Cardiotrophic Growth Factor–Driven Induction of Human Muse Cells Into Cardiomyocyte-Like Phenotype

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Abstract
Multilineage-differentiating stress-enduring (Muse) cells are endogenous nontumorigenic stem cells collectable as stage-specific embryonic antigen 3 (SSEA-3)+ from various organs including the bone marrow and are pluripotent-like. The potential of human bone marrow-derived Muse cells to commit to cardiac lineage cells was evaluated. We found that (1) initial treatment of Muse cells with 5'-azacytidine in suspension culture successfully accelerated demethylation of cardiac marker Nkx2.5 promoter; (2) then transferring the cells onto adherent culture and treatment with early cardiac differentiation factors including wingless-int (Wnt)-3a, bone morphogenetic proteins (BMP)-2/4, and transforming growth factor (TGF) β1; and (3) further treatment with late cardiac differentiation cytokines including cardiotrophin-1 converted Muse cells into cardiomyocyte-like cells that expressed α-actinin and troponin-I with a striation-like pattern. MLC2a expression in the final step suggested differentiation of the cells into an atrial subtype. MLC2v, a marker for a mature ventricular subtype, was expressed when cells were treated with Dickkopf-related protein 1 (DKK-1) and Noggin, inhibitors of Wnt3a and BMP-4, respectively, between steps (2) and (3). None of the steps included exogenous gene transfection, making induced cells feasible for future clinical application.

Keywords
cardiomyocyte, cardiotrophin-1, suspension culture, nontumorigenic cells, pluripotency

Introduction
Cardiovascular disorders are the leading cause of death of noncommunicable diseases, and cell therapy is becoming one of the trends for treatment in this field. The most general stem cells for clinical use might be mesenchymal stem cells (MSCs) because of their easy accessibility and safety. They show a broad spectrum of differentiation potentials across oligolineage boundaries between mesodermal and endodermal, as well as ectodermal lineages whereas the differentiation rate is usually not high, suggesting that a small subpopulation of MSCs are playing a role in this transdifferentiation. MSCs are a heterogeneous population because they are usually collected simply as adherent cells, and a universal surface antigen for their isolation has not been identified.

Within the MSC population, we previously identified multilineage-differentiating stress-enduring (Muse) cells as considerable percentage of total MSCs, which can be isolated as cells positive for a pluripotent stem cell surface marker: stage-specific embryonic antigen (SSEA)-3. They can generate cells representative of all 3 major lineages from a single cell, self-renew, express pluripotency genes, and show triploblastic differentiation, suggesting their pluripotency. Various reports have shown that Muse cells can differentiate into ectodermal cells such as melanocytes, epidermal cells, and neuronal cells; endodermal cells represented by hepatocytes and cholangiocytes; and into mesodermal cells such as osteocytes, adipocytes, and skeletal muscle cells. Adipose-derived Muse cells have also shown spontaneous triploblastic differentiation ability.
Notably, cells negative for SSEA-3 among MSCs, namely, non-Muse cells that correspond to the vast majority of MSCs, do not show such a broad spectrum of differentiation. Promoter regions of octamer-binding transcription factor 3/4 (Oct3/4) and Nanog are highly methylated in non-Muse cells as compared to those of Muse cells, and accordingly, gene expression levels relevant to pluripotency are substantially lower than those in Muse cells. Furthermore, non-Muse cells are not capable of self-renewal, nor do they show differentiation into ectodermal or endodermal lineages even in the presence of appropriate induction cytokines; these are stark differences between Muse and non-Muse cells. Moreover, Muse cells have shown an anti-inflammatory effect by down-regulating tumor necrosis factor-α (TNF) and immunomodulatory effects via spontaneously expressing transforming growth factor β (TGF-β1). For these reasons, Muse cells are suggested to be a better material for generation of cells for transplantation therapy than a crude MSC population.

