Dentin Matrix Protein 1 Regulates Mineralization of MC3T3-E1 Cells via the TNAP-ANK-ENPP1 Axis

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Background: Dentin matrix protein 1 (DMP1) is central to matrix mineralization. Clarification of the function of DMP1 is crucial to understanding normal bone formation and pathological calcification. The tissue-nonspecific alkaline phosphatase (TNAP)-progressive ankylosing enzyme (ANK)-extracellular nucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) axis induces deposition of hydroxyapatite (HA) and pyrophosphate dehydrate (CPPD) by regulating pyrophosphate (PPi). Here, we investigated the mechanism by which DMP1 and the TNAP-ANK-ENPP1 axis participate in mineralization.

Methods: Expression of DMP1, TNAP, NPP1, and ANK genes in MC3T3-E1 cells was detected by RT-qPCR before and after treatment with DMP1 siRNA. An enzyme-linked immunosorbent assay was used to determine expression of DMP1 protein, TNAP activity was detected by SIGMAFAST p-nitrophenyl phosphate tablets, and mineralization of osteoblasts was determined by alizarin red staining. PPi levels were determined radiometrically and equalized for cell DNA. Levels of calcium, inorganic phosphate, zinc, and magnesium were assessed by standard laboratory techniques.

Results: After DMP1 gene silencing, expressions of TNAP, ENPP1, and ANK were correspondingly reduced. DMP1 altered extravesicular and intravesicular ion levels through the TNAP-ENPP1-ANK axis in MC3T3-E1 cells.

Conclusions: DMP1 regulated mineralization of MC3T3-E1 cells via the TNAP-ANK-ENPP1 axis and affected TNAP activity by two processes—rapid regulation of the Zn2+ transporter (ZnT) and transcriptional regulation of hysteresis. However, DMP1 may affect expression of ENPP1 and ANK only via hysteresis transcriptional regulation. DMP1, as a calcium trap or catalytic enzyme, appears to have a role in collagen mineralization. (J Nippon Med Sch 2023; 90: 262–271)

Key words: DMP1, TNAP-ANK-ENPP1 axis, mineralization, Pi/PPi-TNAP-positive feedback

Introduction
Mineralization is a tightly regulated process determined by extracellular matrix (ECM) proteins, ion channels, proteinases, and other promoter and inhibitor molecules. A better understanding of physiological mineralization and the effect of anti-calcification drugs on the mechanisms of mineralization could provide useful information for targeting and treating pathological calcification, defined as deposition of minerals in soft tissues that do not normally mineralize, or premature or excessive calcification of normal mineralized tissues. Many diseases are associated with pathological mineralization/ossification, including anklylosing spondylitis (AS), osteoarthritis (OA), chondrocalcinosis (CC), and pseudogout. AS and OA are mainly characterized by mineralization/ossification associated with hydroxyapatite (HA) deposits1, while CC and

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DMP1 Regulates MC3T3-E1 Cell Mineralization

It is important to gain a firm understanding of the mechanisms involved in the regulation of physiological mineralization before attempting to generate novel therapeutics for treating pathological calcification. MC3T3-E1, a well-established preosteoblastic cell line, is widely used as an in vitro model of osteogenesis that reproduces in vivo conditions. Using the MC3T3-E1 murine preosteoblast cell line, we examined whether there is a direct effect of DMP1 on the regulation of PPi and whether the TNAP-ANK-ENPP1 axis is involved in this response.

Materials and Methods

Cell Culture and Transfection

MC3T3-E1 mouse preosteoblastic cells obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin and streptomycin (Gibco, Gaithersburg, MD, USA) and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA). Stable transfections with small interfering RNA (siRNA) and blank siRNA were performed using Lipofectamine RNAiMAX (Life Technologies Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Transfections were performed on cells at 80-85% confluence in six-well plates. The efficacy of transfection was evaluated via observation of red fluorescent cells versus total cells with a fluorescence microscope. The experiments were repeated at least three times. The sequence of the siRNA-DMP1 was 5’-GGACCGUUCUGAG UUCCGAUGA-3’.

