Original Article

DOI: 10.5582/bst.2016.01153

Polyphosphate-induced matrix metalloproteinase-13 is required for osteoblast-like cell differentiation in human adipose tissue derived mesenchymal stem cells

Nobuaki Ozeki[1,*], Makio Mogi[2], Naoko Hase[1], Taiki Hiyama[1], Hideyuki Yamaguchi[1], Rie Kawai[1], Kazuhiko Nakata[1]

[1] *Department of Endodontics, School of Dentistry, Aichi Gakuin University, Nagoya, Aichi, Japan;*
[2] *Department of Integrative Education of Pharmacy, School of Pharmacy, Aichi Gakuin University, Nagoya, Aichi, Japan.*

Summary **Inorganic polyphosphate [Poly(P)] induces differentiation of osteoblastic cells. In this study, matrix metalloproteinase (MMP)-13 small interfering RNA (siRNA) was transfected into human adipose tissue-derived mesenchymal stem cells (hAT-MSC) to investigate whether MMP-13 activity induced by Poly(P) is associated with osteogenic differentiation. Real-time quantitative polymerase chain reaction, Western blotting, and an MMP-13 activity assay were used in this study. Poly(P) enhanced expression of mature osteoblast markers, such as osteocalcin (BGLAP) and osteopontin (SPP1), osterix (OSX), and bone sialoprotein (BSP), and increased alkaline phosphatase (ALP) activity and calcification capacity in hAT-MSCs. These cells also developed an osteogenic phenotype with increased expression of Poly(P)-induced expression of MMP-13 mRNA and protein, and increased MMP-13 activity. MMP-13 siRNA potently suppressed the expression of osteogenic biomarkers BGLAP, SPP1, OSX, BSP, and ALP, and blocked osteogenic calcification. Taken together, Poly(P)-induced MMP-13 regulates differentiation of osteogenic cells from hAT-MSCs.**

Keywords: Adipose tissue, mesenchymal stem cells, osteogenic cells, matrix metalloproteinase-13

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, lungs, 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 osteocalcin (BGLAP) and osteopontin (SPP1) 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 treatment of pulpitis. We previously reported Poly(P) regulation of differentiation and proliferation in dental pulp fibroblast-like cells (DPFCs) and odontoblast-like cells (*8-10*). Recently, roles of Poly(P) have been suggested in apoptosis and modulation of the mineralization process in bone tissue (*11,12*).

Matrix metalloproteinases (MMPs) play central roles in cell proliferation, migration, differentiation, angiogenesis, apoptosis, and host defenses. Deregulation of MMPs has been implicated in many diseases including rheumatoid arthritis (RA), chronic ulcers, encephalomyelitis, and cancer (*13-15*). MMP-13 is highly overexpressed in pathological situations such as carcinomas, RA, and osteoarthritis (OA). Furthermore, MMP-13 may be involved in articular cartilage turnover and cartilage pathophysiology associated with OA. We previously reported that MMP-13 accelerates bone remodeling following development

Released online in J-STAGE as advance publication October 24, 2016.

**Address correspondence to:*
Dr. Nobuaki Ozeki, Department of Endodontics, School of Dentistry, Aichi Gakuin University, 2-11 Suemori-dori, Chikusa-ku, Nagoya, Aichi 464-8651, Japan.
E-mail: ozeki@g.ag.ac.jp

of periradicular lesions (*16,17*), and presented evidence suggesting that MMP-13 plays a potentially unique physiological role in wound healing and regeneration of alveolar bone. Because alveolar bone tissue consists predominantly of osteoblasts, these cells may represent a potential target cell type for new therapeutic strategies to mitigate these disease states. Moreover, we have reported that the proinflammatory cytokine interleukin (IL)-1 induces MMP-13 activity in purified osteoblast-like cells derived from human stem cells (*16,17*).

