Effect of Tenomodulin overexpression treatment on bone formation activity is dependent on gene transfection vectors

Han Wang
Tohoku University Graduate school of Dentistry
Taichi Tenkumo (taichi.tenkumo.a3@tohoku.ac.jp)
Tohoku University Graduate school of Dentistry
Eiji Nemoto
Tohoku University Graduate School of Dentistry
Toru Ogawa
Tohoku University Graduate school of Dentistry
Yoshiaki Kanda
Tohoku University Graduate School of Dentistry
Keiichi Sasaki
Tohoku University Graduate school of Dentistry

Article

Keywords:

Posted Date: April 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1514134/v1

Abstract

Tenomodulin (Tnmd) has been confirmed in periodontal ligament and reported to inhibit angiogenesis or involved in collagen fibril maturation; however, the effect of Tnmd overexpression treatment on bone regeneration therapy is not clear.

Therefore, in this study we investigated the effect of Tnmd overexpression on bone formation activity in vitro and in vivo, using a calcium phosphate-based gene transfection vector (CaP) or a cationic polymer-based reagent (JetPEI).

Osteogenesis- and chondrogenesis-related gene expression levels in MC3T3E1 and rat bone marrow derived cells (rBMSCs) were detected using qPCR test 3 days after gene transfection with plasmid DNA (Tnmd). The bone filling ratio was evaluated at 28 days after implantation of a collagen sponge scaffold with or without CaP (Tnmd) or JetPEI (Tnmd) in rat calvaria bone defects. The bone filling ratio of JetPEI (Tnmd) was lower than that of the scaffold alone, while that of CaP (Tnmd) was higher. The present study demonstrates that the effect of Tnmd overexpression treatment was dependent on cell lines and a non-viral gene transfection vector in vitro. The gene transfection vector should be used depending on the targeting tissue in the introduction of Tnmd overexpression treatment in tissue regeneration therapy.

1. Introduction

Periodontal tissue is composed of gingiva, cementum, bone, and periodontal ligaments. Among these, periodontal ligaments support the chewing function as a ligament because the end of the collagen fiber is embedded in the cementum and the other side is embedded in the bone [1, 2]. On the other hand, periodontitis is a lifestyle-related disease that causes the loss of alveolar bone or periodontal ligament, resulting in tooth loss. Numerous scaffolds with or without growth factors associated with bone formation like BMP-2 have been developed and applied to regenerate periodontal tissues [3]. However, the periodontal ligament is often not regenerated and ankylosis has been observed on a part of the tooth surface after periodontal regeneration therapy [4, 5]. The consequences of ankylosis include progressive resorption of the root with bone replacement [6]. Therefore, the prevention of ankylosis and the re-formation of functional periodontal ligaments are important for periodontal tissue regeneration therapy at the same time new bone is formed. The localized introduction of an inhibition factor of bone formation around the root surface is an attractive approach to form the periodontal ligament in the periodontal tissue regeneration therapy.

On the other hand, Tenomodulin (Tnmd) expression has been confirmed in the brain, heart, tendon, ligament, and cornea [7–11]. It is reported that Tnmd inhibits angiogenesis or is involved in collagen fibril maturation [8, 12, 13]. Angiogenesis is associated with bone regeneration [14], and Shi et al. reported that Tnmd overexpression significantly reduced the osteogenic and chondrogenic differentiation of bone marrow-derived stem cells and adipose-derived stem cells [15]. Recently, Tnmd was also found in the periodontal ligament [9, 16]. Komiyama et al. reported that Tnmd might play a role in the development

and maintenance of the periodontal ligament [16]. These reports suggest that Tnmd negatively regulates bone formation. Therefore, topical Tnmd overexpression treatment in periodontal regeneration therapy might prevent ankylosis by leading to the coordinated regeneration of bone and periodontal ligament. However, the effect of Tnmd overexpression treatment in bone regeneration therapy is not clear.

The aim of this study was to investigate the effect of Tnmd overexpression treatment on bone formation in vitro and in vivo using two types of non-viral gene transfection vectors: a calcium phosphate-based gene transfection vector (CaP) and a cationic polymer-based reagent (JetPEI).

2. Materials And Methods

2.1. Preparation of gene transfection vectors

In this study, calcium phosphate nanoparticles and a cationic polymer-based reagent (JetPEI® or in vivo-JetPEI™) were used as non-viral gene transfection vectors. Calcium phosphate nanoparticles were prepared according to a previously reported method [17]. Briefly, the core of the calcium phosphate nanoparticles was fabricated by mixing an aqueous solution of calcium nitrate (18mM, pH 9.0, Wako, Tokyo, Japan) and an equal volume of an aqueous solution of diammonium hydrogen phosphate solution (10.8 mM, pH 9.0, Wako, Tokyo, Japan) using a peristaltic pump. Then, 18 μL of the prepared CaP dispersion was immediately mixed with 7.3 μL of aqueous plasmid DNA solution, that is, pcDNA3.1 + C-HA -Tenomodulin (1 mg/mL, GeneScript, Japan) or pUC57-EGFP (enhanced green fluorescent protein; 1 mg/mL, GeneScript, Japan) for gene expression analysis and animal experiments or pcDNA3.1(+)-C-HA mCherry (1 mg/mL, GeneScript, Japan) for gene transfection efficiency tests. After mixing, calcium nitrate (18 mM, pH 9.0; 9 μL,) and diammonium hydrogen phosphate (10.8 mM, pH 9.0; 9 μL, Wako) solutions were added to the prepared dispersion and mixed to cover the CaP core loading the plasmid DNA. Finally, an aqueous solution of protamine sulfate (10 mg/mL; 7.3 μL, Wako, Japan) was added to prepare the CaP nanoparticles. The obtained nanoparticle dispersion was centrifuged at 16,099 ×g at 4 ºC for 10 min to remove excess plasmid DNA or protamine from the prepared CaP nanoparticles. After the supernatant was removed, CaP nanoparticles were re-dispersed into 25 μL of fresh distilled water and denoted as CaP (Tnmd), CaP (EGFP), and CaP (mCherry), respectively. EGFP and mCherry have no osteoinductivity; hence, it was used as a control. We have demonstrated the structure of those CaP nanoparticles in our previous reports [17, 18]. For the fabrication of a cationic polymer-based reagent, JetPEI® (Polyplus, Shanghai, China) was used according to the manufacturer's protocol. Briefly, 0.5 μg of an aqueous solution of pcDNA3.1 + C-HA-Tenomodulin, 0.5 μg of an aqueous solution of pcDNA3.1(+)-C-HA mCherry or μL Jet PEI® reagent was mixed with 24.5 or 24 μL of NaCl (150 mM),respectively. The obtained 25 μL Jet PEI® solution was added to 25 μL of the DNA solution. After incubation for 20 min at room temperature (20–27 ºC), the reacted solution was denoted as JetPEI (Tnmd) or JetPEI (mCherry).

For animal experiments, 7 μL of an aqueous solution of pcDNA3.1 + C-HA-Tenomodulin was mixed with 14 μL of 10% glucose solution, and 7 μL of sterile water was added. 1.4 μL of in vivo JetPEI™ (polyplus, Shanghai, China) was mixed with 13.3 μL of 10% glucose solution, and 13.3 μL of sterile water was

added. The obtained 28 µL of in vivo JetPEI™ solution was added to 28 µL DNA solution and incubated for 15 min at 20–27 ºC of room temperature.

To perform gene transfer experiments using animal experiments, the mixing ratio for all applied solutions in the protocol was the same, but the volume of each solution was different. The volume of attached CaP nanoparticles was calculated by measuring the volume of the remaining plasmid DNA in the supernatant by UV microvolume spectroscopy (Nanodrop2000; Thermo Scientific, Tokyo, Japan). The volume of protamine attached to the CaP nanoparticles was also calculated by UV using a Protein Assay BCA kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol like DNA measurement. The volume of CaP nanoparticles applied in the cell or scaffold was calculated as described in a previous report [17, 19], and as shown in Table 1.