Cardiomyocytes are terminally differentiated cells that lack the ability to proliferate. They have a unique cytoskeleton containing several contractile proteins represented by actin, myosin, titin, troponin, and α-actinin that are arranged into sarcomeres. In addition, intercellular connections through gap junction channels, mainly composed of connexin 43, allow cardiomyocytes to couple electrochemically and to contract synchronously. Various kinds of factors and signaling pathways are known to regulate cardiac differentiation during normal development. Bone morphogenetic proteins (BMPs), wingless-int (Wnt), and TGF-β families are key factors that regulate cardiogenesis in a context-dependent manner. In addition, basic fibroblast growth factor (bFGF) participates in the orchestration of cardiac differentiation. Cardiotrophin-1—member of the interleukin (IL)-6 family—was originally identified as a marker of cardiac hypertrophy, but later it was found to be cardioprotective and is even known to induce differentiation toward the cardiac lineage in MSCs and embryonic stem cells (ESCs). MSCs have been studied for possible regeneration of damaged areas in acute heart conditions via a transplant or intravenous injection. In chronic conditions, however, there may be less chance for stem cells to differentiate into target cells due to a severely hostile microenvironment and damage to the built-in regenerative system. In such a case, injection or transplant of already differentiated stem cell–derived cardiomyocytes may be beneficial. In the present article, we explored the potential of Muse cells to differentiate in vitro into cells with cardiomyocyte characteristics by means of a combination of suspension culture and multistep cytokine induction.

**Materials and Methods**

**Fluorescence-Activated Cell Sorting (FACS) Isolation of SSEA-3+ Muse Cells**

Human bone marrow-derived MSCs (BM-MSCs) were purchased from Lonza. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) low glucose (10567-014; Gibco, Grand Island, NY, USA) containing 10% of fetal bovine serum (FBS) and 2 ng/μL bFGF (Wako, Saitama, Japan). We stained MSCs (from passage 1 to passage 4) with an anti-SSEA-3 IgM antibody (1:100 dilution; Millipore, Billerica, MA, USA) and stained with a secondary antibody: a fluorescein isothiocyanate-conjugated antirat IgM antibody (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as described previously. The SSEA-3+ fraction was sorted by means of a BD FACS Aria™ II cell sorter (Becton Dickinson, San Jose, CA, USA). The sorted fraction was cultured overnight with DMEM low glucose containing 10% of FBS and 2 ng/μL bFGF and then was used for cardiomyocyte induction.

**Cell Culture and Cytokine Cocktails for Induction**

Muse cells were divided into 3 groups and were subjected to each induction scheme as described in Fig. 1.

**Adherent group.** Muse cells were plated at 1.55 × 10⁶/cm² and cultured in the adherent state for the entire period of induction on a laminin-coated surface. The cells were first incubated with DMEM low glucose containing 2% of FBS plus TGF-β1 (2.5 ng/mL, Wako), BMP-4 (5 ng/mL, Wako), BMP-2 (5 ng/mL, Sigma-Aldrich), activin A (10 ng/mL, Wako), Wnt-3a (50 ng/mL, R&D Systems, Minneapolis, MN, USA), and bFGF (10 ng/mL, Wako) for 7 d and then for 2 wk with DMEM low glucose containing 2% of FBS plus TGF-β1, insulin-like growth factor-1 (IGF-1, 5 ng/mL, Sigma-Aldrich), hepatocyte growth factor (HGF; 20 ng/mL, Wako), and cardiotrophin-1 (CT-1; 200 ng/mL, Sigma-Aldrich).

**Sus+Ad group.** Muse cells were first cultured in suspension in DMEM low glucose, 10% of FBS, 2 ng/μL bFGF, and 10 μM 5′-azacytidine (5′-AZA; Sigma-Aldrich) in a poly(2-hydroxyethyl methacrylate) (P9392; Sigma-Aldrich)-coated 24-well plate prepared as described previously at 3 × 10⁴/ well in order to form Muse cell aggregates. After 3 d, the aggregates were transferred to adherent culture, kept overnight to allow the aggregates to attach to the culture dish, and then were treated as described for the adherent group.

**Sus+Ad+DN group.** The protocol is the same as that of the Sus+Ad group except that incubation with Noggin (100 ng/ mL, R&D Systems) and Dickkopf-related protein 1 (DKK-1; 50 ng/mL, R&D Systems) in DMEM low glucose plus 2% of FBS for 2 d was inserted between the 2 incubation steps (Fig. 1). During the induction, the medium was changed every 2 d. The cells were properly trypsinized and subcultured
whenever they reached nearly 100% confluence except for the last 2 wk of induction.

