Gene Expression of Neural Markers in Human Dental Follicle Cells

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Abstract

Neurotrauma and neurodegenerative diseases are associated with the loss of functioning neural cells in the nervous system. Many studies reported that function can be restored by replacing lost cells with stem cells that can mature into neural cells. From this perspective, mesenchymal stem cells represent a valuable tool for regenerative therapy because of their ability to differentiate along several lineages, such as adipocytes, osteoblasts, chondrocytes and neural cells. The dental follicle is an ectomesenchymal tissue surrounding the developing tooth germ. Human dental follicle cells (hDFCs) have the capacity to commit to differentiation into multiple cell types. In this study, we investigated the capacity of hDFCs to differentiate into neural cells, and the efficiency of the neural differentiation process. There was a gene relevant to a neural cell in hDFC. We expanded these findings to address the gene expression of neural markers in hDFCs during neuronal differentiation. The expression levels of *Musashi (MSI)-1* and *-2*, which are neural progenitor cell markers, *microtubule-associated protein 2* (*MAP2*) which is a neuronal cell marker, and *glial fibrillary acidic protein* (*GFAP*) and *myelin basic protein* (*MBP*), which are glial cell markers, were up-regulated in hDFCs undergoing neural differentiation during culture in neuronal differentiation medium. The expression of *tubulin-β-III (TUBB3), which is an early neuronal cell marker,* was peaked on day 3.

Furthermore, expression of *Nestin (NES)* did not change. In conclusion, these *in vitro* data suggest that hDFCs have the capacity to differentiate along neural lineages, raising the possibility that hDFCs may represent a practical and convenient source of adult stem cells for cell-based therapies to treat neurological diseases or trauma.

Introduction

The dental follicle is an ectomesenchymal tissue derived from the neural crest and surrounds the tooth germ. The dental follicle contains stem cells and/or progenitor cells of the periodontium. Human dental follicle cells (hDFCs) have the capacity to differentiate into multiple cell lineages, such as osteoblasts and adipocytes (1-2). hDFCs are a major source of stem cells in human, as they can be easily obtained during the extraction of impacted teeth. Our group previously compared the gene expression profiles of hDFCs and human bone marrow mesenchymal stem cells (hMSCs) to investigate whether hDFCs are a useful cell source for applications in clinical tissue regeneration (3). The expression of MSC markers and growth factor receptors was similar in hDFCs and hMSCs, whereas the expression pattern of homeobox genes differed between the two cell types. hDFCs also express markers for neural stem cells such as *Nestin* (*NES*) and *Notch-1*. Therefore, we hypothesized that hDFCs may have the capacity to differentiate into neural cells.

Neurodegenerative disorders are characterized by the loss or atrophy of neurons, leading to various functional disorders. Different approaches have been proposed to promote peripheral nerve regeneration, including administration of neurotrophic factors, and implantation of stem cells, including embryonic stem cells, neural stem cells, and MSCs (4-7). MSCs are multipotent stem cells that can differentiate into multiple cell types (8, 9). The *in vitro* growth of undifferentiated MSCs is an important modality for cell therapy to treat neurodegenerative disease (10, 11). The aim of this study has been to investigate neuronal differentiation potential of hDFC by microarray and Real-time PCR. Another goal has been to determine whether hDFC are useful as cell source for nervous regeneration in nervous disease and injuries.

Materials and Methods

Isolation and culture of hDFCs

hDFCs were obtained using a previously reported method (1). Briefly, normal human impacted third molars were surgically removed and collected from patients (female: 14 years of age) who gave informed consent. Dental follicle tissues were washed in phosphate-buffered saline, minced with sterilized scalpels, and digested in a solution of 0.1 U/ml collagenase type I and 1 U/ml dispase (Roche, Basel, Switzerland) for 1 h at 37°C. hDFCs attached to 100-mm culture plates and were grown in MSC growth medium (GM; consisting of MSC basal medium supplemented with fetal bovine serum, L-glutamine, and penicillin/streptomycin; Lonza, Basel, Switzerland) in a humidified incubator (CO_2 incubator MCO-175M; Panasonic, Tokyo, Japan) in 5% CO_2 at 37°C. hDFCs from the 5th to 6th passage were used for all experiments. The use of hDFCs was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (Recognition number: EC 15-10-036-1 and EC 15-040).

