Endocrine Journal 2008, **55** (3), 535–543

A Simple Method to Induce Differentiation of Murine Bone Marrow Mesenchymal Cells to Insulin-producing Cells Using Conophylline and Betacellulin-delta4

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Abstract. The present study was conducted to establish a method to induce differentiation of bone marrow (MB)-derived mesenchymal cells into insulin-producing cells. When mouse BM-derived mesenchymal cells were cultured for 60 days in medium containing 10% fetal calf serum and 25 mM glucose, they expressed insulin. Addition of activin A and betacellulin (BTC) accelerated differentiation, and immunoreactive insulin was detected 14 days after the treatment. Insulin-containing secretory granules were observed in differentiated cells by electron microscopy. Treatment of BM-derived mesenchymal cells with conophylline (CnP) and BTC-delta4 further accelerated differentiation, and mRNA for insulin was detected 5 to 7 days after the treatment. Mesencymal cells treated with CnP and BTC-delta4 responded to a high concentration of glucose and secreted mature insulin. When these cells were transplanted into streptozotocin-treated mice, they markedly reduced the plasma glucose concentration, and the effect continued for at least 4 weeks. These results indicate an efficacy of the combination of CnP and BTC-delta4 in inducing differentiation of BM-derived mesenchymal cells into insulin-producing cells.

Key words: Insulin, Beta cell, Differentiation

(*Endocrine Journal* **55**: 535–543, 2008)

ISLET transplantation is becoming a new option for the treatment of type I diabetes [1]. Indeed, according to Ryan *et al.* [2], most of the patients treated with the Edmonton protocol became insulin-independent. Glycemic control became quite stable in all patients after islet transplantation and serious complications were not observed. Although islet transplantation is a promising therapeutic option for the treatment of type I diabetes, a limited supply of islet donors is an increasing problem because the demand for islet transplantation is extremely large. In this regard, sources of pancreatic beta cells other than those from organ donors are expected. Recent advances in the regenerative medicine provide a possibility that beta cells can be obtained from many types of progenitor cells *in vitro*. If well-differentiated beta cells were easily available from progenitor cells *in vitro*, these cells would be a suitable source of islet transplantation.

It is now known that many types of stem/progenitor cells can be converted to insulin-producing cells *in vitro* [3–8]. In this regard, many types of stem/progenitor cells are sources of beta cells for transplantation.

Received: December 27, 2007
Accepted: January 1, 2008
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To obtain glycemic control by transplantation of these *in vitro* differentiated beta cells, however, both the number and quality of beta cells are quite important. To obtain a large number of beta cells *in vitro*, it would be helpful if the number of stem/progenitor cells could be easily expanded *in vitro*. In other words, stem/progenitor cells which can be grown easily *in vitro* are good candidates for the source of beta cells. In this regard, mesenchymal cells in the bone marrow are suitable candidates for the source of beta cells because these cells are obtained easily from donors and even from patients, and they can be easily expanded in a culture system. Moreover, these cells can be differentiated to various types of cells including neuronal cells and most importantly insulin-producing cells [9–11]. Several reports have shown that bone marrow mesenchymal cells can be differentiated to beta cells *in vitro*. There remain, however, several issues to be addressed. For example, long-term culture is required before conversion to insulin-producing cells [9], and transfection of transcription factor gene is necessary [10, 11]. From a practical point of view, a simple and safe method to induce differentiation is favorable. The present study was conducted to establish a simple reproducible method to convert bone marrow (BM)-derived mesenchymal cells *in vitro* to insulin-producing cells. To this end, we used differentiation factors for beta cells, namely, conophylline (CnP) and betacellulin-delta4 (BTC-delta 4) [12].

Materials and Methods

Materials

Recombinant human activin A was provided by Dr. Y. Eto of the Central Research Laboratory of Ajinomoto Inc. (Kawasaki, Japan). Recombinant human BTC and BT-delta4 were prepared as described previously [13]. Conophylline was extracted from the leaves of *Ervatamia microphylla* and purified as described elsewhere [14]. RPMI1640 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen. Penicillin, streptomycin sulphate were obtained from Wako (Kyoto, Japan). Guinea pig anti-insulin antibody was obtained from Dako (Carpinteria, CA).

Culture of Bone Marrow Cells

Eight-week old C57BL/6 male mice were purchased from the Japan SLC (Hamamatsu, Japan). The procedures were performed in accordance with the guidelines for animal experimentation of Gunma University. Mice were sterilized by immersion in 70% ethanol. After removal of the remaining skin and muscles, BM cells were obtained from the femurs and tibias. BM-derived cells were then cultured (37°C, 5% CO_2) in 10 cm plates with RPMI1640 medium containing 11 mM glucose and 10% inactivated fatal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin (basic medium). On the fourth day, culture medium was changed and floating cells were removed. Adherent cells gaining 80% confluence were passaged with the use of 0.05% trypsin. To induce differentiation, cells were used between 5 and 10 passages.

