Novel Embryoid Body–Based Method to Derive Mesenchymal Stem Cells from Human Embryonic Stem Cells

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Application of human embryonic stem cells (hESCs) to stem-cell therapy is not feasible because of the risk of tumorigenicity and rejection. In contrast, human mesenchymal stem cells (hMSCs) are free from the risk of tumorigenicity and also have immune privilege. However, hMSCs obtained from adults have infinite variety in terms of the biological characteristics and functionality. We report here a new derivation method of hMSCs from hESCs. The derivation of hMSCs from three different hESC lines (SNUhES3, CHA3-hESC, and H9) was performed by embryoid bodies formation and subsequent culture with stage-different media without using inductive xenogenic feeder and mechanical selection procedure. The derived cells were morphologically similar to the unique fingerprint-like pattern of hMSCs and grew stably for at least 35 passages *in vitro*. These cells had hMSCs-like immunophenotypes: negative for CD34 and CD45; positive for CD29, CD44, CD73, CD90, and CD105. They could be differentiated into multiple lineages including osteocytes, chondrocytes, adipocytes, and myocytes. They maintained normal karyotype during the long-term cultivation and did not show tumorigenicity when transplanted into the immunodeficient mice. In conclusion, the new embryoid body–based derivation method of hMSCs from hESCs is simple, safe, and reproducible in three different hESC lines. We expect that this method will provide a more effective and powerful tool to derive hMSCs from various hESC lines.

Introduction

STEM-CELL THERAPY is a promising treatment strategy for degenerative disease. However, current stem-cell therapy using human adult stem cells has several serious problems. First, the human adult stem cells have limited differentiation potentials and self-renewing capacities. The human adult stem cells were influenced by aging and diseases that the donors have suffered from.[1] Additionally, harvest of stem cells in human adult tissue frequently requires invasive procedures that can limit applicability and supply of stem cells. Finally, there are wide varieties in the biological characteristics and functionality of the harvested human adult stem cells.

Human embryonic stem cells (hESCs) can be considered as an alternative to overcome the limitations of human adult stem cells. hESCs are believed to be more versatile than tissue-specific human adult stem cells. However, in terms of purity and yield, the current differentiation methods of hESCs are still unsatisfactory, even though it requires lots of complicated manipulations.[2–4] Additionally, there are concerns about the risks of tumor formation and immune rejection after transplantation.[5,6] In contrast to hESCs, human mesenchymal stem cells (hMSCs) have immune privilege[7] and are relatively free from the risk of tumorigenicity. If simple and high purity differentiation methods can be developed, derivation of hMSCs from hESCs will be an attractive platform for stem-cell therapy.

Recently, different groups have reported hMSC derivation from hESCs (hESC-MSC).[8–10] However, these reported methods have the problems for the fine clinical application, such as the employment of animal feeder cells, limitation in

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functional differentiation of derived cells, and the risk of cell damage induced by sorting process.[8–10]

In this study, we developed a novel derivation method for the multipotent hMSCs from hESCs based on the formation of embryoid bodies (EBs) and stage-different media without mechanical selection and xenogenic feeder cells.

Materials and Methods

Culture of hESCs

Three different hESC lines were used in this study: SNUhES3 (Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University, Korea),[11] CHA3-hESC (Stem Cell Research Laboratory, CHA Stem Cell Institute, Pochon CHA University, Korea),[12] and H9 (Wisconsin Regional Primate Research Center, University of Wisconsin). Mitomycin-C-treated (Sigma, St. Louis, MO) SIM mouse embryo-derived thioguanine and ouabain resistant (STO) feeder system and mitomycin-C-treated mouse embryonic fibroblast feeder system were used for SNUhES3, CHA3-hESC, and H9, respectively, in 0.1% gelatin (Sigma)- coated tissue culture dishes at 37°C and 5% CO_2 in an air atmosphere. The medium for the three hESC lines, Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Madison, WI), 20% knock-out serum replacement (Invitrogen), 0.1 mM β-mercapto-ethanol (Sigma), 1% nonessential amino acids (Gibco, Carlsbad, CA), 50 IU/mL penicillin, and 50 mg/mL streptomycin (Gibco), was supplemented with 0.4 ng/mL basic fibroblast growth factor (bFGF; Invitrogen) for SNUhES-3 cells, or 4 ng/mL bFGF for CHA3-hESC and H9 cells, for the maintenance of undifferentiating hESCs, and it was changed daily. These cells were subcultured every 5 to 7 days by gently separating morphologically undifferentiated cells using a dissecting pipette.

New EB-based method to derive MSCs from hESCs

Derivation of MSCs from hESCs (hESC-MSCs) was achieved by multiple steps as described (Fig. 1a). (1) hESC colonies were detached after treatment with 0.5 unit/mL Dispase (Gibco) for 30 min. (2) The detached hESC colonies were incubated in bacterial dishes for 14 days to form EBs. The EB medium, hESC medium without bFGF, was changed every other day. (3) After selection of well-rounded EBs (about $250 \pm 50 \mu m$) under the microscope, the EBs were reattached on a 0.1% gelatin-coated dish. The medium that was composed of DMEM low glucose (Gibco), 10% fetal bovine serum (FBS) (Gibco), and 1% antibiotic–antimycotic (Gibco) was changed every 3 days for 16 days. (4) The outgrowing cells from EBs were expanded by transfer to and culture in a 0.1% gelatin-coated dish with microvascular endothelial cell media-2 (EGM-2 MV) medium (Lonza, Basel, Switzerland). The derived cells were expanded by subculture for more than 150 days. Following the existing criteria, the cells were identified and named as hESC-MSCs; SNU3MSC, CHA3MSC, and H9MSC from SNUhES3, CHA3-hESC, and H9, respectively.

Growth curve of hESC-MSCs

A growth curve was established by multiplying the initial number of cells by the amplification fold for each passage.

Test of teratoma formation by injection of hESC-MSCs to immunodeficient mice

SNUhES3 (3×10^6) and hESC-MSCs derived from SNUhES3 (SNU3MSC-1, 1×10^7) were injected subcutaneously into the back of 5-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (JAX, Bar Harbor, ME). After 12 weeks, the mice were euthanized, and the resulting masses were removed. The excised mass was washed with phosphate-buffered saline (PBS) and fixed in 4% U paraformaldehyde overnight at 4°C. Each tissue was embedded in paraffin or frozen in optimal cutting temperature (OCT) compound (Tissue-Teck; Sakura Finetek, Tokyo, Japan). Tissue sections (7 μm) were stained with hematoxylin–eosin.