Table 1 Concentrations of calcium phosphate nanoparticles loaded with pcDNA3.1 + C-HA–Tenomodulin, pUC57-EGFP and pcDNA3.1(+)-C-HA mCherry

Concentration (µg / well in 48-well plates)	**CaP**	**pcDNA3.1 + C-HA–Tenomodulin**	**pUC57-EGFP**	**pcDNA3.1(+)-C-HA mCherry**	**Protamine**
JetPEI (Tnmd)	0	0.5	0	0	0
CaP (Tnmd)	5.1	1.6	0	0	3.0
CaP (EGFP)	5.1	0	1.6	0	2.8
JetPEI (mCherry)	0	0	0	0.5	0
CaP (mCherry)	5.1	0	0	1.6	2.8
Untransfected cells	0	0	0	0	0
For animal experiments (µg / scaffold)	CaP	pcDNA3.1 + C-HA–Tenomodulin	pUC57-EGFP	pcDNA3.1(+)-C-HA mCherry	Protamine
Scaffold alone	0	0	0	0	0
JetPEI (Tnmd)	0	7	0	0	0
CaP (Tnmd)	23.0	7.3	0	0	11.2
CaP (EGFP)	23.0	0	7.3	0	10.7

2.2 Animals

The study was carried out in compliance with the ARRIVE guidelines. All animals used in this experiment were handled according to the Guide for the Care and Use of Laboratory Animals of Tohoku University, Japan. Animals were obtained from Japan SLC Inc. (Shizuoka, Japan). All animal experimental protocols

were reviewed and approved by the Institutional Animal Experiment Committee of Tohoku University (2020DnA-026-01) before any animal experiments were conducted.

2.3 Cell culture

MC3T3E1 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in alpha-modified Eagle's medium (α-MEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Tokyo, Japan), 100 U/mL penicillin, and 100 U/mL streptomycin (Nacalai Tesque, Kyoto, Japan) at 37 ºC in the presence of 5% CO_2. To collect the rat bone marrow derived cells, five rats were sacrificed by overdose of sodium pentobarbital. Rat femur bones were extracted under general anesthesia and immediately immersed in PBS. The ends of the extracted femur bone were cut, and the bone marrow was collected using a needle and rinsed with PBS. The collected bone marrow was centrifuged at 800 × g for 10 min to remove red blood cells and debris. After re-suspension with α-MEM, the cells were seeded in 10 cm tissue culture dishes with α-MEM containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 ºC in the presence of 5% CO_2. These cells were denoted as rat bone marrow-derived cells (hereinafter called "rBMDCs"). Cells with passage numbers of 2–5 were used.

2.4. Gene transfection efficiency and cell viability

MC3T3E1 cells and rBMDCs were seeded in 48-well at a density of 2×10^4 per well, in the conditions described above. Approximately 24 h later, after the medium was removed, 225 or 200 μL of fresh culture medium with ascorbic acid and 25 μL of CaP (mCherry) or 50 μL of JetPEI (mCherry) solution were added to the cells, respectively. After 3 days, the transfection efficiency was calculated based on the ratio of fluorescing cells (mCherry-expressing cells resulting from successful gene transfection) to the total number of examined cells. The value at no autofluorescence of untransfected cell was used as a threshold. The cells were counted in an area of 557 × 722 μm in three parts per well. Dead cells (as recognized by their shapes) were not included in the computation process. Cell viability was analyzed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, Tokyo, Japan) assay, as described in our previous report [20].

2.5 ELISA and Ca concentration analysis

MC3T3E1 cells and rBMSCs were respectively seeded in 48-well plates at density of 2×10^4 per well under the conditions described above. The supernatant was collected 3 d after transfection. To determine alkaline phosphatase activity in the supernatant, the absorbance of the reaction mixture was measured at 405 nm with Labo Assay ALP (Fujifilm Wako, Tokyo, Japan), according to the manufacturer's instructions, using a microplate reader (iMarktm microplate reader; Bio-Rad, Osaka, Japan).

To determine the calcium concentration in the supernatant, the absorbance of the reaction mixture was measured at 620 nm using the Calcium E-test Wako (Wako, Tokyo, Japan) according to the manufacturer's instructions. The calcium concentration was determined using the culture medium for normalization.

To determine the protein expression levels of Tnmd resulting from gene transfection, the cultured cell was dissolved in RIPA Lysis Buffer solution with Protease inhibitor cocktail and 1% SDS (Nacalai Tesque, Kyoto, Japan) (400 μL), according to the manufacturer's instructions. The reaction solution was collected and centrifugated at 16,099 g for 10 min. The supernatant was collected and subjected to an enzyme-linked immunosorbent assay for quantifying Tnmd (Rat Tnmd (Tenomodulin) ELISA Kit from Wuhan Fine Biotech Co., Ltd, Biocompare, China). The absorbance of the reaction mixture was measured at 450 nm, according to the manufacturer's instructions, using a microplate reader (Spectra MAX 190, Molecular Devices, Japan).

2.6 Gene expression analysis

MC3T3E1 cells and rBMSCs were respectively seeded in 48-well plates at density of 2×10^4 per well under the conditions described above. Approximately 24 h later, the medium was replaced with 250 μL fresh medium with ascorbic acid, and the prepared gene transfection vectors were added to the cells, as shown in Table 1. After 3 days, the cultured media were removed, and total RNA was collected using Cell Lysis RT-qPCR kits (Bio-Rad, Osaka, Japan). After the concentration of the obtained RNA was measured using UV microvolume spectroscopy (Nanodrop2000), RNA (500 ng) was converted into cDNA using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Osaka, Japan). 10 μL of SsoAdvanced Universal SYBR Green system (Bio-Rad Laboratories), 4 μL of DNase-free water, 2 μL of cDNA, and 2 μL of each primer (10 μM) were mixed, followed by polymerase activation and DNA denaturation at 95°C for 30 s, followed by amplification for 40 cycles (denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s) using CFX96 (Bio-Rad, Osaka, Japan). The mRNA expression level of each targeted gene was determined using GAPDH as the control for normalization, using the △△CT method. The primer sequences for osteogenic and chondrogenic markers are shown in Table 2. The primers were purchased from Bio-Rad (Osaka, Japan) or FASMAC (Kanagawa, Japan).

Table 2
Sequences of gene-specific primer used for qPCR analysis

Gene name		For rat bone marrow derived cells Primer sequence 5'-3'	For MC3T3E1 Primer sequence 5'-3'
Tnmd	Fwd	TCCTGTTTTGGGGGAGCAAG	TCCTGTTTTGGGGGAGCAAG
	Rev	GCGTGACGGGTCTTCTCTAC	GCGTGACGGGTCTTCTCTAC
GAPDH	Fwd	GGCAAGTTCAACGGCACAG	GACTTCAACAGCAACTCCCAC
	Rev	CGCCAGTAGACTCCACGAC	TCCACCACCCTGTTGCTGTA
ALP	PrimePCR™ SYBR® Green Assay: Alpp, Rat		PrimePCR™ SYBR® Green Assay: Alpl, Mouse
OCN	PrimePCR™ SYBR® Green Assay: Bglap, Rat		PrimePCR™ SYBR® Green Assay: Bglap, Mouse
Runx2	PrimePCR™ SYBR® Green Assay: Runx2, Rat		PrimePCR™ SYBR® Green Assay: Runx2, Mouse
SP7	PrimePCR™ SYBR® Green Assay: Sp7, Rat		PrimePCR™ SYBR® Green Assay: Sp7, Mouse
Col2a	PrimePCR™ SYBR® Green Assay: Col2a1, Rat		PrimePCR™ SYBR® Green Assay: Col2a1, Mouse
Sox9	PrimePCR™ SYBR® Green Assay: Sox9, Rat		PrimePCR™ SYBR® Green Assay: Sox9, Mouse

2.7 Animal experiments

2.7.1 CaP/collagen or in vivo JetPEI^{TM}/collagen scaffold preparation

The pcDNA3.1 + C-HA -Tenomodulin or pUC57-EGFP loading CaP or pcDNA3.1 + C-HA –Tenomodulin loading in vivo-JetPEI™ dispersion was prepared as described in Section 2.1. The dose of each CaP nanoparticle or in vivo-JetPEI™ with loading plasmid DNA in the scaffolds is shown in Table 1. The bovine-derived atelocollagen sponge (diameter, 5 mm; Atelocollagen sponge MIGHTY, Koken, Tokyo, Japan) was cut at a height of 1.5 mm using a scalpel knife. 10 µL of each CaP dispersion was injected into the cut collagen sponge, freeze-dried, and stored it at -80°C and donated as CaP(Tnmd) or CaP(EGFP). Then, 56 µL of the in vivo-JetPEI™ dispersion was injected into cut collagen sponge within 30 min before the implantation into the rat and denoted as JetPEI (Tnmd).

