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Therapeutic Potential of Human Nasal Inferior Turbinate-Derived Stem Cells: Microarray Analysis of Multilineage Differentiation

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Keywords
Human stem cells · Differentiation · Osteogenesis · Chondrogenesis · Neurogenesis · Gene expression · Microarrays · Gene ontology terms

Abstract
Introduction: Human nasal inferior turbinate-derived stem cells (hNTSCs) are attractive sources of adult stem cells for medical application because they can be easily obtained and cultivated with a highly proliferative capacity. The ability of hNTSCs to differentiate into chondrocytes, osteocytes, and neural cells makes them potential replacement therapeutic candidates in intractable disease. Nevertheless, detailed expression pattern of genes associated with trilineage differentiation (osteogenesis, chondrogenesis, and neurogenesis) in hNTSCs has not been revealed yet. ***Methods:*** In this study, we aimed to evaluate gene expression patterns of various transcription factors and marker genes associated with a particular lineage (osteogenesis, chondrogenesis, and neurogenesis) of differentiation of hNTSCs by DNA microarrays. ***Results:*** In microarrays, 36 transcripts such as E2F transcription factor 1, activating transcription factor 5, and AKR1B10 were upregulated and 36 transcripts such as CA9, PPFIA4, HAS2, and COL4A4 were downregulated in osteogenically differentiated hNTSCs. In chondrogenically differentiated hNTSCs, 3 transcripts (NUDT14, CPA4, and heparin-binding epidermal growth factor-like growth factor) were upregulated and 82 transcripts such as PTGS1, CLEC2D, and TET1 were downregulated. In neurally differentiated hNTSCs, 61 transcripts such as insulin-like growth factor-binding protein-1, nerve growth factor receptor, FGF1, OLFML1, and EPGN were upregulated and 98 transcripts such as ACAN, RUNX2, and C21orf96 were downregulated. In gene ontology (GO) analysis, cell signal-related GO terms were highly expressed. By contrast, catalysis GO terms and GO terms related to oxidoreductase were overrepresented in chondrogenically differentiated hNTSCs and osteogenically differentiated hNTSCs, respectively. ***Conclusion:*** Considering overall results, hNTSCs-specific genetic information may promote further studies on intracellular mechanisms defining key features of these cells.

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Introduction

Mesenchymal stem cells (MSCs) are adult stem cells with the potential to differentiate into various cells lineages [1]. They can be isolated from various tissues of adults [1, 2]. Previously, we have shown that MSCs derived from nasal mucosa (inferior turbinate) (hNTSCs) have a high proliferation potential. In addition, characteristics of hNTSCs do not change regardless of cell passage number [1]. Moreover, hNTSCs show superior potential to differentiate into osteocytes and chondrocytes. These cells have better osteogenic potential regardless of accessorial osteogenic factors, including vitamin D3 and BMP-2, in comparison with MSCs isolated from bone marrow or adipose tissues [3]. These cells can differentiate easily into chondrocytes by in vitro culture without co-culture of MSCs and chondrocytes which can enhance matrix deposition [4]. MSCs are considered as important sources for cellular therapy due to their immunologically privileged properties. Additionally, the isolation of MSCs from tissues normally removed during surgery and storage of them would have significant advantage for patients with various diseases [5]. Although hNTSCs provide a novel opportunity for regenerative medicine, in otorhinolaryngologic parts, core genes during the differentiation process of hNTSCs have not been evaluated sufficiently. Thus, the objective of this study was to determine differentiation phenotypes of hNTSCs under appropriate differentiation-induction media (osteogenic, chondrogenic, and neurogenic differentiation) and perform microarray-based gene expression profiling to evaluate these differentiation phenotypes of cell populations.

Materials and Methods

Statement of Ethics

hNTSCs were isolated from patients who underwent an inferior turbinate surgery. Isolation procedures were approved by the Institutional Review Board of Seoul St. Mary's Hospital, the Catholic University of Korea (KC08TISS0341). Written informed consent was obtained from the subjects.

Cell Isolation

The inferior turbinate tissue obtained after surgery was washed 3 to 5 times with an antibiotic-antimycotic solution (Gibco, Gaithersburg, MD, USA) and 3 times with phosphate buffered saline (PBS). It was then cut into 0.5-mm^3 pieces. These pieces were placed into a culture dish. The dish was covered with a sterilized glass cover slide. Alpha-MEM (WELGENE, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS; WELGENE) and 1% antibiotic-antimycotic solution (Gibco) was added. It was changed every 2 days. After 15 days of culture at 37°C in a 5% CO_2 incubator, the glass cover slide was removed and tissues floating in the culture media were removed by washing. hNTSCs attached to the bottom of the culture dish were then detached using 1 mL of 0.05% trypsin in 1 mM EDTA. After isolation, hTMSCs were seeded at an initial cell density of 1×10^4 cells/cm^2 and amplified in a monolayer culture media. hNTSCs were cultured for 3 passages to evaluate their multidifferentiation potential.

Characterization of Surface Markers on hNTSCs by Flow Cytometry

To investigate the expression of cell surface maker proteins in hNTSCs at passage 3, cells were detached and labeled with FITC- or PE-coupled anti-human antibodies (CD14, CD19, CD29, CD34, CD73, CD90, and HLA-DR; BD Biosciences, San Jose, CA, USA). Ten thousand labeled cells were measured using a FACS calibur flow cytometer (Becton Dickinson, Franklin lakes, NJ, USA). Results were analyzed with a CellQuest software (Becton Dickinson).

Multilineage Differentiation Potential of hNTSCs

To investigate the osteogenic potential of hNTSCs, cells were cultured in a low-glucose DMEM supplemented with 10% (v/v) FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, 50 μM ascorbate-2-phosphate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Culture media were changed twice every week for 4 weeks. Differentiation to osteogenic lineages was induced according to the procedures described by Hwang et al. [1]. A 3-dimensional culture system was used for chondrogenic differentiation with open-cell polylactic acid (BD Biosciences). After 2×10^6 cells were seeded onto the scaffold, cells were cultured in chondrogenic induction media consisting of DMEM supplemented with 10% FBS, 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate (Sigma, St. Louis, MO, USA), 50 mg/mL insulin-transferrin-sodium selenite, 1 mM sodium pyruvate (Gibco), 40 μM proline, and 2 mML-glutamine added to chondrogenic supplements TGF-β1 (10 ng/mL; Sigma) and IGF-1 (10 ng/mL; Sigma). Culture media were changed twice every week for 2 weeks. To induce neurogenic differentiation, hNTSCs were seeded onto 4-well chamber slides (1×10^4 cells/well; Nalgene Nunc International, Rochester, NY, USA) and cultivated in neurobasal media. Cultivated cells were resuspended in DMEM/F-12 (Gibco) with 1 mL B-27 supplement, neurogenic supplements (10 ng/mL glial-derived neurotrophin factor [Invitrogen, Carlsbad, CA, USA], 10 ng/mL neurotrophin-3 [Invitrogen], 10 ng/mL brain-derived neurotrophin factor [Invitrogen], 2 mML-glutamine), 0.5% heparin, and 1% penicillin-streptomycin (Gibco) for 2 weeks. Culture medium was refreshed every 2 days.

