Original Article

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Polyphosphate-induced matrix metalloproteinase-3-mediated proliferation in rat dental pulp fibroblast-like cells is mediated by a Wnt5 signaling cascade

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Summary **Although it is known that inorganic polyphosphate [Poly(P)] induces differentiation of osteoblasts, there are few reports concerning its effects on cell proliferation, especially in fibroblasts. Because we found that Poly(P) stimulates the proliferation of purified rat dental pulp fibroblast-like cells (DPFCs), matrix metalloproteinase (MMP)-3 small interfering RNA (siRNA) was transfected into purified rat DPFCs to investigate whether MMP-3 activity is induced by Poly(P) and/or is associated with cell proliferation in DPFCs. Real-time quantitative polymerase chain reaction, Western blots, an MMP-3 activity assay, and an enzyme-linked immunosorbent assay to assess cell proliferation were used in this study. Poly(P) induced expression of MMP-3 mRNA and protein, and increased MMP-3 activity and cell proliferation. Silencing of MMP-3 expression with siRNA yielded potent and significant suppression of Poly(P)-induced MMP-3 expression and activity, and decreased cell proliferation. Poly(P) also increased mRNA and protein levels of Wnt5 and the Wnt receptor Lrp5/Fzd9. Although exogenous MMP-3 could not induce Wnt5, exogenous Wnt5 was found to increase MMP-3 activity and, interestingly, the proliferation rate of DPFCs. Transfection with Wnt5a siRNA suppressed the Poly(P)-induced increase in MMP-3 expression and suppressed cell proliferation. These results demonstrate the sequential involvement of Wnt5 and MMP-3 in Poly(P)-induced proliferation of DPFCs, and may have relevance in our understanding and ability to improve wound healing following dental pulp injury.**

Keywords: Inorganic polyphosphate, differentiation, osteogenic cells, Lrp5

1. Introduction

Inorganic polyphosphate [Poly(P)] is a linear polymer with tens to hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds. In mammals, Poly(P) is found in cells of the brain, heart, lung, and liver, and in erythrocytes (*1-3*). The most studied and well-known role of Poly(P) is the promotion of intracellular calcification (*4*). Poly(P) induces alkaline phosphate activity and up-regulates osteopontin and osteocalcin gene expression in osteoblastic cells (*5,6*). Because Poly(P) has been identified as a biopolymer in mammalian cells and is being used as an additive in food and cosmetics, it is deemed safe for use in medications. Furthermore, although several studies have focused on Poly(P) as a differentiation factor, only one previous report (*7*) has studied the proliferation of cells treated with Poly(P).

Matrix metalloproteinase (MMP)-3 and interstitial collagenase (MMP-1) are produced by fibroblasts in response to increased levels of inflammatory cytokines caused by disease, such as periodontitis and rheumatoid arthritis, and dental pulp injury (*8,9*). MMP-3 has been implicated in joint and soft tissue destruction associated with these conditions, where it participates in the inflammatory response (*10-13*). Synthesis of MMP-3 is tightly controlled *in vivo* (*12,14*). Although it is intuitive that dental pulp destruction may be a

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function of MMPs, our previous study showed that proinflammatory cytokine-induced MMP-3 actually accelerates wound healing following dental pulp injury (*15-17*) and promotes cell proliferation of odontoblasts derived from mouse induced pluripotent stem (iPS) cells and embryonic stem (ES) cells (*16,18,19*).

Dental pulp is a highly innervated tissue with sensory axons mainly distributed in the dentin-pulp complex. In addition, dental pulp consists predominantly of fibroblasts with a small population of odontoblasts and blood vessels (*20,21*). This heterogeneous mix of cells would likely confound any assessment of the effects of modifiers. Although we previously demonstrated that proinflammatory cytokine-induced MMP-3 regulates cell proliferation of partially purified rat dental pulp cells (*18,19*), the effect of Poly(P) on purified dental pulp fibroblast-like cells (DPFCs) has not been defined.