2.7.2 Surgical procedures

Twenty rats were anesthetized with an intraperitoneal injection of medetomidine (Domitor, 0.375 mg/kg body pon Zenyaku Kogyo, Japan) and midazolam (Sandoz, 2 mg/kg body weight; Meiji Seika Co., Tokyo, Japan) as described in a previous our report [17]. After shaving the head, the flaps, including the periosteum, were revealted. Two circular osseous defects (diameter, 5 mm) were prepared on the cranium using a trephine bur with an external diameter of 5 mm, which was operated at 8,000 rpm with sterile saline irrigation, using a dental electric motor system (VIVAace; NSK, Kanuma, Japan). The prepared scaffolds were then implanted into the prepared defects. After implantation, the flap was rigidly sutured with non-absorbable 4 – 0 silk sutures (Mani, Tochigi, Japan) to prevent infection and loss of scaffolds. Rats were sacrificed by subjecting them to an overdose of sodium pentobarbital at 28 days after surgery, and the crania, including the implants, were extracted, and immediately immersed in PBS.

2.7.3 Micro-CT evaluation of bone defects

All extracted samples were scanned using microcomputed tomography (ScanXmate-E090; 60 kV;80 μA; Comscan Tecno Co. Ltd., Kanagawa, Japan) as described in a previous our report [17]. The bone mineral density (BMD) of newly formed bone tissues was measured at 4000 × 4000 × 2000 μm inside the scaffold using a 3D structural analysis (TRI/3D-VEI; Ra-toc System Engineering Co. Ltd., Tokyo, Japan). The bone defect area was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA), and the ratio of bone formation to original bone defect (volume: 19.625 mm^2) was calculated. The sample number of all groups was ten, as 40 holes were formed in twenty rats. Each rat was treated with a split-mouth design.

2.7.4 Biochemical evaluation

After micro-CT analysis, five samples in each group were used for determination of the yield of Tnmd expression. The implanted area (diameter 5 mm) was extracted using a trephine bur with an external diameter of 5 mm, operated at 10000 rpm with sterile saline irrigation, using a dental electric motor system (VIVAace, NSK, Kanuma, Japan). The extracted tissues were crushed using a multi-bead-shocker MB2000 (3000 rpm, 10 s; 1 cycle; YASUI KIKAI, Japan). The crushed tissue was dissolved in RIPA Lysis Buffer solution (1000 μL; Nacalai, Kyoto, Japan) and homogenized via ultrasonic treatment for 10 s, followed by centrifugation at 16,099 ×g for 15 min, according to the manufacturer's protocol. The supernatant was subjected to an enzyme-linked immunosorbent assay for quantifying Tnmd (Rat Tnmd (Tenomodulin) ELISA Kit from Wuhan Fine Biotech Co., Ltd, Biocompare, China). The absorbance of the reaction mixture was measured at 450 nm, according to the manufacturer's instructions, using a microplate reader (Spectra MAX 190, Molecular Devices, Japan).

2.7.5 Histological and immunohistochemistry analysis

After micro-CT analysis, five samples in each group were used for histological analysis. After fixing with 4% glutaraldehyde for 1 d, the samples were decalcified in 17.7% EDTA (OSTEOSOFT, Merck Millipore, Tokyo, Japan). The samples were dehydrated in an ascending series of ethanol solutions and embedded in paraffin. The tissue sections (6-μm thick) were stained with hematoxylin and eosin (H&E). Histology was examined under a light microscope. To evaluate Tnmd expression, sliced sections were stained with

a Tenomodulin polyclonal antibody (rabbit-poly (anti-Tnmd), diluted 1:2000 in PBS; Bioss, USA). All cells and Tnmd-positive cells were counted in an area of 700 μm × 530 μm in three stained sections at the center of the scaffold, and the ratio of Tnmd-positive cells per all cells in 371,000 μm^2 was calculated.

2.8 Statistical analysis

The statistical analysis was performed the same way as described in our previously reported [17]. All data were expressed as mean ± standard deviation (SD) values. First, normal data distribution was verified using the Shapiro-Wilk test, and the following differences were assessed by one-way analysis of variance or the multiple comparison test. The data that were not normally distributed in gene expression analysis and differences between groups were assessed by Kruskal-Wallis one-way analysis of variance, followed by the Dann-Bonferroni test. The other data can be presumed to have a normal distribution. Thus, statistical differences between groups were assessed using the post-hoc–Tukey Kramer HSD multiple comparison test. Statistical analyses were performed using SPSS 22.0 software (IBM, Tokyo, Japan). Differences were considered significant at $p < 0.05$.

3. Results

3.1 Effect of treatment with gene transfection loaded with plasmid DNA (Tnmd) in MC3T3E1 and rBMDCs

The gene transfection efficiency of JetPEI (mCherry) was significantly higher than that of CaP (mCherry), but the cell viability was lower than that of CaP (mCherry) (Table 3).

Table 3
Transfection efficiency and cell viability of MC3T3E1 and rBMDCs. * $p < 0.05$, compare to JetPEI (Tnmd), respectively

Cell line	group	Transfection efficiency (%)	Cell viability (%)
MC3T3E1	CaP (mCherry)	2 ± 1	99 ± 3
	JetPEI (mCherry)	12 ± 8	46 ± 3
rBMDC	CaP (mCherry)	1 ± 1	138 ± 8
	JetPEI (mCherry)	7 ± 4	24 ± 3

Next, the gene or protein expression levels of Tnmd resulting from gene transfection were investigated. Figure 1A and B show the Tnmd gene expression in MC3T3E1 and rBMDCs and that of CaP(Tnmd) and JetPEI (Tnmd) were significantly higher than those in the other groups ($p < 0.05$). There was no significant difference in the expression levels between CaP (Tnmd) and JetPEI(Tnmd). In the results from ELISA test, the expressed protein level of Tnmd was also the same as that of gene expression (Fig. 1C,D).

Figure 2 shows the gene expression level in MC3T3E1 cells at 3 days after Tnmd overexpression treatment by gene transfection. The gene expression levels of ALP, Runx2, and SP7, which are biomarkers of bone formation, and Col2α and SOX9, which are biomarkers of cartilage, of JetPEI (Tnmd) were significantly lower than those of untransfected cells and CaP (EGFP) ($p < 0.05$). On the other hand, there was no significant difference in the gene expression level of all CaPs (Tnmd) compared to that of the untransfected cell group. Figure 3 shows the gene expression levels in rBMDCs at 3 days after Tnmd overexpression treatment by gene transfection. The gene expression levels of Runx2 and SOX9 in JetPEI (Tnmd) were significantly lower than those in untransfected cells ($p < 0.05$). The gene expression level of OCN in CaP (Tnmd) was higher than that of JetPEI (Tnmd) and untransfected cells, while the gene expression levels of OCN and Col2α of CaP(EGFP) were higher than those of untransfected cells.

The volume of released ALP in the supernatant was not significantly different between all groups in either cell line (Fig. 4A and B). The calcium ion concentration in the supernatant of CaP(Tnmd) and Cap(EGFP) was significantly higher than that of the others in both cell lines (Fig. 4C and D) ($p < 0.05$).

3.2 Tissue reactions of the topical gene transfection with plasmid DNA (Tnmd) in collagen scaffold in the rat cranium bone defect

Table 4 shows the ratio of Tnmd-positive cells at 28 days after implantation of collagen scaffolds, including plasmid DNA (Tnmd)-loaded CaP nanoparticles or JetPEI in the cranium bone defect. The number of Tnmd-stained CaP (Tnmd) and JetPEI (Tnmd) were significantly higher than those of CaP (EGFP) and scaffold alone ($p < 0.05$).