Mesenchymal stem cells (MSCs) are multipotent cells in bone marrow and various other tissues, which are capable of differentiating into osteogenic cells, especially osteoblasts, as well as chondrogenic and adipogenic lineages by culturing in appropriate *in vitro* conditions (*18*). MSCs have been identified in adipose tissue, which are termed adipose tissue-derived MSCs (AT-MSCs) (*19-21*). Compared with bone marrow-derived MSCs (BMSCs), human AT-MSCs are easier to obtain, have relatively lower donor site morbidity, a higher yield at harvest, and can expand more rapidly *in vitro* than BMSCs (*22,23*). Moreover, the proliferation and differentiation potential of AT-MSCs are independent of age (*24,25*). Under appropriate conditions, AT-MSCs can be induced into an osteogenic lineage *in vitro* and can therefore be seeded in proper scaffolds as seed cells to repair bone defects. These advantages suggest that human AT-MSCs are a promising alternative source of seed cells for tissue engineering and regeneration.

We recently established a differentiation method for homogeneous α7 integrin-positive human skeletal muscle stem cell-derived osteoblast-like cells (*26*), and found that IL-1β induces MMP-13-regulated proliferation of these cells (*16,17*). These data suggest that MMP-13 plays a potentially unique physiological role in the regeneration of osteoblast-like cells. We previously reported that Poly(P) induces MMP-3-mediated proliferation of odontoblast-like cells derived from mouse induced pluripotent stem cells (*9*). Although it is known that Poly(P) induces differentiation of osteoblastic cells, the detailed mechanism of Poly(P)-induced differentiation in AT-MSCs remains to be elucidated.

In the current study, we employed purified osteoblast-like cells derived from human AT-MSCs (hAT-MSCs) as an appropriate cell model to examine the mechanism of Poly(P)-induced differentiation *in vitro*. We show, for the first time, that Poly(P)-induced MMP-13 regulates differentiation of osteoblast-like cells from hAT-MSCs.

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 (*27*). As a control, sodium phosphate buffer (pH 6.9) was used instead of Poly(P).

2.2. *Cell culture*

hAT-MSCs were obtained from Takara Bio Inc. (Shiga, Japan) and grown in growth medium (C-28010; Takara Bio Inc.) according to the manufacturer's protocols. hAT-MSCs were tested for cell morphology, proliferation potential, adherence rate, and viability. Furthermore, they were characterized by flow cytometric analysis of a comprehensive panel of markers, namely CD73, CD90, CD105, CD14, CD19, CD34, and CD45 (*28*). Adipogenic, osteogenic and chondrogenic differentiation assays were performed under each appropriate culture condition without antibiotics or antimycotics. Passage 6-9 cells were used in experiments. At 70% confluence, the medium was replaced with osteogenic differentiation medium (ODM) (C-28013; Takara Bio Inc.) or growth medium every 3 days up to 21 days. The first day the medium was replaced with osteogenic culture medium was defined as day 0.

2.3. *Cell proliferation assay and microscopic analysis*

Cell proliferation was evaluated using a bromodeoxyuridine (BrdU)-cell proliferation enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany) as described previously (*29,30*). In addition, cell proliferation was evaluated visually under a BZ-9000 microscope (Keyence, Osaka, Japan) using a BrdU immunohistochemistry kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

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

To assess the phenotype of cultured cells, we measured ALP activity and calcification as markers 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 (*31*) and photographed under the BZ-9000 microscope and/or an IN Cell Analyzer 2000 (GE Healthcare UK Ltd., Buckinghamshire, England).

2.5. *Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis*

qRT-PCR was performed on 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 (human alkaline phosphatase[ALP; *ALPL*]: Hs01029144_m1; human osteocalcin [OC; *BGLAP*]: Hs00609452_g1; human osteopontin [OP; *SPP1*]: Hs00959010_m1; human osterix [*OSX*]: Hs00931793_m1; human bone sialoprotein [*BSP*]: Hs00173720_m1; human *MMP-1*: Hs00899658_m1; human *MMP-2*: Hs01548727_m1; human *MMP-3*: Hs00968305_m1; human MMP-9: Hs00234579_m1; human *MMP-13*: Hs0023392_m1). The standard curve method was used for 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.

2.6. *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). Proteins were separated on 12% SDS-polyacrylamide gels for Western blot analysis using anti-ALP, -OC, -OP, -OSX, -BSP, -MMP-13 and -β-tubulin polyclonal antibodies (sc-271431, sc-30044, sc-10593, sc-22538, sc-73634, 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).

