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Generation of priming mesenchymal stem cells with enhanced potential to differentiate into specific cell lineages using extracellular matrix proteins

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ABSTRACT

Poor understanding of the differentiation of mesenchymal stem cells (MSCs) has resulted in a low differentiation yield, and has hindered their application in medicine. As a solution, priming MSCs sensitive to signaling, thus stimulating differentiation into a specific cell lineage, may improve the differentiation yield. To demonstrate this, priming MSCs were produced by using a gelatin matrix for the isolation of primary MSCs from bone-marrow-derived primary cells. Subsequently, cellular characteristics and sensitivity to specific differentiation signals were analyzed at passage five. Compared to non-priming MSCs, priming MSCs showed no significant differences in cellular characteristics, but demonstrated a significant increase in sensitivity to neurogenic differentiation signals. These results demonstrate that generation of priming MSCs by specific extracellular signaling increases the rate of differentiation into a cell-specific lineage.

1. Introduction

Mesenchymal stem cells (MSCs), which possess the potential to differentiate into multi-lineage precursor cells, have been employed as a therapeutic tool in regenerative medicine [1–5]. Due to the need for a system that induces differentiation into a specific tissue, these cells have been the focus of much cell therapy research [6,7]. To date, MSC differentiation has been associated with a high cost due to the necessity of a continuous supply of differentiation factors and the low yield due to uneven reactivity resulting from the heterogeneity of MSC populations [8–10]. However, these rate-limiting factors in MSC differentiation may be overcome by generation of priming MSCs, which are more sensitive to specific differentiation signals. Accordingly, we provided gelatin-derived extracellular signals using a gelatin-coated matrix during isolation of primary MSCs from bone-marrow-derived primary cells, and produced priming MSCs with high potential to differentiate into neuronal lineage cells; moreover, these cells exhibited normal MSC characteristics.

2. Materials and methods

2.1. Animals

Three-week-old male Sprague–Dawley (SD) rats, which were used as bone marrow cell donors, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animal housing, handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-121101-1) and conducted according to the Animal Care and Use Guidelines of Kangwon National University.

2.2. Retrieval of bone-marrow-derived primary cells

Tibias and femurs were dissected from both legs of SD rats sacrificed by CO_2 asphyxiation, and washed with 1% (v/v) Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea)

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supplemented with 1% (v/v) antibiotic–antimycotic (Gibco Invitrogen, Grand Island, NY). Muscle tissues were removed from the bones, and the spongious end of each bone was cut to expose the marrow cavity. Subsequently, retrieval of bone-marrow-derived primary cells from each bone was conducted by flushing bones with 2% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) containing DPBS. Next, red blood cell (RBC) lysis buffer (Sigma–Aldrich, St. Louis, MO) was used to remove the RBCs from the collected bone-marrow-derived primary cells. The bone-marrow-derived primary cells were then enumerated using a hemocytometer.

2.3. Isolation of MSCs from bone marrow-derived primary cells

Bone-marrow-derived primary cells were cultured in 100-mm dishes coated with or without 1% (wt/v) gelatin in low glucose Dulbecco's modified Eagle's Medium (LG-DMEM; Welgene, Inc.) containing 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic–antimycotic at 37 °C in humidified 95% air and 5% CO_2. After 2 days in culture, non-adherent cells were washed off and fresh culture medium was replaced at 2–3-day intervals. After 14 days of culture, confluent MSCs were dissociated by addition of 0.25% trypsin–EDTA (Gibco Invitrogen). MSCs were then enumerated using a hemocytometer.

2.4. Culture and doubling time calculation of bone marrow-MSCs

BM-MSCs from each group 2×10^5 were cultured on 100-mm dishes coated without gelatin in LG-DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic–antimycotic at 37 °C in humidified 95% air and 5% CO_2 until 80% confluence. Subsequently, BM-MSCs were dissociated with 0.25% trypsin–EDTA and reseeded continuously at the same cell density and cultured under the same conditions until the fifth passage. The doubling time per passage was calculated as follows: $t\log_2/(\log N_t - \log N_0)$. Here, t is time to confluence, N_t is the number of cells at the end of the growth period and N_0 is the initial number of cells.