Flow cytometry of hESC-MSCs

For flow cytometry analysis, the cells were first dissociated by incubation at 37°C for 1 min in 0.25% trypsin/ethylenediaminetetraacetic acid (Invitrogen), washed with PBS containing 2.5% FBS, and incubated for 30 min with antibodies. Antibodies for hMSCs[13,14]: CD29 (Phycoerythrin (PE); BD Biosciences, San Jose, CA), CD44 (Santacruz, Santa Cruz, CA), CD73 (PE; BD Biosciences), CD90 (Fluorescein isothiocyanate (FITC); BD Biosciences), and CD105 (FITC; Serotec, Raleigh, NC) for hESCs; SSEA1 (Santacruz), SSEA4 (e-Bioscience, San Diego, CA), OCT-4 (Chemicon, Temecula, CA), and TRA-1-60 (Chemicon) for other lineage markers; CD31 (PE; BD Biosciences), CD34 (Serotec), CD45 (Dako, Carpinteria, CA), and AC133 (PE; Miltenyi Biotec, Bergisch Gladbach, Germany) for immunogenic-related surface markers; human leukocyte antigen (HLA)-DR (FITC; BD Biosciences), HLA-DQ (FITC; BD Biosciences), B7-1 (FITC; BD Biosciences), and B7-2 (PE; BD Biosciences) were used. Appropriate secondary antibodies (Alexa 488 and 555, all from Invitrogen) were used to detect primary antibodies. After the treatment with antibodies, cells were analyzed with a flow cytometer (FACS calibur; BD Biosciences). Appropriate isotype controls were used for each antibody as a control for nonspecific antibody binding.

Functional differentiation of hESC-MSCs to several lineages

For adipocytic differentiation, hESC-MSCs were cultured in a differentiation medium containing alpha minimum essential medium (MEM) (Invitrogen), 10% FBS (Gibco), 1 mM dexamethasone, 10 μg/mL insulin, and 0.5 mM iso-butylxanthine (all from Sigma). The medium was changed every 3 days for 2 weeks. Adipocytes differentiated from hESC-MSCs were detected by Oil Red O staining.[15]

For chondrocytic differentiation, hESC-MSCs were formed into pellets and cultured in a differentiation medium containing high-glucose DMEM (Invitrogen), 10% FBS (Gibco), 10 ng/mL transforming growth factor-β1 (R&D systems, Minneapolis, MN), 1% BD™ insulin, transferrin and selenium (ITS) + Premix (BD Biosciences, consisting of 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL serum albumin, and 5.35 μg/mL linoleic acid), 37.5 μg/mL ascorbate-2-phospate, 10^{-7} M dexamethasone (all from Sigma), and 1% nonessential amino acids (Invitrogen) for 3 weeks. Chondrocytes differentiated from hESC-MSCs were stained with Alcian Blue.[15]
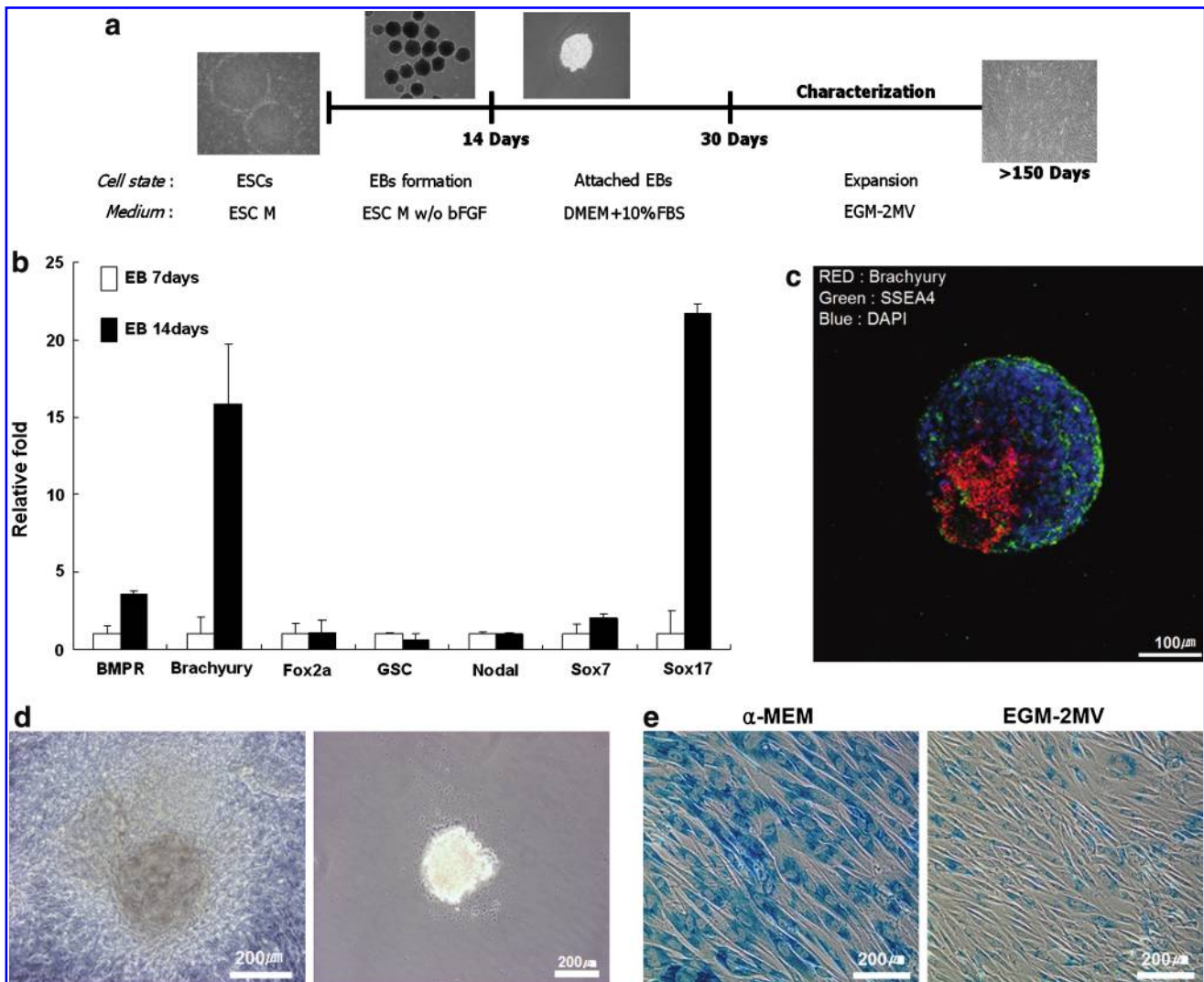
FIG. 1. Derivation of hMSCs from hESCs. (**a**) Experimental scheme for EB-based derivation of hESC-MSCs. Colonies of hESCs were enzymatically detached and then cultured in suspension condition to form EBs. After 14 days suspension culture, EBs were attached on plate and then attached EBs were cultured for following 16 days. Thereafter, hMSC looking cells outgrew from the attached EBs, and these cells were subcultured and expanded. (**b**) Expressions of mesoderm-specific genes in real-time polymerase chain reaction analysis are upregulated in EBs at day 14 of suspension culture compared with day 7. Each experiment was repeated twice, and the results were presented as mean ± standard deviations. (**c**) EBs at day 14 showed expression of brachyury and SSEA4. (**d**) Some attached EBs produced outgrowing hESC-MSCs (day 30, left) and others went to apoptosis (right). (**e**) Beta-gal staining revealed that cells cultured in alpha-minimum essential medium (α-MEM) are prone to senescence than cells in EGM-2 MV. The cells obtained at day 95 were cultured for 1 month in α-MEM or microvascular endothelial cell media-2 (EGM-2 MV). β-Galactosidase activity was detected at pH 6. ESC, embryonic stem cells; ESC M, ESC media; EB, embryoid body; hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell. Color images available online at www.liebertonline.com/ten.