Table 4
ratio of Tnmd stained cell per total cells in an area (371,000 μm^2)

Group	Number of Tnmd staining cells	Tnmd positive ratio (%) (Stained cells/total cells)
Scaffold alone	27 ± 25^c	3 ± 2^c
JetPEI (Tnmd)	230 ± 148^a	26 ± 9^a
CaP (Tnmd)	188 ± 69^a	31 ± 17^a
CaP (EGFP)	60 ± 34^b	7 ± 5^b

Figure 5 shows the histological or immunohistochemical images of all groups at 28 days after implantation. Collagen fibers of the scaffolds were observed in all groups. No infection, severe inflammation, necrosis, or drainage was observed in any group. Newly formed bone tissue was observed around collagen fibers in all groups, and osteocytes were observed inside the newly formed bone tissues (Fig. 5A and B). The brown cell shows Tnmd-stained cells in Fig. 5C. Positive cells were observed in and

outside of the scaffold in CaP (Tnmd) and JetPEI (Tnmd), while few were observed in CaP(EGFP) or scaffold alone.

The results of micro-computed tomography analysis are shown in Fig. 6. The bone filling ratios of CaP (Tnmd) were 66% ± 22%, which was significantly higher than those of JetPEI (Tnmd) (17% ± 12%), CaP (EGFP), and scaffold alone (30% ± 16%) ($p < 0.05$) (Fig. 6A). There were no significant differences between the values for CaP (Tnmd) and CaP (EGFP) (55% ± 17%), and that of JetPEI (Tnmd) was lower than that of scaffold alone, but there was no significant difference. The bone mineral density of CaP (Tnmd) was 1060 ± 126 mg/cm^3 and it was significantly higher than that of JetPEI (Tnmd) (584 ± 378 mg/cm^3) and scaffold alone (514 ± 274 mg/cm^3), with no significant difference compared to that of CaP (EGFP) (886 ± 342 mg/cm^3) (Fig. 6B). Figure 6C shows the expression of Tnmd in the scaffold. Tnmd concentration of CaP (Tnmd) and JetPEI (Tnmd) in scaffold were 9.2 ± 1.0, 9.5 ± 0.9 ng/mL/scaffold, respectively and significantly higher than that of CaP (EGFP) (2.6 ± 1.0 ng/mL/scaffold) and scaffold alone (2.2 ± 0.1 ng/mL/scaffold) ($p < 0.05$). There was no significant difference between CaP (Tnmd) and JetPEI (Tnmd). Figure 6D shows the micro-computed tomography images of each sample.

4. Discussion

It was confirmed that Tnmd was expressed at the gene and protein levels in vitro and in vivo by using calcium phosphate nanoparticles and cationic reagents gene transfection vectors. The volume of expressed Tnmd in CaP(Tnmd) and JetPEI(Tnmd) was approximately same.

Based on these results, osteogenesis-related genes were compared in MC3T3E1 and rBMDCs. Tnmd overexpression by JetPEI(Tnmd) reduced bone formation ability in MC3T3E1. On the other hand, the expression of these osteogenesis-related genes of CaP (Tnmd) was recovered to the same level as that of the untransfected cells group. This result indicates that those expression in MC3T3E1 was influenced by the gene transfection vector. Jeong et al. reported that the release of calcium and phosphorus ions activates the bioactivity of osteoblasts and osteoclasts to accelerate bone regeneration [21]. In this study, a calcium concentration in supernatant of CaP (Tnmd) was higher than that of JetPEI (Tnmd); therefore, the calcium concentration in microenvironment might have influence on the effect of Tnmd overexpression treatment.

In terms of rBMDCs, only Runx2 mRNA of JetPEI (Tnmd) was significantly lower than that of cells alone, and this result was similar to that reported by Jiang et al., who demonstrated that Tnmd overexpression reduced Runx2 and OCN mRNA levels in mouse bone marrow-derived stem cells [22]. In contrast, the expression levels of other osteogenesis-related genes were similar, and the effect of Tnmd overexpression treatment was different between the cell lines.

ChM-I, which has a domain homologous to Tnmd, is located in cartilage [23], and Shi et al. reported that Tnmd overexpression inhibited chondrogenic lineages in bone marrow-derived and adipose-derived stem cells [15]. Therefore, chondrogenesis-related gene expression levels were investigated. Col2α1 and SOX9

play important roles in chondrogenesis; Col2α1, a peculiar regulatory factor of cartilage, is upregulated in proliferating chondrocytes and SOX9 is an indispensable regulator of chondrogenesis [23, 24]. In this study, Tnmd overexpression by JetPEI(Tnmd) inhibited the chondrogenic activity, but the effect of Tnmd overexpression treatment was different between cells, similar to osteogenesis. On the other hand, the chondrogenesis-related gene expression level of CaP (Tnmd) was recovered at the same level of untransfected cells, and this also suggests that the effect of Tnmd overexpression treatment on chondrogenesis activity might be changed by the presence of calcium ions.

In this study, we used type 1 collagen scaffold for the in vivo experiment, because numerous scaffold based collagen were used not only for periodontal regeneration therapy [3], but also as a tendon scaffold [25]. Furthermore, a tendon is composed of 60–85% of Type I collagen in the total dry weight.

Tnmd-positive cells were observed inside the scaffold 28 days after implantation. This indicates that gene transfection using a collagen scaffold including CaP nanoparticles or cationic reagents was conducted inside or around the scaffold 28 days after implantation of the scaffold.

The new bone filling ratio of CaP (Tnmd) was higher than that of JetPEI (Tnmd) and scaffold alone. Tnmd overexpression treatment by gene transfection with calcium phosphate nanoparticles induced new bone formation. Meanwhile, the bone filling ratio of CaP (EGFP) was not different that of CaP (Tnmd). Mutsuzaki et al. reported that new bone on the implanted tendon scaffold was formed by depositing needle-like low-crystalline apatite on the tendon [26]. Olvera et al. demonstrated that the gene expression of mesenchymal stem cells was changed by the addition of apatite on microfiber scaffolds [27]. The calcium and phosphate ions supplied by the CaP nanoparticles in the collagen scaffold might be efficiently deposited on collagen fibrils, resulting in new bone formation.

On the other hand, the bone filling ratio of JetPEI (Tnmd) group was substantially lower, than that of scaffold alone. The ligaments work for the first time by being embedded at both ends of ligaments in hard tissue. Therefore, the microenvironments including bone associated cells or calcium concentration in the regeneration area should be considered for the regeneration of ligament. In this study, the expressed volume of Tnmd protein was approximately 9 ng/mL in both groups. It is not clear whether this 9 ng/mL in the implanted area was too low to affect Tnmd overexpression treatment or not.

As a limitation of this study, Tnmd is a type II transmembrane glycoprotein; therefore, the applied volume of Tnmd to cell or tissue could not be controlled as the application of the released protein like BMP-2. In future, the effect of Tnmd expression with high concentration of over 9 ng / mL should be investigated by using viral gene transfection vectors, which generally have higher gene transfection efficiency compared to that of non-viral transfection vectors.

Based on the in vivo and in vitro investigation, the effect of Tnmd overexpression on bone defects was dependent on the gene transfection vector. Based on the results of this study, the gene transfection vector should be used depending on the formulation of a collagen fiber part alone or collagen fiber part embedded in hard tissue in the introduction of Tnmd overexpression treatment in periodontal

regeneration therapy. Furthermore, the calcium concentration in the microenvironment may be considered for periodontal regeneration therapy.

5. Conclusion

In this study, gene transfection using calcium phosphate nanoparticles and cationic reagents was successfully performed in vitro and in vivo. After gene transfection, the calcium concentration in the supernatant was different between the two gene transfection vectors. The effect of Tnmd overexpression on bone formation activity was dependent on the cell lines and gene transfection vectors used. In vitro, Tnmd overexpression treatment using cationic reagent JetPEI (Tnmd) reduced osteogenesis-related and chondrogenesis-related gene expression, that using CaP (Tnmd) was recovered in MC3T3E1 cells. In a rat calvaria bone defect, the ratio of bone filling was substantially reduced by Tnmd overexpression transfection using JetPEI (Tnmd) compared to that of scaffold alone, while it was enhanced by using CaP (Tnmd).

