INTRODUCTION
Understanding the biology of the dentin-pulp complex has now reached the point at which new treatment modalities aimed at tooth regeneration might be realized in clinical settings. Pulp capping is best described as capping of the exposed pulp and is indicated for reversible pulp tissue injury after physical or mechanical trauma in developing or mature teeth. The control of infection and biocompatibility of pulp-capping materials are important factors in determining treatment outcomes.

Typically, agents based on calcium hydroxide (Ca(OH)₂) and mineral trioxide aggregate (MTA) are used for direct pulp-capping treatment. Ca(OH)₂ dissociates into calcium and hydroxyl ions, which, in turn, results in an increase in pH from neutrality to 12.5 and the release of calcium ions. This high pH resulting from the dissociation of Ca(OH)₂ is thought to produce an envi-

SYNOPSIS
Better understanding of the dentin-pulp complex is now leading to treatment modalities aimed at tooth regeneration, such as pulp capping, in clinical settings. However, the control of infection and biocompatibility of pulp-capping materials are important factors in determining treatment outcomes. The purpose of this study was investigated the effects of CaCO₃ on calcified nodule formation, ALP activity, cell viability and BMP-2 and OCN expression after 30-day culture of human dental pulp cells (hDPCs). The results showed that the number of calcium nodules, ALP activity, cell viability and BMP-2 and OCN expression increased in a CaCO₃-dependent manner. PCR data also confirmed that Smad1 mRNA expression in hDPCs increased in a CaCO₃ dose-dependent manner. This suggests that CaCO₃ is able to induce in vitro cell differentiation of hDPCs into cells capable of mineralization. Thus, treatment of exposed pulp with CaCO₃ is effective for dentinogenesis.

Key words: dental pulp, calcium carbonate, cell differentiation, pulp capping agent, dental pulp calcification

ORIGINAL ARTICLE
Effects of Calcium Carbonate on Odontoblast Differentiation and Calcification Ability of Human Dental Pulp Cells

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ronment that is unfavorable for the survival and proliferation of bacteria, and to stimulate repair by forming a superficial zone of necrosis. In contrast, MTA is considered to enhance the mineralization of dental pulp tissue, as it binds minerals with high biological affinity. However, MTA does not induce necrosis layer formation. Hence, both MTA and Ca(OH)₂ can enhance pulp mineralization, and therefore, MTA may be useful as an alternative to Ca(OH)₂ in dentistry. MTA consists of 50% to 75% calcium oxide and 15% to 25% silicon dioxide.

The two components together comprise 70% to 95% of dental cement. Ca(OH)₂ is produced commercially by treatment with water: CaO+H₂O → Ca(OH)₂. Ca(OH)₂ then reacts with carbon dioxide, resulting in calcium carbonate (CaCO₃) (Ca(OH)₂+CO₂ → CaCO₃+H₂O) (Figure 1). The chemical forms of calcium are changed by interaction with the components of air or with the components of the reaction solution, as shown in Figure 1. Moreover, it has previously been reported that Ca(OH)₂ is also changed to CaCO₃ through a chemical reaction with carbon dioxide in the atmosphere.

Although various theories exist regarding calcium agent-induced odontoblast differentiation and subsequent reparative dentinogenesis, neither the underlying mechanisms nor the reasons for its success are known. Therefore, analysis of the CaCO₃ requirement for odontoblast differentiation, and the biological mechanism by which CaCO₃ modulates dentinogenesis in pulp, is necessary.

Accordingly, our research has focused on investigating the possibility that CaCO₃ functions as a source of cellular calcium. For this purpose, we have performed in vitro investigations involving the treatment of cultured hDPCs with CaCO₃. Here, we investigated the effects of CaCO₃ on expression of mRNAs encoding dentin sialophosphoprotein (DSPP), Smad1, bone morphogenetic protein-2 (BMP-2), alkaline phosphatase (ALP) and osteocalcin (OCN), and of the protein expression of BMP-2 and OCN in hDPCs. All of these molecules are differentiation markers of accelerated calcified nodule formation.

