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Genomic and functional comparison of mesenchymal stromal cells prepared using two isolation methods

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Abstract

Background aims. Mesenchymal stromal cells (MSCs) have been applied to patients in cell therapy for various diseases. Recently, we introduced a novel MSC separation filter device which could yield approximately 2.5-fold more MSCs from bone marrow in a closed system compared with the conventional open density gradient centrifugation method. MSCs isolated with these two methods were phenotypically similar and met the criteria defining human MSC proposed by the International Society for Cellular Therapy. However, these criteria do not reflect the functional capacity of MSCs. It has been shown that the donor, source, isolation method, culture condition and cryopreservation of MSCs have potential to alter their therapeutic efficacy. To determine the equivalency of MSCs isolated by these two methods, we compared their genomic profiles as an index of their biologic potential and evaluated their growth promoting potential as an index of function. *Methods.* The gene expression profiles of human MSCs isolated from 5 healthy donors with two distinct methods were obtained from microarray analyses. The functional activity of freshly expanded/cryopreserved MSCs from these two isolation methods was evaluated using an *in vitro* chondrocyte proliferation assay. *Results.* Freshly expanded MSCs isolated by these two methods were found to exhibit similar gene expression profiles and equivalent therapeutic effects, while freshly thawed, cryopreserved MSCs lacked all measureable therapeutic activity. *Conclusions.* The MSC separation device generates genomically and functionally equivalent MSCs compared with the conventionally isolated MSCs, although freshly thawed, cryopreserved MSCs, isolated by either method, are devoid of activity in our bioassay.

Key Words: *bone marrow MSC, cryopreservation, functional assay, gene expression profile*

Introduction

Mesenchymal stromal cells (MSCs), frequently termed mesenchymal stem cells, are one of the most investigated cell types in cell therapy for various diseases. Accumulating data have demonstrated that MSCs, a heterogeneous population of *ex vivo*–expanded cells [1], exert therapeutic effects by differentiating into target cells, secreting trophic factors which can stimulate or protect endogenous cells, and releasing immunomodulatory molecules which suppress immune cells, even though the detailed mechanism(s) underlying the therapeutic effects has not been fully elucidated [2–5].

The conventional protocol most commonly used in preclinical and clinical studies to isolate MSCs from bone marrow is centrifugation over a density gradient followed by *ex vivo* expansion in culture, which removes hematopoietic cell contamination. However, this conventional procedure is an open system that has risk of bacterial contamination. Moreover, the cell recovery from bone marrow with this method is variable between operators and technical expertise is required to consistently obtain MSCs with high efficiency. We have been using this conventional method to isolate MSCs from bone marrow in our clinical trials of MSC therapy for patients with osteogenesis imperfecta (OI), a genetic bone disorder caused by mutations in type 1 collagen

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[6,7]. After MSC infusion, OI patients demonstrated an acute acceleration of bone growth. In these clinical trials, patients received infusions of 1–5 × 10^6 MSCs/kg body weight, which requires 50 mL of donor bone marrow to isolate sufficient MSCs by the conventional method without excessive expansion in culture [6,7]. If repeated infusions of MSCs are needed to establish this cell therapy, substantially more bone marrow will be required. Thus, it would be of great benefit for donors to use protocols that allow more efficient MSC isolation from bone marrow compared with the conventional methods.

Recently, we introduced a novel MSC separation filter device that allows bone marrow processing in a closed system without centrifugation [8]. Importantly, we showed that the device yielded approximately 2.5-fold more MSCs at passage 2 than the conventional methods from the same initial volume of bone marrow. The processing time using the device was about 20 minutes; the conventional method typically takes more than 1 hour. MSCs isolated by these two methods expressed CD105, CD73 and CD90 but not CD45, CD34, CD11b, CD19 or HLA-DR. Together with the observation that MSCs isolated from both methods successfully differentiated into osteoblasts, adipocytes and chondrocytes *in vitro*, both cell preparations met the defining criteria proposed by the International Society for Cellular Therapy (ISCT) in 2006 [9], suggesting that the device can be used to isolate MSCs from bone marrow for cell therapy in lieu of the conventional method.

Although these two MSC preparations are indistinguishable according to the ISCT criteria, they do not necessarily exert equivalent therapeutic activity, which is a key benchmark for clinical applications, because it has been shown that differences in donor, source, isolation method, and culture condition of MSCs could affect clinical outcomes [10–19].

