Original Article

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Polyphosphate-induced matrix metalloproteinase-3-mediated differentiation in rat dental pulp fibroblast-like cells

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Summary **Inorganic polyphosphate [Poly(P)] induces differentiation of osteoblastic cells. In this study, matrix metalloproteinase (MMP)-3 small interfering RNA (siRNA) was transfected into purified rat dental pulp fibroblast-like cells (DPFCs) to investigate whether MMP-3 activity induced by Poly(P) is associated with cell differentiation into osteogenic cells. Real-time quantitative polymerase chain reaction, western blotting, and an MMP-3 activity assay were used in this study. Poly(P) enhanced expression of mature odontoblast markers dentin sialophosphoprotein (DSPP) and dentin matrix protein (DMP)-1 in DPFCs. These cells also developed an osteogenic phenotype with increased expression of osteocalcin (OC) and osteopontin (OP), high alkaline phosphatase (ALP) activity, and an increased calcification capacity. Poly(P) induced the expression of MMP-3 mRNA and protein, and increased MMP-3 activity. MMP-3 siRNA potently suppressed the expression of osteogenic biomarkers ALP, OC, OP, DSPP, and DMP-1, and blocked osteogenic calcification. Taken together, Poly(P)-induced MMP-3 regulates differentiation of osteogenic cells from DPFCs.**

Keywords: Dental pulp, osteogenic cells, dentin sialophosphoprotein, dentin matrix protein-1

1. Introduction

Inorganic polyphosphate [Poly(P)] is a linear polymer consisting of tens to hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds. In mammals, Poly(P) is found in erythrocytes and cells of the brain, heart, lung, and liver (*1-4*). The most researched and well-known role of Poly(P) is in the promotion of intracellular calcification (*5*). Because Poly(P) induces alkaline phosphate (ALP) activity and up-regulates osteopontin (OP) and osteocalcin (OC) gene expression (*6*), Poly(P) is thought to play an important role in the maturation of bone-related immature cells, and may be involved in the construction of bone tissue by osteoblasts.

In addition to blood vessels and nerves, fibroblasts are a significant component of dental pulp tissue (*7*) and thus might represent a novel therapeutic target for treating pulpitis. However, the effect of Poly(P) regulation of differentiation on dental pulp fibroblast-like cells (DPFCs) has not been well defined.

Matrix metalloproteinase (MMP)-3 and interstitial collagenase (MMP-1) are produced by fibroblasts in response to increased levels of inflammatory cytokines in dental pulp injury and diseases such as periodontitis and rheumatoid arthritis (*8,9*). MMP-3 has been implicated in the joint and soft-tissue destruction associated with these conditions, where it participates in the inflammatory response (*10-14*). MMP-3 synthesis is tightly controlled *in vivo* (*13,15*). Our previous study reported that proinflammatory cytokine-induced MMP-3 actually accelerates wound healing following dental pulp injury (*16-18*) and promotes cell proliferation (*17,19,20*).

Recently, roles of Poly(P) have been suggested in apoptosis and modulation of the mineralization process in bone tissue (*21,22*). We previously reported that Poly(P) induces MMP-3-mediated proliferation of odontoblast-like cells derived from induced pluripotent stem cells (*23*). Although we previously reported that

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Poly(P)-induced MMP-3-mediated proliferation of rat DPFCs is mediated by a Wnt5 signaling cascade (*24*), it is unknown whether Poly(P) is associated with differentiation. Therefore, we investigated the physiological effect of Poly(P) on DPFC differentiation *in vitro*. We reveal a novel role of Poly(P) in the regulation of MMP-3 activity during the differentiation of osteogenic cells from DPFCs.

2. Materials and Methods

2.1. *Materials*

Type-65 Poly(P) with an average chain length of 65 phosphate residues was prepared from sodium tripolyphosphate (Taihei Chemical Industrial Co., Ltd., Osaka, Japan). Concentrations of Poly(P) are shown in terms of phosphate residues (*25*). As a control, sodium phosphate buffer (pH 6.9) was used instead of Poly(P).

