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Direct Differentiation of Bone Marrow Mononucleated Cells into Insulin-Producing Cells by Four Specific Soluble Factors

네 가지 분화유도인자 처리에 의한 골수 유래 줄기세포의 인슐린 생산세포로의 분화

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중개의학과

김 수 빈

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이 논문을 의학석사 학위논문으로 제출함

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의학과 중개의학전공

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김수빈의 의학석사 학위논문을 인준함

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Abstract

Direct Differentiation of Bone Marrow Mononucleated Cells into Insulin-Producing Cells by Four Specific Soluble Factors

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Bone marrow-derived stem cells are self-renewing and multipotent adult stem cells that differentiate into a variety of cell types, including pancreatic endocrine cells. In this study, I aim to identify and investigate a unique combination of four differentiation-inducing factors, putrescine (Put), glucosamine (GlcN), nicotinamide, and BP-1-102, to develop a differentiation method for inducing mature insulin-producing cells (IPCs), and to apply this method to the six-day in vitro-culture protocol in bone marrow mononucleated cells (BMNCs) from mice. BMNCs, when primed with four soluble factors, Put, GlcN, nicotinamide, and BP-1-102, were induced to differentiate into functional IPCs. Specifically, BMNCs cultured under the defined conditions synergistically expressed multiple genes

related to pancreatic beta cell development and function, including *Pdx1*, *Nkx6.1*, *MafA*, *Neeurog3*, *Glut2*, and *Ins*. Insulin/C-peptide and PDX1 production was confirmed using immunofluorescence staining and FACS analysis. In vitro glucose challenge studies showed that the induced cells secreted insulin in a glucose-responsive manner, like normal pancreatic beta cells. Transplantation of the BMNC-derived IPCs could alleviate hyperglycemia in streptozotocin (STZ)-induced diabetic mice. Moreover, I determined the effect of the differentiation-inducing factors on in vivo differentiation of endogenous stem cells into IPCs in STZ-induced diabetic mice and on homing of the stem cells to the pancreas after being exogenously infused. Oral administration with the extrinsic factors lowered blood glucose levels, enhanced glucose tolerance, and improved glucose-stimulated insulin secretion in diabetic mice. I further observed IPCs in the small intestine as well as the pancreas, as assessed by immunohistochemistry. Generation of chimeric C57BL/6 mice harboring BMNCs from the insulin promoter luciferase/GFP transgenic (MIP-Luc/GFP) mice showed more GFP- and insulin-double positive cells in the pancreas of STZ-induced diabetic mice administered with four extrinsic factors, suggesting that endogenous BMNCs were effectively mobilized, differentiated into IPCs, and homed into the pancreas by addition of the differentiation-inducing factors. In addition, when I treated the differentiation-inducing factors in the mouse pancreatic beta cell line, MIN-6, glucose-stimulated insulin secretion was significantly increased, compared to the vehicle-treated control cells, presumably in part due to the increased expression of transcription factors involved in the maturation of beta cells, such as PDX1, MafA, and Nkx6.1. Taken together, these results demonstrated that a unique combination of four

extrinsic factors could induce the in vivo differentiation of endogenous BMNCs into functional IPCs, as well as the in vitro differentiation of BMNCs, thereby indicating the potential for use in the treatment of diabetes caused by beta cell destruction and damage.

Keyword: Bone marrow mononucleated cells (BMNCs), BP-1-102, Differentiation, Glucosamine, Insulin-producing cells (IPCs), Nicotinamide, Putrescine

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List of Abbreviations

Acetyl-CoA: acetyl coenzyme A

BMNC: bone marrow mononucleated cell

G6P: Glucose 6-phosphate

Gcg: glucagon

Gck: glucokinase

GlcN: glucosamine

GlcNAc: N-Acetyl glucosamine

Glut2: glucose transporter 2

GSIS: glucose-stimulated insulin secretion

IPC: insulin producing cell

MIP-Luc/GFP: insulin promoter luciferase/GFP

O-GlcNAc: O-linked N-acetyl glucosamine

OGT: O-GlcNAc transferase

PAO: Polyamine oxidase

Put: putrescine

SAMDC: S-adenosylmethionine decarboxylase proenzyme

SSAT1: Spermidine/spermine N (1)-acetyltransferase

Sst: somatostatin

STAT3: Signal transducer and activator of transcription 3 STZ: streptozotocin

Syt: synaptotagmin

UDP-GlcNAc: Uridine 5′-diphospho-N-acetylglucosamine

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Introduction

Study Background

The need for stem-cell based therapy in diabetes mellitus

Diabetes mellitus (or diabetes) is a chronic metabolic disorder characterized by hyperglycemia, and the prevalence of diabetes mellitus is rapidly increasing worldwide [1, 2]. Diabetes is divided into two major subtypes: type 1 diabetes (T1D) and type 2 diabetes (T2D) [3]. T1D, known as insulin-dependent, has features of early onset in childhood and severe hyperglycemia caused by the autoimmune-mediated beta cell destruction. Impaired beta cell function in the setting of prolonged insulin resistance in the peripheral tissues can lead to T2D, which eventually leads to beta cell failure. The distinction between the two subtypes has been based on four factors: (1) age at onset; (2) degree of loss of beta cell function and insulin resistance; (3) presence of diabetes-associated autoantibodies; and (4) requirement for insulin treatment for survival [4].

Uncontrolled hyperglycemia can cause several acute and chronic complications in diabetic patients. They require continuous injection or administration of exogenous insulin as the first choice; however, it cannot mimic endogenous insulin secreted by functional pancreatic beta cells. Therefore, recent research trends for treatment of diabetes have been based on cell replacement and regeneration such as islet

transplantation or beta cell replacement [5-7]. These therapeutic approaches are thought to hold a great potential for treatment of diabetes.

However, there are several limitations on the pancreatic islet transplantations [8]. A shortage of cadaveric islet cells is the major obstacle. Since these procedures are currently performed in clinical trials, many criteria must have taken into consideration in donor recipient selection process. Moreover, the surgical procedure has a risk of complications such as intraperitoneal bleeding and development of donor-specific antibodies. Also, the recipient are required to take life-long immunosuppressive medications to prevent graft rejection.

To overcome these limitations, stem cell-derived beta cells are emerging as alternate sources of human islet cells [5]. Several studies have shown that they have the unlimited potential to form functional mature insulin-producing beta cells for transplantation in clinical use. In the respect of the cost and availability, stem cell-derived beta cell transplantation has appeared as new alternatives of islet transplantation.

Generation of insulin-producing cells from stem cells: differentiation of stem cells as alternatives for pancreatic transplantation

Stem cells are divided into three main subtypes: adult stem cells, embryonic stem cells, and induced pluripotent stem cells. All types of stem cells have the ability of self-renewal and differentiation into various types of cells. Among them, bone

marrow-derived stem cells are adult pluripotent stem cells which are resided in bone marrow. The bone marrow is an invaluable source as it gives rise to hematopoietic stem cells (HSCs), endothelial progenitor cells, and mesenchymal stem cells (MSCs), amongst others. Such bone marrow-derived stem cells are progenitors of multiple organs, including bone, cartilage, and skeletal tissues and can differentiate into various types of cells such as neuronal cells, hepatocytes, chondrocytes, myeloid-lineage, and lymphoid-lineage blood cells. [9]

Also, bone marrow-derived stem cells have been the most widely used in transplantation therapies for diabetic patients and ongoing clinical trials as a treatment for different types of diseases in stem-cell based clinical approaches [10-12]. The use of bone marrow-derived stem cell transplantation is reported to assist in achieving tissue repair and regeneration, and in modulating immune responses in the context of autoimmunity and transplantation [12, 13]. Specifically, autologous bone marrow–derived stem cell transplantation in patients with type 2 diabetes has shown a significant improvement in both fasting and glucagon-stimulated C-peptide levels [14]. Combination of both bone marrow-derived stem cells and another type of stem cells derived from different origins has been used in clinical trials. Previous studies by Urbán et al. [15] and Cai et al. [16] showed that combined infusion of autologous BMNCs and (umbilical cord) mesenchymal stromal cells resulted in a significant improvement in glycemic and serum insulin (or C-peptide) levels along with tissue regeneration and repair in patients with established diabetes. Our previous studies also demonstrated that adult mouse bone marrow harbors the cells that have the capacity to differentiate into pancreatic endocrine beta cell phenotype

in vivo and that represent a source for cell-based treatment of diabetes [17, 18].

Direct differentiation of bone marrow mononucleated cells into insulin-producing cells using pancreatic beta cell-derived components

Our previous studies confirmed that BMNCs could differentiate into IPCs in vivo, as demonstrated by the neogenesis of the GFP- and insulin-double positive cells in the streptozotocin (STZ)-induced diabetic chimeric mice harboring BMNCs from the insulin promoter luciferase/GFP transgenic (MIP-Luc/GFP) mice [17, 18]. These results demonstrated that BMNCs could differentiate into IPCs in response to the signals or components shed from the damaged beta cells, suggesting that extracellular vesicle shed from beta cells under stress conditions were responsible for the differentiation of BMNCs into IPCs. Based on these findings, we developed a simple and reproducible IPC-generating priming protocol, in which priming with extracellular vesicle-containing conditioned medium prepared from the insulinoma cell lines under stress conditions directed the MIP-Luc/GFP-derived BMNCs to express GFP within 6 days. The transplantation of the in vitro-primed BMNCs contributed to regulate the blood glucose levels in the diabetic mice and improved the fasting blood glucose levels and glucose tolerance [17, 18].

However, the extracellular vesicles are very heterogeneous, and there is no way to effectively separate and purify only the high efficiency-extracellular vesicles. To identify key differentiation-inducing factor(s) in the extracellular vesicles, the metabolomics study was performed along with the proteomic analysis using the

conditioned media from insulinoma cell line MIN-6 and control cell line NIH3T3. We found the possible involvement of polyamine metabolites, spermidine, spermine, and putrescine (Fig. 1).

O-Linked *β*-*N*-acetyl glucosamine (*O*-GlcNAc) is a post-translational modification of nuclear and cytosolic proteins. Its transferase, *O*-GlcNAc transferase (OGT), is highly expressed in pancreatic islets [19]. Previous studies have shown that the transcription factor PDX1 is modified by *O*-GlcNAc and an increase in protein *O*-GlcNAcylation correlates with an increase in the DNA-binding activity of PDX1 and insulin secretion [20]. Further, the levels of *O*-GlcNAc inside the cells depend on the nutrient availability, especially glucose. Downregulation of *O*-GlcNAcylation attenuates insulin secretion in pancreatic beta cells [20]. Accordingly, *O*-GlcNAc is found to act as a glucose sensor to epigenetically regulate the insulin gene in pancreatic beta cells [21]. Thus, we focused on the hexosamine biosynthesis pathway and the role of its end product, *O*-GlcNAc in beta cells to characterize the unique feature of insulin-producing cells as well as the differentiation process into IPCs (Fig. 2).

Purpose of this study

I aim to investigate a single and/or combined effect of the molecules that we identified previously to generate IPCs from BMNCs and optimize an efficient differentiation protocol into IPCs. In this study, I defined four differentiation-inducing factors including Put, GlcN, nicotinamide, and STAT3 inhibitor BP-1-102

and developed an efficient in vitro-6 day-culture protocol using these extrinsic chemicals that directed the differentiation into IPCs. The functionality of these cells was confirmed in vitro by insulin production and release in a glucose-responsive manner. Also, I determined the efficacy of differentiated cells in ameliorating hyperglycemia in a diabetic animal model.

In addition to the role of these extrinsic chemicals as differentiation-inducing factors, I examined the pharmacological effect of the extrinsic chemicals, by which the four chemicals were systemically infused to STZ-induced diabetic mice through an oral gavage. I found that the mice infused with the extrinsic factors showed a hypoglycemic effect, with a significant increase in the insulin-positive cells in the small intestine as well as the pancreas. Moreover, I generated chimeric mice harboring BMNCs from the MIP-Luc/GFP mice, and provided strong evidence that bone marrow includes pancreatic progenitor cells capable of differentiating into functional endocrine cells and homing to pancreas. These results would confirm the effect of exogenous differentiation-inducing factors for the differentiation of endogenous bone marrow-derived cells into IPCs and furthermore, suggest prospects of anti-diabetic drug therapies as well as cell-based therapies to replace insulin administration in diabetes treatment.

Materials and methods

Animals

C57BL/6-Tg (Ins2-Luc/EGFP/TK) 300 Kauf/J (Jackson Laboratories, Bar Harbor, ME, USA; referred to as MIP-Luc/GFP in this study) and wild-type C57BL/6 mice (Orient-Bio Co. Ltd., Seoul, Korea) were maintained on a 12-h light/dark cycle and had ad lib access to both diet and water in a specific pathogen-free facility at the Biomedical Center for Animal Resource Development of Seoul National University. The characteristic of MIP-Luc/GFP mice were described in detail earlier [17, 18]. Briefly, MIP-Luc/GFP mice carried the tri-fusion transgene luciferase (Luc)/enhanced green fluorescent protein (EGFP)/thymidine kinase (TK) under the control of the mouse insulin-2 promoter. Transgene expression determined by Luc enzymatic activity and GFP expression was exclusive to beta cells in the pancreatic islets. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of Seoul National University (Authorization No. SNU-200911-3-2) and efforts were made to minimize animal suffering as well as the number of animals used in the experiments.

Induction of diabetes

Diabetes was induced in male C57BL/6 mice (aged 8-12 weeks) by a single intraperitoneal injection of 150 mg/kg STZ (Sigma-Aldrich, St. Louis, MI, USA) in 0.1 ml of 0.1 M citrate buffer, pH 4.5 within 15 min of dissolution followed by 6 h fasting according to published methods [22-24]. Three-week post STZ stimulation, animals with random blood glucose value ≥ 350 mg/dL for two consecutive days were considered as STZ-induced diabetic mice. Blood glucose levels were monitored using a standard glucometer (Accu-Chek Active; Roche Diagnostics, Mannheim, Germany) during daytime hours (9-11 a.m.) in mice fed ad libitum. The control group received citrate buffer solution without STZ correspondingly.

Chimeric mice

Chimeric mice were generated by transferring BMNCs (1×10^6 cells/mouse) from MIP-Luc/ GFP mice via the retro-orbital vein into lethally (1000 cGy; 500 cGy twice at 4 h interval) irradiated C57BL/6 mice (8–12 weeks old). BMNCs were isolated from tibiae and femurs by flushing with Hank's Balanced Salt Solution (HBSS). Chimeric mice were used for further experiments four weeks after BM reconstitution. Diabetes was induced through intraperitoneal injections at multiple low doses of STZ (Sigma-Aldrich) in 0.1 ml of 0.1 M citrate buffer, pH 4.5 for a consecutive five day schedule according to published methods.

Isolation of BMNCs and cell lines

BMNCs were prepared by flushing the femurs and tibias of MIP-Luc/GFP or wild-type C57BL/6 mice. Whole BMNCs were suspended in BD Pharm Lyse buffer (BD Biosciences, San Jose, CA, USA) to remove red blood cells, washed, and resuspended in CMRL medium, no glucosamine (Thermofisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermofisher Scientific) and 1% penicillin-streptomycin (Thermofisher Scientific). BMNCs (5×10^6 cells/well) were seeded in 12-well non-coated plates and cultured in suspension on a shaking (80 rpm) platform for differentiation. The murine insulinoma MIN-6 and NIH3T3, a murine fibroblast cell line were cultured in DMEM medium (Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 10% FBS and antibiotics. Human BMNCs (Lonza, Basel, Switzerland) were cultured in HPGMTM hematopoietic growth medium supplemented with antibiotics. The murine insulinoma MIN-6 and murine fibroblast NIH3T3 cell lines were cultured in DMEM medium (Hyclone Laboratories Inc.) supplemented with 10% FBS and antibiotics.

In vitro differentiation of murine BMNCs into IPCs

Whole BMNCs (5×10^6 cells/well) were seeded into 12-well non-coated plates and were primed for 6 days in CMRL containing 10% FBS and antibiotics supplemented with 10 mM putrescine (Put; Putrescine dihydrochloride, ≥98% (TLC), Sigma Aldrich, CAS #333-93-7), 10 mM glucosamine (GlcN; D-(+)-Glucosamine hydrochloride, ≥99%, crystalline, Sigma Aldrich, CAS #66-84-2), 10

mM nicotinamide (99.5% (HPLC), Sigma-Aldrich, CAS #98-92-0), and 10 μM STAT3 Inhibitor XVIII, BP-1-102 (3 mg/kg body weight; Merck Millipore, Burlington MA, USA, CAS #1334493-07-0) in suspension on a shaking platform. After 6 day differentiation, cells were collected for further experiments.

Systemic infusion of priming BMNCs in mice having diabetes

BMNCs were isolated from MIP-Luc/GFP mice, seeded into 12-well non-coated plates, and primed for six days in CMRL containing 10% FBS and antibiotics supplemented with 10 mM GlcN. GlcN-primed BMNCs from MIP-Luc/GFP mice (1×10^6 cells/mouse) were infused into STZ-treated diabetic mice through the tail vein.

Transplantation of primed BMNCs in diabetic mice

Primed or non-primed BMNCs (5×10^6 cells/mouse) were resuspended in the same volume of Matrigel (BD Matrigel Basement Membrane Matrix, BD Bioscience, San Jose, CA, USA), and the BMNC-containing Matrigel solution was aspirated into a 1 mL syringe connected to P50 polyethylene tubing and transferred to the head end. The recipient diabetic mouse was anesthetized, the left flank was shaved, and the kidney was exposed through a small lumbar incision. Capsulotomy of the kidney was performed on the causal outer surface, and the tip of the polyethylene tubing was inserted and advanced gently under the kidney capsule. The surface of

the kidney was kept moist with saline during the procedure. The BMNCs in the tubing was pushed out slowly and carefully, and the tube was removed after complete transfer of the cells into the capsule. Random blood glucose levels, body weight, and food and water intake were measured every 3-4 day.

