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Nerve Growth Factor Promotes Differentiation of Odontoblast-Like Cells

Szilvia Arany,* Souichi Koyota, and Toshihiro Sugiyama

Department of Biochemistry, Akita University School of Medicine, Akita, Japan

ABSTRACT

Contemporary strategies in tooth repair markedly rely on the newest findings on the cellular and biological components of dental development. Among several identified bioactive molecules, neurotrophins were recently proposed to affect tooth germ cell proliferation, differentiation, and cell–extracellular matrix interactions. The present study attempted to explore the effect of nerve growth factor (NGF) on a spontaneously immortalized dental papilla mesenchymal cell line. NGF induced differentiation of odontoblast-lineage cells with subsequent biomineralization in vitro. Here we showed that normalized transcript levels of tissue-specific markers such as DSPP and DMP1 were elevated significantly, indicating cell differentiation and maturation processes. We performed innovative gene expression analysis of TM14, a matricellular protein and novel member of the fibulin family. TM14 expression followed a pattern similar to that of DMP1, which suggests its important role in cell–matrix and intercellular interactions during dentin calcification. Alkaline phosphatase enzyme assay confirmed the extracellular matrix calcifications in NGF-supplemented groups. Thus, NGF was characterized as a potent promoter of mineralization during dentin formation. For the first time, we included TM14 in odontoblast genotype analysis and proved that NGF also promotes in vitro odontoblast differentiation. Collectively, these results highlight the importance of NGF during tooth morphogenesis, as well as urge the elaboration of complex epithelial–mesenchymal tissue cultures, where further elucidation of the signaling factor network could be completed. J. Cell. Biochem. 106: 539–545, 2009.

KEY WORDS: ODONTOBLAST; NERVE GROWTH FACTOR; TM14; MINERALIZATION

Experiments exploring the pathways of tooth formation are often used as a model system for organogenesis [Thesleff et al., 1996]. Tooth germ development is based on the reciprocal interactions between epithelial and mesenchymal components and on subsequent cell differentiation, morphogenesis, and extracellular matrix formation. Our understanding of this complex biological process and of the regulating signaling pathways has been rapidly expanding recently. Novel aspects of tooth development, with respect to cell–cell, cell–matrix, and matrix–matrix interactions, shed light on the cascade mechanism of organogenesis, which has been thoroughly investigated during the last several decades. Extending our knowledge of the biochemical and cellular components of dental development represents a promising approach for increased biological applications including tissue engineering.

Although tooth regeneration has been addressed using different strategies, all such attempts share a common feature, that is, that odontogenic capacity is inevitably necessary to generate the tooth structure. This potential has been recognized in isolated epithelial cells [Komine et al., 2007], mesenchymal cells from developing teeth [Yu et al., 2006], oral primordial cells [Bhattacherjee et al., 2007], and even in non-dental cells [Ohazama et al., 2004] when successful recombination with tooth germ components could be achieved. Maintaining the odontogenic potential of various stem cells derived from dental progenitor cells or other tissues (e.g., bone marrow cells, embryonic cells, and neural cells) [Modino and Sharpe, 2005] seems to be a key factor during tooth regeneration experiments. It is generally accepted that odontogenic capacity switching from the dental epithelium to the dental mesenchyme during the early stage of tooth formation is a result of sequential and reciprocal biochemical communication. Regulatory molecules, growth factors, transcription factors, and cell adhesion molecules have been intensively studied in terms of the tissue-specific cross-talk, but the signaling network is still not yet fully understood.

Recently, some neurotrophic factors have been shown to be involved in the differentiation of dental epithelial cells [Yoshizaki et al., 2008] as well as pulp cells [Mizuno et al., 2007]. Members of the neurotrophin family have been found to be expressed in the developing tooth germ [Nosrat et al., 2002] and play an important

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*Correspondence to: Szilvia Arany, Department of Biochemistry, Akita University School of Medicine, Hondo 1-1-1, Akita 010-8543, Japan. E-mail: aszilvia@med.akita-u.ac.jp

