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Differentiation of dental pulp stem cells into a neural lineage

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Abstract We previously investigated whether dental pulp-derived cells possess similar pluripotency to bone marrow cells, and reported their capacity to differentiate into osteoblasts, as well as the characteristics of the stem cells present in dental pulp. In the present study, we hypothesized that neural stem cells would also exist in rat dental pulp, similar to bone marrow and the brain, and attempted to induce their differentiation into a neural lineage by applying an *in vitro* study design previously reported to induce differentiation of human bone marrow cells. Before inducing differentiation, we detected cells expressing nestin (Nes), which is known to be a marker for neural stem cells, within primary cultures of rat dental pulp-derived cells, suggesting the existence of neural stem cells in dental pulp. Quantitative analyses of the mRNA and protein expression levels revealed downregulation of both the Nes mRNA and protein levels to about 68.1% and 12.4%, respectively, after the induction of differentiation compared to the corresponding levels before induction. Conversely, the glial fibrillary acidic protein (Gfap) mRNA level was elevated by 1.3-fold after the induction of differentiation compared with the level before induction. The reduced number of Nes-positive cells and decreased Nes mRNA and protein levels after the induction of differentiation may be attributed to differentiation of neural stem cells into a neural lineage. Moreover, the increased number of Gfap-positive cells and increased Gfap mRNA level after the induction of differentiation most likely support their progressive differentiation into a glial cell lineage, since Gfap is a marker that is upregulated in glial cells. Our present data demonstrate the existence of neural stem cells in tissues other than the central nervous system, and may represent a significant step toward providing more diverse and multiple sources of stem cells for future regenerative medicine.

Key words
Dental pulp,
Differentiation,
Neural lineage,
Stem cell

Introduction

Since the existence of pluripotent stem cells in bone marrow was substantiated, they have been widely used in tissue engineering and genetic therapies[1]. Furthermore, these stem cells have successfully been induced to differentiate into myocytes, glial cells and hepatocytes[2–4]. In the brain, and also bone marrow, neural stem cells with the capacity to differentiate into neural lineages have been identified based on the existence of cells expressing nestin (Nes), a neural stem cell marker. These cells were observed to differentiate into neurocytes and astrocytes[5,6]. Although Nes is mainly expressed at early stages of central nerve system (CNS) development, and then replaced by neurofilaments, it is also expressed in other tissues, such as the heart, testes and neural crest[7]. In most of these tissues, Nes is generally expressed during early development and downregulated in mature tissues[7]. Nes is also accepted to be a marker for odontoblasts that reflects their neural-crest derivation. Therefore, Nes expression in cells could indicate their possible derivation from the neural crest[8]. It is well known that neural stem cells can be obtained from brain tissue and

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bone marrow, but these tissues are difficult to access, and it would be useful to be able to obtain such cells from tissues that are easily available. However, little information has been reported regarding dental pulp stem cells[9,10].

We hypothesize that cells derived from dental pulp may have the potential to differentiate into somatic cells, including neurocytes, myocytes, adipocytes, chondrocytes and osteocytes. Previously, we demonstrated that cells derived from dental pulp could differentiate into osteoblasts[11]. Pluripotent stem cells have also been observed among the cells derived from dental pulp[12]. Many *in vitro* and *in vivo* studies have reported the differentiation of neural stem cells originating from human bone marrow into neural lineages[5,6,13–16]. However, no previous studies have investigated the existence of neural stem cells in dental pulp or their capacity for neural differentiation.

In the present study, we first demonstrated the existence of neural stem cells in rat dental pulp and then investigated their capacity to differentiate into a neural lineage by applying an *in vitro* study design previously reported to induce differentiation of bone marrow cells[5,6].

