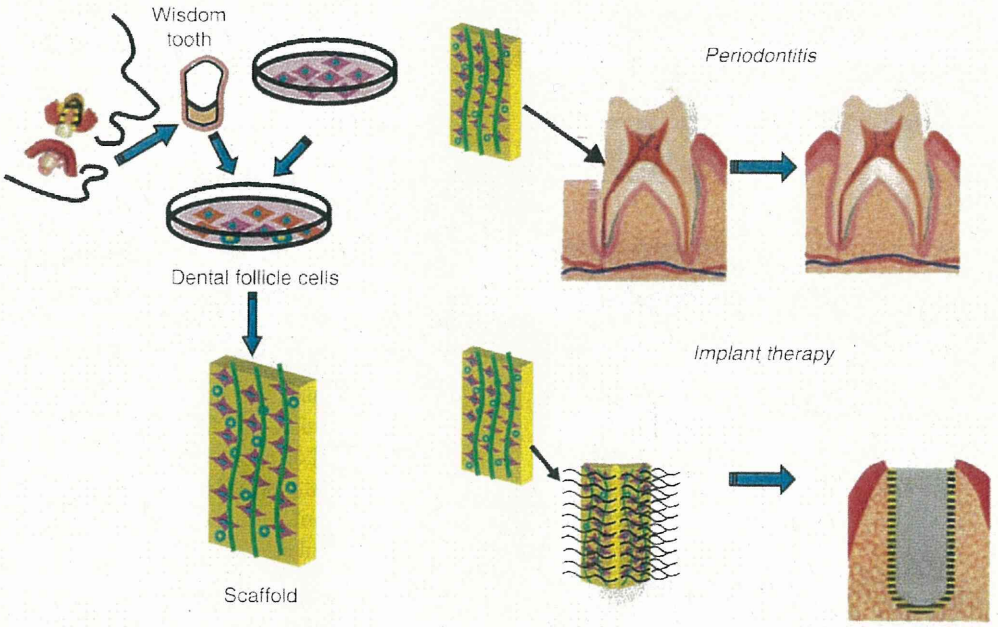

Figure 2. Potential approach to regeneration therapy for periodontal disease. By using cell sheet-engineering, stem cells obtained from the periodontal ligament (PDL) or dental follicle (DF) of wisdom tooth germ are harvested as temperature sensitive sheets for transplantation into periodontal tissue damaged by periodontal disease (upper panel). Stem cell sheets are also applied to dental implants accompanied by a bioengineered PDL that can recover the loss of periodontal tissue including the PDL, cementum and alveolar bone (lower panel).

the efficacy of FGF-2 in stimulating the regeneration of periodontal tissue. These findings collectively suggest that cytokine therapy has great clinical potential for achieving the partial regeneration of periodontal tissue.

4. Novel approaches to periodontal tissue regeneration using ECM administration therapy

ECM components organized in the PDL not only reflect the functional requirements of this matrix such as mechanical stress and storage of signaling molecules, but also regulate the tissue framework during development and regeneration [21]. Diseases affecting ECM function such as MFS have been shown to increase the susceptibility to severe periodontal disease due to a dysfunction of the PDL through a microfibril insufficiency, suggesting that fibrillin-1 microfibril formation plays a central role in PDL formation [68-74]. In addition, a new therapeutic concept has proposed that a fibrillin-1 microfibril insufficiency can be corrected by the administration of ECM components [23].

4.1 Periodontal disease and MFS

MFS is a severe, systemic disorder of connective tissue formation and can lead to aortic aneurysms, ocular lens dislocation, emphysema, bone overgrowth and severe periodontal disease [68,75,76]. MFS has an estimated prevalence of 1 in 5000 – 10,000 individuals [77]. Fibrillin-1 comprises one of the major insoluble ECM components in connective tissue microfibrils which provides limited elasticity to tissues and stores cytokines such as TGF-β [78,79] (Figure 3A). Various mouse models of MFS have now been established via gene targeting or missense mutations in which germline mutations in *fibrillin-1* lead to progressive connective tissue destruction due to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation [72,74,75]. Hence, it is largely accepted that MFS is caused by insufficient fibrillin-1 microfibril formation in various connective tissues [76]. The study of PDL provides a useful experimental model not only for investigating the molecular pathogenesis of MFS, but also for evaluating novel therapeutic strategies for the improvement of microfibril disorders. This is because the principal elastic fiber system of the PDL, the oxytalan fiber, is composed of fibrillin-1 microfibrils and does not contain significant amounts of elastin [80-82]. Indeed, an abnormal PDL in association with progressive destruction of microfibrils is an obvious phenotype in the MFS mouse model [23]. Hence, PDLs will likely be more susceptible to breakdown in MFS compared with other elastic tissues composed of both elastin and fibrillin-1 (Figure 3B).

A structural insufficiency of fibrillin-1 microfibrils arises in MFS and leads to activation of TGF-β and its regulatory targets

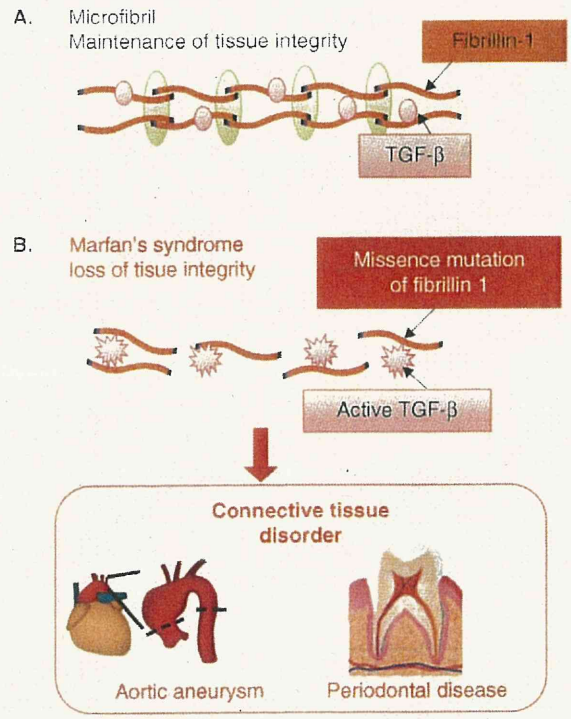

Figure 3. Schematic representation of the pathogenic mechanisms of MFS. A. Fibrillin-1 comprises insoluble extracellular matrix components in connective tissue microfibrils and provides limited elasticity to tissues through fibrillin-1 microfibril formation. **B.** Missense mutations in the *fibrillin-1* gene lead to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation and the pathogenic activation of TGF-β. These abnormalities cause progressive connective tissue destruction including aortic root aneurysms that are life-threatening and severe periodontal disease.

(Figure 3B) [73]. Recently, deregulation of TGF-β activation has been shown to contribute to pathogenesis and systemic antagonism of TGF-β signaling has been observed to have a beneficial effect on MFS symptoms including alveolar septation and muscle hypoplasia [72]. These observations have indicated that the microfibril network plays an important role in not only PDL function but also in the recovery of periodontal tissue integrity and prevention of the pathogenic activation of TGF-β caused by the fibrillin insufficiency that arises in MFS. However, molecular mechanisms governing fibrillin-1 assembly during organogenesis have been hampered because of unanswered issue of the actual factor that drives microfibril assembly.

4.2 Administration of ADAMTSL6β serves as a microfibril therapy for repair of the PDL in an MFS mouse model

A disintegrin-like metalloprotease domain with thrombospondin type I motifs (ADAMTS)-like, ADAMTSL, is a subgroup of the ADAMTS superfamily and its members share particular protein domains with the ADAMTS protease, including thrombospondin type I repeats, a cysteine-rich domain and an ADAMTS spacer, but lack the catalytic and disintegrin-like domains. Among the novel ADAMTSL family molecules, ADAMSL6β is essential for the development and regeneration of the PDL [23]. ADAMTSL6β was recently found to associate with fibrillin-1 microfibrils through its direct interaction with the N-terminal region of fibrillin-1, and thereby promote fibrillin-1 matrix assembly both *in vitro* and *in vivo* [24]. Another study has indicated that fibronectin is an essential component during the assembly of fibrillin-1 through its interaction with the C-terminal region of fibrillin-1, thus suggesting the potential for improved microfibril assembly through the regulation of fibrillin-1-associated proteins including ADAMTSL6β [83,84]. In an animal model of MFS microfibril disorder [85], ADAMSL6β expression can rescue fibrillin-1 microfibril formation through the promotion of fibrillin-1 microfibril assembly (Figure 4A). More importantly, the local administration of ADAMTSL6β was found to be highly effective in accelerating the wound healing of periodontal tissues through the restoration of microfibrils (Figure 4B). Further evidence for the impact of ADAMTSL6β on microfibril assembly is its suppression of TGF-β signaling, a pathway which is known to contribute to elastolysis in MFS.