Wnt signaling plays an important role in the development and maintenance of many organs and tissues by regulating cell growth, differentiation, functions, and death through various signaling pathways (*22*). Wnt proteins constitute a large family of secreted glycoprotein ligands, which are responsible for important developmental processes, and have been increasingly implicated in the tissue homeostasis of adult organisms (*23*). Several Wnt isoforms (Wnt5a, Wnt7a, and Wnt11) are involved in interleukin-1-induced differentiation of articular chondrocytes (*24,25*). Wnt5a activates various signaling cascades in diverse biological systems (*26*), and regulates chondrogenesis and cartilage development by promoting chondrocyte differentiation and inhibiting chondrocyte maturation (*27*). Although Wnt5a has been linked to regulation of MMP-1, MMP-3, and MMP-7 in various cell types (*28*), there is currently no evidence that Wnt5 influences the expression of MMPs in fibroblasts.

Recently, Poly(P) has been suggested to participate in apoptosis as well as modulation of the mineralization process in bone tissue (*29,30*). We previously reported that Poly(P)-induced MMP-3 increases the proliferation of iPS cell-derived odontoblast-like cells (*7*). Here, we examined whether Wnt5a and Wnt5b signals are associated with the expression of MMPs in purified DPFCs, which may occur in inflamed dental pulp. Our study aimed to delineate the degree of involvement of Wnt5 in the expression of MMPs in DPFCs and the factors that regulate this process. We show, for the first time, that Wnt5 up-regulates MMP-3 in DPFCs, leading to enhanced cell proliferation.

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 (*6,7*). Twenty grams of sodium tripolyphosphate was dissolved in 200 mL distilled water, 32 mL of 96% ethanol was added to the solution, and the precipitate was collected as Poly(P). As a control, sodium phosphate buffer (pH 6.9) was used instead of Poly(P).

2.2. *Cell culture*

DPFCs were isolated from rat incisors and cultured using a protocol described previously (*19*). In all experiments, DPFCs were used at passages 2-5. Cells were seeded in six-well tissue culture plates at a density of 1×10^5 cells/cm^2. The study protocol was reviewed and approved by the Animal Experimentation Committee of the School of Dentistry, Aichi Gakuin University, Japan (Approval No: 277). 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 (*31*). As shown in Figure 1A, the purity of the DPFCs was estimated to be 97.4 ± 4.51% (n = 3) by flow cytometric analysis. 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), and then the cells were cultured for 7 days. The culture medium was changed every 3 days.

2.3. *Flow cytometry*

Flow cytometry was conducted using standard procedures (*32,33*). Cells (1×10^6 per mL) were incubated with predetermined optimal concentrations of primary antibodies for 1 h at 4°C, washed, and then incubated with FITC-conjugated secondary antibodies (affinity-purified anti-rat antibodies; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The cells were then stained with propidium iodide (1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 4°C and analyzed using a FACSCalibur (Becton, Dickinson and Co., Franklin Lakes, NJ, USA).

To detect rat PDGFR-α, we used an anti-mouse PDGFR-α polyclonal antibody (sc-338; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The antibodies had no significant cross-reactivity with other proteins (data not shown). For surface marker analysis, data were typically collected from 10,000 cells and analyzed with CellQuest Pro 4.1 software (BD Biosciences, San Jose, CA, USA). Unstained cells and cells incubated with the secondary antibody only were both used as negative controls. Background staining was similar to that using the isotype control antibody.

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

Real-time qPCR was performed in triplicate for all samples and standards with approximately 25 ng

