A New 2-Step Acceleration Protocol Using a Histone Deacetylase Inhibitor to Generate Insulin-Producing Cells From Adipose-Derived Mesenchymal Stem Cells

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Objectives: We aimed to develop a simple protocol for deriving insulin-producing cells (IPCs) from adipose-derived mesenchymal stem cells (ADSCs). We established a 2-step creation method and an acceleration strategy with a histone deacetylase inhibitor that promoted a pro–endocrine pancreatic lineage.

Methods: We seeded ADSCs in 96-well dishes and cultured in Dulbecco's modified Eagle's medium/F12 medium containing 1% fetal bovine serum, 1% B27 supplement, 1% N2 supplement, 50-ng/mL human activin A, and 10-nM exendin-4 for step 1 of differentiation (7 days). Then 10-mM nicotinamide and 50-ng/mL human hepatocyte growth factor, with or without 1 mM histone deacetylase inhibitor, were added for step 2 of differentiation (14 days). After the 2-step differentiation was complete, cell morphology, immunohistochemistry, messenger RNA expression, and function were investigated.

Results: Our new differentiation protocol with the histone deacetylase inhibitor significantly accelerated IPC differentiation compared with the conventional protocol without the histone deacetylase inhibitor (median, 21.6 vs 38.8 days; $P < 0.05$). It also improved the islet morphology score ($P < 0.05$) and the glucose stimulation index (3.1).

Conclusions: By applying our new and easy 2-step protocol using a histone deacetylase inhibitor, ADSCs may be an effective cell source for differentiation of IPCs.

Key Words: insulin-producing cell (IPC), adipose-derived mesenchymal stem cell (ADSC), valproic acid (VA), differentiation protocol, regenerative medicine

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I slet transplantation (ITx) is one of the therapeutic options for type-1 diabetes mellitus and is now used clinically in the United States and Canada.[1] However, ITx has some urgent issues that need to be addressed before wide-spread clinical use, including the requirement of a large islet yield[2] and severe donor shortages in some countries, such as Japan.[3] Islet shipping or changes of the allocation system may help to solve these issues; however, ethical and governmental controversies still remain. Therefore, development of a new insulin-producing cell source is required.

To solve these issues, several options have been considered and we have focused on stem cells for creating insulin-producing cells (IPCs), especially adipose-derived mesenchymal stem cells (ADSCs), as a new cell source.[4,5] The reason we selected this cell type is that ADSCs can be obtained from the patient's own fat tissue with only a mildly invasive procedure and auto-ADSC transplantation has fewer ethical problems compared with the use of induced pluripotent stem or embryonic stem cells. Furthermore, induced pluripotent stem cells can have DNA damage that causes transformation and carcinogenesis.[6] Indeed, ADSCs have already been clinically applied for repair of damage after surgery for breast cancer and regeneration of cardiovascular cells after acute myocardial infarction.[7,8] Moreover, some reports indicate that ADSCs show superior differentiation into various cell types,[9] leading us to select this cell source for our study.

We have also investigated the role of histone deacetylases (HDACs) in cancer[10] and in transplantation.[11] Briefly HDACs, in conjunction with histone acetyltransferases, control the level of acetylation on lysine residues in histones. Treatment of cells with an HDAC inhibitor (HDACi) such as trichostatin A or suberoylanilide hydroxamic acid leads to hyperacetylation of histones, resulting in a more open chromatin architecture and increased access for transcription factors.[12] Histone deacetylases inhibition regulates gene expression as well as the functions of more than 50 transcription factors and nonhistone proteins,[13] and we have used HDAC inhibition to accelerate cancer sphere formation. Furthermore, HDAC inhibition is reported to be strong driver of pancreatic cell lineage progenitors.[14] Therefore, we investigated the use of an HDACi (valproic acid) for acceleration of IPC formation. Here, we established a new 2-step method for IPC production and an acceleration strategy with an HDACi that promotes the pro–endocrine pancreatic lineage.