2.7. *Measurement of MMP-13 activity*

The protocol for measurement of MMP-13 activity has been described previously (*32,33*) and is now a commercially available MMP-13 activity assay kit (SensoLyte™ 520 MMP-13 assay kit; AnaSpec, San Jose, CA, USA).

2.8. *Silencing of the MMP-13 gene by small interfering RNA (siRNA) transfection*

Commercially available MMP-13 siRNA (sc-41559, 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.

2.9. *Statistical analysis*

Data presented in bar graphs are the means ± standard deviation (SD) of four to six independent experiments. Statistical significance was assessed using the Mann-Whitney *U*-test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. *Poly(P) alters hAT-MSC proliferation*

We first analyzed the effect of Poly(P) on the cell proliferation of hAT-MSCs using the BrdU-cell proliferation ELISA. As a result, we found that Poly(P) increased cell proliferation in a dose-independent manner. Poly(P) at a concentration of 0.2 mM was optimal to enhance cell growth ($p < 0.05$) (Figure 1). Both 0.1 and 0.5 mM Poly(P) resulted in potent inhibition of cell proliferation.

3.2. *Poly(P) induces osteogenic differentiation of hAT-MSCs*

We previously analyzed the effect of Poly(P) on cell proliferation of rat DPFCs, and found that 0.2 mM Poly(P) is an optimal concentration to enhance the cell growth, whereas more or less than 0.2 mM Poly(P) results in potent inhibition of cell proliferation (*10*).

To examine whether Poly(P) induced osteogenic characteristics in hAT-MSCs, the cells were cultured in the presence of 0.2 mM Poly(P) for 7 days. Both qRT-PCR and Western blotting revealed higher expression of osteogenic differentiation markers *ALPL*, *BGLAP*, *SPP1*, *OSX*, and *BSP* (Figure 2A-a, b).

The majority of Poly(P)-treated hAT-MSCs showed strong ALP expression, whereas control cells had undetectable ALP expression (Figure 2B-a, b). Extensive deposits of calcified matrix were observed in Poly(P)-treated hAT-MSC cultures, whereas calcified matrix was not apparent in control cell cultures (Figure 2C-a, b). Consistently, Poly(P) treatment induced a marked increase in ARS signals (Figure 2C-a, b). Taken

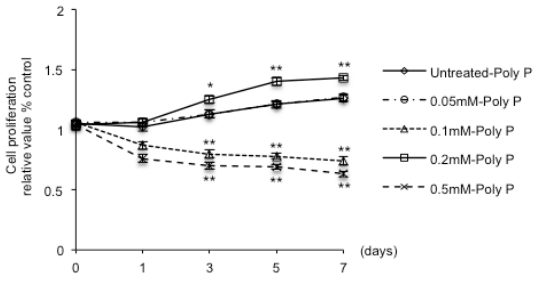

Figure 1. Optimization of Poly(P)-induced proliferation of hAT-MSCs. A BrdU-cell proliferation ELISA was employed to evaluate the proliferation of Poly(P)-treated cells and untreated (control) cells for up to 7 days. Cells were cultured in the absence or presence of the indicated concentrations of Poly(P) in triplicate wells. Data are the means ± SD. Differences between control and Poly(P)-treated groups were assessed by the Mann-Whitney U-test. *$p < 0.05$ and **$p < 0.01$ *vs*. control.