These findings have demonstrated that microfibril assembly induced by ADAMTSL6β is essential not only for fibrillin-1 microfibril restoration but also for the inhibition of the pathological activation of TGF-β. Thus, ECM administration therapy such as microfibril assembly could form the basis of a novel therapeutic approach to PDL regeneration and the treatment of periodontal disease in MFS patients.

5. Conclusions

Regenerative therapies for periodontal disease that use the cells of the patient to repair the periodontal defect have been proposed in a number of studies [86-88]. PDL-derived stem cells such as PDLSCs can differentiate into all of the periodontal lineages that contribute to cell turnover in the steady-state and would thus be useful cell sources for regenerative therapies to treat periodontal disease following tissue injury [89-91]. Treatments that partially regenerate damaged PDLs through the local application of cytokines have now been established, and such regenerative therapies have provided a very useful and feasible clinical study model for the future design of stem cell and cytokine therapies [15,61,92]. Although partial regeneration of the periodontal tissue has been established, methods to achieve the functional regeneration of large defects caused by severe periodontal disease are still lacking. To address this, it is essential to better understand the molecular mechanisms underlying PDL development and to thereby identify the appropriate functional molecules that induce the differentiation of stem cells into periodontal lineage cells for the successful reconstruction of periodontal tissue [31,32]. Investigations of the molecular mechanisms of fibrillin-1 microfibril assembly via ADAMTSL6β during

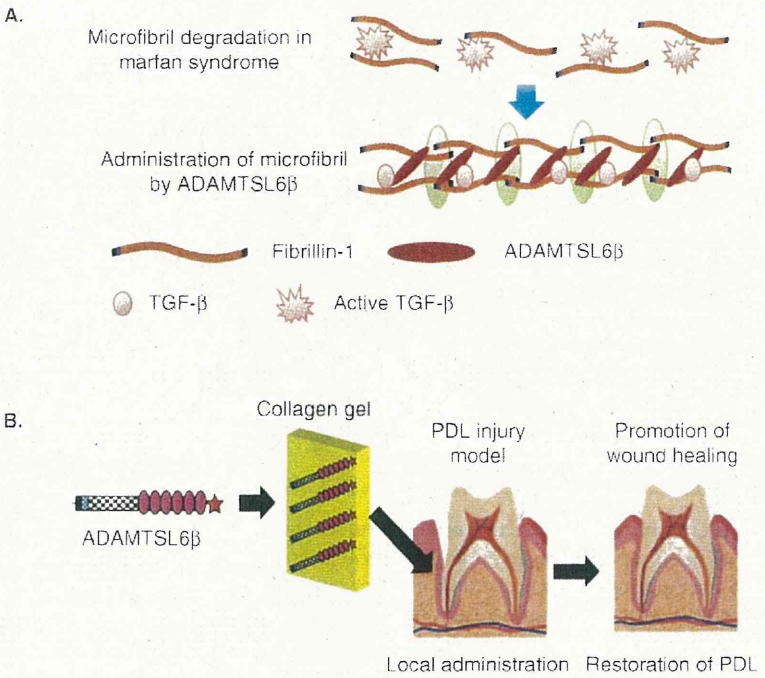

Figure 4. Microfibril administration by administration of ADAMTSL6β. A. Administration of microfibrils by ADAMTSL6β An ECM administration therapy that induces restoration of properly formed microfibrils via ADAMTSL6β is essential not only for improvement of the microfibril disorder, which is a predominant symptom of MFS, but also for the suppression of excessive TGF-β signaling induced by microfibril disassembly. **B.** ADAMTSL6β promotes wound healing of the PDL. A collagen gel containing recombinant ADAMTSL6β is prepared and locally administrated to the injured PDL of Marfan's syndrome mice established via the gene targeting of *fibrillin-1*. In this model, recombinant ADAMTSL6β restores fibrillin-1 microfibril assembly and enhances wound healing.

PDL formation will make substantial contributions to this endeavor [23]. In addition, since microfibrils play an important role in maintaining connective tissue integrity, including the aorta, lung and skin, we are hopeful the ECM administration therapy will in the future encourage the development of PDL regeneration for the treatment of periodontal disease as well as connective tissue disorders such as MFS [75,77].

6. Expert opinion

As described above, the partial regeneration of connective tissue damaged by pathological microflora has been achieved by regeneration therapy using stem cell transplantation and the local application of cytokines. Identification of the stem cells in the PDL or DF has enabled the development of protocols to regenerate the PDL and these have proved to be useful model systems for the development of connective tissue regeneration therapies [15,17]. One of the major research obstacles in PDL regeneration studies is the identification of all of the key functional molecules that drive PDL development. The establishment of ECM administration therapy such as fibrillin-1 microfibril assembly is ultimately critical for the development of new therapeutic approaches for periodontal disease and MFS [76]. MFS fibrillinopathies have been explained by the structural insufficiency of fibrillin-1 microfibrils leading to the activation of TGF-β and its regulatory targets [93]. A variety of MFS therapies have been developed to date, including surgical therapy for aortic root aneurysms that are life-threatening [76], traditional medical therapies such as β-adrenergic receptor blockade for slow aortic growth and to decrease the risk of aortic dissection, and novel approaches based on new insights such as the pathogenesis of insufficient fibrillin-1 microfibril formation and the deregulation of TGF-β activation [77]. In the case of periodontal disease in MFS, surgical therapy or regeneration therapy is performed using stem cells or cytokines to recover damaged periodontal tissue (Figure 5, left panel).

In contrast to these approaches, the administration of ADAMTSL6β to fibrillin-1 microfibrils may represent a new ECM administration therapy which is viable for the treatment of the periodontal disease of MFS [23]. The evidence indicates that ADAMTSL6β is capable of enhancing microfibrils even in the case of a fibrillin-1 haploinsufficiency. Hence, ECM

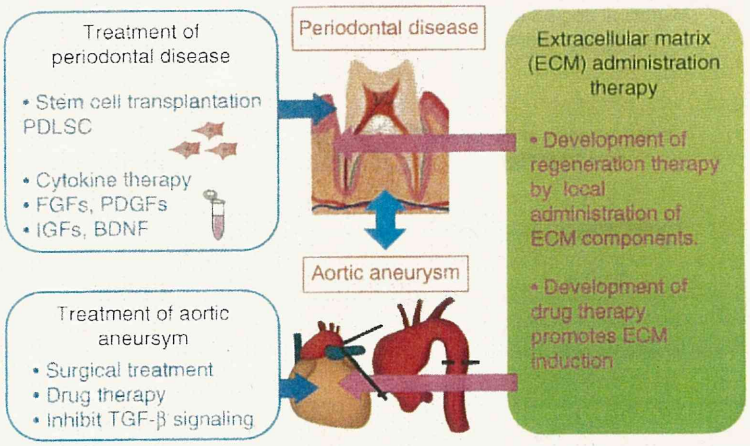

Figure 5. Extracellular matrix (ECM) administration therapy as a novel therapeutic strategy for MFS syndrome. Left panel: A variety of MFS therapies have been developed, including surgical therapy for aortic root aneurysms, traditional medical therapies and mechanisms to deregulate TGF-β activation and thereby decrease the risk of aortic dissection. In the case of periodontal disease, regeneration therapies including stem cell transplantation and cytokine therapy are being performed for the treatment of periodontal disease. Right panel: ECM administration therapy such as ADAMTSL6β administration which induces microfibril assembly should be considered in the development of future mechanism-based therapeutics for the improvement of periodontal disease in MFS. It will also be beneficial to develop drug therapies that promote ADAMTSL6β expression for the treatment of aortic aneurysms.

administration therapy through the promotion of microfibril assembly by ADAMTSL6β may have potentially novel therapeutic benefits for the treatment of periodontal disease and disorders associated with MFS (Figure 5, right panel).

In conclusion, we here introduce the concept that a fibrillin-1-associated protein such as ADAMTSL6β, which induces microfibril assembly, should be considered as an ECM administration agent for the treatment of periodontal disease and improvement of connective tissue disorders such as MFS. The exogenous application of recombinant ADAMTSL6β improves fibrillin-1 microfibril assembly, indicating that the reinforcement of fibrillin-1 microfibrils by ADAMTSL6β may represent a new treatment for periodontal disease which is accessible from oral cavity in MFS patients. Since elastolysis occurs continuously in aortic aneurysms arising in MFS cases, the chronic administration of ADAMTSL6β may be required for the stabilization of microfibrils to prevent progressive tissue destruction. It will also be necessary to develop methodologies for the systemic administration of ADAMTSL6β to induce fibrillin-1 microfibril assembly in connective tissue for the treatment of life-threatening conditions such as an aortic aneurysm (Figure 5, right panel). Hence, an ECM administration therapy involving ADAMTSL6β has the capacity to facilitate drug discovery for treating periodontal diseases, and MFS-associated disorders.