Neuronal differentiation

hDFCs were seeded at 4.0×10^4 cells/dish on 35-mm dishes coated with fibronectin (BioCoat^{TM}, Corning, Corning, NY) in GM in a humidified incubator in 5% CO_2 at 37°C. After the cells reached 50-70% confluency, the medium was replaced with MSC Neurogenic Differentiation Medium (NDM; Promocell, Heidelberg, Germany) and the cells were cultured for a further 7 days, with medium replacement every 2 days.

Total RNA isolation

Total RNA was isolated using miRNeasy Mini Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Microarray analysis

Gene expression profiling was performed with SurePrint G3 Human GE microarray 8×60k (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, Cy3-labelled cRNA was generated from 100 ng of total RNA using Low Input Quick Amp Labeling kit, one-color (Agilent), and hybridized to the array using a Gene Expression Hybridization kit. The array was scanned by an Agilent DNA Microarray Scanner. Raw data obtained from the microarray was loaded into a Gene Spring GX software (Version 11.5; Agilent).

Real-time PCR

Complementary DNA (cDNA) was synthesized from total RNA using a GeneAmp RNA PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed using a DyNAmo SYBR Green qPCR Kit (Thermo Fisher Scientific). The PCR mixture, containing 20 pmol forward and reverse primers and 2 μl cDNA, was subjected to amplification with a DNA Engine Opticon 1 (Bio-Rad, Hercules, CA, USA), with preheating at 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 60°C or 55°C for 30 sec, and 72°C for 30 sec. Primer sequences and the annealing temperatures are shown in Table 1. Gene expression levels were calculated using the $\Delta\Delta C_T$ method with normalization to *GAPDH* (12).

Statistical analysis

Data are shown as mean values ± SD. The two-way ANOVA was used for the analysis of differences and a value of $p < 0.05$ was considered to be statistically significant.

Results

Microarray expression analysis

We used microarray analysis to investigate the expression of neural markers in undifferentiated hDFCs that were not subjected to neuronal differentiation conditions. As shown in Table 2, hDFCs expressed the neural stem cell marker, *Nestin (NES)*, the neural progenitor markers, *Musashi (MSI)-1* and *-2*, a neuronal marker, *tubulin-β-III (TUBB3)*, and the glial cell marker, *myelin basic protein* (*MBP*).

Gene expression of neural stem/progenitor markers during neuronal differentiation

The gene expression of neural markers were examined in hDFCs cultured in GM or NDM on fibronectin coated culture dishes for 0, 3, and 7 days. *NES* was constitutively expressed in hDFCs (Fig. 1a). The expression of *MSI-1* and *-2* was significantly increased in hDFCs cultured with NDM compared with GM (Fig. 1b, and c). In addition, the expression levels of *MSI-1* and *-2* increased in hDFCs cultured with NDM in a time-dependent manner (Fig. 1b, and c).

Gene expression of neuronal markers during neuronal differentiation

The expression of *microtubule-associated protein 2 (MAP2)* was significantly increased in hDFCs cultured with NDM compared with GM (Fig. 2a), and also increased in NDM culture in a time-dependent manner. The expression of *TUBB3* peaked on day 3, and then decreased on day 7 in hDFCs cultured with both GM and NDM (Fig. 2b). *TUBB 3* expression was not up-regulated in NDM culture compared with GM culture (Fig. 2b).

Gene expression of glial cell markers during neuronal differentiation

The expression of *glial fibrillary acidic protein (GFAP)* was significantly increased in NDM culture compared with GM culture on day 3 and 7, although the expression level was similar between GM day 0 and NDM day 7 (Fig. 3a). The expression of *GFAP* was decreased in hDFCs cultured with GM on day 3 and 7 compared with day 0. The expression of *MBP* was significantly increased in NDM culture compared with GM culture on day 3 (Fig. 3b). The expression of *MBP* was decreased in hDFCs cultured with GM on day 3 compared with day 0.