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role in tooth innervation. Nerve growth factor (NGF) has been shown to be specifically associated with tooth morphogenesis [Amano et al., 1999]. NGF is a polypeptide growth factor that serves as a ligand for two kinds of cell surface receptors: the tyrosine kinase receptor A (trkA) and the p75 neurotrophin receptor (p75). NGF signals mediate neurons and non-neuronal cell differentiation and survival through the high-affinity TrkA receptors, while the low-affinity p75 receptor signals can cause apoptosis [Luukko et al., 1996; Woodnutt et al., 2000]. In developing murine teeth, NGF expression has been documented [Nosrat et al., 1998] in the oral epithelium and in condensed mesenchymal cells, then in the inner dental epithelium and in the stratum intermedium. At the cup stage, the dental papilla and the stellate reticulum are positive for NGF transcripts, after which the expression shifts to the epithelial–mesenchymal interface. During the bell stage, NGF mRNA is localized in polarizing preodontoblasts as well as in the sub-odontoblast zone. These early developmental stage observations imply a regulatory role for neurotrophins in tooth formation and their possible modulating function in the reciprocal epithelial–mesenchymal interactions [Yoshizaki et al., 2008]. To investigate whether NGF affects the differentiation of the mesenchymal component of the epithelial–mesenchymal interface, we set up in vitro cultures of an established odontoblast-lineage cell (OLC) line [Arany et al., 2006]. NGF was added to OLC culture for 4 weeks, and in vitro biomineralization was assayed by the alkaline phosphatase (ALP) enzyme activity. We also included in the experiments reference OLC cultures treated by a standard osteogenic differentiation medium (ODM). To further characterize the OLC differentiation model, we attempted to detect transcripts of a newly identified matricellular protein gene known as TM14 or fibulin-7. TM14 is expressed in preodontoblasts and odontoblasts in vivo, and it has been suggested that it plays a considerable role during odontoblast differentiation [de Vega et al., 2007]. Hence, we aimed to determine expression patterns of TM14 and other odontogenic markers, dentin sialophosphoprotein (DSPP), and dentin matrix protein (DMP)-1 over 28 days.

MATERIALS AND METHODS

CELL CULTURES

Immortalized OLCs were maintained as detailed in an earlier report [Arany et al., 2006]. Thus, culturing conditions are only described briefly here. Embryonic (E18.5) dental papilla cells, isolated from C57BL/6-TgN (act-EGFP) mice, were expanded by the method of Hanks et al. [1998]. The odontoblast phenotype, single cell-derived colony cells were routinely transferred over 150 times to the present during the last 3 years. Those subcultures were periodically monitored and confirmed for odontoblastic features.

OLCs were plated in type I collagen-coated 35 mm dishes, at a concentration of 8×10^3/ml, in minimum essential medium with alpha modification (α-MEM) (Sigma, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma), fibroblast growth factor (FGF)-2 (2 ng/ml; Sigma), and gentamicin (50 μg/ml) L-glutamine (2 mM) (Sigma) solution at 37°C in a humidified chamber of 5% (v/v) CO_2 in air.

IN VITRO DIFFERENTIATION EXPERIMENTS

After cells reached confluence, the experimental treatments were initiated. Mouse recombinant NGF (Wako, Japan) was applied to cell cultures in 50 ng/ml (low) and 100 ng/ml (high) concentrations [Amano et al., 1999]. In addition, a commonly used ODM containing 10 mM β-glycerophosphate plus 50 μg/ml ascorbic acid was administered in positive control dishes. Low- and high-concentration NGF, and ODM media, were refreshed every 3 days during the course of the experiment. OLCs were analyzed 7, 14, and 28 days after the initiation of NGF or ODM treatment.

RNA PREPARATION AND GENE EXPRESSION ANALYSIS

Total RNA of confluent, untreated OLC cultures (in duplicate) was prepared for reverse transcription-PCR using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Template RNA was processed to evaluate TM14 expression pattern in OLCs, and in tooth germ controls (embryonic day 17, and postpartum day 3). The Superscript One-Step RT-PCR with Platinum Taq system (Invitrogen, CA) was utilized including specific mouse primer sets (10 pM/μl) for TM14 (5′-CTACAGATGCATCTGTCCTC-3′ and 5′-CGTGATGGGTGTCTTCAACT-3′) and glyceraldehyde-3-phosphate dehyhrogenase (GAPDH). PCR conditions were set up as follows: 40 cycles of 60 s denaturation at 94°C, 60 s annealing at 56°C, and 90 s extension at 72°C. The PCR products were loaded on 1.5% agarose gel for electrophoresis and visualized by GelRed (Biotium, USA) staining under ultraviolet light exposure.