Declaration of interest

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Original article

Gene expression profile of mouse masseter muscle after repetitive electrical stimulation

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Abstract

Purpose: To examine gene expression profile changes in the mouse masseter muscle tissue after repetitive electrical stimulation by using a DNA microarray technique.
Methods: Nine male ICR mice aged 10 weeks were used. Each anesthetized mouse was secured on a platform in a supine position and the masseter muscle tissues on both sides were exposed. Bipolar electrodes were set on the right masseteric fascia to electrically stimulate the masseter muscle (8 V, 10 Hz, 20 ms) for 30 min. After cessation of stimulation bilateral masseter muscle tissues were sampled at 0 h ($n = 3$), 1 h ($n = 3$), 2 h ($n = 3$). Total RNA was isolated from the homogenized muscle tissues and purified mRNA samples (50 μg) were processed and hybridized with microarray slides. Probe arrays were then scanned and analyzed to calculate the signal density. Gene expression profiles were compared at each time point between the right (stimulation side) and left (control side) masseter. When the gene expression levels were different more than 2-fold, the difference was regarded as positive.
Results: Of the 6400 genes assessed, 1733 genes were up-regulated and 515 genes were down-regulated in the stimulation side at least once during the experimental time course. These up- or down-regulated genes were associated with autoimmune/inflammatory disease (28/114), cardiovascular disease (17/61), neuroscience (12/50), apoptosis (27/93), diabetes/obesity (9/28), signal transduction (66/250) and others. 28 genes were up-regulated and 25 genes were down-regulated at all time points.
Conclusions: Dramatic gene expression changes were induced by the repetitive electrical muscle stimulation in mouse masseter.

Keywords: Masseter muscle; Gene expression; Electrical stimulation; Mouse; Microarray

1. Introduction

For many years, it has been argued that oral parafunctions (e.g., tooth clenching or grinding) causes fatigue and pain in masticatory muscles [1–5]. However, since scientific evidence linking oral parafunctions and masticatory muscle pain is still lacking, the premise is still controversial. In order to clarify the relation between parafunction and muscle pain, it is important to know the local biological phenomena induced by muscle hyperactivity. However, local biological phenomena produced during or after muscle contraction in masticatory muscles have been rarely investigated yet.

Regarding the limb muscles, several attempts have been carried out to observe protein or gene expression changes of some candidate molecules during and after experimentally induced muscle activities. Chen et al. (2003) investigated the effect of eccentric exercise on the transcriptome of skeletal muscle in male human subjects who performed 300 concentric contractions with one leg and 300 eccentric contractions with opposite leg [6]. Muscle biopsies were taken from both legs at 4–8 h after exercise and expression was profiled by using microarray with 12,000 genes. The results of their study revealed the great inflammatory responses (chemokine (C–C motif) ligand-2, C/EBP delta, and IL-1 receptor) and vascular remodeling (tenescin C and lipocortin II). While the response is

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smaller and limited than that under eccentric contraction, it is also known that gene expression response is produced under concentric muscle contraction. Chan et al. (2004) studied cytokine gene expression in human skeletal muscle after concentric contraction and showed that IL-6 and IL-8 mRNA expressions significantly increased [7]. Most recently, Mckenzie and Goldfarb (2007) evaluated the gene transcription in rat soleus after aerobic exercise (running on the treadmill for 2 h) using microarray technique and identified that 52 genes significantly altered by the exercise [8]. In their report, the major gene families altered were metabolism, apoptosis, muscle contraction, transcription/cell signaling, tissue generation, and inflammation.

Based on above studies, dramatic molecular responses of masticatory muscles would be predicted during and after contraction. However, since it is known that the anatomical characteristics (e.g., fiber type, diameter) of jaw-closing muscle fibers are different from those of limb and abdominal muscles [9–12], masticatory muscles would be expected to have a wider repertoire of contractile protein expression and function [13]. These anatomical differences lead us to suspect that the biological characteristics of masticatory muscles may also be different from those of the limb muscles. In order to clarify the molecular responses of masticatory muscle to contraction, a specific experiment is needed. We purposed to evaluate the comprehensive gene expression profile changes induced in the mouse masseter muscle tissue after repetitive electrical stimulation, by using a cDNA microarray technique.

2. Materials and methods

2.1. Animals

Nine male ICR mice aged 10 weeks were used in this study. Under general anesthesia (urethane:pentobarbital = 1:1, 0.14 ml/100 g) by intraperitoneal injection, each mouse was set on a platform in a supine position and the upper incisors were secured to the platform. One centimeter skin cut was performed on both cheek region to expose the masseter muscle proper. Bipolar electrodes were set on the right masseteric fascia to electrically stimulate the masseter muscle (8 V, 10 Hz, 20 ms) for 30 min. The left side masseter muscle was kept exposed for 30 min but was not stimulated. To confirm the repetitive muscle contraction was induced, both masseter muscles were observed visually during the experiment. To prevent drying of the muscle tissues, mineral oil (SIGMA, St. Louis, MO, USA) was dropped over both masseter muscles during the electrical stimulation period. After cessation of the stimulation, each mouse was kept on the platform and bilateral masseter muscle tissues were sampled 0 h ($n = 3$), 1 h ($n = 3$) and 2 h ($n = 3$). The obtained muscle tissues were immediately frozen by liquid nitrogen for later RNA isolation. The experimental protocol of this study was reviewed and approved by the animal research control committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (#OKU-2006188).

2.2. RNA extraction and probe preparation

Masseter muscle total RNA was isolated from each individual sample with TRI_{ZOL} Reagent® (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions and pooled group-wise for analysis. Muscle samples weighing approximately 50 mg each were homogenized in 1 mL of TRI_{ZOL}. Following chloroform extraction, RNA was precipitated with isopropyl alcohol and washed with 70% ethanol. The purity and concentration of RNA were determined by measurement of absorbance at 260 and 280 nm.

Total RNA samples (35 μg) were labeled with Cy3-dCTP (as stimulation side) or Cy5-dCTP (as control side) using the Cyscribe First-Strand cDNA Labelling Kit (Amercham Biosciences, Amersham Place Little Chalfont Buckinghamshire, England) according to the manufacture's instructions.

2.3. Microarray hybridization and signal detection

Fluorescently labeled samples were processed and hybridized with cDNA microarray slide (Mouse 6.4 k oligo DNA Microarray, Nippon Laser & Electronics Lab, Nagoya, Japan), which is aminosilane-coated slide-glass, contains the 6400 marker genes representing 12 important functional classifications: (1) apoptosis, (2) stem cell, (3) neuroscience, (4) developmental/regenerative disease, (5) cancer, (6) diabetes/obesity, (7) cell cycle, (8) extracellular matrix and adhesion molecules, (9) autoimmune/inflammatory disease, (10) toxicology and drug metabolism, (11) cardiovascular disease, and (12) signal transduction. Hybridization procedure was performed using UltraGAPS Coated Slides (Corning, Lowell, MA, USA) according to the manufacture's instructions, then poly(A)RNAλ (TAKARA, Otsu, Japan) was mixed with labeled samples as the spiking controls. The hybridized slides were scanned with GenePix 4000B (Molecular Devices, Sunnyvale, CA, USA) using appropriate gains on the photomultiplier tube (PMT) to obtain the highest intensity without saturation. TIFF image was generated for each channel, Cy3 and Cy5. The signal intensity was transformed to numerical value with the Array Vision (IMAGING Research Inc., Ontario, Canada).