Morphological Observations

The cells were evaluated under a phase-contrast microscope. Microphotographs were taken with a Nikon camera and a 310 objective lens (Nikon, Tokyo, Japan).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from osteoblasts by using 0.5 mL TRIzol (Life Technologies) in a 35-mm dish. For each sample, total RNA content was assessed by absorbance at 260 nm, and the purity of the RNA was assessed by the A260/A280 ratio, measured using a NanoDrop spectrophotometer (Fischer Scientific, Loughborough, UK). Total RNA (1 μg/20 μL) was reverse transcribed using the Titanium One-Step real-time quantitative reverse transcription PCR (qRT-PCR) kit (Takara Bio, Inc., Kusatsu, Japan) according to the manufacturer’s instructions. All selected genes were analyzed with the SYBR green detection method using the Stratagene Mx3000P real-time qPCR system (Stratagene, San Diego, CA, USA), and each sam-

pseudogout are primarily characterized by pyrophosphate dehydrate (CPPD) deposits1,2.

Dentin matrix protein 1 (DMP1), a non-collagenous ECM protein, is central to matrix mineralization in the formation of dentin and bone. DMP1 is expressed by odontoblasts and osteoblasts and appears to be crucial for matrix mineralization by regulating crystal size and morphology. Using Fourier transform infrared imaging analysis, Ling et al.7 and Liu et al.8 found that DMP1 depletion decreased bone mineralization in vivo. Moreover, studies by Narayanan et al.9, Bhatia et al.10 and Merkel et al.11 demonstrated that overexpression or endocytic trafficking of DMP1 could induce differentiation of pluripotent and mesenchymal-derived cells and accelerate mineralization to form functional odontoblast-like cells. Taken together, these results suggest that DMP1 has a regulatory function during the formation of the mineralized matrix. Additionally, our previous results indicated that the DMP1 gene is involved in the genetic predisposition to AS in a Chinese Han population from Shandong Province and may contribute to ectopic mineralization or ossification in AS10.

Tissue-nonspecific alkaline phosphatase (TNAP) is an important promoter of mineralization and a well-characterized marker of the osteoblast lineage11. Liu et al.12 highlighted the importance of the HLA-B27-mediated activation of TNAP in AS syndesmophyte pathogenesis. The progressive ankylosing enzyme (ANK) is a transmembrane protein that plays a crucial role in the incorporation of pyrophosphate (PPi) in the mineralized bone matrix. ANK regulates transport of intracellular PPi to the extracellular space12,13 and offsets the activity of TNAP by increasing extracellular PPi levels by providing a substrate for extracellular nucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1)14. Loss of ANK function results in decreased extracellular PPi. ENPP1 is present on the cell surface and in cytoplasm and generates PPi in both regions15,16. The TNAP-ANK-ENPP1 axis induces HA and CPPD deposition by regulating PPi. The actions of TNAP, ENPP1, and ANK maintain the proper PPi/Pi ratio, which is directly related to mineralization: a high PPi/Pi ratio inhibits mineralization, whereas a low PPi/Pi ratio promotes mineralization. However, the mechanism by which DMP1 and the TNAP-ANK-ENPP1 axis participate in mineralization are unknown. Here, we investigated the precise mechanism by which DMP1 and the TNAP-ANK-ENPP1 axis, including TNAP, ANK, and ENPP1, participate in mineralization in cultured MC3T3-E1 cells.

J Nippon Med Sch 2023; 90 (3) 263
Table 1 Primers used for qRT–PCR

<table>
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<th>Gene name</th>
<th>Forward primer (5'- to -3')</th>
<th>Reverse primer (5'- to -3')</th>
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<tr>
<td>DMP1</td>
<td>ggctacgacagaggccaagtga</td>
<td>tctcatgcgaagaaggtgtc</td>
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<td>TNAP</td>
<td>tggctacgacagaggccaagtga</td>
<td>tgaacctcgatccccgtgtg</td>
</tr>
<tr>
<td>ANK</td>
<td>aagctgggaaggcaacaagc</td>
<td>gcaaggcaagaaggccacctc</td>
</tr>
<tr>
<td>NPP1</td>
<td>cagaccacagaagagtgaagctgc</td>
<td>gaagggttagacgcgcgag</td>
</tr>
<tr>
<td>GAPDH</td>
<td>tggcaaagtggaggttgc</td>
<td>ttgcatcgtgggttgtgg</td>
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</tbody>
</table>