Immunocytochemistry of Differentiated hNTSCs

The differentiated hNTSCs were fixed with 4% paraformaldehyde in PBS for 20 min, and nonspecific binding sites were blocked by a 30-min incubation in PBS containing 0.5% Triton X-100 (Promega Co. Medison, WI, USA) at room temperature, then stained to analyze cytologically with Safranin O and Alizarin red for chondrocyte and osteocyte differentiation. For immunocytofluorescence staining, it is followed by antigen retrieval with proteinase K (Abcam) to expose the antigenic sites. After blocking with 5% normal goat serum, cells were each incubated with primary antibodies against NeuN (Abcam) and β-tubulin (Abcam) at room temperature overnight for double staining and washed triply with 0.01 mol/L PBS. After incubating with secondary antibodies, Alexa 488

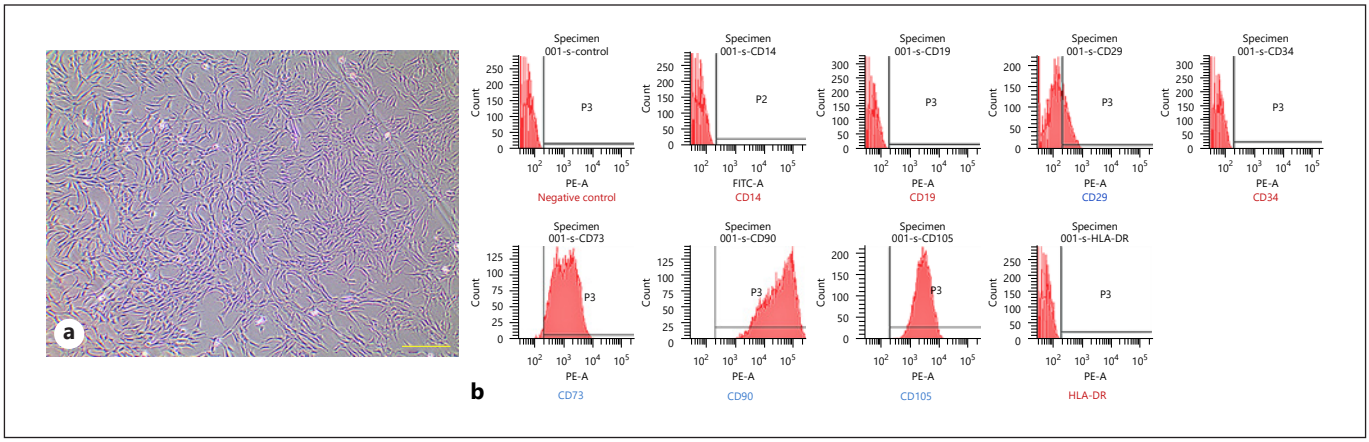

Fig. 1. a FACS analysis of hTMSCs 2 weeks after primary explant culture. The hTMSCs did not show the hematopoietic lineage markers but expressed mesenchymal stem cell markers (after 3 passages). CD14, CD19, and CD34 are markers of hematopoietic stem cells. CD29, CD73, and CD90 are markers of MSCs. HLA-DR is a human leukocyte antigen (**b**). FACS, fluorescence-activated cell sorting; hTMSCs, human turbinate mesenchymal stromal cells; MSCs, mesenchymal stem cells.

(1:100) and Alexa 546 (1:100) at room temperature for 2 h, antigen expression was each identified. For staining nuclei, cells were mounted with mounting medium (Vectashield, Burlingame, CA, USA) conjugated with DAPI and viewed using a fluorescence-attached microscope (OlympusAX70TR62A02, Tokyo, Japan).

RNA Extraction from hNTSCs and Microarray-Based Global Gene Expression

Total RNA was extracted from cells cultured under different induction media for chondrogenic, osteogenic, or neurogenic differentiation with an RNA isolation kit (Kontes, Vineland, NJ, USA). Cy3-labeled cDNA was synthesized using 200 ng of total RNA with a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). cRNAs were hybridized on a Custom Gene Expression Microarray, 4 × 44k (Agilent Technologies) at 65°C for 17 h. After that, microarrays were processed in accordance with the manufacturer's recommended protocol. We applied quantile normalization to compensate interarray variations. Genes with little variation and those with entropy values less than the 10th percentile were excluded. The genes were analyzed for significance by selecting 100 genes with the most significant differences by the SAM method. There are genes with the same significance (delta value), so the specific number is slightly different.

Results

Isolation and Characterization of hNTSCs through Flow Cytometry

Fibroblast-like cells appeared within 3 days, adhered to the culture dish, and formed into a single layer. Flow cytometric analysis was performed for cell surface markers of in vitro-cultured hNTSCs. Results revealed that these cells did not show any expression of hematopoietic cell markers (CD14, CD19, CD34, or HLA-DR). However, they showed expression of MSC markers (CD29, CD73, and CD90) (Fig. 1).

Immunocytochemistry of Differentiated hNTSCs

The cells exposed to osteogenic medium showed direct evidences of calcium mineralization seen by Alizarin Red staining. Cells after neurogenic induction for 14 days were analyzed with immunocytochemistry and the expression of nestin and β-tubulin was detected. Safranin O binds specifically detected the proteoglycan in chondrogenic differentiated hNTSCs (Fig. 2).