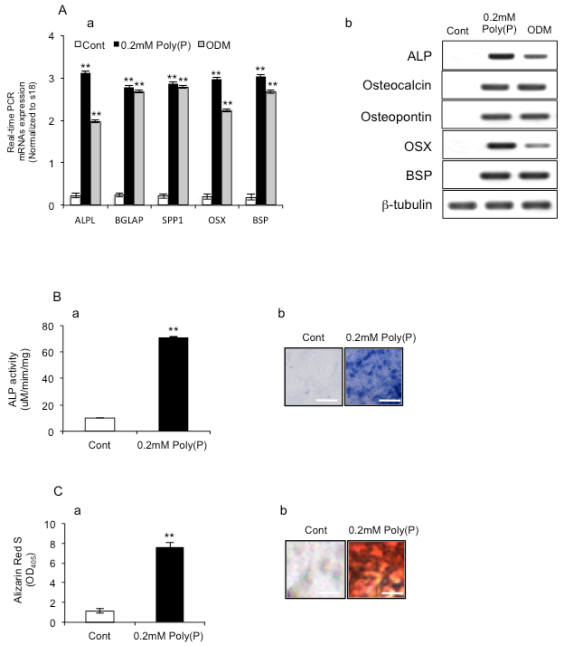

Figure 2. Expression of differentiation markers during osteogenic differentiation induced by Poly(P). (A-a) hAT-MSCs were treated with Poly(P) for 7 days. Expression of osteogenic differentiation markers was assessed by qRT-PCR, including *ALPL*, *BGLAP*, *SPP1*, *OSX*, and *BSP* (**p < 0.01 *vs*. control). Data are presented as the means ± SD 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)**. ODM: osteogenic differentiation medium. ALP activity was measured in hAT-MSCs 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: 100μm. **(C-a, b)** ARS staining of hAT-MSCs treated with Poly(P) (**p < 0.05 *vs*. control). Scale bar: 100 μm.

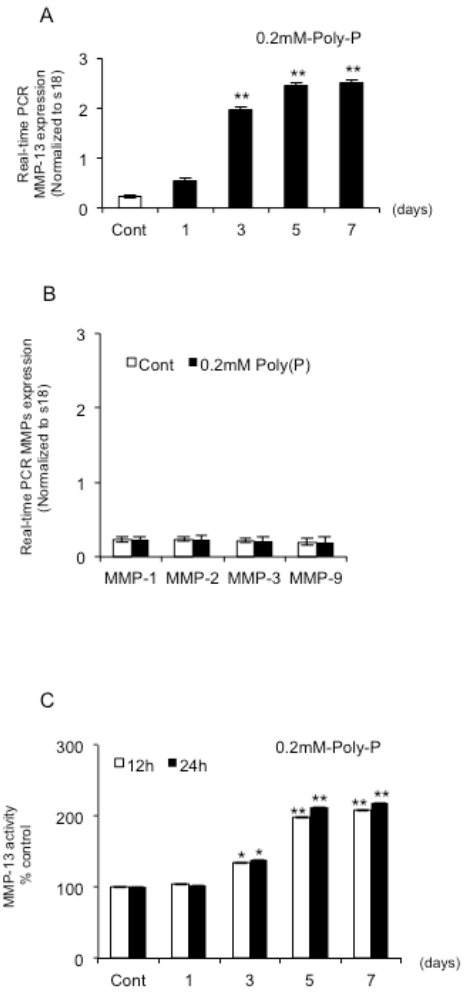

Figure 3. Evaluation of Poly(P)-induced MMP-13 mRNA expression and MMP-13 activity in hAT-MSCs. (A) qRT-PCR analysis of Poly(P)-induced MMP-13 mRNA in hAT-MSCs at 24 h. **(B)** Expression of other MMP mRNAs and proteins in hAT-MSCs. hAT-MSCs were treated with 0.2 mM Poly(P) prior to qRT-PCR analysis of MMP-1, MMP-2, MMP-3, and MMP-9 mRNA expression compared with the control (18S rRNA). Data are the means ± SD of four independent experiments. **(C)** Measurement of active MMP-13 released from cultured hAT-MSCs following treatment with 0.2 mM Poly(P). Cells were incubated in serum-free medium in the absence or presence of 0.2 mM Poly(P) for 12 or 24 h. Data are the means ± SD of at least three independent experiments (*p < 0.05; **p < 0.01).

together, Poly(P) induced osteoblast-like cells from hAT-MSCs.

3.3. *Poly(P) induces expression of MMP-13 mRNA and activity of MMP-13 in hAT-MSCs*

MMP-13 induction by 0.2 mM Poly(P) was assessed using qRT-PCR to measure changes in MMP-13 mRNA expression. The levels of MMP-13 mRNA expression in Poly(P)-treated cells were significantly increased (p < 0.05) at days 3, 5, and 7 of culture (Figure 3A). Bone-associated cells also express other MMPs including MMP-1, MMP-2, MMP-3, and MMP-9 (*34-36*). However, we found no significant changes in their expression levels in hAT-MSCs treated with 0.2 mM Poly(P) (Figure 3B). Furthermore, MMP-13 activity was significantly increased (p < 0.01) at days 3, 5, and 7 following treatment of hAT-MSCs with Poly(P) for 24 h (Figure 3C), and no significant differences were found between Poly(P) treatments for 12 or 24 h.