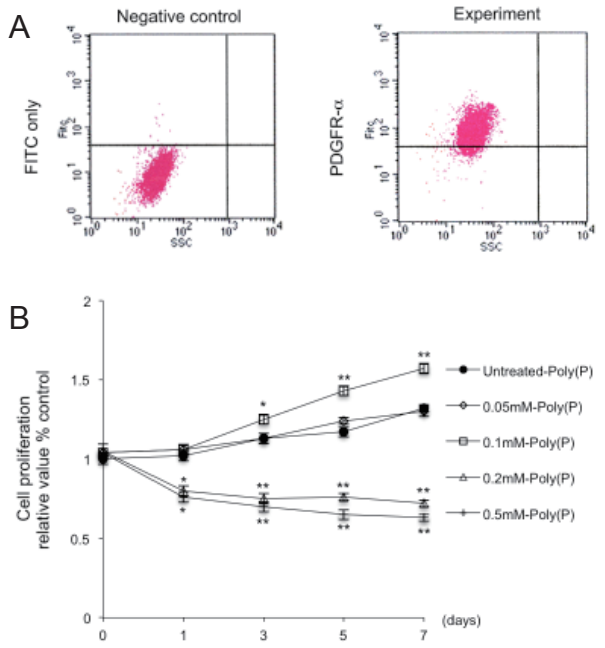

Figure 1. Optimization of Poly(P)-induced proliferation of DPFCs. (A) Flow cytometric analysis of fibroblast-specific PDGFR-α expression. Using an anti-PDGFR-α polyclonal antibody and secondary antibody, flow cytometry was performed to estimate the number of cells expressing PDGFR-α. Negative control values (secondary antibody alone) were subtracted from test values to calculate the mean fluorescence intensity. Data are representative of three independent experiments. **(B)** A BrdU-cell proliferation ELISA was employed to evaluate the proliferation of Poly(P)-treated 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 means ± S.D. 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.

RNA, 0.25 mL RT Mix (Qiagen Quantitect RT Mix, Qiagen Inc., Valencia, CA, USA), and 1.25 mL of 20× Primer/Probe Mix (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; rat *Wnt5a*, Rn01402000_m1; rat *Wnt5b*, Rn01492357_m1; rat *Wnt3a*, Rn01470643_m1; rat *Wnt6*, Rn00437351_m1; rat *Wnt10a*, Rn01401164_m1; rat *Wnt11*, Rn01510237_m1; rat *Lrp5*, Rn01451428_m1; rat *Lrp6*, Rn01492711_m1; rat *Ror1*, Rn01763806_m1; rat *Ror2*, Rn01757507_m1; rat *Ryk*, Rn01403818_m1; rat *Fzd2*, Rn00597004_s1; rat *Fzd9*, Rn00596271_s1). The standard curve method was used for relative quantification of gene expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA served as controls. Analysis was performed using 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, Tokyo, Japan). Protein lysates were separated on SDS-polyacrylamide gels (12%) for Western blot analysis using anti-MMP-3, -tissue inhibitor of metalloproteinase (TIMP)-1, -TIMP-2, -TIMP-3, -Wnt5a, -Wnt5b, -Lrp5, -Fzd9, -MMP-1, -MMP-2, -MMP-9, -MMP-13, and -β-tubulin polyclonal antibodies (sc-6839, sc-5538, sc-6835, sc-6836, sc-365370, sc-109464, sc-21390, sc-33509, sc-13595, sc-6840, sc-30073, and sc-9935, respectively; Santa Cruz Biotechnology Inc.), and an anti-MMP-1 antibody (ab118529; Abcam, Cambridge, UK). Visualization and quantification of blotted protein bands were 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 (*34*) and is now a commercially available MMP-3 activity assay kit (SensoLyte™ 520 MMP-3 assay kit; AnaSpec, San Jose, CA, USA). Prior to detection, MMP-3 was immunoprecipitated from the culture medium using a goat anti-MMP-3 antibody (sc-6839; Santa Cruz Biotechnology Inc.) and protein A/G-agarose for 6 h at 4°C. After centrifugation, the agarose pellet was resuspended in MMP-3 assay buffer (supplied in the assay kit) containing the MMP-3 substrate 5- FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys-QXL^{TM}520-NH_2 fluorescence resonance energy transfer peptide (*35*). MMP-3 activity was then determined according to the manufacturer's instructions.

2.7. *Cell proliferation assay and microscopic analysis*

Cell proliferation was evaluated using a bromo-deoxyuridine(BrdU)-cell proliferation enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany) as described previously (*36,37*). In addition, cell proliferation was evaluated visually with a BZ-9000 microscope (Keyence, Osaka, Japan) using a BrdU immunohistochemistry kit (Abcam) according to the manufacturer's instructions.

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

Commercially available MMP-3 siRNA (Santa Cruz Biotechnology Inc.) was transfected into cultured cells using the siRNA reagent system (Santa Cruz Biotechnology Inc.) according to the manufacturer's protocol. GAPDH siRNA and a control siRNA with no 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 (S.D.) 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 DPFC proliferation*

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

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

MMP-3 induction by Poly(P) was assessed by qPCR and Western blot analyses. Cells were untreated or treated with Poly(P) for 1, 3, 5, or 7 days. Whereas no induction of MMP-3 mRNA was found in untreated cells (Figure 2A, upper left), we did find MMP-3 mRNA and protein expression in Poly(P)-treated cells at days 3, 5, and 7