For osteocytic differentiation, hESC-MSCs were plated at low density in tissue culture dishes in a differentiation medium containing alpha MEM medium (Invitrogen), 10% FBS (Gibco), 10 mM β-glycerol phosphate, 0.1 μM dexamethasone, 200 μM ascorbic acid (all from Sigma). The medium was changed every 3 to 4 days for 3 weeks. Osteocytes differentiated from hESC-MSCs were stained using von Kossa staining.[15]

For myocytic differentiation, hESC-MSCs were cultured in alpha MEM medium (Invitrogen) with 20% FBS (Gibco) for 3 weeks. Differentiated myocytes were stained using *MyoD*.[14]

Real-time reverse transcription–polymerase chain reaction analysis

Total RNAs from the cultured cells were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized from approximately 1 μg of total RNA using the Reverse Transcription System (Promega, Madison, WI) and subjected to polymerase chain reaction (PCR) amplification. The Primer3 software (Whitehead Institute/MIT Center for Genome Research) was used to design all the specific primers used

TABLE 1. REAL-TIME POLYMERASE CHAIN REACTION PRIMER SEQUENCES AND PRODUCT SIZE

Genes		Primer	Product size	Temperature
Srebf1	Forward	ggaaccatcttggcaacagt	249	60°C
	Reverse	aatgtagtcgatggccttgc		
PPARγ	Forward	accaactttgggatcagctc	244	60°C
	Reverse	tctgcaaccactggatctgt		
Aggrecan	Forward	aaaccacctctgcattccac	211	60°C
	Reverse	tctccgctgatttcagtcct		
ALP	Forward	gaaccccaaaggcttcttct	204	60°C
	Reverse	ggggtgtatccaccaaatgt		
Osteocalcin	Forward	tgcagcctttgtgtccaa	171	60°C
	Reverse	tgaaagccgatgtggtca		
Osteopontin	Forward	gcaaccgaagttttcactcc	176	60°C
	Reverse	ccattcaactcctcgctttc		
MyoD	Forward	gcaggtgtaaccgtaaccca	253	60°C
	Reverse	cacacaccatgcctcagaga		
GAPDH	Forward	caacgaatttggctacagca	177	60°C
	Reverse	tgtgaggaggggagattca		

in these experiments (Table 1).[13] PCRs were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with the SYBR® Green PCR Master Mix (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was simultaneously run as a control and used for normalization. Nontemplate control wells without cDNA were included as negative controls. Each test sample was run in duplicate. The results are reported as relative expressions after normalization of transcript amount to the endogenous control using the $2^{-\Delta\Delta C_T}$ method.[16] The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold.

The following formula was used for the analysis.

$$\text{Gene expression level} = 2^{-\Delta\Delta C_T}.$$

Myocardial cryoinjury model and hESC-MSCs transplantation

All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee of the Clinical Research Institute in Seoul National University Hospital and complied with the National Research Council's Guidelines for the Care and Use of Laboratory Animals (revised 1996). C57BL/6J mouse was anesthetized with tiletamine with diazepine (Zoletil, 25 mg/kg; Virbac, Fort Worth, TX) plus Xylazine (10 mg/kg; Bayer, North Rhine-Westphalia, Germany) by intramuscular injection. Experimental myocardial cryoinjury, modified from previous study,[17] was produced by freeze–thaw technique with application of precooled cryoinjury probe (4 mm diameter) on anterior left ventricular wall for 25 s. C57BL/6J mouse was randomized into media injection group (control) and cell injection group. For cell transplantation group, a total of 5×10^4 SNU3MSC-1/60 μL 0.9% saline was transplanted by two intramyocardial injections.

Echocardiographic evaluation

Transthoracic echocardiography was performed 3 days after cryoinjury for baseline evaluation, and then 4 and 8 weeks after cryoinjury with an echocardiographic system (Aplio XG, Toshib, Japan) equipped with a 15-MHz linear-array transducer. Left ventricular end-diastolic dimensions (LVEDD) and left ventricular end-systolic dimensions were measured according to the leading edge method of the American Society of Echocardiography.[18] The percent left ventricular fractional shortening was calculated as 100× (LVEDD − left ventricular end-systolic dimensions)/LVEDD.

Histological preparations and analysis

After echocardiographic evaluation, mice were euthanized, and the hearts were removed. The excised heart was retrograde perfused with PBS for coronary vasculature and left ventricular washing, and fixed with 4% paraformaldehyde overnight at 4°C and then 15% sucrose for overnight at 4°C. Each tissue was embedded in paraffin. Section (7 μm) was stained with Masson's trichrome to detect infarct area and calculated using Image-Pro plus 4.5 software.

Statistical analysis

Data are presented as mean ± standard deviation. Continuous variables are compared between groups using student's t-test. Analysis was performed by SPSS 12.0. A probability value of <0.05 was considered statistically significant.

Results

EB-based derivation methods of hESC-MSCs

hESC-MSC lines were derived from three different hESCs by EB-based differentiation methods. Derivation methods can be summarized by the following four steps (Fig. 1a): (1) enzymatic detachment of hESC colonies, (2) formation of EBs from suspension culture of hESCs for 14 days, (3) reattachment of EBs on plate and culture of outgrowing cells, and then (4) expansion of the outgrowing hMSCs. To determine the optimal timing of reattaching EBs on plate after the formation of EBs from suspension culture, we compared the expression of mesoderm marker genes in EBs at days 7 and 14 of suspension culture, using real-time PCR. Expressions of BMPR2, Brachyury, and Sox17 of EBs are higher