MMP-13 activity is precisely regulated at the post-translational level as a precursor zymogen and by endogenous tissue inhibitors of metalloproteinases (TIMPs) (*37*). Although TIMP-2 and TIMP-3 are known to be induced by cytokines (*37*), we found that TIMP-1–3 proteins were constitutively expressed in all experimental conditions (data not shown).

3.4. *siRNA silencing of MMP-13 blocks osteogenic differentiation*

To examine whether the up-regulation of MMP-13 expression was associated with osteogenic differentiation, hAT-MSCs were transfected with MMP-13 siRNA or a control scrambled siRNA, and then treated with Poly(P) as described above. Transfection of MMP-13 siRNA abrogated the induction of osteogenic

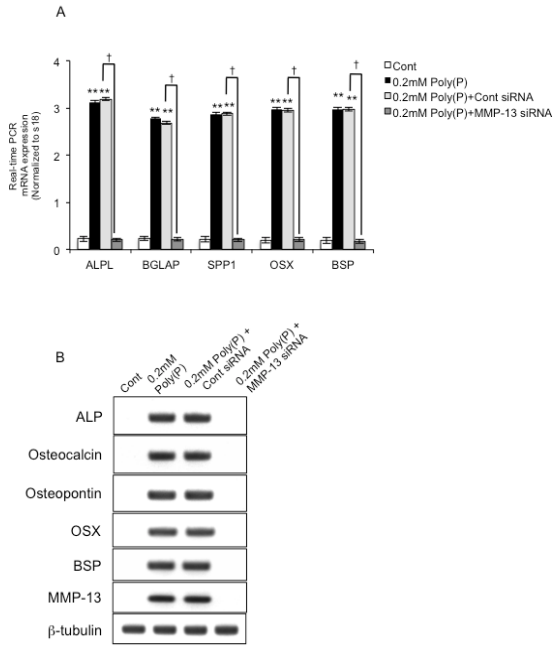

Figure 4. Effect of siRNA silencing on induction of osteogenic markers. (A) The expression of osteogenic marker mRNAs (*ALPL*, *BGLAP*, *SPP1*, *OSX*, and *BSP*) in Poly(P)-treated hAT-MSCs was assessed by qRT-PCR following culture in the presence of MMP-13 siRNA. Data are the means ± SD ($n = 4$). $^{**}p < 0.01$ *vs*. control; $^{\dagger}p < 0.01$ as indicated. **(B)** Western blot analysis of osteogenic marker protein expression in these cells at 24 h after siRNA transfection. Poly(P)-treated hAT-MSCs were treated with MMP-13 siRNA, and then expression of ALP, OC, OP, OSX, BSP, and MMP-13 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.

