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An Increase in Mesenchymal Stem Cells Expressing Nestin in Bone-Marrow-Derived Primary Cells Stimulates Neurogenic Differentiation in Rat

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ABSTRACT

Mesenchymal stem cells (MSCs) have been considered an alternative source of neuronal lineage cells, which are difficult to isolate from brain and expand *in vitro*. Previous studies have reported that MSCs expressing Nestin (Nestin$^+$ MSCs), a neuronal stem/progenitor cell marker, exhibit increased transcriptional levels of neural development-related genes, indicating that Nestin$^+$ MSCs may exert potential with neurogenic differentiation. Accordingly, we investigated the effects of the presence of Nestin$^+$ MSCs in bone-marrow-derived primary cells (BMPCs) on enhanced neurogenic differentiation of BMPCs by identifying the presence of Nestin$^+$ MSCs in uncultured and cultured BMPCs. The percentage of Nestin$^+$ MSCs in BMPCs was measured per passage by double staining with Nestin and CD90, an MSC marker. The efficiency of neurogenic differentiation was compared among passages, revealing the highest and lowest yields of Nestin$^+$ MSCs. The presence of Nestin$^+$ MSCs was identified in BMPCs before *in vitro* culture, and the highest and lowest percentages of Nestin$^+$ MSCs in BMPCs was observed at the third (P3) and fifth passages (P5). Moreover, significantly the higher efficiency of differentiation into neurons, oligodendrocyte precursor cells and astrocytes was detected in BMPCs at P3, compared with P5. In conclusion, these results demonstrate that neurogenic differentiation can be enhanced by increasing the proportion of Nestin$^+$ MSCs in cultured BMPCs.

(Key words: Mesenchymal stem cells expressing Nestin, Neurogenic differentiation, Bone marrow, Rat)

INTRODUCTION

Generally, neurological disorders, such as Parkinson's, Huntington's and Alzheimer's diseases, result from damage or loss of neuronal or glial cells in the brain. Cell therapy is considered a fundamental therapy for these conditions (Kim and de Vellis 2009; Suksuphew and Noisa 2015). While appropriate therapeutic cell types can be obtained from neural stem cells (NSCs) (Fu et al. 2008; Lee et al. 2010), ethical problems (Bae et al. 2011; Ramos-Zúñiga et al. 2012), damage to the brain and limited retrieval numbers (Nam et al. 2015) in the acquisition of NSCs and difficulty of *in vitro* culture (Jakel et al. 2004) have made it difficult to apply NSCs to neuronal disease therapy. Therefore, an alternative method of overcoming the problems associated with NSC-based therapy is needed.

Mesenchymal stem cells (MSCs) derived from bone marrow can differentiate into several cell types, including adipocytes, chondrocytes and osteocytes (Augello and De Bari 2010; Armstrong et al. 2014; Farrell et al. 2015). These bone-marrow-derived MSCs exhibit reduced immune responses because their retrieval from allogeneic or autologous bone marrow is possible. In addition, they have the plasticity to differentiate into neural lineage cells originating from the

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ectoderm under appropriate conditions, although they originate primarily from the mesoderm (Croft and Przyborski 2009; Huat et al. 2014). Therefore, MSCs have been applied not only to basic research but also clinical research in the field of regenerative medicine for neuronal disorders.

Previously, *in vitro* culture of rat MSCs induced the expression of Nestin (Wislet-Gendebien et al. 2003), a neuronal stem/progenitor cell (NSPC) marker, and MSCs not expressing Nestin ($Nestin^-$ MSCs) showed lower expression of genes related to neural development than MSCs expressing Nestin ($Nestin^+$ MSCs), which could differentiate into functional neuron-like cells by co-culture with mouse cerebellar granule neuron (Wislet-Gendebien et al. 2005). These results emphasize the importance of $Nestin^+$ MSCs in the mass production of neuronal-lineage cells from MSCs. Accordingly, in this study, we aimed to identify the presence of MSCs with NSPC characteristics in uncultured and cultured bone-marrow-derived primary cells (BMPCs) and investigate their effects on BMPC differentiation into neuronal-lineage cells.

MATERIALS and METHODS

1. Animals

Six three-week-old male Sprague-Dawley (SD) rats were purchased from DBL (Eumseong, Korea) and used as bone marrow cell donors. All animal housing, handling and experimental procedures were performed according to the Animal Care and Use Guidelines of Kangwon National University and were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval no. KW-121101-1).