Oral administration of four differentiation inducing factors in diabetic mice

Mice were divided into four groups: (1) Control; (2) differentiation cocktail-treated control (Control + diff. cocktail) (3) STZ and (4) differentiation cocktail-treated STZ (STZ + diff. cocktail). Putrescine (200 mg/kg body weight), glucosamine (200 mg/kg body weight), nicotinamide (500 mg/kg body weight), and STAT3 Inhibitor XVIII, BP-1-102 (3 mg/kg body weight) were dissolved in vehicle solution (saline). The dosage of each differentiation-inducing factors administered to mice in this study was based on the one previously established from many studies [25-36]. Mice were daily administered with differentiation cocktail containing four differentiation-inducing factors (referred to as diff. cocktail in the study) by oral gavage for five days twice with a 2-week interval.

Glucose tolerance Test (GTT) and in vivo glucose-stimulated insulin secretion (GSIS)

Mice were fasted overnight and given an intraperitoneal injection either with saline (vehicle) or glucose at a dose 2 g/kg body weight. Blood glucose was measured at

the indicated time points. The degree of glucose intolerance was quantified as area under the curve (AUC). For in vivo GSIS, blood samples were obtained via tail vein at baseline insulin levels (0 min) before and 15, 30 min after glucose loading. Plasma insulin levels were measured using the Mouse Ultrasensitive Insulin ELISA (ALPCO, Salem, NH, USA) according to the manufacturer's instructions.

Insulin content measurement

Pancreatic tissues were cut in half and placed into 5 ml acid-ethanol buffer containing 1.5% HCl in 70% EtOH solution overnight at –20℃ for insulin extraction. Tissues were then homogenized and incubated in the same solution overnight at –20℃. The tissues were then centrifuged at 2000 rpm for 15 min at 4℃, and the supernatants then neutralized with 1M Tris, pH 7.5. Insulin content was determined by the Mouse Insulin ELISA kit (ALPCO) according to the manufacturer's instructions. Pancreatic insulin content was normalized with total protein measured using BCA protein assay (Thermofisher Scientific).

Immunohistochemistry and immunofluorescence staining for tissues

Tissue biopsies were fixed overnight in 4% paraformaldehyde, embedded in paraffin, sectioned in a thickness of 4 μm, and stained with antibodies against insulin (Cell Signaling Technology, Danvers, MA, USA) and PDX1 (Abcam, Cambridge, United Kingdom). All histology was performed by the Pathology Core

Facility at the Seoul National University Hospital Biomedical Research Institute and images were captured using an ECLIPSE Ci-L microscope (Nikon Instruments Inc., Melville, NY, USA). For immunofluorescence staining, sectioned tissues were deparaffinized in xylene, washed with ethanol, re-fixed in pre-cooled acetone (–20℃). Fixed tissues were then antigen-retrieved in Target Retrieval Solution (Dako North America, Inc., Carpinteria, CA, USA), permeabilized with PBS containing 00.03% Triton X-100 (Sigma-Aldrich), immersed in 0.1% Sudan Black B solution in 70% ethanol to reduce auto fluorescence, and blocked with 10% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. The tissues were then incubated with primary antibodies (Table 1) in 1% NDS overnight at 4℃, washed three times with PBS containing 1% NDS, and incubated with secondary antibodies in 1% NGS for 2 h at 4 ℃. The tissue were counterstained with DAPI (1:2,500; Molecular Probes, Eugene, OR, USA) and mounted with fluorescence mounting medium (Dako, North America, Inc., S3023). Images were acquired under a confocal microscope (Leica TCS STED CW, Leica Microsystems, Wetzlar, Germany). To quantify islet areas, the islet areas in images of the pancreatic tissues were manually traced and analyzed using MetaMorph Image Analysis tool (Molecular Devices, San Jose, CA, USA).

Immunocytochemistry

Bone marrow-derived IPCs were harvested and fixed in 4% paraformaldehyde for 15 min. Paraformaldehyde-fixed cells were washed three times with PBS, permeabilized with BD Phosflow Perm Buffer III (BD biosciences) and blocked

with PBS containing 10% normal goat serum (NGS) for 1 h at room temperature. The cells were then incubated with primary antibodies (Table 1) in 1% NGS overnight at 4℃, washed three times with PBS containing 1% NGS, and incubated with secondary antibodies in 1% NGS for 2 h in ice. The cells were counterstained with DAPI (1:2,500; Molecular Probes) and observed using a confocal microscope (Leica TCS SP8, Leica Microsystems) equipped with appropriate filters.

Western blotting for *O*-GlcNAc

Cell lysates from MIN-6 and NIH3T3 were prepared in RIPA buffer (Thermofisher Scientific) containing protease inhibitor cocktail (Thermofisher Scientific). Thirty μg of protein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA), and immunoblotted by monoclonal anti-mouse *O*-linked *N*-acetyl glucosamine (*O*-GlcNAc) antibody (Abcam, Cambridge, UK). The bands were visualized using an Enhanced Chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA). Beta-actin (Sigma-Aldrich) was used as a loading control.

Flow cytometry

Prepared cells were washed twice in staining buffer (HBSS containing 2% FBS). To quantify the GFP-expressing cells controlled by insulin-2 promoter activation, primed BMNC were washed twice with PBS and subjected to flow cytometry using a BD FACS Canto II flow cytometer (BD Biosciences) with subsequent analysis

using the FlowJo data analysis software (FlowJo, Ashland, OR, USA) with at least 50,000 events being acquired. For differentiation efficiency, differentiated cells were fixed with BD Fixation Buffer (BD Biosciences) on ice for 30 min, washed once in FACS buffer (2% FBS, 0.1 mM EDTA in PBS without Ca^{2+} and Mg^{2+}), and permeabilized in BD Phosflow Perm Buffer III on ice for 30 min. Cells were then resuspended in FACS buffer with antibodies against C-peptide Alexa647 (BD Biosciences) and PDX1 6.1 PE (BD Biosciences) and incubated at 4℃ overnight. Cells were washed twice and finally resuspended in 300 µl of FACS buffer, analyzed using BD DACS Canto II flow cytometer with at least 50,000 events recorded. Analysis of the results was performed using FlowJo software.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR)

Total RNA was isolated from cells using TRIzol reagent (Life Technologies, Waltham, MA, USA). cDNA was synthesized from 1 µg total RNA using SuperScript II Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. RT-PCR was performed to amplify each cDNA using the AccuPower PCR premix (Promega). PCR products were analyzed by 2% agarose gel electrophoresis and images were captured by Gel Doc XR+ (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR was performed using SYBR Premix ExTaq (Takara, Shiga, Japan) and the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Each cycle threshold (Ct) values was subtracted

from the glyceraldehyde 3-phosphate dehydrogenase (Gapdh) Ct value of the same samples (dCt) and then subtracted from the dCt value of each control set (ddCt). Relative mRNA levels were expressed as 2-ddCt. Primer sequences are listed in Table 2.

In vitro glucose-stimulated insulin secretion (GSIS)

Insulin secretion was measured using the Krebs Ringer Bicarbonate (KRB) buffer (119 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2mM $KH2PO_4$, 1.2 mM $MgCl_2$, 10 mM HEPES, 25 mM $NaHCO_3$, and 0.2% BSA). Cells were pre-incubated for 2 h in the KRB buffer containing 0.2 mM glucose for glucose starvation. After pre-incubation, cells were incubated in KRB buffer containing 2- or 16.8-mM glucose (Sigma-Aldrich) at 37℃ for 30 min, respectively. Insulin released into the supernatants was measured using the Mouse Ultrasensitive Insulin ELISA kit (ALPCO) according to the manufacturer's instructions. It was normalized with the protein content of the corresponding cell lysates.

Statistical analysis

Data are given as means ± SEM if not specified. Statistical significance ($p < 0.05$) was determined by unpaired Student t-test.

Table 1. List of antibodies used for immunofluorescence staining

Antibodies		Catalog#	Dilution
Unconjugated primary antibodies	Insulin	Cell signaling Technology (#3014S)	1:200
	Insulin	Thermofisher (#PA1-26938)	1:50
	Glucagon	Cell signaling Technology (#2760s)	1:100
	Ki67	Abcam (#ab15580)	1:1000
	PDX1	Abcam (#ab47267)	1:100
	PDX1	Abcam (#ab47308)	1:5000
	GFP	Abcam (#ab6556)	1:500
	O-GlcNAc	Abcam (#ab2739)	1:1000
	O-GlcNAc (CTD110.6)	Santa Cruz (#sc-59623)	1:500
	B-actin	Sigma-Aldrich (#A2228)	1:2000
	GAPDH	Cell Signal (#5174s)	1:5000
Conjugated secondary antibodies	Fluorescein (FITC) AffiniPure Donkey Anti-Guinea Pig IgG (H+L)	Jackson ImmunoResearch (#706-095-148)	1:1000
	Fluorescein (FITC)-AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch (#711-095-152)	1:1000
	Cy3 AffiniPure Donkey Anti Rabbit IgG (H+L)	Jackson ImmunoResearch (#711-165-152)	1:1000
	Alexa Fluor® 594 AffiniPure $F(ab')_2$ Fragment Donkey Anti-Guinea Pig IgG (H+L)	Jackson ImmunoResearch (#706-586-148)	1:1000
	DAPI (4', 6-Diamidino-2-Phenyl indole, Dihydrochloride)	14.3 mM Invitrogen (#D1306)	1:5000

	Goat anti-mouse IgG (HRP)	GeneTex (#GTX21311-01)	1:2000
	Mouse anti-rabbit IgG-HRP	Santa Cruz (#sc-2357)	1:5000

Table 2. Sequences of primers used for qPCR

Gene of interest		Primer sequences (5'→3')	Product size (bp)
Symbol	**Full name**		
Ins1	Insulin	F - CCT GTT GGT GCA CTT CCT AC R - TTG TTC CAC TTG TGG GTC CT	190
Pdx1	Pancreatic and duodenal homeobox 1	F - GAG CAA GAT TGT GCG GTG AC R - CCT CAG ACT GCT GTC CTC AC	199
MafA	V-Maf musculoaponeurotic fibro sarcoma oncogene family, protein A	F - CCC GCC AAC TTC TCG TAT TT R - ATC CGA CTG AAA CAG AAG CG	200
Nkx6.1	NK6 homeobox 1	F - TTC GCC CTG GAG AAG ACT TT R - CTC CGA GTC CTG CTT CTT CT	183
NeuroD1	Neurogenic differentiation 1	F - AGA AGA GGA GGA GGA GGA TCA R - GGG TCT TGG AGT AGC AAG GT	188
Neurog3	Neurogenin-3	F - AGT TGG CAC TCA GCA AAC AG R - TCT GAG TCA GTG CCC AGA TG	192
Cxcr4	C-X-C motif chemokine receptor 4	F - TCC AAC AAG GAA CCC TGC TTC R - TTG CCG ACT ATG CCA GTC AAG	101
Sox17	SRY-box transcription factor 17	F - CGA GCC AAA GCG GAG TCT C R - TGC CAA GGT CAA CGC CTT C	156
FoxA2	Forkhead box protein A2	F - TTC AAC CAC CCG TTC TCC ATC AAC R - TGT TCG TAG GCC TTG AGG TCC ATT	107
Gcg	Glucagon	F – TTA CTT TGT GGC TGG ATT GCT T R – AGT GGC GTT TGT CTT CAT TCA	149
Sst	Somatostatin	F - ATG CTG TCC TGC CGT CTC R - TGC AGA AAC TGA CGG AGT CT	107
Glut2	Glucose transporter 2	F - ATC TTC ACG GCT GTC TCT GT R - ACC TGG CCC AAT CTC AAA GA	198
Gck	Glucokinase	F - GCA TCT CTG ACT TCC TGG ACA AG R - CTT GGT CCA GTT GAG CAG GAT G	122
Syt4	Synaptotagmin 4	F - ATG GCT CCT ATC ACC ACC AG R - AGC AGA TCC AGG CAA AGA GA	112
Gfp	Green fluorescent protein (GFP)	F – GAGCTGAAGGGCATCGACTTCAAG R – GGA CTG GGT GCT CAG GTA GTG G	246
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	F - AGG TCG GTG TGA ACG GAT TTG R - TGT AGA CCA TGT AGT TGA GGT CA	123

F, forward primer; R, reverse primer

Results

Identification of putrescine as a differentiation-inducing factor

Previously, our study confirmed using FACS analysis that BMNCs isolated from MIP-Luc/GFP mice primed with 100 μM spermidine or spermine for six days markedly increased the number of GFP-expressing cells compared to untreated control cells (Fig. 1A). However, the polyamines have strong cytotoxic effects under in vitro culture conditions because they are converted into aldehydes by polyamine oxidase present in FBS of the culture medium. Thus, we investigated whether their precursor putrescine (Put) could replace the differentiation-inducing effect of spermidine and spermine (Fig. 1B).

As shown in the FACS analysis for GFP expression induced from the activation of insulin 2-promoter, BMNCs derived from MIP-Luc/GFP mice primed with 10 mM Put showed higher number of GFP-expressing cells than those primed with vehicle-treated control cells (Fig. 1A). These results suggested that BMNCs, could differentiate into IPCs with a single metabolite. Thus, Put was selected as a differentiation-inducing factor for IPCs.

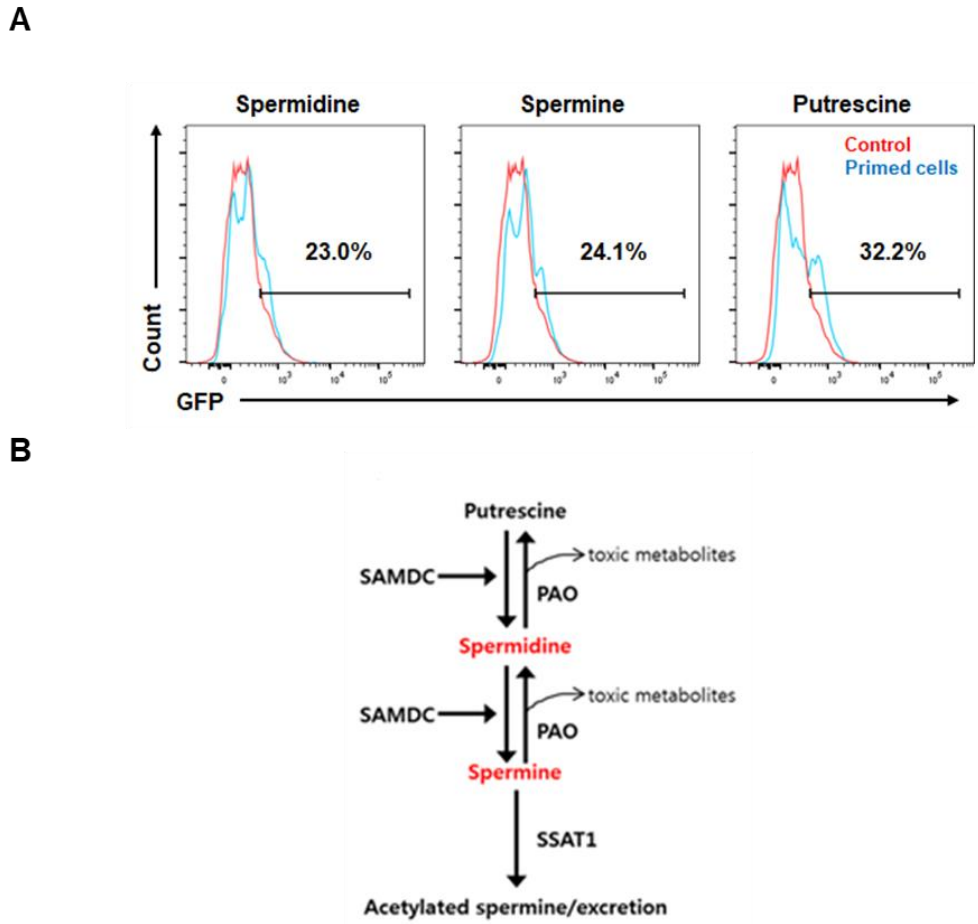


Figure 1. Identification of differentiation-inducing factors into IPCs.

(A) FACS analysis of BMNCs after six day-priming with spermidine, spermine, or putrescine. Representative histograms illustrating GFP expression as a marker of insulin promoter activation. Freshly isolated BMNCs from MIP-Luc/GFP mice were primed with different polyamines (blue) for 6 days and GFP expression was compared to control BMNCs (red) in non-primed culture media. (B) Polyamine biosynthetic pathway.

Glucosamine supplementation improves the differentiation of BMNCs into IPCs

Our previous study confirmed that *O*-GlcNAc levels were considerably higher in the pancreatic beta cell line MIN-6 than in the fibroblast NIH-3T3 cells in Western blotting (Fig. 2A). The result suggested that GlcN could increase *O*-GlcNAc levels by mimicking the hexosamine biosynthesis pathway (Fig. 2C). When BMNCs isolated from MIPLuc/GFP mice were primed in vitro with GlcN (10 mM) for six days, GFP expression was observed by insulin promoter activation in GlcN-primed BMNCs (Fig. 2B).