**Reverse Transcription–Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted from the induced cells on the last day of induction by means of the NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). Then, 400 ng of RNA was used for cDNA synthesis using SuperScript VILO (Invitrogen, Waltham, MA, USA). cDNA was used for Taq polymerase PCR using the primers (Table 1) for GATA-4, Tbx20, atrial natriuretic peptide (ANP), Nkx2.5, MLC1v, HCN-4, Tbx20, and β-actin. Human fetal heart RNA (Clontech Laboratories, Inc., Mountain View, CA, USA) served as positive control for GATA-4, Tbx20, ANP, HCN-4, and Nkx2.5. Human adult heart RNA (Clontech Laboratories, Inc.) was used as positive control for MLC1v and MLC1a. Human adult skeletal muscle RNA (Clontech Laboratories, Inc.) was used as positive control for Myo-D. Human adult liver RNA (Clontech Laboratories, Inc.) was used as a negative control for all markers except Tbx20, ANP, and GATA-4. Human dermal fibroblast RNA (Clontech Laboratories, Inc.) served as negative a control for Tbx20 and ANP, and human adult brain RNA (Clontech Laboratories, Inc.) for GATA-4. The amplification program included the initial denaturation step at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at Tm for 30 s, and extension at 72°C for 30 s; then the final extension step at 72°C for 10 min. For GATA-4, Tbx20, and Myo-D, the denaturation, annealing, and extension intervals were 1 min instead of 30 s, and the final extension step lasted at 7 min instead of 10 min.

**Quantitative PCR (Q-PCR)**

Total RNA was extracted from the induced cells on the final day of induction by means of the NucleoSpin RNA XS Kit (Macherey-Nagel). In addition, total RNA from “sample 1” and “sample 2” of the Sus+Ad+DN group was also collected (Fig. 1). Then, 100 ng of total RNA was used for cDNA synthesis using the SuperScript VILO kit (Invitrogen). GATA-4 (Hs00171403_m1), α-actinin (Hs00153809_m1), MLC2a (Hs00221909_m1), MLC2v (Hs00166405_m1), and HCN4 (Hs00975492_m1) expression levels were analyzed by the ΔΔCT method with the AB systems 7500 Fast real-time PCR (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. β-Actin (Hs99999903_m1) served as an endogenous control. For MLC2a and MLC2v analyses, 2 additional groups for the Sus+Ad+DN group in which either CT-1 or HGF was removed from the induction, named as...
the Sus+Ad+DN(-CT) and Sus+Ad+DN(-HGF) groups, respectively.

For experiments on expression of pluripotency genes, we prepared 3 subsets: the naive adherent Muse, naive suspension Muse, and naive suspension Muse + 5-AZA. Naive adherent Muse cells were prepared directly from adherent culture. Naive suspension Muse + 5-AZA and naive suspension Muse were cultivated for 3 d in suspension culture as described earlier with or without 5-AZA, respectively. Total RNA was extracted from these 3 subsets. Next, Pou5f1 (Hs04260366_g1), Sox-2 (Hs01053049_s1), and Nanog (Hs042 60366_g1) expression levels were analyzed by the ΔΔCT method with β-actin as an endogenous control.

Naive adherent Muse were induced for 10 d under 4 different conditions in order to determine the main signaling pathway(s) involved in CT-1 cardiac induction effect. The 4 induction conditions were either; CT-1 only, CT-1 in presence of phosphatidyl inositol-3 kinase (PI3K) inhibitor (Ly294002, 10 μM, Calbiochem, La Jolla, CA, USA) (CT-1/PI3Ki), CT-1 in presence of mitogen activated protein kinase 1,2 (MEK1,2) inhibitor (U0126, 10 μM, Calbiochem) (CT-1/MEKi), or glycogen synthetase kinase 3 (GSK3) inhibitor only (CHIR99021, 10 μM, Wako) (GSK3i). After 10 d, β-actin expression level was determined using the same conditions described above.

**Global DNA Methylation**

DNA was extracted from naive adherent Muse, naive suspension Muse, naive suspension Muse + 5-AZA, and suspension Muse + 5-AZA/adherent Muse (naive suspension Muse + 5-AZA group transferred onto adherent culture overnight) by means of the QIAamp® DNA Mini Kit (Qiagen, Düsseldorf, Germany). The relative percentage of DNA methylation was determined using the Methylated DNA Quantification Kit (cat. #ab117128; Abcam, Cambridge, UK).