MATERIALS AND METHODS

Adipose-Derived Mesenchymal Stem Cell Preparation (Step 1)

The protocols for creating IPCs are based on D'Amour et al,[15] and we modified that protocol into a 2-step differentiation protocol. We purchased ADSCs (RAWMD-01001) from Cyagen Biosciences Inc (Santa Clara, Calif), and the ADSCs were cultured according to the manufacturer's guidelines. After thawing using established procedures, cells were cultured until passages 5 and 6, then 5×10^5 cells were seeded into 12-well ultra-low attachment plates (Sigma-Aldrich Japan Co, LLC, Tokyo, Japan). For step 1, cells were cultured for 7 days in a differentiation cocktail of Dulbecco's modified Eagle's medium/F12 (Thermo Fisher Scientific Inc, Waltham, Mass), 1% fetal bovine serum, 1% B27 supplement (Thermo Fisher Scientific Inc), 1% N2 supplement (Thermo Fisher Scientific Inc), 50-ng/mL activin A (Sigma-Aldrich Japan), and 10-nM exendin-4 (Sigma-Aldrich Japan), then these cells were subjected to step 2 (IPC differentiation phase, Fig. 1).
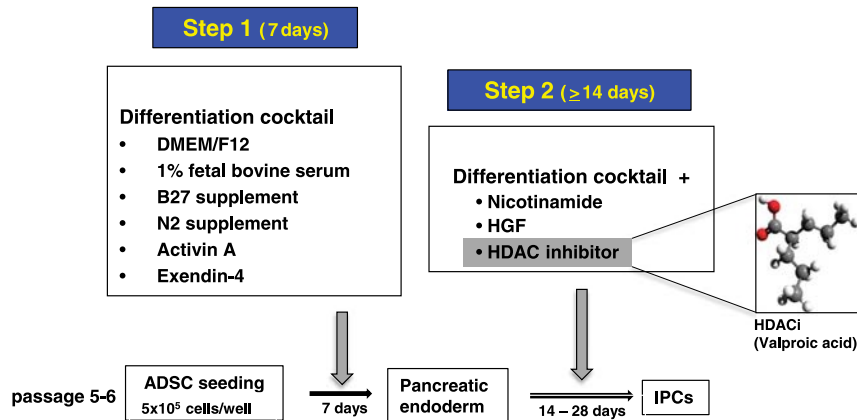
New 2-step protocol

FIGURE 1. The scheme of the new 2-step protocol for generating IPCs from ADSCs.

Insulin-Producing Cell Differentiation (Step 2)

Briefly, in step 1 undifferentiated ADSC spheres were generated and in step 2, differentiated pancreatic endoderm was derived by culturing in the same differentiation medium as above, but with the addition of 50-ng/mL hepatocyte growth factor (HGF; Funakoshi Co, Ltd, Tokyo, Japan) and 10-mM nicotinamide (Sigma-Aldrich Japan), with or without HDACi (valproic acid; Fig. 1).

Cell Counts, Purity, and Viability

Cell number, purity, and viability were determined as described previously.[2] Briefly, at least 3 samples of the generated cells were assessed for cell number and purity by staining with dithizone dye (Sigma-Aldrich Japan) dissolved in dimethyl sulfoxide (Burdick and Jackson, Morristown, NJ) and a propidium iodide (PI) staining kit (TAKARA Bio Inc, Tokyo, Japan), and assessment by fluorescence microscopy.

Morphological Analysis

We assessed morphological changes of IPCs using a morphological score according to islet status. Insulin-producing cells were scored for shape (flat vs spherical), border (irregular vs well-rounded), integrity (fragmented vs solid/compact), uniformity of stain (not uniform vs perfectly uniform), and diameter (all < 100 μm vs more than 10% > 200 μm), as previously described.[16,17]

Immunohistochemical Staining

The cells were pelleted by centrifugation and incubated with primary antibody against insulin (aa287-299, LS-B129; LifeSpan BioSciences, Inc, Seattle, Wash) at a dilution of 1:100 in phosphate-buffered saline for 1 hour at room temperature. Then cells were incubated with biotinylated secondary antibody, followed by a streptavidin-biotin-horseradish peroxidase complex. Finally, positive staining was visualized with diaminobenzidine and cell nuclei were counterstained with Mayer's hematoxylin.

