Isolation and characterization of multipotent mesenchymal stem cells in nasal polyps

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Abstract

Mesenchymal stem cells (MSCs) are multipotent progenitor cells in adult tissues. This study aimed to investigate nasal polyp (NP) tissues as a potential new source of multipotent MSCs that maintain their stemness and differentiation potential following multiple rounds of passaging. NP tissues were obtained from 10 patients during endoscopic sinus surgery. After isolating and culturing NP-derived MSCs (npMSCs), the expression levels of the surface markers CD34, CD44, CD45, CD73, CD90, CD105, CD106, CD146 and human leukocyte antigens-class II DR antigen (HLA-DR) were estimated by flow cytometry. npMSCs were cultured in chondrogenic, osteogenic, adipogenic, or neurogenic differentiation medium. The differentiation potential of npMSCs was analyzed by Alcian blue, alizarin red S, oil red O, and immunocytochemical staining and reverse transcription-polymerase chain reaction. The clonogenic potential of npMSCs was measured using a colony-forming unit assay. Cell proliferation of npMSCs was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Flow cytometry analysis revealed that npMSCs were negative for hematopoietic lineage markers (CD34, CD45, and HLA-DR) and positive for MSC markers (CD44, CD73, CD90, and CD105). The npMSCs differentiated into osteogenic, adipogenic, chondrogenic, and neurogenic lineages, respectively. Chondrogenically differentiated npMSCs were stained with Alcian blue, osteogenically differentiated npMSCs were stained with alizarin red S, and adipogenically differentiated npMSCs were stained with oil red O. Real-time polymerase chain reaction results showed that the differentiated npMSCs expressed the respective differentiation markers (*Sox 9* and *Col2A* for chondrogenesis, *Runx2* and *osteocalcin* for osteogenesis, *fatty acid-binding protein 4* and *peroxisome proliferator-activated receptor γ* for adipogenesis, *TuJ1*, *neurofilament light chain*, and *neurofilament heavy chain* for neurogenesis). There were no significant differences in the clonogenic potential and proliferation rate between early and late passage npMSCs. These results show that npMSCs possess the characteristics of MSCs in terms of morphology, multipotent differentiation capacity, cell surface marker expression, and clonogenicity. Thus, npMSCs may represent an alternative source of MSCs.

Keywords: Nasal polyp, mesenchymal stem cell, osteogenesis, adipogenesis, chondrogenesis, neurogenesis

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Introduction

Stem cells are undifferentiated and unspecialized cells that can differentiate into specialized cells. Stem cells are originated from various organs, including embryonic, fetal, adult, amniotic, and cord blood.¹ Multipotent mesenchymal stem cells (MSCs) are a population of adult stem cells.² MSCs are excellent candidates for cell therapy because they are easily accessible, their isolation is relatively simple, they can be biopreserved with minimal loss of potency, and no adverse reactions to allogeneic versus autologous MSCs

transplants have been reported.³ Three major accessible sources of autologous adult stem cells in the clinic are the bone marrow, adipose tissue, and blood. Stem cells can be isolated from various tissues and organs, including nervous tissues,⁴ skin,⁵ adipose tissue,⁶ tendons,⁷ and synovial membranes.⁸ Bone marrow-derived MSCs are the best-characterized MSC population.⁹ However, for various reasons, including the trauma caused by bone marrow extraction procedures¹⁰ and bone marrow-related diseases, much stem cell research has focused on finding alternative

sources of adult stem cells that can be isolated noninvasively or by minimally invasive collection procedures.¹¹

MSCs from the human olfactory mucosa have been recently isolated and characterized.¹² It has been reported that olfactory mucosa-MSCs have morphologic and phenotypic similarities with bone marrow-derived MSCs.¹³ Human olfactory mucosa-MSCs have also the capacity to differentiate into ectoderm and mesoderm cells.¹⁴ Human turbinate MSCs were obtained from turbinate tissue discarded during turbinate surgery. Human turbinate MSCs have been characterized and their capacity to differentiate into chondrogenic, osteogenic, adipogenic, and neurogenic cells has been tested.¹⁵ The Schneiderian membrane is the mucosa that covers the inner part of the maxillary sinus cavities. Human Schneider stroma derived from the Schneiderian membrane has been shown to contain mesenchymal progenitor cells.¹⁶ In these recent studies, mesenchymal stromal cells from otorhinolaryngological tissues have been characterized. However, studies of mesenchymal stromal cells derived from nasal polyps (NPs) have not been reported. NPs are a common chronic inflammatory disease of mucous membranes in the nose and paranasal sinuses.¹⁷ The prevalence of NPs is 1–4% in the general population. NPs are removed by endoscopic sinus surgical procedures.

