Original Article

J Korean Soc Transplant 2015;29:118-129
http://dx.doi.org/10.4285/jkstn.2015.29.3.118

JKST

Retinoic Acid-induced Differentiation of Rat Mesenchymal Stem Cells into β-Cell Lineage

Graduate Program of Nano Science and Technology, Graduate School of Yonsei University[1], Departments of Medical Engineering[2], Surgery[3], and Orthopedic Surgery[4], Yonsei University College of Medicine, Department of Transplantation Surgery, Severance Hospital, Yonsei University Health System[5], Seoul, Korea

Jae Hyung Kim, Ph.D.[1,2], Kyung Sik Kim, M.D.[1,3], Sang Woo Lee, B.S.[1,2], Hyun Woo Kim, M.D.[1,4], Dong Jin Joo, M.D.[3,5], Yu Seun Kim, M.D.[1,3,5] and Hwal Suh, D.D.S.[1,2]

Backgrounds: Type I diabetes mellitus (T1DM), an autoimmune disease, is associated with insulin deficiency due to the death of β-cells. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are capable of tissue repair and thus are a promising source of β-cell surrogates.

Methods: In this study, the therapeutic potential of BM-MSCs as β-cell replacements was analyzed both *in vitro* and *in vivo*. First, we used retinoic acid (RA) to induce rat BM-MSCs to differentiate into cells of endodermal/pancreatic lineage. Then, differentiated rat BM-MSCs were syngeneically injected under the renal capsule of rats.

Results: Analysis of gene expression revealed that rat BM-MSCs showed signs of early pancreatic development, and differentiated cells were qualitatively and quantitatively confirmed to produce insulin *in vitro*. *In vivo* study was performed for short-term (3 weeks) and long-term (8 weeks) period of time. Rats that were injected with differentiated MSCs exhibited a reduction in blood glucose levels throughout 8 weeks, and grafted cells survived *in vivo* for at least 3 weeks.

Conclusions: These findings show that RA can induce differentiation of MSCs into the β-cell lineage and demonstrate the potential of BM-MSCs to serve as therapeutic tools for T1DM.

Key Words: Type 1 diabetes mellitus, Insulin-secreting cells, Mesenchymal stromal cells, Tretinoin, Insulin

중심 단어: 1형 당뇨병, 췌장 베타세포, 중간엽줄기세포, 레티노산, 인슐린

INTRODUCTION

Type 1 diabetes mellitus (T1DM), an autoimmune disease associated with certain genetic human leukocyte antigen configurations, results in the death of pancreatic β-cells due to continued autoimmune activity, thereby leading to insulin deficiency(1). During embryonic development, the pancreas originates from the foregut endoderm as ventral and dorsal buds(2). Pancreatic progenitor cells within the foregut epithelium are marked by the expression of pancreatic-duodenal homeobox 1 (PDX-1), which can be activated by forkhead box A2 (FoxA2) and is required for pancreatic bud growth(3). PDX-1 expression is down-regulated in acinar and ductal cells, maintained in differentiated endocrine

Received June 13, 2015
Revised July 6, 2015
Accepted July 6, 2015

Corresponding authors: Yu Seun Kim
Department of Transplantation Surgery, Severance Hospital, Yonsei University Health System, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea
Tel: 82-2-2228-2115, Fax: 82-2-313-8289
E-mail: yukim@yuhs.ac
Hwal Suh
Department of Medical Engineering, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea
Tel: 82-2-2228-1918, Fax: 82-2-363-9923
E-mail: hwal@yuhs.ac

This work was mainly performed by the laboratory expense of Department of Medical Engineering, Yonsei University College of Medicine. It was supported in part, to purchase reagents and animals, by Yonsei University (http://research.yonsei.ac.kr) IACF (2013-31-0834).

cells, and up-regulated in β-cells(4). Also, Huang et al.(5) found that a pro-endocrine transcription factor, neurogenin 3 (Ngn3), induces expression of essential β-cell transcription factors, such as paired box gene 4 (Pax4), during pancreatic development.

Adult stem cells are widely used in medical research due to their unique properties of self-renewal and ability to differentiate into multiple lineages(6). Two popular types of stem cell populations are contained in bone marrow (BM): hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs are favored for cell-based therapies because of their multipotency and immune-modulatory properties, with several groups reporting the ability of MSCs to minimize the risk of graft-versus-host disease after allogeneic transplantation in patients(7-11). MSCs can potentially be induced to differentiate into β-cell-like, insulin-producing cells via exposure to specific differentiation factors or genetic modification(12). Oh et al.(13) found that introducing MSCs into diabetic mice via subcapsular renal transplantation lowered blood glucose levels, demonstrating that BM-derived cells are capable of differentiating into insulin-producing cells and suggesting their potential application for T1DM treatment.

The expansion of β-cells from a progenitor/stem cell source is a practical solution to the shortage of donor islets, as several research groups have shown that multipotent MSCs are capable of giving rise to functional endoderm(13-16), and numerous studies show that stem cells of various origins can differentiate into islet-like cells(17,18). Furthermore, several studies have demonstrated that progenitor/stem cells, including BM-derived cells, can differentiate into insulin-secreting cells *in vitro*, and transplantation of these differentiated cells into diabetic mice reduces hyperglycemia (13,19-21).

All-trans retinoic acid (RA) is a signaling molecule involved in the development of neuroectoderm and mesoderm in vertebrates. RA also regulates early pancreatic bud formation during embryogenesis and enhances insulin expression in pancreatic β-cells(22), and RA signaling is involved in the emergence of Pdx1-expressing progenitor cells(23). Cavallari et al.(24) demonstrated that RA, coupled with hyaluronic and butyric acids, can improve islet revascularization when islets are co-transplanted with stem cells into diabetic rats. Here, we examined the potential of RA to induce differentiation of rat BM-derived MSCs into the β-cell lineage *in vitro* without using growth factors. Furthermore, we investigated the potential *in vivo* therapeutic use of differentiated, insulin-secreting MSCs for T1DM.