2. Experimental design

Firstly, to determine whether MSCs with NSPC characteristics exist in a heterogeneous cell population derived from bone marrow, combinatorial expression of CD90 (an MSC marker) and Nestin (an NSPC marker) was measured in uncultured BMPCs. Subsequently, to investigate the effects of *in vitro* culture of BMPCs on the generation of MSCs with NSPC characteristics, combinatorial expression of CD90 and Nestin was measured at each passage during the culture of BMPCs up to the fifth passage. Finally, to identify the effects of different population ratios of MSCs expressing NSPC marker proteins in cultured BMPCs on differentiation into neuronal lineage cells, BMPCs at passages showing the highest and lowest percentages of MSCs with NSPC characteristics were differentiated into three neuronal lineage cells, and the efficiencies of neurogenic differentiation were compared.

3. Isolation of BMPCs

Tibias and femurs obtained from both legs of SD rats sacrificed by CO_2 asphyxiation were washed with Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) supplemented with 1% (v/v) antibiotic-antimycotic (Welgene). Subsequently, the spongious end of each bone experiencing removal of muscle tissue was cut to expose the marrow cavity, and BMPCs were retrieved by flushing bones with DPBS supplemented with 2% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) through the marrow cavity. Elimination of red blood cells (RBCs) from flushed BMPCs was then conducted using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO).

4. Culture of BMPCs

Culture of 1×10^7 BMPCs on 100-mm dishes was conducted in low-glucose Dulbecco's Modified Eagle's Medium (LG-DMEM; Welgene) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic at 37ºC in humidified 95% air and 5% CO_2. After 2 days in culture, buoyant cells were discarded and medium was changed at 2-3-day intervals for 2 weeks. Confluent cells were then dissociated with 0.25% trypsin-EDTA (Welgene), and harvested cells were reseeded on culture dishes. Subsequently, reseeded cells were cultured under the same conditions until the fifth passage.

5. Neurogenic differentiation of cultured BMPCs

Prior to neurogenic differentiation, cells were seeded onto 0.01% (wt/v) poly-L-lysine (Sigma-Aldrich)-coated dishes. After incubation for 24 h, attached cells were washed with DPBS and incubated for 5 h in pre-induction medium consisting of high-glucose DMEM (HG-DMEM; Welgene) supplemented with 2% (v/v) dimethylsulfoxide (DMSO; Sigma-Aldrich) and 0.1 mM 2-mercaptoethanol (Gibco Invitrogen, Grand Island, NY) at 37ºC

under 5% CO_2 in a humidified air atmosphere. Subsequently, to guide neurogenic differentiation, incubation of pre-induced cells in neural induction medium consisting of HG-DMEM supplemented with 10% (v/v) heat-inactivated FBS, 0.5 mM isobutylmethylxanthine (IBMX; Sigma-Aldrich), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma-Aldrich), 10 μg/L human basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ), and 10 μg/L human epidermal growth factor (hEGF; Peprotech) was conducted for 7 days. Cells of neural lineage were rinsed with DPBS, retrieved using 0.25% trypsin-EDTA, and adjusted for flow cytometric analysis.

6. Flow cytometry

Fixation of cells was conducted with 4% (v/v) formaldehyde (Junsei Chemical Co., Ltd, Chuo-ku, Japan). Fixed cells were washed with ice-cold DPBS. BMPCs in DPBS containing 0.01% (v/v) Triton-X-100 (Sigma-Aldrich) were double-stained with APC-conjugated anti-rat CD90 (an MSC marker; Biolegend, San Diego, CA) and FITC-conjugated anti-Nestin (a neural stem/progenitor cell marker; Santa Cruz Biotechnology, Dallas, TX) antibodies for 45 min at 4ºC. Moreover, BMPCs differentiated into neural-lineage were stained with anti-NeuN (a neuron-specific marker; Millipore, Billerica, MA), anti-NG2 chondroitin sulfate proteoglycan (an oligodendrocyte precursor cell marker; Millipore), and anti-GFAP (an astrocyte marker; Abcam, Cambridge, UK) antibodies diluted in DPBS containing 0.01% (v/v) Triton-X-100 for 45 min at 4ºC. Subsequently, cells stained with primary antibodies were washed with ice-cold DBPS and detected using Alexa Fluor 488 chicken anti-rabbit IgG (Molecular probe, Eugene, OR) for 45 min at 4ºC. Table 1 shows detailed information regarding the antibodies and dilutions used. Stained cells were then rinsed with DPBS and sorted using a FACSCalibur (Becton, Dickinson and Co., Franklin Lakes, NJ). Data analysis was conducted using the BD CellQuest Pro Software (Becton, Dickinson and Co).