Materials and Methods

Animals and primary cultured cells

Five-week-old male Wistar rats (Shimizu Laboratory Supplies, Kyoto, Japan) were used in this study. Following administration of pentobarbital sodium (20 mg; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) into the peritoneal cavity, the rats were sacrificed without pain. After removal of the mandible, it was resected at the distal site of the third molar. The pulp tissue was gently extracted from the cut section of the mandible using a dental instrument (reamers, 25 mm #10; Morita Corporation, Osaka, Japan), minced in phosphate-buffered saline (PBS(−)) and incubated in PBS(−) containing 3 mg/mL collagenase type I (Invitrogen, Carlsbad, CA, USA) and 4 mg/mL dispase (Invitrogen) for 30 min at 37°C. The released cells were inoculated onto polylysine-coated Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY, USA) and low-cell-binding 24-well plates (Nalge Nunc International). The cells were cultured for 3 days in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% fetal calf serum (Invitrogen) for primary cultures. Animal experiments were conducted in accordance with the guidelines of Osaka Dental University for the care and use of laboratory animals.

In vitro neurogenic induction assay

Cells were maintained in D-MEM/F-12 (Sigma-Aldrich) supplemented with 1.55 mg/mL glucose (Sigma-Aldrich), 0.073 mg/mL L-glutamine (Sigma-Aldrich), 1.69 mg/mL $NaHCO_3$ (Sigma-Aldrich), N-2 Plus Media Supplement (25 μg/mL bovine insulin, 100 μg/mL human transferrin, 16.1 μg/mL putrescine, 5.2 ng/mL selenite and 6.3 ng/mL progesterone; R&D Systems Inc., Minneapolis, MN, USA), 20 ng/mL EGF (R&D Systems) and 20 ng/mL FGF (R&D Systems) at 37°C in a 5% humidified CO_2 atmosphere. The media were changed twice a week for varying periods up to 2 weeks.

Immunofluorescence staining

Cells cultured on chamber slides were fixed with 4% paraformaldehyde (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 20 min, placed in 0.1 M phosphate buffer containing 0.1% Triton X-100 (Sigma-Aldrich) for 5 min to increase the antibody permeability, and then washed twice with 10 mM glycine in PBS (Sigma-Aldrich). Non-specific binding was blocked by treatment with 3% BSA-PBS (Pierce Biotechnology Inc., Rockford, IL, USA) for 30 min at room temperature, followed by washing with 10 mM glycine-PBS. Next, the cells were incubated with primary antibodies against nestin (Nes; Chemicon International Inc., Temecula, CA, USA), a neural stem cell marker, or glial fibrillary acidic protein (Gfap; Spring Bioscience, Fremont, CA, USA), a glial cell marker, for 1 h at room temperature. Subsequently, the cells were washed 3 times with 0.1% BSA-PBS and incubated with Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. After 3 washes with 0.1% BSA-PBS, the nuclei were counterstained with SYTOX Green (Invitrogen), and the cells were observed using a laser confocal microscope (FLUOVIEW FV300; Olympus, Tokyo, Japan).

Quantitative real-time RT-PCR assay

Total RNA was isolated using an RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. Part of each sample was subjected to real-time RT-PCR analysis by Takara Bio Inc. (Otsu, Japan) to examine the expression

Table 1 Primers used in the quantitative real-time RT-PCR assays

Gene	Primer nucleotide sequence	Accession number
Gapdh	Forward: 5′-GACAACTTTGGCATCGTGGA-3′; Reverse: 5′-ATGCAGGGATGATGTTCTGG-3′	NM_017008
Tubb3	Forward: 5′-CAGCTGGAACGCATCAGTGTC-3′; Reverse: 5′-CAGGCCTGAATAGGTGTCCAAAG-3′	NM_139254
Mtap2	Forward: 5′-TGTACCTGGAGGTGGTAACGTGAA-3′; Reverse: 5′-ACCTGCTTGGCGACTGTGTG-3′	NM_013066