Another strategic element that could alter the therapeutic effect of MSC is cryopreservation. With the cell therapy industry supplying Good Manufacturing Practice (GMP)-prepared MSCs, most investigators have utilized cryopreserved MSCs immediately after thawing in their clinical trials [20–24]. The availability of these "off-the-shelf" MSCs is necessary to provide MSC therapy to patients at hospitals without GMP facilities. In contrast to freshly expanded MSCs, however, Francois *et al.* [25–28] reported that cryopreserved MSCs failed to achieve therapeutic effects in acute graft-versus-host disease (GvHD), raising questions about the therapeutic equivalency of freshly expanded and freshly thawed, cryopreserved MSCs.

In this study, we compared gene expression profiles obtained by microarray analysis between MSCs isolated with the MSC separation filter device and the conventional density gra... thod to examine the fundamental character and biologic potential of these MSCs. Additionally, we evaluated the therapeutic potential for growth promotion and bone regeneration using our *in vitro* chondrocyte proliferation assay, which we developed in the course of studying the mechanism of MSC-stimulated bone growth in patients with OI [6,7]. Finally, by use of the proliferation assay, we examined whether cryopreserved MSCs have the potential to maintain their therapeutic activity in the treatment of OI.

Methods

Mesenchymal stromal cells

MSCs were isolated and expanded as previously described [8]. Briefly, nucleated cells were isolated from bone marrow of five healthy donors (ages between 22–52 years, sample 3 is from a male donor and other samples are from female donors) through the use of two distinct methods, density centrifugation with lymphocyte separation medium (LSM; MP Biomedicals, LLC, Solon, OH, USA) and the Bone Marrow MSC Separation Device (KANEKA CORPORATION, Tokyo, Japan). Isolated bone marrow nucleated cells were cultured in Dulbecco's modified of Eagle's medium (DMEM; Corning Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA, USA) to establish MSCs. MSCs at passage 3 were collected and used for RNA isolation and for infusion into mice.

Chondrocyte isolation

Primary chondrocytes were isolated from 2–5 days old neonatal C57BL/6 mice as previously described [7]. Briefly, femoral and humeral heads were dissected under a stereo microscope and treated with 0.25% Trypsin solution (Corning Cellgro) for 20 minutes at 37°C to remove attached soft tissues, followed by digestion with 86.5 U/mL collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C overnight. After dissociation by pipetting and passing through a cell strainer, the isolated chondrocytes were used for the chondrocyte proliferation assay.

Chondrocyte proliferation assay

Freshly expanded or cryopreserved MSCs, 1 × 10^6 in 300 μL of phosphate-buffered saline (PBS), or 300 μL of PBS as a control were infused intravenously into 6- to 8-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA), and serum was

collected after 7 days. Freshly isolated chondrocytes were plated in 96-well plates at 5000 cells/well and incubated in DMEM with 10% FBS overnight. Chondrocytes were then washed and maintained in DMEM containing 0.3% FBS overnight. The culture medium was replaced with DMEM supplemented with 1% of serum samples collected from MSC or PBS infused mice. After 6 days of incubation, the chondrocyte proliferation was evaluated with the use of a CyQuant NF Cell Proliferation Assay Kit (Life Technologies). Fluorescence intensity was measured with a Synergy HT microplate reader (BioTek, Winooski, VT, USA).

RNA isolation

RNA was isolated from 80% confluent MSCs at passage 3 with the use of Trizol Reagent (Ambion, Life Technologies, Grand Island, NY, USA). RNA was quantified with the use of a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA quality was evaluated with the use of an Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA). RNA preparations with an RNA integrity number ≥9.0 were used for complementary DNA (cDNA) synthesis.

Microarray

Sense-strand single-stranded cDNA was prepared from 200 ng of total RNA with the use of the Ambion WT Expression Kit (Ambion, Life Technologies). Both complementary RNA and cDNA were quantified with the use of a NanoDrop spectrophotometer. Single-stranded cDNA (5.5 μg) was fragmented and labeled using the Affymetrix GeneChip WT Terminal Labeling kit (Affymetrix, Santa Clara, CA, USA). Affymetrix GeneChip Hybridization Wash and Stain Kit protocol (Affymetrix) was used for all hybridization, wash and staining steps. Fragmented and labeled cDNA (130 μL at 25 ng/μL) was hybridized for 17 hours at 45°C to Affymetrix GeneChip Human Gene 2.0ST Arrays (Affymetrix). Chips were washed and stained on an Affymetrix Genechip Wash Station 450 using the FS450_0002 protocol and scanned on the Affymetrix GeneChip Scanner 3000 (Affymetrix). Cell files were generated for analysis with the use of Affymetrix GeneChip Command Console v3.2.