Table 2 Number of mineralized nodules

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<th>Group</th>
<th>12 days</th>
<th>16 days</th>
<th>20 days</th>
<th>24 days</th>
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</thead>
<tbody>
<tr>
<td>Blank control</td>
<td>22.67 ± 6.53</td>
<td>62.33 ± 6.08</td>
<td>99.00 ± 12.00</td>
<td>159.67 ± 20.02</td>
</tr>
<tr>
<td>siRNA control</td>
<td>21.00 ± 8.00*</td>
<td>61.00 ± 11.00*</td>
<td>96.00 ± 11.00*</td>
<td>155.33 ± 22.69*</td>
</tr>
<tr>
<td>siRNA</td>
<td>1.33 ± 0.58#</td>
<td>17.00 ± 5.00#</td>
<td>35.00 ± 7.00#</td>
<td>60.67 ± 9.08#</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD. *P > 0.05 compared to the blank control. #P < 0.01 compared to the siRNA control.

ple was assayed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping/reference gene for target gene normalization, and gene expression levels were compared using relative quantification with corrected amplification efficiency for all primers. The primers are shown in Table 1. Data were analyzed using the ΔΔCt method.

TNAP Activity Assay

We determined the protein content and specific activity of TNAP as described previously[18]. TNAP activity was measured after washing the MC3T3-E1 osteoblast cultures twice with phosphate-buffered saline before harvesting in 10 mM Tris (pH 7.4)/0.2% IGEPAL (Sigma-Aldrich, St. Louis, MO, USA). MC3T3-E1 cell lysates were obtained by sonication, and TNAP activity was measured using SIGMAFAST p-nitrophenyl phosphate tablets (Sigma-Aldrich) according to the manufacturer’s instructions. TNAP from bovine intestinal mucosa (Sigma-Aldrich) was used as a standard, and TNAP activity was normalized to the protein content.

Alizarin Red Staining for Matrix Mineralization

MC3T3-E1 cells were fixed with 4% paraformaldehyde for 30 min at room temperature before staining with 2% alizarin red (Sigma-Aldrich) at pH 4.2. After the images were captured, the cells were destained with 10% cetylpyridinium chloride and the optical density was determined at 570 nm[19]. The conditions of nodule formation were routinely checked via phase-contrast microscopy (Nikon, Tokyo, Japan) at 100× magnification. Ten fields of view were retrieved. The experiments were performed in triplicate.

Matrix Vesicle Isolation

MC3T3-E1 cells were cultured for 10 days, and the medium was changed daily until day 7, after which 10 mL of medium was left in each plate until day 10. The medium was collected and initially centrifuged at 20,000 × g for 20 min at 4°C to precipitate cellular debris. Subsequently, the supernatant was retrieved and centrifuged at 100,000 × g for 1 h to isolate the matrix vesicle fraction, which was later resuspended in 0.4 mL lysis buffer (LB; 1% Triton X-100 in 0.2 M Tris base with 1.6 mM MgCl₂, pH 8.1)[18,20].

Analysis of PPI, Calcium, Inorganic Phosphate, Zinc, and Magnesium Levels

PPI levels were determined radiometrically and normalized to the DNA content of the cell, as described previously[21]. To determine intracellular PPI levels, the cells were rinsed and heated at 65°C for 45 min, before rinsing again and lysing in LB. Extracellular PPI levels were determined from conditioned media treated in the same manner[18]. Levels of calcium (Ca²⁺), inorganic phosphate (P), zinc (Zn²⁺), and magnesium (Mg²⁺) were assessed using standard laboratory techniques (BioVision, Milpitas, CA, USA; Roche/Hitachi 747, Tokyo, Japan).