In this study, we report that nasal polyp-derived mesenchymal stem cells (npMSCs) isolated from NP tissues have the potential for multilineage differentiation (chondrogenic, osteogenic, adipogenic, and neurogenic differentiation capacity) and maintain high clonogenicity during long-term *in vitro* culture.

Materials and methods

Reagents

Fluorescein isothiocyanate- or phycoerythrin-conjugated mouse monoclonal antibodies (antihuman CD34, CD44, CD45, CD73, CD90, CD105, CD106, CD146, and HLA-DR) were purchased from BD Bioscience (San Diego, CA).

NpMSCs isolation and culture

NP tissues were obtained from 10 patients (5 men and 5 women; average age: 31.5y) with chronic rhinosinusitis with NP who were recruited from the Department of Otorhinolaryngology at the Korea University Medical Center. Investigations were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each patient, and the study was approved by the Korea University Medical Center Institutional Review Board (KUGH13136) before surgery. Average tissue volume of biopsied NP was approximately 0.8 g and dissected for the isolation of npMSCs. To isolate npMSCs, NP tissues were washed once with a gentamicin solution (Invitrogen, Carlsbad, CA) and twice with phosphate-buffered saline (PBS), and then dissected into 1 mm³ pieces for the isolation of npMSCs. After dissection, all tissue pieces were resuspended in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) and placed in culture dishes. After one week, the tissue pieces were attached and the

cultured medium was changed every three days. npMSCs began to migrate from the explants as plastic-adherent cells. The attached npMSCs were detached using 0.05% trypsin/Ethylene diaminetetraacetic acid (EDTA) solution (Invitrogen) and subcultured at a ratio of 1:3. The number of npMSCs is approximately 6×10^6 each biopsied tissue and the cultured npMSCs were expanded *in vitro* until passage 15. Cell morphology was continuously assessed throughout *in vitro* expansion and differentiation. Cryopreservation does not affect the stem characteristics and damage of npMSCs.

Flow cytometry

Adherent npMSCs (passage 2–3 and passage 13–15) were harvested in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, 1% penicillin (10,000 U/mL), and 10,000 µg/mL streptomycin (Invitrogen) and resuspended at a concentration of 1×10^6 cells/mL in PBS containing 5% FBS. Aliquots containing 1×10^6 npMSCs were incubated with fluorescein isothiocyanate-conjugated mouse-specific monoclonal antibodies directed against CD34, CD44, CD45, CD73, CD90, CD105, CD106, CD146, and HLA-DR for 30 min at room temperature. npMSCs were washed three times in PBS solution containing 5% FBS. Labeled npMSCs were fixed in 1% formaldehyde in PBS prior to analysis. At least 10,000 gated events per acquisition were acquired using a flow cytometer (FC 500; Beckman Coulter, Fullerton, CA).

Multilineage differentiation of npMSCs

Briefly, 1×10^4 cells/well of npMSCs were plated in 12 well tissue culture plates and cultured using osteogenesis, adipogenesis, chondrogenesis differentiation kits (Gibco, Carlsbad, CA) and neurogenic differentiation medium (PromoCell, Heidelberg, Germany). Medium was replaced every three days for three weeks. For osteogenic differentiation, the induced cells were analyzed by alizarin red S staining (ScienCell, Carlsbad, CA) to assess mineralization and used for reverse transcription-polymerase chain reaction (RT-PCR).¹² For adipogenic differentiation, the induced cells were stained with oil red O (Sigma, St. Louis, MO) and used for RT-PCR.¹² For chondrogenic differentiation, the induced cells were stained with Alcian blue (Sigma) for detecting glycoproteins in the extracellular matrix and used for RT-PCR.¹² For neurogenic differentiation, the induced cells were stained with fibronectin (green, Santa Cruz, CA) and TuJ1 (red, Millipore, Billerica, MA) antibodies by immunofluorescence method and used for RT-PCR.¹¹