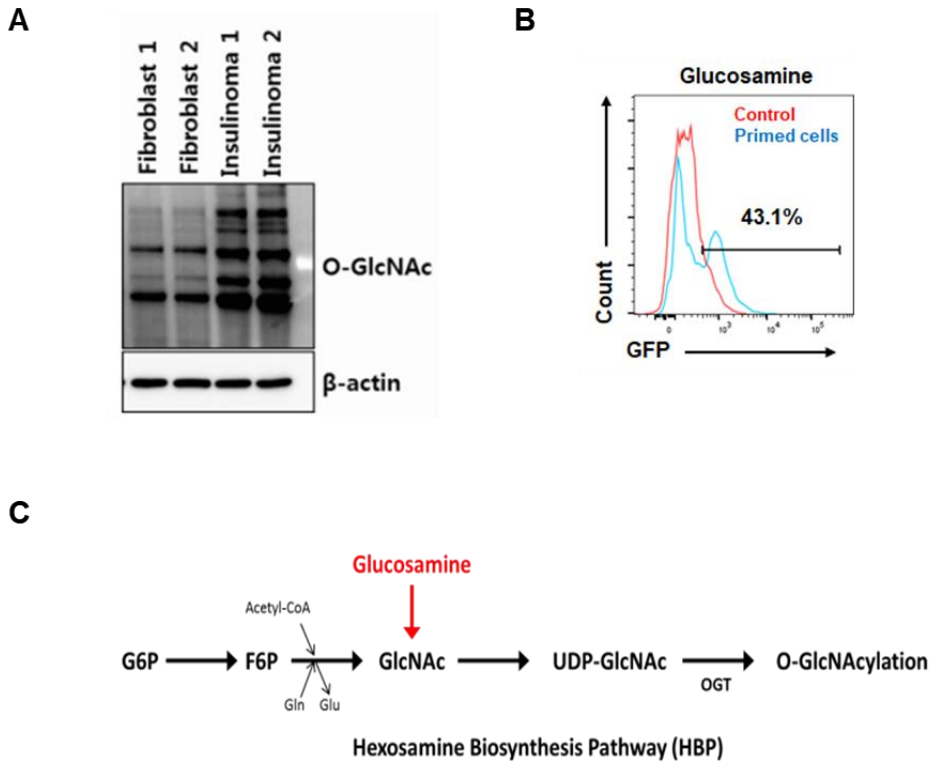


Figure 2. Glucosamine supplementation improves the differentiation of BMNCs into IPCs.

(A) The levels of *O*-GlcNAcylation in NIH3T3 and pancreatic beta cell line MIN-6 cells were verified by immunoblotting using anti-*O*-GlcNAc antibody. Beta-actin was used as a protein loading control. (B) FACS analysis of BMNCs isolated from MIP-Luc/GFP mice after six day-priming with glucosamine. Representative histograms illustrating GFP expression as a marker of insulin promoter activation. Freshly isolated BMNCs from MIP-Luc/GFP mice were primed with glucosamine (blue) for 6 days and GFP expression was compared to control BMNCs (red) in non-primed culture media. (C) Role of glucosamine in hexosamine biosynthesis pathway. GlcN, glucosamine.

Establishment of the optimal condition for differentiation of BMNCs-derived IPCs using four extrinsic factors

Then, I found that co-treatment with Put and GlcN improved the differentiation of BMNCs into IPCs, as assessed by qPCR (Fig. 3). Nicotinamide, a poly (ADP-ribose) synthetase inhibitor, has been reported to promote IPC maturation after the endocrine progenitor stage. To promote further differentiation, nicotinamide (10 mM) was added to the medium containing Put and GlcN. A combination of the three factors showed the synergistic effect on in vitro differentiation of BMNCs toward IPCs. Since the suppression of STAT3 signaling efficiently enhances the reprogramming efficiency into beta cells induced by the defined transcription factors PDX1, NEUROG3, and MAFA, BMNCs were treated with STAT3inhibitor BP-1-102, together with Put, GlcN, and nicotinamide, which resulted in the most effective mRNA induction of beta cell markers (Fig. 3). GFP expression using BMNCs isolated from MIP-Luc/GFP mice also confirmed a synergistic increase in insulin promoter activation with the combination of four factors (Fig. 4).

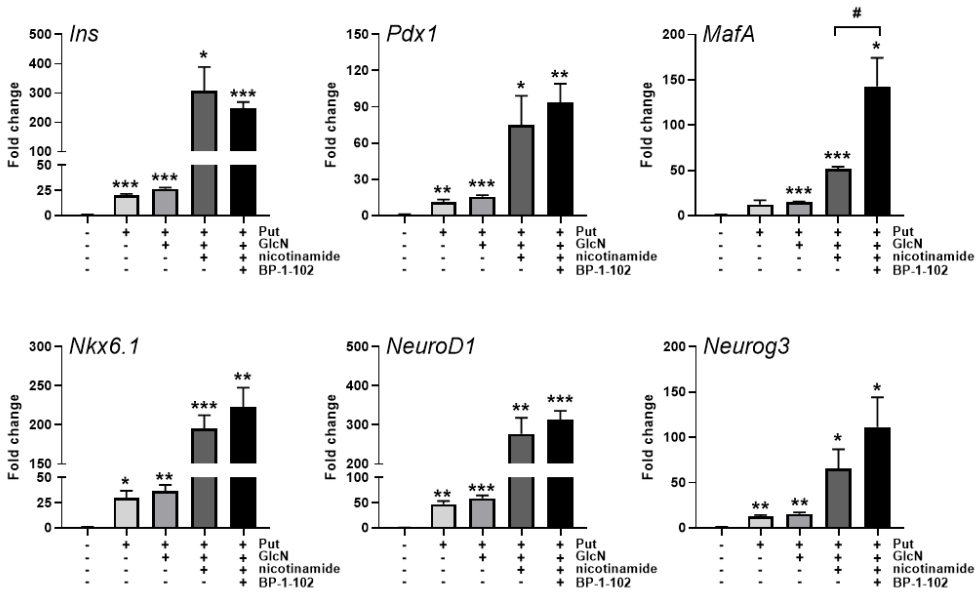


Figure 3. Relative expression of beta cell-specific genes in mouse bone marrow-derived cells treated with four differentiation-inducing factors.

Isolated BMNCs were treated with Put, GlcN, nicotinamide, and BP-1-102 for 6 days. mRNA expression levels were analyzed using qPCR. Results are presented as mean ± SEM, obtained from at least three independent experiments. Put, putrescine; GlcN, glucosamine. *Means are significantly different from non-treated BMNCs at $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. $^{\#}$Means are significantly different from BMNCs primed with Put + GlcN + nicotinamide at $p < 0.05$.

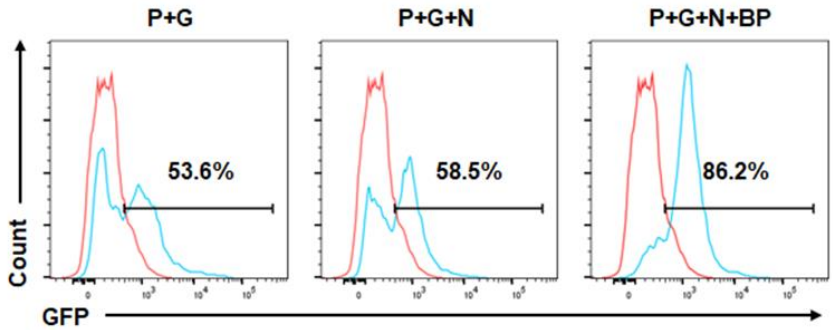


Figure 4. Synergistic effect of four differentiation-inducing factors in BMNC-derived IPCs.

FACS analysis of MIP-Luc/GFP-derived BMNCs after 6 days of priming with four differentiation-inducing factors in the indicated combinations. GFP expression by insulin promoter activation in primed- (blue) *vs.* non-primed (red) cells was monitored. P+G, combination of putrescine and glucosamine; P+G+N, combination of putrescine, glucosamine, and nicotinamide; P+G+N+BP, combination of putrescine, glucosamine, nicotinamide, and BP-1-102.

Characterization of IPCs following in vitro culture of murine BMNCs with four differentiation-inducing factors

To determine whether primed BMNCs produce PDX1 and synthesize insulin protein, BMNCs differentiated with the four extrinsic factors Put, GlcN, nicotinamide, and BP-1-102, were stained with anti-insulin and anti-PDX1 antibodies and visualized using confocal microscopy. Primed BMNCs were strongly stained with anti-insulin and anti-PDX1 antibodies (Fig. 5A). However, these proteins were rarely detected in the control cells. Furthermore, differentiation efficiency was evaluated using FACS analysis of C-peptide- and PDX1-positive cells, revealing that the percentage of C-peptide^{+} /PDX1^{+} cells increased to 30.9% of total cells by day 6 (Fig. 5B).

Next, to determine whether the BMNCs primed with four differentiation-inducing factors underwent pancreatic differentiation, gene expression profiles for beta cell development, differentiation markers, and hormones were assessed using RT-PCR (Fig. 6A). Primed BMNCs underwent pancreatic endocrine differentiation, as evidenced by a gradual decrease in mRNA expression of definitive endodermal markers (*Cxcr4*, *Sox17*, and *FoxA2*) and an increase in mRNA expression of *Pdx1*, *Nkx6.1*, *MafA*, *Neurog3*, *NeuroD1*, and *Ins* until day 9. Primed BMNCs expressed somatostatin (*Sst*) for delta cells; however, glucagon (*Gcg*) for alpha cells was not observed, suggesting that this protocol induced differentiation into Pax4-positive endocrine cells, including beta and delta cells, divergent from the Arx-positive alpha cell lineage (Fig. 6B). Thus, this differentiation protocol successfully differentiated BMNCs into endocrine progenitors.

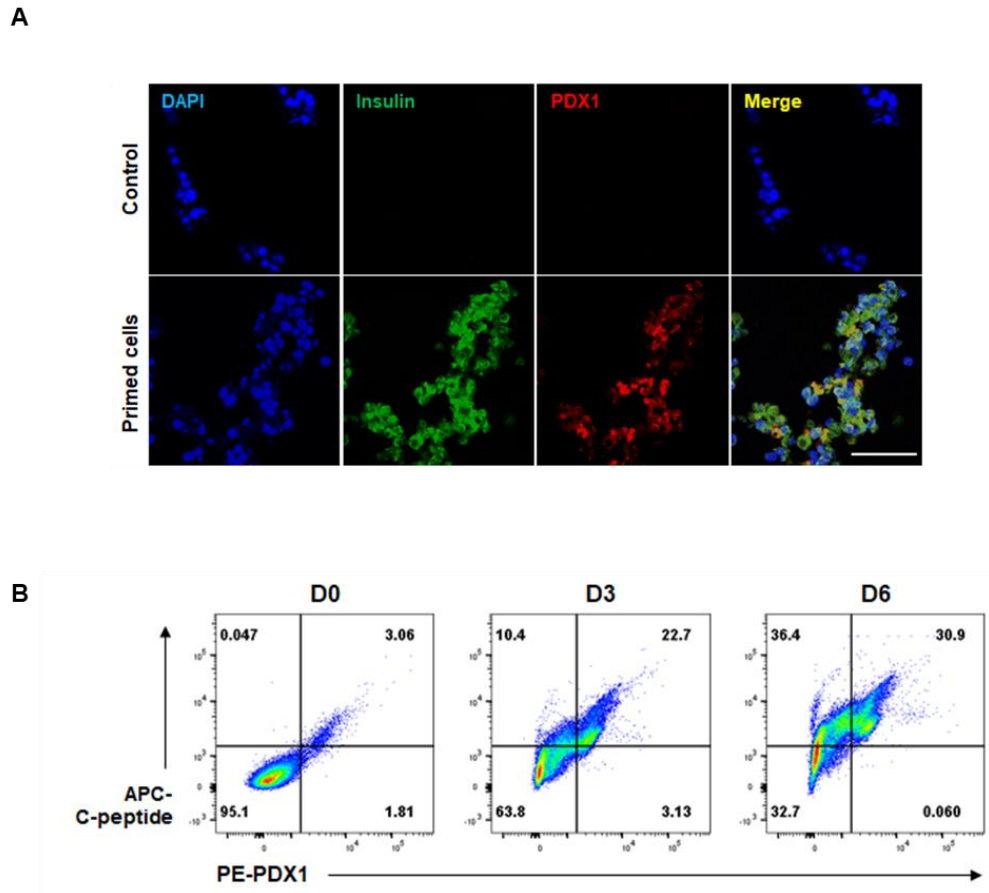
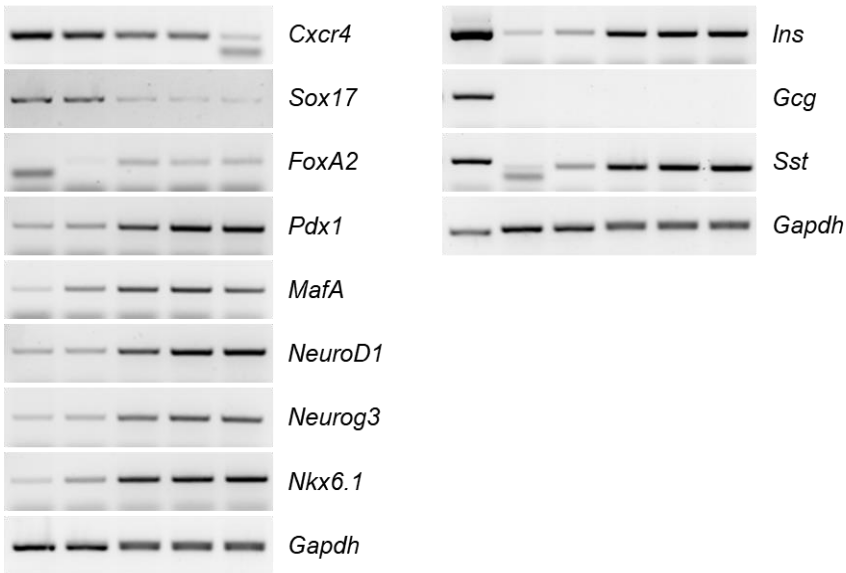


Figure 5. Characterization of IPCs following in vitro culture of murine BMNCs with four differentiation-inducing factors.

(A) Immunofluorescence staining of cells stained with anti-insulin (green) and anti-PDX1 (red), followed by DAPI staining (blue) for nuclei and observed under a confocal fluorescence microscope. Scale bar, 50 μm; original magnification, 1,200X. (B) Representative FACS plots illustrating protein expression of C-peptide and PDX1 in BMNCs-derived IPCs. Numbers mark the percentage of the cells in each quadrant. Data shown are representative of three independent experiments.

A


0 1 3 6 9 (day)
Cxcr4
Sox17
FoxA2
Pdx1
MafA
NeuroD1
Neurog3
Nkx6.1
Gapdh
NIT1 0 1 3 6 9 (day)
Ins
Gcg
Sst
Gapdh

B

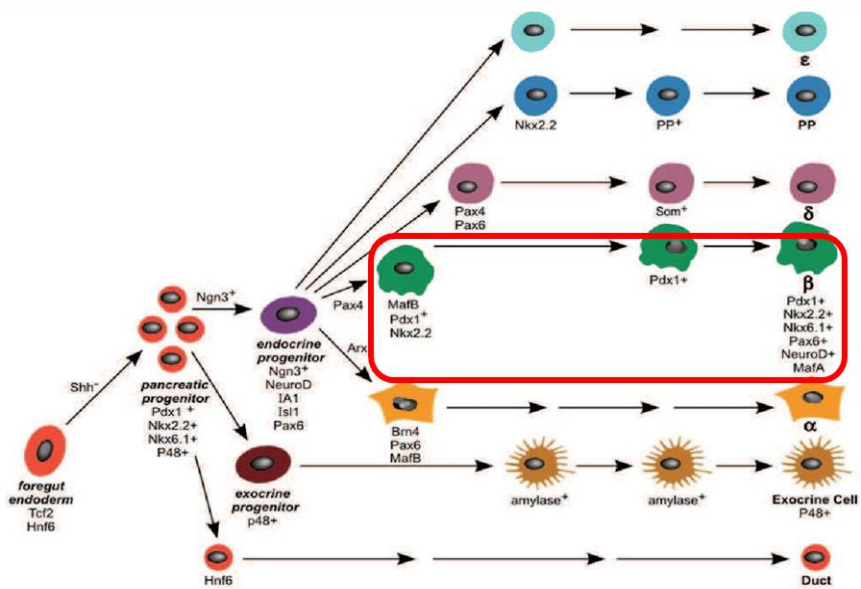

foregut endoderm
Tcf2
Hnf6
Shh+
pancreatic progenitor
Pdx1 +
Nkx2.2+
Nkx6.1+
P48+
Ngn3+
endocrine progenitor
Ngn3+
NeuroD
IA1
Isl1
Pax6
Pax4
Arx
exocrine progenitor
p48+
Hnf6
MafB
Pdx1+
Nkx2.2
Brn4
Pax6
MafB
Nkx2.2
PP+
PP
Pax4
Pax6
Som+
δ
Pdx1+
β
Pdx1+
Nkx2.2+
Nkx6.1+
Pax6+
NeuroD+
MafA
α
amylase+
amylase+
Exocrine Cell
P48+
Duct

Figure 6. Expression of pancreatic developmental markers during the progression of differentiation.

(A) RT-PCR analysis for pancreatic development-related genes. GAPDH was served as a loading control for template cDNA. Expression of pancreatic developmental markers during the progression of differentiation was determined by PCR with reverse transcription. All the data shown here are representative of three independent experiments. *Ins*, insulin; *Gcg*, glucagon; *Sst*, somatostatin. (B) Schematic diagram of lineage development in the pancreas showing role of defined transcription factors adopted from *Stem Cells in Clinic and Research* titled by Pancreatic Stem Cells: Unresolved Business by Jiang FW and Morahan G.

Insulin secretion in response to glucose stimulation of BMNC-derived IPCs by a combination of four extrinsic factors

To determine whether the primed BMNCs were responsive to glucose challenge, insulin release from vehicle-treated control or differentiation-inducing factors-treated (primed) BMNCs was measured using ELISA. Insulin released from the differentiated BMNCs was markedly higher in both basal and glucose-stimulated conditions than control BMNCs (Fig. 7A). Insulin secretion in the primed cells was approximately-6 fold higher than that in the vehicle-treated cells under high glucose conditions (control vs primed cells, 1.48 ± 0.45 vs. 8.64 ± 0.67) and about 2.5-fold higher under low glucose conditions (control vs. primed cells, 1.3 ± 0.37 vs. 3.45 ± 12 0.73). In contrast, control BMNCs showed no significant release of insulin in the presence or absence of glucose (Fig. 7A). These results suggested that differentiation-inducing factors play an indispensable role in the differentiation of BMNCs into IPCs and that differentiated BMNCs are responsive to glucose challenge.