Gene Expression Profile

In osteogenic differentiated hNTSCs, 36 transcripts such as E2F transcription factor 1 (E2F1), activating transcription factor 5 (ATF5), and AKR1B10 were upregulated and 36 transcripts such as CA9, PPFIA4, HAS2, and COL4A4 were downregulated. In chondrogenic differentiated hNTSCs, 3 transcripts (NUDT14, CPA4, and heparin-binding epidermal growth factor-like growth factor [HBEGF]) were upregulated and 82 transcripts such as PTGS1, CLEC2D, and TET1 were downregulated. In neural differentiated hNTSCs, 61 transcripts such as insulin-like growth factor-binding protein (IGFBP)-1, nerve growth factor receptor (NGFR), FGF1, OLFML1, and EPGN were upregulated and 98 transcripts such as ACAN, RUNX2, and C21orf96 were downregulated. These up- and downregulated genes in trilineage differentiated hNTSCs are listed in Tables 1–3 and online suppl. Table 1; see www.karger.com/doi/10.1159/000516016 for all online suppl. material, (fold change in expression). Ad-

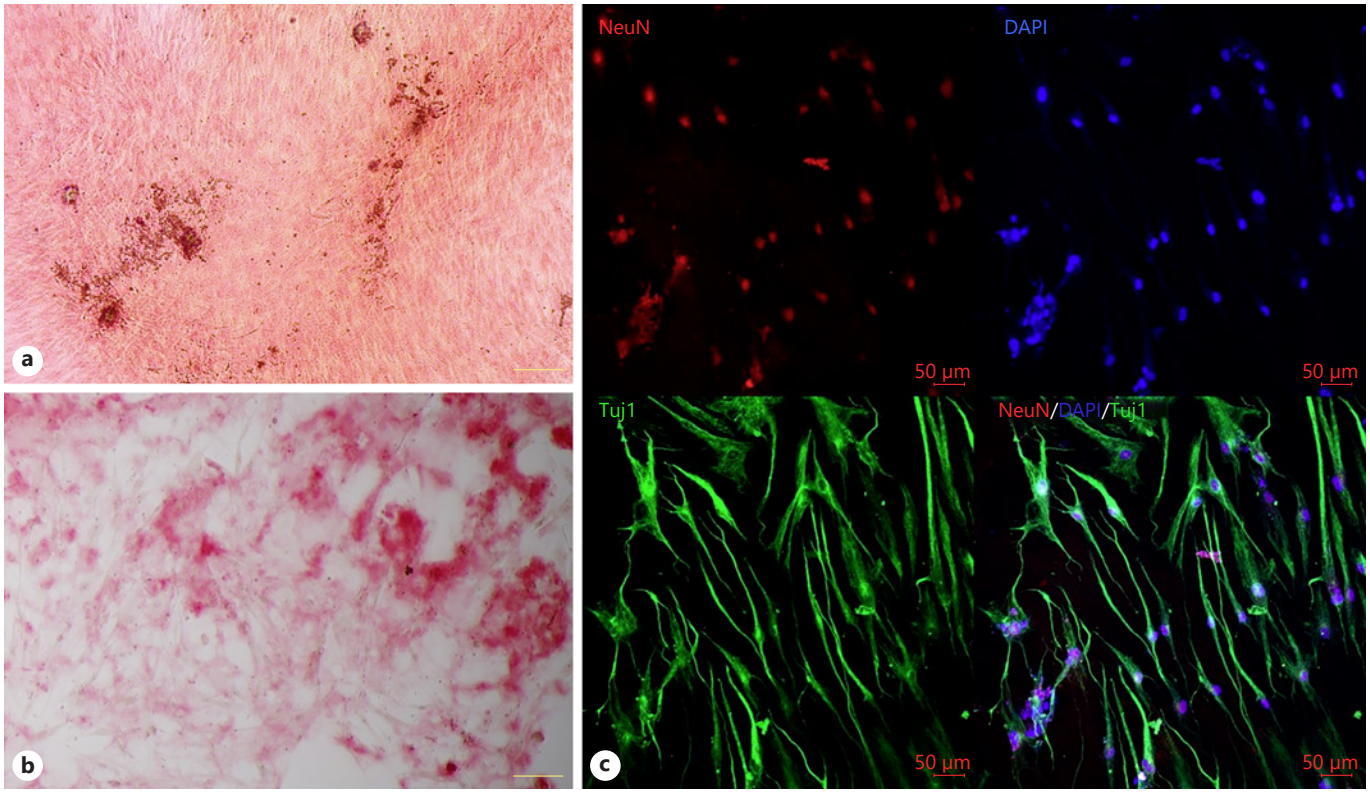

Fig. 2. Histologic analysis of hTMSCs 2–3 weeks after trilineage differentiation. The cells exposed to chondrogenic (**a**) and osteogenic (**b**) medium showed direct evidences of proteoglycan and calcium mineralization seen by Safranin O staining and Alizarin Red staining. Cells after neurogenic induction expressed nestin and β-tubulin (**c**). hTMSCs, human turbinate mesenchymal stromal cells.

ditionally, principal component analysis plot for comparing differences of cell groups according to differentiation and a heatmap for comparing genes differentially expressed in undifferentiated and trilineage differentiated hNTSCs were prepared. Results are shown in Figures 3, 4.

Gene Ontology Analysis

Major over- and underrepresented gene ontology (GO) classes are shown in Tables 4–6. In neural differentiated hNTSCs, major GO classes of cytokine or chemokine were abundant. In chondrogenic differentiated hNTSCs, catalysis GO terms were overrepresented. In osteogenic differentiated hNTSCs, GO terms related to oxidoreductase were overrepresented.

Discussion

Results of the present study verified specific gene expression patterns in hNTSCs according to trilineage differentiation. These results were compared to expression profiles of undifferentiated hNTSCs as a reference. Expression profiles of osteo-, chondro-, and neuro-differentiated hNTSCs were found to be different from each other. Based on expression profile analysis, many genes were shown to be up- or downregulated. Furthermore, GO analyses of regulated gene products showed over- or underexpressed GO classes. These results provide a first step for revealing core molecules associated with characteristics of hNTSCs.

Microarrays have become vital tools for detecting the expression of thousands of genes and for identifying differentially expressed genes. They enable us to explain and compare gene expression patterns of cells during or after differentiation. Based on microarray data, we can comprehend mechanisms controlling the expression of individual cells' characteristics. Some microarray studies have been conducted to check new specific markers and investigate the origin of various diseases including cancers [6–8].