MATERIALS AND METHODS

1. Cell isolation and expansion

Rat BM-derived MSCs (rBM-MSCs) were isolated from male 10-week-old Sprague Dawley (SD) rats (Orient, Seongnam, Korea). After sacrifice, the femora and tibiae were removed. Both ends of the bones were cut off, and BM aspirate was obtained by irrigation of phosphate buffered saline (PBS) through the bone using a syringe. Aspirate was suspended in PBS, filtered through a cell strainer with 100-μm pore size, and centrifuged at 800 ×g for 10 minutes and centrifuged at 800 ×g for 10 minutes. Supernatant was removed, and the remaining pellet was washed by re-suspending in PBS and centrifuging at 800 ×g for 5 minutes. After removal of supernatant, the pellet was re-suspended in low glucose Dulbecco's Modified Eagle's Medium (DMEM-lg, Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Antibiotic-Antimycotic, Life Technologies). Re-suspended cells were plated onto a 60-mm dish and cultivated at 37°C in air containing 5% CO_2. The next day, the medium was removed, and cells were washed with PBS six times to completely remove debris. Attached cells were expanded, and the medium was changed every 3～4 days. Cells at passage 3 were used in the experiments.

2. Induction of pancreatic differentiation

rBM-MSCs were initially plated at a concentration of 2,000～2,500 cells/cm^2. The next day, serum-free medium was applied for 48 hours prior to induction of differentiation. The "base" differentiation medium consisted of DMEM-lg supplemented with FBS, antibiotics, 10 mM nicotinamide (Sigma-Aldrich, St. Louis, MO, USA), and 2 mM L-glutamine (Sigma-Aldrich). Cells were cultivated with

this base medium for 4 days, and then 10 μM RA (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added of the medium (1% of total volume) for 3 days. This 4/3 day cycle was repeated weekly. Cell morphology was monitored using an Olympus IX71inverted microscope (Olympus Optical Co., Tokyo, Japan).

3. Total RNA isolation and reverse-transcriptase polymerase chain reaction

The RNeasy Kit (Qiagen, Venlo, Netherlands) was used to extract total RNA following the manufacturer's instructions. One microgram isolated total RNA was treated with 0.5 μg OligodT primer 12～18 mer (Life Technologies) and incubated at 70°C for 5 minutes and then 4°C for 5 minutes. Four microliters 5×reaction buffer, 2.4 μL $MgCl_2$, 1 μL deoxyribonucleotide triphosphates, and 1 μL ImProm-II reverse transcriptase (Promega, Fitchburg, WI, USA) were added to the mixture. The mixture was then incubated at 25°C for 5 minutes, 42°C for 1 hour, and 70°C for 5 minutes to synthesize cDNA. The product was maintained at 4°C.

Synthesized cDNA product was quantified and transferred into AccuPower polymerase chain reaction (PCR) premix (Bioneer, Daejeon, Korea) with primers. PCR was performed in a DNA thermal cycler 2720 (Life Technologies). An initial denaturation condition of 94°C for 5 minutes was applied to all primer sets, followed by 35 cycles of 94°C for 30 seconds, specific annealing temperatures for 30 seconds, and 72°C for 30 seconds or 1 minute for primers with product sizes of less or greater than 500 bp, respectively (Table 1). A final extension step of 72°C for 5 minutes was completed after the last cycle. PCR products were loaded into wells in 1.8% agarose gel for electrophoresis.

4. Fluorescence-activated cell sorting analysis

MSCs were harvested after detachment with 0.25% trypsin-ethylenediaminetetraacetic acid (Life Technologies). Cells were washed with 0.2% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in PBS and collected by centrifugation at 2,000 rpm for 5 minutes. Fluorescein isothiocyanate-conjugated CD29, CD45, CD90 (BD Biosciences, Franklin Lakes, NJ, USA), and CD34 (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used for rBM-MSCs. Analysis was performed for at least 10,000 cells/sample using fluorescence-activated cell sorting Calibur and Cell Quest software (BD Biosicences).

5. Immunocytochemistry

rBM-MSCs at week 3 were fixed in 10% formaldehyde solution for 30 minutes at room temperature after washing with PBS. Fixed cells were washed three times with PBS, permeabilized by incubation in 100% ice-cold methanol for 5 minutes, and washed again washed three times with PBS. Non-specific binding was blocked with 5% (w/v) BSA solution in PBS by incubation at room temperature for 45 minutes. Cells were then incubated in insulin (H-86) pri-

Table 1. Primers for rat bone marrow-derived mesenchymal stem cells

Gene	Sequence	Accession number	Product size (bp)	Annealing Temperature (°C)	Cycles
FoxA2	Sense: 5'-AAG GGA AAT GAC AGG CTG AGT GGA-3' Antisense: 5'-TGT GGA ACT CTG GCA TTC TAG CCA-3'	NM_012743.1	581	63	30
PDX-1	Sense: 5'-TGC CAC CAT GAA TAG TGA GGA GCA-3' Antisense: 5'-CGC GTG AGC TTT GGT GGA TTT CAT-3'	NM_022852.3	394	63	30
Ngn3	Sense: 5'-TTA CAA AGA TCG AGA CCC TGC GCT-3' Antisense: 5'-AAG AGC CAG TGA GGT AAG ACG CAA-3'	NM_021700.1	670	63	30
Pax4	Sense: 5'-AGC TGA GGC ACT GGA GAA AGA GTT-3' Antisense: 5'-AGC ACA GCT GAC AGA AGG AAG GAT-3'	NM_031799.1	305	63	30
C-peptide	Sense: 5'-TGT CAA ACA GCA CCT TTG TGG TCC-3' Antisense: 5'-AGT GGT GGA CTC AGT TGC AGT AGT-3'	NM_019129.3	500	63	30

Abbreviations: FoxA2, forkhead box A2; PDX-1, pancreatic-duodenal homeobox 1; Ngn3, neurogenin 3; Pax4, paired box gene 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

mary antibody (Santa Cruz Biotechnology) diluted 1:200 in 1% BSA solution overnight on a shaking plate in a cold room. Cells were washed three times with PBS for 5 minutes at room temperature and then incubated with CruzFluo (CFL)-conjugated (maximum excitation wavelength of 488 nm) goat anti-rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology) secondary antibody diluted 1:200 in 1% BSA solution for 1 hour at room temperature in a container covered with foil. Cells were washed three times with PBS for 10 minutes. Prior to observation, cell nuclei were stained with 4',6-diamidino-2-phenyindole (Sigma-Aldrich). Immunostained cells were observed using an Olympus IX71fluorescence light microscope, and images were obtained and analyzed using a CCD DP71 digital camera and DP Controller software (Olympus Optical Co.).