In vitro Differentiation

To induce differentiation of BM-derived mesenchymal cells to pancreatic endocrine cells, the cells were cultured in the basic medium in the presence of factors such as activin A, CnP, BTC and BT-delta4 for various periods. Cellular differentiation was monitored by measuring the expression of genes expressed in pancreatic beta cells and by measuring the expression of insulin by immunostaining.

A mouse insulinoma cell line MIN6 [15] was a generous gift from Dr. Jun-ichi Miyazaki of Osaka University (Osaka, Japan). The cells were maintained in DMEM containing 25 mM D-glucose, 15% inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C under a humidified atmosphere. MIN6 cells were used as a positive control for measurement of insulin content and insulin release.

RNA Extraction and RT-PCR

Total RNA was prepared from bone marrow derived cells using TRIzol reagent (Invitrogen). Gene expression related to pancreatic endocrine development was determined by RT-PCR according to the manufacturer's protocol. Contaminating genomic DNA was removed with RNase-free DNase 1 (Invitrogen). Four micrograms of DNase-treated RNA were incubated with 1 microliter of dNTP mix (10 mM) and 1 microliter of

Table 1. List Primers Used RT-PCR Analysis

Genes	Forward	Reverse	Size (bp)	Cycle
insulin 1, 2	GGC TTT TGT CAA AVA GCA CCT TTG	AGC AGA TGC TGG TGC AGC A	240	35
glucagon	ACT CAC AGG GCA CAT TCA CC	CCA GTT GAT GAA GTC CCT GG	353	32
somatostatin	ATG CTG TCC TGC CGT CTC CA	GCC TCA TCT CGT CCT GCT CA	251	32
PP	TAG TGC TGC CTC TCC CTG TT	CCA GGA AGT CCA CCT GTG TT	224	32
neurogenin 3	CAT ACC TAG GGA CTG CTC CGA	CAT ACA AGC TGT GGT CCG CTA	320	34
neuro D	GCT CCA GGG TTA TGA GAT CG	CTC TGC ATT CAT GGC TTC AA	205	35
Pax 6	AAC AAC CTG CCT ATG CAA CC	ACT TGG ACG GGA ACT GAC AC	206	35
Pax 4	CTC ACG GAG CCT TAA GGT ATC	TGA ATC TGG ATA CTG CCC ACG	463	35
Isl 1	CAC TAT TTG CCA CCT AGC CAC	AAA TAC TGA TTA CAC TCC GCA C	256	35
Nkx 2.2	GTC CGG AAC CAT GTC GCT GA	GAC TTG GAG CTC GAG TCT TG	318	35
Nkx 6.1	ACT TGG CAG GAC CAG AGA GA	AGA GTT CGG GTC CAG AGG TT	224	35
PDX-1	TGT AGG CAG TAC GGG TCC TC	CCA CCC CAG TTT ACA AGC TC	325	32
GLUT 2	CAT TCT TTG GTG GGT GGC	CCT GAG TGT GTT TGG AGC G	221	32
glucokinase	GCA GAT CCT GGC AGA GTT CCA	GGA AGG AGA TGA AGC CCA	175	32
Kir 6.2	TAG GCC AAG CCA GTG TAG TG	GTG GTG AAC ACA TCC TGC AG	248	35
SUR 1	GGA TGA ATG CCT TCA TCA AG	GGA AGA CGT GGT TCT CCT TC	309	35
PC 1/3	ATG GAG CAA AGA GGT TGG AC	GCT GCA GTC ATT CTG GTA TC	419	35
PC 2	TCG CCA AGT TGC AGC AGA AC	CTT CGG CCA CGT TCA AGT CTA	314	35
GAPDH	CAT GAC CAG AGT CCA TGC CAT C	CAC CCT GTT GCT GYA GCC ATA TTC	451	17

oligo dT or 1 microliter of 2 pmol gene-specific primer at 65°C for 5 min. Four microliters of a 5 × first strand buffer, 2 microliter of DTT (0.1 M), and 1 microliter of RNaseOUT Recombinant RNase Inhibitor (Invitrogen) were added to each reaction. After incubation for 2 min at 42°C, 1 microliter of reverse transcriptase was added. Samples were incubated at 42°C for 50 min, then at 70°C for 15 min. One microliter RNase H was added to each reaction, and samples were incubated at 37°C for 20 min. The forward and reverse primers of each PCR set and the sizes of PCR products, cycles, and annealing temperature are listed in Table 1. Reactions contained 5 microliter of a 10 × PCR buffer, 1 microliter of $MgCl_2$ (50 mM), 1 microliter of dNTP mix, 1 microliter of forward primer, 1 microliter of reverse primer, 0.5 μl of Taq polymerase, and 2 microliter of cDNA. In addition, DNA-PCR without RT was performed to determine any DNA contamination. MIN6 cell cDNA was used as a positive control in each experiment.