In the otolaryngologic part, partial turbinate resection is one of the most popular and efficient surgery for reduc-

Table 1. Identification of genes differentially expressed during chondrogenesis

RefSeq ID	Gene name
Upregulated genes	
NC_000014.9	Nudix-type motif 14
NC_000007.14	Carboxypeptidase A4
NC_000005.10	HBEGF
Downregulated genes	
NC_000009.12	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
NC_000012.12	C-type lectin domain family 2 member D
NC_000077.6	SH3 and cysteine-rich domain 2
NC_000019.8	Hypothetical protein LOC100128567
NC_000010.11	Tet methylcytosine dioxygenase 1
NC_000003.12	Ceruloplasmin (ferroxidase)
NC_000007.14	Hypoxia inducible lipid droplet associated
AK094436.1	Uncharacterized LOC284219
NP_775819.1	Ensembl 59 human genes, GRCh37 assembly-ZNF584
NC_000007.14	Leptin
XM_001123368	Similar to cholinergic receptor, muscarinic 3
NC_000016.10	Proline-rich transmembrane protein 2
NC_000006.12	POU class 5 homeobox 1
NC_000005.10	Family with sequence similarity 153, member B
NC_000005.10	NLR family, apoptosis inhibitory protein
NC_000006.12	Forkhead box Q1
NC_000005.10	Liver-expressed antimicrobial peptide 2
NC_000014.9	FBJ murine osteosarcoma viral oncogene homolog
NC_000019.10	Intercellular adhesion molecule 5, telencephalin
NC_000014.7	Uncharacterized LOC440200
NC_000022.11	Galactose-3-O-sulfotransferase 1
NC_000007.14	CCZ1 homolog B, vacuolar protein trafficking and biogenesis associated
NC_000007.14	Leucine-rich single-pass membrane protein 1
NC_000022.11	Uncharacterized LOC729461
NC_000005.10	Phosphodiesterase 8B
NC_000010.11	RUN and FYVE domain containing 2
NC_000009.11	Uncharacterized MGC24103
NC_000019.10	Zinc finger protein 615
NC_000011.10	H19, imprinted maternally expressed transcript
NC_000011.10	KCNQ1 opposite strand/antisense transcript 1
NC_000016.10	Guanine nucleotide binding protein
NC_000011.10	Mucin 5AC, oligomeric mucus/gel-forming
NC_000020.11	Family with sequence similarity 65, member C
NC_000017.11	Ring finger protein 112
NC_000001.11	Phosphodiesterase 4B, cAMP-specific
NC_000009.12	Carbonic anhydrase IX
NC_000010.9	Uncharacterized protein BC008131
NC_000017.11	RAS, dexamethasone-induced 1
NC_000002.12	Ankyrin repeat domain 36B
NC_000006.12	Tenascin XB
NC_000007.14	WD repeat domain 86
AL110203.1	Uncharacterized LOC158863
NC_000010.11	Uncharacterized LOC399715
NC_000007.14	Nicotinamide phosphoribosyltransferase
NC_000009.12	Uncharacterized protein PRO2852
NC_000006.12	Suppressor APC domain containing 1
NC_000017.11	Gasdermin B
NC_000001.11	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
NC_000015.10	Long intergenic nonprotein coding RNA 597
NC_000004.10	Uncharacterized LOC100131829

Table 1 (continued)

RefSeq ID	Gene name
NC_000006.12	Tudor domain containing 6
NC_000007.14	Cyclin D binding myb-like transcription factor 1
NC_000002.11	Uncharacterized LOC100128563
NC_000006.12	Wilms tumor 1-associated protein
NC_000011.10	Dopamine receptor D4
NC_000020.10	Uncharacterized LOC29053
NC_000023.11	Apelin
NC_000003.12	Sarcolemma-associated protein
BX647230.1	Uncharacterized LOC399832
NC_000002.12	Frizzled-related protein
NC_000019.10	Cartilage intermediate layer protein 2
NC_000008.11	Metastasis suppressor 1
NC_000014.9	RAS (RAD and GEM)-like GTP binding 2

Nudix, nucleoside diphosphate-linked moiety X; HBEGF, heparin-binding epidermal growth factor-like growth factor.

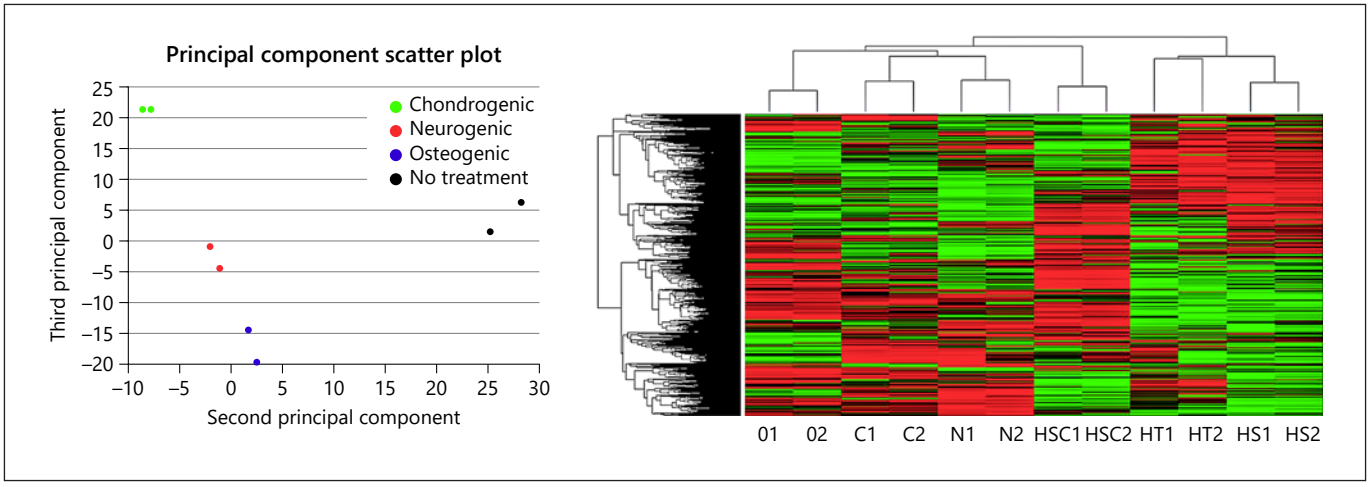

Fig. 3. PCA plot. A PCA plot showed the differences for cell groups according to differentiation. Each spot represented a single array sample. PCA, principal component analysis.

ing nasal symptoms. MSCs derived from the inferior tubinate can be obtained in ease and abundance. Due to this feature, hNTSCs could serve as important resources of adult stem cells. However, characteristics and differentiation of hNTSCs using genome-wide research techniques have not been reported yet. Studies on MSC derived from different tissues have shown a phenotype analogous to each other. Nevertheless, considerable differences in the molecular phenotype among MSCs from different tissues have been reported, showing ontological and functional differences [9]. Thus, it would be important to perform genome-wide gene expression profiling and GO analysis for hNTSCs. Thus, this is the first study to obtain gene expression profiles of hNTSCs through microarray.