MATERIALS AND METHODS
1. Cell culture and CaCO₃ treatment
This study was approved by the ethics committee of the Nihon University School of Dentistry at Matsudo (approval number EC 03-025). hDPCs were obtained from unerupted teeth extracted from young patients in the course of orthodontic treatment. Patients gave informed consent before providing samples. After the dental pulp had been extracted under sterile conditions, it was washed twice with Hank’s balanced salt solution (pH 7.4). Pulpal tissue was then minced, placed on a 35-mm tissue culture dish, and covered with a sterilized glass coverslip.

The culture medium used was α-minimal essential medium (MEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum and 100
μg/mL penicillin G (Meiji Seika, Ltd., Tokyo, Japan), 100 μg/mL gentamicin sulfate (Meiji Seika) and 0.3 μg/mL fungisone (Gibco). The culture was maintained in an atmosphere of 5% CO₂, 90% N₂, and 5% O₂ at 37°C.

When the explanted cells had reached confluence, cells were detached with 0.05% trypsin (580 BAEE units/mg; Gibco) in phosphate-buffered saline (PBS) and were subcultured in culture dishes. Phase-contrast microscopic observation indicated that these confluent cells had not formed the small mats typical of epithelial cells.

For experimental use, hDPCs of 3 to 6 passages were counted using a Coulter Counter ZM (Coulter Electronic Ltd., Luton, England) and were plated at a density of 1×10⁵ cells (in 1.5 mL of medium) per dish. After a 24-hours culture, hDPCs were treated with α-MEM supplemented with 10% FCS, antibiotics, 50 μg/mL ascorbic acid (Wako, Osaka, Japan), and 0.1 or 10 μg/mL CaCO₃ (control, 1 μg/mL CaCO₃ and 10 μg/mL CaCO₃ groups, respectively) (Wako). Culture medium was changed every 3 days throughout the 28-day experimental period.

The concentrations of CaCO₃ used were determined based on cell number, ALP activity and von Kossa staining data obtained in a pilot study (data not shown).

2. von Kossa staining and number of calcified nodules
Calcified nodule formation was examined in hDPCs cultured for 30 days in vitro. These hDPCs were washed with PBS, fixed in 10% buffered formalin for 1 hour, stained using the von Kossa technique 9-11 and calcified nodules were then analyzed using a light microscope. One microscopic field consisted of a 5-mm² grid and the calcified nodules in 10 fields were counted at 40 magnification.

3. Alizarin red S staining and calcium content
hDPCs cultured for 30 days in vitro were washed with PBS and were then fixed in 10% buffered formalin for 1 hour. Cells were washed again and stained with Alizarin Red S 9,11,15. Calcium in calcified nodules was dissolved in 0.5 N HCl (1.0 mL) for 12 hours, and the amount of calcium was then measured using the Calcium C test Wako (Wako) 9,11,13.

4. Assay of cell viability
hDPCs (1×10⁴ cells/well) were cultured in 96-well plates containing 100 μL of α-MEM supplemented with 0, 1 or 10 μg/mL CaCO₃. Cell viability was measured 1, 2 or 3 days later using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). This assay is based on conversion of the water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] to a water-soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases. Medium (100 μL) was incubated with 10 μL of WST-8 solution for 1 h at 37°C. Absorbance was recorded at 450 nm using a microplate reader. Cell viability was expressed as a percentage of the control culture value 16.

5. ALP staining
On day 30 of hDPC culture in the presence or absence of CaCO₃, cells were fixed with 10% neutral formalin for 10 min at room temperature and were then rinsed 3 times in PBS. ALP activity was determined by enzyme histochemistry using naphthol AS-MX phosphate and Fast blue BB salt 12-14.