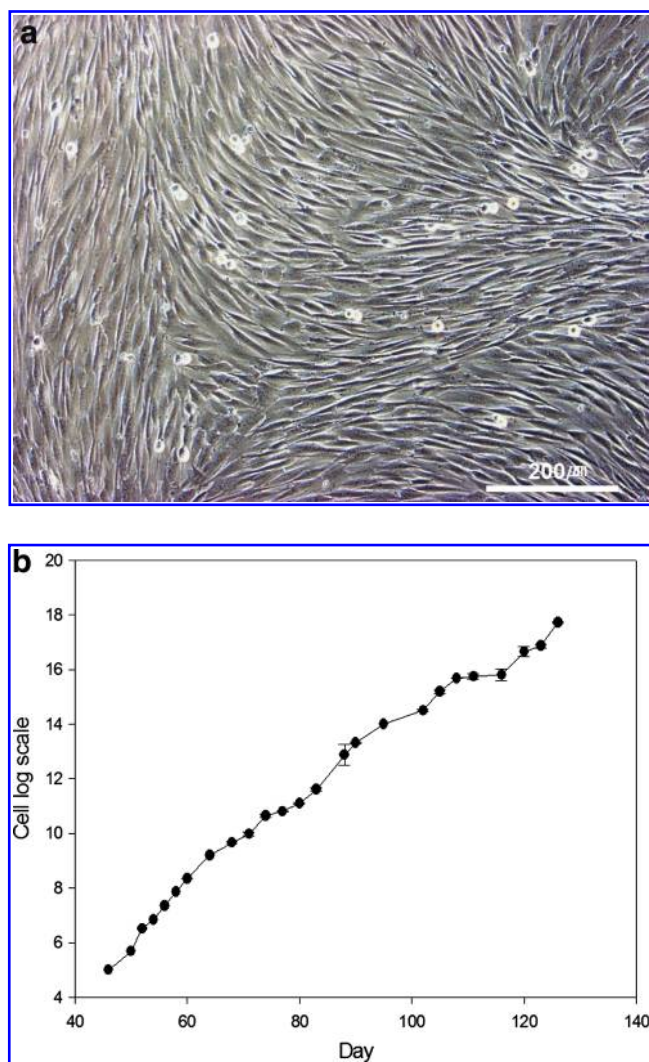**FIG. 2.** Morphology and growth curve of hESC-MSCs. (**a**) Morphology of SNU3MSC-1 at confluence under phase-contrast microscopy exhibits a typical fingerprint-like pattern. (**b**) SNU3MSC-1 was subcultured every 3–4 days over 120 days. Color images available online at www.liebertonline .com/ten.

at day 14 of suspension culture than day 7 (Fig. 1b, c). Timing of reattachment was determined based on the level of mesoderm marker expression in EBs and yield of outgrowing hESC-MSCs from the attached EBs. Around 16 days after reattachment, EBs went different courses; some EBs produced the outgrowing cells, whereas the others

went to apoptosis (Fig. 1d). At this time (day 30 from the start of EBs suspension culture), the cells outgrowing from EBs were transferred to a 0.1% gelatin-coated tissue culture dish in EGM-2 MV medium. EGM-2 MV medium prevents senescence of outgrowing cells during the long-term cultivation compared with α-MEM (Fig. 1e), but the underlying mechanism is still unclear. Outgrowing cells, named hESC-MSCs, could be stably expanded by subcultures and showed uniform characteristic morphology of hMSCs. Proliferative capacity of hESC-MSCs was maintained till 35 passages (Fig. 2).

Characterization of hESC-MSCs

The three different hESC lines of SNUhES3, CHA3-hESCs, and H9 were used in this study to derive hESC-MSC lines. hMSCs derived from SNUhES3, CHA3-hESCs, and H9 were named SNU3MSC-1~5, CHA3MSC-1~2, and H9MSC-1~2 respectively (Table 2). After the derivation of hESC-MSCs, characteristics of derived cells were identified by the typical morphology, cell surface markers, and proliferative and differentiation potentials into mesenchymal lineages such as adipocyte, chondrocyte, and osteocyte.[13]

SNU3MSC-1 expressed CD29, CD44, CD73, CD90, and CD105 (Fig. 3). Expressions of CD73 and CD105 were gradually increased as passages went. SNU3MSC-1 did not express hESC-specific marker; SSEA1, SSEA4, TRA-1-60, and OCT-4 after 95 days from start of EBs suspension culture. These results suggested that contamination of hESCs is minimized, and the risk of tumorigenicity also can be minimized in SNU3MSC-1. SNU3MSC-1 was also negative for other lineage markers of CD31, CD34, CD45, and AC133 (Fig. 3b). Other hESC-MSC lines, CHA3MSC-1 and H9MSC-1, also showed similar patterns of marker expression and morphology to SNU3MSC-1, which suggests EB-based hESC-MSCs derivation method can be reproducibly applied to CHA3-hESCs and H9 (Supplemental Fig. 1A–D available online at www.liebertonline.com/ten).

To prove the multipotency of SNU3MSC-1, we tried to induce SNU3MSC-1 to differentiate into adipogenic, chondrogenic, osteogenic, and myogenic lineage under specialized culture conditions. After 14 to 21 days of culture in the adipogenic differentiation medium, fat granules appeared and grew in size (Fig. 4a). Expression of adipocyte-specific markers of *PPARγ* and *Srebf1* also increased in real-time PCR (Fig. 4b). Chondrogenic differentiation of SNU3MSC-1 was induced using the pellet culture method. After 3 weeks of induction, more than 90% of all cells stained positive with Alcian Blue, a specific marker for extracellular matrix proteoglycans (Fig. 4a). The expression

Table 2. Three Different Human Embryonic Stem Cell Lines Used and Human Embryonic Stem Cell–Mesenchymal Stem Cell Lines Established in This Study

	Culture condition			
ESC line	*Feeder type*	*bFGF con.(ng/mL)*	*Race/sex*	*Established hESC-MSCs*
SNUhES3	STO	0.4	Oriental/XY	SNU3MSC-1~5
CHA3-hESC	MEF	4	Oriental/XY	CHA3MSC-1, 2
H9	MEF	4	Caucasian/XX	H9MSC-1, 2