HBEGF is a mitogenic and chemotactic molecule related to tissue repair and other tissue-modeling phenomena. Krampera et al. [10] have found that HBEGF can increase the proliferation of bone marrow MSCs but prevent chondrogenic differentiation reversibly. However, in our study, hNTSCs showed increasing HBEGF expression during chondrogenic differentiation. Transforming growth factor beta can induce extracellular matrix proteins during chondrogenic differentiation in vitro. In

Table 2. Identification of genes differentially expressed during osteogenesis

RefSeq ID	Gene name
Upregulated genes	
NC_000009.12	Mmicroseminoprotein, prostate associated
NC_000020.11	E2F transcription factor 1
NC_000008.11	Scavenger receptor class A, member 5
NC_000019.10	Activating transcription factor 5
NC_000002.12	LY6/PLAUR domain containing 1
NC_000003.12	Acyl-CoA oxidase 2, branched chain
NC_000015.10	Aldehyde dehydrogenase 1 family, member A3
NC_000003.12	Family with sequence similarity 107, member A
NC_000012.12	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54
NC_000002.12	Vitrin
NC_000011.10	Matrix metallopeptidase 1
NC_000012.12	G protein-coupled receptor, class C, group 5, member A
NC_000014.9	Nudix-type motif 14
NC_000002.12	Kynureninase
NC_000001.11	Vav 3 guanine nucleotide exchange factor
NC_000019.10	Anti-silencing function 1B histone chaperone
NC_000016.10	Periplakin
NC_000010.11	Interferon-induced protein with tetratricopeptide repeats 3
NC_000017.11	Chemokine (C-X-C motif) ligand 16
NC_000007.14	Carboxypeptidase A4
NC_000007.14	Aldo-keto reductase family 1, member B10 (aldose reductase)
NC_000023.11	Retinoic acid-induced 2
NC_000006.12	Transcription factor AP-2 beta (activating enhancer binding protein 2 beta)
NC_000014.9	Deiodinase, iodothyronine, type II
NC_000007.14	STEAP family member 4
NC_000019.10	Aldehyde dehydrogenase 16 family, member A1
NC_000009.12	Phytanoyl-CoA dioxygenase domain-containing 1
NC_000023.11	Nuclear receptor subfamily 0, group B, member 1
NC_000004.12	Toll-like receptor 3
NC_000009.12	Glycine dehydrogenase (decarboxylating)
NC_000016.10	GINS complex subunit 2 (Psf2 homolog)
NC_000011.10	Purinergic receptor P2Y, G-protein coupled, 2
NC_000017.11	ATP-binding cassette, subfamily A (ABC1), member 6
Downregulated genes	
NC_000009.12	Carbonic anhydrase IX
NC_000001.11	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4
NC_000008.11	Hyaluronan synthase 2
NC_000002.12	Collagen, type IV, alpha 4
NC_000007.14	Leptin
NC_000020.11	Fer-1-like family member 4, pseudogene (functional)
NC_000007.14	Hypoxia inducible lipid droplet-associated
NC_000001.11	Dual specificity phosphatase 5 pseudogene 1
NC_000009.12	Very low-density lipoprotein receptor
NC_000009.12	Prostaglandin D2 synthase 21 kDa (brain)
NC_000005.10	Family with sequence similarity 153, member B
NC_000014.7	Uncharacterized LOC440200
NC_000012.12	Cysteine and glycine-rich protein 2
NC_000017.11	Serine/arginine-rich splicing factor 1
NC_000001.11	Thioredoxin interacting protein
NC_000017.11	Arachidonate 15-lipoxygenase, type B
NC_000012.12	C-type lectin domain family 2, member D
NC_000017.11	Asialoglycoprotein receptor 1
NC_000011.10	KCNQ1 opposite strand/antisense transcript 1 (nonprotein coding)
NC_000006.12	Collagen, type XXI, alpha 1

Table 2 (continued)

RefSeq ID	Gene name
NC_000023.11	Apelin
NC_000006.12	Vascular endothelial growth factor A
NC_000017.11	Ring finger protein 112
NC_000015.10	Prader Willi/Angelman region RNA, SNRPN neighbor
NC_000001.11	Uncharacterized LOC100131564
NC_000004.12	Chromosome 4 open reading frame 47
NC_000002.12	Ankyrin repeat domain 36B
NC_000009.12	Prune homolog 2 (Drosophila)
NC_000013.11	Periostin, osteoblast-specific factor
NC_000022.11	Galactose-3-O-sulfotransferase 1
NC_000006.12	Wilms tumor 1-associated protein
NC_000019.10	Cartilage intermediate layer protein 2
NC_000019.10	Intercellular adhesion molecule 5, telencephalin
NC_000021.9	Junctional adhesion molecule 2

Nudix, nucleoside diphosphate-linked moiety X.

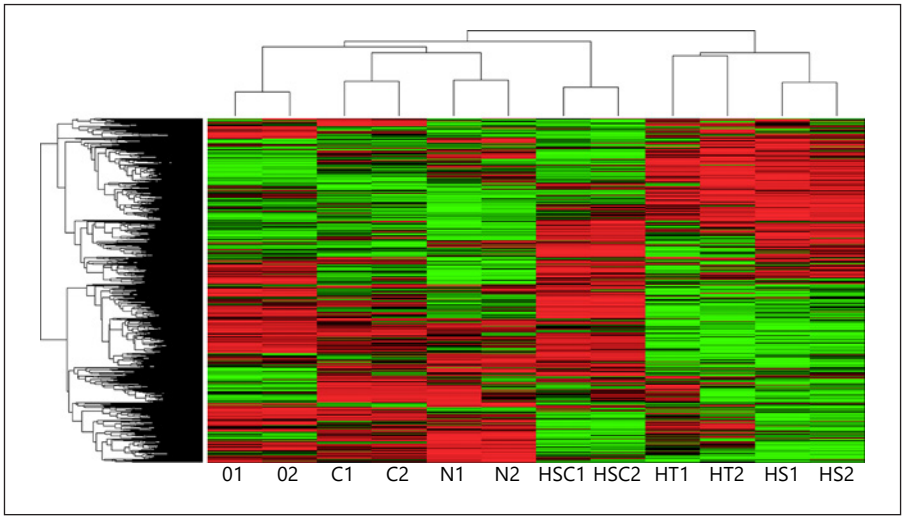

Fig. 4. Gene expression microarray analysis of hTMSCs according to differentiation. Heatmap comparing the genes differently expressed in the groups. Each row and columns matched with a transcript and sample, respectively. Color changed from green, for the lowest degree of expression, to red for the highest degree of expression. hTMSCs, human turbinate mesenchymal stromal cells.