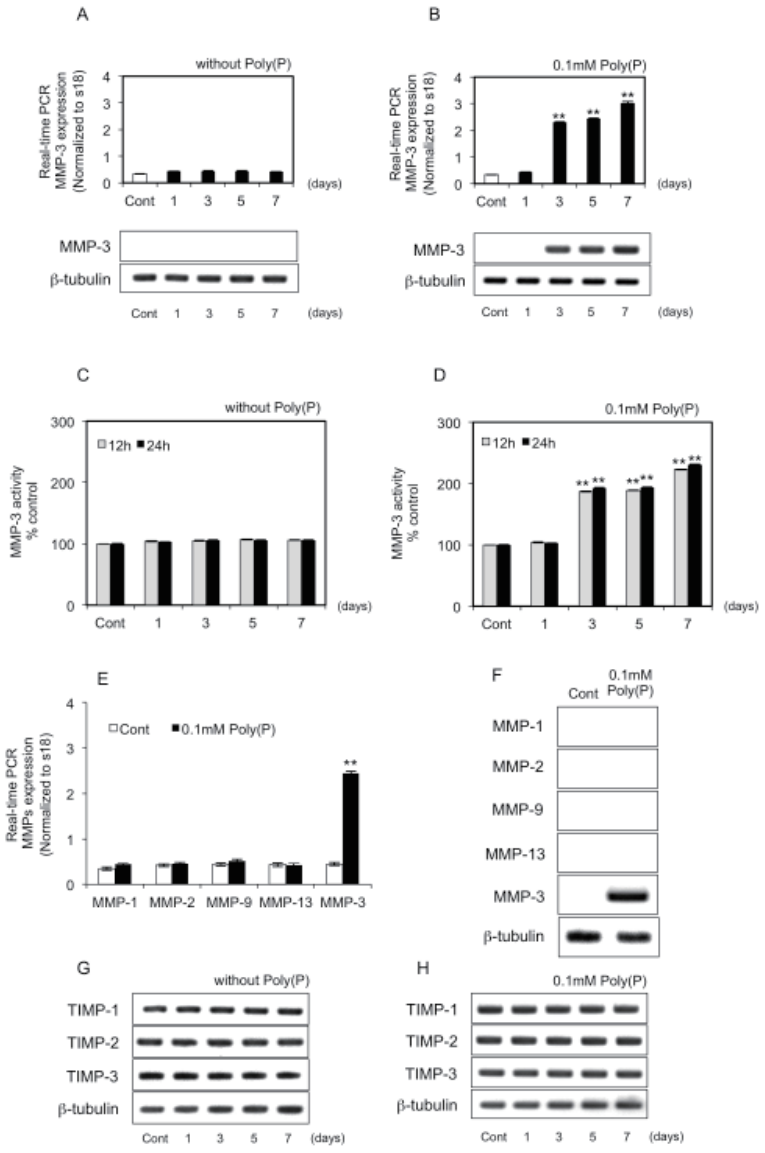

Figure 2. Evaluation of Poly(P)-induced MMP-3 mRNA and protein expression, and MMP-3 activity in DPFCs. (A and B) qPCR analysis of Poly(P)-induced MMP-3 mRNA expression compared with untreated cells (left upper panels) at 24 h. Western blot analysis of MMP-3 and β-tubulin protein levels following stimulation with Poly(P) (lower panels) compared with untreated cells (lower left panels). Representative blots of three independent experiments are shown. **(C and D)** Effects of 0.1 mM Poly(P) on the amount of active MMP-3 released from cultured cells. The cells were incubated in serum-free medium in the absence or presence of 0.1 mM Poly(P) for 12 h (grey bars) or 24 h (black bars). Data are means ± S.D. of three independent experiments. ** $p < 0.01$. **(E)** mRNA levels of other MMPs in DPFCs. Cells were incubated with 0.1 mM Poly(P) and then subjected to qPCR analyses of MMP-1, MMP-2, MMP-9, MMP-13, and MMP-3 mRNA expression relative to the control (18S rRNA). Data are means ± S.D. of four independent experiments. **(F)** Images below each panel show Western blot analysis of MMP-1, MMP-2, MMP-9, MMP-13, MMP-3, and β-tubulin protein expression. Images are representative of three independent experiments. **(G and H)** Western blot analyses of TIMP-1, TIMP-2, and TIMP-3 compared with untreated cells (left panels). Data are representative of three independent experiments.

of culture ($p < 0.01$) (Figure 2B, upper right and lower row).