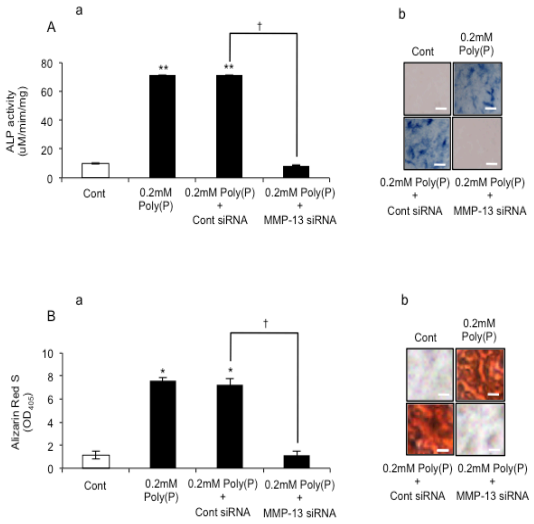

Figure 5. siRNA silencing of MMP-13 blocks osteogenic differentiation. (A-a, b) Effect of MMP-3 siRNA on the functional activities of hAT-MSCs. ALP activity was measured in control and MMP-13-depleted hAT-MSCs treated with 0.2 mM Poly(P). Data are presented as the means ± SD ($n = 4$) normalized against total protein, ($^{**}p < 0.01$ *vs*. control; $^{\dagger}p < 0.05$ as indicated). Scale bar: 100 μm. **(B-a, b)** Effect of MMP-13 siRNA on the mineralization capacity of hAT-MSCs. Cells were prepared and mineralization was assessed by ARS staining with quantification performed by measuring absorbance at 405 nm. Data are the means ± SD ($n = 4$). $^{*}p < 0.05$ *vs*. control; $^{\dagger}p < 0.05$ as indicated. Scale bar: 100 μm.

differentiation markers *ALPL*, *BGLAP*, *SPP1*, *OSX*, and *BSP* ($p < 0.05$, Figure 4A). Similar changes in the protein levels of each marker were observed in Western blot analyses (Figure 4B). Furthermore, MMP-13 siRNA blocked induction of ALP activity in Poly(P)-treated cells ($p < 0.01$, Figure 5A-a, b). Similarly, the induction of calcification was markedly suppressed ($p < 0.05$) by Poly(P) treatment of MMP-13-depleted cells (Figure 5B-a, b). Collectively, these data show that expression of MMP-13 is required for osteoblast-specific functions in hAT-MSCs.

4. Discussion

First, this study is the first report of Poly(P)-induced, MMP-13-mediated responses in the differentiation of hAT-MSCs. We demonstrated that Poly(P)-treated hAT-MSCs could be a novel *in vitro* model of bone tissue regeneration. Poly(P) at a concentration of 0.2 mM induced MMP-13 expression in hAT-MSCs (Figure 2A) and led to enhanced hAT-MSC differentiation into osteoblast-like cells (Figure 2A-C), although we were unable to precisely determine how many hAT-MSCs had differentiated. However, phenotypic characterization based on calcification and the levels of *ALPL*, *BGLAP*, *SPP1*, *OSX*, and *BSP* suggested that a large proportion of the hAT-MSCs had differentiated into osteoblast-like cells (Figure 2A-a, b).

We have previously demonstrated that the inflammatory cytokine IL-1β or a cytokine mixture induces MMP-3-regulated cell proliferation and suppresses apoptosis in rat DPFCs (*38,39*). Moreover, we previously reported that Poly(P)-induced, MMP-3-mediated proliferation in rat DPFCs is mediated by a Wnt5 signaling cascade (*10*). Considering the effect of MMP-13 on osteogenic cell differentiation, the present findings suggest that targeting the *MMP-13* gene in these osteogenic cells may have utility in the treatment of periradicular lesions. Additionally, Poly(P)-treated hAT-MSCs could serve as an effective model to explore the pathophysiological mechanisms of wound healing. Furthermore, our current evidence suggests that Poly(P)-induced MMP-13 has previously unrecognized physiological functions in wound healing and bone tissue regeneration.

We showed that Poly(P)-induced cells acquired osteoblast-specific functions following differentiation from hAT-MSCs. Poly(P)-treated hAT-MSCs appeared to be predominantly osteoblasts. A major concern is that we were unable to identify the differentiated cells as osteoblasts because these cells also expressed specific osteoblastic markers including osteocalcin and osteopontin.