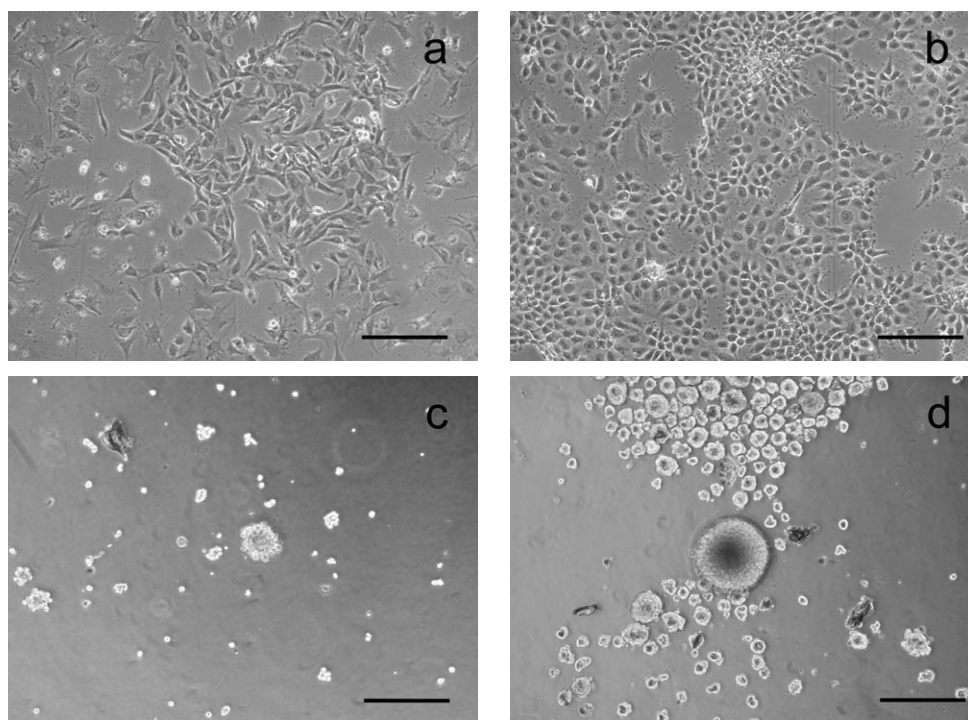

Fig. 1 Cell morphologies

The cell morphologies of the rat dental pulp cells at 0W (a) and 2W (b) after the induction of differentiation are shown. On the low-cell-binding plates, some of the cells have differentiated into a neurosphere-like body at 2W (d) compared to the morphology at 0W (c). Scale bars, 200μm.

levels of tubulin-βIII (Tubb3) and microtubule-associated protein 2 (Mtap2). Rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control to correct the quantitative analysis. The rat-specific PCR primers used are listed in Table 1. Moreover, the expression levels of Nes (TaqMan Gene Expression Assays, Rn00564394_m*l*) and Gfap (TaqMan Gene Expression Assays, Rn00566603_m*l*) were examined by real-time RT-PCR analyses using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Ribosomal RNA Control (18S rRNA) MGB (Applied Biosystems) was used as an internal control for the quantitative analysis using the ABI system.

Preparation of whole cell lysates

For detection of the Nes protein expression level, whole cell lysates of dental pulp cells were prepared before and at 2 weeks after the induction of differentiation. Briefly, dental pulp cells on 35-mm dishes were washed twice with ice-cold PBS, and then harvested in PBS. After a brief centrifugation, the cells were lysed by the addition of 20μL of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS and