**DNA Bisulfite Sequencing**

DNA was extracted from naive adherent Muse, naive suspension Muse + 5-AZA, and sample 1 (Fig. 1) using QIAamp® DNA Mini kit. One microgram of DNA was treated with bisulfite using a MethylEasy™ Xceed Kit (cat. # ME002; Human Genetic Signatures, Sydney, Australia) according to the manufacturer’s instructions. Primers were designed for a CpG island with 30 CpGs at Nkx2.5 proximal promoter29. Primers used were forward (5'-TCGCGAGGATTTTTATAGTTG-3') and reverse (5'-AACAATCTACCAAAAAATTCTAAAAAC-3'). Finally, ligation with pGEM-T Easy Vector System (Promega, Madison, WI, USA), bacterial transformation with *Escherichia coli* DH5α, and sequencing with ABI 3500xl. Genetic Analyzer (Life Technologies, Camarillo, CA, USA) was done.

**Western Blot**

Induced Muse cells were collected at the end point of induction in the adherent, Sus+Ad, and Sus+Ad+DN groups. Cell lysis was done by incubation for 15 min on ice with buffer containing 20 mM Tris–HCL, 1% Triton, 150 mM NaCl, and 1× protease inhibitor cocktail (Roche, Mannheim, Germany). Protein separation was done by 10% polyacrylamide gel (Supersep™ Ace, Wako), then transferred to a polyvinylidene difluoride (PVDF) membrane (0.45 μm Immobilon-P, Millipore). Blocking was done by incubation with 5% skim milk in TBST for 1 h with shaking at 4°C. The membrane was incubated with the primary antibody diluted in 1% skim milk overnight with shaking at 4°C, then washed with TBST 3 times each for 5 min at room temperature (RT). Then, the membrane was incubated with the secondary antibody diluted in 1% skim milk for 1 h with shaking at RT, followed by same washing step done after primary antibody incubation. Visualization of the membrane was done by chemiluminescence (Pierce ECL Plus, Thermo Fisher,
Immunocytochemistry

Muse cells from the adherent, Sus+Ad, and Sus+Ad+DN groups were fixed on the last day of induction by means of 4% paraformaldehyde (PFA) at 4°C for 2 h. Then, the cell samples were washed twice with phosphate-buffered saline (PBS). The cells were fixed with a block solution, which consisted of 20% Block Ace (DS Pharma Biomedical, Taito, Tokyo, Japan), 5% bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan), and 0.3% Triton X-100 (Wako) in PBS for 2 h at 4°C. After the blocking, the samples were incubated with primary antibodies at 4°C overnight. Primary antibodies were the following: a mouse anti-troponin-I antibody (1:200 dilution; Chemicon), mouse anti α-actinin antibody (1:150 dilution; Sigma-Aldrich), and a rabbit anti-connexin 43 antibody (1:250 dilution; Abcam). The antibody diluent was the same as the block solution but with 5% Block Ace and 1% BSA. Then, the samples were washed 3 times for 5 min each at RT with PBS/Tween 20 (0.05%). Secondary antibodies were diluted with PBS/Triton X-100, and the cells were incubated for 1.5 h at RT. Secondary antibodies were the following: an Alexa 488-conjugated donkey antimouse antibody (Invitrogen) and an Alexa 568-conjugated donkey antirabbit antibody (Invitrogen) both at 1:1,000 dilution. After the secondary antibody incubation, 3 washes for 5 min each at RT were done. The cells were then counterstained with a 1:500 dilution of 4’,6-diamidino-2-phenylindole (DAPI) in PBS/Triton X-100 and were examined under a Nikon C2 Eclipse laser confocal microscope (Nikon, Tokyo, Japan). The percentage of troponin-I+ cells was calculated at the end point of the 3 induction groups using ~2,000 cells for each group.
comparisons using Microsoft® Excel 2007 software and (GraphPad InStat 3, San Diego, CA, USA) programs.

Results

Pluripotency and DNA Methylation

After isolation, Muse cells had a spindle shape similar to that of mesenchymal cells (Fig. 2A). When they were transferred to suspension culture, Muse cells aggregated to form spheroids (Fig. 2B).