In conclusion, the current findings presented here support our previous reports (38,39) and indicate that MMP-13 may have a previously unrecognized physiological function in wound healing and bone tissue regeneration. Because Poly(P) induces MMP-13-regulated hAT-MSC differentiation into osteoblast-like cells, the use of Poly(P) represents a potentially superior therapeutic approach for treatment of periradicular lesions combined with root canal treatment.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (A) (Grant No. 25253101 to SY), a Grant-in-Aid-for Scientific Research (C) (Grant No. 26462905 to KN), a Grant-in-Aid-for Scientific Research (C) (Grant No. 26462904 to NO), and a Grant-in-Aid-for Young Scientists (B) (Grant No. 15K20418 to NH) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Kornberg A, Rao NN, Ault-Riche D. Inorganic polyphosphate: A molecule of many functions. Annu Rev Biochem. 1999; 68:89-125.
2 Kumble KD, Kornberg A. Inorganic polyphosphate in mammalian cells and tissues. J Biol Chem. 1995; 270:5818-5822.
3 Leyhausen G, Lorenz B, Zhu H, Geurtsen W, Bohnensack R, Muller W E, Schroder HC. Inorganic polyphosphate in human osteoblast-like cells. J Bone Miner Res. 1998; 13:803-812.
4 Schroder HC, Kurz L, Muller WE, Lorenz B. Polyphosphate in bone. Biochemistry (Mosc). 2000; 65:296-303.
5 Shiba T, Nishimura D, Kawazoe Y, Onodera Y, Tsutsumi K, Nakamura R, Ohshiro M. Modulation of mitogenic activity of fibroblast growth factors by inorganic polyphosphate. J Biol Chem. 2003; 278:26788-26792.
6 Kawazoe Y, Shiba T, Nakamura R, Mizuno A, Tsutsumi K, Uematsu T, Yamaoka M, Shindoh M, Kohgo T. Induction of calcification in MC3T3-E1 cells by inorganic polyphosphate. J Dent Res. 2004; 83:613-618.
7 Teles JC. Study on the dentin-pulp complex. Rev Bras Odontol. 1967; 25:305-310.
8 Hiyama T, Ozeki N, Hase N, Yamaguchi H, Kawai R, Kondo A, Mogi M, Nakata K. Polyphosphate-induced matrix metalloproteinase-3-mediated differentiation in rat dental pulp fibroblast-like cells. Biosci Trends. 2016; 9:360-366.
9 Ozeki N, Hase N, Yamaguchi H, Hiyama T, Kawai R, Kondo A, Nakata K, Mogi M. Polyphosphate induces matrix metalloproteinase-3-mediated proliferation of odontoblast-like cells derived from induced pluripotent stem cells. Exp Cell Res. 2015; 333:303-315.
10 Ozeki N, Yamaguchi H, Hase N, Hiyama T, Kawai R, Kondo A, Nakata K, Mogi M. Polyphosphate-induced matrix metalloproteinase-3-mediated proliferation in rat dental pulp fibroblast-like cells is mediated by a Wnt5 signaling cascade. Biosci Trends. 2015; 9:160-168.
11 Kawano MM. Inorganic polyphosphate induces apoptosis specifically in human plasma cells. Haematologica. 2006; 91:1154A.
12 Orriss IR, Key ML, Brandao-Burch A, Patel JJ, Burnstock G, Arnett TR. The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of p2x receptors. Bone. 2012; 51:389-400.
13 Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J Biol Chem. 2005; 280:41342-41351.
14 Paula-Silva FW, da Silva LA, Kapila YL. Matrix metalloproteinase expression in teeth with apical periodontitis is differentially modulated by the modality of root canal treatment. J Endod. 2010; 36:231-237.
15 Tseng WY, Huang YS, Chiang NY, Chou YP, Wu YJ, Luo SF, Kuo CF, Lin KM, Lin HH. Increased soluble CD4 in serum of rheumatoid arthritis patients is generated by matrix metalloproteinase (MMP)-like proteinases. PLoS One. 2013; 8:e63963.
16 Ozeki N, Kawai R, Yamaguchi H, Hiyama T, Kinoshita K, Hase N, Nakata K, Kondo A, Mogi M, Nakamura H. IL-1beta-induced matrix metalloproteinase-13 is activated by a disintegrin and metalloprotease-28-regulated proliferation of human osteoblast-like cells. Exp Cell Res. 2014; 323:165-177.
17 Ozeki N, Mogi M, Hase N, Hiyama T, Yamaguchi H, Kawai R, Kondo A, Nakata K. Wnt16 Signaling Is Required for IL-1beta-Induced Matrix Metalloproteinase-13-Regulated Proliferation of Human Stem Cell-Derived Osteoblastic Cells. Int J Mol Sci. 2016;17:221
18 Wong SP, Rowley JE, Redpath AN, Tilman JD, Fellous TG, Johnson JR. Pericytes, mesenchymal stem cells and their contributions to tissue repair. Pharmacol Ther. 2015; 151:107-120.
19 Minteer DM, Marra KG, Rubin JP. Adipose stem cells: Biology, safety, regulation, and regenerative potential. Clin Plast Surg. 2015; 42:169-179.
20 Zuk P A, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002; 13:4279-4295.
21 Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz H P, Hedrick MH. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng. 2001; 7:211-228.
22 Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, Quarto N, Contag CH, Wu B, Longaker MT. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol. 2004; 22:560-567.
23 De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo J L, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs. 2003; 174:101-109.
24 Buschmann J, Gao S, Harter L, Hemmi S, Welti M, Werner CM, Calcagni M, Cinelli P, Wanner GA. Yield and proliferation rate of adipose-derived stromal cells as a function of age, body mass index and harvest site-increasing the yield by use of adherent and supernatant fractions? Cytotherapy. 2013; 15:1098-1105.