Real-time PCR

NpMSCs were differentiated into osteogenic, adipogenic, chondrogenic, or neurogenic lineages. After differentiation, total RNA was isolated with Trizol reagent (Invitrogen). For real-time RT-PCR, 2 µg of total RNA was reverse transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Beijing, China) following the manufacturer's protocols. Quantitative PCRs were then performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA)

using 3 μ L of cDNA template, 1 nmol of primers, and 12.5 μ L of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), in a total reaction volume of 25 μ L. The sequences of the forward and reverse primers were as follows: *sex determining region Y-box 9* (*Sox9*) (sense sequence, 5'-AGG TGC TCA AAG GCT ACG ACT-3'; antisense sequence, 5'-AGA TGT GCG TCT GCT CCG TG-3'), *collagen type 2A* (*Col2A*) (sense sequence, 5'-ACT TGC GTC TAC CCC AAT CC-3'; antisense sequence, 5'-ACA GTC TTG CCC CAC TTA CC-3'), *runt-related transcription factor 2* (*Runx2*) (sense sequence, 5'-CCA CGA CAA CCG CAC CAT-3'; antisense sequence, 5'-CAC TCC GGC CCA CAA ATC TC-3'), *osteocalcin* (sense sequence, 5'-CAC TCC TCG CCC TAT TGG C-3'; antisense sequence, 5'-CCC TCC TGC TTG GAC ACA AAG-3'), *fatty acid-binding protein 4* (*FABP4*) (sense sequence, 5'-GCA TGG CCA AAC CTA ACA TGA T-3'; antisense sequence, 5'-CCT GGC CCA GTA TGA AGG AAA-3'), *peroxisome proliferator-activated receptor γ* (*PPAR γ*) (sense sequence, 5'-AGC CTC ATG AAG AGC CTT CCA-3'; antisense sequence, 5'-ACC CTT GCA TCC TTC ACA AGC-3'), *neurofilament light chain* (*NF-L*) (sense sequence, 5'-GAC CAA GCC CGA CCT TTC-3'; antisense sequence, 5'-CAT TCC TCA GCG TTC TGC AT-3'), *neurofilament heavy chain* (*NF-H*) (sense sequence, 5'-CAA GGA ACC CAG CAA ACC-3'; antisense sequence, 5'-GCC TCT GTC TTG GGT TTC TC-3') and *glyceraldehyde-3-phosphate dehydrogenase* (sense sequence, 5'-GCA AAT TCC ATG GCA CCG T-3'; antisense sequence, 5'-TCG CCC CAC TTG ATT TTG G-3'). The cDNA templates were amplified with an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 58°C for 10 s. After cooling at 72°C for 10 s, a melting curve was generated. To confirm the amplification specificity, the PCR products obtained from each primer pair were subjected to melting curve analysis. Analysis of relative gene expression was performed by evaluating the quantitative RT-PCR data using the $2^{-\Delta\Delta C_t}$ method. The gene expression levels were normalized to that of the *glyceraldehyde-3-phosphate dehydrogenase*.

Immunofluorescence staining

NpMSCs were cultured in neurogenic differentiation medium for neurogenic differentiation. After neurogenic differentiation, npMSCs were fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in 1% bovine serum albumin for 10 min, blocked with 5% bovine serum albumin for 1 h at room temperature and incubated with antifibronectin (Santa Cruz) or TuJ1 (Millipore) antibody. npMSCs were then incubated with goat antirabbit Alexa 488 (Invitrogen) or goat antimouse Alexa 555 (Invitrogen) secondary antibodies. Stained npMSCs were captured and visualized using a LSM700 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Colony-forming units

Fibroblast colony-forming units were assessed by crystal violet staining (Sigma). NpMSCs were seeded at 1×10^4 cells in 100-mm culture dishes. The seeded npMSCs were cultured for 14 days. The cells formed several colonies and

these colonies were stained with crystal violet. The stained colonies were counted under a microscope.