Quantitative Reverse Transcription-Polymerase Chain Reaction for Messenger RNA Expression

We investigated the gene expression profile of the transcription factor NeuroD1 and the endocrine hormone insulin as we reported previously.[18] Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and complementary DNA was synthesized from 2.5 μg of total RNA by reverse transcription using a SuperScript RT kit (Promega, Madison, Wis),

following the manufacturer's instructions. Then, quantitative reverse transcription—polymerase chain reaction was performed using the Applied Biosystems 7500 real-time polymerase chain reaction system, TaqMan Gene Expression Assays on demand, and a TaqMan Universal Master Mix (gene-specific TaqMan probes on a StepOne Plus; Applied Biosystems, Foster City, Calif). NeuroD1 (NM_019218) and Insulin1 (NM_019129) primers (Qiagen) were used. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for normalization. Expression levels of all genes were calculated as a ratio to glyceraldehyde-3-phosphate dehydrogenase. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1 (Thermo Fisher Scientific Inc).

Glucose-Stimulated Insulin Secretion

The glucose stimulation index (SI) of IPCs was calculated as previously described.[19] First, IPCs were cultured in RPMI-1640 medium with 5-mM glucose for 1 hour and then in medium containing 45-mM glucose for 1 hour, before being cultured in medium containing 5-mM glucose for an additional 1 hour. The insulin concentration of the supernatant was analyzed by enzyme-linked immunosorbent assay (AKRIN-011H, Shibayagi, Japan), with a microplate reader at a wavelength of 450 nm. Total DNA was extracted to determine the cell count. Then SI was calculated by dividing the amount of insulin secretion from the high-glucose incubation by the insulin secretion from the low-glucose incubation. Three independent experiments were undergone for this calculation.

Statistics

Descriptive statistics were presented as mean (standard deviation), median with range for quantitative variables and number (percentages) for qualitative variables. Univariate analysis was performed by using 1-way analysis of variation, paired and unpaired *t*-tests, Scheffe's test, or the Log-rank test, as appropriate. $P < 0.05$ was considered statistically significant, and all *P* values reported were 2-sided. All analyses were performed with State Mate III version 3.14 (ATMS Co, LTD, Tokyo, Japan) for Windows.