6. RT-PCR
hDPCs (1×10⁶) were cultured in tissue culture dishes (10 cm) containing 10 mL of α-MEM supplemented with 0, 1 or 10
μg/ml CaCO₃ for 1, 3 or 6 days. CaCO₃ medium was added when cells were sub-confluent. Total cellular RNA was extracted from the cells using an RNeasy mini kit (QIAGEN, Hilden, Germany), and RNA isolation was performed in accordance with the protocol provided with the RNeasy mini kit. cDNA synthesis and amplification by RT-PCR were conducted using a One-Step RT-PCR kit. For PCR mixture, RNA (200 ng) and oligonucleotide primers (500 nM) were used. PCR primers for DSPP for 27 cycles, Smad1 for 27 cycles, BMP-2 for 24 cycles, ALP for 30 cycles, OCN for 27 cycles and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for 27 cycles were designed and synthesized with reference to the reported cDNA sequences. These primers were as follows: GAPDH (forward) 5.-ATCACCATCTTCCAGGAG-3., (reverse) 5.-ATGGACTGTGGTCATGAG-3.; DSPP (forward) 5.-ATGGGACTAGTGGTCTAGAG-3.; BMP-2 (forward) 5.-GCTGTACTACGACACCCAC-3., (reverse) 5.-TCATAAAACCTGCAACCACT-3.; ALP (forward) 5.-GAAAGAGACCCCAG-3., (reverse) 5.-ACCACCATGATCACATC-3.; and OCN (forward) 5.-GATACCTATCAATGGCTGGGAGCC-3., (reverse) 5.-GTCGACATAGGCCTCCCTGAAGGC-3.. The GeneAmp PCR system 9600 (PerkinElmer) was programmed for cDNA synthesis, and the procedure involved pre-denaturation for 30 min at 95°C, followed by 30 thermal cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, chain elongation for 30 s at 72°C, and final extension for 10 min at 72°C. PCR fragments were electrophoresed on 1.5% agarose gels and were subsequently stained with ethidium bromide.

7. Quantification of BMP-2 and OCN protein production
BMP-2 released into the culture medium over 3 to 9 days of incubation of hDPC cells was quantified using a Quantikine BMP-2 ELISA kit® (R&D Systems, Minneapolis, MN, USA). The results were analyzed using spectrophotometry at 450 nm. OCN released into the culture medium after 24 days of incubation of hDPC cells was measured using an Instant OCN ELISA kit (Bender MedSystems, Burlingame, CA, USA). Results were analyzed using spectrophotometry at 570 nm on a model 2550 microplate reader.

8. Assay of ALP activity
hDPCs of all 3 CaCO₃ groups were cultured in 35-mm dishes in standard medium at a density of 1×10⁵ cells/well for 1, 3, 5, 8, 12, 16 or 20 days after transfection, respectively. Cultured hDPCs were then rinsed three times with PBS. Glycine (100 mM, pH 10.5) buffer containing 2 mM MgCl₂, and 8 μM p-nitrophenylphosphate (p-NPP) was then added and ALP activity was determined based on the amount of p-NP released, which was spectrophotometrically measured at 415 nm. One unit of ALP activity was defined as the activity that liberated 1 mM p-NP/min at 37°C, and ALP activity was expressed as mU/dish.