Immunocytochemistry

Cells were seeded on non-coated sterile glass coverslips at a density of 1×10^4/ml. Cells were fixed in 4% paraformaldehyde, treated with 0.1% (vol/vol) Triton X-100 in PBS for 5 min, and incubated sequentially with Block Ace (Dainippon Seiyaku, Osaka, Japan) and polyclonal guinea pig anti-insulin (1 : 100) (Dako) for 1 h. After washing, the cells were incubated with Alexa Fluor^R 568 goat anti-guinea pig IgG (H + L) conjugate (1 : 1000) (Invitrogen) and 4' diamino-2-phenylindoke (DAPI: PIERCE, IL). Immunofluorescence images were recorded with an Olympus AX70 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a PXL 1400 cooled-CCD camera system (Photometrics, Tucson, AR), which was operated with IP Lab Spectrum software (Signal Analysis, Vienna, VA). For DAB staining, cells were incubated with biotynylated anti-guinea pig IgG (1 : 200) (Vector Lab) for 30 min and horseradish perioxydate (HRP)-conjugated streptavidin (1 : 500) (Vector Lab) for 30 min following the reaction with primary antibody. DAB served as chromagen. Nuclei were counterstained with Carazzi's hematoxylin (Wako).

In vitro Differentiation of Bone Marrow Cells into Adipocytes

Cells were seeded at 5×10^4 cells per 35-mm dish, and on the next day, 1 μM insulin, 1 μM dexamethazone, and 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine were added, and the cells were cultured for ten days. Lipid was stained with oil-red O.

Immunoelectron Microscopy

BM cells were processed for immunoelectron microscopic analyses [16]. BM cells grown in 100 mm dishes were fixed first with 0.1% glutaraldehyde-4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 minutes at 4°C, and were then rinsed three times in 0.1 M phosphate buffer (pH 7.2) containing 7.5% sucrose at 4°C. After fixation, the cells were gently scraped from the dishes and centrifuged. Pellets of fixed cells were subsequently dehydrated with 70% ethanol and infiltrated into pure LR White resin (London Resin) for 12 hrs at 4°C. The pellets were placed in gelatin capsules with fresh LR White resin, and polymerized for 24 hrs at 55°C.

Immunogold labeling was performed as described previously [16]. The primary anti-insulin antibody was diluted at 1 : 300. Intracellular localization of insulin was distinguished by labelling with colloidal gold particles conjugated to guinea pig anti-insulin IgG (purchased from British Biocell International; particle size 10 nm in diameter). Following the immunoreaction, the sections were contrasted with saturated aqueous solutions of uranyl acetate and lead citrate, and examined with the JOEL electron microscope (JEM-1010).

Measurement of Insulin Content, Insulin secretion and Fractionation of Mature Insulin

To measure insulin content, differentiated cells were homogenized using a glass homogenizer. Insulin was extracted by using 0.1 M HCl. After neutralization of the sample with NaOH solution, samples were stored at –20°C. Insulin was measured by radioimmunoassay using a kit obtained from Amersham (Back, UK). For measurement of insulin secretion, cells were preincubated in Krebs-Ringer bicarbonate buffer containing 5 mM glucose. Various stimulants were then added and cells were further incubated for 60 min. Insulin released to the medium was measured by radioimmunoassay.

To measure production of mature insulin, culture medium was lyophilized and dissolved in 50 mM sodium acetate buffer (pH 5.0). Gel filtration was performed with a 1.0 × 120 cm column of a Sephadex G-50 (superfine) gel (Pharmacia LKB, Piscataway, NJ) equilibrated with 50 mM sodium acetate buffer (pH 5.0). Fractions of 1.5 ml were collected. Size calibration was carried out with blue dextran (void volume) and potassium ferricyanide (total volume).

Transplantation of Differentiated Cells

Differentiated cells were detached and suspended in a soft agar. The soft agar was then collected in a syringe, and agar string containing differentiated cells was prepared by using a 16 G needle. Differentiated cells in a soft agar tube were embedded in the omentum of the nude mice pretreated with streptozotocin (STZ). Plasma glucose concentration was measured at 9 a.m. in the morning.