For real-time RT-PCR assay, cultures were washed twice in phosphate-buffered saline (PBS), and total RNA was extracted (cell culture experiments were performed in triplicate) on days 7, 14, and 28. RNA concentration was calculated by the absorption ratio (OD_{260}/OD_{280}) using a NanoDrop Spectrophotometer (Thermo Scientific, USA). Quantitative reverse-transcriptase-mediated polymerase chain reaction assays were performed in triplicate using the LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics, Switzerland). In the LightCycler 480 real-time PCR System, a 50 ng cDNA template from untreated cultures as well as from cultures treated with 50 or 100 ng/ml of NGF and cultures treated with ODM was prepared in 20 μl reaction mix containing the primer pairs for DSP, DMP1, and TM14. We designed target-specific primer pairs (the nucleotide sequences are listed in Table I) using information from

TABLE I. Primer Sequences for Real-Time PCR Analysis

Target gene	Forward	Reverse
Dentin sialophosphoprotein (DSPP)	5′-ACATGAAACGACGCCTCAGA-3′	5′-CACCAGAGCCTGTATCTTCA-3′
Dentin matrix protein-1 (DMP-1)	5′-ACAGCCTGAACACATTCTCC-3′	5′-ATGTTCTTGGGACGGATGTC-3′
TM14	5′-TGGTTCCTACCGATGTACCT-3′	5′-CGTGATGGGTGTCTTCAACT-3′
18S	5′-CGGCTACCACATCCAAGGAA-3′	5′-GCTGGAATTACCGCGGCT-3′

the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) to monitor cell differentiation. The primer sets were screened in normal RT-PCR before use in real-time RT-PCR analysis. The following cycling parameters were used: pre-incubation (95°C for 5 min), 45 cycles of amplification (denaturing at 95°C for 10 s, annealing at 56°C for 15 s, and elongation at 72°C for 15 s), and melting curve analysis with continuous fluorescent measurement. Standard curves from control dilution series were created, and the relative quantity of target genes was calculated by the normalization method versus the 18S endogenous reference gene for each sample [Nolan et al., 2006].

ALKALINE PHOSPHATASE ASSAY

Alkaline phosphatase activity was assessed in untreated control dishes, as well as in NGF- (50 and 100 ng/ml), or ODM-treated culture dishes. Three culture samples were measured per group and time on days 7, 14, and 28 after initiation of the treatment. Quantitative analysis of ALP activity was achieved using the SensoLyte FDP Alkaline Phosphatase Assay Kit (AnaSpec, USA) by fluorometric measurements on a Fluoroskan Ascent plate reader (Thermo Fischer Scientific). ALP activity (values were calculated from an ALP calibration curve) mean values and standard deviations were calculated.

To confirm biomineralization, parallel OLC cultures from days 14 and 28 were observed by light microscope (in duplicates). Calcium deposition was identified by 1% alizarin red S (Sigma) staining after 4% formaldehyde neutral buffer fixation.

STATISTICAL ANALYSIS

The results of assessment of real-time PCR expression levels and ALP assays were obtained using an unpaired two-tailed Student's test. Values are given as mean values ± standard deviation. *P*-values <0.05 were considered statistically significant results. Statistical analyses were performed using the JMP7 (SAS Institute, Inc., Cary) software for Windows.