6. Enzyme-linked immunosorbent assay

Culture media from differentiated hBM- and rBM-MSCs at week 3 was collected and stored at −20°C. Enzyme-linked immunosorbent assay (ELISA) analysis was performed using the Rat Insulin ELISA Kit (Shibayagi, Shibukawa, Japan) following the manufacturers' instructions. Reactivity at 450 nm was measured using the Versa Max ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Measured values were converted to insulin concentrations using references supplied by the manufacturers.

7. Syngeneic graft of rBM-MSCs

Animal study was approved by Department of Laboratory Animal Resources, which is an Institutional Animal Care and Use Committee and a designated Association for Assessment and Accreditation of Laboratory Animal Care International facility, Yonsei Biomedical Research Institute, Yonsei University College of Medicine, and the entire procedure was performed under control. Meloxicam (1 mg/kg body weight) was daily applied to ameliorate suffering, and gentamicin was used as antibiotics. Animals were sacrificed by CO_2 gas.

Male SD rats (250～300 g, 8～9 weeks old) were housed at the Department of Laboratory Animal Medicine at Yonsei University College of Medicine, under a 12-hour-day/night cycle with access to water and standard rat chow. Experimental diabetes was induced by intraperitoneal injection of 80 mg/kg streptozotocin (STZ; Sigma-Aldrich) dissolved in 100 mmol/L citrate buffer (pH 4.5) at a volume of 1 L/kg body weight. Glucose levels were checked every three days with an Optium blood glucose monitoring device (Abbott Laboratories, Abbott Park, IL, USA) using blood obtained by cutting the tip of the tail. Blood glucose levels above 500 mg/dL were recorded as 500.

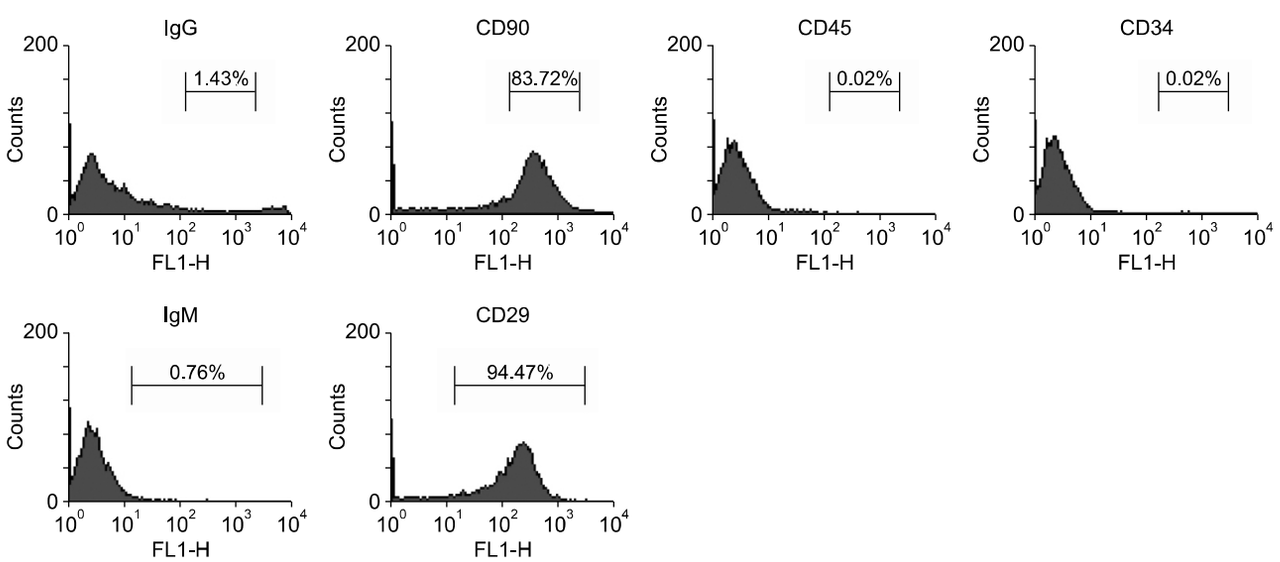

Fig. 1. Fluorescence-activated cell sorting analysis for characterization of rat mesenchymal stem cells (MSCs) at passage 3. Abbreviation: IgG, immunoglobulin G.

Diabetic rats (glucose level >450 mg/dL) received syngeneic grafts of rBM-MSCs that had been cultured for 3 weeks. Cells were labeled with the red fluorescent cytoplasmic stain PKH26 (Sigma-Aldrich), and 1×10^6 cells suspended in 50 μL medium were injected under the surface of the left kidney capsule using a 30-gauge needle. Rats injected with medium (sham; n=4) or rBM-MSCs (n=9) were observed over a 3-week period, and rats injected with dif-