To investigate whether the four differentiation-inducing factor-mediated insulin secretion is induced by the expression of genes associated with the insulin secretion machinery, qPCR was performed to examine the mRNA expression of *Glut2*, *Gck*, and *Syt* in the differentiated cells. Consistent with the increased insulin release in primed cells, the mRNA levels of these genes were markedly increased in the primed cells (Fig. 7B). These results indicated that insulin secretion and dose-dependent glucose responsiveness of differentiated cells might be due to an increase in the expression of molecules involved in the insulin secretion machinery.

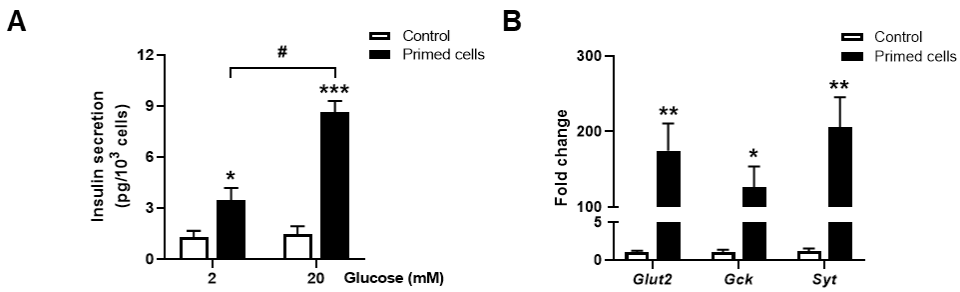


Figure 7. Functionality of BMNC-derived IPCs.

(A) Insulin secretion in response to glucose stimulation in BMNC-derived IPCs. Insulin secretion from differentiated IPCs on day 6 was measured by ultrasensitive ELISA in the presence of low (2 mM) and high (20 mM) glucose concentrations. Results shown here represent those of five separate experiments. (B) The mRNA expression of *Glut2*, *Gck*, and *Syt* in differentiated IPCs was measured by qPCR. Results represent those of at least three separate experiments. Data are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. $^{\#}$Means are significantly different from primed cells in the presence of low glucose concentrations at $^{\#}$, $p < 0.001$. *Glut2*, glucose transporter 2; *Gck*, glucokinase; *Syt*, synaptotagmin.

Transplantation of primed BMNCs ameliorates hyperglycemia in STZ-induced diabetic mice

In C57BL/6 mice with STZ-induced hyperglycemia, BMNC-derived IPCs were transplanted under the kidney capsule (Fig. 8A) and blood glucose levels were measured every 3–4 days for 42 days after grafting BMNC-derived IPCs. The STZ-treated control mice implanted with non-primed BMNCs exhibited metabolic parameters including a significant increase in both food and water intake (Fig. 8B), blood glucose levels (Fig. 8C) and persistent weight loss (Fig. 8D).

However, the IPC-implanted mice showed decreased blood glucose levels as early as day 18 following grafting (Fig. 8C). Aberrant food and water intake decreased after grafting (Fig. 8B). In addition, the IPC-grafted mice maintained their body weight; however, the control mice showed a marginal decrease in body weight (Fig. 8D). These results suggested that BMNC-derived IPCs are functional in vivo and capable of lowering hyperglycemia in diabetic mice.

Most of all, mice implanted with BMNC-derived IPCs in diabetic states showed significantly lower fasting blood glucose levels and improved glucose tolerance 27 days post grafting compared to the matched controls. The area under the glucose clearance curves was significantly reduced in IPC-grafted mice following intraperitoneal glucose challenge (Fig. 8E). Further, in vivo GSIS from IPC-transplanted mice displayed statistically higher levels ($p = 0.017$) of plasma insulin at 15 min post-glucose injection compared with the corresponding controls in STZ-treated mice (Fig. 8F).

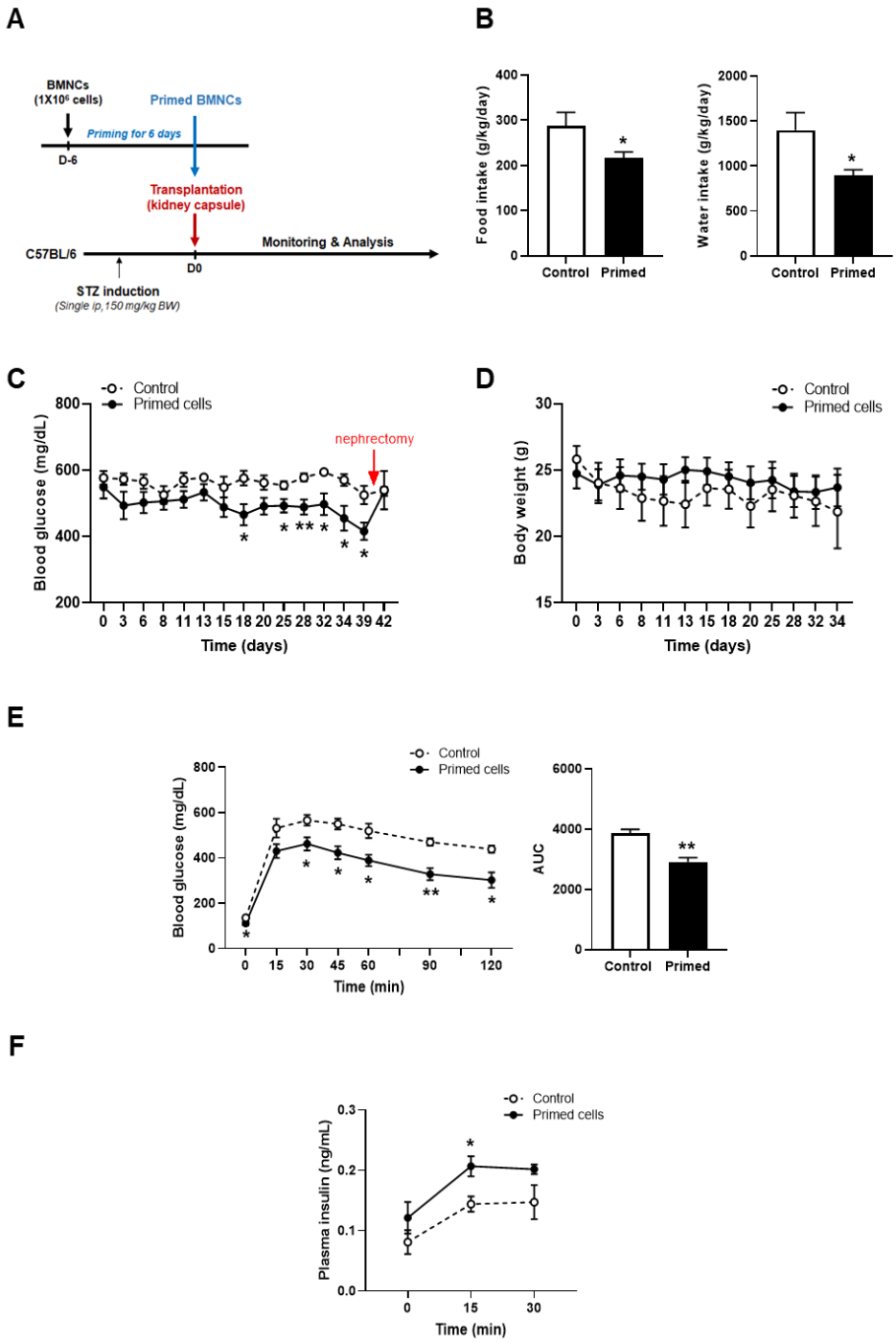

A
BMNCs
(1X10⁶ cells)
Primed BMNCs
Priming for 6 days
D-6
Transplantation
(kidney capsule)
C57BL/6
Monitoring & Analysis
D0
STZ induction
(Single ip,150 mg/kg BW)
B
Food intake (g/kg/day)
Control
Primed
Water intake (g/kg/day)
Control
Primed
C
Control
Primed cells
nephrectomy
Blood glucose (mg/dL)
Time (days)
D
Control
Primed cells
Body weight (g)
Time (days)
E
Control
Primed cells
Blood glucose (mg/dL)
Time (min)
AUC
Control
Primed
F
Control
Primed cells
Plasma insulin (ng/mL)
Time (min)

Figure 8. Transplantation of primed BMNCs improves hyperglycemia in STZ-induced diabetic mice.

(A) Summarized scheme of animal experiments. Male C57BL/6 mice were injected intraperitoneally with STZ. After 7 days, primed or non-primed BMNCs (1×10^6 cells/mouse) were transplanted under the kidney capsule, followed by monitoring for 42 days. (B) Food (left panel) and water (right panel) intake was measured every 3–4 day. Changes in random feeding blood glucose levels (C) and body weight (D) of mice transplanted with primed BMNCs (filled circle, straight line, n=6) or comparable non-primed BMNCs (empty circle, dotted line, n=5) were measured at the indicated days. (E) Blood glucose responses from the intraperitoneal glucose tolerance test (ipGTT). Glucose (1 g of /kg body weight) was injected after overnight fasting 27 days post grafting. AUC (right panel) was measured. (F) Plasma insulin levels at the indicated time points post glucose injection (2 g/kg, body weight) followed by overnight fasting was measured by ultrasensitive mouse insulin ELISA. Data are presented as mean ± SEM. *, *p* value < 0.05; **, *p* value < 0.01 for non-primed- vs. primed BMNC-transplanted mice. STZ, streptozotocin.

The presence of insulin- and PDX1-expressing cells in IPC-transplanted kidney corrected hyperglycemia

To confirm that the IPCs transplanted under the kidney capsule were indeed responsible for the correction of hyperglycemia, the kidney sections from the nephrectomized mice were subjected to immunofluorescence staining using anti-insulin and anti-PDX1 antibodies. Immunofluorescence staining confirmed the presence of insulin- and PDX1-expressing cells in IPC-transplanted kidney (Fig. 9A).

Finally, to eliminate the possibility of endogenous pancreatic beta cell regeneration, immunohistochemistry with anti-insulin antibody from the pancreatic tissues was performed on day 42 from the STZ-treated mice grafted with BMNC-derived IPCs. This demonstrated that the pancreas from the mice grafted with IPCs revealed near complete loss of pancreatic islets, which is comparable to the diabetic controls, and no evidence of endogenous beta cell regeneration (Fig. 9B). As an additional assessment, I quantitatively evaluated the pancreatic insulin content in these animals, which resulted in no significant difference between the groups (control vs. primed cells, 1.77 ± 1.46 vs. 2.20 ± 0.91; $p = 0.598$) (Fig. 8C). Thus, IPCs respond to glucose challenge in vivo by releasing insulin. Analysis of IPC graft sites at 42 days after transplantation revealed no substantial tumor formation. Collectively, the engrafted IPCs remained differentiated and survived up to a month after transplantation. This data suggested that BMNC-derived IPCs are responsible for the reversal of hyperglycemia in diabetic mice.

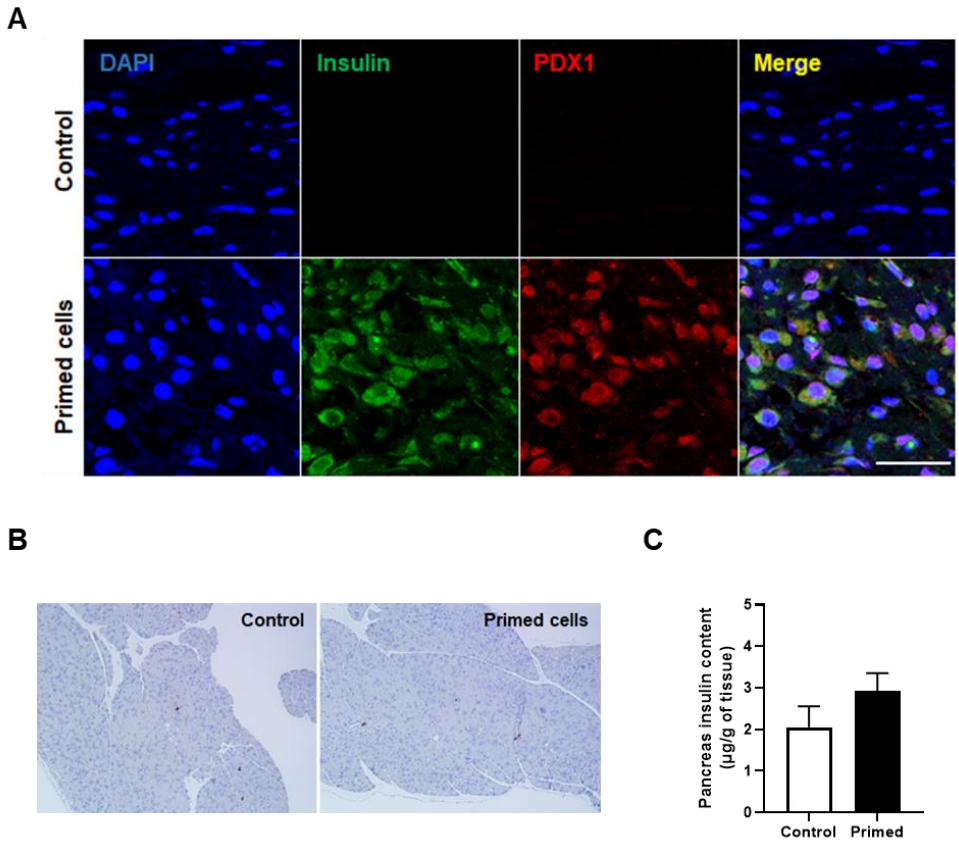


Figure 9. Analysis of grafts following kidney excision via immunofluorescence staining for insulin and PDX1 expression.

(A) Representative immunofluorescent images of the nephrectomized grafts stained with anti-insulin (green) and anti-PDX1 (red), followed by DAPI staining (blue) for nuclei and observed under a confocal fluorescence microscope. Scale bar, 50 μm; original magnification, 1,200X. (B) Representative immunohistochemical images of the pancreas for insulin harvested 42 days after transplantation. All images were acquired with an ECLIPSE Ci-L microscope. Scale bar, 100 μm; original magnification, 200X. (C) Pancreatic insulin content in mice was also determined from the pancreatic tissues harvested 42 days after transplantation

using high-range mouse insulin ELISA. Data are presented as mean ± SEM, obtained from mice transplanted with primed BMNCs (n=6) and non-primed BMNCs (n=5).

Cytotoxic effect of four differentiation-inducing factors in pancreatic beta cell line, MIN-6

Next, I would like to investigate the effect of four differentiation-inducing factors on in vivo differentiation of endogenous BMNCs into IPCs in STZ-induced diabetic mice and on homing of the BMNCs to the pancreatic tissues, as well as the small intestine, after being exogenously infused. Accordingly, the cytotoxic effect of four differentiation-inducing factors in pancreatic beta cell line, MIN-6 was first examined. The cells were treated with various concentrations of each differentiation-inducing factor or the combination of the factors for 24 h, and the cell viability was determined by Cellometer *K*2 Fluorescent Viability Image Cytometer followed by staining with Acridine Orange (AO) and Propidium Iodide (PI). As shown in Fig. 10, there was no toxicity to MIN-6 cells even at high concentrations up to 40 mM for Put, GlcN, and nicotinamide and 20 μM for BP-1-102. Thus, four differentiation-inducing factors at the indicated concentrations used in this study were confirmed to be safe, indicating that the combination could be used as a pharmaceutical composition for preventing or treating diabetes.

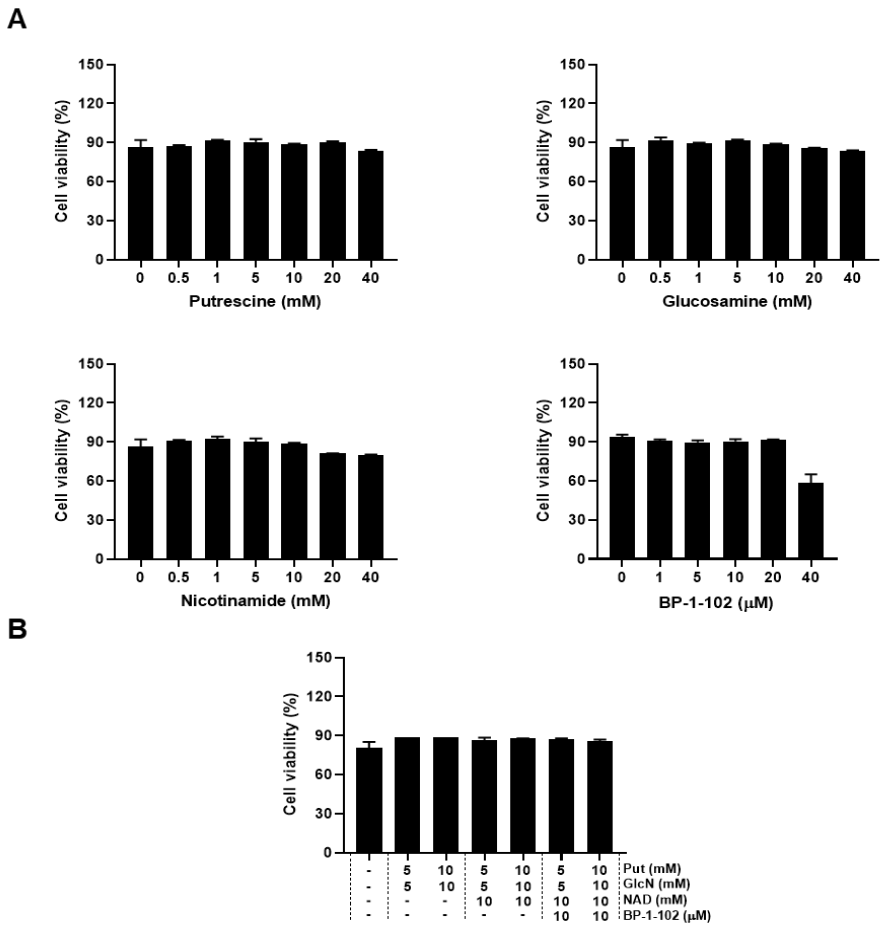


Figure 10. Cytotoxic effect of four differentiation-inducing factors on MIN-6 cell viability.