MTT assay

The proliferation capacity of npMSCs was evaluated with the MTT assay. NpMSCs were plated at a density of 1500 cells/well in 96-well culture plates and cultured for five days. On each day, the cells were treated with MTT solution (5 mg/mL in PBS, Sigma) for 4 h, and the formed formazan crystals were resuspended in dimethyl sulfoxide. The plates were agitated on a plate shaker and the optical density was then observed at 540 nm using a fluorescence microplate reader (F2000; Hitachi, Ltd., Tokyo, Japan).

Statistical analysis

Results were obtained from at least three independent samples of 10 patients. The statistical significance of the differences between control and experimental data was analyzed with unpaired *t*-test (GraphPad Prism, version 5, GraphPad Software, San Diego, CA). The values are expressed as the mean $\pm$ standard error of mean (SEM) of independent experiments. Significance was established at the 95% confidence level. *P* values less than 0.05 were accepted as statistically significant.

Results

Isolation and characterization of human npMSCs

Isolated human npMSCs were observed under an inverted light microscope during the culture period. Adherent npMSCs were cultured in a confluent monolayer. With regard to the morphology, npMSCs had a flat, elongated, spindle-like, and fibroblast-like shape (Figure 1(a)). For the characterization of npMSCs, flow cytometry analysis was used to assess cell surface markers of the expanded npMSCs. As expected, the isolated npMSCs expressed MSC markers (CD44, CD73, CD90, and CD105). However, they did not express hematopoietic lineage markers (CD34, CD45, and HLA-DR). CD106 and CD146 were slightly expressed (16.4%) (Figure 1(b)). These results indicated that the isolated npMSCs are MSCs.

Multilineage differentiation potential of npMSCs

The plasticity and capacity of npMSCs to differentiate into chondrogenic, osteogenic, adipogenic, and neurogenic cells were investigated under *in vitro* conditions. To verify chondrogenesis, npMSCs were cultured in chondrogenic induction medium. After 21 days of induction in chondrogenic induction medium, npMSCs had acquired a rounded and enlarged morphology (Figure 2(a)). Chondrogenic npMSCs were identified by Alcian blue staining. Alcian blue staining is used to stain acidic polysaccharides such as glycosaminoglycans in cartilages and proves the ability of these cells to secrete proteoglycans. Compared to control npMSCs, chondrogenic npMSCs showed strong Alcian blue staining (Figure 2(b)). We examined the mRNA expression of the chondrogenic differentiation markers *Sox9* and *Col2A* by RT-PCR in chondrogenic npMSCs. The mRNA expression

Figure 1 Morphology and characterization of human npMSCs. (a) Morphology of npMSCs under an inverted photomicroscope. (b) Flow cytometry analysis of npMSCs shows the expression of CD34, CD44, CD45, CD73, CD90, CD105, CD106, and HLA-DR. Scale bar = 400 μ m. (A color version of this figure is available in the online journal.)

Note: FITC: fluorescein isothiocyanate; PE: phycoerythrin

levels of *Sox9* and *Col2A* were significantly increased in chondrogenic npMSCs (Figure 2(c)).

To verify osteogenesis, npMSCs were cultured in osteogenic induction medium. After osteogenic induction, the morphology of npMSCs had changed from spindle-shaped to cubical (Figure 3(a)). Because calcium forms complexes with alizarin red S in the chelation process, calcium deposition of osteogenic lineages can be detected with alizarin red staining. No mineralization of calcium phosphate was observed in control npMSCs. However, positive alizarin red S staining was detected in osteogenic npMSCs (Figure 3(b)). The mRNA levels of the osteogenic markers *Runx2* and *osteocalcin* were estimated using RT-PCR (Figure 3(c)). The mRNA expression levels of *Runx2* and *osteocalcin* were increased in osteogenic npMSCs.