DMP1 Enzyme-Linked Immunosorbent Assay

To measure DMP1 protein levels, we developed an enzyme-linked immunosorbent assay method that uses plates coated with a monoclonal antibody against native DMP1 (Chemicon, Temecula, CA, USA) diluted 1:2,500 in 100 μL/well of 0.1 mol/L sodium bicarbonate at pH 9.0 and 4°C overnight. The wells were blocked with 10 mmol/L Tris, and 150 mmol/L NaCl and 0.05% Tween-
DMP1 Regulates MC3T3-E1 Cell Mineralization

Fig. 1  Efficiency and optimal transfection concentrations of siRNA at (A) 20 nM, (B) 40 nM, and (C) 80 nM for MC3T3-E1-E1 cells viewed under fluorescence microscopy.

Fig. 2  DMP1 protein concentration in each group at 24 h after transfection (ng/mL). *P<0.01.

Laboratory Assessment
Serum levels of Ca\(^{2+}\), Pi, Zn\(^{2+}\), and Mg\(^{2+}\) were assessed by standard laboratory techniques (BioVision; Roche/Hitachi 747).

Statistical Analysis
Statistical analysis was performed using SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA). All quantitative data are presented as mean ± SD. The methods and assays were repeated at least three times to ensure the accuracy of the results. The statistical significance of differences among groups was determined by analysis of variance (ANOVA) and Scheffé’s multiple-comparison techniques. Comparisons between time-based measures within each group were performed using repeated measures ANOVA. P-values <0.05 were considered statistically significant.

Results

Efficiency and Optimal Transfection Concentration of siRNA for MC3T3-E1 Cells under Fluorescence Microscopy after Successful Transfection with siRNA
To determine the optimal transfection concentration, we tested red fluorescent dye at three concentrations (20, 40, and 80 nM), according to the manufacturer’s instructions. Red fluorescence was emitted when the dye was successfully transfected into the cells. The transfection efficiencies were 70%, 90%, and 84%, respectively (Fig. 1 A~C). The transfection efficiency was highest at a dye concentration of 40 nM.

DMP1 Gene and Protein Expression after Transfection with siRNA Targeting DMP1
We measured the DMP1 protein concentration (ng/mL) in the cultured cells at 24 h after successful transfection. The results were as follows: blank control group, 2.497 ± 0.026; negative siRNA control group, 2.491 ± 0.025; and siRNA interference group, 0.257 ± 0.003 (Fig. 2).

Gene Expression of DMP1, TNAP, ANK, and ENPP1 in Cells after Transfection into Osteoblast MC3T3-E1 Cells with siRNA Targeting the DMP1 Gene
qRT-PCR was used to analyze gene expression levels of DMP1, TNAP, ANK, and ENPP1, and the data were analyzed using the ΔΔCt method. We also applied repeated measurements and ANOVA to analyze the data. Statistical significance (P < 0.01) is indicated by an asterisk in the figures. After DMP1 gene silencing, DMP1 gene expression decreased, along with expression levels of TNAP, ENPP1, and ANK (Fig. 3A~D).
Effect of Transfection with siRNA Targeting the DMP1 Gene on TNAP Activity

MC3T3-E1 cells were cultured in a mineralization medium. Throughout the induction period, TNAP activity first increased and then decreased, reaching a peak on day 9. TNAP activity was lower in the siRNA group than in the blank control and siRNA control groups. A P value of < 0.01 was considered statistically significant. After transfection with siRNA, TNAP activity underwent a refractory period from day 3 through day 6 (Fig. 4).

Mineralization Analysis

On day 7 of culture with mineralization medium, an inverted microscope was used to observe cells at the bottom of the bottle. On days 12, 16, 20, and 24 of culture, cells were stained with alizarin red and observed with an inverted microscope, before capturing images with a camera (data not shown). On day 12 of culture, scattered orange mineralized nodules were observed in the blank control and siRNA control groups. However, the siRNA group showed a few mineralized nodules. As the mineralization induction time progressed, the number of mineralized nodules increased (Fig. 5, Table 2).