(A) Pancreatic beta cell line, MIN-6 cells were treated with each differentiation-inducing factor at the indicated different concentrations for 24 h. (B) MIN-6 cells were treated with different combinations of four differentiation-inducing factors at the indicated concentrations for 24 h. Cells were harvested and stained with Acridine Orange (AO) and Propidium Iodide (PI). Cell viability measurement was assessed by Cellometer *K*2 fluorescent viability Image cytometer. Data is presented as mean ± SEM.

Functional effect of four differentiation-inducing factors in MIN-6 cells

To determine whether treatment of the four extrinsic factors increase insulin secretion in MIN-6 cells, insulin release from vehicle-treated control or differentiation-inducing factor-treated cells was measured using ELISA. Insulin released from the cells treated with the combination of four differentiation-inducing factors was markedly higher in both basal and glucose-stimulated conditions than controls (Fig. 11A). Insulin secretion in the treated cells was approximately-2 fold higher than that in the vehicle-treated cells under both low and high glucose conditions.

To investigate whether the four differentiation-inducing factor-mediated insulin secretion is induced by the expression of beta cell-specific genes, qPCR was performed to examine the mRNA expression of *Pdx1*, *MafA*, and *Nkx6.1* in MIN-6 cells. After 16 h treatment with the factors, mRNA expression of those beta cell-related genes was investigated. As shown in Fig. 11B, the expression levels of transcription factors involved in the maturation of beta cells, such as *Pdx1*, *MafA*, and *Nkx6.1*, were increased in differentiation-inducing factor-treated MIN-6 cells. Specifically, PDX1 and NKX6.1 are suggested to be essential transcription factors for beta cell development, and when they are deficient, beta cell maturation does not occur in an appropriate manner. MafA is known as one of the key transcription factors for insulin secretion. Therefore, these results suggested that four differentiation-inducing factors enhanced the insulin secretion process even in the normal pancreatic beta cells, regardless of the STZ stimulation, and increased expression of transcription factors associated with insulin secretion and beta cell

maturation might play a role in the increase in insulin secretion.

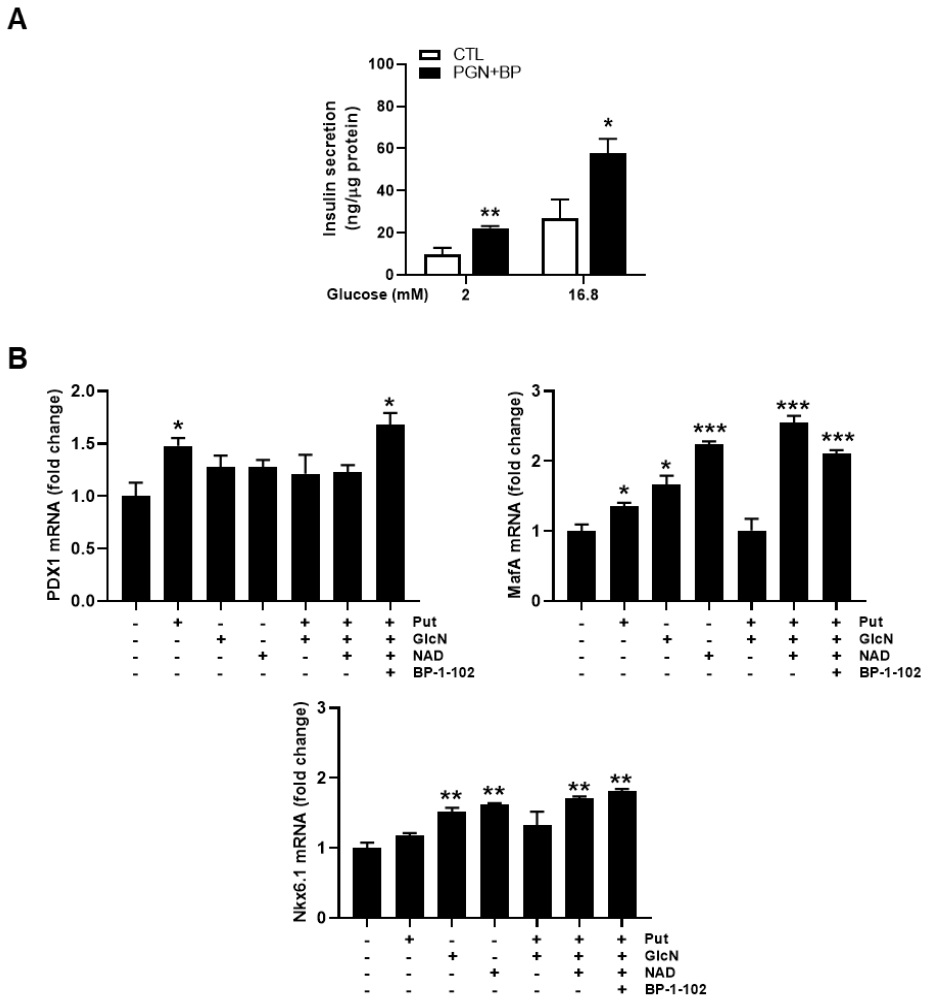


Figure 11. Functional effect of four differentiation-inducing factors in MIN-6 cells in glucose-stimulated insulin release.

(A) MIN-6 cells were treated with Put, GlcN, nicotinamide, and BP-1-102 for 4 h. Followed by 2 h-glucose starvation, MIN-6 cells were stimulated for 30 min with low (2 mM) and high (16.8 mM) glucose concentrations, respectively. Insulin secretion was measured by ultrasensitive ELISA. Results shown here represent those of five separate experiments. (B) MIN-6 cells were treated with Put, GlcN, nicotinamide, and BP-1-102 for 16 h. Relative mRNA expression levels were

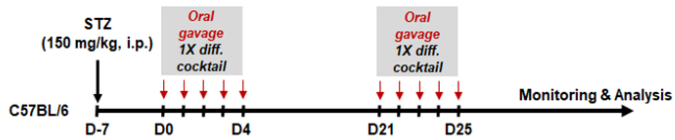
analyzed using qPCR. Results are presented as mean ± SEM, obtained from at least three independent experiments. Put, putrescine; GlcN, glucosamine. *Means are significantly different from non-treated BMNCs at $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Systemic infusion of four differentiation-inducing factors (diff. cocktail) improved hyperglycemia in diabetic mice

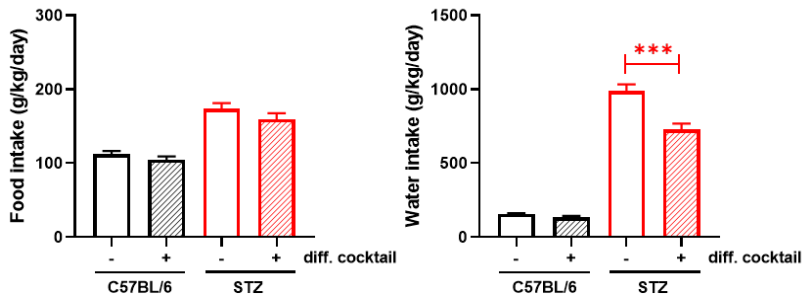
To verify the safety and efficacy of four differentiation-inducing factors as pharmaceutical drugs for prevention and/or treatment of diabetes, animal experiments were designed as indicated in Fig. 12A. I administered physiologically relevant concentrations of the four factors (200 mg/kg of Put, 200 mg/kg of GlcN, 500 mg/kg of nicotinamide, 3 mg/kg of BP-1-102; referred to as 1X differentiation cocktail (diff. cocktail) described here) for the consecutively 5 days twice at two-week intervals and monitored the mice for 55 days. The animals in STZ-treated diabetic group showed significant increase in food and water intake after receiving STZ stimulation. The abnormal water intake was significantly improved after diff. cocktail treatment compared to vehicle-administered STZ–induced diabetic mice, but the increased food intake was not changed regardless of the presence of diff. cocktail (Fig. 12B). Also, the diff. cocktail-administered mice in STZ-treated group showed a modest increase but no significant change in body weight. In normal C57BL/6 group, body weight of the mice administered with diff. cocktail tended to be lower, but it was not statistically different (Fig. 12C). Random blood glucose levels in mice of STZ-treated group were dramatically elevated compared to those in normal C57BL/6 mice group. Systemic infusion of diff. cocktail significantly decreased blood glucose levels in STZ-induced diabetic mice from day 16 to the end of the study (Fig. 12D). Moreover, mice infused with diff. cocktail in STZ-treated group improved glucose tolerance compared to the matched controls and the area under the glucose clearance curves were remarkably reduced (Fig. 13A).

In vivo GSIS from diff. cocktail-administered mice displayed higher levels of plasma insulin at 1 h post glucose injection when compared to the corresponding controls in STZ-treated group (Fig. 13B). Fasting blood glucose levels were lower not only in STZ-induced diabetic group but also in normal C57BL/6 group infused with diff. cocktail (Fig. 13C). However, I didn't observe that diff. cocktail supplementation itself affect the liver damage imposed by possible toxicities derived from the chemical compounds as assessed by plasma ALT and AST activities, although STZ-induced diabetes significantly increased plasma ALT and AST activities (Fig. 13D).

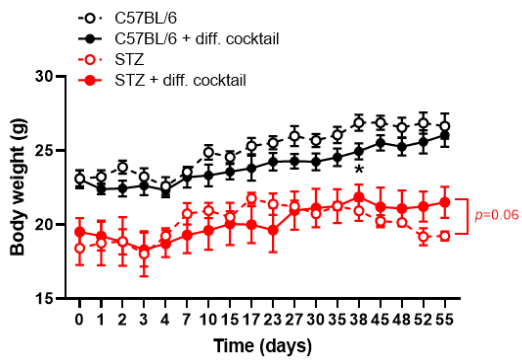
A


STZ
(150 mg/kg, i.p.)
Oral gavage 1X diff. cocktail
Oral gavage 1X diff. cocktail
C57BL/6
Monitoring & Analysis
D-7
D0
D4
D21
D25

B


Food intake (g/kg/day)
300
200
100
0
- + - +
diff. cocktail
C57BL/6
STZ
Water intake (g/kg/day)
1500
1000
500
0
***
- + - +
diff. cocktail
C57BL/6
STZ

C


C57BL/6
C57BL/6 + diff. cocktail
STZ
STZ + diff. cocktail
Body weight (g)
30
25
20
15
p=0.06
0 1 2 3 4 7 10 15 17 23 27 30 35 38 45 48 52 55
Time (days)

D

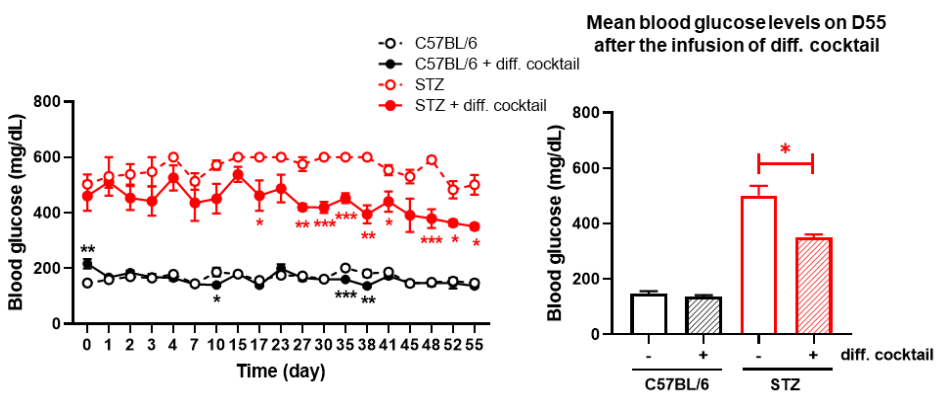

C57BL/6
C57BL/6 + diff. cocktail
STZ
STZ + diff. cocktail
Blood glucose (mg/dL)
800
600
400
200
0
0 1 2 3 4 7 10 15 17 23 27 30 35 38 41 45 48 52 55
Time (day)
Mean blood glucose levels on D55
after the infusion of diff. cocktail
Blood glucose (mg/dL)
800
600
400
200
0
*
- + - +
diff. cocktail
C57BL/6
STZ

Figure 12. Characterization of STZ-induced diabetic mice systemically infused by four differentiation-inducing factors (diff. cocktail).

Summarized scheme of animal experiments. Male C57BL/6 mice were injected intraperitoneally with STZ (diabetic; red lined in the panels, n=4 for STZ; n=6 for STZ + diff. cocktail) or C57BL/6 (normal; black lines in the panels, n=4 for C57BL/6; n=5 for C57BL/6 + diff. cocktail). After 7 days, 1×differentiation cocktail (diff. cocktail) containing 200 mg/kg of Put and GcN, 500 mg/kg of nicotinamide, and 3 mg/kg of BP-1-102, was systemically infused via oral gavage for 5 consecutive days twice at intervals of 2 weeks. (B) Food (left panel) and water (right panel) intake was measured every 3–4 day. Body weight (C), changes in random blood glucose levels, and mean blood glucose levels at day 55 (D) of mice given daily with diff. cocktail (filled circle, straight line, n=5 for C57BL/6 + diff. cocktail; n=6 for STZ + diff. cocktail) or comparable vehicle (empty circle, dotted line, n=4 for C57BL/6; n=4 for STZ) were measured at the indicated days. Data are presented as mean ± SEM. *, p value < 0.05; **, p value < 0.01 for non- vs. differentiation cocktail-orally treated mice. STZ, streptozotocin.

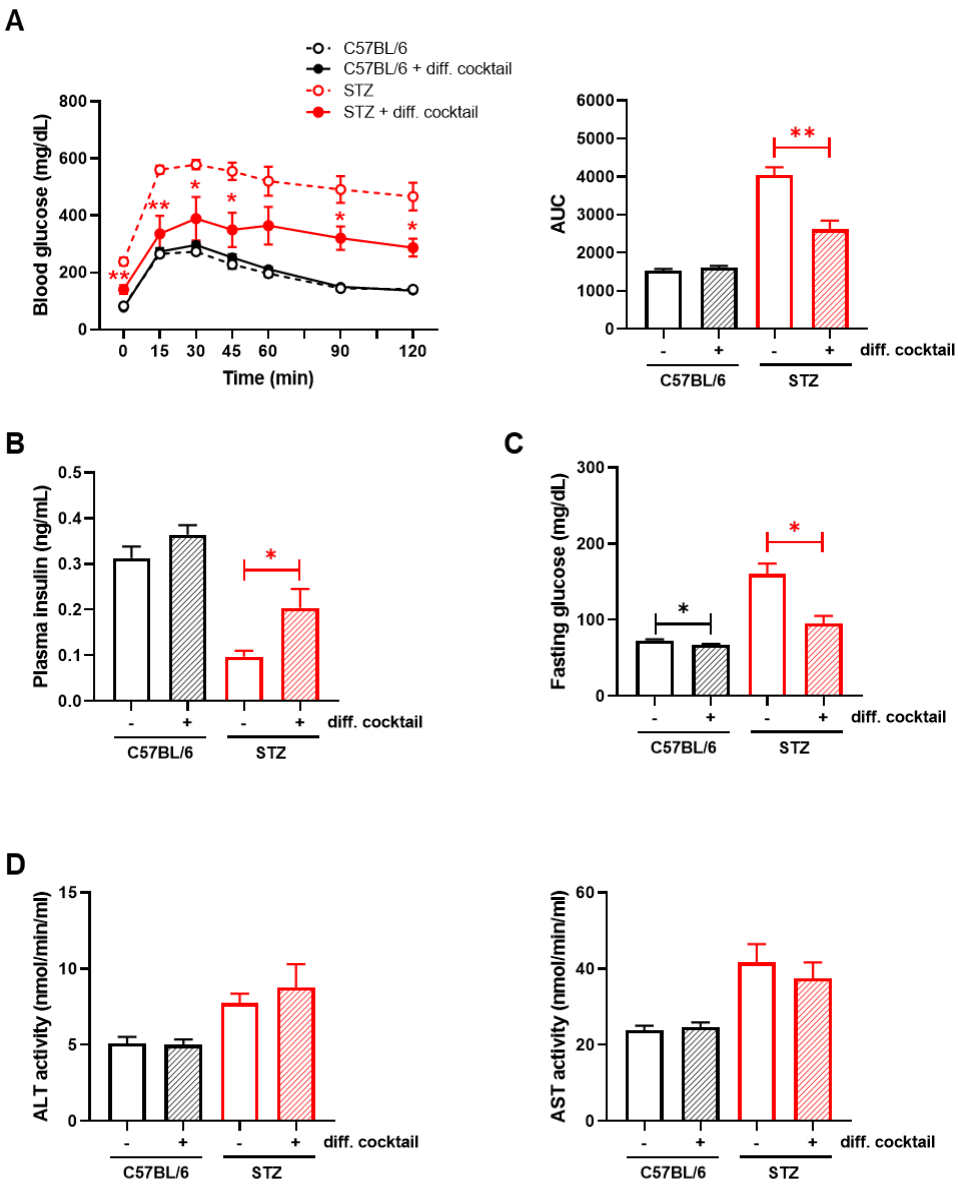

A
C57BL/6
C57BL/6 + diff. cocktail
STZ
STZ + diff. cocktail
Blood glucose (mg/dL)
Time (min)
AUC
diff. cocktail
C57BL/6
STZ
B
Plasma insulin (ng/mL)
C
Fasting glucose (mg/dL)
D
ALT activity (nmol/min/ml)
AST activity (nmol/min/ml)

Figure 13. Systemic infusion of diff. cocktail improves hyperglycemia in the diabetic mice.

(A) Blood glucose responses from the intraperitoneal glucose tolerance test (ipGTT). Glucose (1 g/kg body weight) was injected after overnight fasting at 42 days followed by oral administration. AUC (right panel) was measured. (B) Plasma insulin levels at 60 min post glucose injection (2 g/kg, body weight) followed by overnight fasting at 50 days after oral administration was measured by ultrasensitive mouse insulin ELISA. Fasting glucose levels (C) and serum ALT and AST levels (D) were measured at 57 days before sacrifice. Data are presented as mean ± SEM. *, p value < 0.05; **, p value < 0.01 for non- vs. diff. cocktail-treated mice. STZ, streptozotocin.