Discussion

Our observation that undifferentiated hDFCs expressed several neural makers by microarray analysis suggesting that hDFCs have neurogenic potential. This study was initiated to explore the neuronal differentiation potential of hDFCs cultured in NDM on fibronectin-coated plates. The gene expression of markers of neural cells, including neuronal stem cell/progenitors, neuronal cells, and glial cells was examined in hDFCs during neuronal differentiation.

The expression levels of *MSI-1* and *-2* were significantly increased in hDFCs during neurogenic differentiation, whereas *NES* expression was expressed constitutively and did not change. *NES* expression by pluripotent stem cells is considered to be a prerequisite for the commitment of cells toward the neural lineage (13, 14). *MSI-1* and *-2* are mammalian neural RNA-binding proteins that are highly enriched in neural precursor cells that can generate both neurons and glia during embryonic and postnatal central nervous system development (15, 16). Previous reports have shown that *MSI-1* was expressed during early neuronal development of neural stem/progenitor cell cultures from mouse brain and human umbilical cord blood cells (16). Consistent with the microarray data, these results suggest that hDFCs contain a neural stem/progenitor cell population. *MAP2*, a neuronal marker, is associated with actin during early axonal development (17). *TUBB3*, a phosphorylated tubulin that is considered to be a neural-specific marker, is expressed during the initial stages of brain development (18). In this study, the expression of *MAP2* was significantly increased in hDFCs during neuronal differentiation in a time-dependent manner. In contrast, the expression of *TUBB3* was increased in hDFCs cultured with NDM on day 3 compared with day 0, and then decreased on day 7. It has been reported that the expression of *TUBB3* is slightly up-regulated in porcine neural progenitor cells treated with ciliary neurotrophic factor compared to standard proliferation conditions, and then slightly down-regulated during the treatment of ciliary neurotrophic factor (19). These results suggest that *TUBB3* expression may be increased transiently in the early phase of neuronal induction. *GFAP*, encoding an intermediate filament protein, and *MBP*, encoding a structural protein in myelin, are markers of glial cells (20). The expression of *GFAP* and *MBP* was up-regulated in hDFCs cultured with NDM compared with GM, although expression was decreased on day 3 in both GM and NDM culture compared with day 0. These results suggest that cultured hDFCs display heterogeneous phenotypes during neurogenic differentiation. This possibility is consistent with several studies that showed that although MSCs from several tissues, including bone marrow, umbilical cord, and dental pulp cells, have the potential for neuronal differentiation, only a subpopulation of MSCs are capable of differentiating into neuron-like cells *in vitro.* This study shows that hDFCs have neural progenitor-like properties and express neural markers in an undifferentiated state. hDFCs up-regulated several neural markers in appropriate neural stimulation conditions. In conclusion, we suggest that hDFCs have the capacity to differentiate along neural lineage and hDFCs are appropriate candidates for treatment of nervous diseases and injuries.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

(1) Morsczeck C, Götz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, Sippel C, Hoffmann KH: Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol, 24: 155-165, 2005.

(2) Kémoun P, Laurencin-Dalicieux S, Rue J, Farges JC, Gennero I, Conte-Auriol F, Briand-Mesange F, Gadelorge M, Arzate H, Narayanan AS, Brunel G, Salles JP: Human dental follicle cells acquire cementoblast features under stimulation by BMP-2/-7 and enamel matrix derivatives (EMD) in vitro. Cell Tissue Res, 329: 283-294, 2007.

(3) Aonuma H, Ogura N, Takahashi K, Fujimoto Y, Iwai S, Hashimoto H, Ito K, Kamino Y, Kondoh T: Characteristics and osteogenic differentiation of stem/progenitor cells in the human dental follicle analyzed by gene expression profiling. Cell Tissue Res, 350: 317-331, 2012.