bFGF, basic fibroblast growth factor; hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast.
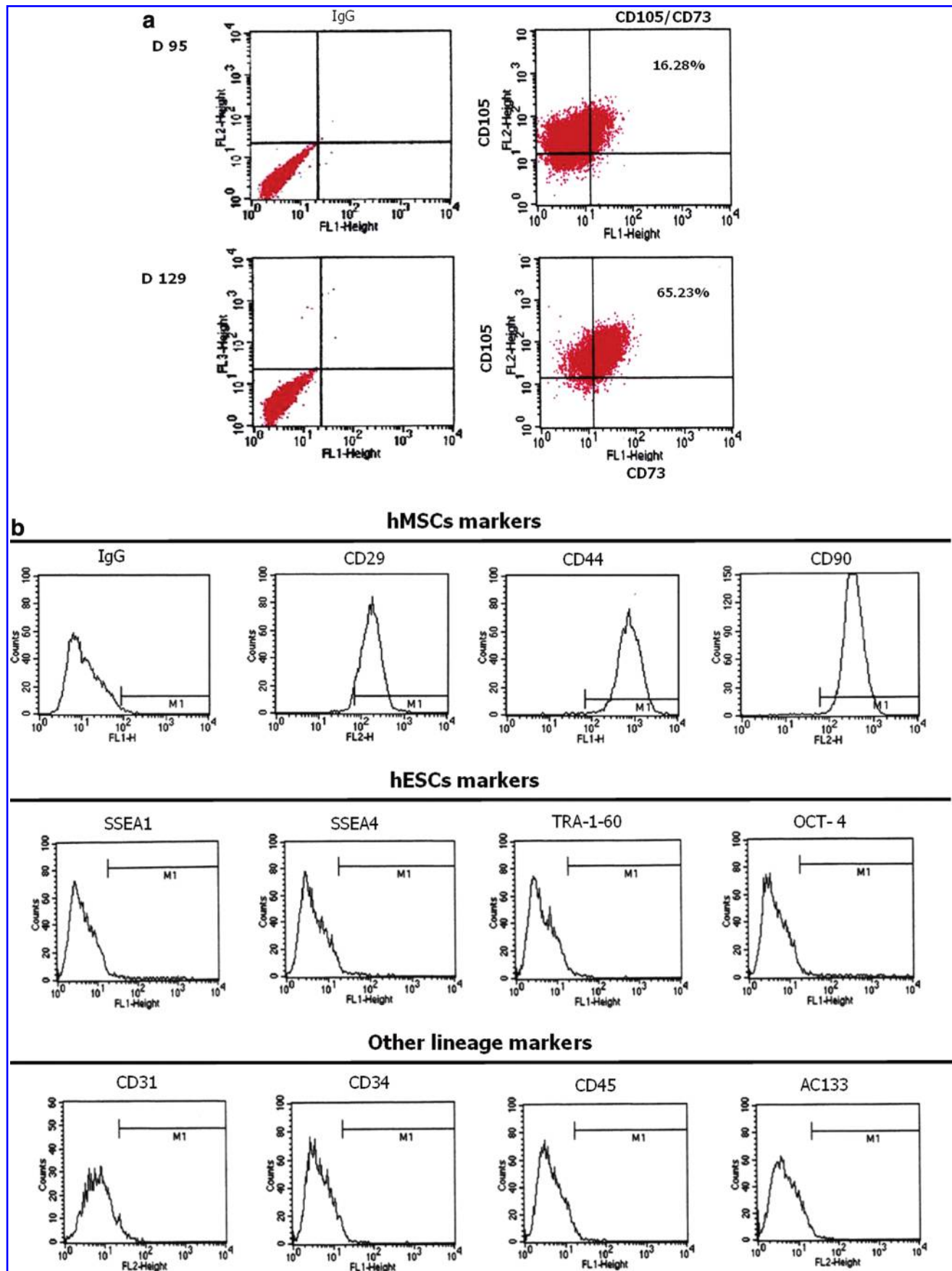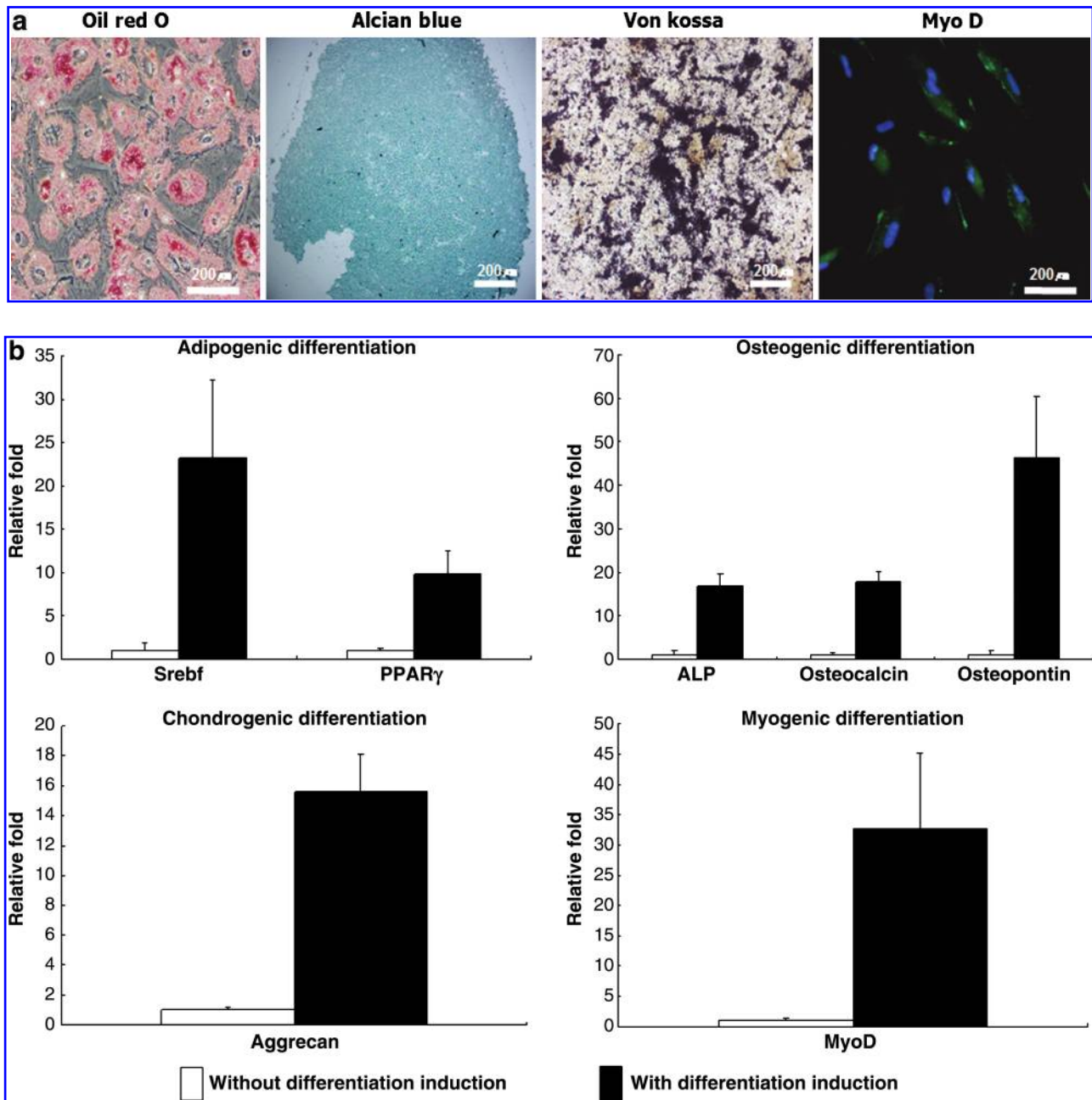
FIG. 4. Functional differentiation of hESC-MSCs into several lineages. (**a**) SNU3MSC-1 differentiated into adipocytes, chondrocytes, osteocytes, and myocytes by culture in specified differentiation media, respectively. (**b**) Lineage-specific gene expression was determined by real-time polymerase chain reaction. *Srebf1* and *PPARγ* for adipocytes; *aggrecan* for chondrocytes; *ALP*, *osteocalcin*, and *osteopontin* for osteocytes; and *MyoD* for myocytes. Each experiment was repeated twice, and the results were presented as mean ± standard deviations. Color images available online at www.liebertonline.com/ten.