Increased expression of insulin- and PDX1-positive cells in pancreas and small intestine in diabetic mice infused by diff. cocktail

Then, I analyzed the pancreas and the small intestine of the mice by immunohistochemistry with antibodies against insulin and PDX1 to identify the possibility of endogenous pancreatic beta cell regeneration. The result revealed that the pancreas from the diabetic mice infused with diff. cocktail showed more insulin- and PDX1-positive cells, compared to those from the diabetic controls, and there was an evidence of endogenous beta cell regeneration (Fig. 14A). In addition, more insulin-positive cells were increased in the lamina propria of the intestinal villi in the presence of diff. cocktail in diabetic animal group. As an additional assessment, I quantitatively evaluated the pancreatic insulin content in these animals, which resulted in significant increase in diabetic mice treated with diff. cocktail (Fig. 14B). Thus, differentiation-inducing factors improves glucose tolerance by responding to glucose challenge in vivo and releasing insulin. This data suggest that four differentiation-inducing factors themselves are responsible for the reversal of hyperglycemia in diabetic mice, at least in part, due to endogenous pancreatic beta cell regeneration.

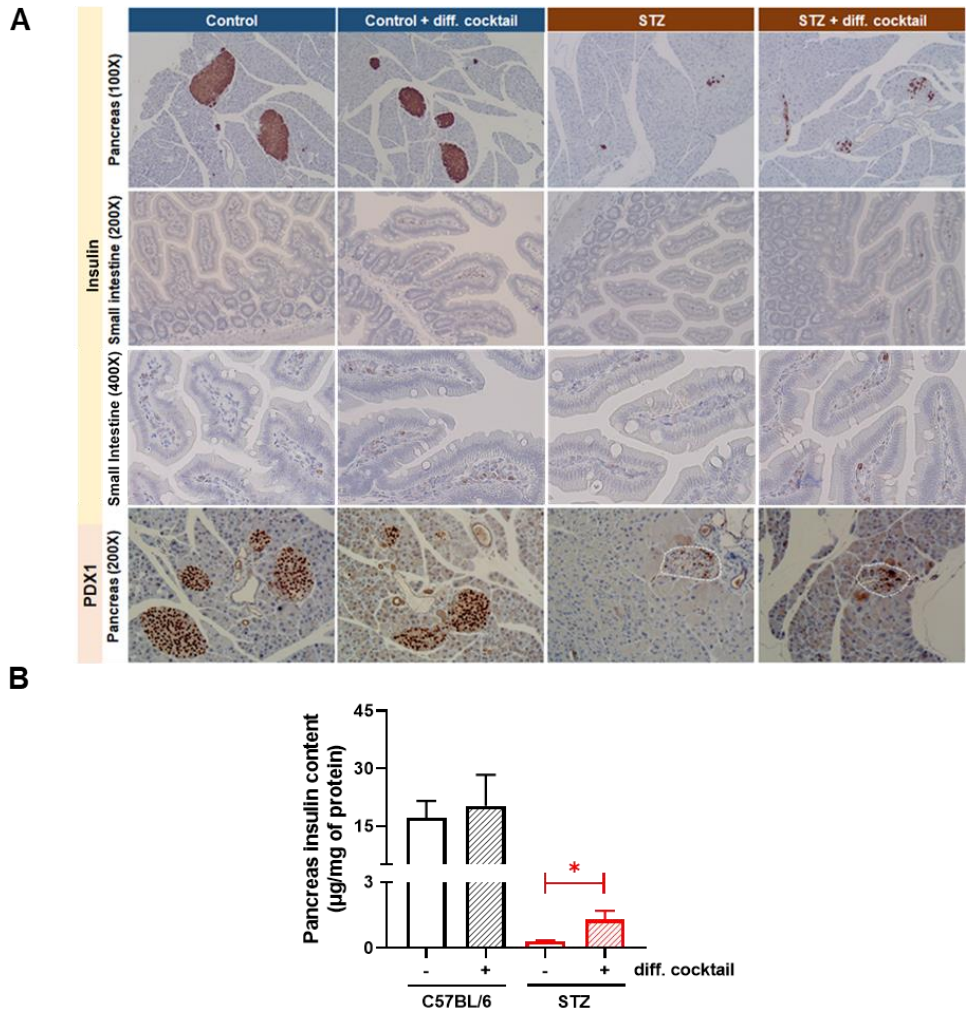


Figure 14. Increased numbers of insulin-/PDX1- positive cells in pancreas and small intestine in diabetic mice infused by diff. cocktail.

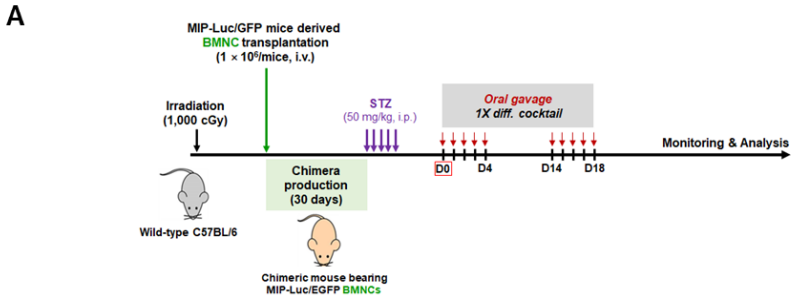
(A) Representative immunohistochemical images for Insulin and PDX1 in the sectioned pancreas and small intestine harvested at 57 days after oral administration. All images were acquired with an ECLIPSE Ci-L microscope. Original magnification, 100X for insulin staining in pancreas; 200X for insulin staining in pancreas and small intestine and PDX1 staining in pancreas; 400X for

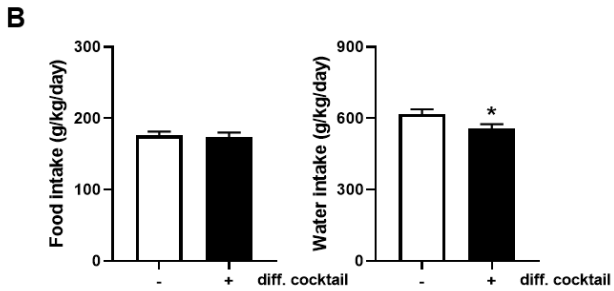
insulin staining in small intestine. (B) Pancreatic insulin content in mice was also determined from the pancreatic tissues harvested at 57 days after oral administration using high-range mouse insulin ELISA. All data are presented as mean ± SEM, obtained from 4 mice of each group.

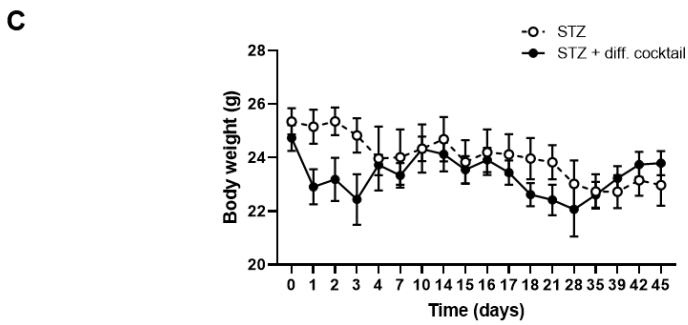
In vivo differentiation of BMNCs into IPCs.

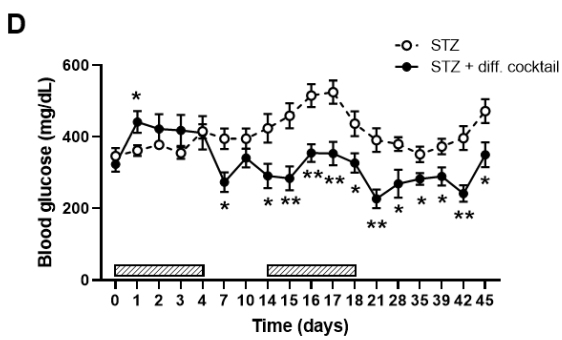
I demonstrated that diff. cocktail supplementation not only effectively differentiated endogenous BMNCs into IPCs, but also was involved in beta cell regeneration in the diabetic animal group. Next, to confirm whether endogenous BMNCs indeed differentiate into IPCs, home to the pancreatic tissues as well as the small intestine, and is involved in beta cell regeneration, I generated chimeric mice harboring BMNCs isolated from the MIP-Luc/GFP mice. C57BL/6 mice were irradiated to destroy their own BMNCs, and BMNCs (1×10^6 cells/mouse) derived from MIP-Luc/ GFP mice were exogenously injected via the retro-orbital vein.

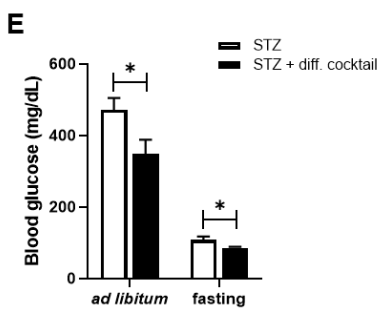
In chimeric mice with STZ-induced hyperglycemia, mice were orally administrated with diff. cocktail for 5 consecutive days twice at 2-week intervals in the same way as indicated in Fig. 12 (Fig. 15A). The blood glucose levels were measured every 3–4 days for 45 days after oral administration of diff. cocktail. Chimeric mice treated with diff. cocktail reversed the aberrant water intake and recovered the body weight from day 28 (Fig. 15B, C). Random blood glucose levels were significantly lower and the fasting glucose levels at day 45 after oral administration were reduced in diff. cocktail-administered diabetic mice (Fig. 15D, E). Consistent with the results shown in Fig. 13A, chimeric mice treated with diff. cocktail in hyperglycemic conditions showed improved glucose tolerance. Further, the fasting plasma insulin levels in mice were significantly increased in the presence of diff. cocktail (Fig. 15G). These results suggested that differentiation-inducing factors are functional in vivo to differentiate endogenous BMNCs and correct hyperglycemia in mice.


A
MIP-Luc/GFP mice derived
BMNC transplantation
(1 × 10⁶/mice, i.v.)
Irradiation
(1,000 cGy)
STZ
(50 mg/kg, i.p.)
Oral gavage
1X diff. cocktail
Monitoring & Analysis
D0
D4
D14
D18
Chimera
production
(30 days)
Wild-type C57BL/6
Chimeric mouse bearing
MIP-Luc/EGFP BMNCs


B
Food intake (g/kg/day)
300
200
100
0
-
+
diff. cocktail
Water intake (g/kg/day)
900
600
300
0
*
-
+
diff. cocktail


C
STZ
STZ + diff. cocktail
Body weight (g)
28
26
24
22
20
0 1 2 3 4 7 10 14 15 16 17 18 21 28 35 39 42 45
Time (days)


D
STZ
STZ + diff. cocktail
Blood glucose (mg/dL)
600
400
200
0
0 1 2 3 4 7 10 14 15 16 17 18 21 28 35 39 42 45
Time (days)


E
STZ
STZ + diff. cocktail
Blood glucose (mg/dL)
600
400
200
0
*
*
ad libitum
fasting

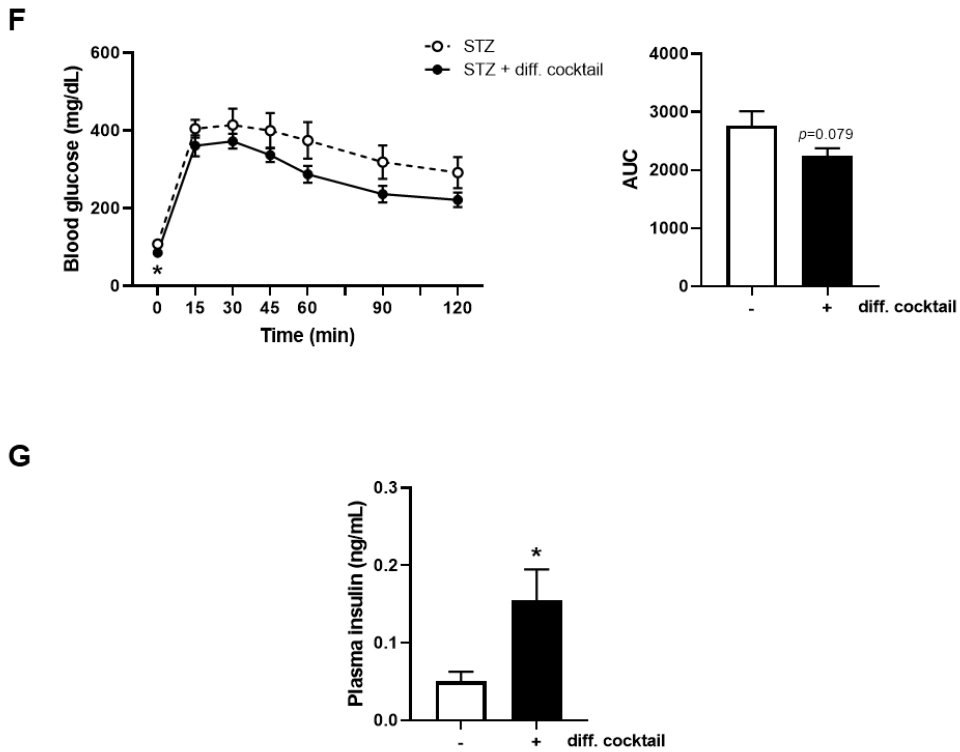


Figure 15. In vivo differentiation of BMNCs into IPCs.

(A) Summarized scheme of animal experiments. Chimeric mice were generated by injecting BMNCs (1×10^6 cells/mouse) derived from MIP-Luc/ GFP mice into lethally (500× 2 cGy) irradiated male C57BL/6 mice (8–12 weeks old) *via* the retro-orbital vein. After monitoring for four weeks, mice were given 1X diff. cocktail for 5 consecutive days twice at intervals of 2 weeks. (B) Food (left panel) and water (right panel) intake was measured every 3–4 day. Changes in body weight (C), random feeding blood glucose levels (D) and fasting blood glucose levels of mice given daily 1X diff. cocktail (filled circle, straight line, n=8) or comparable vehicle (empty circle, dotted line, n=6). (F) Blood glucose responses from the intraperitoneal glucose tolerance test (ipGTT). Glucose (1 g of /kg body weight) was injected after overnight fasting at 36 days after oral administration. AUC (right panel) was measured. (G) Plasma insulin levels following overnight

fasting at 46 days after oral administration was measured by ultrasensitive mouse insulin ELISA. Data are presented as mean ± SEM, obtained from 5-6 mice of each group. *, p value < 0.05 for non- vs. diff. cocktail-treated mice.

Insulin- and GFP-double positive cells were increased in the pancreas and small intestine of chimeric mice administrated with diff. cocktail

Immunofluorescence staining of the pancreatic sections from chimeric mice confirmed the presence of more insulin- and GFP-double positive cells in mice with diff. cocktail (Fig. 16A). The islets appeared larger compared to islets from untreated diabetic mice. Also, the islets had an increase in mouse insulin immunoreactivity, and there was an increase in number of islets per section. More GFP-positive cells were co-stained with anti-insulin antibody. I also determined the pancreatic insulin content in chimeric mice and the insulin content of pancreas in mice with diff. cocktail was significantly increased (Fig. 16B). To confirm whether insulin-positive and GFP-positive cell area are increased in pancreas from chimeric mice administrated with diff. cocktail, I quantified immunofluorescence images using Metamorph image analysis tool in chimeric mice, which resulted in significant increase of mean beta cell area and GFP-positive cell area in the group administrated with diff. cocktail. The ratio of GFP- to insulin-positive cell area was also increased in the pancreas of group administrated with diff. cocktail. In addition, the GFP^{-}/$Insulin^{+}$ cell area was also significantly increased in the group administered with diff. cocktail. Based on this finding, the differentiation of bone marrow-derived cells was not only induced more in the pancreas of the group administered with diff. cocktail, but also the recovery of beta cells was increased (Fig. 16C).

Consistent with these findings, qPCR analysis from the pancreatic tissues of diff. cocktail-administered mice showed a significant increase in mRNA levels of *Gfp*, *Ins1*, and *Ins2* (Fig. 17A). In addition to the pancreas, qPCR analysis from three parts of the small intestine - duodenum, jejunum, and ileum - of diff. cocktail-administered mice showed a significant increase in mRNA levels of *Ins2*. However, no statistical significance in mRNA levels of GFP was observed in the small intestine (Fig. 18B). Moreover, I found that in other tissues, especially spleen and lung, mRNA levels of Ins2 were increased in the diff. cocktail-administered chimeric mice (Fig. 18C), although it should be confirmed in co-localization with the antibodies against GFP and insulin in each tissue. These results indicated that endogenous BMNCs were homing to multiple tissues including pancreas in the damaged states and some of GFP-positive BMNCs indeed differentiated into IPCs. It confirms that BMNCs are self-renewing and multipotent stem cells that differentiate into pancreatic endocrine cells. Immunofluorescence staining of each part of the small intestine from chimeric mice confirmed the presence of more insulin- and GFP-double positive cells in mice with diff. cocktail (Fig. 18). GFP- and/or insulin-positive cells were observed in intestinal villi and crypt, and GFP- and insulin-double positive cells were further increased in the lamina propria of the villi.