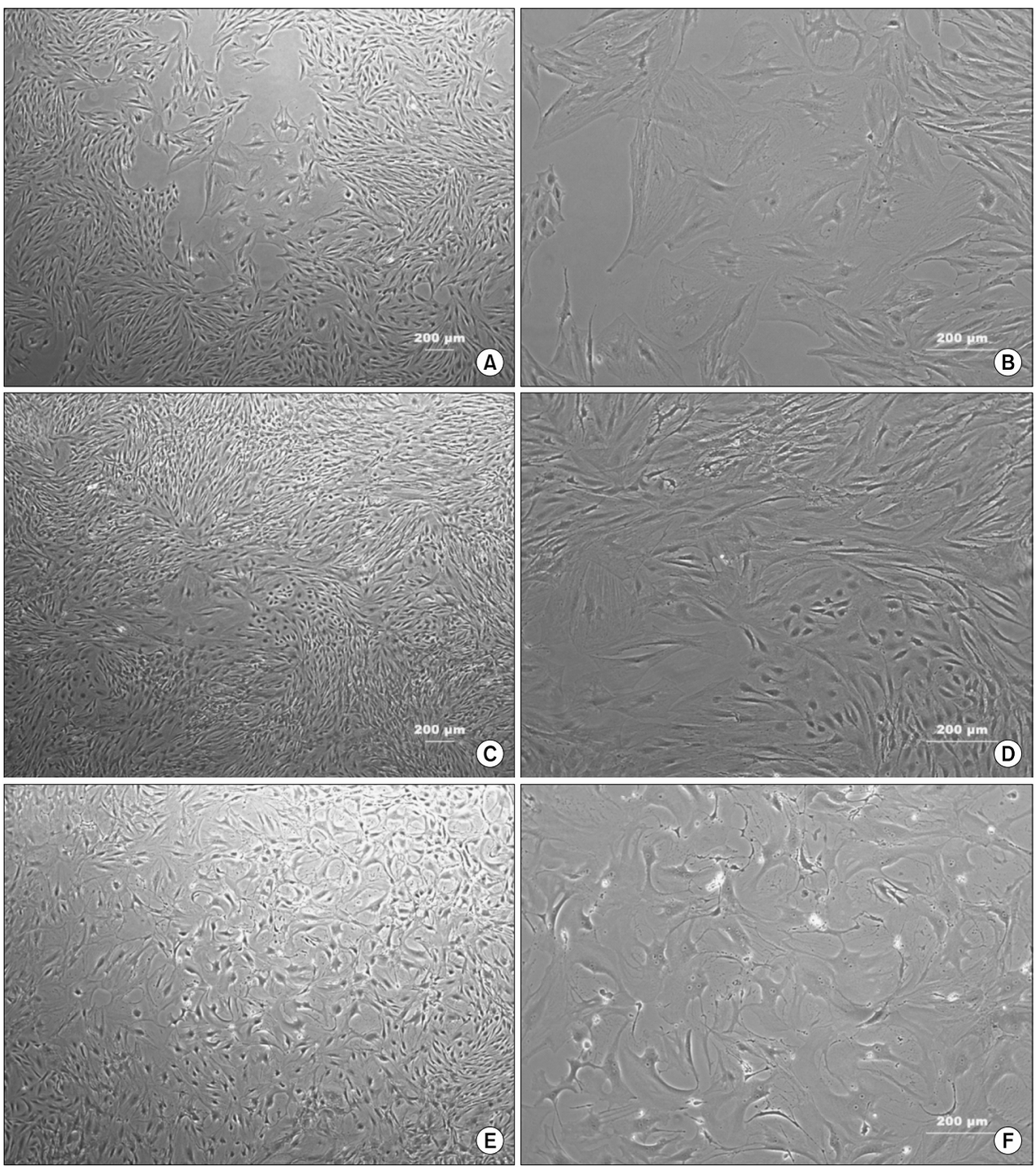

Fig. 2. Morphological changes of differentiated rat bone marrow-derived mesenchymal stem cells. (A, B) Week 1. (C, D) Week 2. (E, F) Week 3 (A, C, E: ×40; B, D, F: ×100).

ferentiated MSCs (n=8) were observed over an 8-week-period. Non-diabetic rats were observed as positive controls, and untreated STZ-induced rats were observed as negative controls. Glucose levels were checked every 3 days. Nephrectomy of the graft-bearing kidney was performed at the end of the observation period, and rats were monitored for an additional 6 days.

8. Immunohistochemistry

Removed graft-bearing kidneys were frozen in plastic molds filled with Tissue-Tek OC compound at −70°C and cryosected at a thickness of 6 μm. Slides were incubated in acetone for 10 minutes and then washed twice with distilled water on a shaking plate. ImmEdg Pen (Vector Laboratories, Burlingame, CA, USA) was applied to slides around the tissue prior to antibody binding. Tissue was incubated with insulin antibody (H86) diluted 1:200 at 4°C overnight. Slides were washed three times in PBS on a shaking plate for 5 minutes. Tissue was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Life Technologies) diluted 1:250 for 1 hour at room temperature. Slides were washed three times in PBS on a shaking plate for 5 minutes. Streptavidin (Dako, Glostrup, Denmark) was diluted 1:200 and reacted with the tissue for 1 hour. Slides were washed twice in PBS on a shaking plate for 5 minutes. The 3,3'-diaminobenzidine substrate kit (Vector Laboratories) was used to stain tissue sections following the manufacturer's instructions. Slides were then washed with distilled water twice for 5 minutes on a shaker, and Accustain Harris Hematoxylin Solution was applied for 1 minute as a counterstain. Slides were washed with flowing water followed by absolute alcohol and xylene and then cover-slipped with Permount (Fisher Scientific, Hampton, NH, USA). Tissue was observed using a fluorescence light microscope.

9. Statistical analysis

Quantitative results are expressed as mean±standard deviation. Paired t-tests were used to compare groups using GraphPad Prism 6 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was set at $P<0.05$.

RESULTS

1. Characterization of rBM-MSCs

rBM-derived cells were predominately positive for CD29 and CD90 (94.47% and 83.72%, respectively) (Fig. 1) but negative for CD34 and CD45 (both 0.02%). Prior to induction of differentiation, cells were spindle-shaped with typical MSC-like morphology (Fig. 2).

2. Morphology of differentiated rBM-MSCs

After RA treatment, morphological changes of rBM-MSCs were monitored on a weekly basis. rBM-MSCs showed considerable alterations in their appearance across weeks (Fig. 2). Cells typically exhibited polygonal or round shapes with radial nuclei.