of chondrogenic gene such as *aggrecans*, which are components of the extracellular matrix selectively expressed in chondrocytes, was confirmed by real-time PCR (Fig. 4b). Osteogenic differentiation was observed in the presence of β-glycerol phosphate. After 3 weeks of induction, calcium deposits were observed in the matrix and stained with von Kossa (Fig. 4a). The gene levels of *ALP*, *osteocalcin*, and *osteopontin*, which are bone specific markers, also increased (Fig. 4b). Additionally, myogenic differentiation of SNU3MSC-1 was successfully achieved using alpha MEM

FIG. 3. Flow cytometry analysis of SNU3MSC-1. After expansion for 95 and 129 days, cells were trypsinized and stained with specific markers for hMSCs (CD73, CD105) (**a**), hESCs surface markers, and other lineage markers (**b**). They were positive for hMSC markers, whereas negative for hESCs or other lineage markers. Color images available online at www.liebertonline.com/ten.
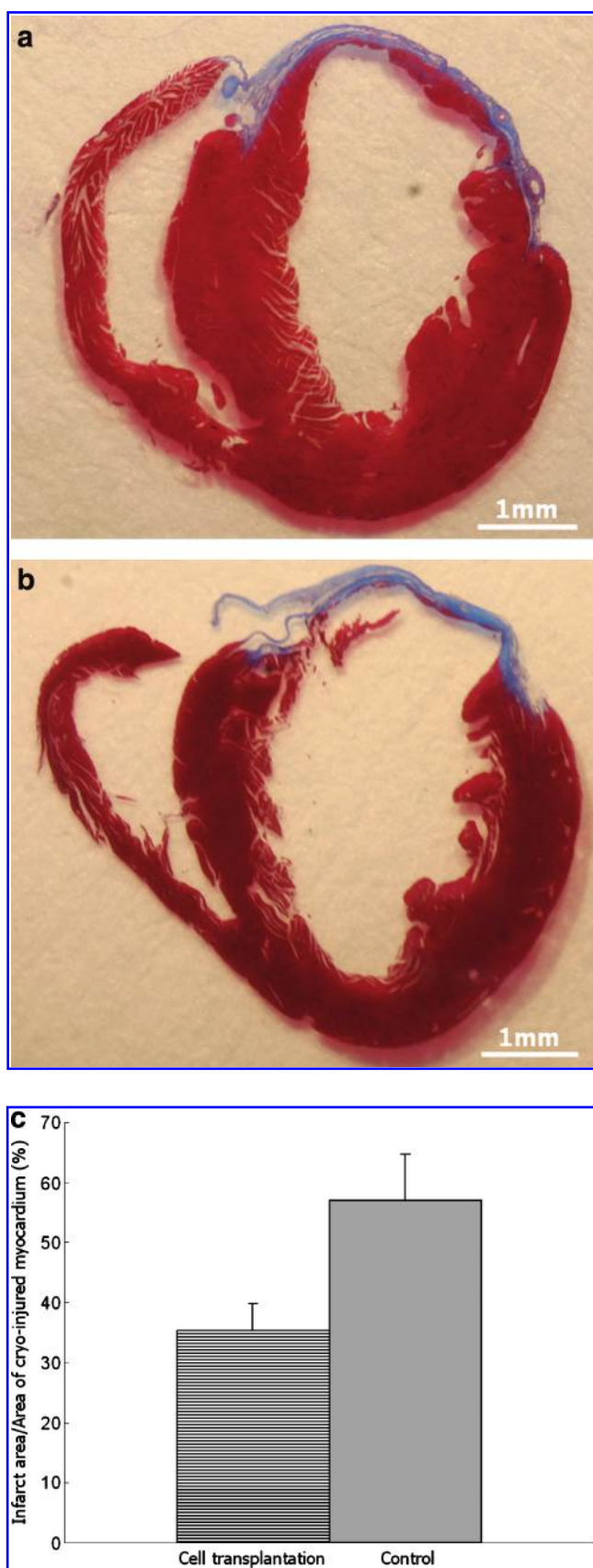
medium with 20% heat-inactivated FBS. After 2 weeks of induction, myocyte-like cells appeared and expressed *MyoD* (Fig. 4). From these results, we can conclude that hESC-MSCs have differentiation potential to multiple mesenchymal derivatives.

Feasibility in therapeutic application

In myocardial cryoinjury model, mice which received transplantation of SNU3MSC-1 showed significantly better cardiac function measured by echocardiography compared with the injury-only group at 4 weeks after transplantation (left ventricular fractional shortening at 4 weeks in control [$n = 5$] vs. cell transplantation group [$n = 5$]: $20.3 \pm 2.9\%$ vs. $25.0 \pm 2.2\%$, $p < 0.05$). Although statistical significance of difference in cardiac function between groups was not maintained till 8 weeks ($20.5 \pm 1.0\%$ vs. $23.7 \pm 3.7\%$, $p = 0.097$, Table 3), cell transplantation showed smaller infarct in injured area compared with the control group at 8 weeks ($35.5 \pm 4.4\%$ vs. $57.0 \pm 7.8\%$; $p < 0.001$, Fig. 5a–c).

Safety and stability of hESC-MSCs in therapeutic application

To investigate the safety and stability of hESC-MSCs for therapeutic application, we performed karyotypeing, *in vivo* teratoma formation assay, and fluorescence-activated cell sorting (FACS) analysis of immune-related surface markers. After prolonged culture up to 161 days, SNU3MSC-1 showed normal karyotype identical to that of the original hESCs (Fig. 6a). CHA3MSC-1 and H9MSC-1 also showed normal karyotype after long-term culture (Supplemental Fig. 1E, F). These results confirmed the chromosomal stability of hESC-MSCs in long-term cultivation. We evaluated tumorigenicity of hESC-MSCs (SNU3MSC-1) in NOD/SCID mice in comparison to original hESC line (SNUhES3). We observed that SNUhES3 formed teratoma containing three-germ layer derivatives in NOD/SCID mice (Fig. 6b). In contrast, SNU3MSC-1 did not form teratoma or any tumor after transplantation of threefold higher number of cells than hESCs, which is significantly higher than the threshold dose that was required to form teratoma.[19]

Moreover, SNU3MSC-1 showed immunotolerant phenotypes like other hMSCs obtained from adults in FACS analysis (Fig. 6c). Expressions of HLA-DR, DQ, and costimulators B-7.1 and B-7.2 were not detected on the surface of SNU3MSC-1. In previous reports, maintenance with bFGF resulted in increased HLA-DR expression in hMSCs.[20] But, SNU3MSC-1 did not express HLA-DR, HLA-DQ, and costimulators during and after long-term culture with bFGF-containing culture media. Further, SNU3MSC-1 showed stable and constant immunophenotypes even after multiple cycles of freezing–thawing.