2.2. *Cell culture*

The study protocol (No. 63) was reviewed and approved by the Animal Experimentation Committee, School of Dentistry, Aichi Gakuin University, Japan. DPFCs were isolated from rat incisors and cultured using a previously described protocol (*20*). The proportion of platelet-derived growth factor receptor (PDGFR)-α positive cells in the total fibroblast-like cell population is a measure of the purity of DPFCs (*24*). Therefore, using flow cytometry, we evaluated the ratio of PDGFR-α-positive cells as a percentage of the total differentiated cells to determine the purity of the differentiated cell population. Our DPFC cultures showed 98.64 ± 5.2% homogeneity (% total, $n = 3$). In all experiments, DPFCs were used at passages 2-5. Cells were seeded into six-well tissue culture plates at a density of 1×10^5 cells/cm^2. To expose the cells to Poly(P), the culture medium was replaced with alpha-minimal essential medium containing 10% fetal bovine serum and Poly(P), followed by 7 days of culture.

2.3. *Functional assay for assessment of the osteogenic phenotype*

To assess the phenotype of the cultured cells, we measured ALP activity and calcification (as a marker of differentiation). ALP activity was determined using an ALP Staining Kit (Primary Cell Co., Ltd., Hokkaido, Japan). Mineralization from the Poly(P)-treated cells was quantified using an Alizarin red S (ARS) assay (Sigma-Aldrich, St. Louis, MO, USA). ARS staining was quantified using a previously reported method (*26*) and photographed using a BZ-9000 microscope (Keyence, Osaka, Japan) and/or an IN Cell Analyzer 2000 (GE Healthcare UK Ltd, Buckinghamshire, England).

2.4. *Real-time quantitative polymerase chain reaction (qRT-PCR) analysis*

qRT-PCR was performed for all samples and standards in triplicate with approximately 25 ng RNA, 0.25 mL Quantitect RT Mix (Qiagen Inc., Valencia, CA, USA), and 1.25 mL of 20× Primer/Probe Mix (rat ALP [*ALPL*]: Rn00575319_g1; rat OC [*BGLAP*]: Rn00566386_g1; rat OP [*SPP1*]: Rn01449972_m1; rat DSPP [*DSPP*]: Rn02132391_s1; rat DMP-1 [*DMP-1*]: Rn01450120_m1; rat *MMP-3*: Rn00591740_m1; human *MMP-1* (rat available): Hs00899658_m1; rat *MMP-2*: Rn01538170_m1; rat *MMP-9*: Rn00579162_m1; rat *MMP-13*: Rn01448194_m1). The standard curve method was used to determine the relative quantification of gene expression with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA as controls. Analysis was performed by the delta-delta Ct method.

2.5. *Western blot analysis*

Cells were cultured for 6 h with or without Poly(P) and then lysed using cell lysis buffer (Cell Signaling Technology Japan, K.K., Tokyo, Japan). Protein lysates were separated on SDS-polyacrylamide gels (12%) in preparation for western blot analysis using anti-ALP, -OC, -OP, -MMP-3, -DMP-1, and -β-tubulin polyclonal antibodies (sc-271431, sc-30044, sc-10593, sc-6839, sc-5538, sc-13595, sc-6840, sc-30073, and sc-9935, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Visualization of blotted protein bands was performed using a Multi Gauge-Ver3.X (Fujifilm, Tokyo, Japan).

2.6. *Measurement of MMP-3 activity*

The protocol for measurement of MMP-3 activity has been described previously (*27,28*) and is now a commercially available MMP-3 activity assay kit (SensoLyte™ 520 MMP-3 assay kit; AnaSpec, San Jose, CA, USA).

2.7. *Silencing of the MMP-3 gene by small interfering RNA (siRNA) transfection*

Commercially available MMP-3 siRNA (sc-61874, Santa Cruz Biotechnology, Inc.) was transfected into cultured cells using an siRNA reagent system (sc-45064, Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocol. GAPDH siRNA and a control siRNA without known homology to any vertebrate sequence (Thermo Scientific, Lafayette, CO, USA) were used as positive and negative controls, respectively.

2.8. *Statistical analysis*

Data presented in bar graphs are the means ± standard

deviation (S.D.) of four to six independent experiments. Statistical significance was assessed using the Mann-Whitney *U*-test. A *p*-value of < 0.05 was considered statistically significant.

3. Results

3.1. *Poly(P) induces osteogenic differentiation of DPFCs*

We previously analyzed the effect of Poly(P) on cell proliferation of DPFCs, and found that 0.1 mM Poly(P) is an optimal concentration to enhance the cell growth, whereas > 0.2 mM Poly(P) results in potent inhibition of cell proliferation (*24*).