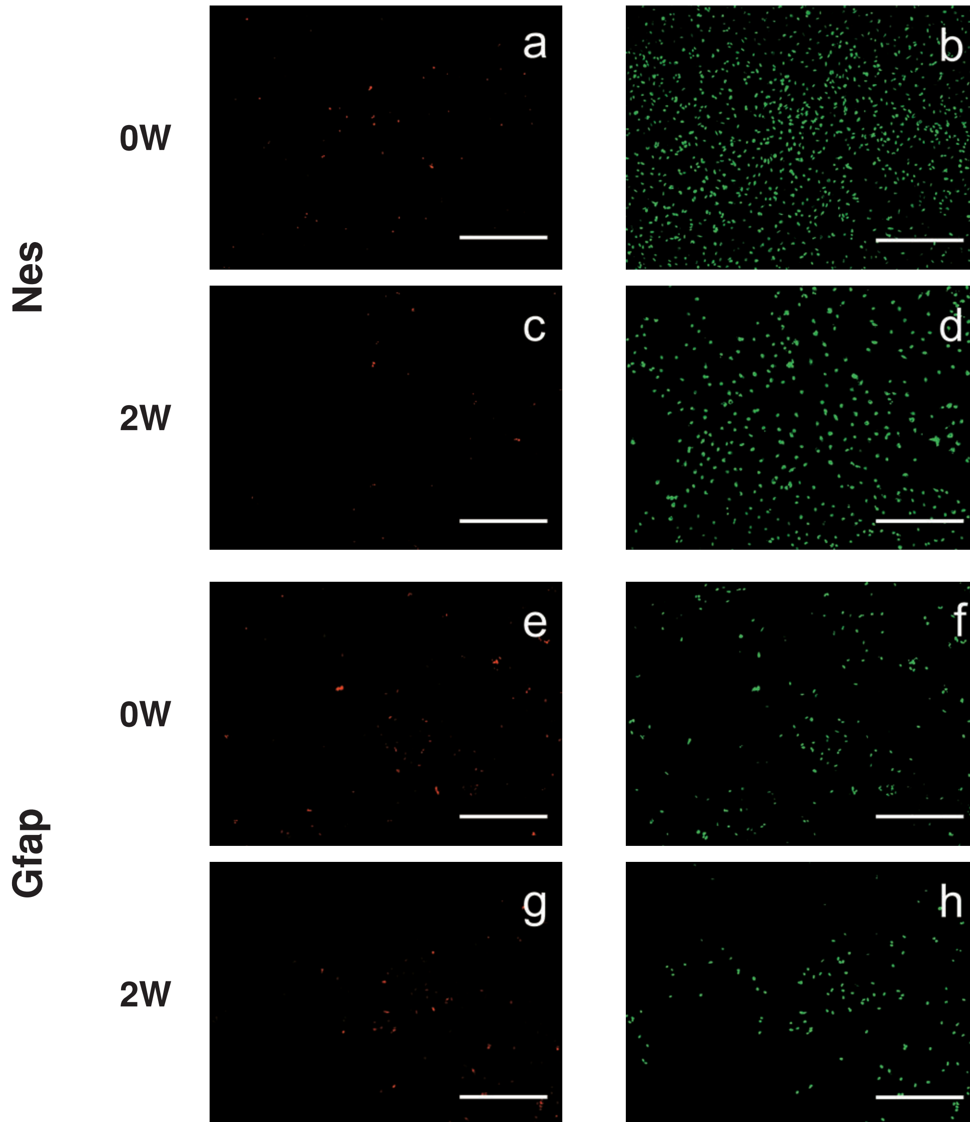

Fig. 2 Immunofluorescence staining

Cells obtained from rat dental pulp before (0W: a, b, e, f) and after (2W: c, d, g, h) the induction of differentiation were immunofluorescently stained for Nes, a neural stem cell marker (a–d), or Gfap, a glial cell marker (e–h). The left panels show the numbers of cells expressing each protein. The right panels show counterstaining of the nuclei. Scale bars, 200μm.

protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)) for 1 h at 4°C with occasional suspension. Whole cell lysates were obtained as the supernatants following centrifugation at 10,000 × *g* for 10 min at 4°C. The protein concentrations of the whole cell lysates were determined by the Bradford method.