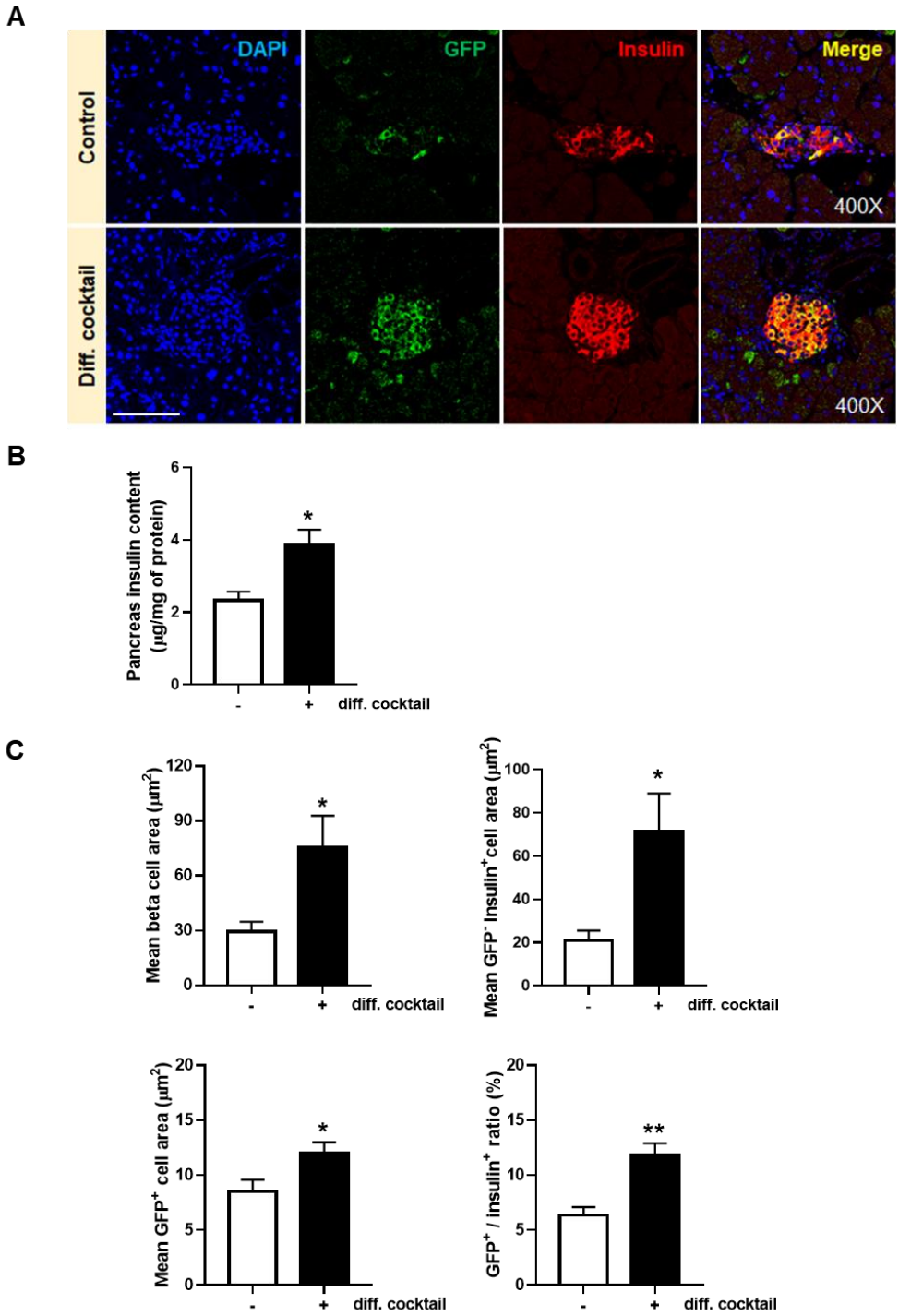

A
DAPI
GFP
Insulin
Merge
Control
Diff. cocktail
400X
400X
B
Pancreas insulin content (μg/mg of protein)
6
4
2
0
*
-
+
diff. cocktail
C
Mean beta cell area (μm2)
120
90
60
30
0
*
-
+
diff. cocktail
Mean GFP- Insulin+ cell area (μm2)
100
80
60
40
20
0
*
-
+
diff. cocktail
Mean GFP+ cell area (μm2)
20
15
10
5
0
*
-
+
diff. cocktail
GFP+ / Insulin+ ratio (%)
20
15
10
5
0
**
-
+
diff. cocktail

Figure 16. Appearance of insulin- and GFP-double positive cells in the pancreas of chimeric mice.

(A) Representative immunofluorescence staining of the pancreatic tissues harvested from chimeric mice. More GFP- and insulin-double positive cells in the pancreatic tissues were observed in the diff. cocktail-treated group compared to the control group. Note the appearance GFP (green) and insulin (red) in cytoplasm of pancreatic islet cells. (B) Pancreatic insulin content in mice was determined from the pancreatic tissues using high-range mouse insulin ELISA. (C) Quantification of GFP- and insulin-positive cell area. Mean GFP-negative and insulin-positive cell area and ratio for GFP- and insulin-double positive cell are also measured. All data are presented as mean ± SEM, obtained from diabetic mice given daily with 1 × diff. cocktail (n=8) and comparable vehicle (n=6). Scale bar, 50 μm; original magnification, 400X.

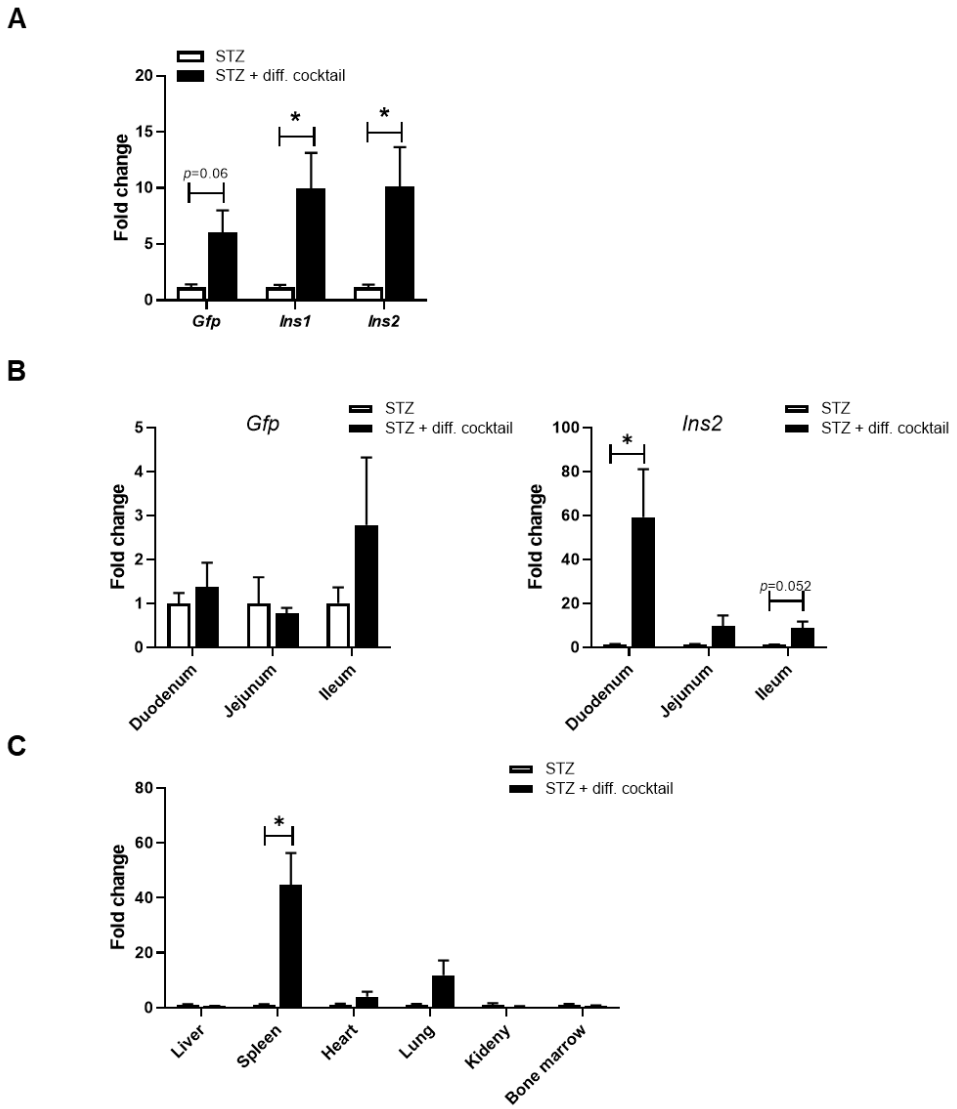


Figure 17. Relative mRNA expression of *Gfp, Ins1,* and *Ins2* in pancreas and *Gfp* and *Ins2* in small intestine and *Ins2* mRNA expression in multiple tissues of chimeric mice.

Isolated tissues from chimeric mice were homogenized and RNA was extracted. Relative mRNA expression levels were analyzed using qPCR. (A) qPCR analysis for *Gfp, Ins1, Ins2* gene expression in the pancreas of chimeric mice. (B) qPCR analysis for *Gfp* and *Ins2* gene expression in duodenum, jejunum, and ileum of the

small intestine. (C) qPCR analysis for *Ins2* gene expression in the various tissues of chimeric mice. Results are presented as mean ± SEM. *Means are significantly different from the control STZ group at $p < 0.05$.

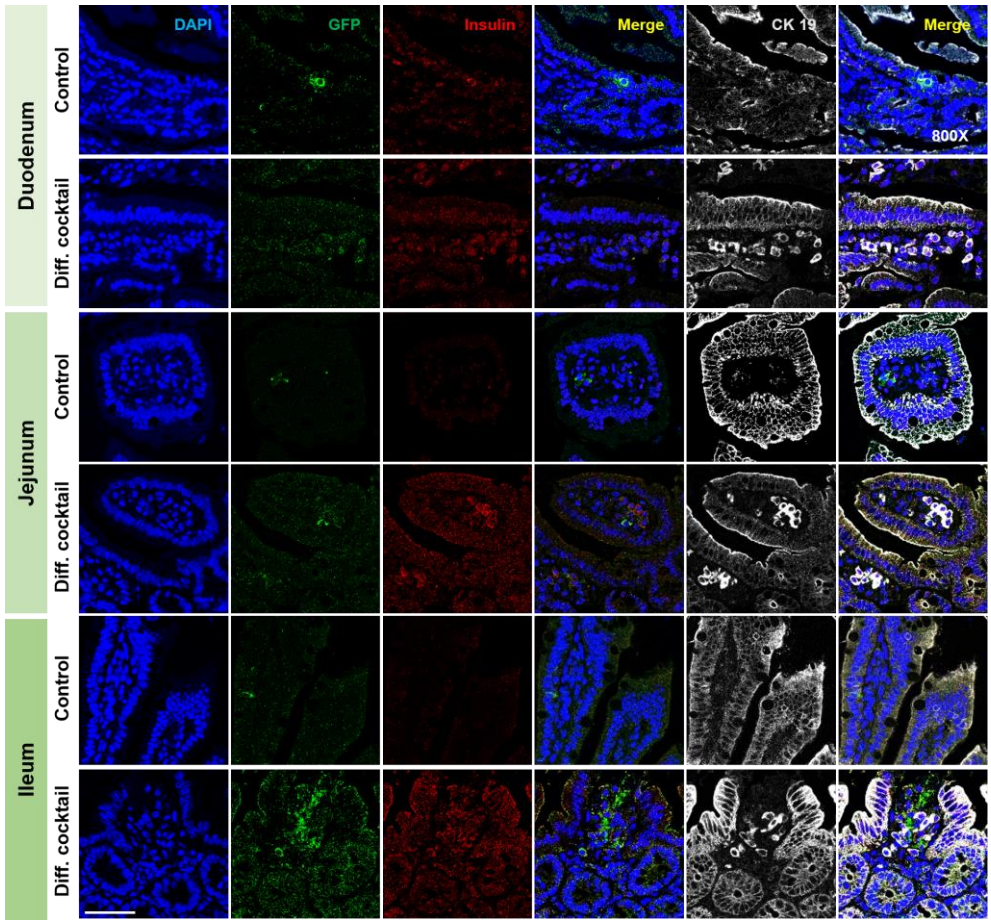


Figure 18. Appearance of insulin- and GFP-double positive cells in the small intestine of chimeric mice.

Representative immunofluorescence staining of the small intestines harvested from chimeric mice. More GFP- and insulin-double positive cells in three parts of small intestine, duodenum, jejunum, and ileum were observed in the diff. cocktail-treated group (n=8) compared to those in the control group (n=6). Note the appearance GFP (green), insulin (red), and cytokeratin 19 (CK19, grey) in intestinal villi and crypt. Fluorescent images were obtained using Leica TCS STED CW confocal microscope. Scale bar, 50 μm; original magnification, 800X.

Discussion

Bone marrow-derived cells (BMNCs) have been recognized as a safe and abundant source for large quantities of multipotent adult stem cells. Accumulating evidence suggest that bone marrow-derived cells can provide potential cell-based therapeutic agents for diabetes mellitus in two aspects; (1) transplantation of in vitro differentiated BMNC-derived IPCs, (2) mobilization and proliferation of endogenous bone marrow-derived cells. Previous studies show that transplanted bone marrow-derived stem cells home to injured tissues and repair them by several different mechanisms, including differentiation into multiple cellular phenotypes providing cytokines and chemokines [37] and enhanced proliferation of endogenous stem progenitor cells in injured tissues [38].

This work demonstrated that mouse BMNCs could differentiate toward glucose-competent IPCs and pancreatic endocrine beta cell phenotype by four differentiation-inducing factors under specific in vitro culture conditions. A unique combination of four differentiation-inducing factors, namely Put, GlcN, nicotinamide, and BP-1-102 was identified to develop straightforward 6-day differentiation protocol to generate IPCs from BMNCs without progressively changing differentiation-inducing factors and/or culture media. This simplified approach successfully differentiated BMNCs into definitive endoderm (cells expressing *Cxcr4*, *Sox17*, and *FoxA2*), followed by pancreatic endoderm (cells expressing *Pdx1* and *Nkx6.1*) and endocrine progenitors (cells expressing *Nkx6.1*,

Neurog3, and *NeuroD1*). The expression of multiple genes involved in beta cell development and insulin production was confirmed by using qPCR, immunofluorescence staining, and FACS analysis. The functionality of in vitro BMNC-derived IPCs was also confirmed by measuring insulin secretion in response to a glucose challenge. Furthermore, BMNC-derived IPCs were transplanted into STZ-induced diabetic mice under the kidney capsules to confirm in vivo effect of the differentiated cells and found to ameliorate diabetic state upon introduction of the BMNC-derived IPCs. Besides, this study showed that systemic infusion of four extrinsic differentiation-inducing factors themselves improved hyperglycemia in the STZ-induced diabetic mice and the possibility of *in vivo* differentiation of BMNCs. Chimeric mice with GFP-labeled bone marrow-derived cells under the activation of Ins2 promoter were used to find GFP- and insulin-double positive cells in the multiple tissues, mainly the pancreas, of the diabetic states. Collectively, these results affirm that the bone marrow contains pluripotent cells capable of being programmed into functional IPCs *in vitro* and *in vivo* by defined factors and endogenous bone marrow stem cells could be mobilized for tissue recovery and differentiated by extrinsic small molecules for tissue recovery.

Previous studies have reported that a single treatment of Put or GlcN is involved in glucose-stimulated insulin secretion. Micromolecular concentrations of Put in MIN-6 increased basal and glucose- stimulated insulin secretion due to increased expression of MafA involved in insulin production [39]. GlcN supplementation also potentiates the differentiation of adipose-derived stem cells (ADSCs) into

glucose-responsive IPCs [40]. Addition of GlcN at any stage during the 12-day stepwise differentiation significantly increased the expression of beta cell-specific genes and insulin secretory genes, thereby increasing insulin secretion. Addition at a later stage of differentiation particularly affected the gene expression and insulin secretion, indicating that GlcN might affect endocrine lineage commitment and maturation of beta cells. Here, co-treatment with Put and GlcN had been shown to have a greater effect on the expression of beta cell markers and insulin promoter activation using BMNCs derived from MIP-Luc/GFP mice, and specific *in vitro* culture conditions in combination with nicotinamide and BP-1-102 enhance insulin secretion under basal and glucose-stimulated conditions. Moreover, an increased expression of genes associated with the insulin secretion machinery (*Glut2*, *Gck*, and *Syt*) in differentiated IPCs was observed. Glucose transporters, such as GLUT2 and GCK are essential regulators of glucose responses in beta cells, and expression of these regulators in BMNC-derived IPCs suggests that IPCs respond to glucose. SYT is also known to be involved in exocytosis during insulin secretion when insulin granules are fused to the surface of the plasma membrane [41]. Therefore, increased insulin secretion and dose-dependent glucose responsiveness of differentiated IPCs using these factors is due to the increase in the expression of molecules involved in the insulin secretion machinery. Consistent with these findings, this study demonstrated that insulin secretion was dramatically increased in differentiated BMNCs in the presence of differentiation inducing factors including Put and GlcN. Nicotinamide has been widely used for pancreatic endocrine differentiation. Nicotinamide has been shown to be a specific differentiation regulator of NEUROG3^{+} islet progenitor cells [42]. Nicotinamide-

regulated beta cell differentiation required a shorter period of 4–6 days, and a transcriptome profile of differentiated IPCs on day 4 exhibited a correlation of 94% with the transcriptome of adult islets. Consistent with this finding, this study showed a robust increase in insulin expression, along with the expression of other beta cell markers, as demonstrated using qPCR and RT-PCR. Thus, simultaneous exposure to Put, GlcN, and nicotinamide optimized insulin expression on day 6. STAT3 plays a role in maintaining cellular identities and differentiation of various cell types, including of neuronal, endothelial, epithelial, and muscular phenotype. Neurogenin3 (NEUROG3) is known to be a critical downstream effector of STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia [36]. Although NEUROG3, as a pro-endocrine gene, is an important transcriptional regulator that determines pancreatic endocrine fate, its expression is reduced at the mature beta cell stage. A recent study by Miura et al. demonstrated that STAT3 inhibition and exogenous expression of PDX1, NEUROG3, and MAFA promotes cellular reprogramming into beta cells [43]. Particularly, the small molecule STAT3 inhibitor BP-1-102 and genetic deletion of STAT3 significantly increased the efficiency of beta cell formation *in vivo* and ameliorated hyperglycemia in diabetic mice. Similarly in my study, IPC maturation from primed BMNCs up to day 9 was shown to be accompanied by increased insulin expression, reduced Neurog3 expression, and increased or maintained levels of *Pdx1*, *MafA*, *NeuroD1*, and *Nkx6.1* expression. Based on these findings, it is probable that a combination of nicotinamide and BP-1-102 effectively promotes further maturation of BMNC-derived IPCs in this experimental system.