2.5. Flow cytometry

Cultured BM-MSCs were fixed with 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd, Chuo-ku, Japan) and rinsed in ice-cold DPBS. Fixed cells in DPBS containing 2% (v/v) FBS were stained for 45 min at 4 °C with APC-conjugated anti-CD90 (mesenchymal stem-cell-specific marker; BioLegend, San Diego, CA), APC-conjugated anti-CD29 (mesenchymal-stem-cell-specific marker; BioLegend), FITC-conjugated anti-CD45 (hematopoietic-cell-specific marker; BioLegend) and FITC-conjugated anti-CD31 (endothelial-cell-specific marker; Abcam, Cambridge, UK) antibodies, respectively. BM-MSCs differentiated into neuronal lineage cells were stained using an APC-conjugated anti-Nestin (BD Biosciences, San Jose, CA) antibody diluted in DPBS containing 0.01% (v/v) Triton-X-100 (Sigma–Aldrich) for 45 min at 4 °C. Supplementary Table 1 shows details of the primary antibodies and the dilutions used. After washing with DPBS, stained cells were sorted using a FACSCalibur (Becton, Dickinson and Co., Franklin Lakes, NJ); the BD CellQuest pro software (Becton–Dickinson Co.) was used for data analysis.

2.6. Adipogenic differentiation potential of BM-MSCs

Differentiation into adipocytes was carried out for 3 weeks in adipogenic differentiation medium, consisting of high-glucose DMEM (HG-DMEM; Welgene Inc.) supplemented with 10% (v/v) heat-inactivated FBS, 0.5 mM isobutylmethylxanthine (Sigma–Aldrich), 200 μM indomethacin (Sigma–Aldrich), 1 μM dexamethasone (Sigma–Aldrich), 10 μg/mL human insulin (Sigma–Aldrich), and 1% (v/v) antibiotic–antimycotic; fresh medium was replaced every third day. BM-MSCs undergoing adipogenic differentiation were fixed with 10% (v/v) paraformaldehyde for 1 h at room temperature, and then washed with 60% (v/v) isopropyl alcohol (Sigma–Aldrich) and dried completely. Subsequently, fixed cells were incubated for 10 min at room temperature in Oil-Red-O (Sigma–Aldrich) solution, consisting of distilled water supplemented with 60% (v/v) isopropyl alcohol, and 0.3% (wt/v) Oil-Red-O reagent. Isopropyl alcohol was used to wash off excess Oil-Red-O stain. After transferring the extracted solution into 96-well plates, absorbance