Western blot analysis

Aliquots of the whole cell lysates (containing 25μg protein) were separated by 2–15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli[17]. After electrophoresis at 20 mA for 2 h, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (ATTO Co., Tokyo, Japan) at 5 V for 120 min. Nonspecific reactions were blocked with 5% skim milk in 0.01 M PBS containing 0.03% Triton X-100 (PBST) for 1 h. Next, the membrane was washed with PBST for 3 × 5 min, incubated with the anti-Nes antibody diluted (1:2,000) in PBST containing 1% skim milk, washed again with PBST for 3 × 5 min and incubated with horseradish peroxidase-conjugated anti-mouse IgG diluted (1:10,000) in PBST containing 1% skim milk for

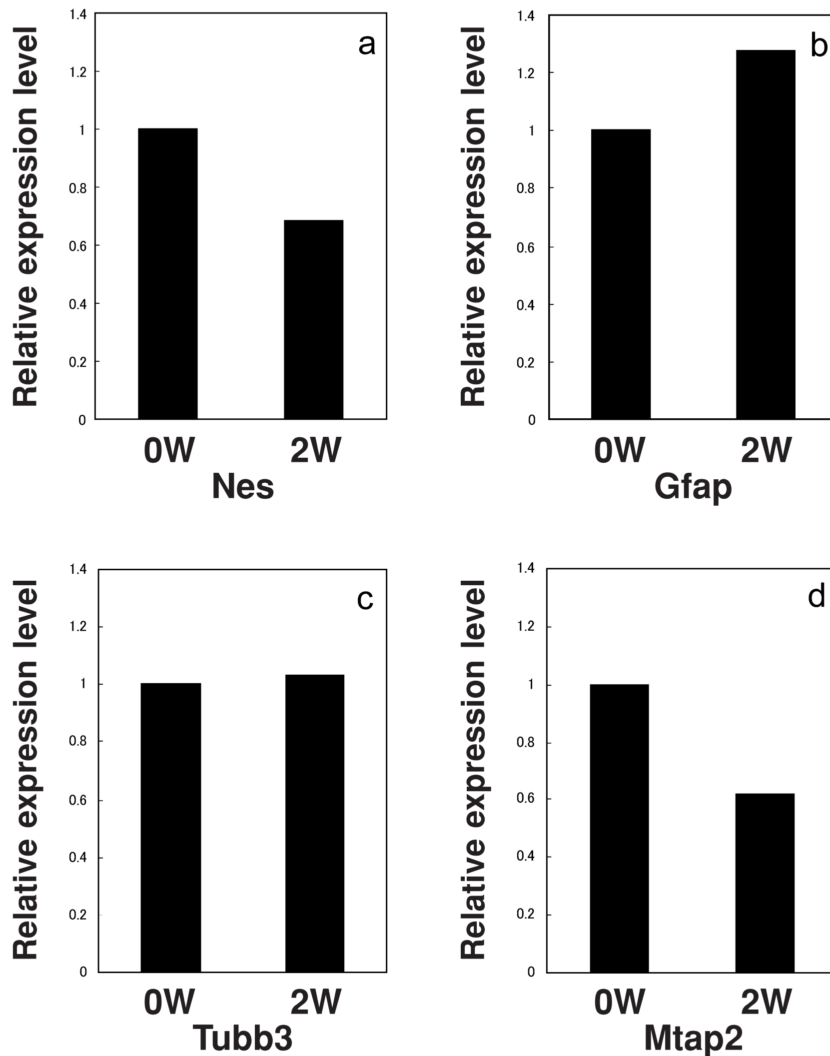

Fig. 3 Analysis of the mRNA expression levels by quantitative real-time RT-PCR

The levels at 2W after the induction of differentiation relative to the corresponding levels at 0W are shown (a: Nes; b: Gfap; c: Tubb3; d: Mtap2). The levels were corrected by the corresponding Gapdh level as a reference. The Y-axis and X-axis represent the relative expression level and the time in weeks, respectively.