(4) Pollock M: Nerve regeneration. Curr Opin Neurol, 8: 354-358, 1995.

(5) Murakami T, Fujimoto Y, Yasunaga Y, Ishida O, Tanaka N, Ikuta Y, Ochi M: Transplanted neuronal progenitor cells in a peripheral nerve gap promote nerve repair. Brain Res, 974: 17-24, 2003.

(6) Pan HC, Yang DY, Chiu YT, Lai SZ, Wang YC, Chang MH, Cheng FC: Enhanced regeneration in injured sciatic nerve by human amniotic mesenchymal stem cell. J Clin Neurosci, 13: 570-575, 2006.

(7) Pan HC, Cheng FC, Chen CJ, Lai SZ, Lee CW, Yang DY, Chang MH, Ho SP: Post-injury regeneration in rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells. J Clin Neurosci, 14: 1089-1098, 2007.

(8) Herzog EL, Chai L, Krause DS: Plasticity of marrow-derived stem cells. Blood, 102: 3483-3493, 2003.

(9) Keating A: Mesenchymal stromal cells: new directions. Cell Stem Cell, 10: 709-716, 2012.

(10) Martino G, Franklin RJ, Van-Evercooren AB, Kerr DA: Stem cell transplantation in multiple sclerosis: current status and future prospects. Nat Rev Neurol, 6: 247-255, 2010.

(11) Maltman DJ, Hardy SA, Przyborski SA: Role of mesenchymal stem cells in neurogenesis and nervous system repair. Neurochem Int, 59: 347-356, 2011.

(12) Livak KJ, Schmittgen TD: Analysis of Relative Gene Expression Data Using

Real-Time Quantitative PCR and the 2DD*C*T Method. METHODS, 25: 402-408, 2001.

(13) Shiota M, Heike T, Haruyama M, Baba S, Tsuchiya A, Fujino H, Kobayashi H, Kato T, Umeda K, Yoshimoto M, Nakahata T: Isolation and characterization of bone marrow-derived mesenchymal progenitor cells with myogenic and neuronal properties. Cell Res, 313: 1008-1023, 2007.

(14) Wislet-Gendebien S, Leprince P, Moonen G, Rogister B: Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. J Cell Sci, 116: 3295-3302, 2003.

(15) Sakakibara S, Nakamura Y, Datoh H, Okano H: RNA-Binding Protein Musashi2: Developmentally Regulated Expression in Neural Precursor Cells and Subpopulations of Neurons in Mammalian CNS. J Neurosci, 21: 8091-8107, 2001.

(16) Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR: Expression of Neural Markers in Human Umbilical Cord Blood. Exp Neurol, 171: 109-115, 2001.

(17) Tondreau T, Lagneaux L, Dejeneffe M: Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. Differentiation, 72: 319-326, 2004.

(18) Jiang YQ, Oblinger MM: Differential regulation of beta III and other tubulin genes during peripheral and central neuron development. J Cell Sci, 103: 643-651, 1992.

(19) Yang J, Gu P, Menges S, Klassen H: Quantitative changes in gene transcription during induction of differentiation in porcine neural progenitor cells. Mol Vis, 18: 1484-1504, 2012.

(20) Lamers KJB, Vos P, Verbeek MM, Rosmalen F, van Geel WJA, van Engelen BGM: Protein S-100B, neuron-specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) in cerebrospinal fluid (CSF) and blood of neurological patients. Brain Res Bull, 61: 261-264, 2003.

Table 1 Primer sequences for real-time PCR.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	annealing temperature (˚C)
・Neural stem/progenitor markers			
NES	AACAGCGACGGAGGTCTCTA	TTCTCTTGTCCCGCAGACTT	60˚C
MSI-1	GCCCAAGATGGTGACTCG	ATGGCGTCGTCCACCTTC	60˚C
MSI-2	TTAGGTGATGTCCTCAGACC	GAGAGGGAAACCATCAAGA	60˚C
・Neuronal marker			
TUBB 3	AGTGATGAGCATGGCATCGA	AGGCAGTCGCAGTTTTCACA	60˚C
MAP2	AGGCGTATGATCTCTTTGAG	GTTTGCTCCTAGGGTTTCTT	55˚C
・Glial cell marker			
GFAP	GAGGCGGCCAGTTATCAGGA	GTTCTCCTCGCCCTCTAGCA	60˚C
MBP	CTATAAATCGGCTCACAAGG	ATTAGGTAACAGGGGCAAGT	55˚C

Table 2 Gene expression of neural markers in undifferentiation hDFC.