To examine whether Poly(P) induced osteogenic characteristics in DPFCs, the cells were cultured in the presence of 0.1 mM Poly(P) for 7 days. Both qRT-PCR and western blotting revealed higher expression of osteogenic differentiation markers *ALPL*(ALP), *BGLAP*(OC), *SPP1*(OP), *DSPP*, and *DMP-1* (Figure 1A-a, b).

The majority of Poly(P)-treated DPFCs showed strong ALP expression, whereas control cells had undetectable ALP expression (Figure 1B-b). Extensive deposits of calcified matrix were observed in Poly(P)-treated DPFC cultures, whereas calcified matrix was not apparent in control cell cultures (Figure 1C-b). Consistently, Poly(P) treatment induced a marked increase in ARS signals (Figure 1C-a, b). Taken together, Poly(P) induced osteogenic cells from DPFCs.

3.2. *Poly(P) induces expression of MMP-3 mRNA and MMP-3 activity in DPFCs*

MMP-3 induction by 0.1 mM Poly(P) was assessed using qRT-PCR to measure changes in MMP-3 mRNA expression. The levels of MMP-3 mRNA expression in Poly(P)-treated cells were significantly increased ($p < 0.05$) at days 3, 5, and 7 of culture (Figure 2A). Furthermore, MMP-3 activity was significantly increased ($p < 0.01$) at days 3, 5, and 7 following treatment of DPFCs with Poly(P) for 24 h (Figure 2C).

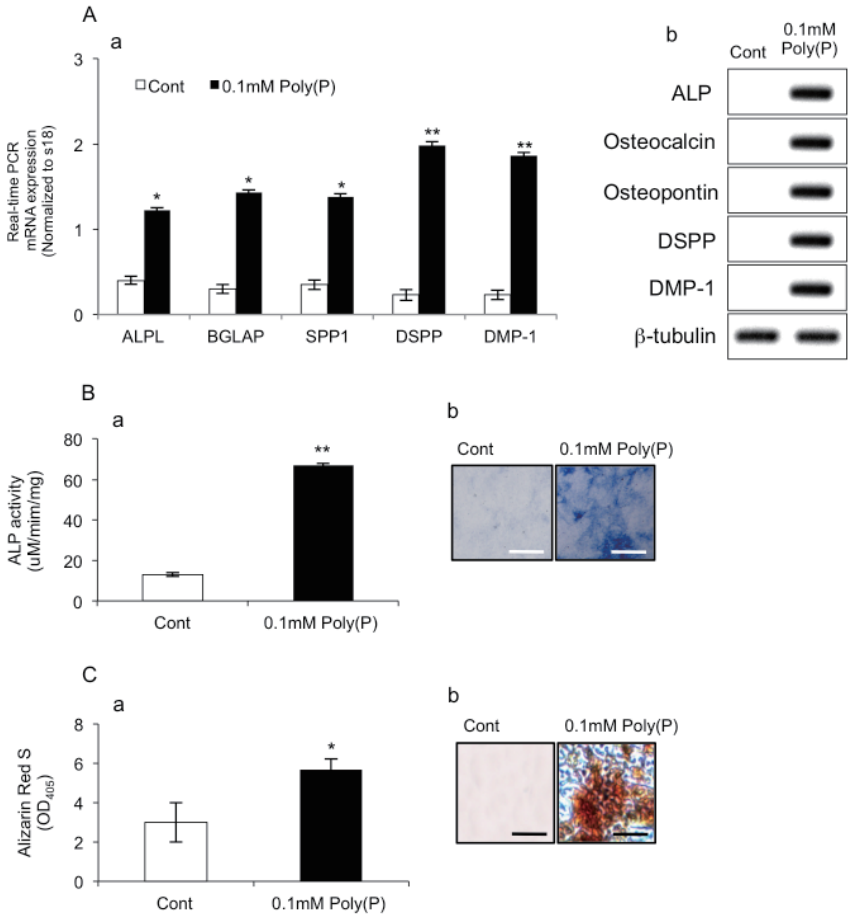

Figure 1. Expression of differentiation markers during osteogenic differentiation induced by Poly(P). (A-a) DPFCs were treated with Poly(P for 7 days. Expression of osteogenic differentiation markers was assessed by qRT-PCR, including ALPL, BGLAP, SPP1, DSPP, and DMP-1. (*$p < 0.05$, **$p < 0.01$ *vs*. control). Data are presented as means ± S.D. and are representative of at least three independent experiments. Similar changes in the protein expression levels of these markers were observed in western blot analyses **(A-b)**. ALP activity was measured in DPFCs treated with or without Poly(P). **(B-a,b)** ALP activity was measured by absorbance at 405 nm and normalized against total protein (**$p < 0.01$ *vs*. control). Scale bar: 100 μm. **(C-a,b)** ARS staining of DPFCs treated with Poly(P) (*$p < 0.05$ *vs*. control). Scale bar: 100 μm.