Declarations

Acknowledgements

This work was financially supported by a Grant-in-Aid for Scientifc Research from the Japan Society for the Promotion of Science (19K10181). The funders had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

Data Availability

The datasets used during the current study available from the corresponding author on reasonable request.

Author contributions (names must be given as initials)

H. W., T.T., Y. K., E.N., K.S. designed the experiment. H.W., T.T. and K. S. wrote the main manuscript text. H. W., Y. K. and T.T. prepared figures 1-5 and Table 1,3. H.W., T.T., Y. K. and T.O. prepared figures 6. All authors reviewed the manuscript.

Conflict of interest

The authors declare no competing interests.

References

1. Kinane, D.F., Berglundh, T. Lindhe, J. Host-Parasite Interactions in Periodontal Disease, Clinical Periodontology and Implant Dentistry, 4th ed., Wiley-Black- well, Oxford. pp. 150–178. (2008).

2. Liu J, et al. Periodontal Bone-Ligament-Cementum Regeneration via Scaffolds and Stem Cells. Cells. 8.537. (2019).
3. Fa-Ming C., Yan, J. Periodontal tissue engineering and regeneration: current approaches and expanding opportunities. Tissue Eng Part B Rev. 16. 219-255. doi: 10.1089/ten.TEB.2009.0562. (2010).
4. Caton. J.G., DeFuria, E.L., Polson,A.M., Nyman,S. Periodontal regeneration via selective cell repopulation. J Periodontol. 58. 546-552. doi:10.1902/jop.1987.58.8.546. (1987).
5. Kato, A., et al. Periodontal Healing by Implantation of Collagen Hydrogel-sponge Composite in One-wall Infrabony Defects in Beagle Dogs. J oral tissue engine. 8. 39-46. (2010).
6. Andersson, L., Malmgren, B. The problem of dentoalveolar ankylosis and subsequent replacement resorption in the growing patient. Aust Endod J. 25. 57-61. doi: 10.1111/j.1747-4477.1999.tb00088.x. (1999).
7. Brandau, O., Meindl, A., Fa¨ ssler, R., Aszo´di, A. A novel gene, tendin, is strongly expressed in tendons and ligaments and shows high homology with chondromodulin-I. Dev Dyn. 221. 72–80. (2001).
8. Shukunami, C, Oshima, Y., Hiraki, Y. Molecular cloning of tenomodulin, a novel chondromodulin-I related gene. Biochem Biophys Res Commun. 280.1323–1327. (2001).
9. Itaya, T., et al. Characteristic changes of periodontal ligament-derived cells during passage. J Periodontal Res. 44. 425–433. (2009).
10. Shukunami, C., Takimoto, A., Oro, M., Hiraki, Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Dev Biol. 298. 234–247. (2006).
11. Oshima, Y., et al. Expression and localization of tenomodulin, a transmembrane type chondromodulin-I-related angiogenesis inhibitor, in mouse eyes. Invest Ophthalmol Vis Sci 44: 1814–1823. (2003)
12. Wang, W., Liu, G.X., Li, Y.H., Li, X.D., He, Y. Int J Ophthalmol. Inhibitory effect of tenomodulin versus ranibizumab on *in vitro* angiogenesis. 10. 1212-1216. doi: 10.18240/ijo.2017.08.04. (2017).
13. Docheva, D., Hunziker,E.B., Fässler, R., Brandau,O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol. 25. 699-705. doi: 10.1128/MCB.25.2.699-705.(2005).
14. Percival CJ, Richtsmeier JT. Angiogenesis and intramembranous osteogenesis. Dev Dyn. 242. 909-22. (2013).
15. Shi, Y., et al.Conditional tenomodulin overexpression favors tenogenic lineage differentiation of transgenic mouse derived cells. Gene. 598. 9-19. doi: 10.1016/j.gene.2016.10.028. (2016).
16. Komiyama, Y., et al. Tenomodulin expression in the periodontal ligament enhances cellular adhesion. PLoS One. 8. e60203.(2013).
17. Xiang, C., et al. Gene transfection achieved by utilizing antibacterial calcium phosphate nanoparticles for enhanced regenerative therapy. Acta Biomater. 119.375-389.(2021).
18. Vanegas, Sáenz. J.R, Tenkumo, T., Kamano, Y., Egusa, H., Sasaki, K. Amiloride-enhanced gene transfection of octa-arginine functionalized calcium phosphate nanoparticles. PLoS One. 12.

e0188347. (2017).

19. Sokolova, V., Knuschke,T., Buer,J., Westendorf,A.M., Epple,M. Quantitative determination of the composition of multi-shell calcium phosphate-oligonucleotide nanoparticles and their application for the activation of dendritic cells. Acta Biomater. 11.4029-4036. doi: 10.1016/j.actbio.2011.07.010. (2011).
20. Tenkumo, T., et al. Gene transfection of human mesenchymal stem cells with a nano-hydroxyapatite-collagen scaffold containing DNA-functionalized calcium phosphate nanoparticles. Genes Cells. 7. 682-695. doi: 10.1111/gtc.12374. (2016).
21. Jeong, J., Kim, J.H., Shim, J.H., Hwang, N.S., Heo, C.Y. Bioactive calcium phosphate materials and applications in bone regeneration. Biomater Res. 23. 4. doi: 10.1186/s40824-018-0149-3. (2019).
22. Jiang, Y. et al. Enhanced tenogenic differentiation and tendon-like tissue formation by tenomodulin overexpression in murine mesenchymal stem cells. J Tissue Eng Regen Med. 11.2525-2536. doi: 10.1002/term.2150. (2017).
23. Bell, D.M. et al. SOX9 directly regulates the type-II collagen gene. Nat Genet 16.174–178. (1997).
24. Ng, L.J.,et al. SOX9 binds DNA, activates transcription, and coexpresseswith type II collagen during chondrogenesis in the mouse. DevBiol 183. 108–121. (1997).
25. Jaiswal, D. et al. Tendon tissue engineering: biomechanical considerations.Biomed Mater. 15.052001. doi: 10.1088/1748-605X/ab852f.(2020).
26. Mutsuzaki, H. et al. The interaction between osteoclast-like cells and osteoblasts mediated by nanophase calcium phosphate-hybridized tendons. Biomaterials. 26.1027-1034.(2005).
27. Olvera D, Sathy BN, Kelly DJ. Spatial Presentation of Tissue-Specific Extracellular Matrix Components along Electrospun Scaffolds for Tissue Engineering the Bone-Ligament Interface. ACS Biomater Sci Eng. 6.5145-5161. doi: 10.1021/acsbiomaterials. (2020).