7. Statistical analysis

Statistical Analysis System (SAS) Software was used to statistically analyze numerical data. Significant differences were determined by analysis of variance (ANOVA), and a least-square difference or Duncan's method was used for comparisons among groups. A p value < 0.05 was considered to indicate statistical significance.

RESULTS

Identification of MSCs expressing the NSPC marker in BMPCs

We first investigated the type of neurogenic cells present in heterogeneous primary cells derived from bone marrow. As shown in Figure 1, depending on the expression of MSC (CD90) and/or NSPC marker (Nestin), the presence of each of four cell types was identified in BMPCs. The majority of cells (61.72 ± 11.87) expressed only MSC marker protein ($CD90^+/Nestin^-$ cells), whereas 35.84 ± 13.14% of cells showed no expression of both MSC and NSPC marker proteins ($CD90^-/Nestin^-$ cells). Moreover, MSCs expressing NSPC marker protein ($CD90^+/Nestin^+$ cells) was also identified at a low percentage (2.43 ± 2.11), whereas NSPCs ($CD90^-/Nestin^+$ cells) was negligible (0.01 ± 0.01). Accordingly, we conclude that MSCs expressing an NSPC marker protein were present among primary cells retrieved directly from bone marrow, albeit at a low frequency.

Effects of in vitro culture of BMPCs on the generation of MSCs expressing NSPC marker

Next, to identify the destiny of each cell type in retrieved BMPCs upon *in vitro* culture, we investigated the ratio of each cell type per subculture. In the case of MSCs expressing NSPC marker protein ($CD90^+/Nestin^+$ cells) (Figure 2A), the highest proportion was detected at the third passage (4.06 ±

Table 1. Primary and secondary antibodies

Antibody Name	Catalog Number	Company	Dilution Rate
APC conjugated mouse anti-CD90 IgG1	202526	Biolegend	1:500
FITC conjugated mouse anti-Nestin IgG1	Sc-33677 FITC	Santa cruz biotechnology	1:20
Rabbit anti-NeuN IgG	ABN78	Millipore	1:500
Rabbit anti-NG2 IgG	AB5320	Millipore	1:500
Rabbit anti-GFAP IgG	ab7260	Abcam	1:200
Alexa Fluor® 488 chicken anti-rabbit IgG	A-21441	Molecular probe	1:150