Bone-associated cells also express other MMP proteins including MMP-1, MMP-2, MMP-9, and MMP-13 (*29-31*). However, we found no significant changes in their expression levels in DPFCs treated with 0.1 mM Poly(P) (Figure 2B).

MMP-3 activity is precisely regulated at the post-translational level as a precursor zymogen and by endogenous tissue inhibitors of metalloproteinases (TIMPs) (*32*). Although TIMP-2 and TIMP-3 are known to be induced by cytokines (*32*), we found that TIMP-1, TIMP-2, and TIMP-3 proteins were constitutively expressed in all experimental conditions (data not shown).

3.3. *siRNA silencing of MMP-3 blocks osteogenic differentiation*

To examine whether the up-regulation of MMP-3 expression was associated with osteogenic differentiation, DPFCs were transfected with MMP-3 siRNA or a control scrambled siRNA, and then treated with Poly(P) as described above. Transfection of MMP-3 siRNA abrogated the induction of osteogenic differentiation markers ALPL, BGLAP, SPP1, DSPP, and DMP-1 ($p < 0.05$, Figure 3A). Similar changes in the protein levels of each marker were observed in western blot analyses (Figure 3B). Furthermore, MMP-3 siRNA blocked

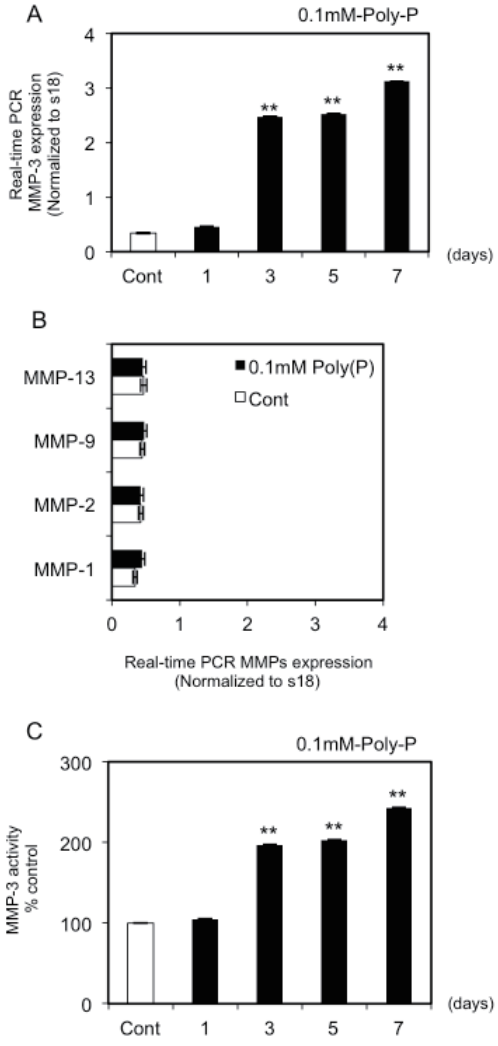

Figure 2. Evaluation of Poly(P)-induced MMP-3 mRNA expression and MMP-3 activity in DPFCs. (A) qRT-PCR analysis of Poly(P)-induced MMP-3 mRNA in DPFCs at 24 h. **(B)** Expression of other MMP mRNAs and proteins in DPFCs. DPFCs were treated with 0.1 mM Poly(P) prior to qRT-PCR analysis of MMP-1, MMP-2, MMP-9, and MMP-13 mRNA expression compared with the control (18S rRNA). Data are the means ± S.D. of four independent experiments. **(C)** Measurement of active MMP-3 released from cultured DPFCs following treatment with 0.1 mM Poly(P). Cells were incubated in serum-free medium in the absence or presence of 0.1 mM Poly(P) for 24 h. Data are the means ± S.D. of at least three independent experiments (**$p < 0.01$).