30 min. Immune complexes were visualized using ECL plus reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) according to the manufacturer's instructions. The expression levels were analyzed quantitatively using Image J 1.34 software.

Results

Cell morphologies

The rat dental pulp-derived cells had a fibroblastic appearance (Fig. 1a). At 2 weeks (2W) after the induction of differentiation, most of the cells did not show any morphological changes (Fig. 1b). However, some of the cells appeared to have formed neurosphere-like bodies (Fig. 1d), although the number of these bodies was not estimated because they were aggregated on the low-cell-binding plates.

Immunofluorescence staining

The cell preparations were fluorescently stained with anti-Nes or anti-Gfap antibodies before (0W) and at 2W after the induction of differentiation. The positive cells were stained red (Fig. 2a, c, e, g), while the nuclei were counterstained green (Fig. 2b, d, f, h). At 2W after the induction of differentiation, the total cell number had decreased to about 70% (Fig. 2d, h), indicating that the induction agents

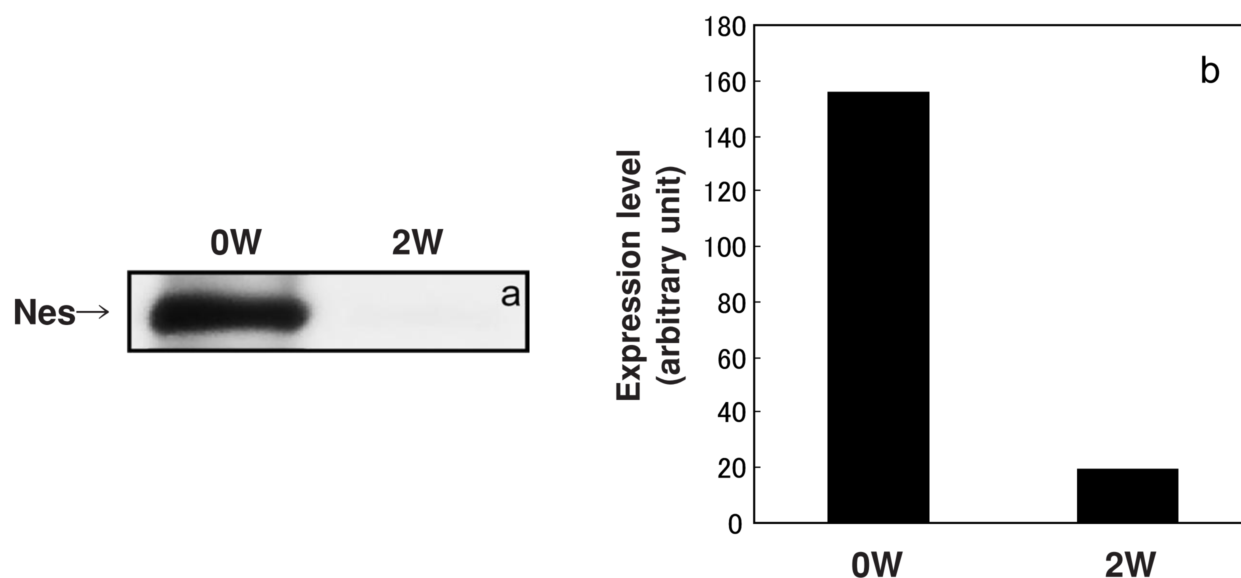

Fig. 4 Quantitative analysis of the protein expression levels

Quantitative analysis of the Nes protein expression levels before and after the induction of differentiation. (a) Detection of a 200-kDa band corresponding to Nes before (0W) and at 2 weeks (2W) after the induction of differentiation. (b) The intensities of the bands in (a) were analyzed quantitatively. The Y-axis and X-axis represent the band intensity and the time in weeks, respectively.

used were somewhat cytotoxic. The Nes-positive cells represented about 3.5% of the cells in one field of view before the induction (Fig. 2a), and decreased to about 2.7% at 2W after the induction (Fig. 2c). Conversely, the number of Gfap-positive cells represented about 10.2% of the cells in one field of view before the induction (Fig. 2e), and increased to about 13.0% at 2W after the induction (Fig. 2g).