3. Reverse-transcriptase PCR of differentiated rBM-MSCs

Primers were selected from representative markers from each step of pancreatic differentiation of rBM-MSCs: FoxA2, PDX-1, Ngn3, Pax4, and C-peptide. Primers for rBM-MSCs corresponded to those for hBM-MSCs: FoxA2, PDX-1,

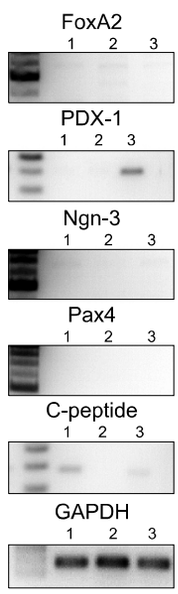

Fig. 3. Reverse-transcriptase polymerase chain reaction of rat bone marrow-derived mesenchymal stem cells (rBM-MSCs). Lanes 1 to 3: differentiated rBM-MSCs from week 1 to 3. Abbreviations: FoxA2, forkhead box A2; PDX-1, pancreatic-duodenal homeobox 1; Ngn3, neurogenin 3; Pax4, paired box gene 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

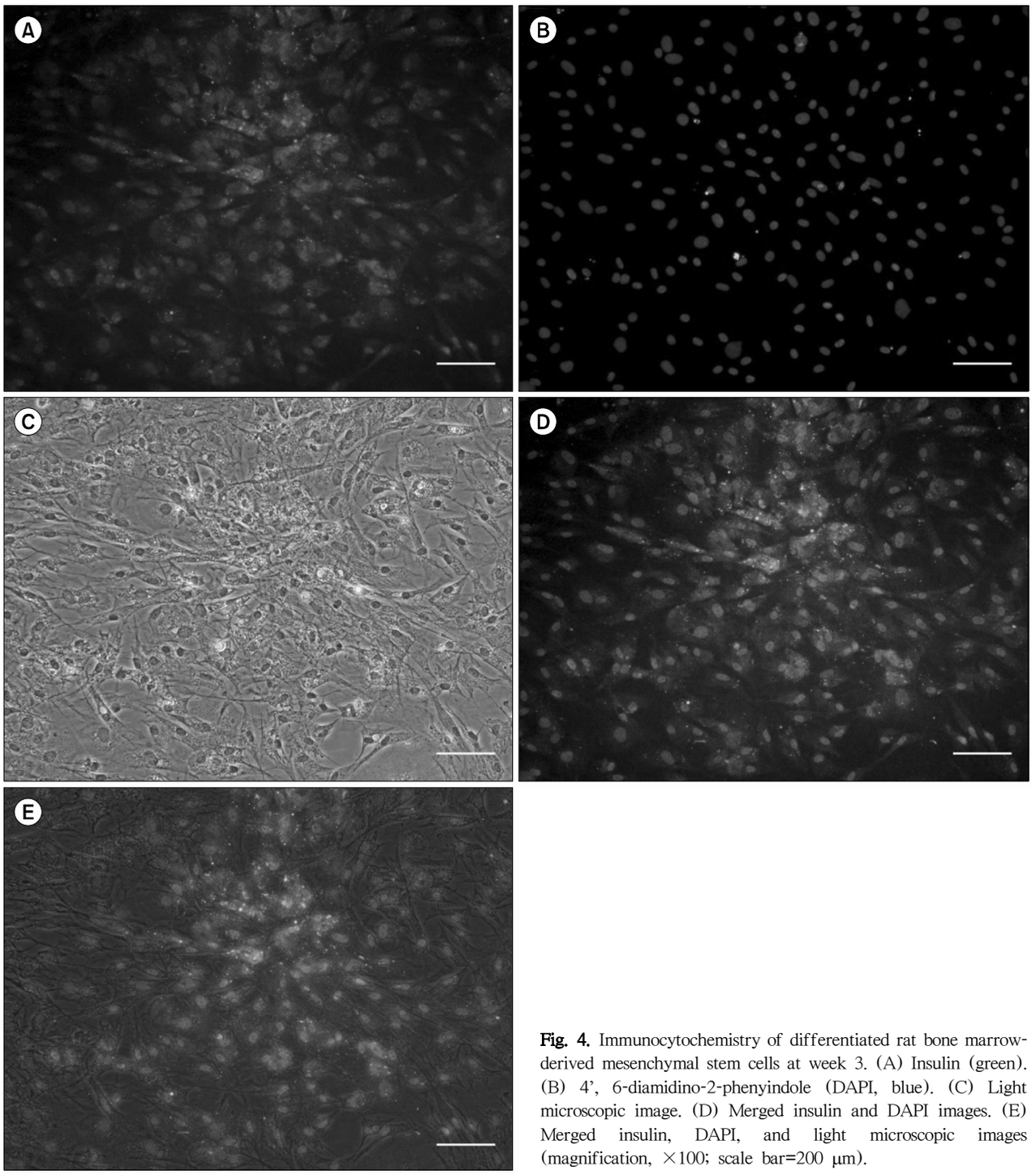

Fig. 4. Immunocytochemistry of differentiated rat bone marrow-derived mesenchymal stem cells at week 3. (A) Insulin (green). (B) 4', 6-diamidino-2-phenyindole (DAPI, blue). (C) Light microscopic image. (D) Merged insulin and DAPI images. (E) Merged insulin, DAPI, and light microscopic images (magnification, ×100; scale bar=200 μm).

Ngn3, Pax4, and C-peptide for rodents. The expression patterns of differentiated rBM-MSCs had no expression of Pax4, minimal expression of FoxA2 and Ngn3, and up-regulation of PDX-1 and C-peptide at week 3 (Fig. 3).

4. Qualitative and quantitative analysis of insulin expression in hBM- and rBM-MSCs

Differentiated rBM-MSCs were incubated with insulin primary antibody and labeled with green fluorescent CFL-488-conjugated secondary antibody. Insulin expression

was clearly observed in differentiated cells (Fig. 4).

ELISA revealed that differentiated rBM-MSCs showed a 2.063-fold increase in insulin secretion, which was significantly higher than that observed for MSCs cultivated in normal medium ($P<0.05$; Fig. 5).