Results and Discussion

BM cells were obtained from C57BL/6 male mice. On the second day of culture, spindle-shaped cells were observed. On the fourth day, unattached BM cells were removed. After a week, spindle-shaped adherent cells reached 70–80% confluence (Fig. 1A). The cells were then released from the substrate surface using trypsin-EDTA, replated under the same culture condition and cultured for several passages. We determined the growth curve by using MTT. As shown in Fig. 1B, growth of BM-derived mesenchymal cells was exponential over the period studied.

We cultured BM-derived mesenchymal cells in the adipogenic differentiation medium. One week later, a few adipocytes appeared (data not shown). These BM mesenchymal cells thus could be differentiated into adipocytes. These findings are consistent with the notion that BM-derived mesenchymal cells possess stem cell properties.

To determine whether BM-derived mesenchymal cells had a potential to differentiate into insulin-producing cells, we examined the expression of insulin by immunocytochemistry and RT-PCR. As shown in Fig. 2A, BM-derived mesenchymal cells expressed immunoreactive insulin when they were cultured in DMEM containing 10% FBS and 25 mM glucose for 60 days. RT-PCR revealed that those cells expressed mRNA for insulin (Fig. 2B). The size of the PCR product obtained in BM-derived mesenchymal cells was the same as that in MIN6 cells. We confirmed that the PCR product was insulin by sequence analysis (data not shown).

To efficiently induce differentiation, BM-derived mesenchymal cells were cultured in DMEM containing

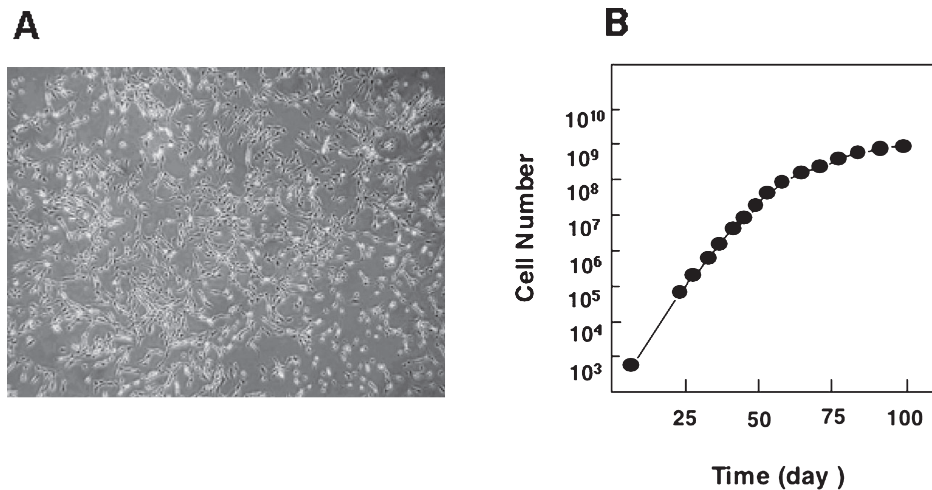

Fig. 1. Morphology and Growth Curve of BM-derived Mesenchymal Cells
A: Morphology of BM-derived mesenchymal cells.
B: Growth curve of BM-derived mesenchymal cells: Cells were cultured for indicated periods and the cell number was counted.

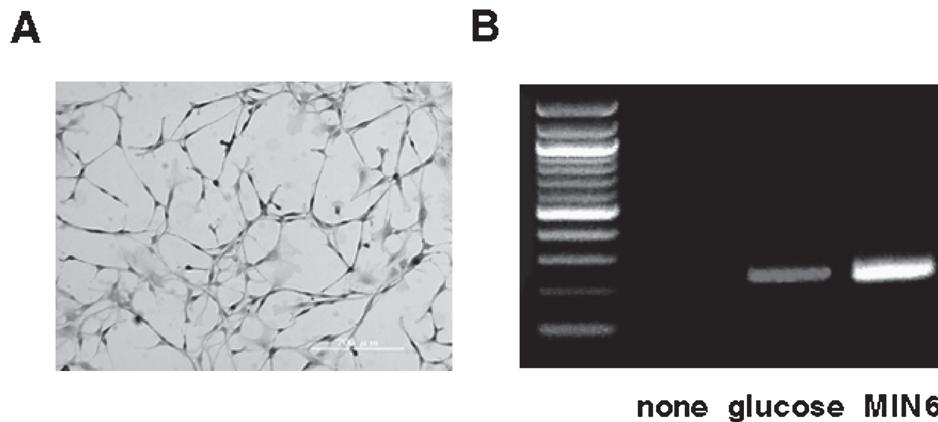

Fig. 2. Expression of Insulin in BM-derived Mesenchymal Cells
BM-derived mesenchymal cells were cultured for 60 days in DMEM containing 10% FBS and 25 mM glucose.
A: Cells were stained with anti-insulin antibody.
B: Expression of mRNA for insulin was measured by RT-PCR. RNA samples obtained from cells cultured with 10% FBS (none) or 10% FBS and 25 mM glucose (Glucose). MIN6: RNA sample obtained from MIN6 cells.