Pluripotency gene expression levels were compared between the naive adherent and naive suspension Muse cells. As shown in Fig. 2C, Pou5f1, Sox2, and Nanog were all substantially upregulated in the naive suspension Muse cells as compared to the naive adherent Muse cells with statistically significant differences. We next tested whether the presence of 5'-AZA, a known factor for DNA demethylation, might further upregulate pluripotency genes in suspension culture. Expression levels of Pou5f1, Sox2, and Nanog in Q-PCR became higher after the addition of 5'-AZA. Notably, Pou5f1 and Nanog showed statistically significant differences (P < 0.01; Fig. 2D).

The global DNA methylation level was further investigated among 4 conditions: (1) naive adherent Muse, (2) naive suspension Muse, (3) naive suspension Muse+5-AZA, and (4) suspension Muse+5-AZA/adherent (Fig. 2E). The naive adherent Muse group showed the highest percentage of DNA methylation (1.45%) and was less methylated when these cells were simply cultured in suspension (0.74%), while that was under detection level in the naive suspension Muse+5-AZA group, suggesting that suspension culture combined with 5'-AZA accelerated DNA demethylation. Nonetheless, when the native suspension Muse+5-AZA group was transferred into adherent culture (suspension Muse+5-AZA/adherent), their DNA methylation percentage increased.

Moreover, the effect of 5'-AZA on Muse cell viability (naive adherent Muse + 5-AZA) was evaluated by the trypan blue exclusion method at 3 d. There was no significant difference in cell viability between the naive adherent Muse with 5'-AZA and without 5'-AZA (Fig. 3A). Based on these findings, we set 3 induction systems, namely, the adherent, Sus+Ad, and Sus+Ad+DN groups in which either adherent or suspension culture were combined with a cocktail of cytokines relevant to cardiac differentiation as shown in Fig. 1. Passage 4 MSCs showed a low percentage of senescence compared to passage 1 (Fig. 3C and D), therefore we used MSCs at passage 4 in our experiment.

DNA Bisulfite Sequencing

Bisulfite sequencing at the Nkx2.5 promoter region showed a significant decrease in the percentage of methylated CpGs in the naive suspension Muse + 5-AZA compared to the naive adherent Muse (Fig. 4A and B). In addition, the percentage of methylated CpGs of the sample 1 induced Muse was significantly lower than that of the naive adherent Muse, with no significant difference in the naive suspension Muse+5-AZA group (Fig. 4B).
Immunocytochemical Analysis

Phase contrast microscopic images at the final time point of each group are shown in Fig. 5A. Cells of the adherent group had morphological features that were different from those of the original naive adherent Muse group (Fig. 2A). Some of the cells of the Sus+Ad and Sus+Ad+DN groups contained striation-like patterns (Fig. 5A). A striation-like pattern was also observed in troponin-I immunocytochemistry in both the Sus+Ad+DN and Sus+Ad groups while that was not evident in the adherent group (Fig. 5B). The results showed 45.7% ± 1.5% troponin-I+ cells in the Sus+Ad+DN group, 30.3% ± 1.6% in the Sus+Ad group, and 24.1% ± 1.6% in the adherent group (Fig. 3E and F).

In double staining with α-actinin and connexin 43, the α-actinin staining pattern was random and not well organized in the adherent group while that was more organized in the Sus+Ad group (Fig. 6). The Sus+Ad+DN group showed the most organized staining pattern. In all of the 3 groups, connexin 43 seemed to be located mainly at cell–cell contact sites (Fig. 6).

Western Blot Analysis

While all 3 induction groups expressed α-actinin and desmin proteins in Western blot (Fig. 7A), densitometry revealed that the Sus+Ad+DN group showed the highest expression level of α-actinin with statistical significance compared to the adherent group. Furthermore, the Sus-Ad+DN group showed the highest expression level of desmin with significant differences compared with both the adherent and the Sus+Ad groups. On the other hand, the adherent and Sus+Ad groups did not show significantly different expression levels in either marker (Fig. 7B). HCN4—a marker for pacemaker cardiomyocytes—was not detected in all groups.

Cardiac Marker Expressions in RT-PCR and Q-PCR

For assessment of cardiac differentiation in each group, RT-PCR analysis was performed at the final time point of each group (Fig. 7C). GATA-4, an early cardiac marker, was not detected in the 3 groups, probably because it is an early cardiac marker and might have been already downregulated at the final time point. Cardiac progenitor markers—Tbx20, ANP, and Nkx2.5—were detected in all 3 groups. Additionally, MLC1a and MLC1v, myosin light chain variants expressed by cardiomyocytes, were detected in all 3 groups. MyoD, a marker for skeletal muscle and not for cardiomyocytes, was not detected in any of the 3 groups.