RESULTS

The 2-Step Protocol Generated Cells With IPC Morphology

Morphological changes from ADSCs to IPCs (Fig. 2A) were examined at day 14 (Fig. 2B), at day 35 (Fig. 2C), and at
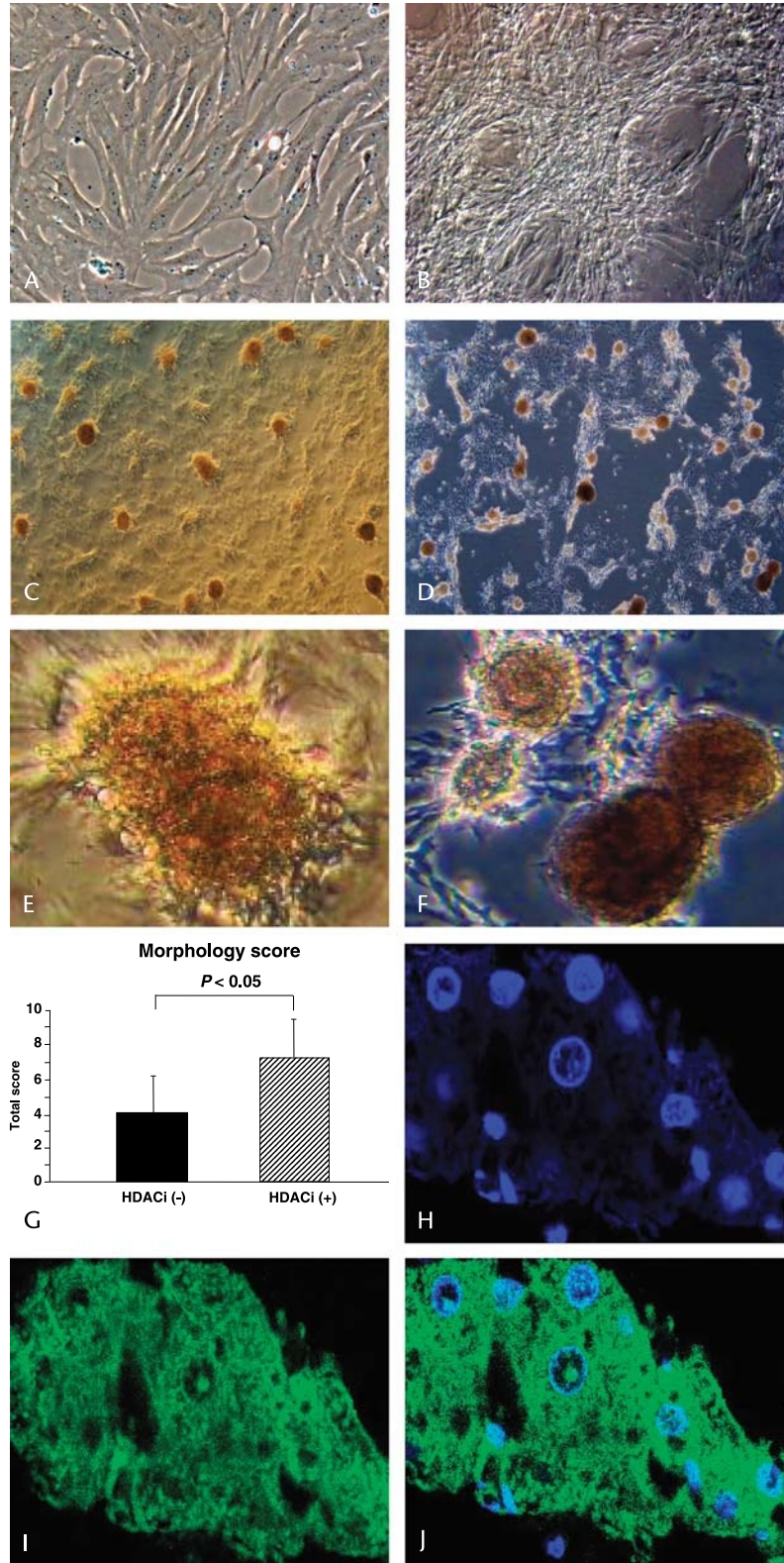
FIGURE 2. Morphological changes from ADSCs to IPCs. A, ADSC, not differentiated; B, at 14 days; C, at 35 days; D, at 90 days of differentiation; E, dithizone staining at day 35, original magnification × 400, high power field; F, dithizone staining at day 90, original magnification × 400, high power field. G, cell morphological scores were significantly higher when we added HDACi (unpaired *t*-test, *P* < 0.05). H–J, the differentiated IPCs with this 2-step protocol using HDACi retained good cell viability and function, as shown by nuclear, cytoplasmic (insulin) staining and the marge image, respectively.

day 90 (Fig. 2D). Islet-like cell formations were confirmed at around day 14 (Fig. 2B, not stained), and these cells strongly stained with dithizone at day 35 (Fig. 2E, [HPF]) and at day 90 (Fig. 1F: HPF). Then we compared morphology of IPCs cultured and differentiated with or without HDACi. Cell morphological scores were significantly higher when HDACi was added ($P < 0.05$, Fig. 2G).