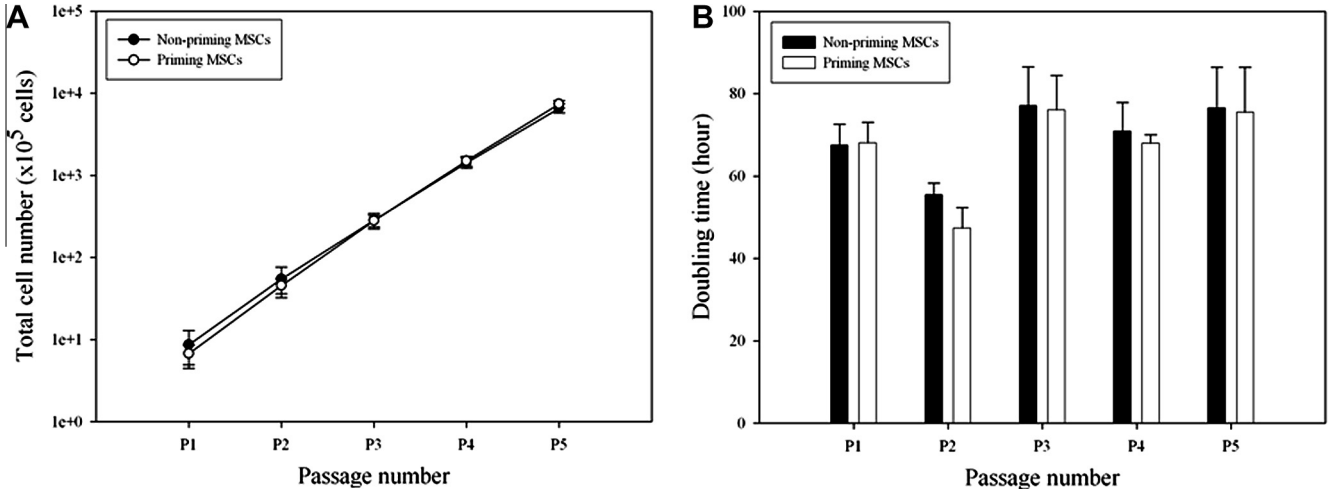

Fig. 1. Effects of gelatin-derived extracellular signals on proliferation of bone-marrow-derived mesenchymal stem cells (BM-MSCs). Bone-marrow-derived primary cells were cultured on plates coated with (priming MSCs) or without 1% (wt/v) gelatin (non-priming MSCs). After 14 days of culture, 2×10^5 BM-MSCs from each two-dimensional (2D) matrix condition per passage continued to be cultured on gelatin-free 100-mm culture plates until passage five. BM-MSCs at each passage were enumerated using a hemocytometer, and doubling time per passage was calculated using the following: equation $t\log_2/(\log N_t - \log N_0)$, where t is time to confluence, N_t is final cell number and N_0 is initial cell number. Exposure to gelatin-derived extracellular signals during primary culture had no effect on either the total number (A) or doubling time (B) per passage of BM-MSCs. Data represent means ± standard deviation (s.d.) of five independent experiments.

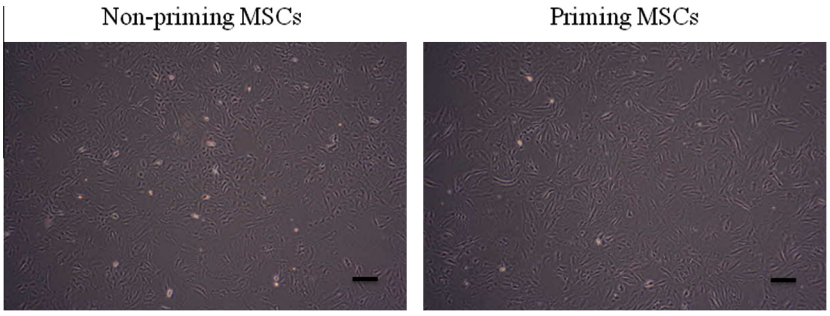

Fig. 2. Comparison of the morphologies of passage-five BM-MSCs cultured on 2D gelatin-free matrix post-culture of bone-marrow-derived primary cells on 2D matrix coated with (priming MSCs) or without 1% (wt/v) gelatin (non-priming MSCs). Exposure of MSCs to gelatin signals during primary culture had no effect on the typical fibroblast-like morphology. Scale bar, 200 μm.

measurements at 490 nm were performed using a microplate reader (Epoch; Biotek, Winooski, VT).

2.7. Osteogenic differentiation potential of BM-MSCs

Differentiation of BM-MSCs into osteoblasts was performed by incubation in osteogenic differentiation medium, consisting of LG-DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 nM dexamethasone, 10 mM β-glycerolphosphate (Sigma–Aldrich), 50 μM ascorbate-2-phosphate (Sigma–Aldrich), and 1% (v/v) antibiotic–antimycotic. After 2 weeks, differentiated cells were rinsed with DPBS, fixed with cold 70% (v/v) ethanol and washed with distilled water. Next, Alizarin Red staining (ARS) was conducted for 10 min in a 2% (wt/v) ARS solution (Sigma–Aldrich). Unincorporated dye was removed by washing four times with distilled water. Two millimolar $NaHPO_4$ (Sigma–Aldrich) supplemented with 10% (wt/v) cetyl-pyridinium chloride (CPC; Sigma–Aldrich) was used to extract the deposited alizarin-calcium complexes, and the extracted solution was transferred into 96-well plates. Alizarin Red dye was quantified by measuring absorption at 550 nm using a microplate reader (Biotek).