10% FBS, 25 mM glucose, and a combination of 2 nM activin A and 1 nM BTC. Cellular differentiation was then monitored by Immunocytochemistry, and the time course of insulin expression was monitored by RT-PCR. Treatment of BM-derived mesenchymal cells with the combination of activin A and BTC accelerated differentiation, and immunoreactive insulin was detected histochemically 14 days after the addition of these factors (Fig. 3A). Immunoreactive C-peptide was also detected (data not shown). Fig. 3B shows the time course of the expression of mRNA for insulin. mRNA for insulin became detectable on day 9. Hence, a combination of activin A and BTC was effective in accelerating differentiation of BM-derived mesenchymal cells. The expression of insulin became detectable much earlier compared to the cells treated with a high concentration of glucose alone. We performed morphological analysis of differentiated cells. In particular, we examined whether or not secretory granules were formed in differentiated cells. To identify secretory granules, we used electron microscopy. As shown in Fig. 3C-a, some granules were observed in differen-

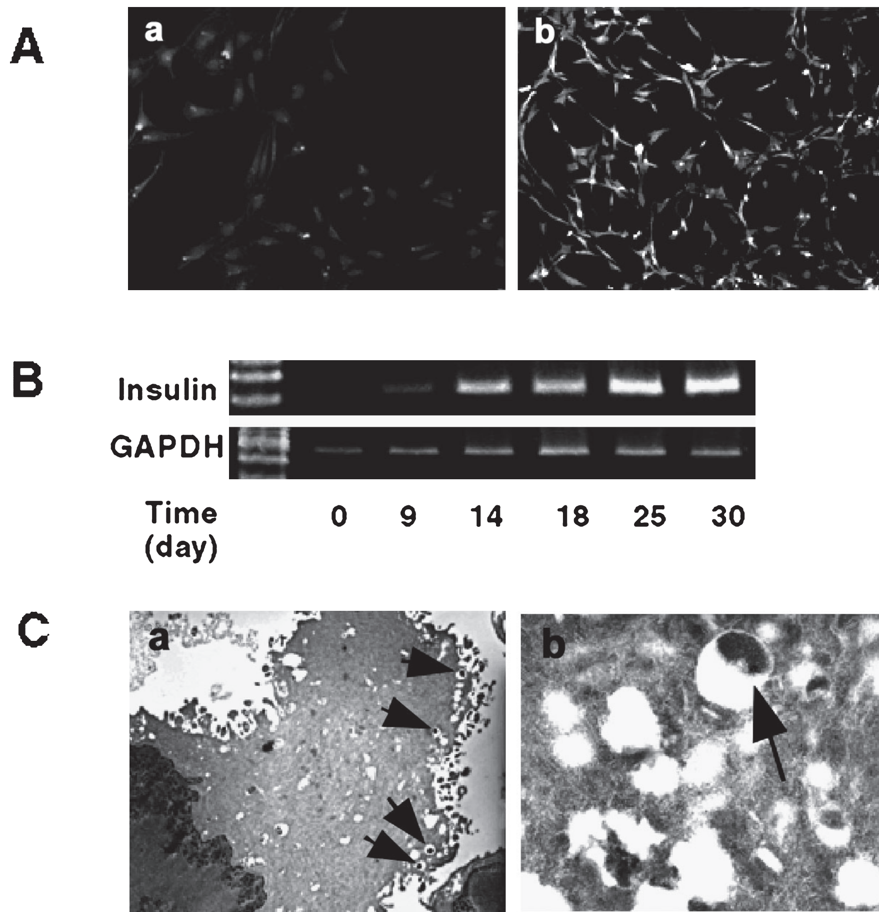

Fig. 3. Expression of Insulin in BM-derived Mesenchymal Cells Treated with Activin A and BTC
BM-derived mesenchymal cells were cultured for various periods with 10% FBS, 25 mM glucose and 2 nM activin A and 1 nM BTC.
A: Immunohistochemistry: Cells were incubated for 14 days with 10% FBS and 25 mM glucose in the presence (b) and absence (a) of 2 nM activin A and 1 nM BTC. Immunohistochemistry was done by using anti-insulin antibody.
B: Cells were cultured for various periods with 25 mM glucose, activin A and BTC and the expression of mRNA for insulin was measured by RT-PCR.
C: Electron micrograph: Cells were incubated for 14 days with 25 mM glucose, activin A and BTC.
a: electron micrograph. Arrows indicate secretory granules.
b: immunoelectron micrograph. The arrow indicates cluster of immunogold particles.