Figures

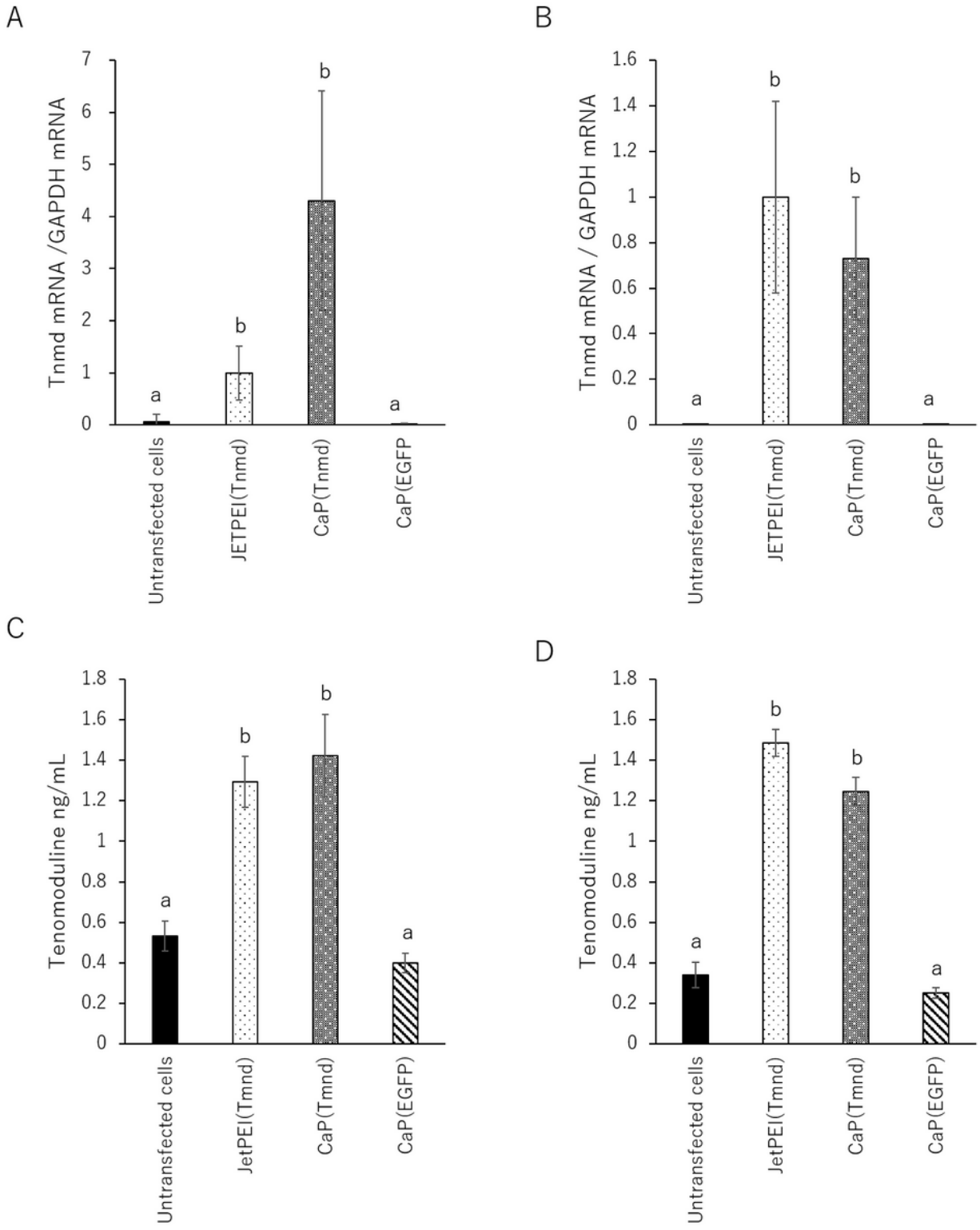

Figure 1

Determination of Tnmd expression level at 3 days after gene transfection in MC3T3E1 and rat bone marrow derived cells (rBMDCs). Tnmd gene expression level in MC3T3E1 (A) in rBMDCs (B). Tnmd protein expression levels in MC3T3E1 (C) and rBMDCs (D). Values and error bars indicate mean and standard deviation values, respectively. Significant differences ($p < 0.05$) between groups are denoted by different superscript letters (i.e., bars with the same letter are not significantly different).

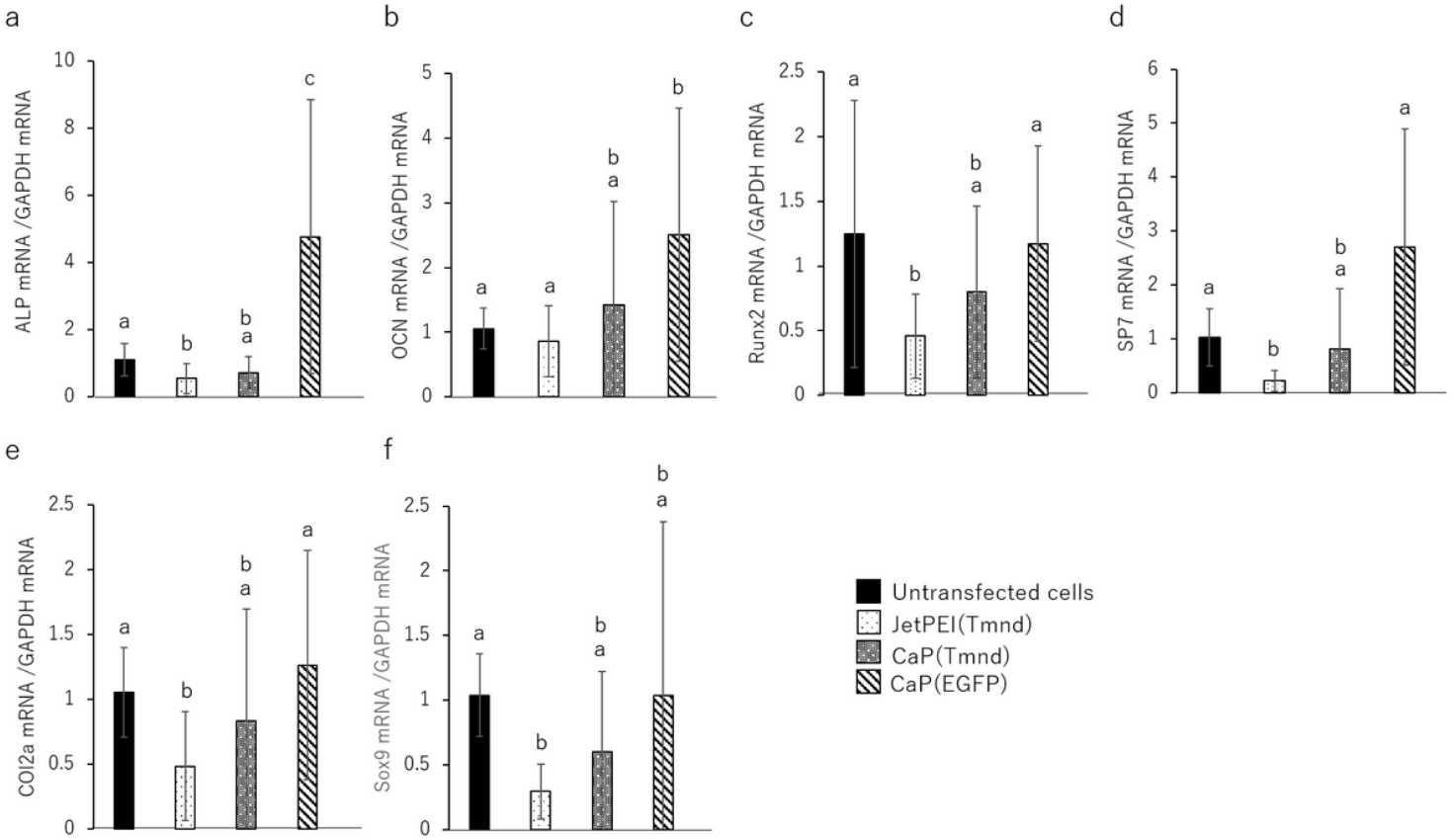

Figure 2

Gene expression analyses in MC3T3E1 3 days after gene transfection. (a) ALP mRNA expression, (b) OCN mRNA expression, (c) Runx2 mRNA expression. (d) SP7 mRNA expression, (e) Col2αmRNA expression, (f) SOX9 mRNA expression. Values and error bars indicate the mean and standard deviation, respectively. Significant differences ($p < 0.05$) between groups are denoted by different superscript letters (i.e., bars with the same letter are not significantly different).