2.8. Neurogenic differentiation potential of BM-MSCs

BM-MSCs were incubated in pre-induction medium, consisting of HG-DMEM supplemented with 0.1 mM 2-mercaptoethanol (Gibco Invitrogen) and 2% (v/v) dimethylsulfoxide (DMSO; Sigma–Aldrich), for 5 h. Next, cells were exposed to neuronal induction medium, consisting of HG-DMEM supplemented with 10% (v/v) heat-inactivated FBS, 10 μg/L basic fibroblast growth factor (b-FGF; R&D Systems, Inc., Minneapolis, MN), 10 μg/L human epidermal growth factor (hEGF; R&D Systems, Inc.), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma–Aldrich), and 0.5 mM isobutylmethylxanthine (IBMX; Sigma–Aldrich) for 7 days. Cells differentiated into neurogenic lineage cells were rinsed with DPBS and harvested using 0.25% Trypsin–EDTA. Harvested cells were then subjected to flow cytometry.

2.9. Statistical analysis

All numerical data were analyzed using the Statistical Analysis System (SAS) software. Comparison among treatment groups was conducted using Duncan's method or the least-square difference, and the significance of main effects was evaluated by analysis of variance (ANOVA) using the SAS software. A value of $p < 0.05$ was regarded to indicate a statistically significant difference.

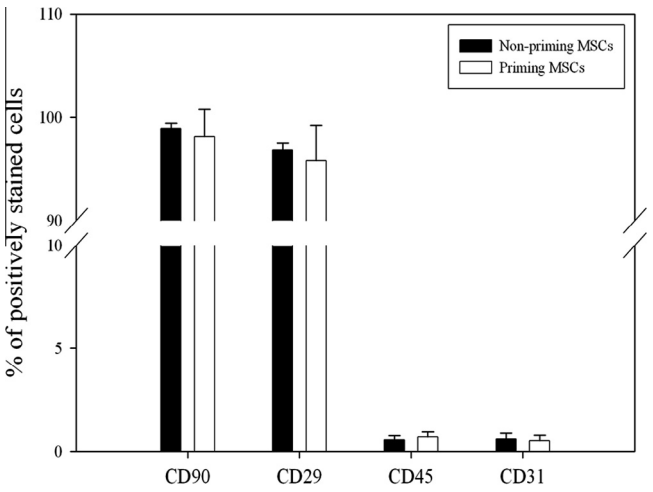

Fig. 3. Effects of gelatin-derived extracellular signaling on the expression of mesenchymal-stem-cell-specific marker proteins by BM-MSCs. After culturing of bone-marrow-derived primary cells on a 2D culture plate-coated with (priming MSCs) or without 1% (wt/v) gelatin (non-priming MSCs) for 14 days, cells from each condition were cultured on dishes coated without gelatin until passage five. Subsequently, flow cytometric analysis was conducted by staining cultured BM-MSCs with fluorescently conjugated primary antibodies against CD90 and CD29 (mesenchymal-stem-cell-specific markers), CD45 (hematopoietic-stem-cell-specific marker), and CD31 (endothelial-cell-specific marker). Gelatin-derived extracellular signals had no effects on the expression of BM-MSC-specific, hematopoietic-stem-cell-specific and endothelial-cell-specific marker proteins. Data represent the means ± s.d. from three independent experiments.

3. Results

3.1. Effects of gelatin-derived extracellular signaling on the characteristics of MSCs

We determined the effect of exposure of MSCs to gelatin-derived extracellular signals during isolation on their characteristics. The proliferation, doubling time per passage, cellular morphology and surface marker expression at passage five of MSCs cultured on plates without gelatin post-culture of bone-marrow-derived primary cells on gelatin-coated (priming MSCs) or -free (non-priming MSCs) culture plate were investigated.

Priming MSCs showed no significant difference in proliferation per passage compared to non-priming MSCs (Fig. 1A); moreover, no significant difference in doubling time per passage was found (Fig. 1B). In addition, no alteration of the typical fibroblast-like morphology was found in MSCs exposed to gelatin signaling (Fig. 2). Regarding surface marker expression, priming MSCs showed positive staining for the MSC-specific marker proteins