tiated BM-derived mesenchymal cells. The number of granules was, however, far less than those observed in MIN6 cells used as a positive control. These granules contained immunoreactive insulin as detected by immunogold particles (Fig. 3C-b), but the morphology of the granules was still immature and different from that of insulin granules found in beta cells. Although we detected immunoreactive insulin in differentiated cells, secretion of insulin was not detectable probably because of the low amount of secreted insulin.

We reported recently that conophylline (CnP) and BTC-delta4 reproduce differentiation activity of activin A and BTC, respectively, and a combination of CnP and BTC-delta4 is much more efficient in inducing differentiation of pancreatic progenitor cells compared to the combination of activin A and BTC [12]. We therefore examined the effect of CnP and BTC-delta4 in BM-derived mesenchymal cells. As shown in Fig. 4A, mRNA for insulin was detected 5 to 7 days after the treatment. mRNA for glucagon also became detectable on day 5 to 14 but disappeared thereafter. Similarly, mRNA for somatostatin and pancreatic polypeptide was observed on day 7 to 14, and it tended to decrease thereafter. Fig. 4B demonstrates the

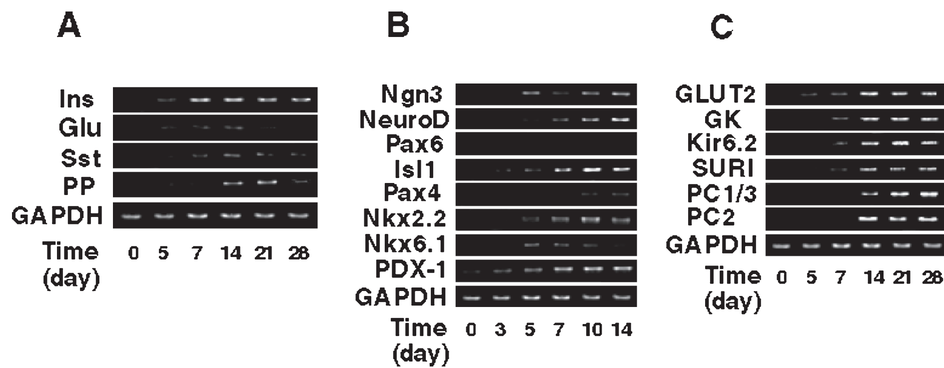

Fig. 4. Expression of Hormones and Transcription Factors
Cells were incubated for various periods in DMEM containing 10% FBS, 25 mM glucose, 100 ng/ml CnP and 1nM BTCdelta4. Expression of mRNA was determined by RT-PCR.