Array data analysis

All microarray data were analyzed (correlations/clustering) with the use of the R (2.15.3) statistical language [29] and Bioconductor [30]. Data were processed and normalized by the RMA algorithm with the use of the oligo package [31] and visualizations were generated using the ggplot2 [32], pheatmap [33] and scatterplot3d [34] packages. For the heat map, euclidean distance and complete linkage options were used for clustering the data.

Cryopreservation

MSCs at passage 3 were harvested and resuspended in cell-freezing medium containing 70% (vol/vol) DMEM, 20% (vol/vol) FBS and 10% (vol/vol) dimethylsulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) at a density of 1×10^6 cells/mL into Cryogenic tubes (Nunc, Thermo Scientific, Waltham, MA, USA). Cells were frozen at −80°C overnight in freezing containers (Thermo Scientific) followed by transfer to a liquid nitrogen Locator System (Thermo Scientific). Frozen MSCs stored in liquid nitrogen for approximately 1 week were thawed in a water bath at 37°C and resuspended in DMEM with 10% FBS. Cell viability was assessed using trypan blue staining. After centrifugation at 300*g* for 5 minutes, cells were resuspended in PBS for injection at a density of 1×10^6 live cells per 300 μL or were re-plated in DMEM with 10% FBS at a density of 4000 cells/cm^2. These re-plated cells were cultured for 3 days, 7 days or 21 days and were then collected for injection.

Statistical methods

Data are presented as mean ± standard error of the mean. Differences were considered statistically significant by use of the paired or unpaired Student's *t*-test for comparison of two samples, and by one-way analysis of variance for multiple samples followed by Tukey's multiple comparisons test if they attained $P < 0.05$ (Prism, v6, GraphPad Software, Inc, La Jolla, CA, USA).

Results

Differential gene expression between MSCs isolated by means of two methods

To evaluate the similarities between the transcriptomes of MSCs isolated by two distinct methods from the same marrow donor, the gene expression profile of each MSC sample was acquired from Affymetrix GeneChip Human Gene ST Arrays, which contain 30,654 coding transcripts as well as 11,086 long intergenic non-coding transcripts. The microarray analysis was performed in duplicate for each MSC sample and the results were averaged to reduce the technical deviations. The gene expression level of MSCs collected with the MSC separation filter device (Device) was plotted against that of

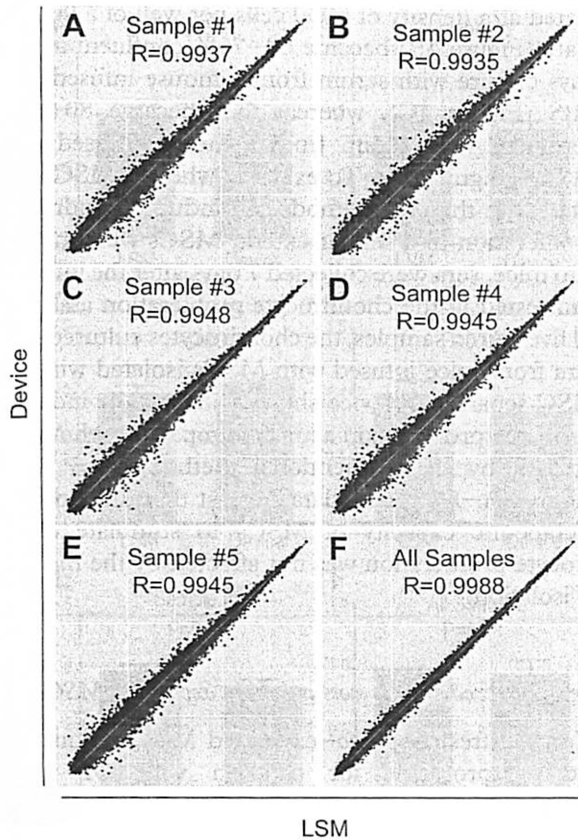

Figure 1. Comparison of gene expression profiles. The gene expression profiles of MSCs were obtained from cDNA microarray performed in duplicate. The profile of MSCs isolated with the conventional centrifugation method with the use of LSM was compared with that of MSCs isolated with the MSC separation device (Device) in dot plots and was analyzed by correlation coefficients (*R*). MSCs were isolated from five donors (A–E). Profiles of all five samples are combined (F).