To verify adipogenesis, npMSCs were cultured in adipogenic induction medium. After adipogenic induction for 21 days, npMSCs showed adipocyte morphology, i.e. lipid droplets and oily vesicles (Figure 4(a)). The adipogenic

npMSCs were assessed with oil red O staining. Oil red O staining is used for staining of neutral triglycerides and lipids on frozen sections and some lipoproteins. Lipid-containing npMSCs have the potential to undergo adipogenesis (Figure 4(b)). The mRNA levels of the adipogenic markers *PPAR γ* and *FABP4* were assessed by RT-PCR and were found to be significantly upregulated in adipogenic npMSCs compared to control npMSCs (Figure 4(c)).

To verify neurogenesis, npMSCs were cultured in neurogenic induction medium. After differentiation into neural lineages, npMSCs showed neuron-like morphology, i.e. they were bipolar and extending neurites from the cells (Figure 5(a)). TuJ1 (class III β -tubulin) is expressed in neurons of the peripheral and central nervous systems. It contributes to microtubule stability in neuronal cell bodies and axons, and plays a role in axonal transport. The extracellular matrix including collagens and fibronectin has a crucial role in tissue structure and function, interactions between stem cells and the surrounding extracellular matrix are

Figure 2 Chondrogenic differentiation of human npMSCs. NpMSCs were cultured in chondrogenic medium for 21 days. (a) Morphological change of npMSCs after chondrogenic differentiation under an inverted photomicroscope. (b) Alcian blue staining of the chondrogenic monolayer of cultured npMSCs. (c) mRNA expression levels of the chondrogenic markers *sex determining region Y-box 9* (*Sox9*) and *collagen type 2A* (*Col2A*) determined by reverse transcript-polymerase chain reaction. * $P < 0.05$ compared to control cells. Scale bar = 400 μ m. Values are expressed as means $\pm$ SEM of least three separate experiments. (A color version of this figure is available in the online journal.)

Figure 3 Osteogenic differentiation of human npMSCs. NpMSCs were cultured in osteogenic medium for 21 days. (a) Morphological change of the npMSCs after osteogenic differentiation under an inverted photomicroscope. (b) Alizarin red S staining of cultured osteogenic npMSCs. (c) mRNA expression levels of the osteogenic markers *runx-related transcription factor 2* (*Runx2*) and *osteocalcin* determined by reverse transcription-polymerase chain reaction. * $P < 0.05$ compared to control cells. Scale bar = 400 μ m. Values are expressed as means $\pm$ SEM of least three separate experiments. (A color version of this figure is available in the online journal.)

Figure 4 Adipogenic differentiation of human npMSCs. npMSCs were cultured in adipogenic medium for 21 days. (a) Morphological change of npMSCs after adipogenic differentiation under an inverted photomicroscope. (b) Oil red O staining of cultured osteogenic npMSCs. (c) mRNA expression levels of the adipogenic markers *fatty acid-binding protein 4 (FABP4)* and *peroxisome proliferator-activated receptor γ (PPAR γ)* estimated using reverse transcription-polymerase chain reaction. Arrows represent lipid droplets. * $P < 0.05$ compared to control cells. Values are expressed as means $\pm$ SEM of least three separate experiments. (A color version of this figure is available in the online journal.)

likely to participate in decisions regarding the stem cell fate.¹⁸ Differentiated human neural progenitor cells were stained for TuJ1 and decreased fibronectin expression (Figure 5(b)). The results of RT-PCR showed that npMSCs expressed the neuronal markers *NF-L* and *NF-H* (Figure 5(c)). The expression levels of *NF-L* and *NF-H* were increased in npMSCs following induction of neurogenesis.

These results demonstrate that npMSCs can be induced to differentiate into chondrogenic, osteogenic, adipogenic, and neurogenic lineages.