Immunohistochemical Staining Showed that Viable IPCs Secreted Insulin

Insulin-producing cells differentiated with this 2-step protocol including HDACi showed good cell viability, as determined by nuclear staining (Fig. 2H). Moreover, their cytoplasm was well stained by an anti-insulin antibody (Figs. 2I, J).
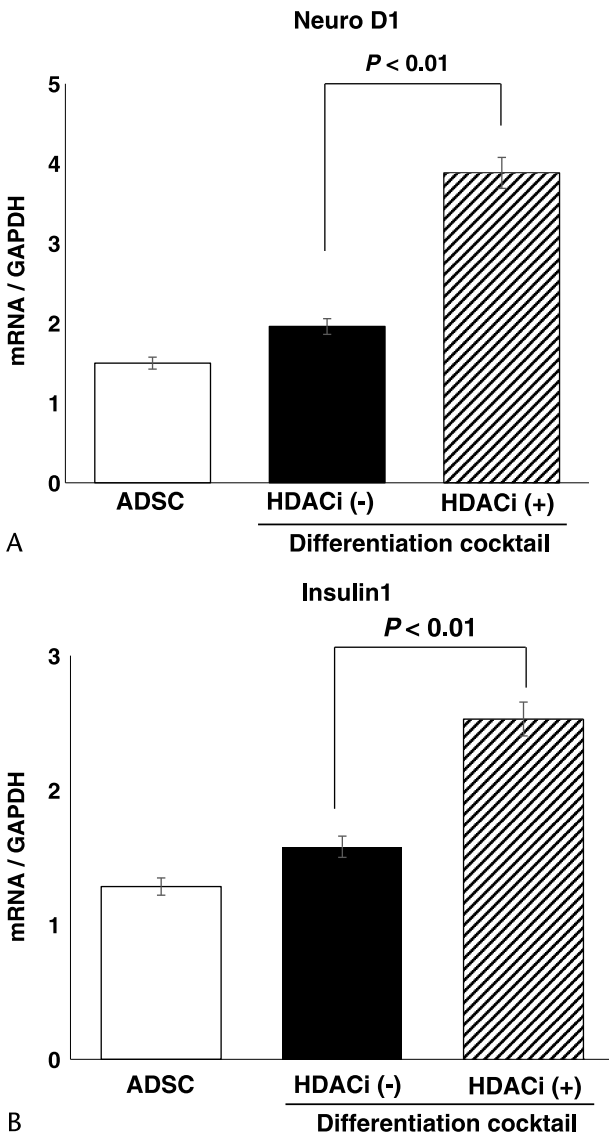
Insulin and NeuroD Messenger RNA Expression by IPCs

The messenger RNA (mRNA) expression of NeuroD significantly increased when ADSCs were differentiated with HDACi compared with cells without HDACi or the normal control (Fig. 3A), as did that of the insulin gene INS1 (Fig. 3B).

The 2-Step Protocol With HDACi Accelerated the Differentiation of IPCs

We defined the completion of differentiation as the time when cells were strongly stained with Dithizone. Differentiation completion (differentiation culture duration) was compared between IPCs cultured and differentiated with or without HDACi. The duration was significantly shortened when HDACi was added to the culture medium compared with no HDACi (median, 21.6 vs 38.8 days; $P < 0.05$; Fig. 4).

The Generated IPCs Showed a Good Glucose SI

The insulin concentration in the supernatant of the IPCs at day 28 significantly increased when these cells were incubated in "low" glucose medium (5 mM, $P < 0.05$) and when moved to "high" glucose medium (45 mM, $P < 0.01$). The final glucose SI was 3.2 (Fig. 5).

DISCUSSION

Islet transplantation (ITx) is a useful option for treatment of type-1 diabetes mellitus, which was refined by Shapiro et al[20]; however, these authors also noted that ITx has limitations. One of them is the low rate of insulin independence after a single ITx; in one study, after 5 years, only 9% of patients had achieved insulin-free status.[21] Thus, repeated ITx was recommended to