Analysis of Intravesicular, Extravesicular, and Extracellular Matrix Levels of Ppi, Pi, Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\)

Next, the effect of transfection with siRNA on the ability of DMP1 to influence intravesicular, extravesicular, and ECM levels of Ppi, Pi, Ca\(^{2+}\), Zn\(^{2+}\), and Mg\(^{2+}\) was investigated in MC3T3-E1 cells. We transfected MC3T3-E1...
DMP1 Regulates MC3T3-E1 Cell Mineralization

Fig. 5 Mineralized nodules (40×) stained with alizarin red were observed under an inverted microscope.

Fig. 6 ECM (or extravesicular) and intravesicular matrix levels of PPI, Pi, Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\) in MC3T3-E1 cells. (A) ECM (or extravesicular) ion concentration (percentage); (B) intravesicular ion concentration (percentage).

cells with siRNA targeting DMP1 or blank siRNA, as described in the Materials and Methods, after 48 h of culture. Extravesicular and ECM levels of PPI, Mg\(^{2+}\), and Zn\(^{2+}\) remained constant but Pi and Ca\(^{2+}\) levels decreased. Moreover, intravesicular levels of Zn\(^{2+}\) and PPI decreased, while Pi, Mg\(^{2+}\), and Ca\(^{2+}\) levels remained constant, as compared with the siRNA control group (Fig. 6A, B; \( ^{*} P < 0.01 \) vs siRNA controls).

Discussion

DMP1 Promotes Expression of TNAP, ENPP1, and ANK through Transcriptional Pathways

The functional differentiation of odontoblasts requires unique sets of genes to be turned on and off in a differentiation-specific manner. We observed that after gene silencing of DMP1, expression of DMP1, TNAP, ENPP1, and ANK was reduced. Narayanan et al.\(^{22}\) reported that DMP1 is primarily located in the nuclei, where it acts as a transcription factor for activation of osteoblast-specific genes and is responsible for transcription of matrix genes involved in the formation of mineralized tissue. On the basis of findings from overexpression studies, Narayanan et al.\(^{7}\) speculated that DMP1 may directly activate transcriptional pathways, leading to expression of TNAP in osteoblasts. In the nucleus, DMP1 is responsible for transcription of matrix genes involved in mineralized tissue formation. However, the mechanism by which DMP1 is taken up by osteoblasts is un-
known. TNAP, ENPP1, and ANK are well-characterized markers of osteoblast lineage mineralization, differentiation, and maturation. Previous findings, including those of our group, suggest that DMP1 may directly activate transcriptional pathways, leading to expression of TNAP, ANK, and ENPP1 in MC3T3-E1 cells, and that DMP1 may combine with an enhancer or transcription factor of TNAP, ANK, and ENPP1 in the nucleus of MC3T3-E1, thereby affecting expression of the three genes, which should be verified in further research.

**DMP1 May Affect TNAP Activity via Regulation of Zn\textsuperscript{2+} Transporters**

TNAP is a compact, dimeric enzyme with four metal-binding sites in or near the catalytic region of each subunit. The catalytic site of each subunit contains two Zn\textsuperscript{2+} ions and one Mg\textsuperscript{2+} ion, while a stabilizing Ca\textsuperscript{2+} ion is located 10 Å from the active site\textsuperscript{23}. Fujimoto et al.\textsuperscript{24} suggested that cooperative Zn\textsuperscript{2+} handling by zinc transporter 1, metallothionein, and zinc transporter 4 in cytoplasm is required for full activation of TNAP, demonstrating that the activation process of TNAP is elaborately controlled. Importantly, Zn\textsuperscript{2+} is remarkably abundant in matrix vesicles\textsuperscript{23}.

In our study, TNAP activity in MC3T3-E1 cells treated with βGP and ascorbic acid increased, reached a peak on day 9, and decreased thereafter. This finding demonstrates that βGP (a source of phosphate for mineralization) can promote TNAP activity. TNAP activity was lower in the DMP1 siRNA interference group than in the blank control and siRNA control groups. Notably, after transfection with siRNA, TNAP activity underwent a refractory period from day 3 through day 6 (Fig. 4), and the concentration of intravesicular Zn\textsuperscript{2+} decreased after transfection with siRNA (Fig. 6B). This finding suggests that DMP1 affects TNAP activity by regulating Zn\textsuperscript{2+} transporters, especially in the early stage of interference. Nishito et al.\textsuperscript{25} observed that zinc transporter 1 expression on the cell surface was elaborately controlled by intracellular zinc levels. DMP1 may regulate these transporters involved in Zn\textsuperscript{2+} uptake, likely in the matrix vesicles before mineralization formation. Therefore, DMP1 affects the activity of TNAP in two ways—by rapid ZnT regulation, in the initial stage, and by hysteresis transcriptional regulation, during the later stage.