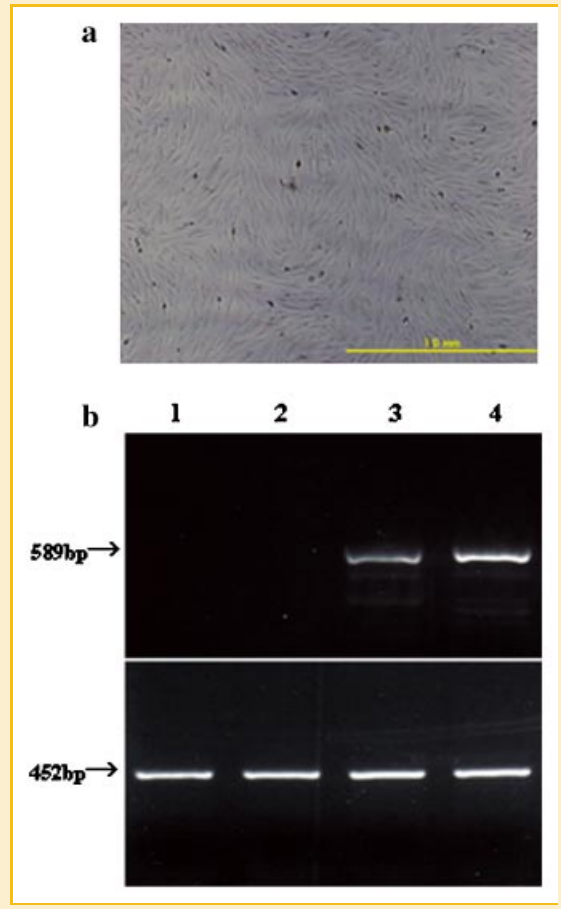

Fig. 1. a: Phase contrast microscopic image of fibroblast-like OLCs at confluence, 7 days in culture (experiments were done in duplicate). b: RT-PCR analysis of TM14 gene expression (589 bp) in odontoblast-lineage cell culture after 1-week culture. Lane 1, 3T3 cells (negative control); lane 2, E17 tooth germ; lane 3, OLC cultured in α-MEM; lane 4, ppd3 tooth germ (positive control). In all lanes GAPDH internal controls are shown, 452 bp.

RESULTS

TM14 DETECTION IN ODONTOBLAST-LIKE CELLS

To investigate whether immortalized odontoblast-like cells express TM14, we seeded cells at low density and cultured them under routine conditions for 1 week (Fig. 1a). Then OLCs were collected and their TM14 gene expression, along with that of tooth germ control samples, was investigated. The OLC line was found to be positive for TM14 transcripts, as shown in Figure 1b. In E17 tooth germs, TM14 mRNAs were not detected, although TM14 gene expression could be detected in ppd3 tooth germs.

EFFECT OF NGF ON IN VITRO CALCIFICATION AND ALP ACTIVITY

For in vitro mineralization investigation, cultures were treated with NGF, applied in low and high concentrations, that is, 50 and 100 ng/ml, respectively. Additionally, positive control cultures were grown supplemented with biomineralization factors in standard ODM. Untreated controls were maintained in the absence of both exogenous NGF and ODM. Cells in every experimental group were grown overconfluent, followed by the formation of multilayer. In ODM- and NGF-treated cultures, calcification initialized promptly after the application of inductive substances. A representative image of calcified matrix deposition is shown in Figure 2b,c after 14 and 28 days, respectively. Microscopic observation revealed the occurrence of mineralization centers on collagen I-coated surfaces of the culture dishes.

As demonstrated in Figure 2a, during the 4-week culture period, a statistically significant increase in alkaline phosphatase activity was recorded both in ODM- and NGF-treated dishes compared to untreated control cells. Specifically, a quick response was detected in the ODM-treated dishes at day 7. In contrast, NGF only modestly affected ALP activity. At day 14, NGF induced a marked increase in ALP activity to an almost equivalent high level in both the low- and high-concentration groups, compared to ODM cultures. At the end of the treatment, steadily boosted enzyme activity was recorded in NGF-treated groups; moreover, cultures treated with 100 ng/ml of NGF manifested the highest ALP activity levels.