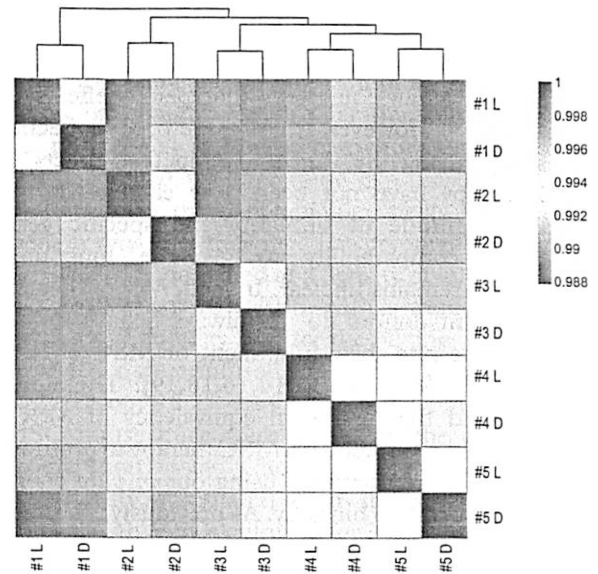

Figure 2. Heat map and hierarchical clustering analysis. Based on the correlation coefficients, the comparison of gene expression profiles between all MSCs was presented in a heat map. The hierarchical clustering analysis demonstrated the genomic similarity between MSCs. Samples labeled with an L were isolated with the use of LSM and samples labeled with a D were isolated using the filter device.

MSCs isolated by the conventional density gradient method with the use of LSM. The correlation coefficients (R) of all five samples ranged from 0.9935 to 0.9948 (Figure 1A–E) and that of overall device versus LSM was 0.9988 (Figure 1F), suggesting that MSCs isolated with two different methods from the same donor bone marrow are essentially identical.

A heat map based on the correlation coefficients of all comparable MSC combinations similarly demonstrated that gene expression profiles of MSCs isolated with the device (D) paralleled those isolated by the conventional method (L) from the same donor (Figure 2). Hierarchical clustering analysis of whole transcripts revealed that MSCs isolated from the same donor by use of each of the two methods clustered significantly closer to each other than MSCs from different donors irrespective of the isolation method (Figure 2), suggesting that different donors provide a greater diversity of gene expression and potentially biologic/therapeutic activity in the *ex vivo*–expanded MSCs than the specific method of isolation. Principal component analysis, used to visualize the distance between MSCs in gene expression profiles, corroborated the results of the hierarchical clustering analysis, demonstrating that MSCs isolated from the same donor but with two distinct methods clustered closely to each other, whereas MSCs from different donors were considerably more separated (Figure 3).

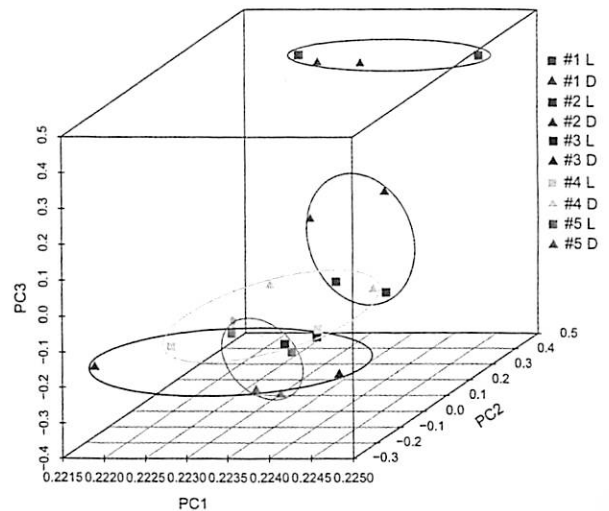

Figure 3. Principal component analysis. Duplicated profiles of each sample from both LSM and Device are shown in (□) and (Δ), respectively. Individual samples are grouped in a circle.