5. Syngeneic graft of rBM-MSCs *in vivo*

Non-diabetic (+control) and STZ-injected rats (sham) exhibited obvious differences in blood glucose levels, with non-diabetic rats maintaining normoglycemia but diabetic rats exhibiting hyperglycemia (Fig. 6A). Diabetic rats that received normal MSCs showed no reduction of hyperglycemia across the 3-week observation period, whereas diabetic rats that received differentiated MSCs showed a modest decrease in blood glucose level (Fig. 7B). Linear regression analysis revealed that differentiated MSCs reduced blood glucose levels more rapidly than normal MSCs (slope for the differentiated MSC-injected group=-1.728 ± 0.7725 vs. slope for the normal MSC-injected=-0.4935 ± 0.5103) (Fig. 6D).

When we continued to observe differentiated MSC-injected rats for an additional 5 weeks, we found that blood glucose levels remained below 400 mg/dL throughout the entire observation period (Fig. 6C). Furthermore, rats exhibited a rise in blood glucose levels after nephrectomy of the grafted kidney.

6. Histological observation of grafted rBM-MSCs

rBM-MSCs were labeled with red fluorescent PKH26 prior to their injection. Three weeks after injection, we observed labeled cells mostly along the membrane near the injection site, with some cells partially migrating into the kidney (Fig. 8). Insulin- and hematoxylin-stained images show that grafted cells successfully survived along the inner surface of the tissue membrane (Fig. 7).

DISCUSSION

The generation of insulin-producing β-cell surrogates from progenitor/stem cells *in vitro* has remarkable potential for a cell-based therapy for T1DM(13,17-21). However, the *in vivo* therapeutic success of mature β-cell alternatives has yet to be demonstrated. BM-MSCs exhibit great capacities for differentiation and plasticity. Furthermore, they may be an ideal cell type for clinical use because they are more accessible for harvesting and adaptable to new environments compared with pancreatic islets(17). Over the past several years, we have genetically modified cells for T1DM treatment and established insulin-producing cells *in vitro*(25,26). However, two major obstacles we encountered were low transfection efficiency and insufficient viability of cells to establish populations for *in vivo* trials. Therefore, in the present study, we focused on enhancing differentiation of cells *in vitro* while endeavoring to reduce the use of cytotoxic materials and/or growth factors.

Three non-toxic reagents were used to induce β-cell differentiation of cultivated MSCs: nicotinamide, L-glutamine, and RA. Nicotinamide, also known as vitamin B_3, has been reported to induce endocrine differentiation in pancreatic cells(27). Also, several groups report that L-glutamine induces stem cells to become functional endoderm in humans(28). Moreover, several groups show that RA receptor signaling is required for the development of pancreatic progenitor cells(29,30). As growth factors may induce uncontrollable cellular activities *in vivo* such as oncogenesis(31), we eliminated growth factors from the cultivation medium and attempted to use a limited number of reagents to achieve sufficient β-cell differentiation.

RA, in particular, is an essential substance for β-cell differentiation and is closely associated with expression of PDX-1(29,32). PDX-1, a marker of early pancreatic pro-

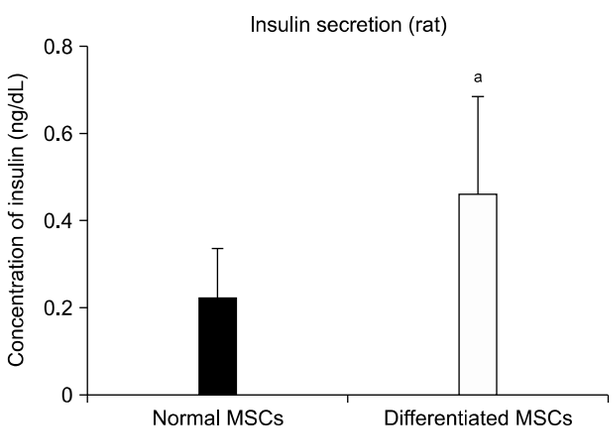

Fig. 5. Insulin secretion from differentiated rat bone marrow-derived mesenchymal stem cells (MSCs) measured by enzyme-linked Immunosorbent Assay at week 3 (n=9). [a]$P<0.05$.