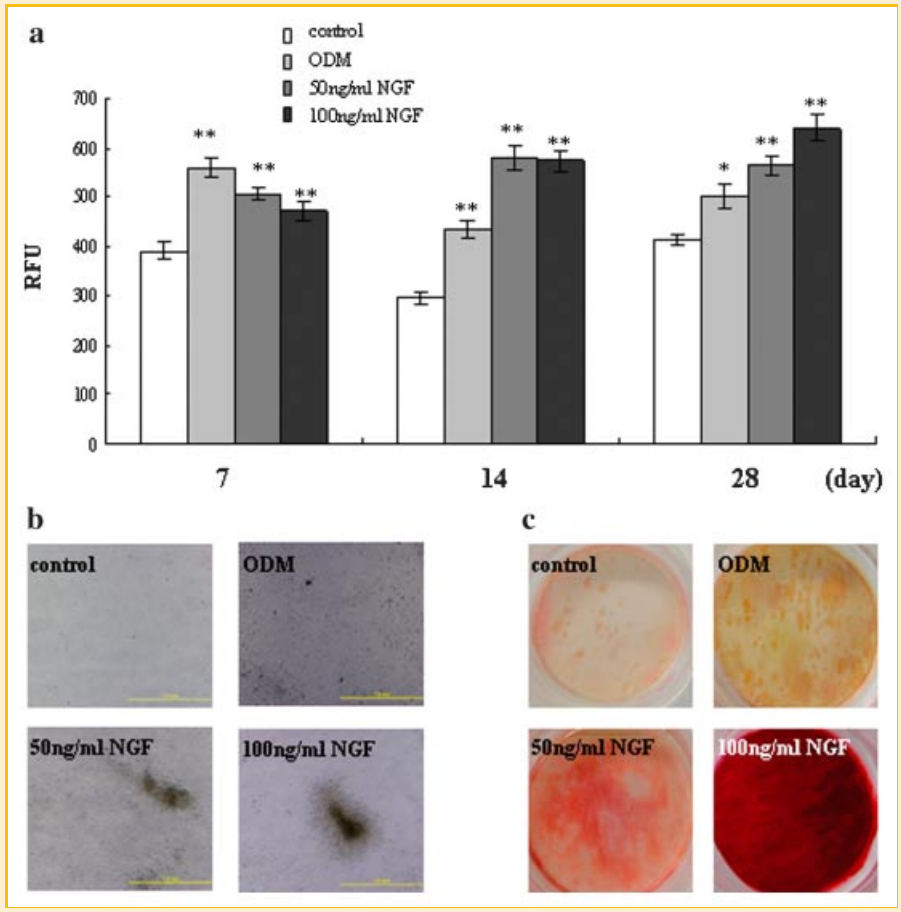

Fig. 2. Extracellular matrix mineralization under differentiation culture conditions in vitro (experiments were done in triplicate). a: Graphical image of ALPase activity in untreated controls and in cell cultures treated either with osteogenic differentiation medium (ODM; positive control) or with low (50 ng/ml) and high (100 ng/ml) concentrations of NGF. The values represent the average of three replicates from each time point (RFU, relative fluorescence units). Standard deviations are indicated by error bars, $^{*}P < 0.05$, $^{**}P < 0.0001$. b: Effects of ODM (positive control), 50 ng/ml NGF, and 100 ng/ml NGF supplementation on OLC cultures on day 14 (light microscopy, treatment initiated after confluence). Controls are represented by untreated cells. NGF-treated (50 and 100 ng/ml) dishes show prominent focal mineral depositions. c: Representative images of progressing calcification in different experimental groups detected by alizarin red S staining after 28-day culture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EFFECT OF NGF ON ODONTOBLAST MARKER GENE EXPRESSION DURING DIFFERENTIATION

On days 7, 14, and 28, the relative expression ratios of the odontogenic markers were calculated based on PCR efficiency (E) and the crossing points (CP) of every sample versus untreated controls (set as baseline controls), and expressed in comparison to 18S reference gene. As demonstrated in Figure 3, the addition of exogenous NGF induced a statistically significant increase in DMP1 and TM14 expression after 7 days. Subsequently, this tendency was maintained during the second week of the experiment. DMP1 was apparently up-regulated in low- and-high concentration NGF cultures, as well as in the positive control ODM-treated cultures by 53-, 76-, and 65-fold, respectively. TM14 also showed up-regulation for low (14-fold) and high (21-fold) concentration NGF and ODM groups (16-fold). In the cells treated with 50 ng/ml of NGF, DSPP expression (1.6-fold) reached the highest level as well. On day 28, DMP1 and TM14 mRNA levels were still prominently higher in NGF- and ODM-treated cultures as compared with those of the untreated controls, and an apparent induction of DSPP transcripts in ODM cultures (1.6-fold) was recorded.

Comparing NGF-treated cultures with the positive control ODM-treated cultures, results showed that NGF treatment yielded similar profile of the regulation of odonto-specific gene expression as ODM treatment. Specifically, at the end of the experiment course on day 28, DMP1 mRNA levels (~43-fold) and TM14 levels (~21-fold) showed an even up-regulation in both of the NGF-treated groups as well as the ODM-treated group. However, DSPP was down-regulated on day 28 in both of the NGF-treated groups, although ODM showed up-regulation at the same time.