Functional analysis of MSCs isolated with two methods

MSCs with similar gene expression profiles are expected to show equivalent therapeutic effects in MSC therapy. However, because the precise mechanisms underlying the various therapeutic effects of MSC therapy have not been fully elucidated, the overall magnitude of similarity and specific gene expression comparability to generate functional equivalency remains unclear. Indeed, MSCs isolated from different donors do not always behave in the same way despite their fulfillment of MSC criteria proposed by ISCT [10,12,14,16,18,19]. Therefore, we examined the functional equivalency of MSCs isolated with two methods in MSC therapy to promote growth in children with OI using our *in vitro* chondrocyte proliferation bioassay. As previously reported, we developed this assay in the course of studying the underlying mechanism of bone growth after MSC infusion observed in our clinical trial of MSC therapy for OI patients [7]. When MSCs are intravenously infused into mice, we found that factor(s) secreted from the MSCs induced production of second factor(s) released into the serum, which stimulated chondrocyte proliferation both *in vitro* and *in vivo*. We also demonstrated that the serum concentration of this chondrocyte proliferation factor was maximal at 7 days after MSC infusion [7]. In this assay, MSC activity was tested indirectly by measuring the chondrocyte proliferation factor activity in serum. Cell number was determined by measuring the level of fluorescent dye incorporated into cellular DNA using a fluorescent plate reader (Figure 4A). Chondrocytes plated at a density of 5000 cells per well of a 96-well plate (Figure 4B) became 60–70% confluent after 6 days culture with serum from a mouse infused with PBS (Figure 4C), whereas they became 80–90% confluent with serum from a mouse infused with MSCs (Figure 4D). To examine whether MSCs isolated with the two methods can induce chondrocyte proliferation to a similar extent, MSCs were infused into mice, sera were collected 7 days after the infusion and tested in the chondrocyte proliferation assay. In all five paired samples, the chondrocytes cultured with sera from mice infused with MSCs isolated with the MSC separation device showed statistically indistinguishable proliferation activity compared with MSCs isolated by the conventional method ($P = 0.38$, Figure 5A–F). These data suggest that the biologic/therapeutic capacity of MSCs to stimulate chondrocyte proliferation was not affected by the method of isolation.

Cryopreserved MSCs versus freshly expanded MSCs

We used fresh, *ex vivo*–expanded MSCs in our cell therapy protocols for children with OI [6,7]. However, banked "off-the-shelf" cryopreserved MSCs would simplify delivery of this treatment and would obviate the need of GMP facilities rendering it more widely available. However, this approach raises critical questions as to whether freshly thawed cryopreserved MSCs are therapeutically equivalent to freshly expanded MSCs. Thus, using the chondrocyte proliferation assay, we examined whether