Clonogenic potential and proliferation rate of npMSCs

The colony-forming unit assay was used to assess the proliferative potential of isolated cells at different passages. No significant difference was observed between npMSCs at passage 1 and those at passage 15. A representative colony of npMSCs was assessed by crystal violet staining. After 100 cells of passages 1 and 15 were seeded in 10-cm plates, the number of colonies was counted. However, there was no significant difference in the growth rate between cultured npMSCs of early and late passages (Figure 6(a)). These data suggest that npMSCs have clonogenic potential until passage 15. Cell proliferation was monitored using the MTT assay at the indicated time points for five days. Passages 1 and 15 npMSCs proliferated at a similar rate; furthermore, the numbers of passage 1 npMSCs and passage 15 npMSCs increased from one day to five days (Figure 6(b)). There was no statistically significant difference in the proliferation rate between early and late passage npMSCs. These results indicate that the proliferation rate of npMSCs remains the same until passage 15.

Discussion

Previous studies characterized mesenchymal stromal cells isolated from otorhinolaryngeal tissues. MSCs derived from inferior turbinate and palatine tonsils were negative for hematopoietic stem cell markers and positive for MSC markers.^{15,19} However, studies of mesenchymal stromal cells derived from NPs have not been reported. NPs are polypoidal masses that arise mainly from mucous membranes of the nose and paranasal sinuses.¹⁷ The prevalence of NPs is 1–4% in the general population.²⁰ NPs are removed by endoscopic sinus surgical procedures.

We isolated adherent spindle-shaped cells from human NP tissues. The aim of the present study was to determine whether adherent spindle-shaped cells from human NP tissues have characteristic features of MSCs and whether these characteristics are influenced by the passage number. This paper is the first study that identified MSCs from human NP tissues.

Because of the lack of a specific marker, MSCs are characterized by a set of negative and positive surface markers. Here, by using flow cytometry, we show that npMSCs expressed MSC-specific antigens such as CD44, CD73, CD90, and CD105, but that they were negative for hematopoietic markers such as CD34, CD45, and HLA-DR.²¹ CD106 Vascular Cell Adhesion Molecule 1 (VCAM-1) is a surface marker and expressed in bone marrow-MSCs and chorionic villi-MSCs, not adipose-MSCs and umbilical cord-MSCs.²² CD146 is a marker of clonal multipotent cultured MSCs and expressed in vascular luminal and abluminal cells.²³ npMSCs might weakly express CD106 and CD146.

In addition, we found no significant passage-related changes in the expression levels of these cell surface markers.

Figure 5 Neurogenic differentiation of human npMSCs. npMSCs were cultured in neurogenic medium for 21 days. (a) Morphological change of npMSCs after neurogenic differentiation under an inverted photomicroscope. (b) Immunocytochemical staining of cultured npMSCs for fibronectin (green) and TuJ1 (red). (c) mRNA expression levels of the neuronal markers *neurofilament light chain (NF-L)* and *neurofilament heavy chain (NF-H)* estimated using reverse transcription-polymerase chain reaction. * $P < 0.05$ compared to control cells. Values are expressed as means $\pm$ SEM of least three separate experiments. (A color version of this figure is available in the online journal.)

Therefore, we concluded that adherent spindle-shaped cells from human NP tissues (npMSCs) are very similar to MSCs. The npMSCs have no *in vitro* genetic aberrations or risk of *in vivo* tumorigenicity (data not shown). The multilineage potential of MSCs has been proven by demonstrating their ability to differentiate into chondrogenic, osteogenic, adipogenic, and neurogenic lineages.²¹ We found that npMSCs maintained under appropriate culture conditions could be induced to differentiate *in vitro* into chondrocyte-, osteocyte-, adipocyte-, and neuron-like cells.

Chondrogenic differentiation of npMSCs was proved by the change to a rounded and enlarged morphology, positive staining for Alcian blue, and the expression of the chondrogenic differentiation markers *Sox9* and *Col2A*. *Sox9* is a transcription factor that is required for chondrocyte differentiation and *Col2A* is expressed in proliferating

chondrocytes.²⁴ In our study, the mRNA expression levels of *Sox9* and *Col2A* were increased. This result suggests that culturing npMSCs in chondrogenic differentiation medium for 21 days is sufficient to obtain chondrocyte-like cells.