DISCUSSION

In vitro investigations of tooth germ development and of the differentiation of dental progenitor cells represent a considerable challenge. The experiments require consistent and homogenous

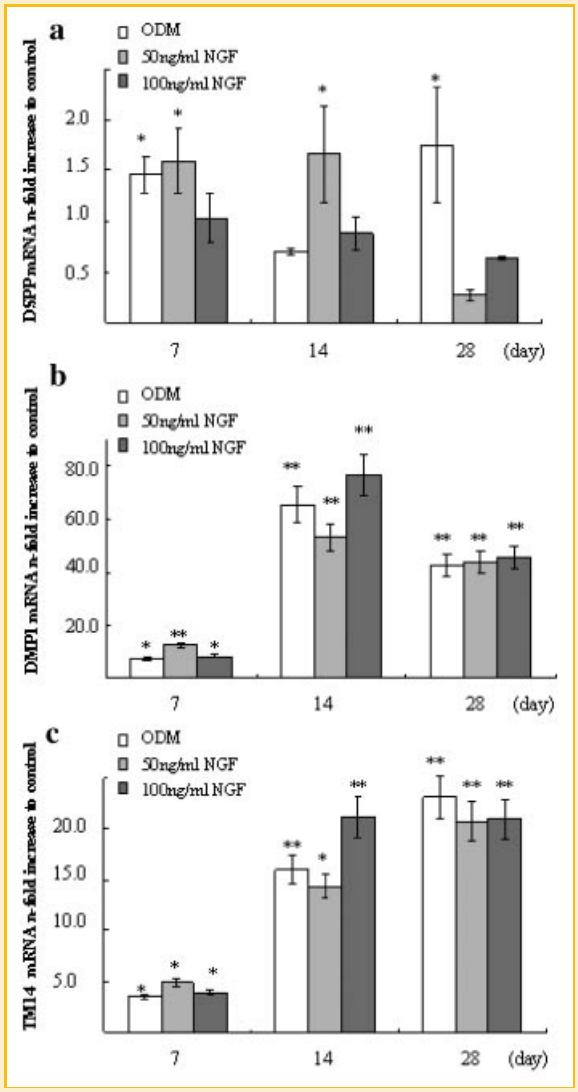

Fig. 3. Quantification of tissue-specific mRNA in OLC cultures using real-time RT-PCR. In vitro cell culture experiments were performed in triplicate. a: DSPP, (b) DMP1, and (c) TM14 expression detected after 7, 14, and 28 days of ODM (positive control) or NGF (50, 100 ng/ml) treatment, respectively. The graphs show the relative expression ratio, calculated by the method of Pfaffl [2001], of a target gene in comparison to a reference gene (18S). Control values (basement lines) are defined by untreated cell culture dishes. Statistical significance and standard deviation indicate values from three replicates of every measurement ($^{*}P < 0.05$, $^{**}P < 0.0001$).

research material for repetitive, reliable data collection and continuous cell lines retaining odontogenic features. Since matured odontoblasts are postmitotic cells and the isolation of preodontoblasts from the dental papilla is indeed a laborious process [Guo et al., 2000], we studied odontoblast differentiation in a spontaneously immortalized OLC line. We set up the culture conditions for OLCs in the presence of NGF, a presumptive mediator for pulp cell differentiation, and compared the results with a well-known differentiation medium (ODM). In order to determine NGF's effect on odontoblast differentiation, we assayed tissue non-specific ALPase activity, which serves as an early sign of calcification. Previous results have demonstrated that neurotrophins stimulate the calcification of human pulp cells [Mizuno et al., 2007]. As expected, NGF significantly increased ALPase levels in OLC cultures. Notably, ALPase response of ODM-treated positive controls showed a prompt increase followed by regression, compared to those in NGF-treated cells. The results confirmed that NGF brings about a long-lasting induction of in vitro biomineralization, which exceeds the effect of the "conventional" ODM. Additionally, the time course of the increased ALPase activity was analogous to that of earlier reports on dental pulp stem cell differentiation [Yu et al., 2006].