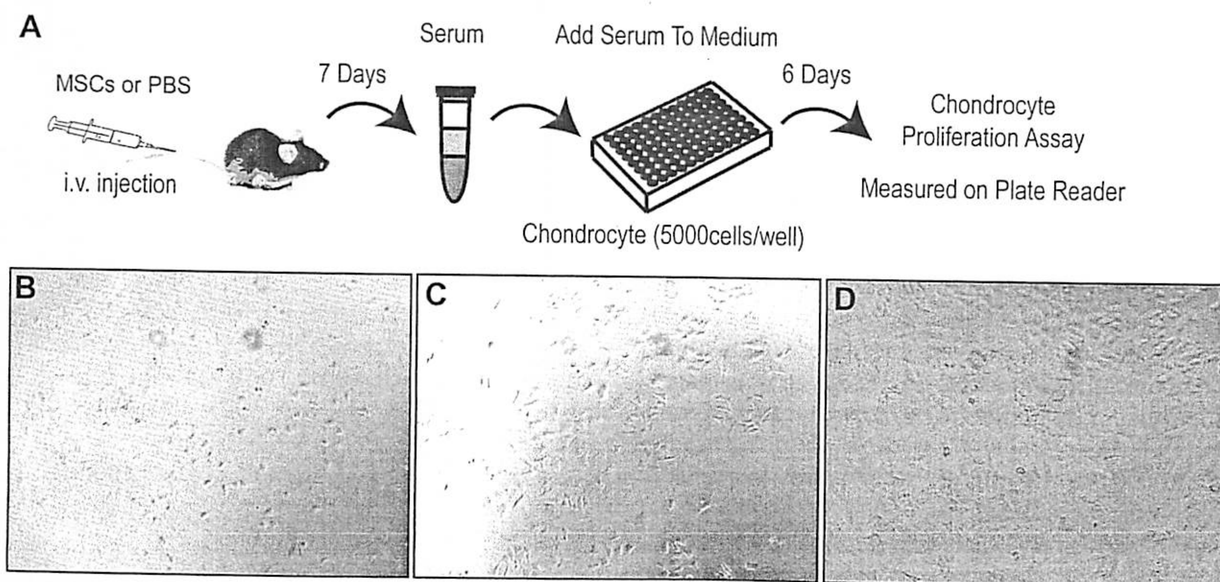

Figure 4. Schematic protocol of *in vitro* chondrocyte proliferation assay. 1×10^6 MSCs or PBS as a control were intravenously infused into a mouse. The serum was collected 7 days after the infusion. Freshly isolated chondrocytes were cultured in the presence of the serum for 6 days, and proliferation was measured on a plate reader (A). Chondrocytes plated at a density of 5,000 cells/well (B) proliferated up to 60–70% or 80–90% confluent after 6-day culture with serum from a mouse infused with PBS (C) or MSC (D), respectively.

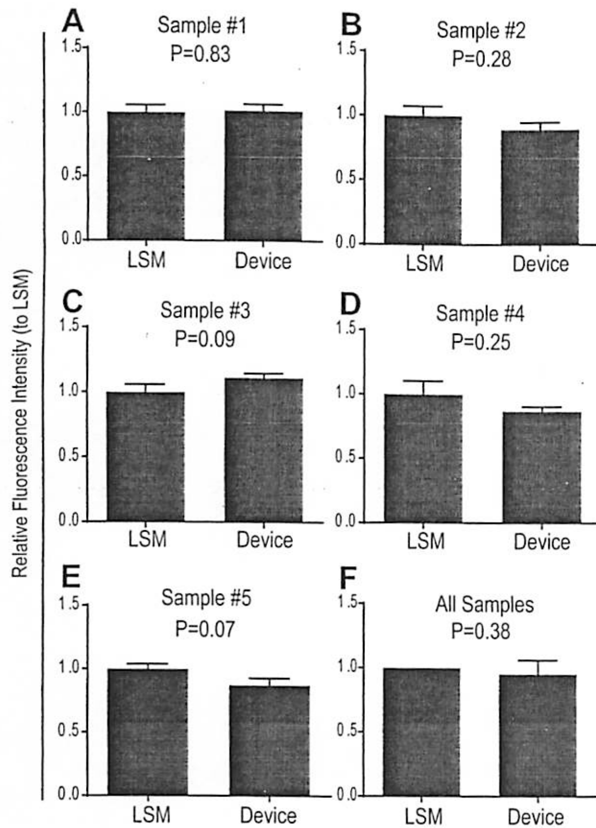

Figure 5. Functional comparison of MSCs. Using the *in vitro* chondrocyte proliferation assay, MSC activity to induce chondrocyte proliferation was evaluated. The activity of MSCs isolated with the device was compared with that of MSCs isolated the use of LSM. The proliferation of chondrocytes was normalized to the LSM group, and the relative proliferation is shown. Five individual samples (A–E) and the combined result of all five samples (F) are presented as mean ± standard error of the mean.

cryopreserved MSCs can induce chondrocyte proliferation equivalently to freshly expanded MSCs. MSCs at passage 3 were collected and cryopreserved and stored in a −80°C freezer for 1 day, then in liquid nitrogen for 6 days. The cryopreserved MSCs were thawed, viability confirmed with trypan blue staining (ranged from 87.6% to 93.3%), and then infused into mice at a dose of 1×10^6 live cells per mouse. Flow cytometric analysis demonstrated that cryopreservation did not alter the phenotype of the MSC (Supplementary Figure 1). Fresh, *ex vivo*–expanded MSCs and PBS were also infused into mice as positive and negative controls, respectively. Serum was collected 7 days after the infusion for analysis. Chondrocyte proliferation was significantly stimulated with the sera from mice infused with fresh, *ex vivo*–expanded MSCs, whereas the sera from cryopreserved MSC infused mice failed to stimulate chondrocyte proliferation, using either isolation method (Figure 6A,B). These results were replicated in two MSC samples from different donors ($P = 0.004$ and $P < 0.0001$, respectively, Figure 6A,B), suggesting that cryopreserved MSCs are functionally altered and cannot exert therapeutic effects on chondrocyte proliferation in our bioassay and presumably in MSC therapy for OI patients. We then examined whether these cryopreserved MSCs could recover their therapeutic effects by culturing them *ex vivo* after thawing. The cryopreserved MSCs were thawed and plated at a density of 4000 cells/cm^2 in DMEM with 10% FBS. MSCs were collected at day 3, day7 and day 21 after the plating, and 1×10^6 live MSCs were infused into a mouse (cell viability ranged from 95.2% to 98.9%). The sera from these MSC infuse mice were tested on the chondrocyte proliferation assay. Chondrocyte proliferation was significantly stimulated with the sera from mice infused with MSCs cultured for 3 days as well as for 7 and 21 days after thawing (Figure 6C). The similar recovery of the stimulatory activity by culturing after thawing was observed in different donors irrespective of the isolation methods (Figure 6D–F). MSCs cultured for longer periods appeared to have higher activity; however, the chondrocyte proliferation with the sera from mice infused with 21-day cultured MSCs was not statistically different from that with the sera from mice infused with 3-day cultured MSCs, suggesting that 3 days in culture is long enough for cryopreserved MSCs to recover their therapeutic effects. Consistent with our initial findings (Figure 5), the isolation method did not affect the outcome, demonstrating that MSCs isolated with two different methods are functionally equivalent as measured in the chondrocyte proliferation bioassay.