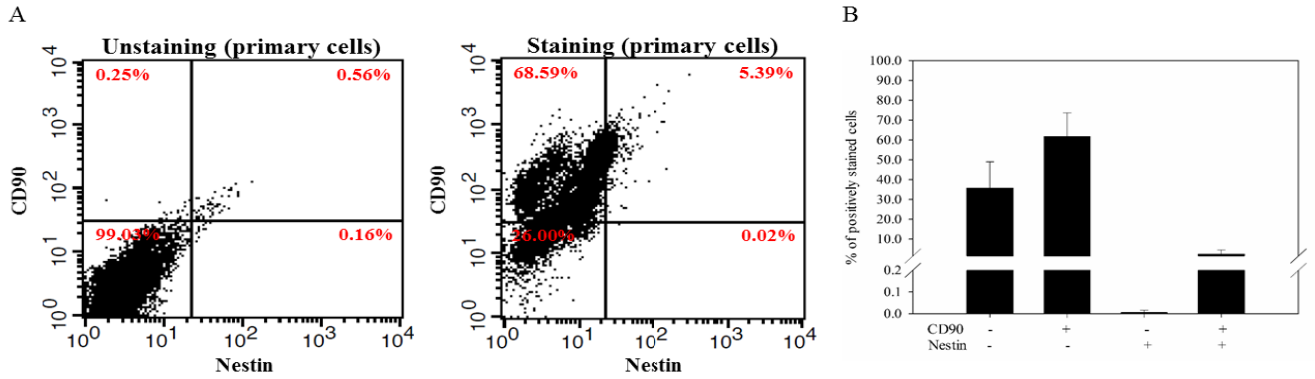

Figure 1. The presence of MSCs expressing Nestin (Nestin$^+$ MSCs) in a heterogeneous cell population derived from rat bone marrow. BMPCs isolated from tibias and femurs were double-stained with anti-CD90 (an MSC marker) and anti-Nestin (an NSPC maker) antibodies. Dual-color analysis was conducted by flow cytometry. Nestin$^+$ MSCs (CD90$^+$/Nestin$^+$ cells) were present among BMPCs at an extremely low level, but NSPCs (CD90$^-$/Nestin$^+$ cells) were not. Moreover, most cells in the population showed MSC characteristics (CD90$^+$/Nestin$^-$ cells); those without the characteristics of both MSCs and NSPCs (CD90$^-$/Nestin$^-$ cells) were also detected at a high level. A is representative FACS analyses of the percentage of CD90 and/or Nestin$^+$ cells in BMPCs and B is composite average (means ± standard deviation) of the percentage of CD90 and/or Nestin$^+$ cells in BMPCs from three independent experiments.

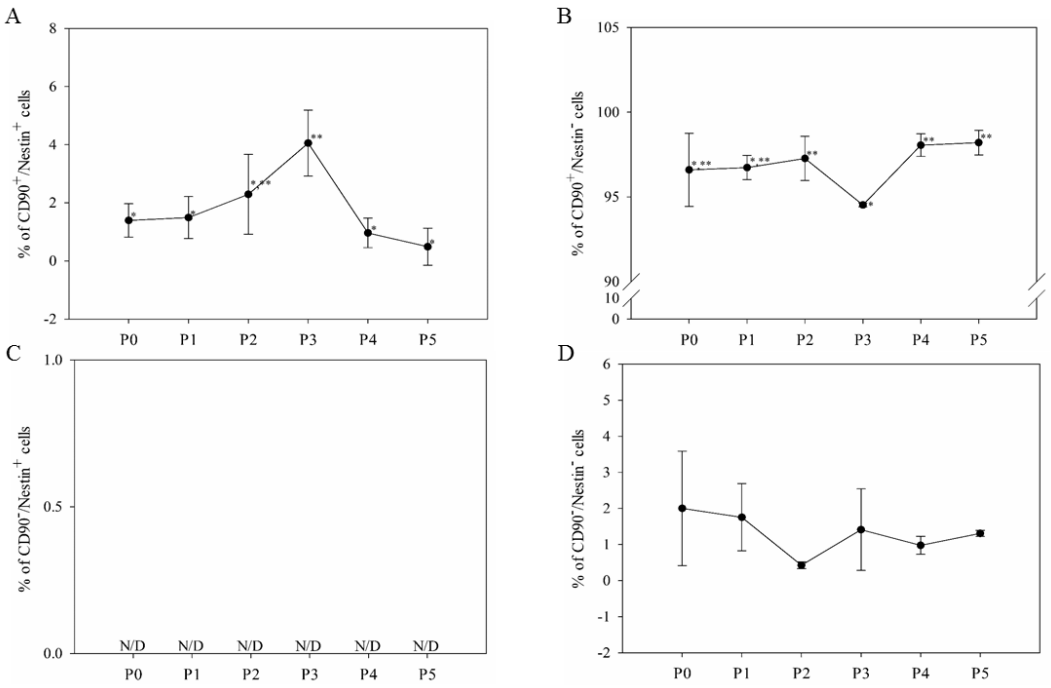

Figure 2. Alterations in the Nestin$^+$ MSCs proportion among BMPCs during *in vitro* culture. Isolated BMPCs were cultured for 2 weeks and dissociated enzymatically. Harvested BMPCs were repeatedly reseeded, cultured and detached under the same conditions up to the fifth passage. Confluent BMPCs at each passage were double-stained with anti-CD90 (an MSC marker) and anti-Nestin (an NSPC marker) antibodies. The stained cells were analyzed by flow cytometry. The highest proportion of Nestin$^+$ MSCs (CD90$^+$/Nestin$^+$ cells) was observed at the third passage, and the lowest proportion at the fifth passage. In contrast, Nestin$^-$ MSCs (CD90$^+$/Nestin$^-$ cells) at the third passage showed significantly the lowest proportion and the highest proportion value among passages showing significantly higher proportion than the third passage was identified at the fifth passage. Moreover, no NSPCs were detected, and an extremely low proportion of cells without characteristics of both MSCs and NSPCs (CD90$^-$/Nestin$^-$ cells) was observed in BMPCs cultured confluently per passage. Data represent the means ± standard deviation (s.d.) of three independent experiments. $^{*,**}P < 0.05$.