FIGURE 3. The gene expression profile of the transcription factor NeuroD1 and the endocrine hormone insulin. A, mRNA expression of NeuroD in controls and differentiated ADSCs without and with HDACi at step 2 of differentiation. B, mRNA expression of INS1 compared with controls and differentiated ADSCs without and with HDACi at step 2 of differentiation.
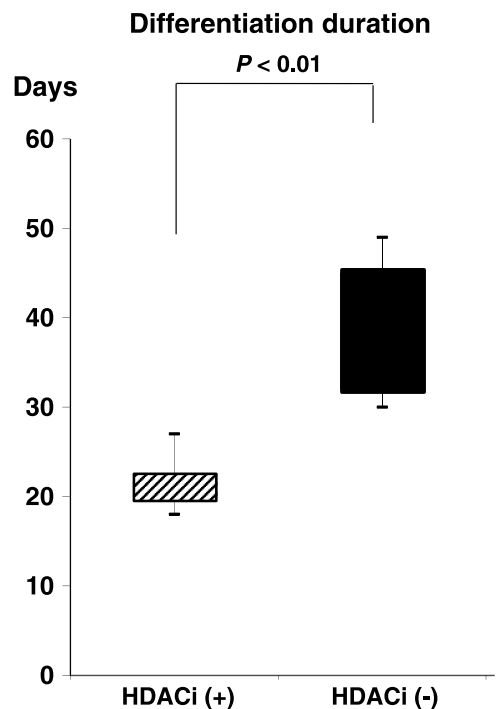

FIGURE 4. Differentiation completion time. The total duration of differentiation from ADSCs to mature IPCs was significantly shortened when HDACi was added to the culture medium (median, 21.6 vs 38.8 days; unpaired *t*-test, $P < 0.05$).
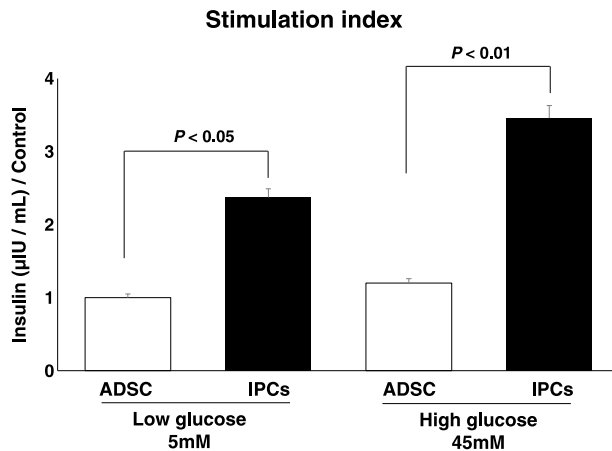
Stimulation index

FIGURE 5. Glucose SI. The insulin concentration in the supernatant of IPCs at day 28 significantly increased when these cells were put in "low" glucose medium (5 mM; paired *t*-test, $P < 0.05$) and when moved into "high" glucose medium (45 mM; paired *t*-test, $P < 0.01$).

increase the possibility of achieving insulin-free status. However, although enough cadaveric donors are available for multidonor–one-recipient ITx in North America or some countries in Europe, it is very difficult to find enough cadaveric donors for ITx in counties such as Japan, even though the Japanese law regarding transplantation dramatically changed in 2007. Thus, transplant surgeons in such countries have struggled to find a new cell source. Consequently, we focused on mesenchymal stem cells that can be used for autotransplantation.

Among mesenchymal stem cells, we focused on ADSCs because they have some advantages as a cell source, such as significant potential as stem cells, better yield compared with bone marrow, less invasive procurement, and no ethical issues for autotransplantation when clinical application is considered. However, even though ADSCs have these advantages, some urgent issues need to be solved before clinical application. The differentiation protocol is very complex; culture duration exceeds 30 days, and the cell expansion rate and actual cell function as IPCs are extremely poor. Thus, we developed a new 2-step differentiation protocol for generating IPCs that solves some of the issues regarding differentiation duration and poor functional ability. However, some issues remain to be resolved, including IPC function and long term fate in vivo after transplantation. We have already commenced in vivo research to address these issues (data not shown).

In conclusion, ADSCs may be an effective cell source for generating functional IPCs through our new established 2-step protocol using an HDACi that promotes cells of the pancreatic lineage. This strategy may provide a breakthrough for clinical ITx in countries that are affected by severe donor shortage.

ACKNOWLEDGMENTS

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