In addition to the in vitro differentiation of BMNCs toward IPCs, I determined a pharmacological effect of four differentiation-inducing factors, as demonstrated by a reversal of diabetes upon oral supplementation of the extrinsic factors into diabetic mice. Through the successive experiments using chimeric mice, I found that exogenously transplanted BMNCs derived from MIP-Luc/GFP mice were co-localized with insulin in multiple tissues, mainly the pancreas. This result is consistent with other reports that multipotent bone marrow-derived cells home to injured sites and promote tissue repair [13, 44-46]. Bone marrow-derived cells have been shown to promote migration to pancreatic islets by expressing functionally active chemokine receptors [47] or combination of cytokine treatment [48]. Earlier studies demonstrated that bone marrow-derived cells contribute to pancreatic beta cell regeneration at a low frequency, ranging from 1 to 3% [49]. Studies by others and us confirmed that allogenic bone marrow transplantation with as low as 1% chimerism in the pancreatic islets could reverse the diabetogenic process in diabetic mice [18, 50]. Inspired by these observations, I regard it reasonable to assume that oral supplementation of diff. cocktail in the current study facilitates more efficiently differentiation of endogenous BMNCs into functional IPCs in diabetic mice. In parallel, it is possible that multipotent stem cells within pancreatic islets may join the differentiation toward IPCs, as observed by more insulin-positive but GFP-negative cells in the pancreas of diff. cocktail-administered diabetic mice, and an increase in glucose-dependent insulin secretion and mRNA expression of related transcription factors in MIN-6 cells treated with the extrinsic factors.

Despite that bone marrow-derived cells have been shown to differentiate into functional mature beta cells both in vitro and in vivo, mechanisms involved in the extrinsic factor-mediated differentiation process to facilitate maturation and differentiation of BMNCs into functional beta-like cells should be elucidated. Going further, it is essential to understand mechanisms underlying transcription factors or signaling molecules controlling the regulation of beta cell-associated gene expression.

In addition, in the case of the crypt (intestinal gland), the basal part of the intestinal epithelium, the intestinal stem cells are distributed, and they differentiate into several types of cells constituting the intestinal epithelium and play an important role in the regeneration of the intestinal epithelium [51]. Through this, it is thought that the bone marrow cells that have moved to the small intestine will be differentiated after they move to the crypt, and it is thought that a follow-up study related to this mechanism is necessary. Combining with the results of previous studies, bone marrow cells have been mobilized into the small intestine, and it has been confirmed that these cells can be differentiated into insulin-expressing cells through differentiation inducing factors. Furthermore, the destination of BMNCs and their fate have not been clearly determined. Therefore, mechanisms on the homing of BMNCs in the pancreas and other tissues should be thoroughly studied in the future.

In conclusion, a simple protocol using a novel combination of four extrinsic factors, Put, GlcN, nicotinamide, and BP-1-102, to differentiate BMNCs into functional IPCs that produce physiologically active insulin was identified in my study.

BMNCs primed with these factors in vitro followed sequential developmental pathways through physiological intermediate cells. Moreover, in vitro and in vivo findings indicate that the bone marrow includes pancreatic progenitor cells capable of differentiating into functional endocrine cells. Approximately 30% of the total cells were mature IPCs that secreted insulin in response to glucose stimulation. Evidence suggests that producing IPCs from stem cells is feasible and promising but variations in stem cell sources and induction techniques pose a challenge because there is no standard protocol for IPC generation. In this aspect, the hypoglycemic effect of the four differentiation-inducing factors administered in diabetic mice may bypass this challenge. Finally, these results suggest that a combination of four differentiation-inducing factors established here can be beneficial for the differentiation of BMNCs into functional IPCs. Pharmacological application will improve prospects of cell-based therapies as well as anti-diabetic drug therapies to replace insulin administration in diabetes treatment.

Acknowledgements

First and foremost, I am deeply grateful to my research supervisor, Professor Kyong Soo Park, whose insight and encouragement were invaluable to bring my thinking to a higher level. And I would like to gratefully acknowledge Dr. Seung-Ah Lee and Sung Soo Chung. I am extremely thankful for sharing their expertise and sincere guidance.

I would also like to thank Jong Yeon Park, Jinyan Nan, Hyunsuk Lee, and Eun Kyung Joo for helping me with experiments including bone marrow renal transplantation, glucose-stimulated insulin secretion and glucose tolerance test involved in this project. I would express my gratitude to Ho Seon Park for helpful advice on experiments.

Without their assistance and dedicated involvement in every step throughout my master's degree process, this paper would have never been accomplished. I would like to appreciate their support and understanding.

국문초록

네 가지 분화유도인자 처리에 의한 골수 유래 줄기세포의 인슐린 생산세포로의 분화

골수 유래 줄기세포는 췌장 내분비세포를 비롯한 다양한 세포 유형으로 분화되는 다능성 성체 줄기세포로 알려져 있다. 본 연구에서는 Putrescine (Put), Glucosamine (GlcN), Nicotinamide 및 BP-1-102 의 네 가지 분화유도인자의 조합으로 성숙한 인슐린 생산세포 (Insulin-producing cell; IPC)로의 분화 방법 개발을 목표로 하였다.

마우스의 골수 단핵구 세포를 네 가지 분화유도인자인 Put, GlcN, Nicotinamide 및 BP-1-102 를 사용하여 6 일 동안 시험관 배양하여 인슐린 생산세포로의 분화를 유도하였다. 그 결과, 마우스 골수세포 유래 인슐린 생산세포가 단계적인 췌장 발달 과정을 보이며, 포도당 자극에 의한 인슐린 분비능 및 관련 유전자들의 발현이 증가됨을 관찰하였다. 또한, kidney capsule 이식을 통하여 마우스 골수 유래 인슐린 생산세포의

생체 내 혈당 조절 능력을 검증하였다. 이는 내인성 베타세포의 재생과는 무관하며 이식된 분화 유도된 세포의 특성에 기인함을 의미한다.

더불어, 네 가지 분화유도인자를 당뇨병 동물 모델에 경구로 직접 투여하여 당뇨병 예방 또는 치료용 약학적 조성물로서의 분화유도인자의 효과를 알아보았다. 그 결과, 분화유도인자가 투여된 당뇨병 동물 모델에서 혈당 강하 및 내당능 개선이 관찰되었으며, 췌장 및 소장에서의 인슐린 생산세포의 출현이 대조군 대비 현저히 증가한 것을 확인하였다. 또한 인슐린 프로모터의 활성화로 GFP 가 발광 되는 형질전환 마우스의 골수세포를 이식한 키메라 마우스 모델을 제작하여 골수 유래 세포가 인슐린 생산세포로 분화하며, 분화된 세포의 췌장으로의 귀소성 (homing)을 통해 생체 내 인슐린 분비가 증가되어 고혈당을 완화시킴을 규명하였다. 이러한 결과를 통해, 분화유도인자 조성물의 생체 내 유효성 및 상기 확립된 분화유도인자 조성물이 내재성 줄기세포의 활성을 조절하는 인자로서 기능함을 볼 수 있다. 이는 궁극적으로 베타세포 파괴 및 손상에 의한 당뇨병 치료에 확립된 분화유도인자가 세포치료제로서뿐만 아니라, 약학적 조성물로서도 활용될 가능성을 시사한다고 볼 수 있다.

주요어: 골수유래 줄기세포, 분화, 인슐린 생산 세포, **BP-1-102, Glucosamine, Nicotinamide, Putrescine**

학번: **2020-20283**

Bibliography

1. Lin, X., et al., *Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025.* Sci Rep, 2020. **10**(1): p. 14790.
2. Lin, J., et al., *Projection of the future diabetes burden in the United States through 2060.* Popul Health Metr, 2018. **16**(1): p. 9.
3. American Diabetes Association Professional Practice, C., *2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2022.* Diabetes Care, 2022. **45**(Suppl 1): p. S17-S38.
4. Leslie, R.D., et al., *Diabetes at the crossroads: relevance of disease classification to pathophysiology and treatment.* Diabetologia, 2016. **59**(1): p. 13-20.
5. Chhabra, P. and K. L. Brayman, *Stem Cell Strategies to Promote Islet Transplantation Outcomes.* OBM Transplantation, 2018. **2**(2): p. 1-1.
6. Hamza, A.A., et al., *Mechanistic insights into the augmented effect of bone marrow mesenchymal stem cells and thiazolidinediones in streptozotocin-nicotinamide induced diabetic rats.* Sci Rep, 2018. **8**(1): p. 9827.
7. Chen, S., K. Du, and C. Zou, *Current progress in stem cell therapy for type 1 diabetes mellitus.* Stem Cell Res Ther, 2020. **11**(1): p. 275.
8. de Klerk, E. and M. Hebrok, *Stem Cell-Based Clinical Trials for Diabetes Mellitus.* Front Endocrinol (Lausanne), 2021. **12**: p. 631463.
9. Wilson, A. and A. Trumpp, *Bone-marrow haematopoietic-stem-cell niches.* Nat Rev Immunol, 2006. **6**(2): p. 93-106.
10. Esmatjes, E., et al., *Regeneration of insulin production by autologous bone marrow blood autotransplantation in patients with type 1 diabetes.* Diabetologia, 2010. **53**(4): p. 786-9.
11. Bhansali, A., et al., *Efficacy and safety of autologous bone marrow-derived stem cell transplantation in patients with type 2 diabetes mellitus: a randomized placebo-controlled study.* Cell Transplant, 2014. **23**(9): p. 1075-85.
12. Mesples, A., Y. Zhang, and X. Hu, *Long Term Benefit of Autologous Bone Marrow Stem Cell Transplantation without Immunosuppression in Chronic Type 1 Diabetic Patients.* Stem Cell Discovery, 2020. **10**(01): p. 1-14.
13. RH, L., et al., *Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli.* Proc Natl Acad Sci U S A, 2006. **14**: p. 17438-43.
14. Bhansali, A., et al., *Efficacy of autologous bone marrow-derived stem cell transplantation in patients with type 2 diabetes mellitus.* Stem Cells Dev, 2009. **18**(10): p. 1407-16.

15. Urban, V.S., et al., *Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes.* Stem Cells, 2008. **26**(1): p. 244-53.
16. Cai, J., et al., *Umbilical Cord Mesenchymal Stromal Cell With Autologous Bone Marrow Cell Transplantation in Established Type 1 Diabetes: A Pilot Randomized Controlled Open-Label Clinical Study to Assess Safety and Impact on Insulin Secretion.* Diabetes Care, 2016. **39**(1): p. 149-57.
17. Oh, K., et al., *In vivo differentiation of therapeutic insulin producing cells from BM cells via Extravesicle-mimetic nanovesicles.* ACS Nano, 2015. **9**: p. 11718-11727.
18. Oh, J.E., et al., *Direct differentiation of bone marrow mononucleated cells into insulin producing cells using pancreatic beta-cell-derived components.* Sci Rep, 2019. **9**(1): p. 5343.
19. Akimoto, Y., et al., *Localization of the O-linked N-acetylglucosamine transferase in rat pancreas.* Diabetes, 1999. **48**(12): p. 2407-13.
20. Gao, Y., J. Miyazaki, and G.W. Hart, *The transcription factor PDX-1 is post-translationally modified by O-linked N-acetylglucosamine and this modification is correlated with its DNA binding activity and insulin secretion in min6 beta-cells.* Arch Biochem Biophys, 2003. **415**(2): p. 155-63.
21. Durning, S.P., et al., *O-Linked beta-N-acetylglucosamine (O-GlcNAc) Acts as a Glucose Sensor to Epigenetically Regulate the Insulin Gene in Pancreatic Beta Cells.* J Biol Chem, 2016. **291**(5): p. 2107-18.
22. Deeds, M.C., et al., *Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models.* Lab Anim, 2011. **45**(3): p. 131-40.
23. Brosius, F., *Low dose streptozotocin induction protocol (Mouse).* Diacomp Protocols, 2015.
24. Furman, B.L., *Streptozotocin-Induced Diabetic Models in Mice and Rats.* Curr Protoc Pharmacol, 2015. **70**: p. 5 47 1-5 47 20.
25. Yang, S.F., et al., *Nicotinamide Facilitates Mesenchymal Stem Cell Differentiation Into Insulin-Producing Cells and Homing to Pancreas in Diabetic Mice.* Transplant Proc, 2015. **47**(6): p. 2041-9.
26. al., J.Y.K.e., *Inhibition of diabetes in Non-obese diabetic mice by nicotinamide treatment for 5 weeks at the early age.* J Korean Med Sci., 1997(4): p. 293-7.
27. Bergin, D.H., et al., *Safety and neurochemical profiles of acute and sub-chronic oral treatment with agmatine sulfate.* Sci Rep, 2019. **9**(1): p. 12669.
28. Carames, B., et al., *Glucosamine activates autophagy in vitro and in vivo.* Arthritis Rheum, 2013. **65**(7): p. 1843-52.
29. Fukaya, M., et al., *Protective effects of a nicotinamide derivative, isonicotinamide, against streptozotocin-induced beta-cell damage*

and diabetes in mice. Biochem Biophys Res Commun, 2013. **442**(1-2): p. 92-8.
30. Jiang, Z., et al., *Protective effects of BP-1-102 against intracranial aneurysms-induced impairments in mice.* J Drug Target, 2021. **29**(9): p. 974-982.
31. Jiang, Z., et al., *Pharmacological inhibition of STAT3 by BP-1-102 inhibits intracranial aneurysm formation and rupture in mice through modulating inflammatory response.* Pharmacol Res Perspect, 2021. **9**(1): p. e00704.
32. Kibe, R., et al., *Upregulation of colonic luminal polyamines produced by intestinal microbiota delays senescence in mice.* Sci Rep, 2014. **4**: p. 4548.
33. Olenyik, T., C. Gilroy, and B. Ullman, *Oral putrescine restores virulence of ornithine decarboxylase-deficient Leishmania donovani in mice.* Mol Biochem Parasitol, 2011. **176**(2): p. 109-11.
34. Sanchez-Sevilla, L., E. Mendieta-Condado, and R. Hernandez-Munoz, *Putrescine treatment reverses alpha-tocopherol-induced desynchronization of polyamine and retinoid metabolism during rat liver regeneration.* J Transl Med, 2016. **14**(1): p. 307.
35. Tahara, A., A. Matsuyama-Yokono, and M. Shibasaki, *Effects of antidiabetic drugs in high-fat diet and streptozotocin-nicotinamide-induced type 2 diabetic mice.* Eur J Pharmacol, 2011. **655**(1-3): p. 108-16.
36. Zhang, X., et al., *Orally bioavailable small-molecule inhibitor of transcription factor Stat3 regresses human breast and lung cancer xenografts.* Proc Natl Acad Sci U S A, 2012. **109**(24): p. 9623-8.
37. DJ Prockop., e.a., *One strategy for cell and gene therapy: Harnessing the power of adult stem cells to repair tissues.* Proc Natl Acad Sci U S A, 2003. **30** p. 11917-23.
38. JR Munoz., e.a., *Human stem, progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice.* Proc Natl Acad Sci U S A, 2005. **103**(13): p. 18171-6.
39. HE Park., e.a., *Putrescine and Cadaverine enhance insulin secretion of mouse pancreatic B-cell line. .* J Exp Biomed Sci, 2012. **18** p. 193-200.
40. al., Y.W.e., *Adipose derived mesenchymal stem cells transplantation via portal vein improves microcirculation and ameliorates liver fibrosis induced by CCl4 in rats* Journal of Translational Medicine 2012.
41. Wu, B., et al., *Synaptotagmin-7 phosphorylation mediates GLP-1-dependent potentiation of insulin secretion from beta-cells.* Proc Natl Acad Sci U S A, 2015. **112**(32): p. 9996-10001.
42. Jiang, F.X., et al., *Differentiation of Islet Progenitors Regulated by Nicotinamide into Transcriptome-Verified beta Cells That Ameliorate Diabetes.* Stem Cells, 2017. **35**(5): p. 1341-1354.

43. Miura, M., et al., *Suppression of STAT3 signaling promotes cellular reprogramming into insulin-producing cells induced by defined transcription factors.* EBioMedicine, 2018. **36**: p. 358-366.
44. Zhai, R., et al., *Pharmacological Mobilization of Endogenous Bone Marrow Stem Cells Promotes Liver Regeneration after Extensive Liver Resection in Rats.* Sci Rep, 2018. **8**(1): p. 3587.
45. Watanabe, T., et al., *Bone marrow contributes to the population of pancreatic stellate cells in mice.* Am J Physiol Gastrointest Liver Physiol, 2009. **297**(6): p. G1138-46.
46. Scarlett, C.J., *Contribution of bone marrow derived cells to the pancreatic tumor microenvironment.* Front Physiol, 2013. **4**: p. 56.
47. Sordi, V., et al., *Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets.* Blood, 2005. **106**(2): p. 419-27.
48. Luo, L., et al., *Cytokines inducing bone marrow SCA+ cells migration into pancreatic islet and conversion into insulin-positive cells in vivo.* PLoS One, 2009. **4**(2): p. e4504.
49. D Hess., e.a., *Bone marrow derived stem cells initiate pancreatic regeneration.* Natuer Biotechnology 2003 **21**: p. 763-770.
50. Milanesi, A., et al., *beta-Cell regeneration mediated by human bone marrow mesenchymal stem cells.* PLoS One, 2012. **7**(8): p. e42177.
51. Meran, L., A. Baulies, and V.S.W. Li, *Intestinal Stem Cell Niche: The Extracellular Matrix and Cellular Components.* Stem Cells Int, 2017. **2017**: p. 7970385.