2.4. Data analysis

Probe arrays were then scanned and analyzed to calculate the signal density using the computer software (Mouse DNA Chip Consortium ver.1, INTEC Web and Genome Informatics Corporation, Tokyo, Japan). Spots with background-subtracted intensity, which were lower than 0 in either Cy3 or Cy5 channel were filtered out. Global normalization was then applied to correct the artifacts caused by different dye incorporation rates or scanner settings for two dyes. Additionally, the digital signals were normalized with the Lowess algorithm (0.33). Gene expression profiles were compared at each time point between the right (stimulation side) and left (control side) masseter. The ratio of each side gene expression (right/left) was indicated as a logarithm in the data table. When the gene

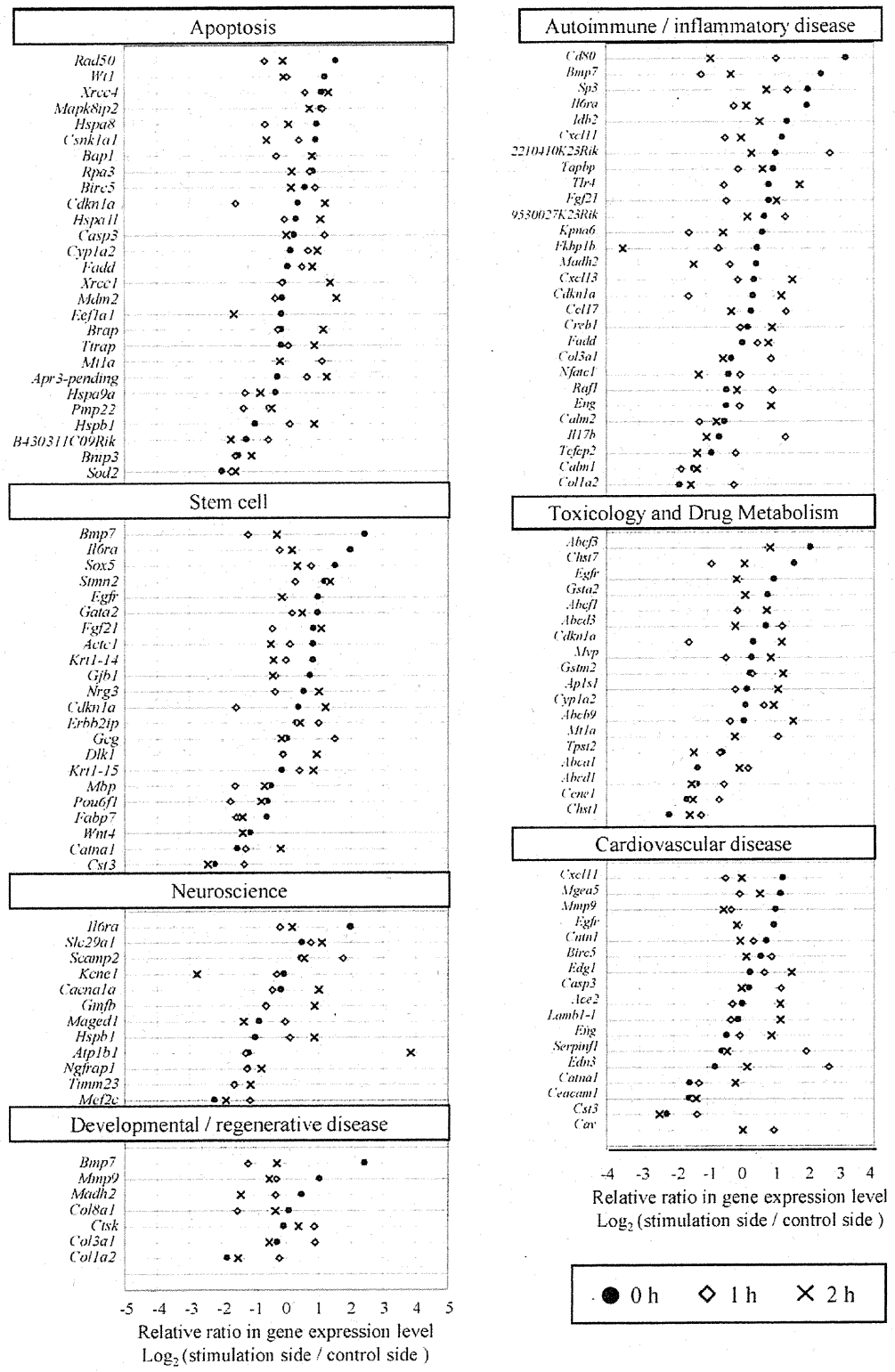

Fig. 1. Functional classification of muscle genes that showed a more than 2-fold change in expression level by electrical stimulation or not. The horizontal axis shows fold of changes (log 2) at each time point. Functional classification of muscle genes was based on the annotation system (Mouse DNA Chip Consortium ver.1, INTEC Web and Genome Informatics Corporation, Tokyo, Japan).

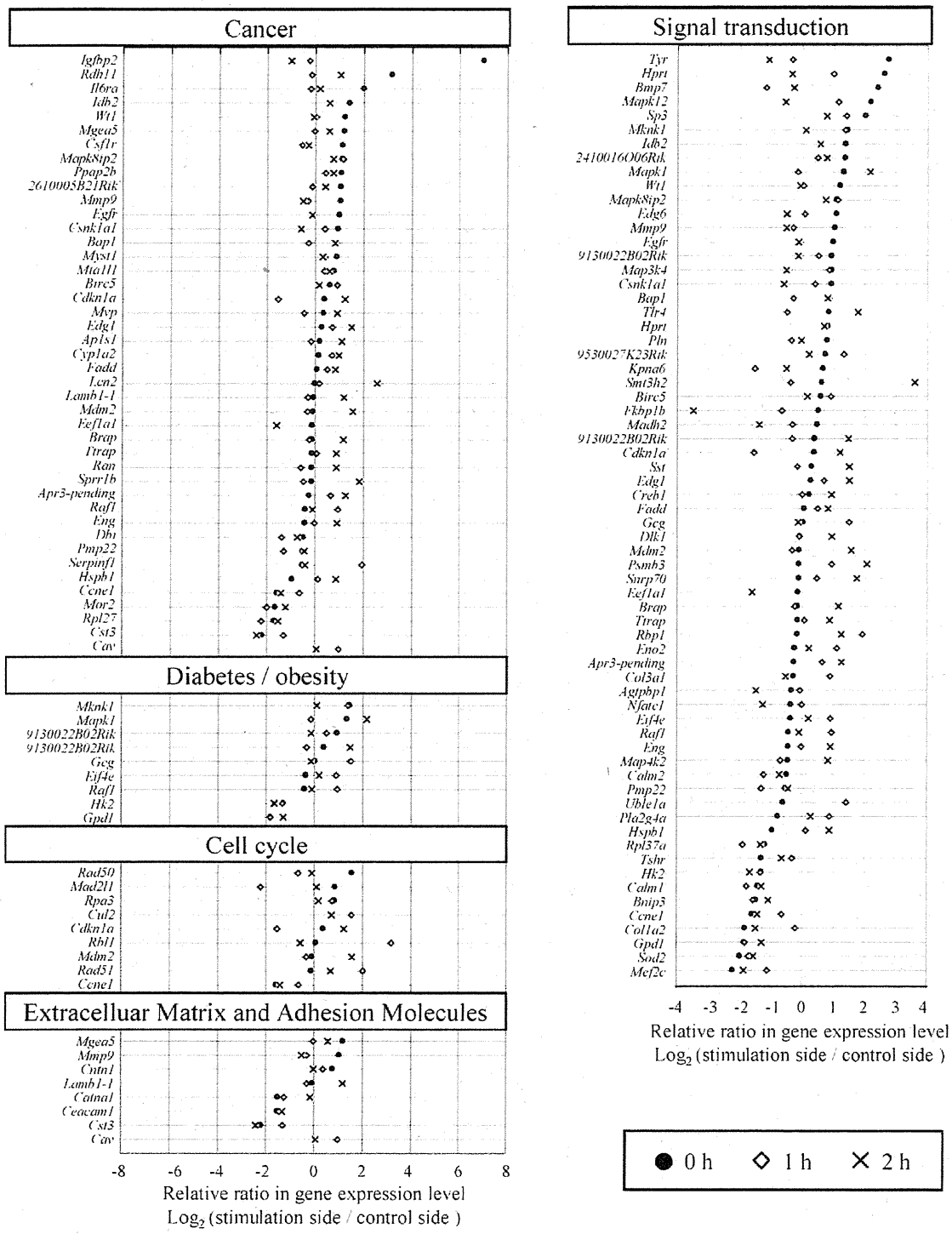

Fig. 1. (*Continued*).

expression levels were different more than 2-fold ($\log 2 \geq 1$: up-regulated or $\log 2 \leq -1$: down-regulated), the difference was regarded positive [14].