chondrocyte progenitor cells stimulated by transforming growth factor beta, HBEGF was strongly expressed during induction, suggesting that HBEGF could be important in extracellular matrix manufacture as with cell function [11]. Our results showed that hTMSCs after differentiation were most similar to chondrocyte progenitor cells. This could explain why HBEGF is an important role during the chondrogenic differentiation of hNTSCs. ATF5 is a member of the ATF/CREB family of beta-ZIP transcription factors. It is strongly expressed in various neoplasms. It also controls stem cell functions [12]. During osteogenic differentiation of MSCs from human adipose tissue, ATF5 was highly expressed, reaching a peak of expression at the stage of bone mineralization. Vicari et al. [13] have suggested that ATF5 could play an interesting regulatory role during osteogenesis. These results were similar to our results showing that ATF5 was expressed highly during osteogenic differentiation.

E2F1 functions as a DNA-binding domain of pRb. Signaling via the interaction of pRb-E2F is considered to be associated with cell cycle regulation, cell fate, and differentiation of MSCs [14]. pRB is an activator of tissue-specific gene expression along diverse lineages including osteoblast differentiation [15]. In addition, the transcription start site of the ATF5 gene with promoter activity retaining has potential binding sites for several transcrip-

Table 3. Identification of genes differentially expressed during neurogenesis

RefSeq ID	Gene name
Upregulated genes	
NC_000002.12	LON peptidase N-terminal domain and ring finger 2
NC_000004.12	Annexin A10
NC_000004.12	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1
NC_000002.12	Adapter-related protein complex 1, sigma 3 subunit
NC_000005.10	Family with sequence similarity 169, member A
NC_000009.12	TEK tyrosine kinase, endothelial
NC_000007.14	Insulin-like growth factor binding protein 1
NC_000017.11	Chemokine (C-X-C motif) ligand 16
NC_000007.14	Fibrinogen-like 2
NC_000004.12	Chemokine (C-X-C motif) ligand 6
NC_000005.10	Proprotein convertase subtilisin/kexin type 1
NC_000012.12	Parathyroid hormone-like hormone
NC_000001.11	Lipid phosphate phosphatase-related protein type 4
NC_000004.12	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
NC_000017.11	Netrin 1
NC_000012.12	Retinol dehydrogenase 5 (11-cis/9-cis)
NC_000011.10	Chordin-like 2
NC_000001.11	Hydroxysteroid (11-beta) dehydrogenase 1
NC_000005.10	Cytoplasmic FMR1 interacting protein 2
NC_000002.12	Sperm-associated antigen 16
NC_000004.12	Chemokine (C-X-C motif) ligand 2
NC_000009.12	Tumor necrosis factor (ligand) superfamily, member 15
NC_000009.12	Microseminoprotein, prostate associated
NC_000001.11	Regulatory subunit of type II PKA R-subunit (RIIa) domain containing 1
NC_000012.12	Serine dehydratase-like
NC_000002.12	Kynureninase
NC_000017.11	Chemokine (C-C motif) ligand 7
NC_000011.10	Olfactomedin-like 1
NC_000003.12	Acyl-CoA oxidase 2, branched chain
NC_000020.11	Potassium voltage-gated channel, modifier subfamily S, member 1
NC_000010.11	Internexin neuronal intermediate filament protein, alpha
NC_000004.12	Epithelial mitogen
NC_000014.9	Long intergenic nonprotein coding RNA 643
NC_000001.11	Chitinase 3-like 2
NC_000003.12	Claudin 1
NC_000013.11	Glypican 5
NC_000014.9	Somatostatin receptor 1
NC_000017.11	Nerve growth factor receptor
NC_000017.11	Family with sequence similarity 20, member A
NC_000012.12	Spexin hormone
NC_000017.11	Chemokine (C-C motif) ligand 2
NC_000007.14	Tissue factor pathway inhibitor 2
NC_000016.10	Chromosome 16 open reading frame 89
NC_000004.12	Chemokine (C-X-C motif) ligand 3
NC_000007.14	Carboxypeptidase A4
NC_000007.14	Calcium channel, voltage-dependent, alpha 2/delta subunit 1
NC_000003.12	Solute carrier family 7, member 14
NC_000001.11	Vascular cell adhesion molecule 1
NC_000018.10	Collectin sub-family member 12
NC_000005.10	Fibroblast growth factor 1 (acidic)
NC_000011.10	Dedicator of cytokinesis 1 pseudogene
NC_000012.12	TAP binding protein-like
NC_000004.12	TLR3
NC_000011.10	Midkine (neurite growth-promoting factor 2)

Table 3 (continued)