Even though GATA-4 was not detected by RT-PCR at the final time point of all 3 groups, Q-PCR revealed that GATA-
4 was not strongly expressed immediately after 7-d incubation with 6 factors (Fig. 8A; corresponding to sample 1 in Fig. 1) but was substantially expressed after inhibition of Wnt and BMP by addition of DKK-1 and Noggin, respectively (the point corresponding to sample 2 in Fig. 1), in the Sus+Ad+DN group (Fig. 8A). However, GATA-4 was not detected at the final time point in any of the 3 groups (Fig. 8A). Expression of α-actinin in Q-PCR was the highest in the Sus+Ad+DN group compared to the other groups (Fig. 8A). The Sus+Ad group showed the highest expression of MLC2a with statistically significant differences compared with other groups (Fig. 8A). Expression of α-actinin in Q-PCR was the highest in the Sus+Ad+DN group compared to the other groups (Fig. 8A).

The Sus+Ad group showed the highest expression of MLC2a with statistically significant differences compared with other groups (Fig. 8B). The adherent group showed the lowest expression, which significantly increased in the Sus-Ad+DN group. MLC2a expression was under the detection level in the Sus+Ad+DN(-CT-1) group.

The Sus+Ad+DN group showed the highest expression of MLC2v with statistically significant differences compared with other groups (Fig. 8C). The adherent group showed the lowest expression, which was significantly increased after DKK-1 and Noggin treatment (sample 2) and also in the Sus+Ad group.

CT-1 Signaling in Cardiac Differentiation Illustrated by Q-PCR

Muse cells induced with either CT-1 only or CT-1/MEKi showed nearly the same α-actinin expression level (Fig. 8D). In contrast, α-actinin expression was abrogated in the CT-1/PI3Ki group. Induction with a GSK3 inhibitor partially suppressed α-actinin expression. However, the expression level was significantly (P < 0.01) lower than in either CT-1 only or the presence of a MEK1,2 inhibitor.

We tried adding small molecules known to enhance programming of stem cells. Since the induction was dependent on the PI3K pathway, we thought that inhibition of the PI3Kγ subtype—known to interfere with cardiomyocyte maturation31—during last 2 wk of induction might be beneficial. In addition, Oct4 activator; OAC-2 and GSK3 inhibitor; CHIR99021 were added to both the Sus+Ad and Sus+Ad+DN groups on the first week and last two weeks of induction, respectively. These modified protocols showed an increase in the incidence of cells with a striation-like pattern (Fig. 7D&E).
Discussion

In the present article, we tested 3 protocols for cardiomyocyte induction. Q-PCR analysis of α-actinin, GATA-4, MLC2a, and MLC2v expression as well as Western blot of α-actinin and desmin expression levels were used to compare the efficiency of cardiac differentiation among the 3 protocols. Immunocytochemical analysis confirmed the expression of troponin-I, α-actinin, and connexin 43 proteins. We selected early cardiac markers including Tbx20, GATA-4, Nkx2.5, and ANP as well as late cardiac markers such as MLC1a and MLC1v for RT-PCR assays.

Several attempts to induce MSCs into cardiomyocytes were based on coculture of MSCs with cardiomyocytes rather than cytokine-driven induction.32-34 In addition, some investigators used a cardiomyocyte extract for induction.33,35,36 These experimental approaches showed that MSCs have the potential for cardiac differentiation. Nonetheless, the involvement of nonhuman cells or tissue extracts might delay their progression toward clinical applications.