3. Results

Of the 6400 genes assessed, 1733 genes were up-regulated and 515 genes were down-regulated from the stimulation side at least once during the experimental time points. 28 genes were up-regulated and 25 genes were down-regulated at all time points. However, those 53 genes could not be classified into any functional categories, because they all were not listed in the gene annotation database (FANTOM II, GenBank and UniGene). The genes which showed positive differences between right and left masseter at each time point are shown in Fig. 1 in each functional category. As shown in Fig. 1, the up-

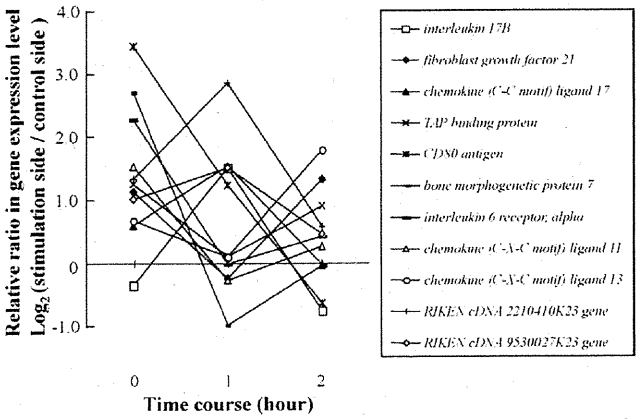

Fig. 2. Changes of muscle genes related to "cytokine and inflammatory response" which indicated a more than 2-fold changes ($\log 2 \geq 1$ or $\log 2 \leq -1$) in expression levels at least once during the experimental time course.

or down-regulated genes at least once during the experimental time course were associated with autoimmune/inflammatory disease (28/114), cardiovascular disease (17/61), neuroscience (12/50), apoptosis (27/93), diabetes/obesity (9/28) and signal transduction (66/250) and others.

Since previous studies provided the data to support the notion that several kinds of cytokines play important role for muscle metabolism changes related to muscle contraction [7,15–20], the data on 'cytokine and inflammatory response' were also analyzed in detail. As a result, 11 genes in relation to 'cytokine and inflammatory response' were up-regulated at least once during the three time points in this study (Fig. 2). In particular, marked expression changes were observed in CD80 antigen, bone morphogenetic protein 7, interleukin 6 receptor alpha, and RIKEN cDNA 2210410K23 gene during the experimental time course. The expression levels of these genes were over 4-fold when compared with those in not stimulated contralateral side.

4. Discussion

In order to improve our understanding of the molecular events underlying masticatory muscle contraction, mRNA expression profiling in mouse masseter after repetitive muscle contraction was performed. In this study, concurrent measurement of the magnitude of masseter contraction during electrical stimulation was not elected since EMG needle electrodes could induce local inflammation in muscle proper. However, we did insert the needle electrode into the masseter muscle tissue in our pilot study using several mice to confirm proper muscle activity was consistently induced by the level of electrical stimulation used in this experiment. Additionally, repetitive muscle contractions were certainly confirmed visually during the experiments. For these reasons, it is considered that our electrical stimulation method used in this study actually induced masseter muscle activity.

Overall a fairly dramatic molecular response in mouse masseter muscles were observed and of the 6400 genes assessed, 1733 genes were up-regulated and 515 genes were down-regulated on the stimulation side at least once during the experimental time points. This data does not allow us to determine the functional meaning of these gene expression changes at this stage. Also, the mRNA expression levels of cytokines were carefully analyzed, because it is known that the several cytokines play important role for muscle metabolism changes related to muscle contraction [7,15–21]. Several kinds of cytokine mRNA levels changed after the cessation of the electrical stimulation. Previous study demonstrated that IL-6 gene expression levels significantly increased after cessation of the repetitive contraction using rat masseter muscle [21]. Other research findings also suggested that up-regulation of IL-6 and IL-6 receptor gene expression in muscle tissues can be a good marker of muscle contraction [15,17–23]. Therefore, we paid attention to the IL-6 receptor alpha gene levels. Since the up-regulation of IL-6 receptor alpha gene was certainly observed, these results suggest the excellent validity of this experimental muscle contraction model. Regarding the function of IL-6 produced in contracting muscle tissues, several reports suggest it may work as an energy sensor within the muscle cells [24]. Other organs also release IL-6 during exercise; however, muscle-derived IL-6 seems to play an important role in signaling between the muscles and other organs in order to maintain energy supply. Moreover, muscle-derived IL-6 is likely to initiate many of the exercise associated immune changes, as IL-6 can increase plasma levels of the cytokines IL-1 ra and IL-10, together with cortisol and blood neutrophils [19]. It is also known that IL-6 inhibits low-level TNF-α production [23], thus anti-inflammatory effect of IL-6 is widely speculated by numerous researchers.

Before closing the manuscript, we would like to mention the shortcomings of the study. Since the array slides utilized in this study could not measure the every gene which is known to express in skeletal muscle tissues, we need to recognize that there will be other genes that show larger expression changes by electrical stimulation. Additional research studies, which are under consideration of these matters, would be desirable.

In summary, the present study found that the dramatic gene expression changes were induced by the repetitive electrical muscle stimulation in mouse masseter using microarray technique. These data will provide useful information for the scientists whose research target is a further understanding of masticatory muscle biology. In addition to the mRNA expression levels analyzed in this study, evaluation of other gene expression levels, which were not involved on the microarray slide utilized in current study, is also needed. Furthermore, by using the assessment of protein production levels in masticatory muscles during and after muscle contraction is promising to get better understanding of the masticatory muscle biology.

5. Conclusions

Within the limitation of this study, the results suggested that the dramatic gene expression changes were induced by the repetitive electrical muscle stimulation in mouse masseter muscle.

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Simvastatin Induces the Odontogenic Differentiation of Human Dental Pulp Stem Cells *In Vitro* and *In Vivo*

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Abstract

Statin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, is known to promote bone formation. However, it is not clear whether statin affects the differentiation of pulp cells. This study used a cell proliferation assay, cell cycle analysis, quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and *in vivo* transplantation to examine the effects of simvastatin on human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Simvastatin at 1 μmol/L was able to significantly suppress the proliferation of DPSCs without inducing apoptosis. Quantitative RT-PCR revealed both osteocalcin and dentin sialophosphoprotein to be significantly up-regulated when DPSCs were cultured with simvastatin in comparison to bone morphogenetic protein-2 treatment. The *in vivo* transplantation data showed that simvastatin treatment promoted mineralized tissue formation. Taken together, these results suggest that statin might be an ideal active ingredient to accelerate the differentiation of DPSCs. *(J Endod 2009;35:367–372)*

Key Words

Cell differentiation, cell proliferation, dental pulp stem cells, dentin regeneration, pulp capping material, reparative dentin formation, simvastatin

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Nonsurgical endodontic treatment, eg, a pulpectomy, is initially selected for the treatment of irreversible pulpitis. It is reported that 97% of teeth were retained in the oral cavity 8 years after the initial nonsurgical endodontic treatment (1). However, the frequency of clinical complications, including apical periodontitis and root fracture, has been reported to increase in endodontically treated teeth in comparison to vital teeth (2, 3). Therefore, treatment to maintain pulp vitality and function (vital pulp therapy) is used to avoid totally irreversible pulpitis that would certainly result in a pulpectomy. Although calcium hydroxide is conventionally used as a pulp capping material for vital pulp therapy, its long-term success rate varies among reports and is sometimes unsatisfactory (4, 5). One of the reasons for these unpredictable results is that calcium hydroxide does not biologically activate odontoblasts or accelerate reparative dentin formation (6). Therefore, the development of pulp capping materials with the biologic ability to activate odontoblasts and accelerate dentin formation is important. However, so far, no optimal pulp capping materials with excellent long-term clinical outcome have been developed.

In an experimental setting, several growth factors were investigated and proved to induce odontogenic differentiation of dental pulp cells *in vitro* and to accelerate dentin formation *in vivo* (7–12). Among them, bone morphogenetic proteins (BMPs) were considered to be promising growth factors capable of promoting odontogenic differentiation (9–11). However, the possibility of unexpected side effects and the production cost can be obstacles for their clinical application.

Statin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, is the first-line drug for hyperlipidemia, and it has been recognized to be a safe and low-priced drug as a result of its worldwide longtime usage. Statin has multiple functions including anti-inflammation, induction of angiogenesis, and improvement of the vascular endothelial cell function (13–16). Another interesting and important function of statin is its effect on bone formation. Statin improves the osteoblast function via the BMP-2 pathway and suppresses osteoclast function, resulting in enhanced bone formation (17–19). Therefore, statin might improve the function of odontoblasts, thus leading to improved dentin formation. However, the effects of statin on odontoblastic cells have not yet been reported.

The purpose of this study was to examine the effects of statin on the behavior of human dental pulp stem cells (DPSCs) (20–22) in terms of cell proliferation, cell cycle, gene expression patterns, and *in vivo* tissue formation.