9. Statistical Analysis
The results were analyzed with one way analysis of variance (ANOVA). When appropriate, ANOVA was followed by post-hoc Tukey’s test to compensate for multiple comparisons (P<0.05).
Figure 2  Calcified nodule formation, calcium contents and ALP staining in hDPCs. hDPCs were treated with 0.1 or 10 μg/mL CaCO₃ for 30 days. Calcified nodules in hDPCs were then visualized by von Kossa staining (A) and by alizarin red S staining (B) on day 30. The number of calcified nodules on day 30 is shown in (A) and cellular calcium content is indicated in (B). The strength of von Kossa and alizarin red S staining of hDPC groups increased with CaCO₃ concentration. The number of calcified nodules in both the 1 and 10 μg/mL CaCO₃ groups was significantly greater than that in the control group (*P<0.05) (A). Calcium content was also significantly greater in the 1 and 10 μg/mL CaCO₃ groups than in the control group (*P<0.05) (B). ALP in the cultures was immunohistochemically stained on day 30. The strength of the ALP staining in the different hDPC groups was in the order of 10μg/mL CaCO₃ > 1 μg/mL CaCO₃ > control group. (C) The number of mineralization positive cells present in the 35-mm dishes on day 30 was greater in both the 1 and 10 μg/mL CaCO₃ groups than in the control group.
RESULTS
The effects of CaCO₃ on odontoblast differentiation and cellular calcium were analyzed by treatment of cultured hDPC cells with 0 (control), 1 or 10 μg/mL CaCO₃ for various times. These cells were then analyzed with regard to the following parameters.

1. Formation of mineralized nodules
The formation of mineralized nodules in the cultures was first analyzed by staining of calcium deposits using von Kossa stain and was quantified by microscopic counting of these nodules. The strength of von Kossa staining of the hDPC groups was in the order of: 10 μg/mL CaCO₃ > 1 μg/mL CaCO₃ > control group (Figure 2A). The number of calcified nodules present in 35-mm dishes of the 1 or 10 μg/mL CaCO₃ groups was significantly greater than that in the control group (*P<0.05, n=8) (Figure 2A).

We further confirmed the calcium contents of the 30-day hDPC cultures by staining of the calcified nodules present in the 35-mm dishes with alizarin red S. The intensity of this staining strongly increased in a CaCO₃ concentration-dependent manner (Figure 2B).

Quantification of calcium level using the Calcium C test indicated that the calcium contents of the 1 and 10 μg/mL CaCO₃ groups was significantly greater than that in the control group (*P<0.05, n=8) (Figure 2B).

2. ALP staining
Intensity of the ALP staining of hDPC groups was in the order of 10 μg/mL CaCO₃ > 1 μg/mL CaCO₃ > control group. The number of mineralization-positive cells present in the 35-mm dishes on day 30 was greater in the 1 and 10 μg/mL CaCO₃ groups than in the control group (Figure 2C).

3. Assay of cell viability
In order to understand the mechanism by which the formation of mineralized nodules was stimulated, we first assayed the cell viability of the hDPC groups on hours 24, 48 and 72 after CaCO₃ addition using the Cell Counting Kit-8 (CCK-8) assay. Viability of the hDPCs increased with CaCO₃ addition in a dose-dependent manner. The observed increase in both CaCO₃ groups was significantly different when compared with the control group after 24 or 48 hours of CaCO₃ treatment (*P<0.05, n=6) (Figure 3A).

4. Analysis of differentiation marker expression
It was next determined that the effects of CaCO₃ treatment on the mRNA expression of the odontoblast differentiation markers DSPP, Smad1, BMP-2, ALP and OCN in hDPCs, using RT-PCR analysis. The mRNA expression of all markers was increased in a CaCO₃ dose-dependent manner, as compared to the control group; Smad1, BMP-2 and DSPP (on days 1, 3 and 6), ALP (on day 6) and OCN (on days 3 and 6) (Figure 4).

We further analyzed the levels of BMP-2 and OCN protein released into the culture medium during incubation using ELISA. BMP-2 production increased in both the control and the CaCO₃ groups over 24 to 72 hours of incubation. CaCO₃ addition increased BMP-2 production in a CaCO₃ dose-dependent manner. The levels of BMP-2 produced were significantly greater in the 10 μg/mL CaCO₃ group than in the control group on 24, 48 and 72 hours (Figure 3B) (*P<0.05, n=8).