Gene Symbol	GeneBank accsion	Signal intensity	Flag	Gene Title
・Neural stem/progenitor markers				
NES	NM_006617	4976.7	Detected	Nestin
MSI 1	NM_002442	255.2	Detected	Musashi Drosophila Homolog 1
MSI 2	NM_138962	139.8	Detected	Musashi Drosophila Homolog 2
・Neuronal marker				
TUBB 3	NM_006086	14016.4	Detected	Tubulin, BETA-3
・Glial cell marker				
MBP	NM_001025101	25.1	Detected	Myelin Basic Protein

Neural stem/progenitor cell markers

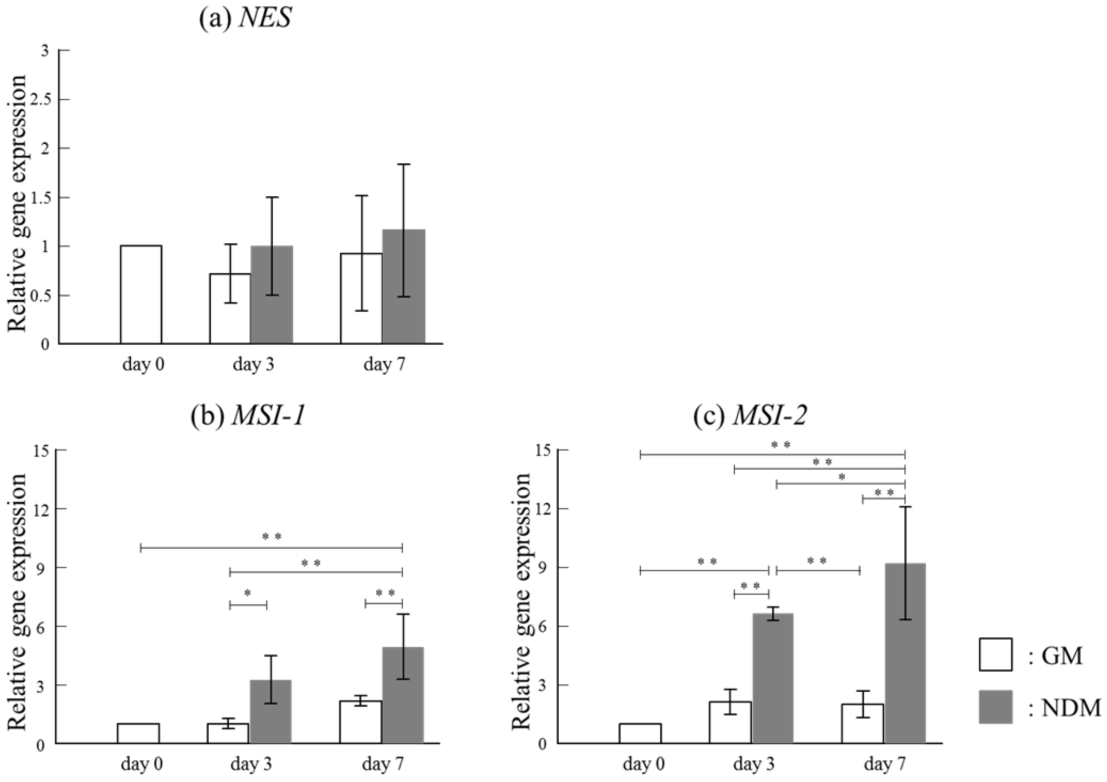


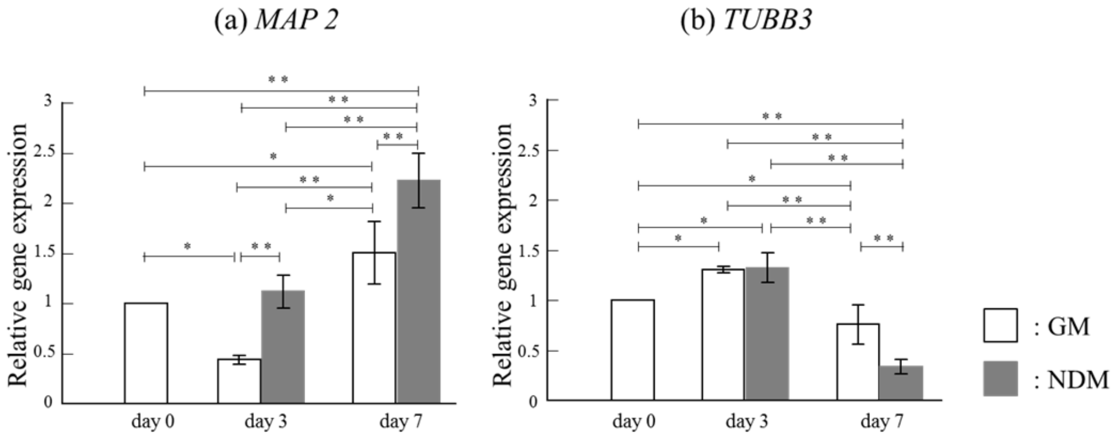
Figure 1:

Gene expression of neural stem/progenitor markers during neuronal differentiation

Expression of neural stem/progenitor cell marker was examined in hDFCs during neuronal differentiation in monolayer culture.