To characterize NGF's ability to enhance odontoblast differentiation, we included a quantitative investigation of the odontoblast representative gene expression in vitro. Normalized transcript levels from the small integrin binding ligand *N*-linked glycoproteins (SIBLING) family, such as DSPP and DMP1, displayed up-regulated expression during the 1 month of treatment; however, the expression levels did not increase in a dose-dependent manner. On the contrary, the tooth volume in mouse first branchial arch explants showed dose-dependency with exogenous NGF concentration [Amano et al., 1999]. It is noteworthy to add that a recent publication [Yamakoshi et al., 2005] indicated that dentin sialoprotein (DSP; posttranslational cleavage product of DSPP macromolecule) glycosaminoglycans chains bind to growth factors. The expression of these two SIBLING family members is considered to be the hallmark of odontoblast differentiation [Gronthos et al., 2000] during tooth development. Having established that the up-regulation of these odontoblast prototype genes evidence terminal differentiation of preodontoblasts [Hao et al., 2005], our preliminary results highlight NGF's effect on OLC differentiation. This influence resembles the standard osteogenic differentiation treatment, which was represented in our study by positive controls. According to the latest research communications, DMP1 macromolecules enhance transcription of the DSPP gene [Narayanan et al., 2006]. Thus, DSPP expression follows DMP1 expression. Our findings are consistent with in vivo observations [Hao et al., 2004], suggesting the early onset of DMP1 expression during the differentiation of presumptive odontoblasts. Then, as noticed in developing teeth [Begue-Kirn et al., 1998], DMP1 transcripts later manifest a decreasing tendency, while transcripts of DSPP continue to increase. Conversely, under our in vitro conditions, DMP1 transcripts maintained a significant accumulation, which might be due to the absence of epithelial stimuli and the prolonged application of osteoinductive supplementation. Latest evidence supports this idea since ameloblast differentiation is inducible by NGF supplementation [Yoshizaki et al., 2008]. Since epithelial–mesenchymal interactions lead to cell division, differentiation, and biomineralization in vivo, this inductive milieu would be inevitably necessary for further progression of odontoblast differentiation and dentin matrix production. In conclusion, these results fortify the modulatory function of NGF in cell–cell interactions.

This study included the first attempt, to our knowledge, to demonstrate the expression of a new matricellular protein, TM14, in OLC in vitro cultures during biomineralization. We sought to verify OLCs further on the basis that epithelial components of developing teeth are negative for TM14, and that TM14 is not expressed by the dental mesenchyme at early stages of tooth formation. RT-PCR results validated OLCs as neural-crest-origin, preodontoblast-like,

dental papilla cells. Our pioneer data suggest that exogenous NGF supplementation, analogous to the standard osteogenic treatment, promotes TM14 gene expression in OLCs. Our preliminary observations are supported by the investigators [de Vega et al., 2007] who first described and classified this new fibulin member protein. Accordingly, TM14 is believed to be odontoblast-specific in teeth, with a definitely stronger expression in differentiated odontoblasts than in preodontoblasts. In light of this, we suppose that NGF enhanced the differentiation of dental mesenchymal cells, and thus the highest amount of TM14 mRNA could be detected after 28 days of cell culturing. The expression pattern resembles that of DMP1. DMP1 plays an important role in dentin mineralization through hydroxyapatite nucleation [He et al., 2003]. Having proposed that TM14 co-localized with DMP1 in the predentin matrix [de Vega et al., 2007], which signifies the mineralization front [Narayanan et al., 2006], and that TM14 interacts with ECM molecules (e.g., DSPP), we might conclude that TM14 is the product of odontoblast cells and is an essential factor in the protein–protein interactions of the dentin matrix.

In summary, the present study affirmed the potential of using OLCs for dental developmental research, because OLCs maintain recently updated dentinogenic cytological and functional properties. NGF enhanced OLC biomineralization as well as differentiation and resulted in significantly up-regulated odontoblast-specific gene expression of DSPP, DMP1, and TM14. These findings provide a new perspective on the physiological and non-neural tasks of neurotrophins. However, ambiguity remains regarding the molecular pathway of NGF-triggered differentiation. Besides, our recent understanding of TM14 biological functions is particularly reserved. Our data, consistent with the pioneer study, implied that TM14 deposited in the ECM during mineralization. Consecutive experiments are required to explore whether TM14 is involved in epithelial–mesenchymal interface reciprocal signaling and to clarify its binding mechanism to other ECM molecules. These results, however, are encouraging in terms of future possibilities such as the practical application of various bioactive molecules for prospective treatment. A proposal regarding clinical application of neurotrophins such as use of pulp capping agent after dentin injury has already been introduced [Mizuno et al., 2007]. Hence, further three-dimensional scaffold investigations on possible odontoinductive molecules in vitro and in vivo are necessary.

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