Osteogenic differentiation of npMSCs was proved by the morphological change from spindle shaped to cubical, positive staining for alizarin red S, and the expression of the osteogenic differentiation markers *Runx2* and *osteocalcin*. *Runx2* is a transcription factor that is required for osteoblast differentiation and positively regulates the expression of *osteocalcin*.²⁵ *Osteocalcin* is thought to be indicative of a more osteoblast phenotype of osteocytes.²⁶ In our study, the mRNA expression levels of *Runx2* and *osteocalcin* were significantly increased. This result suggests that culturing npMSCs in osteogenic differentiation medium for 21 days is sufficient to obtain osteocyte-like cells.

Figure 6 Clonogenic potential and proliferation rate of human npMSCs. npMSCs (1×10^4 cells) of passages 1 and 15 were cultured for 14 days in 100 mm culture dishes. (a) The crystal violet-stained colonies were counted under an inverted photomicroscope. (b) The cellular proliferation rates of early and late passage npMSCs were monitored for five days using the MTT assays and compared. Values are expressed as means $\pm$ SEM of least three separate experiments.

Note: CFU: colony-forming unit

Adipogenic differentiation of npMSCs was proved by the change to an adipocyte-like morphology, i.e. formation of lipid droplets and oily vesicles, positive staining for oil red O, and the expression of the adipogenic differentiation markers *PPAR γ* and *FABP4*. *PPAR γ* is a transcription factor that is required for adipocyte differentiation and *FABP4*, which is a carrier protein for fatty acids, is primarily expressed in adipocytes.²⁷ In our study, the mRNA expression levels of *PPAR γ* and *FABP4* were significantly increased in npMSCs that had differentiated into adipogenic cells. This result indicates that culturing npMSCs in adipogenic differentiation medium for 21 days is sufficient to obtain adipocyte-like cells.

Neurogenic differentiation of npMSCs was proved by the change to a neuron-like morphology, e.g. cells were bipolar and neurites extended from them, positive staining for TuJ1 (class III β -tubulin), and the expression of the neurogenic differentiation markers *NF-L* and *NF-H*.²⁸ TuJ1 is a microtubule element of the tubulin family found almost exclusively in neurons. Neurofilaments are intermediate filaments found in neurons and they are believed to function primarily to provide structural support for axons.²⁹ In our study, the expression levels of *NF-L*, *NF-H*, and *TuJ1* were significantly increased in npMSCs that had differentiated into neurogenic cells. This result suggests that npMSCs have the potential to differentiate into neuron-like cells.

In previous studies, some MSCs such as turbinate-derived and tonsil-derived stem cells have been shown to have multilineage potential.^{15,19} Because MSCs originate from particular tissues, they have an original niche. Nasal septal progenitors express high levels of bone morphogenetic protein 2; thus, nasal septal progenitors have high osteogenic and chondrogenic potential compared to bone marrow-derived MSCs.³⁰ The higher osteogenic, adipogenic, and chondrogenic potential has been particularly emphasized for periosteal progenitor cells, adipose tissue-derived MSCs, and progenitor cells from the synovial

membrane, respectively. MSCs that originate from otolaryngological regions (inferior turbinate-derived stem cells, palatine tonsil-derived stem cells, and NP-derived stem cells) will have particular differentiation potential. It will be necessary to compare the multilineage differentiation capacity for the development of effective regenerative therapies.

The number of colony-forming units did not decline until passage 15. The MTT assay showed that there was no significant difference in the proliferation rates of npMSCs of passages 1 and 15. These findings indicate that npMSCs were still highly clonogenic, that they retained their self-renewal ability until passage 15, and that their proliferative activity was not affected.

Our data demonstrated that (1) npMSCs have characteristics of MSCs; (2) npMSCs express MSC surface markers until passage 15; (3) npMSCs differentiate into chondrocytes, osteocytes, adipocytes, and neuron-like cells without losing their multilineage potential until passage 15; and (4) npMSCs retain their clonogenic potential and maintain their proliferative activity until passage 15. We conclude that npMSCs could be used as a new alternative cell source for the development of regenerative therapies.

Author contributions: All authors contributed to the design, interpretation of the studies, analysis of the data, and review of the manuscript. J-SC conducted the experiments and wrote the manuscript. H-ML supervised the project.

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