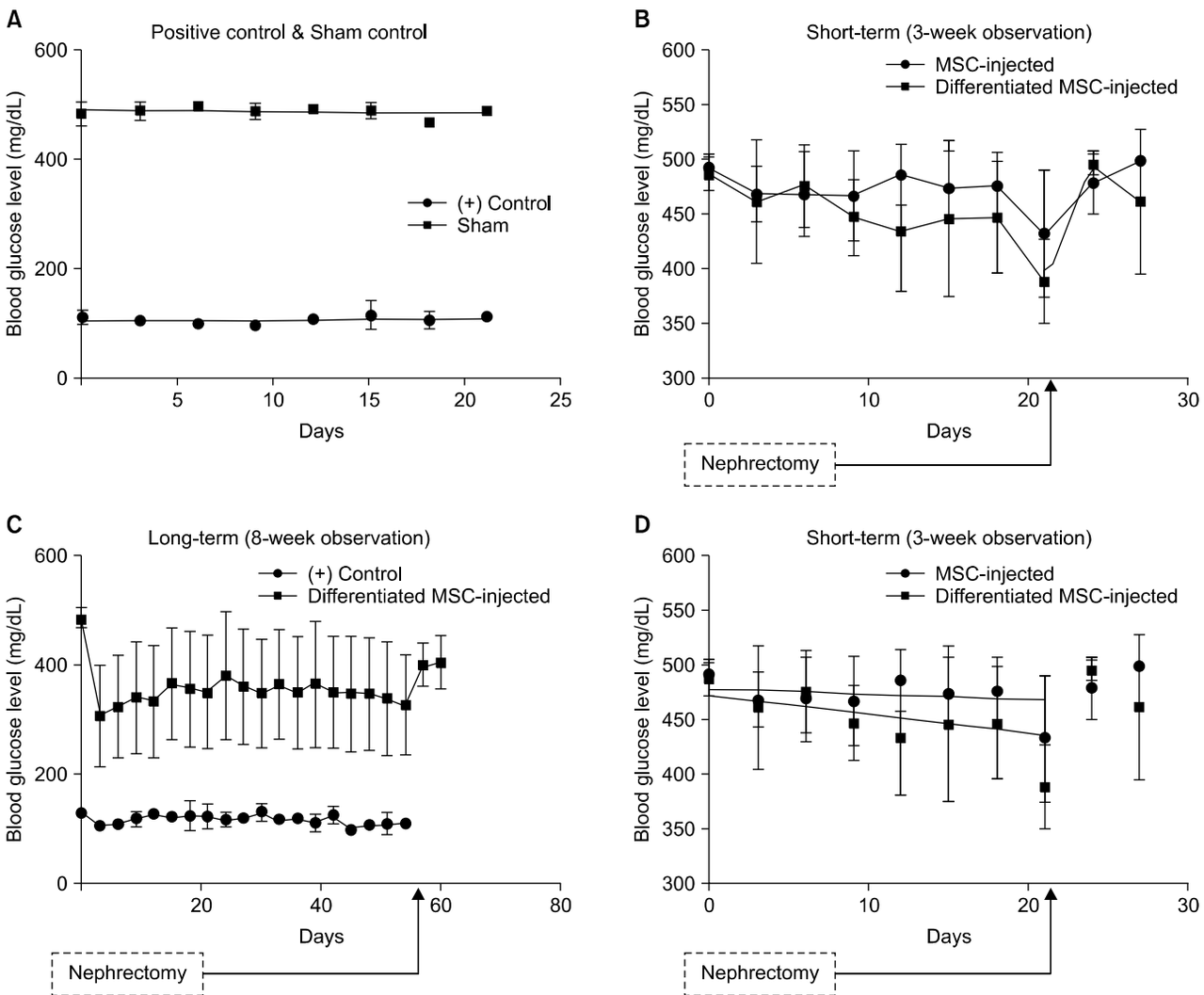

Fig. 6. Blood glucose levels of rats observed for 3 (A, B) or 8 (C) weeks. (A) Positive control (n=4) and sham control (n=4). (B) Blood glucose level of normal mesenchymal stem cell (MSC)-injected (n=9) and differentiated MSC-injected (n=8) rats. (C) Positive control (n=4) and differentiated MSC-injected (n=8) rats. (D) Linear regression of blood glucose levels from MSC-injected (n=9) and differentiated MSC-injected (n=8) rats. Slope for MSC-injected group=−0.4935±0.5103; slope for differentiated MSC-injected group=−1.728±0.7725.

genitor cells and mature β-cells, is a well-established sign of β-cell differentiation(32-34), and overexpression of PDX-1 alone may induce differentiation of MSCs into insulin-producing cells that are responsive to glucose stimulation(17). Consistent with these findings, our reverse-transcriptase PCR and ELISA results show that PDX-1 expressed with insulin secretion (Fig. 5) in rat (Fig. 3) MSCs *in vitro*. As Ostrom et al.(30) reports, RA is at least partly required for the development of dorsal pancreatic mesenchyme during early pancreas development. Our results show that MSCs from rats secreted insulin (Fig. 5) and express C-peptide (Fig. 3). Nevertheless, we conclude that these cells cannot be considered as β-cells but only as candidates with potential. Limitations clearly remain, which we are seriously taking into account to resolve in the next study.

T1DM is primarily an autoimmune disease, and, as a consequence, graft function progressively declines due to the loss of functional islets during the early post-transplantation period(35). Therefore, the present study focused on MSCs, which could play a major role in tissue regeneration through localized immune-suppressive effects. To increase the functional activity of MSCs *in vivo*, we induced their differ-

entiation by culturing MSCs in a customized differentiation medium for 3 weeks before their graft into the left kidney capsule of rats. Differentiated MSCs were modestly more effective than undifferentiated cells in reducing blood glucose levels (Fig. 6), and differentiated MSCs successfully survived in the kidney for at least 3 weeks (Fig. 7, 8).

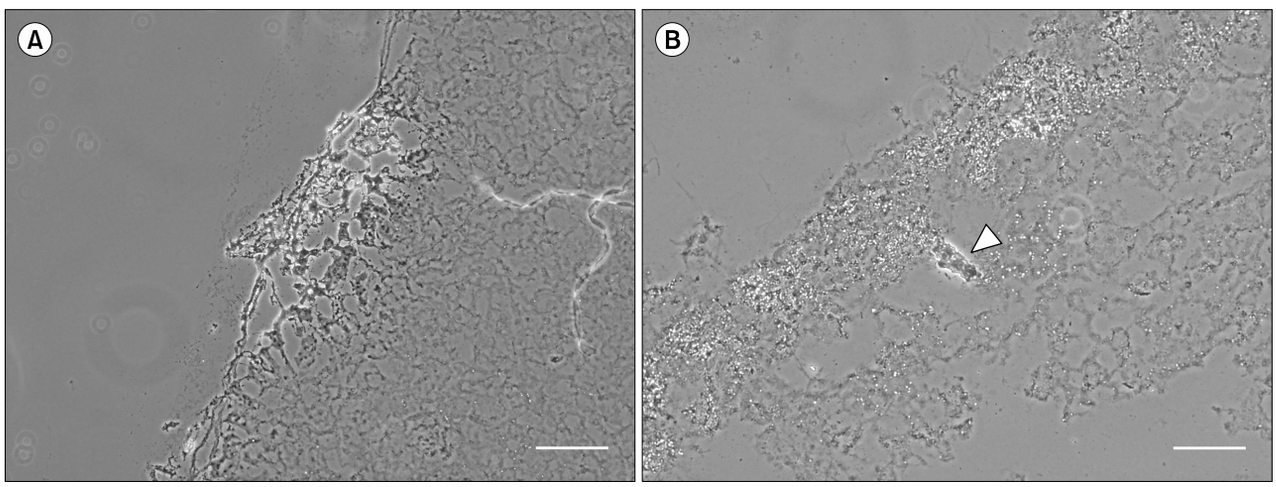

Fig. 7. 3,3'-Diaminobenzidine-stained tissue. (A) Cells located along the inner surface of the tissue membrane. (B) Cell that migrated a short distance into the tissue (marked by yellow arrowhead) (magnification: ×100; scale bar=200 μm).