RefSeq ID	Gene name
NC_000010.11	Interferon-induced protein with tetratricopeptide repeats 3
NC_000008.11	Musculin
NC_000020.11	Peptidase inhibitor 3, skin-derived
NC_000007.14	Interleukin 6
Downregulated genes	
NC_000011.10	Insulin-like growth factor 2
NC_000010.11	Zinc finger protein 91 pseudogene
NC_000001.11	Glutamate-ammonia ligase
NC_000011.10	H19, imprinted maternally expressed transcript (nonprotein coding)
NC_000012.12	Taste receptor, type 2, member 30
NC_000015.10	Aggrecan
NC_000016.10	Metallothionein 1X
NC_000009.12	Uncharacterized protein PRO2852
NC_000007.14	Leptin
NC_000003.12	Sarcolemma associated protein
NC_000017.11	Ankyrin repeat and FYVE domain containing 1
NC_000005.10	Family with sequence similarity 153, member B
NC_000001.11	Cell adhesion molecule 3
NC_000019.10	Cartilage oligomeric matrix protein
NC_000013.11	Collagen alpha-1(II) chain-like
AB007954.1	Uncharacterized LOC57235
NC_000022.11	POM121 transmembrane nucleoporin-like 8 pseudogene
NC_000006.12	FK506 binding protein 5
NC_000020.11	Junctophilin 2
NC_000007.14	Pyruvate dehydrogenase kinase, isozyme 4
NC_000017.11	Hepatic leukemia factor
NC_000019.10	Cytokine receptor-like factor 1
NC_000008.11	Hair growth associated
NC_000017.11	Noggin
NC_000010.11	Tet methylcytosine dioxygenase 1
NC_007869.1	Chemokine (C-X-C motif) receptor 7
NC_000010.11	Calcium channel, voltage-dependent, beta 2 subunit
NC_000017.11	Chromosome 17 open reading frame 67
NC_000010.11	Sorbin and SH3 domain containing 1
NC_000017.11	Arachidonate 15-lipoxygenase, type B
NC_000001.11	HNRNPU antisense RNA 1
NC_000023.11	TSC22 domain family, member 3
NC_000022.11	POM121 transmembrane nucleoporin-like 1, pseudogene
NC_000002.12	Frizzled-related protein
NC_000002.12	Methyltransferase like 21A
NC_000006.12	POU class 5 homeobox 1
NC_000004.10	Uncharacterized LOC100131829
NC_000014.9	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
NC_000010.11	Nebulette
NC_000013.11	Regulator of cell cycle
NC_000017.11	Coronin 6
NC_000006.12	Wilms tumor 1 associated protein
NC_000011.10	Platelet derived growth factor D
NC_000021.9	RUNX1 intronic transcript 1
AL110203.1	Uncharacterized LOC158863
NC_000007.1	SND1 intronic transcript 1
NC_000017.11	Serine/arginine-rich splicing factor 1
NC_000008.11	PHD finger protein 20-like 1
NC_000011.10	Zinc finger and BTB domain containing 16
NC_000006.12	MyoD family inhibitor

Table 3 (continued)

RefSeq ID	Gene name
NC_000007.14	WD repeat domain 86
NC_000020.11	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
NC_000020.11	Acyl-CoA synthetase short-chain family member 1
NC_000009.11	Uncharacterized MGC24103
NC_000005.10	CD14 molecule
NC_000015.10	Putative ubiquitin-conjugating enzyme E2Q2-like protein
NC_000020.11	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
NC_000006.12	Runt-related transcription factor 2
NC_000007.14	Leucine-rich single-pass membrane protein 1
NC_000011.10	BH3-like motif containing, cell death inducer
NC_000003.12	Long intergenic nonprotein coding RNA 312
NT_113901.1	Uncharacterized LOC645566
NC_000005.10	Liver expressed antimicrobial peptide 2
NC_000014.9	FBJ murine osteosarcoma viral oncogene homolog
NC_000012.12	Inositol 1,4,5-trisphosphate receptor, type 2
NC_000014.7	Uncharacterized LOC440200
NC_000016.10	Copine VII
NC_000019.10	Cartilage intermediate layer protein 2
AK094436.1	Uncharacterized LOC284219
NC_000022.11	POM121 transmembrane nucleoporin-like 9, pseudogene
NC_000008.11	ASAP1 intronic transcript 1
NC_000013.11	Mitochondrial inner membrane organizing system 1 pseudogene 1
NC_000002.12	Ankyrin repeat domain 36B
NC_000022.11	Galactose-3-O-sulfotransferase 1
NC_000017.11	RAS, dexamethasone-induced 1
NC_000001.11	Endothelin 2

Table 4. Major over or underrepresented GO classes during chondrogenesis

Reporter ID	Reporter name	*p* value
Upregulated		
GO:0008768	UDP-sugar diphosphatase activity	0.001193246
GO:0047631	ADP-ribose diphosphatase activity	0.002623259
GO:0009046	Zinc D-Ala-D-Ala carboxypeptidase activity	0.004407889
GO:0061473	Murein tripeptide carboxypeptidase activity	0.004407889
GO:0005515	Protein binding	0.004645598
GO:0008317	Gurken receptor binding	0.005477128
GO:0004180	Carboxypeptidase activity	0.00784908
GO:0005154	Epidermal growth factor receptor binding	0.008204381
GO:0004181	Metallocarboxypeptidase activity	0.008559554
Downregulated		
GO:0004118	cGMP-stimulated cyclic-nucleotide phosphodiesterase activity	0.000558946
GO:0004120	Photoreceptor cyclic-nucleotide phosphodiesterase activity	0.000558946
GO:0004119	cGMP-inhibited cyclic-nucleotide phosphodiesterase activity	0.000593312
GO:0004117	Calmodulin-dependent cyclic-nucleotide phosphodiesterase activity	0.000628668
GO:0004115	3′,5′-cyclic-AMP phosphodiesterase activity	0.000740648
GO:0047555	3′,5′-cyclic-GMP phosphodiesterase activity	0.000740648
GO:0004114	3′,5′-cyclic-nucleotide phosphodiesterase activity	0.000861435
GO:0004112	Cyclic-nucleotide phosphodiesterase activity	0.001539249
GO:0004130	Cytochrome-c peroxidase activity	0.001946968

GO, gene ontology.

Table 5. Identification of genes differentially expressed during neurogenesis

Reporter ID	Reporter name	*p* value
Upregulated		
GO:0008009	Chemokine activity	1.92E−08
GO:0042379	Chemokine receptor binding	1.93E−08
GO:0045236	CXCR chemokine receptor binding	1.98E−08
GO:0005125	Cytokine activity	1.02E−05
GO:0031725	CXCR6 chemokine receptor binding	1.24E−05
GO:0005153	Interleukin-8 receptor binding	1.36E−05
GO:0031723	CXCR4 chemokine receptor binding	1.36E−05
GO:0031724	CXCR5 chemokine receptor binding	2.82E−05
GO:0031732	CCR7 chemokine receptor binding	2.94E−05
Downregulated		
GO:0010314	Phosphatidylinositol-5-phosphate binding	0.001318916
GO:0001010	Sequence-specific DNA binding transcription factor recruiting transcription factor activity	0.002113322
GO:0001011	Sequence-specific DNA binding RNA polymerase recruiting transcription factor activity	0.002113322
GO:0001034	Sequence-specific DNA binding RNA polymerase III transcription factor activity	0.002113322
GO:0001073	DNA binding transcription antitermination factor activity	0.002113322
GO:0001130	Sequence-specific DNA binding bacterial-type RNA polymerase transcription factor activity	0.002113322
GO:0001142	Sequence-specific DNA binding mitochondrial RNA polymerase transcription factor activity	0.002113322
GO:0001167	Sequence-specific DNA binding RNA polymerase I transcription factor activity	0.002113322
GO:0001199	Metal ion regulated sequence-specific DNA binding transcription factor activity	0.002113322