After 24 days of culture, the level of OCN protein in the media had increased in all groups, but was higher in the CaCO₃ groups than in the control group. This increase occurred in a CaCO₃ dose-dependent manner. The level of CaCO₃ produced was significantly greater in the 10 μg/mL CaCO₃ group than in the control group (Figure 3C).
Figure 3  Effects of CaCO₃ on cell viability, and BMP-2 and OCN production in hDPCs. hDPCs were cultured with 0.1 or 10μg/mL CaCO₃. Cell viability was measured 24, 48 or 72 hours later using a Cell Counting Kit-8. Viability was measured as the absorbance at 450 nm and is expressed as a percentage of the control culture value. Cell viability was enhanced by CaCO₃ treatment in a dose-dependent manner. The viability of the CaCO₃-treated groups was significantly elevated when compared with that of the control group after 24 or 48 hours of treatment (*P<0.05) (A). hDPCs were cultured with 0.1 or 10μg/mL CaCO₃ for 24 days. BMP-2 (B) and OCN (C) protein in the media was then analyzed by ELISA on 24, 48 and 72 hours, and on day 24, respectively. BMP-2 production was significantly greater in the 10μg/mL CaCO₃ group or in the 1μg/mL CaCO₃ group than in the control group on 24, 48 or 72 hours (*P<0.05) (B). OCN production was significantly greater in the 10μg/mL CaCO₃ group or in the 1μg/mL CaCO₃ group than in the control group on day 24 (*P<0.05) (C).
Figure 4  Effects of CaCO₃ on odontoblast- and dentinogenesis-related mRNA expression in hDPCs. hDPCs were cultured with 0, 1 or 10 μg/mL CaCO₃ for 1, 3 or 6 days, after which the mRNA expression of the indicated genes was analyzed using RT-PCR and specific primers. The mRNA expression of DSPP, Smad1, BMP-2, ALP and OCN was greater in the 10 μg/mL CaCO₃ group than in the control group. mRNA expression of Smad1, BMP-2 and DSPP was greater on days 1, 3 and 6, ALP was greater on day 6, and OCN was greater on days 3 and 6.

Figure 5  Effects on ALP activity in hDPCs. hDPCs were cultured with 0.1 or 10 μg/mL CaCO₃ for 25 days. The ALP increase was higher in the CaCO₃ groups in a CaCO₃ dose-dependent manner. ALP activity was significantly greater in the 10 μg/mL group than in the control group on days 15 (*P<0.05).
5. ALP activity
ALP activity of the hDPC groups was determined using an enzyme histochemical method. ALP activity, which was measured using a colorimetric assay, increased in both the CaCO₃ treatment and control groups and peaked on day 15. The ALP increase was higher in the CaCO₃ groups in a CaCO₃ dose-dependent manner. ALP activity was significantly greater in the 10 μg/mL group than in the control group (*P<0.05, n =8) (Figure 5).

DISCUSSION
We analyzed the effects of CaCO₃ treatment during odontoblast differentiation in long-term mineralized cultures of hDPCs. It was found that CaCO₃ is intimately involved in dentinogenesis, which may provide important clues for further investigation of the mechanisms of odontoblast differentiation.

We assayed the formation of mineralized nodules in hDPCs after 30-day culture in the presence or absence of CaCO₃ treatment using von Kossa staining and Alizarin red S staining. Both the number of calcified nodules and the calcium content in the 1 and 10 μg/mL CaCO₃ groups differed significantly from those in the control group. These results are in agreement with our previous report that, when the mineralization ability of hDPCs was enhanced, there was an increase in both the number of calcified nodules and calcium content. Our results are also supported by the report of Maeda et al. that treatment of human periodontal ligament cells with MTA or CaCl₂ in the absence of osteogenic induction media induces a positive von Kossa staining reaction. In addition, they showed that a calcium-rich environment stimulated the production of higher concentrations of phosphorus, and that these higher external concentrations of calcium and phosphorus were important for higher intracellular concentrations of phosphorus and calcium. The combined results suggest that CaCO₃ enhances the formation of calcium phosphate in hDPCs.