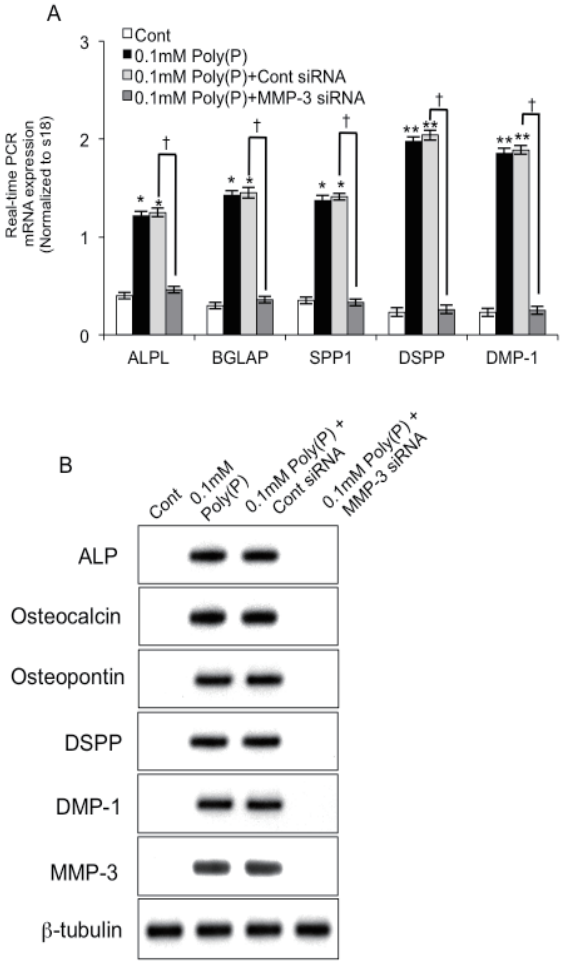

Figure 3. Effect of siRNA silencing on induction of osteogenic markers. (A) The expression of osteogenic marker mRNAs (ALPL, BGLAP, SPP1, DSPP, and DMP-1) in Poly(P)-treated DPFCs was assessed by qRT-PCR following culture in the presence of MMP-3 siRNA. **(B)** Western blot analysis of osteogenic marker protein expression in these cells at 24 h after siRNA transfection. Poly(P)-treated DPFCs were treated with MMP-3 siRNA, and then expression of ALP, OC, OP, DSPP, DMP-1, and MMP-3 proteins was measured. No significant cross-reactivity with other proteins was observed for the antibodies used in the analyses. Images are representative of at least three independent experiments.