Quantitative analysis of the mRNA expression levels

A relative quantitative method ($\Delta\Delta$Ct method) was used for quantitative analysis of the Nes, Gfap, Tubb3 and Mtap2 mRNA expression levels at 2W relative to the corresponding levels at 0W (Fig. 3a–d). The Nes mRNA level was decreased to about 68.1% at 2W compared to the level at 0W (Fig. 3a), whereas the Gfap mRNA level had increased by approximately 27.5% at 2W compared to the level at 0W (Fig. 3b). The Tubb3 mRNA level remained unchanged between 0W and 2W (Fig. 3c). The Mtap2 mRNA level was decreased to about 61.8% at 2W compared to the level at 0W (Fig. 3d).

Quantitative analysis of the protein expression levels

Western blot analysis revealed that the Nes protein level was decreased at 2W after the induction of differentiation compared to the level at 0W (Fig. 4a). A quantitative analysis confirmed that the high level of Nes protein expression at 0W was decreased to about 12.4% at 2W after the induction of differentiation (Fig. 4b).

Discussion

To date, neural stem cells have been proven to exist in bone marrow and the brain[5,6,13,14,18–20]. However, by extending the interpretation of neural progenitors, recent investigations have revealed that their sources are not limited to tissues of the CNS. Among cultured rat or human parenchymal cells derived from the cerebral cortex, spinal cord and hippocampus, pluripotent cells with the ability to proliferate have been identified[21–23]. These findings clearly demonstrate that neural progenitor cells exist in more tissues than expected. Revealing the existence of neural stem cells in tissues outside the CNS will contribute toward meeting the needs of modern regenerative medicine seeking alternative sources of stem cells in mature tissues. Dental pulp has the advantage of being relatively easier to handle than tissues from the CNS, brain and spinal cord, and may represent a possible alternative source of neural stem cells. Therefore, dental pulp-derived

cells can offer very useful materials for cell replacement in the treatment of tissues known to contain neural stem cells.

In previous reports, Nes expression has been used as an evaluation criterion for the existence of neural stem cells[5,6,19], which are associated with the proliferation, differentiation and maturation of neural lineages. During the course of development and maturation into neural lineages, neural stem cells generally disappear, as evidenced by the downregulation of Nes and upregulated expressions of markers for neural cells, such as Gfap, Tubb3 and Mtap2[5,6,18–20,24]. These findings may be due to differentiation into glial cells and neurons, resulting in upregulation of Gfap expression and Tubb3 and Mtap2 expressions, respectively. Regarding Tubb3 and Mtap2, Tubb3 is an earlier marker for neurons than Mtap2. Subcutaneous transplantation of neural stem cells into immunosuppressed mice has been investigated for the induction of successful differentiation into neural lineages *in vivo*[13–15,18]. However, the capacity of stem cells originating from dental pulp for *in vitro* neural differentiation has not yet been studied. Therefore, we investigated the distribution of neural stem cells in dental pulp and attempted to induce neural differentiation *in vitro* to demonstrate their capacity to generate neural lineages. The *in vitro* induction assay used in this study was designed according to a previously reported system for cells derived from human bone marrow[6]. Furthermore, the N-2 Plus Media Supplement we added to the medium as the induction factor has been optimized for neural stem cell expansion[25]. In addition, FGF was included to promote the *in vitro* proliferation of neural precursors, while EGF was added since it may be useful for optimizing human neural precursor growth in culture. These added chemicals appear to stimulate the gene expressions required for neural differentiation in a cooperative manner.