FIG. 5. Therapeutic potentials in mice myocardial cryoinjury model. The mice received SNU3MSC-1 transplantation (**a**) showed smaller infarct size compared with the control mouse (**b**) at 8 weeks after transplantation. Each slide was stained with Masson's trichrome. (**c**) Infarct area (blue) was calculated by Image-Pro plus 4.5 software (cell transplantation; $35.5 \pm 4.4\%$ vs. control; $57.0 \pm 7.8\%$; $p < 0.001$). Color images available online at www.liebertonline.com/ten.
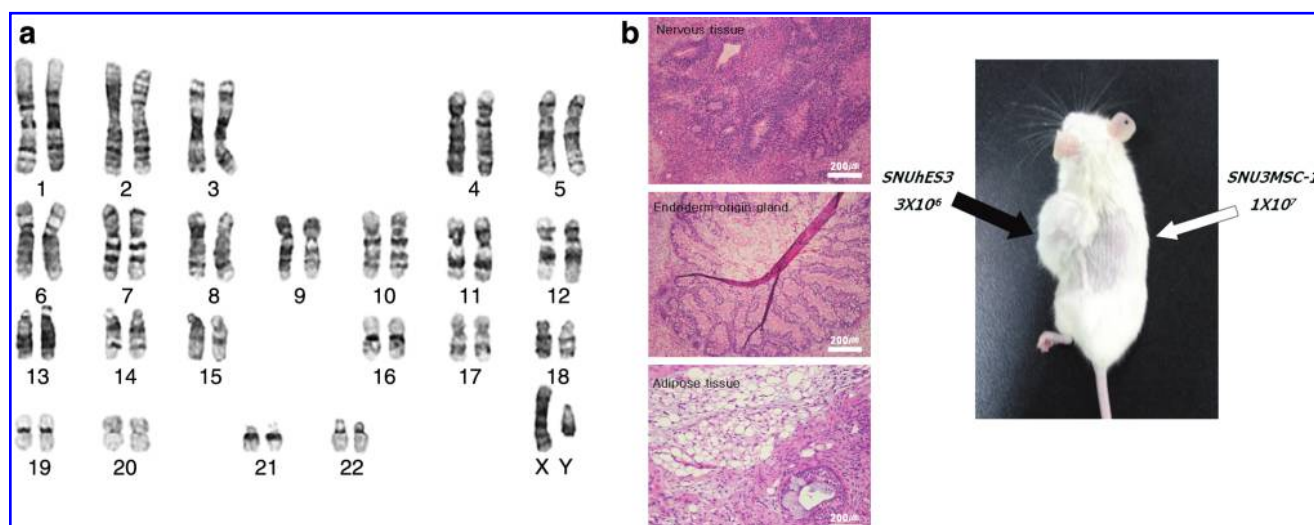
FIG. 6. Karyotyping, tumorigenic potentials, and immunophenotypes. (**a**) SNU3MSC-1 maintained normal karyotype after 161 days of culture in expansion conditions, which was proved by G-band staining. (**b**) Hematoxylin–eosin staining: the potential of teratocarcinoma formation was tested by injection of cells into nonobese diabetic/severe combined immuno-deficient mice. Aliquots of 1×10^7 and 3×10^6 cells of SNU3MSC-1 and SNUhES3, respectively, were injected. Although SNUhES3 formed teratocarcinoma, SNU3MSC-1 did not produce tumor. (**c**) Expression of human leukocyte antigen (HLA) type II and costimulators were not observed in SNU3MSC-1 by FACS analysis after 95 and 129 days of cultivation. Color images available online at www.liebertonline.com/ten.

Discussion

In this study, we established a novel, simplified derivation method of hESC-MSCs. Our EB-based derivation method is reproduced in three different hESC lines. We also proved the therapeutic efficacy of derived hESC-MSCs in mice myocardial cryoinjury model. Our protocol did not require mechanical selection and separation.

Several derivation methods of MSCs from hESCs were reported recently.[8–10] These reports bear some problems such as the risk of animal cell or pathogen contamination,[8] the limited differentiation potency of derived cells,[9] and the requirement of FACS sorting that has risks of damaging the cells.[8,10] In addition, these methods used only one or two hESC lines in their experiments. Our derivation protocol has several advantages compared to the previously reported methods.

First, our protocol has a potential to be a standard derivation method that can be generally be applicable to various hESC lines. Previous protocols may have forced researchers to develop customized derivation protocols for each hESCs. The process is time- and labor consuming. In this study we attempted to develop a generally applicable method to derive hMSCs from hESCs and successfully derived hESC-MSC lines from three different hESCs: SNUhES3, CHA3-hESCs, and H9. These three hESCs are derived from donors of different races and sexes (Table 2). However, our simple derivation protocol reproducibly established hESC-MSCs without individualized modification for each hESC line.

Second, hESC-MSCs obtained by our protocol have shown the genetic stability and biologic safety. Although we could not evaluate possibility of gene translocation in this study, we did not observe any chromosomal abnormality with G-banding in all hESC-MSC lines even after multiple cycles of freezing–thawing. Additionally, they did not provoke a tumor in NOD/SCID mice. Previous studies reported tumorigenic risk of cells derived from hESCs. Even the terminally differentiated cells derived from hESCs such as cardiomyocytes still have the possible risk of tumorigenicity.[21] In our study even though significantly higher numbers of hESC-MSCs than previously reported tumorigenic threshold were transplanted in NOD/SCID mice,[19] we did not observe the formation of tumors. Moreover, our protocol did not require xenogenic feeder. Xenogenic feeder is one of the major limiting factors for the application of hESCs to clinical field as they possess the risk of transmitting pathogens and other unidentified risks.[10]

Third, our hESC-MSCs have powerful self-renewal and proliferative capacities. They can be stably cultured and expanded at least up to 35 passages and after multiple freeze–thaw cycles. It makes possible to obtain and store sufficient amounts of hESC-MSCs from a single derivation. The derived cells in turn can be prepared and stored by HLA type