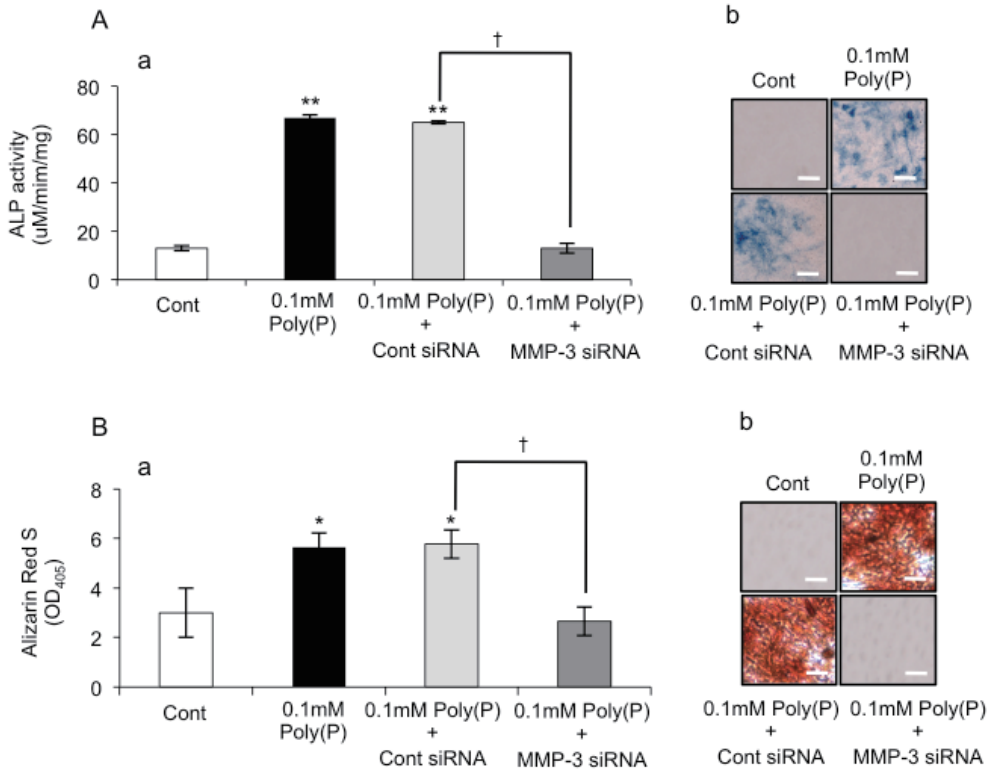

Figure 4. siRNA silencing of MMP-3 blocks osteogenic differentiation. (A-a,b) Effect of MMP-3 siRNA on the functional activities of DPFCs. ALP activity was measured in control and MMP-3-depleted DPFCs treated with 0.1 mM Poly(P). Data are presented as the means ± S.D. ($n = 4$) normalized against total protein, (**$p < 0.01$ *vs*. control; †$p < 0.05$ as indicated). Scale bar: 100 μm. **(B-a,b)** Effect of MMP-3 siRNA on the mineralization capacity of DPCs. Cells were prepared and cell mineralization was assessed by ARS staining with quantification performed by measuring absorbance at 405 nm. Data are the means ± S.D. ($n = 4$). *$p < 0.05$ *vs*. control; †$p < 0.05$ as indicated. Scale bar: 100 μm.

induction of ALP activity in Poly(P)-treated cells ($p < 0.01$, Figure 4A-a, b). Similarly, the induction of calcification was markedly suppressed ($p < 0.05$) by Poly(P) treatment of MMP-3-depleted cells (Figure 4B-a, b). Collectively, these data show that expression of MMP-3 is required for osteogenic-specific functions in DPFCs.

4. Discussion

This study indicated that Poly(P)-treated DPFCs can be a novel *in vitro* model of dental pulp regeneration. Poly(P) at a concentration of 0.1 mM induced MMP-3 expression in DPFCs (Figure 2A) and led to enhanced DPFC differentiation into osteogenic cells (Figure 1A–C), although we were unable to precisely determine how many DPFCs had differentiated. However, phenotypic characterization based on calcification and the levels of ALPL (ALP), BGLAP (OC), SPP1 (OP), DSPP, and DMP-1 suggested that a large proportion of the DPFC population differentiated into osteogenic cells (Figure 1A-a, b).

We have previously demonstrated that the inflammatory cytokine interleukin-1β or a cytokine mixture induces MMP-3-regulated cell proliferation and suppresses apoptosis in rat DPFCs (*19,20*). Moreover, we previously reported that Poly(P)-induced, MMP-3-mediated proliferation in rat DPFCs is mediated by a Wnt5 signaling cascade (*24*). Because we had no definite data on Poly(P)-induced MMP-3 in terms of IGF-1/PI3K/Akt and MAPK signaling pathways (*33,34*) in osteogenic differentiation, it remains to be shown. This study is the first report of Poly(P)-induced, MMP-3-mediated responses in the differentiation of DPFCs. Considering the effect of MMP-3 on osteogenic cell differentiation, the present findings suggest that targeting the *MMP-3* gene in these osteogenic cells may have a utility in the treatment of suppurative pulpitis. Additionally, Poly(P)-treated DPFCs could serve as an effective model to explore the pathophysiological mechanisms of wound healing. Furthermore, our current evidence suggests that Poly(P)-induced MMP-3 has previously unrecognized physiological functions in wound healing and dental pulp tissue regeneration.

We showed that Poly(P)-induced cells acquired osteogenic-specific functions following differentiation from DPFCs. Poly(P)-treated DPFCs appeared to be predominantly odontoblasts and osteoblasts. A major concern is that we were unable to identify the differentiated cells as odontoblasts because these cells also expressed specific osteoblastic markers including OC and OP. We speculate that DPFCs contain a small population of dental pulp stem cells. Therefore,

osteogenic cells may be predominantly derived from dental pulp stem cells, which remains to be elucidated.

The findings presented here support our previous reports (*19,20*) and indicate that MMP-3 may have a previously unrecognized physiological function in wound healing and tissue regeneration. Because Poly(P) induces MMP-3-regulated DPFC differentiation into osteogenic cells, the use of Poly(P) represents a potentially superior therapeutic approach for treatment of dental pulp injury instead of applying pulp-capping materials.

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