Values represent means ± SD (n = 3). *p < 0.05, ** p < 0.01

Neuronal cell markers


(a) *MAP 2*

(b) *TUBB3*

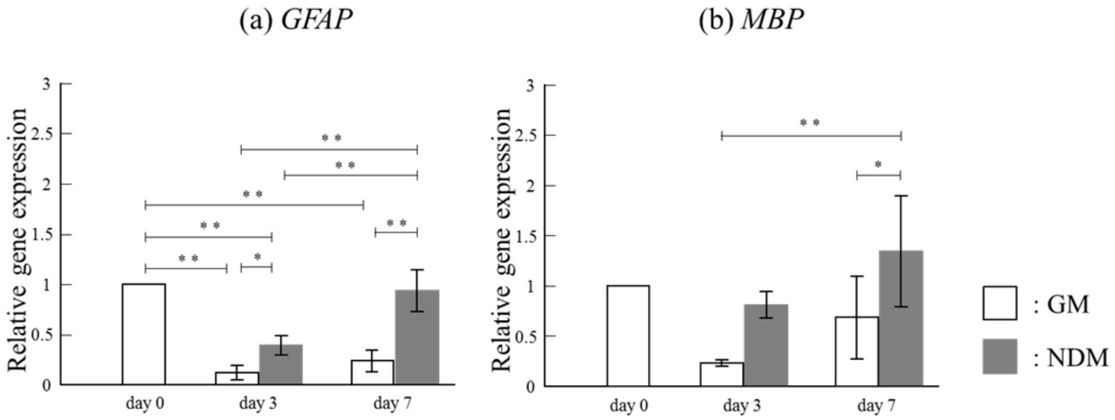
Figure 2:

Gene expression of neuronal markers during neuronal differentiation

Expression of neuronal cell markers was examined in hDFCs during neuronal differentiation by monolayer culture.

Values represent means ± SD (n = 3). *$p < 0.05$, ** $p < 0.01$

Glial cell markers


(a) *GFAP*

(b) *MBP*

Figure 3:

Gene expression of glial cell markers during neuronal differentiation

Expression of glial cell markers was examined in hDFCs during neuronal differentiation by monolayer culture.

Values represent means ± SD (n = 3). *$p < 0.05$, ** $p < 0.01$