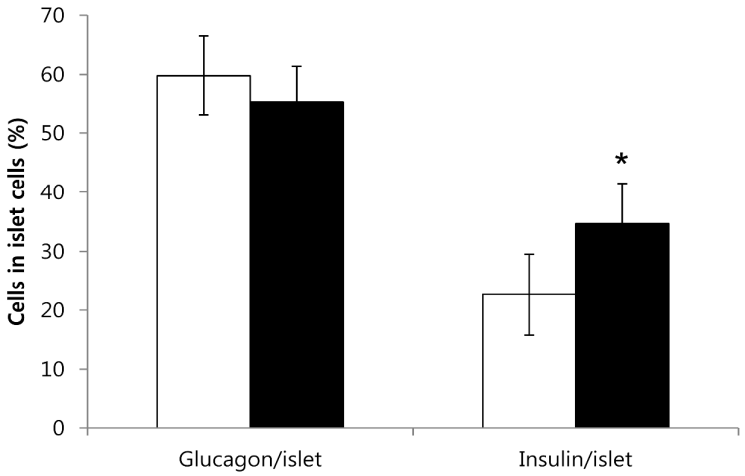
Supplemental Figure 4. **Remission of diabetes in rAd-GLP-1-injected Glucagon-rtTA;Tet-O-Cre;R26-YFP mice.** Blood glucose levels of rAd-GLP-1-injected (black circles, n = 4) or rAd-βgal-injected (white squares, n = 4) Glucagon-rtTA;Tet-O-Cre;R26-YFP mice. Data are means ± SE. * $P<0.05$ compared with rAd-βgal-treated mice.



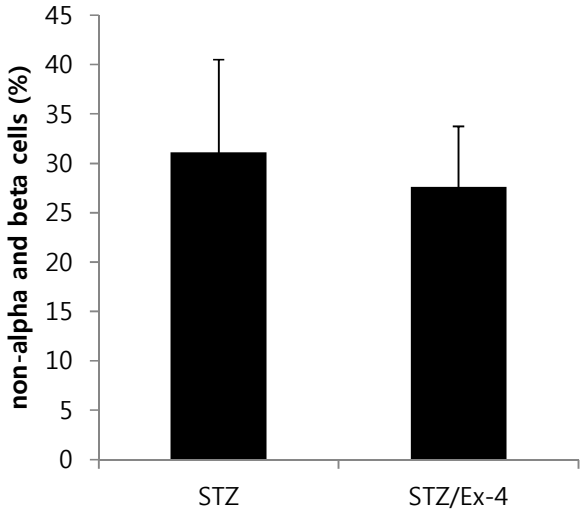
Supplemental Figure 5. GLP-1 increases FGF21 expression in islet cells. The diabetic Glucagon-rtTA;Tet-O-Cre;R26-YFP mice were injected with rAd-βgal or rAd-GLP-1. After 1 week, pancreatic sections were prepared and stained with anti-FGF21 antibody. $FGF21^{+}$ cells were counted and expressed as a percentage of the cell number in the islet. N = 4~5 mice, islet number = 23~44 islet. Data are means ± SE. * $P < 0.05$ compared with rAd-βgal treated mice.



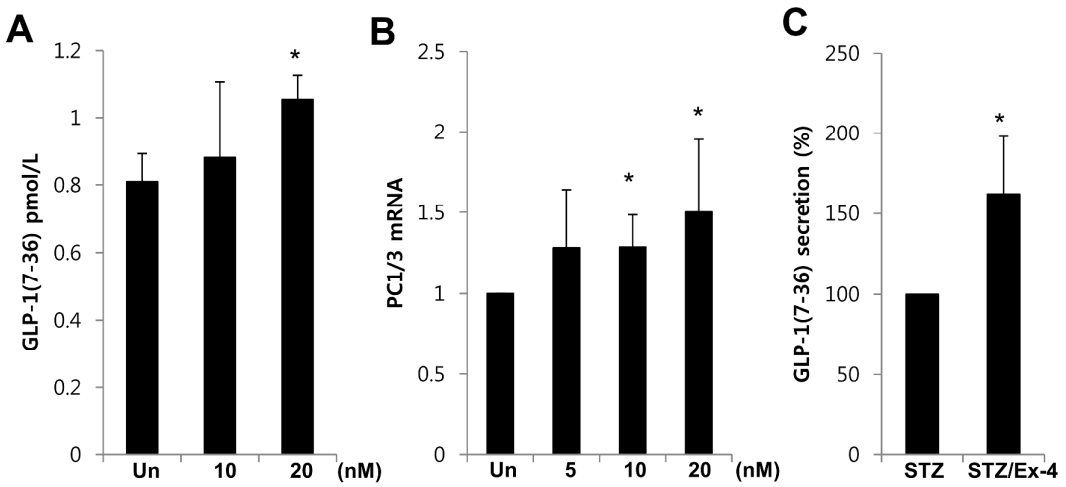
Supplemental Figure 6. **Concentration of glucagon in serum of rAd-GLP-1 or rAd-βgal injected RIP-CreER/YFP mice.** STZ-induced diabetic RIP-CreER/YFP mice were injected with Ad-GLP-1 or rAd-βgal; 4 weeks later, these mice were fasted for 4 h and the serum was collected. Serum glucagon was measured using an RIA glucagon kit. Data are means ± SD. N=5~13.