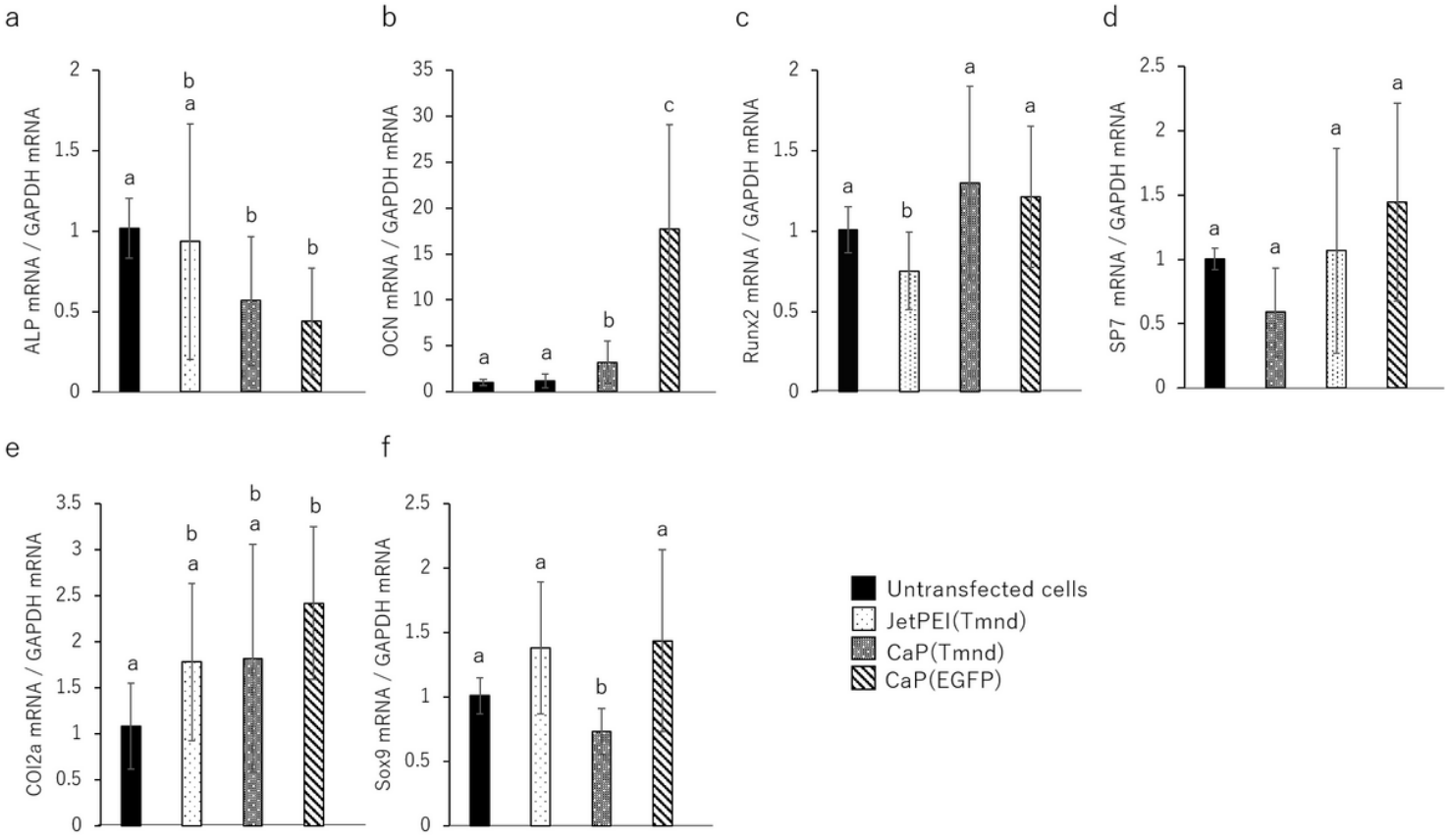

Figure 3

Gene expression analyses in rBMDCs 3 days after gene transfection. (a) ALP mRNA expression, (b) OCN mRNA expression, (c) Runx2 mRNA expression. (d) SP7 mRNA expression, (e) Col2α mRNA expression, (f) SOX9 mRNA expression. Values and error bars indicate the mean and standard deviation, respectively. Significant differences ($p < 0.05$) between groups are denoted by different superscript letters (i.e., bars with the same letter are not significantly different).

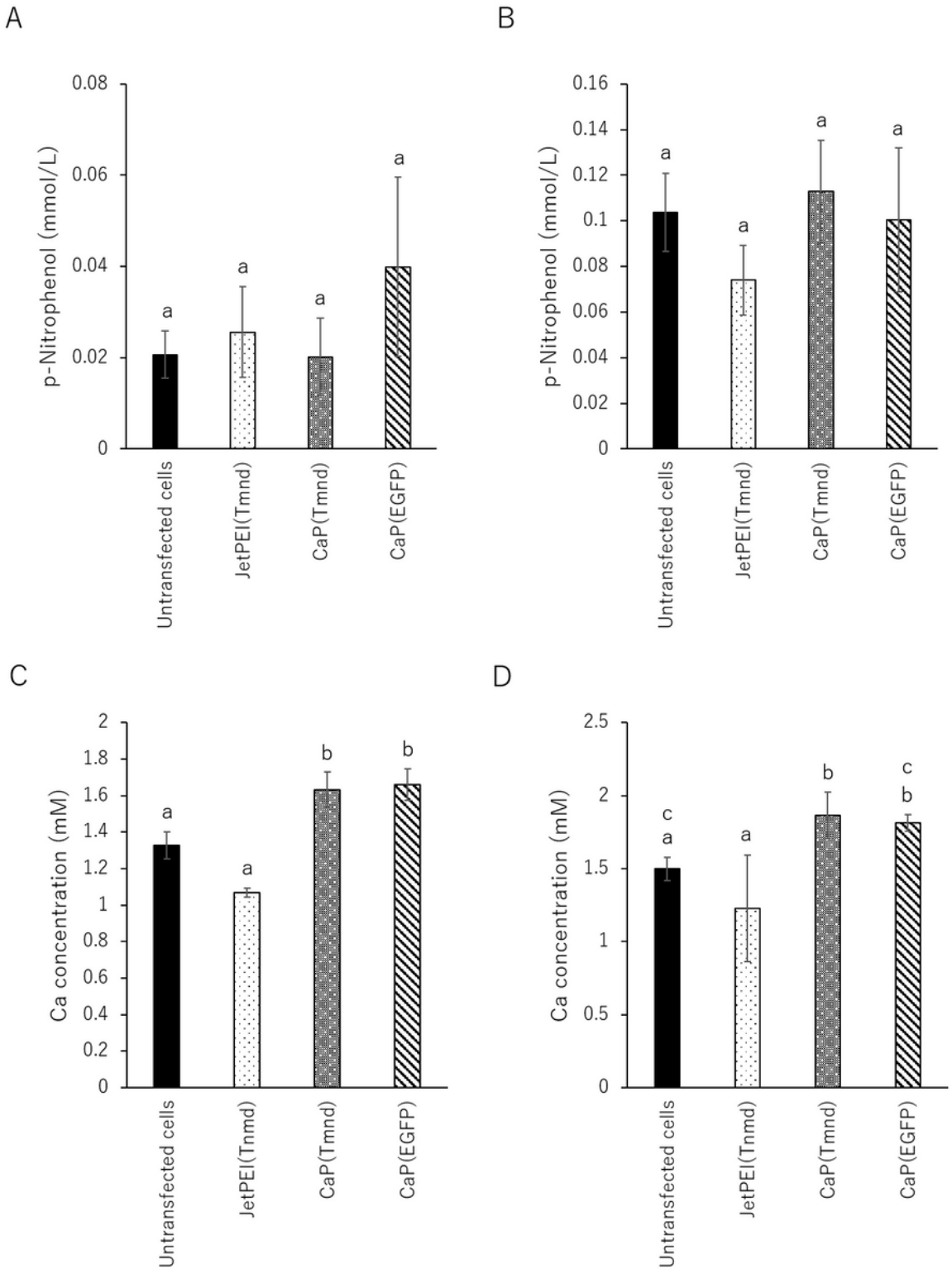

Figure 4

The concentration of released ALP in supernatant of culture medium of MC3T3E1 (A) and rBMDCs (B) 3 days after gene transfection. The concentration of released Ca ion concentration in supernatant of culture medium of MC3T3E1 (C) and rBMDCs (D) 3 days after gene transfection. Values and error bars indicate the mean and standard deviation, respectively. Significant differences ($p < 0.05$) between groups are denoted by different superscript letters (i.e., bars with the same letter are not significantly different).