Suspension Culture and 5′-AZA

Some reports suggested that 5′-AZA induces dedifferentiation and enhances cell plasticity in some types of somatic cells37 and facilitates cardiac differentiation in rat MSCs by inducing DNA demethylation38. Regarding suspension culture, Kuroda et al. demonstrated that Muse cell self-renewal and pluripotency become apparent when they are cultured in suspension4. We confirmed that suspension culture substantially upregulated the expression level of genes related to pluripotency in Muse cells when compared to adherent Muse cells, and administration of 5′-AZA to suspension culture
Cytokines for Cardiac Differentiation

Several reports have described the induction of cardiomyocyte-like cells from BM-MSCs with various sets of cytokines. Behfar et al. used single-step cytokine induction with TGF-β1, BMP-4, activin A, retinoic acid, bFGF, IGFR-1, α-thrombin, and IL-6, assessing the induction effect by means of Nkx2.5 and MeF2c. Siegel et al. used different cytokine cocktails: those containing 5'-AZA, bFGF, vascular endothelial growth factor (VEGF), BMP2, and Noggin and validated differentiation by Q-PCR analysis of troponin-I, α-actinin, and myosin light chain. Although cardiac lineage genes were detected, those authors stated that the cells did not differentiate into mature functional cardiomyocytes. Shim et al. used insulin, dexamethasone, and ascorbic acid and reported cardiomyocyte-like cells with the formation of a striation-like pattern.

Indeed, the cardiac specification is a complex process of differentiation that is controlled by several cytokines in a context-dependent manner. A combination of TGF-β1 and BMP4 induces differentiation toward the mesendodermal lineage via Smad2/3 and Smad1/5/8, respectively, whereas either of them alone does not work sufficiently. BMP/Smads facilitate expression of cardiac progenitor cell markers such as GATA-4 and Mef2, whereas Wnt/β-catenin induces Nkx2.5 and Islet-1. In our protocol for the Sus+Ad and Sus+Ad+DN groups, TGF-β1, BMP2, BMP4, activin A, Wnt3a, and bFGF might have synergistically activated cardiac differentiation of Muse cells, which were strongly potentiated by suspension culture plus 5'-AZA. What is unique to the Sus+Ad+DN group is that the protocol contains an extra step of DKK-1 and Noggin incubation. Some studies on murine cardiac development showed that Noggin, a BMP pathway blocker, promotes the cardiac commitment of meso/endodermal lineage cells. Long-term exposure to Noggin halts cardiac development in mice. Wnt/β-catenin signaling during early development facilitates mesodermal cell differentiation into cardiomyocytes rather than a hematopoietic lineage; however, at late developmental stages, it has the opposite effect. DKK-1, a Wnt pathway blocker, promotes specification of cardiac precursors in the anterolateral mesoderm that forms the cardiac crescent. Consequently, effects of DKK-1 and Noggin on cardiac development depend on developmental status. These data suggest that the Sus+Ad+DN protocol is rationally designed as compared to the other 2 protocols.

CT-1 and HGF might be key factors for the late step induction in our protocols. When the Sus+Ad+DN group induction was performed in the absence of either CT-1 or HGF, MLC2a and MLC2v gene expression was substantially lowered; MLC2a expression was even under the detection level in the Sus+Ad+DN(-CT) group. Moreover, CT-1 cardiac induction is mainly dependent on the PI3K pathway since α-actinin gene expression level became undetectable in the presence of the PI3K inhibitor. The importance of GSK3 inhibition—a downstream signaling pathway of PI3K—in cardiac programming has been described previously. However, GSK3 inhibition yielded a lower α-actinin gene expression level compared to CT-1. This may suggest that GSK3 inhibition is not the only downstream pathway of PI3K responsible for cardiac programming.

Cardiac Marker Expression in Induced Muse Cells

All of the groups expressed cardiac progenitor markers: Nkx2.5, Tbx20, and ANP. GATA-4 is an early marker of cardiac development, the expression of which is observed before linear heart development. Although GATA-4 expression could not be detected at the final time point of any of the 3 induction groups, Q-PCR revealed that the cells in the Sus+Ad+DN group expressed GATA-4 only after DKK-1 and Noggin induction (sample 2). The same group showed the highest α-actinin expression at the final time point when compared to the same time point in the adherent or Sus+Ad groups. These results may eventually help to understand why DKK-1 and Noggin promote maturation of cardiac progenitor cells, causing an increase in the expression of α-actinin at the final time point of induction.

All groups tested negative for Myo-D; this finding suggested that all the induced cells were not skeletal muscle cells.