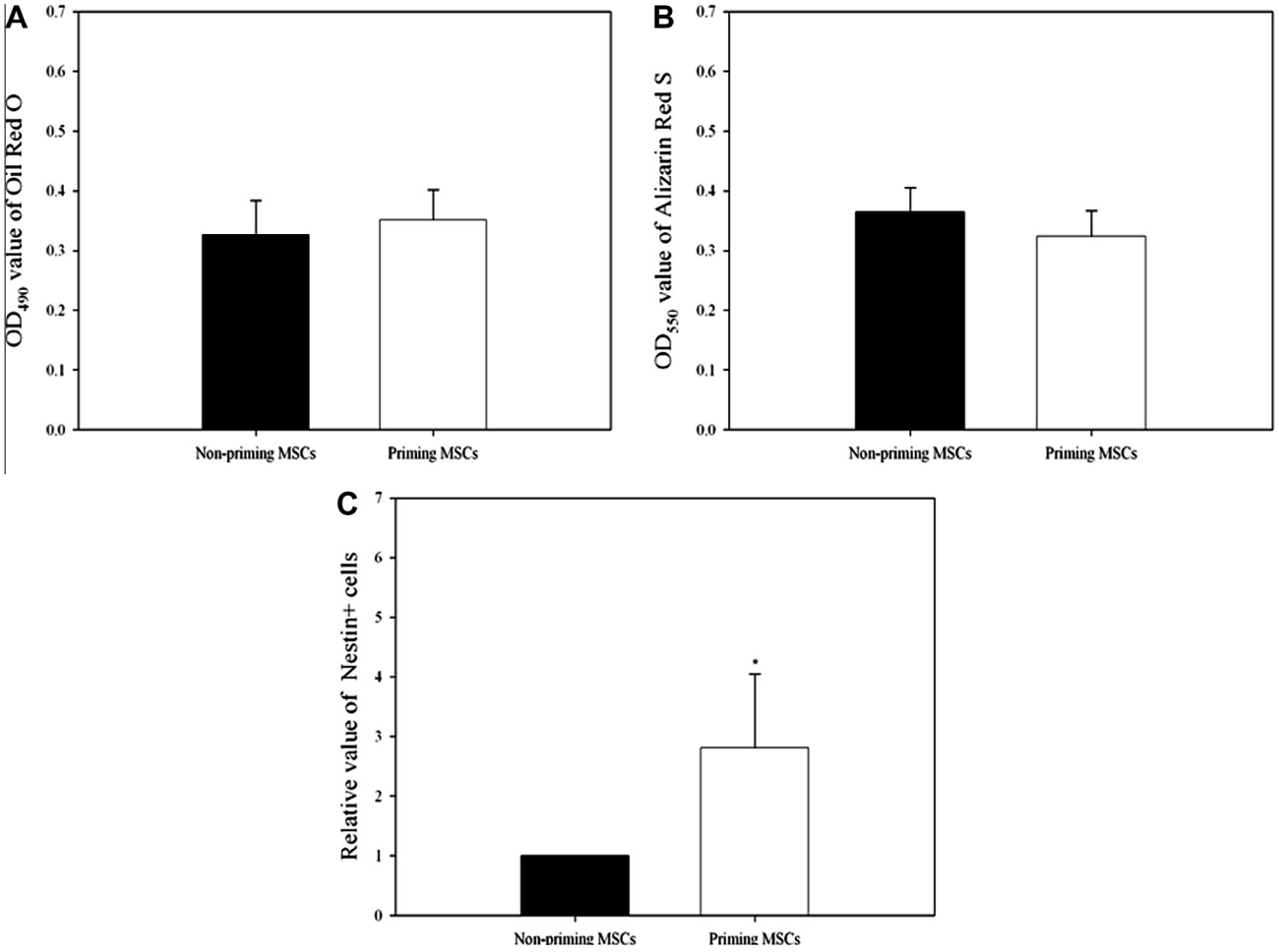

Fig. 4. Effects of gelatin-derived extracellular signals on the differentiation potential of BM-MSCs. MSCs were isolated by culturing bone-marrow-derived primary cells on 2D culture plates coated without (non-priming MSCs) or with 1% (wt/v) gelatin (priming MSCs) for 14 days. Subsequently, primary MSCs from each group continued to be cultured on a 2D matrix without gelatin-derived extracellular signaling, and the differentiation potentials of BM-MSCs into adipogenic-, osteogenic- and neurogenic-lineage cells were quantified at passage five. (A) In the case of adipogenic differentiation, after culture in the presence of adipogenic differentiation medium for 3 weeks, differentiation into adipocytes was assayed by Oil-Red-O staining and quantified by measuring the absorbance at 490 nm. Gelatin-derived extracellular signals had no effect on primary cells' differentiation potential into adipocytes, compared to non-priming MSCs. (B) Regarding osteogenic differentiation, BM-MSCs were incubated for 2 weeks in osteogenic differentiation medium, and BM-MSC osteoblastic differentiation was assayed by identifying calcium deposits stained positively by alizarin red staining (ARS), by measuring the absorbance at 550 nm. No significant difference in osteogenic differentiation potential between non-priming MSCs and priming MSCs was detected. (C) In the case of neurogenic differentiation, differentiation of BM-MSCs into neurogenic-lineage cells was conducted by incubating cells for 7 days in neurogenic differentiation medium. Next, flow cytometric analysis was conducted after staining with a fluorescence-conjugated primary antibody against Nestin (neural lineage marker). Priming MSCs showed a significantly higher percentage of Nestin-positive cells than did non-priming MSCs. Data represent means ± s.d. from three (C) or four (A and B) independent experiments. $^{*}p < 0.05$.

CD90 (98.14 ± 2.62%) and CD29 (95.81 ± 3.44%), but not for a hematopoietic lineage marker protein (CD45; 0.72 ± 0.24%) or an endothelial lineage marker protein (CD31; 0.54 ± 0.25%) (Fig. 3). Moreover, no significant increase or decrease in total surface marker protein expression was detected in priming MSCs compared to non-priming MSCs. Therefore, gelatin-derived extracellular signals during MSC isolation had no effect on their cellular characteristics.

3.2. Effects of gelatin-derived extracellular signals on MSC sensitivity to differentiation signals