In order to clarify the mechanism by which cells are stimulated to form pulpal calcified nodules following CaCO₃ treatment, we determined cell viability, ALP staining and the expression of differentiation markers. Our results show that cell viability, and both ALP staining and activity, were greatly enhanced by CaCO₃ in a dose-dependent manner. Furthermore, the mRNA expression of all the differentiation markers assayed was enhanced in a CaCO₃ dose-dependent manner. Of the differentiation markers analyzed, DSPP in particular is known to be specifically expressed in odontoblast differentiation and is the most important marker of odontoblast differentiation. DSPP is also known to be intimately involved in dentin matrix mineralization. DSPP is a developmentally regulated protein that specifically appears in mature odontoblasts prior to the onset of mineralization. Therefore, our results demonstrate that CaCO₃ regulates DSPP mRNA expression and that this regulation is likely to be related to the function of DSPP as a transcription factor.

Our RT-PCR data also show that the mRNA expression of Smad1 was increased on days 1, 3 and 6 following CaCO₃ treatment of hDPCs, in a CaCO₃ dose-dependent manner. These data are consistent with a previous report that Smad1 mRNA expression was enhanced, and that the mRNA expression of BMP-2 was promoted, in hDPCs during mineralization, indicating that Smad1 plays an important role as a mediator in a series of processes that lead to the mineralization of hDPCs. These data are consistent with Smad1 mRNA being a BMP-activated Smad, and this result may support the hypothesis that CaCO₃ treatment of
hDPCs activates BMP-Smad signaling, thereby inducing Smad1 mRNA expression.

In accordance with this hypothesis, our data indicate that both BMP-2 mRNA expression and protein production are enhanced in DPCs in a CaCO₃ dose-dependent manner. Furthermore, this increase in BMP-2 expression was accompanied by a CaCO₃ dose-dependent increase in the mRNA expression and enzymatic activity of ALP and in the mRNA expression and protein production of OCN. BMP-2, ALP and OCN are all considered to play important roles in a series of processes that lead to osteogenesis and dentinogenesis. BMP-2 is also believed to play important roles in odontoblast differentiation and dentin formation, growth, and dentinogenesis. It has previously been reported that ALP activity is promoted in mouse preosteoblastic cell lines by treatment with BMP-2. In addition, we previously reported that BMP-2 mRNA expression is upregulated, and ALP activity is promoted, in hDPCs following exposure to low concentrations of reactive oxygen species. These findings suggest that BMP-2 is also involved in the mineralization of bone and pulp. OCN is known to be a protein that adjusts cellular calcium levels during excessive calcification.

Our combined data demonstrate that CaCO₃ induces the production of markers of the initial stage of odontogenic differentiation such as BMP-2 and ALP, as well as late-stage markers including OCN. Up-regulation of these genes is suggestive of the differentiation of human DPSCs into odontoblast-like cells. Our data are supported by those of Paranjpe et al., who reported that MTA up-regulates the levels of OCN, ALP, odontoblast-specific markers in DSP cells. Thus, our results suggest that CaCO₃ up-regulates odontogenic markers, and that treatment of hDPCs with CaCO₃ induces their differentiation into cells with the ability to undergo dentinogenesis. These data also suggest a possible signaling pathway by which pulpal cells differentiate into odontoblast-like cells.

CONCLUSIONS
These data in this study suggest that CaCO₃ is able to induce in vitro cell differentiation. Our findings indicate that hDPCs differentiate into cells with the ability to exhibit mineralization, and that this effect is mediated by stimulation with calcium derived from CaCO₃. We therefore speculate that treatment of exposed pulp with CaCO₃ is effective for dentinogenesis. In addition, as Ca(OH)₂ is also converted into CaCO₃ through a chemical reaction with carbon dioxide in the atmosphere, we believe that our results show one possible mechanism by which Ca(OH)₂ and MTA modulate pulp mineralization. Our results suggest that hDPC cell differentiation and dentinogenesis are activated by the release of calcium from CaCO₃ into the culture environment.

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