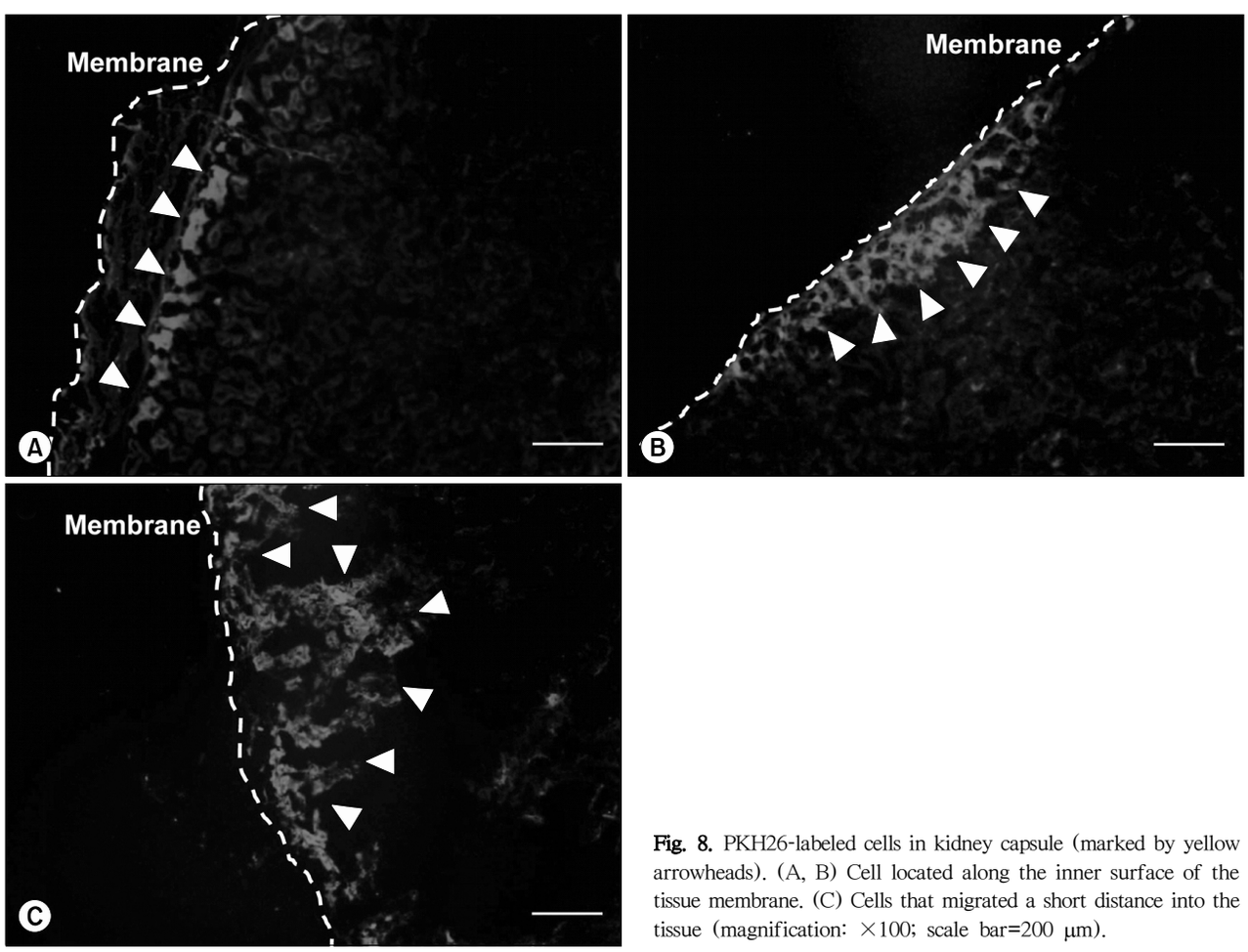

Fig. 8. PKH26-labeled cells in kidney capsule (marked by yellow arrowheads). (A, B) Cell located along the inner surface of the tissue membrane. (C) Cells that migrated a short distance into the tissue (magnification: ×100; scale bar=200 μm).

Furthermore, after nephrectomy of the grafted kidney, rats exhibited a return of high blood glucose levels. Although T1DM is an acute disease, we assume that grafted MSCs may enable chronic recovery, aiding in secretion of insulin.

Several research groups report that RA can regulate the commitment of embryonic stem cells to establish pancreatic endoderm(29,30,36). However, detailed *in vivo* evidence of recovered function does not exist, and there has been no attempt to use MSCs, which entail fewer ethical concerns and are more clinically applicable. Thus, the results of the present study are compelling, as they clearly show a reduction in blood glucose levels and the survival of MSCs 3 weeks after injection. We conclude that RA-induced β-cell differentiation of BM-MSCs may have great therapeutic potential for T1DM.

One may argue that the sustained reduction of blood glucose levels in the present study was still too high to be considered normoglycemia. However, we find this data to be meaningful because only a very low number of cells (1× 106 cells per rat) were injected into rats. Increasing the number of injected cells could be explored in future studies as a way to improve the effectiveness of this potential therapeutic tool. Furthermore, diabetic rats initially exhibited excessively high levels of blood glucose, (i.e., typically above 500 mg/dL), meaning that the observed reduction in blood glucose levels could be considered evidence that the differentiated cells aided in the recovery of insulin secretion. It is also noteworthy that blood glucose level appeared to be continuously regulated, without a single instance of a rapid return to hyperglycemia throughout both the short- and long-term observation periods.

CONCLUSION

In summary, we showed that RA can induce the differentiation of rBM-MSCs into the β-cell lineage, resulting in cells that have the ability to secrete insulin. Furthermore, the success of grafted MSCs in a rat model of diabetes suggests that MSCs can potentially serve as β-cell surrogates within therapies for T1DM.

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