Materials and Methods

Sample Collection and Cell Culture

Seven third molars were collected from 5 adults (22–26 years old) at Okayama University Hospital under the approved guidelines and protocol (Okayama University Ethics Committee #418 and 433), with written informed consent obtained from all subjects . The isolation and culture of human DPSCs were performed according to the method previously described (20–22). In brief, pulp tissue was gently and aseptically separated from the extracted teeth, minced, and digested in a solution of 3 mg/mL collagenase type I (Invitrogen, Carlsbad, CA) and 4 mg/mL dispase (Invitrogen) for approximately 1 hour at 37°C. Single-cell suspensions were obtained by passing the

solution through a 70 μm strainer, seeded at 1×10^4 cells/10-cm culture plate, and cultured with complete medium consisting of α-MEM (Sigma Chemical Company, St Louis, MO), 15% fetal bovine serum (FBS; Cancera International Inc, Ontario, Canada), 100 mmol/L L-ascorbic acid 2-phosphate (Sigma Chemical Company), 2 mmol/L L-glutamine (Sigma Chemical Company), 100 units/mL penicillin (Meiji Seika, Tokyo, Japan), and 100 mg/mL streptomycin (Meiji Seika) at 37°C under 5% CO_2 in air. DPSCs at 3–6 passages were confirmed to express dentin sialophosphoprotein (*dspp*) by reverse transcriptase polymerase chain reaction (RT-PCR) and used in this study (23).

Simvastatin (Calbiochem, San Diego, CA) was used as a typical statin in this study, and its stock solution was prepared according to the manufacturer's instructions. BMP-2 was purchased from R&D Systems Inc (Minneapolis, MN) and used as a positive control. Each experiment *in vitro* and *in vivo* was repeated at least twice, and the reproducibility for each was also confirmed.

Evaluation of Cell Proliferation

DPSCs were seeded onto 96-well plastic culture plates at 2.0×10^4 cells/well with complete medium. Twenty-four hours after seeding, the medium was changed to complete medium supplemented with simvastatin (0.1, 1, or 10 μmol/L) or BMP-2 (100 ng/mL). At 1, 3, and 5 days after simvastatin or BMP-2 addition, the MTS assay was carried out to evaluate the number of viable cells (CellTiter 96 Aqueous One Solution; Promega, Madison, WI) according to the manufacturer's instructions (24). The proliferation efficiency was determined from data obtained by measuring the optical absorbance at a wavelength of 490 nm with a microplate reader (Bio Rad, Hercules, CA). The mean values obtained from 8 wells under each condition were computed and statistically analyzed.

Mevalonate (Sigma Chemical Company) was used to confirm whether the effect of statin on DPSCs was mediated via the mevalonate pathway. DPSCs were cultured with complete medium supplemented mevalonate (1 mmol/L) (25) and/or simvastatin (1 μmol/L) for 5 days. Thereafter, an MTS assay was performed to evaluate the number of viable cells.

Evaluation of Fiber Formation

To evaluate the effect on fiber formation, DPSCs at the subconfluent stage were cultured with serum-free medium for 3 days. Next, simvastatin (1 or 10 μmol/L) or BMP-2 (100 ng/mL) was added to the medium, and the cells were cultured for 2 days. The actin fibers were stained with phalloidin-TRITC (Sigma Chemical Company), and the nuclei were stained with DAPI (Invitrogen), and thereafter they were observed under a fluorescence microscope.

Cell Cycle Analysis

The cell cycle and DNA fragmentation was analyzed by fluorescence-activated cell sorter (FACS) (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). In brief, DPSCs cultured with simvastatin (1 μmol/L) for 3 days were collected and stained with propidium iodide (200 ng/mL) (Calbiochem) and then subjected to a FACS analysis. H_2O_2 (0.3 mmol/L) was used as an apoptosis inducer (26) or a positive control in this experiment.

Quantitative RT-PCR Analysis

Total cellular RNA was extracted from DPSCs cultured with simvastatin (0.1, or 1 μmol/L) for 7 days by RNeasy (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA samples were reverse transcribed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Biochemicals, Mannheim, Germany). Quantitative RT-PCR was performed to quantify the gene expression level of odontoblastic markers by using a specified thermal cycler (LightCycler; Roche Molecular Biochemicals) with SYBR Green reagent (Roche Molecular Biochemicals). These genes included glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), *dspp*, and osteocalcin (*ocn*). The samples were subjected to 40 cycles of amplification at 95°C for 15 seconds followed by 64°C for 20 seconds and 72°C for 25 seconds by using the specific primers (Table 1). The results of the assays were normalized to the level of *gapdh*.

TABLE 1. Primer Sequences for Quantitative RT-PCR

Gene name (accession no.)		Primer sequences
gapdh (NM002046)	Forward	5′-CCATGGAGAAGGCTGGG-3′
	Reverse	5′-CAAAGTTGTCATGGATGACC-3′
dspp (NM14208)	Forward	5′-GTGATAGAGGAAGGCAAGAG-3′
	Reverse	5′-ATTCCAGCCCTCAATATTCC-3′
ocn (NM199173)	Forward	5′-CAAAGGTGCAGCCTTTGTGTC-3′
	Reverse	5′-TCACAGTCCGGATTGAGCTCA-3′

In Vivo Transplantation and Histologic Examination

DPSCs were cultured with simvastatin (0.1 or 1 μmo/L) or BMP-2 (100 ng/mL) for 7 days. Next, approximately 4.0 x 10^6 *ex vivo* expanded DPSCs were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc, Warsaw, IN) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised mice (NIH-bg-nu/nu-xid; Harlan Sprague Dawley, Indianapolis, IN) as previously described (20, 21, 27). These procedures were performed in accordance with the specifications of an approved animal protocol (Okayama University #OKU-2007226 and University of Southern California #10874). The transplants were recovered at 8 weeks after transplantation, fixed with 4% paraformaldehyde, decalcified with buffered 10% ethylenediaminetetraacetic acid, and then embedded in paraffin. Sections were deparaffinized and stained with hematoxylin-eosin. For the quantification of newly formed mineralized tissue *in vivo*, NIH Image software (http://rsb.info.nih.gov/nih-image) was used. The mineralized tissue area rate was calculated as the percentage of mineralized tissue area per total area at 4 representative areas from each group.

Statistical Analysis

One-way factorial analysis of variance (ANOVA) followed by Fisher PLDS tests were used for the statistical analysis (StatView; SAS Institute, Cary, NC). *P* values of less than .05 were considered to be statistically significant. All statistical data were presented as the mean ± standard deviation.

Results

Simvastatin Suppressed the Proliferation of DPSCs

Fig. 1 represents the effect of simvastatin on the proliferation of DPSCs. Simvastatin at 0.1 μmol/L and BMP-2 had no effects on the proliferation until 5 days in culture. However, simvastatin at 10 μmol/L significantly suppressed the proliferation in day-3 and day-5 cultures in comparison to the control. Simvastatin at 1 μmol/L also significantly suppressed the proliferation in a day-5 culture. These cells were stained by 0.5% trypan blue, and the stained cells were counted to evaluate the ratio of dead cells in each group. The ratio of dead cells in the cells treated by simvastatin at 10 μmol/L groups was 56.4% and

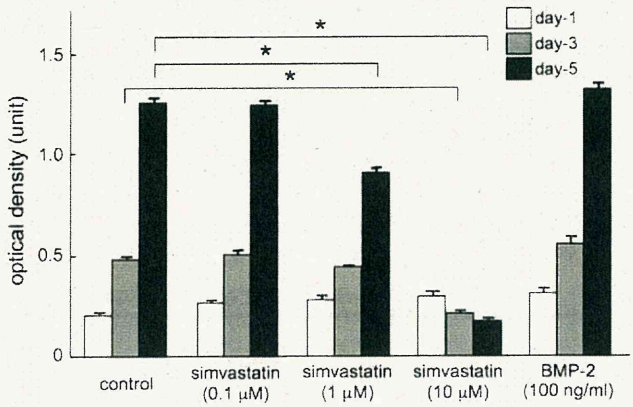

Figure 1. Simvastatin suppressed the proliferation of DPSCs. When simvastatin was added to the culture of DPSCs, the proliferation was significantly suppressed in day-3 (at 10 μmol/L) and day-5 cultures (at 1 and 10 μmol/L). BMP-2 did not affect the proliferation of DPSCs (n = 8, One-way factorial ANOVA followed by Fisher PLDS tests; *$P < .0001$).

much higher than other groups (control, 21.2%; simvastatin at 0.1 μmol/L, 25.3%; simvastatin at 1 μmol/L, 20.2%; BMP-2, 30.5%).