1.13) and, although there were no significant differences among the remaining groups, the lowest proportion was observed at the fifth passage (0.49 ± 0.64). Diametrically, the proportion of MSCs not expressing NSPC marker protein ($CD90^+/Nestin^-$ cells) was lowest at the third passage (94.53 ± 0.11) and highest at the fifth passage (98.21 ± 0.72) (Figure 2B). Moreover, following culture of BMPCs, NSPC ($CD90^-/Nestin^+$ cells) weren't discovered (Figure 2C), and cells not expressing both MSC and NSPC marker proteins ($CD90^-/Nestin^-$ cells) showed no proliferation, with a dramatic decrease in the cell population ratio (Figure 2D). These results demonstrate that *in vitro* culturing stimulates the proliferation of MSCs expressing an NSPC marker protein for a short-term in BMPCs. Simultaneously, we speculate that BMPCs at the third passage, which exhibited the highest proportion of NSPC marker protein-expressing MSCs, may undergo effectively neurogenic differentiation.

Effects of NSPC marker-expressing MSC populations among BMPCs on differentiation into neural-lineage cells

Subsequently, by comparing the efficiency of neural differentiation in BMPCs between the third, (highest ratio of $CD90^+/Nestin^+$ cells) and fifth passages (lowest ratio of $CD90^+/Nestin^+$ cells), we investigated whether the differentiation efficiency of BMPCs into neural-lineage cells can be regulated by the ratio of an MSC population expressing an NSPC marker protein in BMPCs. Overall, BMPCs at the third passage showed significantly higher efficiency of differentiation into neurons (NeuN-positive, Figure 3A and B), oligodendrocytes (NG2-positive, Figure 3A and C) and astrocytes (GFAP-positive, Figure 3A and D) than those at the fifth passage. Accordingly, these results suggest that an increased population of NSPC