**After Treatment of MC3T3-E1 Cells with siRNA, Extravesicular Low Pi Switches Off the Pi/Pi-TNAP-Positive Feedback Loop before a High Pi/Pi Ratio Is Reached**

The concentration of extracellular Pi is primarily regulated by the action of TNAP, ENPP1, and ANK. TNAP decreases Pi levels by cleaving Pi to generate P, resulting in a high Pi/Pi ratio; this promotes mineralization and TNAP activity, which is a positive feedback process (Fig. 7). ENPP1 and ANK assist with inhibiting calcification, TNAP activity, and the emergence of Pi/Pi-TNAP-positive feedback by increasing the concentration of extracellular Pi\textsuperscript{26}. Mice lacking ANK exhibit an arthritis-like phenotype characterized by ectopic calcifications in cartilage and joint tissues\textsuperscript{27}. Moreover, mice lacking ENPP1 exhibit mineralization in the arteries and kidneys and ectopic formation of cartilage in the joints and spine\textsuperscript{28}. A previous study showed that oral SBI-425, a selective TNAP inhibitor, inhibited development of arterial media calcification in mice, without affecting bone mineralization\textsuperscript{29}. Thus, lack of ENPP1 and ANK promotes mineralization, TNAP activity, and the emergence of Pi/Pi-TNAP-positive feedback. In other words, a high Pi/Pi ratio promotes positive feedback, whereas a low Pi/Pi ratio inhibits it. Reduced expression of ENPP1 and ANK decreases the production of P. Additionally, hydrolysis of Pi is reduced because of the decreased function of TNAP. Therefore, in the present study, Pi concentration was constant in the ECM (Fig. 6A). DMP1 affects the activity of TNAP in two ways—by rapid ZnT regulation, in the initial stage, and hysteresis transcriptional regulation, in the later stage. However, DMP1 may only affect expression of ENPP1 and ANK by hysteresis transcriptional regulation. Thus, the change in Pi/Pi ratio occurs earlier than that of Pi/Pi caused by ENPP1 and ANK. Low extravascular Pi switches off the Pi/Pi-TNAP-positive feedback after treatment of MC3T3-E1 cells with siRNA, before reaching a high Pi/Pi ratio, because of the lack of ENPP1 and ANK. This explains why, after transfection with siRNA, the activity of TNAP underwent a refractory period from day 3 through day 6 (Fig. 4).

In summary, DMP1 may regulate mineralization of MC3T3-E1 cells via the TNAP-ANK-ENPP1 axis, which results in unique sets of genes being turned on and off, low and high levels of ions, Pi/Pi-TNAP-positive feedback being switched on and off, self-phosphorylation, and conformational changes (Fig. 7). These results highlight a promising target for regulating HA crystal growth during mineralization and lay the foundation for further studies of potential therapeutic targeting of these proteins in the treatment of disorders of mineralization, such as AS and OA. An understanding of the regulatory mechanisms that control the physiological process of
Fig. 7 DMP1 regulated mineralization of MC3T3-E1 cells via the TNAP-ANK-ENPP1 axis. MV = matrix vesicle.

biomineralization is necessary to better understand skeletal disorders and guide the development of therapeutics for treating pathological calcification.

Author contributions: JML and JHZ conceived the research and designed the experimental work with support from LZ; JML and JHZ performed the experiments with support from ZL (optimization of methods), HMW (experiments), and BBW and WL (results analysis); JML and LZ wrote and revised the manuscript for the final version to be published. All authors agree to be accountable for all aspects of the work.

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Conflict of Interest: All authors declare that they have no competing interests.

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DMP1 Regulates MC3T3-E1 Cell Mineralization

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