To confirm the putative pathway of this suppression, 3 experiments were conducted. At first, mevalonate was added to the culture in addition to simvastatin (1 μmol/L) and found to restore the proliferation (Fig. 2*A*). Next, actin fiber formation, as a downstream pathway of Rho, was confirmed by phalloidin staining (Fig. 2*B*). In a day-3 culture, simvastatin at 1 μmol/L slightly suppressed the actin fiber formation. Obvious suppression of fiber formation was observed in the culture treated by simvastatin at 10 μmol/L. The effect of BMP-2 on fiber formation was not evident. Third, the cell cycle was analyzed by FACS (Fig. 2*C*). The treatment by simvastatin at 1 μmol/L led to a decrease of the peak of the cells in the G2/M phase. H_2O_2 treatment also led to a decrease of the peak of the cells in the G2/M phase. However, at the same time, H_2O_2 treatment resulted in the accumulation of the cells in the apoptotic sub-G1 phase, which was not observed in the simvastatin-treated cells.

Simvastatin Induced Odontogenic Gene Expression in DPSCs

The gene expression levels of odontogenic markers, *dspp* and *ocn*, were analyzed in a day-7 culture by quantitative RT-PCR (Fig. 3). Simvastatin at 1 μmol/L induced *dspp* gene expression up to 3.7 times significantly in comparison to the control. The induction of *dspp* gene expression by BMP-2 was not significantly different in comparison to the control. The *ocn* gene expression was also up-regulated by simvastatin at 1 μmol/L up to 18.1 times in comparison to the control. BMP-2 also up-regulated *ocn* gene expression significantly

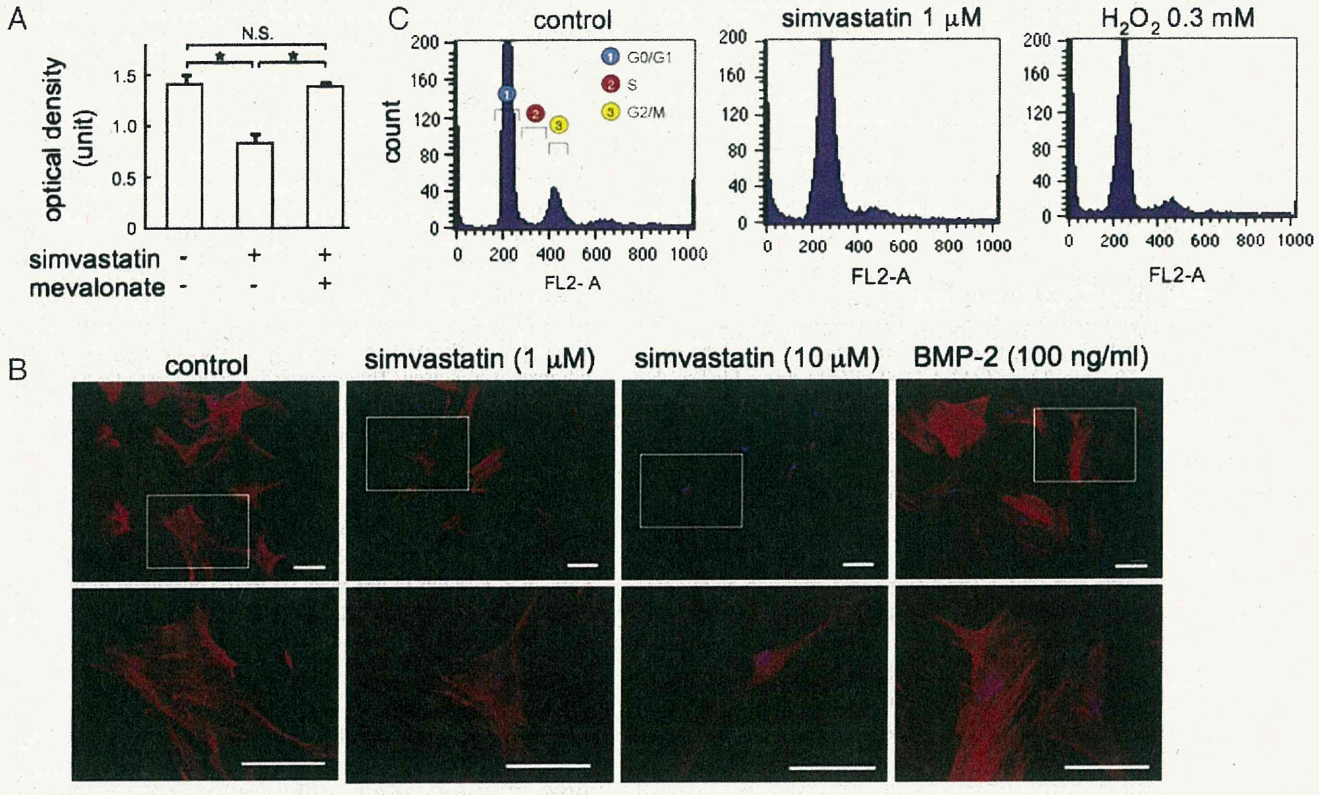

Figure 2. The suppression of the proliferation was a result of G1 arrest but not apoptosis that was mediated via the mevalonate and Rho pathways. (*A*) The proliferation was suppressed when simvastatin was added at 1 μmol/L to the culture for 5 days. However, when mevalonate was added at 1 mmol/L to the culture together with simvastatin, the suppression was restored (n = 8, one-way factorial ANOVA followed by Fisher PLDS tests; *$P < .001$, NS). (*B*) The actin fiber formation was suppressed slightly by simvastatin at 1 μmol/L and significantly by simvastatin at 10 μmol/L. BMP-2 did not affect the actin fiber formation. The white square in each upper panel indicates the region of each corresponding lower panel. Representative images are shown (scale bar, 100 μm). (*C*) The treatment by simvastatin at 1 μmol/L led to a decrease of the peak of the cells in the G2/M phase. H_2O_2 treatment also led to a decrease of the peak for the cells in the G2/M phase. However, at the same time, H_2O_2 treatment resulted in the accumulation of the cells in the apoptotic sub-G1 phase that was not observed in the simvastatin-treated cells.

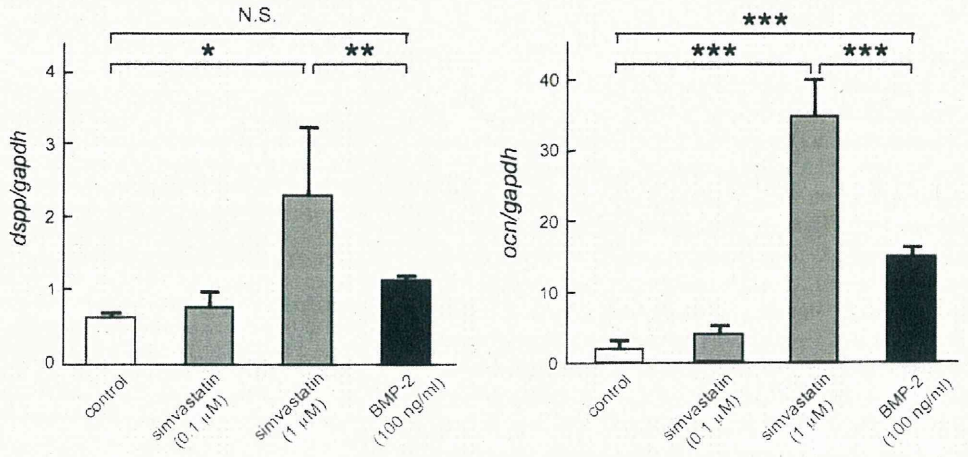

Figure 3. Simvastatin induced odontogenic gene expression in DPSCs. Simvastatin at 1 μmol/L induced *dspp* gene expression in a day-7 culture up to 3.7 times in comparison to the control. The induction of *dspp* gene expression by BMP-2 was not significant in comparison to the control. *ocn* gene expression was also up-regulated by simvastatin at 1 μmol/L in day 7 culture up to 18.1 times in comparison to the control. BMP-2 up-regulated *ocn* gene expression significantly higher than the control. However, the induction by simvastatin was significantly higher than that by BMP-2 (n = 8, one-way factorial ANOVA followed by Fisher PLDS tests: $*P < .01$, $**P < .05$, $***P < .0001$, N.S.=not significant).

higher than the control. However, the induction by simvastatin was significantly higher than that by BMP-2.

Simvastatin Accelerated DPSC-Mediated Mineralized Tissue Formation *In Vivo*

DPSCs were treated by simvastatin or BMP-2 and then transplanted into immunocompromised mice to evaluate the effects on mineralized tissue formation (Fig. 4). Although all groups, including the control, showed mineralized tissue formation after 8 weeks, DPSCs pretreated by simvastatin at 1 μmol/L or BMP-2 for 7 days formed a significantly larger amount of mineralized tissue in comparison to others (Fig. 4*E*).