Discussion

The minimal criteria to define human MSC proposed by ISCT in 2006 have been accepted and widely utilized by many investigators to characterize their cells of study [9]. However, it has also been demonstrated that MSCs from different sources, donors, and culture conditions do not always behave in the same way in cell therapy even though they meet the ISCT criteria [10–19]. One possible reason for this discrepancy is that MSCs have many features such as multipotency, secretion of trophic factors and immunomodulatory function, which are critical to exert their therapeutic effects; however, the ISCT criteria do not reflect these functional aspects of MSCs [12]. Recently, we introduced a novel filter device which allowed the isolation of approximately 2.5-fold more MSCs than the conventional density gradient centrifugation protocol from the same initial volume of bone marrow [8]. Moreover, MSCs obtained from the device are phenotypically

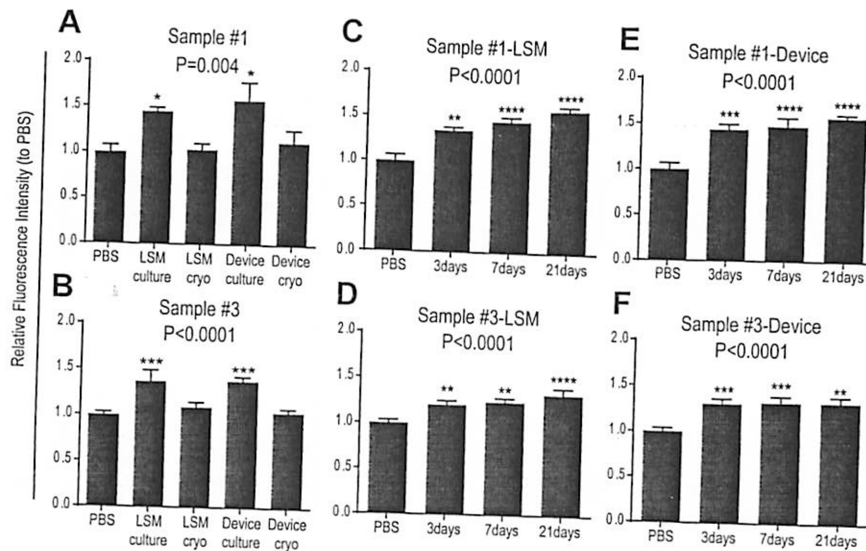

Figure 6. Functional comparison between freshly expanded MSCs and cryopreserved MSCs. Freshly expanded MSCs (culture) and cryopreserved MSCs (cryo) isolated both with LSM and the device from two donor bone marrow were infused into mice, and the sera were tested by the *in vitro* chondrocyte proliferation assay (A and B). Freshly thawed cryopreserved MSCs isolated with two methods from two donors were cultured for 3 days, 7 days and 21 days followed by infusion into mice. The chondrocyte proliferation assay was performed with the sera from these mice (C–F). All data were normalized to the PBS control and are shown as mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$, compared with PBS.