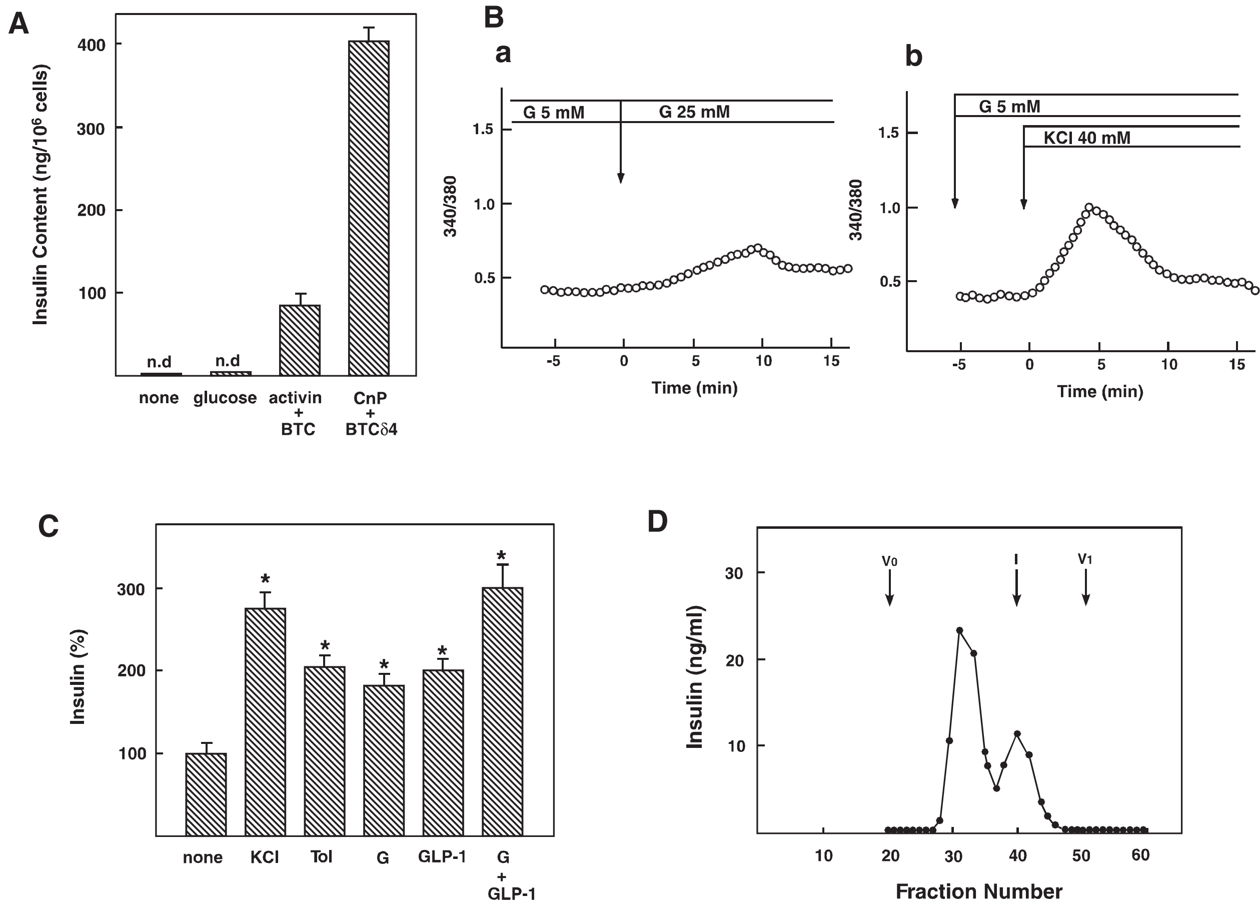

Fig. 5. Differentiated Functions of BM-derived Mesenchymal Cells Treated with CnP and BTC-delta4
A: Cells were incubated for 14 days in DMEM containing 10% FBS in the presence of various factors and insulin content was measured. Values are the mean ± S.E. for four experiments.
B: Cells treated with CnP and BTC-delta4 for 14 days were loaded with fura-2, and changes in $[Ca^{2+}]c$ in response to 25 mM glucose (a) or 40 mM KCl (b) were monitored. Note that undifferentiated cells did not respond to 25 mM glucose nor 40 mM KCl.
C: Cells treated with CnP and BTC-delta4 for 14 days were incubated for 1 h with 40 mM KCl, 10 μM tolbutamide (Tol), 25 mM glucose (G), 100 nM GLP-1, and a combination of 25 mM glucose and 100 nM GLP-1. Secreted insulin was measured. Values are the mean ± S.E. for four experiments. *: $p<0.05$ vs none.
D: Fractionation of Immunoreactive Insulin. Insulin was extracted from cells treated with CnP and BTC-delta4 and applied to Sephadex G-50 column chromatography, and elution of mature insulin (I) was detected by radioimmunoassay.

changes in the expression of various transcription factors before and after the treatment with CnP and BTC-delta4. As depicted, PDX-1 was detected at a low level before the treatment, and the expression level was increased as a function of time. Neurogenin 3 and NeuroD were induced after the treatment with CnP and BTC-delta4. As shown in Fig. 4C, mRNA for GLUT2 was induced by CnP and BTC-delta4. After differentiation, the expression of glucokinase, Kir6.2, SURI, PC1/3 and PC2 was observed.

We measured the insulin content of the BM-derived mesenchymal cells treated with various factors. As shown in Fig. 5A, the insulin content was undetectable in naïve cells or cells treated with a high concentration of glucose. Insulin content became detectable when cells were treated with a combination of activin A and BTC. In accordance with our previous report [12], the insulin content was much higher when BM-derived mesenchymal cells were treated with a combination of CnP and BTC-delta4.