Discussion

DPSCs possess postnatal stem cell characteristics, including multipotent differentiation, self-renewal, clonogenic capacity, and expression of multiple mesenchymal stem cell surface markers (20, 21, 28). DPSCs are also a heterogenic stem cell analogous to bone marrow mesenchymal stem cells (BMMSCs) (20–22). One of the unique characteristics of DPSCs is their capacity to form dentin pulp–like tissue when transplanted into immunocompromised mice by using HA/TCP as a carrier (20–22). Therefore, DPSCs are considered to be suitable cells to evaluate odontogenic differentiation both *in vitro* and *in vivo*.

This is the first study to show that statin induces odontoblastic differentiation of DPSCs *in vitro* and *in vivo* (Figs. 3 and 4). The ability of statin to accelerate mineralized tissue formation *in vivo* was

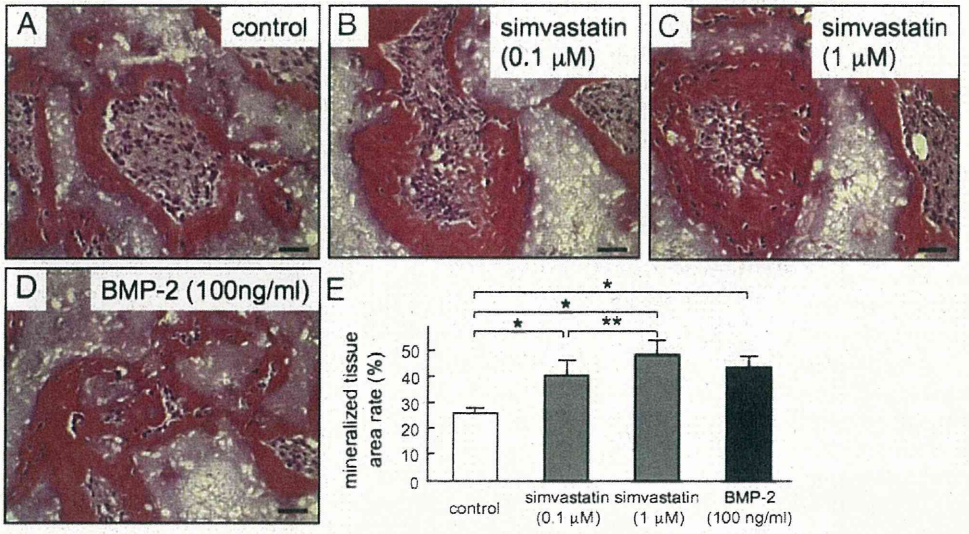

Figure 4. Simvastatin accelerated DPSC-mediated mineralized tissue formation *in vivo*. (*A–D*) All groups, including the control, showed mineralized tissue formation 8 weeks after transplantation. Importantly, mineralized tissue formed by simvastatin-treated cells formed tubular-like structures perpendicular to the surface of the carrier. Representative images are shown (scale bar, 100 μm). (*E*) DPSCs pretreated by simvastatin at 1 μmol/L or BMP-2 for 7 days formed significantly larger amount of mineralized tissue in comparison to the others (n = 4, one-way factorial ANOVA followed by Fisher PLDS tests: $*P < .0001$, $**P < .01$).

equivalent to BMP-2 (Fig. 4). Statin is known to accelerate BMP-2 expression and then enhance bone formation (18). However, our results showed the different effects of statin and BMP-2 on DPSCs in gene expression patterns (Fig. 3). Especially, statin significantly accelerated *dspp* gene expression in DPSCs. However, BMP-2 only slightly accelerated *dspp* gene expression, and the difference was not significant (Fig. 3*A*). The effect of BMP-2 on *dspp* gene expression observed in this study was inconsistent with a former report (29). The reason for this inconsistency might be due to the experimental conditions. In that report, a pellet culture was used, and the accelerated *dspp* gene expression was observed under BMP stimulation for more than 10 days (29). On the other hand, in this study, a monolayer culture was used, and the accelerated *dspp* gene expression was observed in a day-7 culture. In another report, an accelerated *dspp* gene expression was observed in a day-21 culture with the osteoinduction medium (containing dexamethasone, β-glycerophosphate, and ascorbic acid) (23). On the basis of these findings, statin might be a strong accelerator of *dspp* gene expression. Whether other pathways independent from BMP-2 stimulation could mediate the effects of statin on DPSCs, however, still needs further investigation.

The suppression of cell proliferation observed in this study (Fig. 1) is common to other cell types including osteoblastic cells, vascular smooth muscle cells, and neuronal cells (30–32). This suppression is mediated through the inhibition of the mevalonate and Rho pathways (25, 30, 32). Indeed, in DPSCs, mevalonate also effectively restored the proliferation suppressed by statin (Fig. 2*A*). Furthermore, statin inhibited actin fiber formation (Fig. 2*B*) and cell cycle progression (Fig. 2*C*) that is regulated by Rho. These results indicate that the suppression of proliferation in DPSCs is also mediated through the inhibition of the mevalonate and Rho pathways.

Pulp tissue contains a large amount of blood vessels and peripheral nerves. Statin is known to induce angiogenesis (13, 33) and to regulate the survival and increase neurogenesis of neuronal cells (34, 35), indicating the possible effectiveness of statin in pulp regeneration along with dentin regeneration. Furthermore, statin has an anti-inflammatory effect in various tissues. This could help restore the inflamed pulp tissue. Taken together, these results suggest that statin might be an ideal active ingredient in pulp capping material to accelerate reparative dentin formation. However, at the same time, attention has to be paid to the cell death observed in the cells treated with a high concentration of statin (Figs. 1 and 2*B*). This fact suggests that cells in pulp tissue might be damaged if statin acts on the cells at high concentration. Therefore, a careful evaluation is required before clinical application to determine the suitable concentration when applied indirectly to a cavity or directly to pulp tissue.

Acknowledgments

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Polyphosphoric acid treatment promotes bone regeneration around titanium implants

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SUMMARY **This study was conducted to evaluate the effect of polyphosphoric acid (PPA) treatment on bone regeneration around titanium (Ti) implants *in vivo*. Adsorption of PPA by Ti was achieved by immersing Ti implants (2 mm in diameter, 4 mm in length) in different concentrations of PPA solution (0, 1 and 10 wt%) for 24 h at 37 °C after proper Ti surface cleaning. The treated Ti implants were implanted on 8-week-old-male rat (n = 30) tibiae. Two or four weeks after implantation, all animals were deeply anaesthetized and underwent perfusion fixation. Ten specimens in each condition were further immersed in the same fixative for 1 week and eventually embedded in polyester resin. Afterwards, undecalcified sections were ground to a thickness of approximately 70 μm parallel to the long axis of the implant. The sections were stained with basic fuchsine and methylene blue and then examined by light microscopy. For quantitative evaluation of bone regeneration around the implants, the bone-implant contact ratio (BICR) was determined. Polyphosphoric acid treatment of the Ti implant surface significantly enhanced direct bone contact to the Ti surface. Especially, the BICRs of the 1 wt% PPA-treated Ti implants were significantly higher than those of the control untreated Ti implants, both 2 and 4 weeks after implantation. At 4 weeks, 10 wt% PPA-treated implants also significantly increased the BICR as compared to that of the untreated Ti implants. These results suggest that PPA treatment promotes osteoconductivity of Ti *in vivo*.**

KEYWORDS: **implant, titanium, polyphosphoric acid, bone formation, rat**

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Introduction

It is well known that the surface structure of the implant material that interfaces with bone plays an important role in osseointegration (1). Therefore, much *in vitro* and *in vivo* research is devoted to surface modifications of titanium (Ti) as well as to their biocompatibility with bone cells. Titanium surface modifications experimented include mechanical and/or chemical alterations of surface hydrophilicity, roughness, texture and morphology (2–6). In addition to such modifications, recent research interest has gradually shifted towards biological modification of implant surfaces by using e.g. cell growth factors, cell attachment factors and Arg-Gly-Asp peptides (7–10). Many bioactive surface coatings have been developed in an attempt to promote early osseointegration and bone formation around implants.

While such surface coatings have been shown to be effective with varying success, most of these procedures are rather complicated and/or costly for clinical use; virtually, none of them have currently been accepted as routine treatments to bioactivate implant surfaces. In this respect, a recent study has provided evidence that inorganic polyphosphate enhances human fibroblast proliferation as well as it promotes the mitogenic

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