Supplemental Figure 7. **Insulin-producing cells increased in rAd-GLP-1-injected RIP-CreER;R26-YFP mice.** The percentage of glucagon-producing cells in rAd-GLP-1-injected mice (black bars, n=3, 71 islets) was similar to rAd-βgal-treated mice (white bars, n=3, 56 islets) in spite of an increase in BrdU-incorporating glucagon cells. However, the percentage of insulin-producing cells was significantly increased in rAd-GLP-1-treated RIP-CreER;R26-YFP mice. Data are means ±SE. * $P<0.05$ compared with rAd-βgal-treated mice.



Supplemental Figure 8. **The percentage of non-alpha and beta cells did not increase in STZ/Ex-4 treated islet cells.** Mouse islets were treated with STZ (1 mM) for 15 h and then with or without exendin-4 (10 nM) for 2 days (add per 24 h). Islets were double-stained with anti-glucagon and anti-insulin antibodies. The non-stained ($insulin^-glucagon^-$) cells were counted and expressed as percentage of the number of islet cells (n = 713 STZ-treated cells, 733 STZ-exendin-4-treated cells). Data are means ± SE from four independent experiments.



Supplemental Figure 9. GLP-1(7-36) secretion increased in exendin-4-treated αTC1-9 cells and rat islets. (A) GLP-1 (active 7-36) secretion was measured in untreated (Un) or exendin-4 (Ex-4) -treated (10 or 20 nM for 24 h) αTC1-9 cells. (B) αTC1-9 cells were treated without (Un) or with exendin-4 (5, 10, 20 nM) for 6 h. The expression of PC1/3 mRNA was measured by RT-qPCR and is presented as a ratio of the control. (C) Rat islets were isolated and treated with STZ (1 mM) for 15 h and then with or without exendin-4 (Ex-4, 10 nM) for 2 days. Active GLP-1 secretion was measured and expressed as a percentage of the STZ-treated islet value. Data are means ± SE from three to four independent experiments. * $P<0.05$ compared with untreated or STZ-treated islets.

Supplementary Table 1. List of qPCR primers and sequences.

Gene	sense	anti-sense
cyclin D2	5’-CCGTCAAGAGCAGCATAACG-3’	5’-TGGCTTGGTCCGGATCTTC-3’
cyclin A2	5’-AAGAGGCAGCCAGACATCACTAA-3’	5’-CTCAACCAGCCAGTCCACAA-3’
cyclin E	5’-GTTCCGTTCGCCATGGTTAT-3’	5’-CCCGGAAGTGCTTGAGCTT-3’
cyclin D3	5’-TCCAAGCTGCGCGAAAC-3’	5’-GGTCCGTATAGATGCAAAGCTTCT-3’
glucagon	5’-TGCCACCACGCCCTTC-3’	5’-GCGCTTCTGTCTGGGA-3’
PDX-1	5’-GAAATCCACCAAAGCTCACG-3’	5’-CGGGTTCCGCTGTGTAAG-3’
Arx	5’-TTCCAGAAGACGCACTACCC-3’	5’-TCTGTCAGGTCCAGCCTCAT-3’
insulin	5’-AGCTCCAGTTGTGCCACTTGT-3’	5’- TCAAGCAGCACCTTTGTGGT-3’
PC1/3	5'-ATTTTGGTGCTGCTGCTCTT-3'	5'-GGAGTGCTCGTCTCAACCA-3'
FGF21	5'-ACCTGGAGATCAGGGAGGAT-3'	5'-CACCCAGGATTTGAATGACC-3'
PPAR-α	5'-ACAAGGCCTCAGGGTACCA-3'	5'-GCCGAAAGAAGCCCTTACAG-3'
FGFR-1	5'-TGTTTGACCGGATCTACACACA-3'	5'-CTCCCACAAGAGCACTCCAA-3'
FGFR-2	5'-TCGCATTGGAGGCTATAAGG-3'	5'-CGGGACCACACTTTCCATAA-3'
FGFR-3	5'-GCATCCTCACTGTGACATCAAC-3'	5'-CCTGGCGAGTACTGCTCAAA-3'
FGFR-4	5'-CGCCAGCCTGTCACTATACAAA-3'	5'-CCAGAGGACCTCGACTCCAA-3'
β-klotho	5'-TGGGGTCCCATTGGATAGAG-3'	5'-ACTCAGGGTAGTCGCCGTC-3'
MafA	5'-CCAGCTGGTATCCATGTCC-3'	5'-TTCTGTTTCAGTCGGATGACC-3'
MafB	5'-CAACAGCTACCCACTAGCCA-3'	5'-GGCGAGTTTCTCGCACTTGA-3’
18S rRNA	5’- CCATCCAATCGGTAGTAGCG-3’	5’-GTAACCCGTTGAACCCCATT-3’
cyclophilin	5’-TGGAGAGCACCAAGACAGACA-3’	5’–TGCCGGAGTCGACAATGAT-3’