Nes, a 200-kDa intermediate filament protein, was originally identified as a protein predominantly expressed in the stem cells of the neuronal tube, but absent from all mature CNS cells. Upon terminal neural differentiation, Nes is downregulated and replaced by neurofilaments. In the present study, Nes expression at 0W was evidenced by the detection of Nes-positive cells, as well as the presence of both Nes mRNA and protein. These results suggest the existence of neural stem cells in primary cultured cells derived from rat dental pulp. At 2W after the induction of differentiation, the number of Nes-positive cells as well as the Nes mRNA and protein expression levels were all reduced, although its expression was slight but quite detectable at both the mRNA and protein levels. This downregulation is most probably due to differentiation into neural lineages, since Nes is a known marker for neural stem cells and was actually downregulated in the course of induced differentiation in a previous study[5]. Meanwhile, Nes can be detected in odontoblasts, the stratum intermedium and in some of the apical processes of ameloblasts[8]. The expression of Nes could indicate a similarity between the functions of oligodendrocytes and odontoblasts, which are both derived from the neural crest. In other words, both odontoblasts and neural cells in dental pulp have the same origin in the neural crest. In our culture system, we cannot exclude possible contamination by odontoblast-like cells, since we cultured a heterogeneous population of cells derived from dental pulp tissues[7]. However, as shown in Fig. 1, our primary cultured cells showed the morphology of fibroblast-like cells. Moreover, after the neurogenic induction for 2W, the cells downregulated their Nes expression and were considered to be derived from stem cells, since Nes has been shown to be constitutively expressed in odontoblasts of permanent teeth under normal and pathological conditions[7]. Since Nes is rather upregulated in cultured primary human pulp cells during their differentiation into odontoblasts, we consider it appropriate to regard our cells that downregulated their Nes expression after the induction as being derived from stem cells, but not odontoblasts. On the other hand, a quantitative analysis of the mRNA expression levels after the induction revealed upregulation of Gfap, a glial cell marker[5], downregulation of Mtap2, a mature neurocyte marker[5], and unchanged expression of Tubb3, an immature neurocyte marker[19], corresponding to the Nes downregulation, suggesting that the cells tended toward a glial cell lineage, rather then a neurocyte lineage. An increase in the number of Gfap-positive cells after the induction of differentiation was observed by immunofluorescence staining, consistent with the results of the quantitative analysis of the mRNA expression levels demonstrating a high level of Gfap after the induction. The sequential changes in the Nes and Gfap expressions in the rat dental pulp-derived cells according to the progress of differentiation are quite similar to the changes

observed in neural stem cells from bone marrow and the brain. The results for the differentiation-induction model used in the present study suggest that rat dental pulp-derived cells differentiate into a glial lineage. This study has demonstrated the first successful induction of neural differentiation of rat dental pulp-derived cells, particularly into a glial cell lineage. Successful *in vitro* and *in vivo* differentiation of neural stem cells obtained from bone marrow into neurons and/or oligodendrocytes has previously been reported. However, definitive differentiation into neurocytes, astrocytes and oligodendrocytes has not yet been achieved. The present data do not exclude the potential of neural stem cells in dental pulp to differentiate into other neural lineages besides the glial cell lineage. Further investigations concerning the differentiation of these cells into the neurocyte, astrocyte and oligodendrocyte lineages in addition to the glial cell lineage are expected in the future. There are previous reports of induced differentiation of CNS-derived neural stem cells into neural cells[6,13]. Moreover, by substituting the cells used for the induction of neural cell differentiation, the method can be applied to the treatment of Parkinson disease[26]. On the other hand, the functions of the neural cells differentiated from dental pulp stem cells have not yet been investigated, and will be analyzed in future studies. Taken together, these findings suggest the possible pluripotency of rat dental pulp cells. Such pluripotent dental pulp stem cells will benefit future tissue engineering and regenerative medicine.

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