Sensitivity of MSCs to signals inducing adipogenic, osteogenic and neurogenic differentiation was investigated by evaluating the ratios of differentiation into adipogenic-, osteogenic- and neurogenic-lineage cells at passage five. A significant increase in the ratio of differentiation into neurogenic (Fig. 4C) lineages was detected in priming MSCs, whereas gelatin-derived signals had no effect on adipogenic (Fig. 4A) or osteogenic differentiation (Fig. 4B). Therefore, exposure of primary MSCs to gelatin-derived extracellular signals contributes to production of priming MSCs highly sensitive to differentiation signals, thus promoting differentiation into neurogenic-lineage cells.

4. Discussion

In this study, we exposed MSCs to gelatin-derived extracellular signaling by culturing on a gelatin-coated matrix during isolation from bone marrow-derived primary cells. This resulted in a significant increase in their differentiation into neurogenic lineage cells, together with maintenance of original MSC characteristics. Therefore, priming MSCs with high potentiality to differentiate into neuronal lineage cells can be generated by short-term gelatin-derived signals. These results demonstrate that extracellular signaling derived from specific extracellular components may provide guidance as a starting point of differentiation-related signaling pathways activated preferentially by specific differentiation stimulation in MSCs, which results in a marked increase in the ratio of

MSCs that differentiate into specific cell lineages according to the specific differentiation conditions.

Stem cell fate can be determined by the type and/or intensity of signaling derived from the extracellular matrix [10,11] or cell-to-cell interaction proteins [12,13], suggesting that extracellular components could be an important tool for regulating stem cell fate. In the case of MSCs – which are known to be the best source of cells for therapeutic purposes [14–16] – their multipotency makes their application in regeneration of damaged tissues difficult, with the exception of mesodermal lineage tissues. However, enhancement of MSC plasticity by extracellular component-derived signaling, as reported here, will increase their potential as cell therapeutics for regenerative medicine.

In this study, we increased MSC plasticity; for example, their ability to undergo neuronal lineage differentiation, using gelatin-derived extracellular signals, which may benefit patients with neuronal disorders. Moreover, application of different extracellular signals to MSCs, will likely result in generation of priming MSCs with high potential to differentiate into specific cell lineages. Therefore, simultaneous studies of the cytoplasmic events associated with each signaling pathway must be conducted. Moreover, these achievements will make a notable contribution to improving the applicability of MSCs in cell therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.116.

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