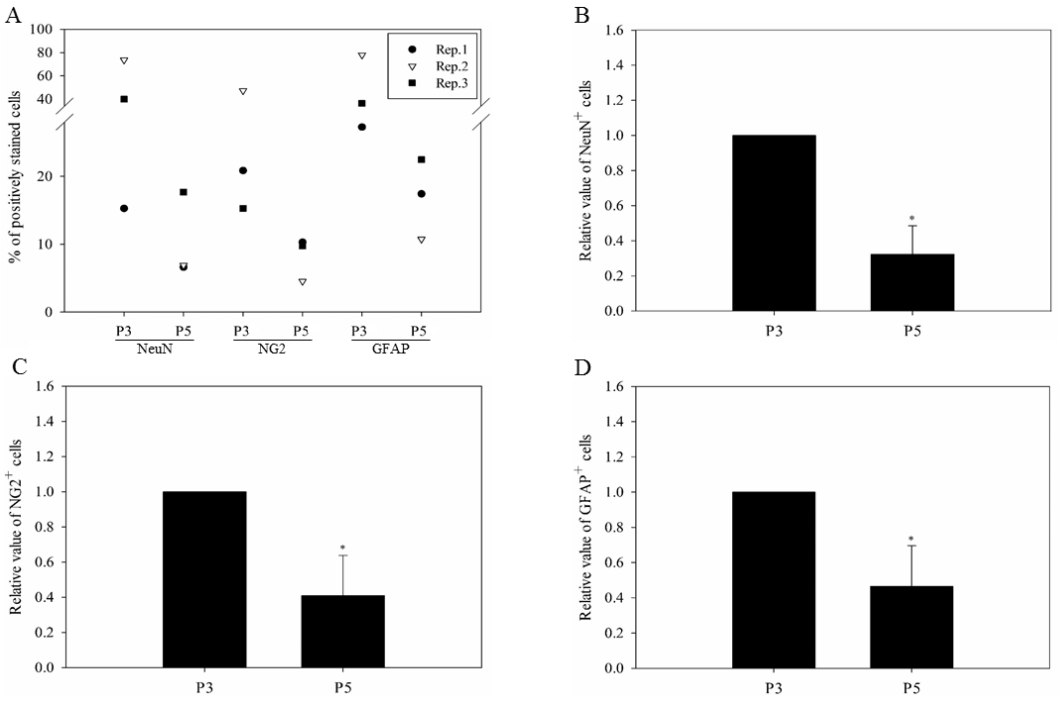

Figure 3. Neural differentiation capacity of BMPCs between the third and fifth passages. Confluent BMPCs cultured at the third and fifth passages were differentiated into neural-lineage cells by sequential incubation in pre-induction medium for 5 h and neural induction medium for 7 days. Subsequently, flow cytometric analysis (A) was conducted after staining with antibodies against NeuN (a neuron-specific marker), NG2 (an oligodendrocyte precursor cell marker) and GFAP (an astrocyte marker). The relative value of each marker protein-positive cell was then calculated using the following equation: $A_{target}/A_{reference}$, where A_{target} represents the percentage of cells stained positively with each antibody post-neural differentiation of BMPCs at each passage, and $A_{reference}$ represents the percentage of cells stained positively with each antibody post-neural differentiation of BMPCs at the third passage (B, C and D). In all repeated experiments, BMPCs at the third passage showed a numerically and significantly higher percentage of NeuN-, NG2- and GFAP-positive cells than those at the fifth passage. Data represent the means ± standard deviation (s.d.) of three independent experiments. $^*P < 0.05$.

marker protein-expressing MSCs among BMPCs can enhance the differentiation of BMPCs into neural-lineage cells.

DISCUSSION

In this study, a small number of $Nestin^+$ MSCs were included in heterogeneous primary cells retrieved from bone marrow, and their proportion in BMPCs increased gradually by *in vitro* culture, resulting in improved efficiency of neurogenic differentiation. Accordingly, we determined that neurogenic differentiation of MSCs could be enhanced by increasing the number of MSCs with NSPC characteristics in the MSC population.

As shown in Figure 2A, the percentage of $Nestin^+$ MSCs ($CD90^+/Nestin^+$ cells) in BMPCs was increased consistently by the third passage and decreased greatly thereafter. However, the percentage of $Nestin^-$ MSCs ($CD90^+/Nestin^-$ cells) in BMPCs increased gradually following culture within an extremely small range (from 96.6 ± 2.16 to 98.21 ± 0.72%), with the exception of a significant decrease at the third passage (Figure 2B). Previous studies have reported that NSPCs can be routinely cultured in specialized culture medium supplemented with a variety of factors to stimulate self-renewal and inhibit the differentiation of NSPCs into neuronal-specific lineage cells (Smith et al. 2003; Mothe et al. 2011). Therefore, a small number of MSCs with NSPC characteristics among BMPCs may be difficult to proliferate in MSC culture medium lacking factors supporting NSPC self-renewal. This is supported by the fact that a minority of NSPCs in heterogeneous primary cells isolated from bone marrow was undetected following culture in MSC culture medium (Figure 2C), and the number of MSCs with NSPC characteristics was greatly decreased after the third passage in MSC culture medium (Figure 2A). Accordingly, we speculate that the increase in $CD90^+/Nestin^+$ cells by the third passage may be a result of transformation of $CD90^+/Nestin^-$ cells into $CD90^+/Nestin^+$ cells, rather than $CD90^+/Nestin^-$ cell proliferation. This is supported by previous reports that the specific type of cells was transformed into different type of cells by external stimulation (Eshghi and Schaffer 2008; Lee et al. 2013). However, the exact mechanism of MSC transformation should be investigated in further studies.

BMPCs at the third passage showed significantly higher neurogenic differentiation efficiency than those at the fifth passage (Figure 3). This difference may be related to the presence of different proportions of MSCs with NSPC characteristics in BMPCs at each passage. In previous studies, the expression of Nestin, an NSPC marker, has been considered a prerequisite for differentiation into neuronal lineage cells (Wislet-Gendebien et al. 2003), which may explain BMPCs at the third passage (highest proportion of $Nestin^+$ MSCs) differentiate into neuronal-lineage cells more efficiently than those at the fifth passage.

In conclusion, the co-existence of MSCs with NSPC characteristics in an MSC population enhances the neurogenic differentiation of MSCs. Therefore, mass production of neuronal lineage cells from MSCs, the best tool for cell therapy, is possible by enhancing neurogenic differentiation by increasing the proportion of MSCs with NSPC characteristics in an MSC population. Moreover, development of an *in vitro* culture system that stimulates the generation of MSCs with NSPC characteristics in an MSC population will contribute to the advancement of MSC-based therapy for neurological disorders.

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