There was a gradual increase in α-actinin protein expression in the Sus+Ad+DN and adherent groups, showing the highest and lowest expression, respectively. Also, the Sus+Ad+DN showed a higher desmin protein level compared to other groups. However, none of the groups expressed the pacemaker cardiomyocyte marker HCN4 as shown in the Western blot.
Troponin-I positive cells with a striation-like pattern were regularly organized in the Sus+Ad and Sus+Ad+DN groups and were positive for α-actin which is characteristic of Z line responsible for actin–myosin cross-linking. Additionally, the striations were positive for troponin-I, the troponin subunit responsible for binding the troponin–tropomyosin complex to actin. The presence of connexin 43 is suggestive of functional characteristics of the Muse cells converted into cardiomyocyte-like cells. Preda et al. reported that IGF-1 and bFGF, both of which were included in our protocol, enhance connexin 43 mRNA and protein expression in murine BM-MSCs.

### Ventricular and Atrial Subtypes

Directed atrial and ventricular differentiation of human or murine ESCs has been shown previously. Nonetheless, atrial and ventricular differentiation from MSCs has never been attempted before. The expression pattern of different variants of myosin light is used to differentiate atrial and ventricular cardiomyocytes. MLC2a is expressed throughout the linear heart during cardiac development, then becomes restricted to fetal atria. On the other hand, MLC2v is considered as a marker for ventricular specification in the linear heart tube and adult heart.

The adherent group showed the lowest MLC2a or MLC2v gene expression level. This result is suggestive of incomplete or weak differentiation of Muse cells toward either atrial or ventricular cardiomyocytes. On the other hand, the Sus+Ad group showed the highest expression level of MLC2a. This expression profile indicates directed differentiation toward atrial cardiomyocytes. Sample2—which is indicative of a DKK-1/Noggin effect—showed significantly higher MLC2v gene expression compared to the adherent group. The Sus-Ad+DN group showed the highest MLC2v gene expression level among all groups, suggesting directed differentiation toward ventricular cardiomyocytes.

The induced Muse cells in our article—particularly the Sus+Ad+DN group—expressed major markers of mature cardiomyocytes. On the other hand, they did not show spontaneous beating nor did they express the pacemaker cardiomyocyte marker HCN4 as shown by Western blotting and Q-PCR. It is suggested that the induction method described in our study preferentially induced Muse cells into working cardiomyocytes but not into pacemaker cells, or that Muse cells have a lower potential for differentiation into pacemaker cells rather than into working cardiomyocytes. Usually, mature working cardiomyocytes do not spontaneously beat in the absence of the pacemaker cell stimulus. This might partly explain the lack of spontaneous beating in the induced cells. From another view point, this might be beneficial for regenerative medicine, since the induced cells will simply follow the original rhythm of the host pacemaker cells avoiding ectopic foci of contraction that may lead to arrhythmias.

Embryonic and induced pluripotent stem cells have been the focus of cardiac regeneration studies because of their high pluripotency and the ability to stably proliferate in vitro into a large number of cells. However, their clinical applications are still under scrutiny, hindered by their tumorigenicity and ethical issues associated with ESCs. We used Muse cells at a low in vitro passage. This is because high growth in vitro may induce senescence that impairs the therapeutic potential of these cells. Indeed, the failure in some BM-MSC-based therapies has to be ascribed to the use of cultures with a high percentage of senescent cells. Currently, several investigators recommend evaluation of the senescent cell percentage in samples that have to be used for clinical purposes. Being nontumorigenic and available from easily accessible sources (such as bone marrow aspirates or adipose tissue), Muse cells seem to be suitable for clinical applications.

### Conclusion

In the present article, we showed a system for in vitro cardiac lineage induction of Muse cells. In addition, the directed differentiation toward human atrial or ventricular cardiomyocyte-like cells in a simple 3- or 4-step induction procedure is described. Neither the Muse cell collection nor the induction method involved any genetic manipulations or unethical procedures. We believe that this article would be beneficial for clinical applications in cardiac diseases including personalized regenerative treatment.

### Ethical Approval

The statement of Ethical Approval is not applicable for this article.

### Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects and Statement of Human and Animal Rights is not applicable.

### Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Yoshihiro Kushida, Shohei Wakao and Mari Dezawa are affiliated with the Department of Stem Cell Biology and Histology at Tohoku University Graduate School of Medicine, which is party to a codevelopment agreement with Life Science Institute, Inc. (LSII). Mari Dezawa and Shohei Wakao have a patent Muse cells and isolation method thereof licensed to LSII.

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