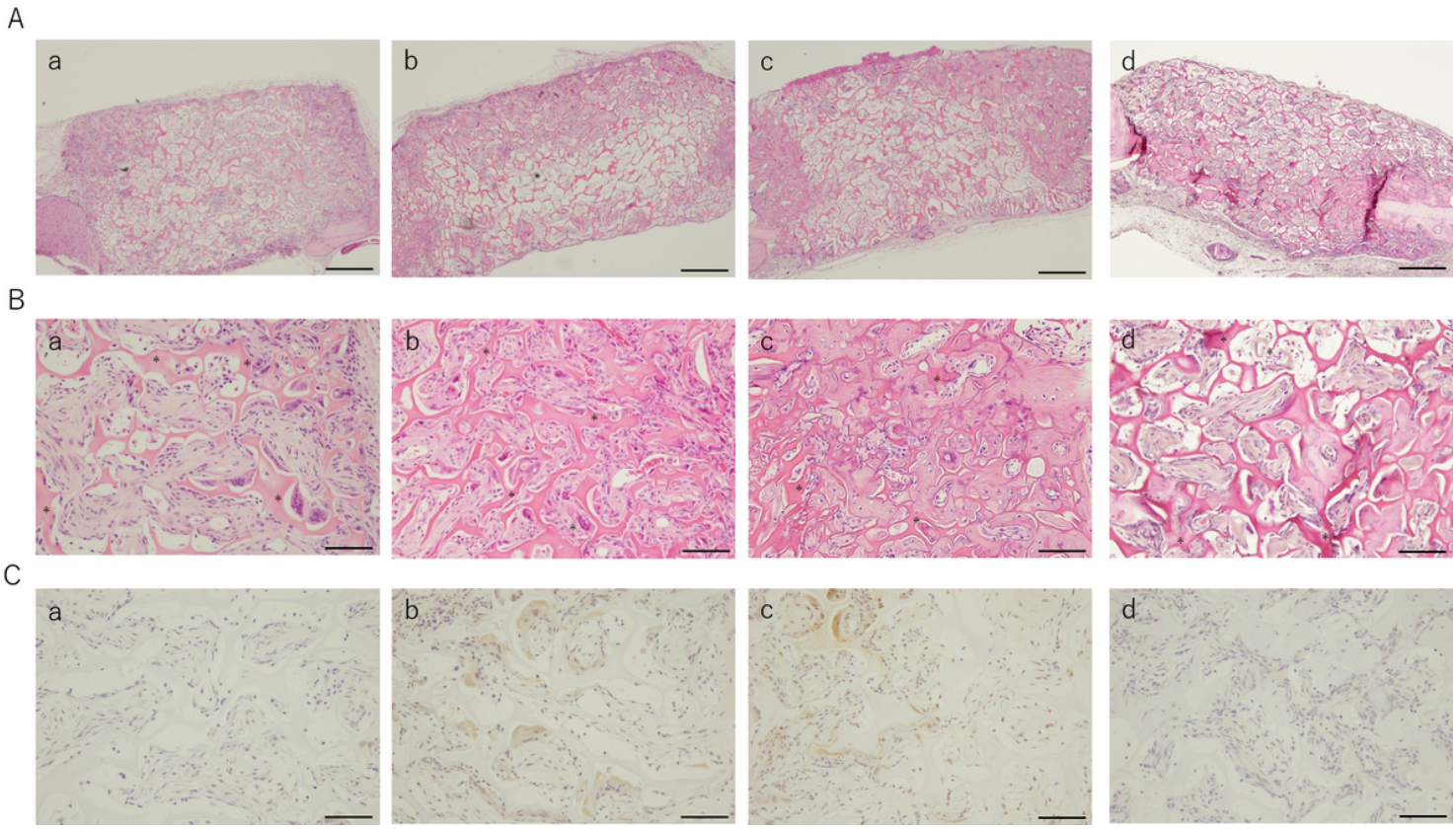

Figure 5

Histological analysis of cells stained with A: Hematoxylin and eosin (H&E) at low magnification, B: high magnification, C: Tnmd at 28 days in the cranium bone defect. (a) Scaffold alone, (b) JetPEI (Tnmd), (c) CaP (Tnmd), (d) CaP(EGFP). The symbol * indicates the remaining collagen fibers. Scale bars: 200 μm in A, 100 μm in B and C.

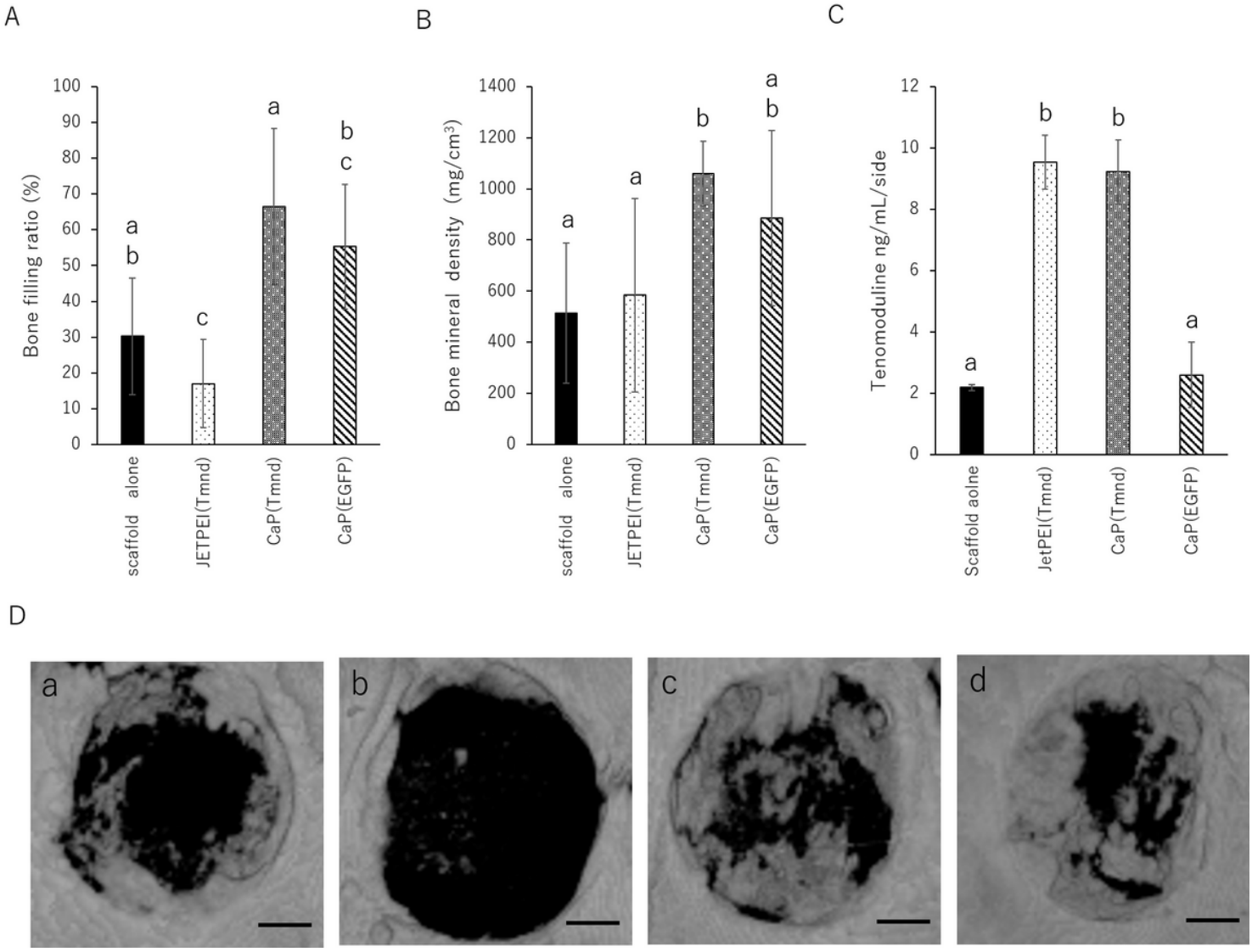

Figure 6

The ratio of newly formed bone tissues in the bone defect (A) and bone mineral density (BMD) (B) determined after micro computed tomography analysis, 28 days after implantation including gene transfection vector into the cranium bone defect. Comparison of yields of expressed Tnmd (C) inside scaffold, 28 days after collagen scaffolds, including gene transfection vectors, were implanted. Values and error bars indicate the mean and standard deviation, respectively. Significant differences ($p < 0.05$) between groups are denoted by different superscript letters (i.e., bars with the same letter are not significantly different).

Micro CT images (□) are shown; scale bars= 1000 μm. The sample number in all groups was ten, as there were ten bone defects.