25 Ding DC, Chou HL, Hung WT, Liu HW, Chu TY. Human adipose-derived stem cells cultured in keratinocyte serum free medium: Donor's age does not affect the proliferation and differentiation capacities. J Biomed Sci. 2013; 20:59.
26 Ozeki N, Lim M, Yao CC, Tolar M, Kramer RH. Alpha7 integrin expressing human fetal myogenic progenitors have stem cell-like properties and are capable of osteogenic differentiation. Exp Cell Res. 2006; 312:4162-4180.
27 Tsutsumi K, Saito N, Kawazoe Y, Ooi HK, Shiba T. Morphogenetic study on the maturation of osteoblastic cell as induced by inorganic polyphosphate. PLoS One. 2014; 9:e86834.
28 Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006; 8:315-317.
29 Mogi M, Togari A. Activation of caspases is required for osteoblastic differentiation. J Biol Chem. 2003; 278:47477-47482.
30 Ozeki N, Mogi M, Nakamura H, Togari A. Differential expression of the Fas-Fas ligand system on cytokine-induced apoptotic cell death in mouse osteoblastic cells. Arch Oral Biol. 2002; 47:511-517.
31 Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: Comparison with cetylpyridinium chloride extraction. Anal Biochem. 2004; 329:77-84.
32 Candelario-Jalil E, Taheri S, Yang Y, Sood R, Grossetete M, Estrada EY, Fiebich BL, Rosenberg GA. Cyclooxygenase inhibition limits blood-brain barrier disruption following intracerebral injection of tumor necrosis factor-alpha in the rat. J Pharmacol Exp Ther. 2007; 323:488-498.
33 Koyama Y, Tanaka K. Endothelins stimulate the production of stromelysin-1 in cultured rat astrocytes. Biochem Biophys Res Commun. 2008; 371:659-663.
34 Hayami T, Kapila YL, Kapila S. MMP-1 (collagenase-1) and MMP-13 (collagenase-3) differentially regulate markers of osteoblastic differentiation in osteogenic cells. Matrix Biol. 2008; 27:682-692.
35 Huang CY, Pelaez D, Dominguez-Bendala J, Garcia-Godoy F, Cheung HS. Plasticity of stem cells derived from adult periodontal ligament. Regen Med. 2009; 4:809-821.
36 Xu J, Wang W, Kapila Y, Lotz J, Kapila S. Multiple differentiation capacity of STRO-1+/CD146+ PDL mesenchymal progenitor cells. Stem Cells Dev. 2009; 18:487-496.
37 Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res. 2003; 92:827-839.
38 Ozeki N, Yamaguchi H, Hiyama T, Kawai R, Nakata K, Mogi M, Nakamura H. IL-1beta-induced matrix metalloproteinase-3 regulates cell proliferation in rat dental pulp cells. Oral Dis. 2015; 21:97-105.
39 Yamaguchi H, Ozeki N, Kawai R, Tanaka T, Hiyama T, Nakata K, Mogi M, Nakamura H. Proinflammatory cytokines induce stromelysin-1-mediated cell proliferation in dental pulp fibroblast-like cells. J Endod. 2014; 40:89-94.

(Received August 4, 2016; Revised September 21, 2016; Accepted September 30, 2016)