TABLE 3. ECHOCARDIOGRAPHIC VARIABLES IN MICE CRYOINJURY MODEL

	Baseline		4 weeks		8 weeks	
	LVEDD (mm)	LVFS (%)	LVEDD (mm)	LVFS (%)	LVEDD (mm)	LVFS (%)
Control	39.1 ± 3.8	22.1 ± 0.2	41.6 ± 3.7	20.3 ± 2.9	42.6 ± 4.7	20.5 ± 1.0
Cell transplantation	38.0 ± 0.9	22.8 ± 0.4	40.0 ± 1.5	25.0 ± 2.2	41.1 ± 2.5	23.7 ± 3.7

LVEDD, left ventricular end-diastolic dimension; LVFS, left ventricular fractional shortening.
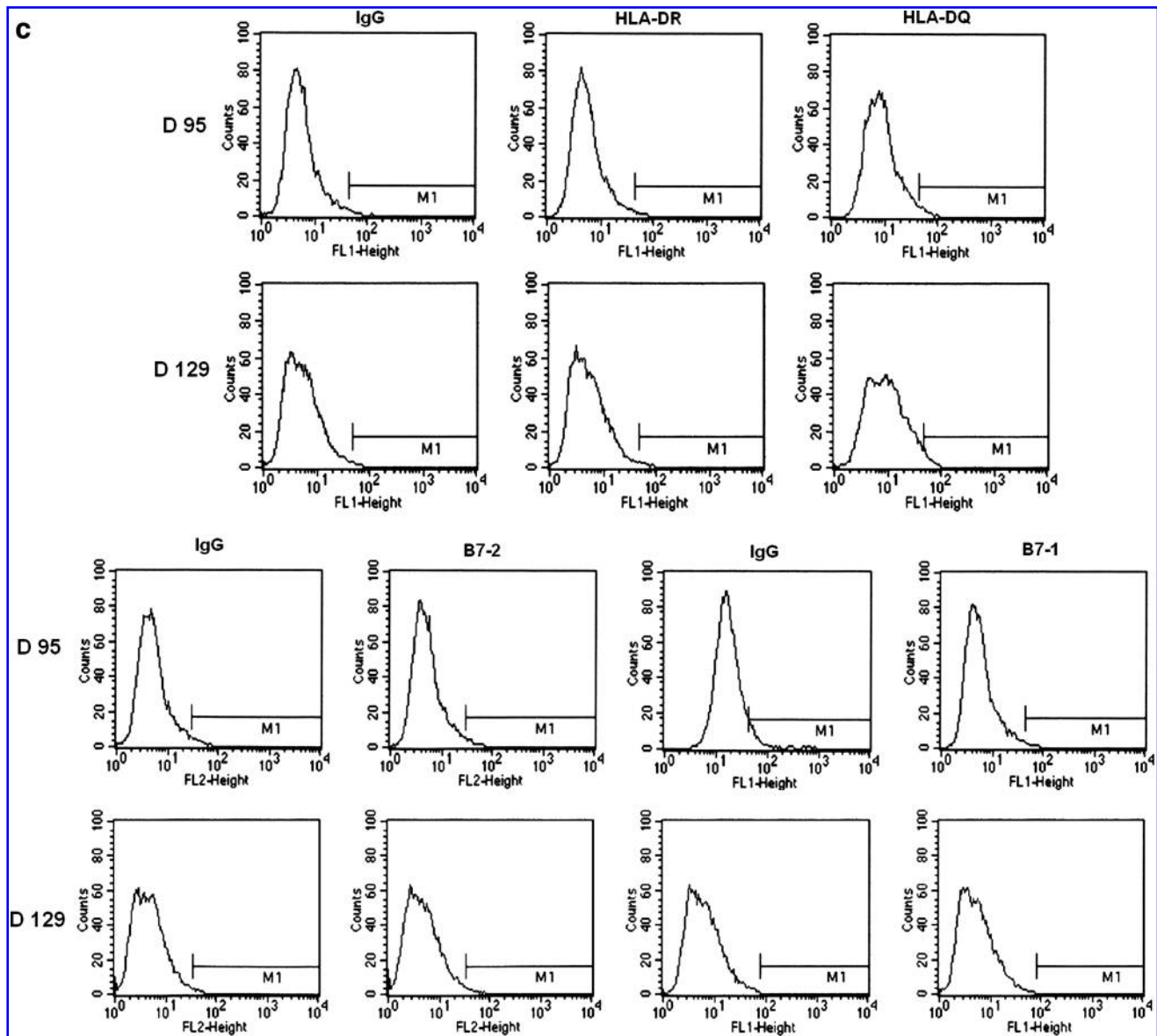
FIG. 6. (Continued).

to satisfy future demands and thus serve well as off the shelf allogenic stem-cell sources. Although relatively longer derivation time than previously reported protocols can be a limitation of our protocol, expansibility and storability of hESC-MSCs obtained by our protocol offset the potential problems related with longer derivation time.

Fourth, our hESC-MSCs have potentials to obtain immune privileges like other hMSCs.[7] In small pilot study using myocardial cryoinjury, improvement of cardiac function after SNU3MSC-1 transplantation is comparable between immunocompetent mice and athymic nude mice. These results suggest that SNU3MSC-1 is immunetolerant *in vivo* model like most of the adult MSCs. Immune tolerability of hMSCs makes HLA-mismatched allogenic hMSC transplantation possible in clinical trials. Our hESC-MSCs did not express HLA-DR, DQ, and costimulators B-7.1 and B-7.2. Nonexpression of these surface antigens might contribute to immune tolerability of hESC-MSCs. However, it should be confirmed in the future studies.

In the future clinical application of hESC-MSCs, the clonal derivation from single cells is very useful. However, most reported hESC lines are not clonally derived and also have a potential to contain multiple precursor cells that are committed to certain lineages. In addition, several hESC lines are reported to develop abnormal karyotype under certain culture conditions. Recently, Heins *et al.* reported clonal derivation of hESC line that had karyotype of trisomy.[22] Although we could not detect abnormal karyotype in hESC lines used, we cannot clearly exclude the possibility of heterogeneity of original cells. For that reason, we tried clonal derivation from early outgrowing cells at protocol day 40 without selection by a specific marker. However, in this experiment we failed to achieve consistent clonal expansion of hESC-MSCs from a single cell. In animal study, we observed improvement of cardiac function in cell transplantation group. However, improvement of cardiac function in cell-transplanted group could not be maintained till later phase of observation. Mechanism of hESC-MSCs func-

tionality in *in vivo* model needs to be analyzed in further studies.

In summary, our new EB-based method to derive hMSCs from hESCs is characterized as generalized applicability and safety. This protocol will provide a more effective platform for the production of large amount of genetically identical hMSCs from each hESC line.

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Disclosure Statement

The authors have no conflicts of interest.

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