BM-derived mesenchymal cells treated with CnP and BTC-delta4 expressed mRNA for GLUT2, glucokinase and ATP-sensitive potassium channel, molecules important for glucose sensing. We then studied whether or not differentiated BM-derived mesenchymal cells were able to respond to a high concentration of glucose. To this end, we first monitored changes in cytoplasmic free calcium concentration ($[Ca^{2+}]_c$) in response to the high concentration of glucose. As shown in Fig. 5B-a, 25 mM glucose induced a gradual increase in $[Ca^{2+}]_c$. $[Ca^{2+}]_c$ response to a high concentration of glucose was, however, smaller than that to a depolarizing concentration of potassium (Fig. 5B-b). We next determined whether or not differentiated BM-derived mesenchymal cells secreted insulin. As shown in Fig. 5C, differentiated BM-derived mesenchymal cells treated with the combination of CnP and BTC-delta4 secreted immunoreactive insulin. Cells treated with CnP and BTC-delta4 responded to a depolarizing concentration of potassium and tolbutamide, an inhibitor of the ATP-sensitive potassium channel. Most importantly, differentiated cells also responded to a high concentration of glucose. However, glucose-induced insulin secretion was less than 2-fold. Glucagon-like peptide-1 (GLP-1) also induced insulin secretion and potentiated glucose-induced secretion. When cells were stimulated by a combination of a high concentration of glucose and GLP-1, insulin secretion was approximately 300% of the basal secretion. As shown in Fig. 4C, differentiated cells expressed mRNA for PC1/3 and PC2, enzymes catalyzing the conversion of proinsulin to mature insulin. We then investigated whether differentiated BM-derived mesenchymal cells actually produced mature insulin. As shown in Fig. 5D, differentiated cells secreted mature insulin, but the secretion of mature insulin was less than that of proinsulin.

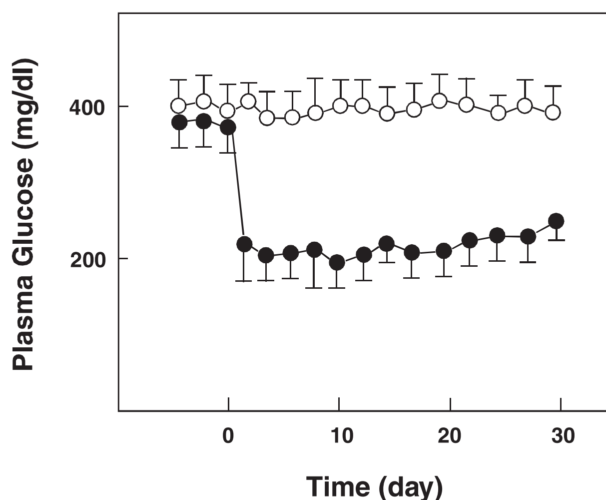

Fig. 6. Effect of Transplantation of Differentiated BM-derived Mesenchymal Cells on the Plasma Glucose in STZ-treated Nude Mice
Nude mice were injected with 200 mg/kg STZ. Five days later, BM-derived mesenchymal cells treated with CnP and BTC-delta4 for 14 days or naïve mesenchymal cells were transplanted. Changes in the plasma glucose were measured. Values are the mean ± S.E. for three experiments.

To determine whether BM-derived mesenchymal cells secrete bioactive insulin and have the ability to correct hyperglycemia in diabetic mice, nude mice were treated with STZ to become diabetic. Differentiated BM-derived mesenchymal cells (5×10^7/mouse) were then transplanted. As demonstrated in Fig. 6, the plasma glucose levels in the diabetic mice transplanted with undifferentiated BM-derived mesenchymal cells remained elevated. In contrast, the plasma glucose levels in mice tranplanted with differentiated BM-derived mesenchymal cells were improved. The plasma glucose concentration remained decreased for at least four weeks.

These results indicate that mouse BM-derived mesenchymal cells can be induced to differentiate into insulin-secreting cells *in vitro* by a simple reproducible method using CnP and BTC-delta4. Although differ-

entiated cells were able to secrete insulin, these cells were still immature in many respects. Nevertheless, differentiated cells could secrete mature insulin and respond to physiological stimuli including glucose and an incretin. Furthermore, they secreted bioactive insulin, and hyperglycemia could be reversed in insulin-deficient mice. In this regard, these cells can be used for the treatment of hyperglycemia in insulin-deficient animals. It is, however, obvious that further studies are needed to obtain insulin-secreting cells with additional differentiated functions. In particular, a method should be established to form pseudoislets using differentiated BM-derived mesenchymal cells since pseudoislets have advantages over isolated cells for transplantation [17].

Acknowledgments

This study was supported by a grant from the Project of Realization of Regenerative Medicine. Authors are grateful to Mayumi Odagiri for secretarial assistance.

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