indistinguishable from MSCs isolated by use of the conventional method and both preparations yield cells that meet the ISCT criteria for MSCs [8]. In this study, we have shown that the global gene expression profile and at least one biologic activity of MSCs, chondrocyte proliferation in our bioassay as an indicator of growth-promoting potential in children with OI, are indistinguishable between the two preparations. Biostatistical analyses of gene expression profiles of over 30,000 genes showed that MSCs isolated by both methods from the same donor were indistinguishable (correlation coefficients: $R > 0.99$); however, substantial variance, far greater than that caused by isolation method, was found due to differing donors. Indeed, as shown in Figure 3, sample 1 isolated with the two methods was genomically distinct from the other samples. Further biostatistical analyses identified 29 upregulated and 13 downregulated genes in sample 1 compared with the other samples. However, given that sample 1 and sample 3 showed functional similarity in the chondrocyte proliferation assay in Figure 6, these are less likely to be the specific genes encoding key molecules necessary for therapeutic effects. With the known variability among donors, more samples and further examination will be necessary to identify the critical molecules within the mechanism of therapeutic action. However, comparing the two methods of initial isolation evaluated in our study, the isolation method did not affect the quality of the final cell product. Additionally, these data suggest that MSCs from the same marrow donor in separate flasks are transcriptionally identical, justifying the use of a sentinel flask when expanding cells in a multi-layer cell culture vessel.

Importantly, we have developed a laboratory bioassay measuring the capacity of MSCs to stimulate chondrocyte proliferation [7] which presumably underlies the growth promoting activity observed in patients with OI [6]. Thus, these data suggest that these two cell isolation procedures may be equally suited for clinical applications, but donor selection remains vital.

In this study, human MSCs infused into mice induced the second factor, in the serum, capable of chondrocyte proliferation *in vitro* (Figure 6). These data suggest that the single infusion of human cells may not elicit an immune response consistent with others showing a therapeutic effect in animal models of diseases treated with human MSCs [35,36]. Alternatively, the interval required to mount a primary immune response might be sufficient for the MSC-secreted factor to induce the secondary murine serum factor prior to immunologic elimination.

As cell banking systems are becoming globally available, the application of cryopreserved MSCs in cell therapy is attracting increasing attention [37]. The advantages of using cryopreserved MSCs immediately after thawing are (i) to make MSC therapy available even at institutes that do not have GMP facilities, (ii) to make MSCs available when needed without the time-consuming process of preparation from bone marrow or other tissues, which is beneficial for urgent

demands such as in ischemic cardiac diseases and (iii) to allow the selection of the most suitable MSCs for diseases and patients from the banked "off-the-shelf" MSCs. It has been shown that MSCs recovered from cryopreservation are phenotypically indistinguishable from continuously cultured MSCs and maintain tri-lineage differentiation potential [38–42]. However, several recent reports demonstrated that, contrary to freshly expanded MSCs, freshly thawed cryopreserved MSCs failed to show immune suppressor activity, resulting in no therapeutic effect on patients with GvHD [25–28]. Similarly, we have demonstrated in this study that cryopreserved MSCs were not able to induce the production of chondrocyte proliferation factor when infused immediately after thawing. Interestingly, the therapeutic effects of cryopreserved MSCs were restored by 3 days of *ex vivo* culture after thawing. This functional recovery of cryopreserved MSCs in immune suppressor activity was also reported by Francois *et al.* [26], in which they showed that 24 hours of culture was enough to restore the activity. Taken together with the results from the GvHD studies, our findings indicate that cryopreserved MSCs are functionally distinct from freshly expanded MSCs and further identification of the mechanism underlying the therapeutic effects provided by MSCs is necessary to uncover the functional discrepancy between cryopreserved and freshly expanded MSCs. Interestingly, we have always used freshly expanded MSCs in our clinical trials based on an unfounded, at the time, concern regarding the consequences of cryopreservation. In retrospect, the use of freshly expanded MSCs seems to have been essential to the success of our first MSC clinical trial, which was the first application of allogeneic MSCs in human subjects [6].

Given that the use of banked "off-the-shelf" MSCs is critical to advance the use of MSC therapy and make it more widely available as one of regular treatments, novel methods of cryopreserving MSCs that retain their functional activities and/or new protocols to retrieve their activities immediately after MSC thawing without further culture are critically needed.

This study advances our previous findings that the MSC separation filter device generated nearly 2.5-fold more MSCs than the conventional density gradient centrifugation method, that both MSC preparations meet criteria proposed by ISCT, that the resulting MSCs were genomically and functionally nearly identical, and that the variance from the isolation methods was much smaller than that from donor differences, suggesting that this MSC separation filter device can be a more efficient option to isolate MSCs from bone marrow than the conventional method. Moreover, as seen in the GvHD studies, cryopreserved MSCs failed to provide the stimulatory activity on chondrocyte proliferation and, under current technology, cannot replace cultured MSCs in the treatment of OI.

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Supplementary data

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