Table 6. Identification of genes differentially expressed during osteogenesis

Reporter ID	Reporter name	*p* value
Upregulated		
GO:0001758	Retinal dehydrogenase activity	3.27305E−06
GO:0016620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	2.55861E−05
GO:0003942	N-acetyl-gamma-glutamyl-phosphate reductase activity	0.000203654
GO:0004030	Aldehyde dehydrogenase [NAD(P)+] activity	0.000203654
GO:0004043	L-aminoadipate-semialdehyde dehydrogenase activity	0.000203654
GO:0004073	Aspartate-semialdehyde dehydrogenase activity	0.000203654
GO:0008774	Acetaldehyde dehydrogenase (acetylating) activity	0.000203654
GO:0008863	Formate dehydrogenase (NAD+) activity	0.000203654
GO:0008883	Glutamyl-tRNA reductase activity	0.000203654
GO:0008886	Glyceraldehyde-3-phosphate dehydrogenase (NADP+) (nonphosphorylating) activity	0.000203654
Downregulated		
GO:0004551	Nucleotide diphosphatase activity	0.000113085
GO:0016462	Pyrophosphatase activity	0.001860999
GO:0030229	Very low-density lipoprotein particle receptor activity	0.003572689
GO:0043183	Vascular endothelial growth factor receptor 1 binding	0.003572689
GO:0043185	Vascular endothelial growth factor receptor 3 binding	0.003572689
GO:0030021	Extracellular matrix structural constituent conferring compression resistance	0.004052636
GO:0030020	Extracellular matrix structural constituent conferring tensile strength	0.004196858
GO:0030197	Extracellular matrix constituent, lubricant activity	0.004196858
GO:0030023	Extracellular matrix constituent conferring elasticity	0.004269826
GO:0005539	Glycosaminoglycan binding	0.004273952

tion factors, including EBF1, Sp1, and E2F1. In particular, mutation of the E2F1-binding site obviously impairs the activity of the ATF5 promoter, showing that this site is the principal cis-element for the transcriptional activation of human ATF5 gene [12]. These facts could support our results showing that E2F1 was expressed highly during induction for osteogenic differentiation. IGFBP coordinate and regulate biological activities of IGF, a powerful neurotrophic factor that provokes proliferation, migration, and differentiation of glial and neuronal cells and prevents these cells from going through apoptosis [16]. Some authors have proposed that MSCs might have neurotrophic properties to differentiate into neuron and repair nerve. A previous study using a nerve injury model has shown a considerable increment in the release of IGFBP-1 from transplanted MSCs and IGFBP-1 during repair of peripheral nerves has significant influence on engraftment and neural differentiation of transplanted cells. Therefore, it has been suggested that IGFBP-1 is an essential neurotrophic factor released from MSCs that can promote regeneration of damaged neural tissues [17]. NGFRs are a group of growth factor receptors that can specifically bind to neurotrophins. They are also used as cell surface markers to define MSC phenotype [18]. In particular, in several studies using monoclonal antibodies to NGFR for isolation of NGFR positive MSCs, this subset of cells has a higher neuronal differentiation property than the whole MSCs population [19, 20]. These results were similar to our results showing that IGFBP-1 and NGFR were expressed highly during induction for neurogenic differentiation.

GO is a method to comprehend massive-scale gene expression data. In the GO database, top-level ontologies are molecular function, biological process, and cellular component [21]. In our study, according to GO analysis, GO terms related to molecular functions were overrepresented in all trilineage differentiation of hNTSCs. However, GO terms related to cytokine or chemokine were overrepresented in neural differentiated hNTSCs while GO terms related to catalysis and GO terms related to oxidoreductase activity were mainly overrepresented in chondrogenic and osteogenic differentiated hNTSCs, respectively. These facts clearly showed that differently differentiated MSCs had other specific characteristics. In the future, further studies are needed to find the meaning of other major overrepresented GO terms in differentiated hNTSCs.

We performed microarray analysis during trilineage differentiation of hNTSCs in vitro. We found that over 300 genes were expressed differentially in hNTSCs during differentiation. However, certain limitations need to be noted in this study. Of over 300 genes in hNTSCs we found, we could not evaluate characteristics of the majority of genes regulated during in vitro differentiation. Additional studies are needed to determine changes of most genes detected by microarray analysis and characterize these genes more precisely. Additionally, it would be important to identify sequential gene expression during differentiation. Previously, temporal changes of cell growth and osteoblast phenotype-associated genes in human and rodent models have been reported [22, 23]. In future, we will evaluate sequential expression profiling of hNTSCs during differentiation. However, gene expression profiling and GO analysis of this study are useful for defining genes responsible for characteristics of hNTSCs and for analysis of responses of hNTSCs to signals that drive different differentiation processes. Our results may provide novel information of genes involved in the differentiation of hNTSCs.

Conclusions

We described genome-wide gene expression patterns of hNTSCs during trilineage differentiation. Differences in expression profiles of trilineage differentiated hNTSCs were found and 316 candidate genes were shown to be definitely up- or downregulated. Further analytical studies should be conducted to comprehensively determine functional roles of these differentially expressed genes detected by microarray analysis. In the future, gene expression data of hNTSCs could offer important understanding for cellular differentiation processes in MSCs.

Statement of Ethics

This study was approved by the institutional review board committee (IRB No. KC08TISS0341), and informed consent was obtained from all patients.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Sun Hwa Park: data analysis, drafting, final approval, and accountability for all aspects of the work; Do Hyun Kim: data analysis, drafting, final approval, and accountability for all aspects of the work; Mi Hyun Lim: data analysis, drafting, final approval, and accountability for all aspects of the work; Sang A Back: data analysis, drafting, final approval, and accountability for all aspects of the work; Byeong Gon Yun: data analysis, drafting, final approval, and accountability for all aspects of the work; Jung Ho Jeun: data analysis, drafting, final approval, and accountability for all aspects of the work; Jung Yeon Lim: data analysis, drafting, final approval, and accountability for all aspects of the work; Su Young Kim: data analysis, drafting, final approval, and accountability for all aspects of the work; Se Hwan Hwang: data analysis, drafting, final approval, and accountability for all aspects of the work; Sung Won Kim: data analysis, drafting, final approval, and accountability for all aspects of the work.

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