To assess MMP-3 activity induced by Poly(P), we used an immunoprecipitation-MMP-3 activity assay. MMP-3 activity was significantly induced in Poly(P)-treated cells at days 3, 5, and 7 of culture ($p < 0.01$) (Figure 2D), whereas no induction of MMP-3 was found in untreated cells (Figure 2C).

Bone-associated cells also express other MMPs such as MMP-1, MMP-2, MMP-9, and MMP-13 (*38,39*). Although we assessed whether other MMPs were induced by 0.1 mM Poly(P) in DPFCs, except for MMP-3, there were no significant increases in their mRNA or protein expression in DPFCs (Figures 2E and 2F).

MMP-3 activity is regulated precisely after secretion at the post-translational level as a precursor zymogen and by TIMPs (*40*). Although it is known that TIMP-2 and TIMP-3 are inducible by cytokine stimulation (*40*), we confirmed that TIMP-1, TIMP-2, and TIMP-3 proteins were continuously expressed at stable levels under all experimental conditions [untreated or treated with Poly(P)] (Figures 2G and 2H).

3.3. *Effect of MMP-3 siRNA on cell proliferation*

We found that Poly(P) increased cell proliferation in a time-dependent manner (Figure 3A). The cells were cultured in the absence or presence of Poly(P) for 1, 3, 5, and 7 days, and then cell proliferation was examined by the BrdU-cell proliferation ELISA ($p < 0.05$) (Figure 3A). Next, we examined the effect of MMP-3 siRNA on Poly(P)-induced changes in cell proliferation. Compared with untransfected and control siRNA-transfected cells, MMP-3 silencing considerably decreased the number of proliferating DPFCs following Poly(P) stimulation ($p < 0.05$) (Figure 3B). The reduced proliferative potential was estimated to be similar to the control level. These results were confirmed by microscopic analysis of cell proliferation (images in Figure 3B, lower row).

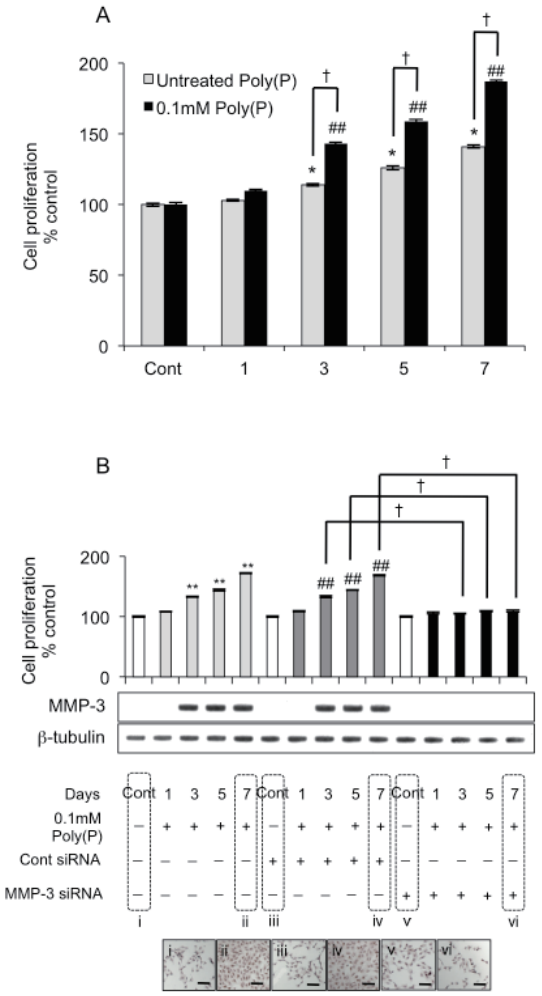

Figure 3. Effect of MMP-3 siRNA on cell proliferation. **(A)** To evaluate the effect of 0.1 mM Poly(P) on DPFC growth, the cells were cultured in the absence or presence of Poly(P) for 1, 3, 5, and 7 days, and then cell proliferation was examined by the BrdU-cell proliferation ELISA. * $p <$ 0.05 and ## $p < 0.01$ *vs*. control day 0; † $p < 0.05$. Data are presented as means ± S.D. of three independent experiments. **(B)** DPFCs were transfected with MMP-3 siRNA for 24 h before evaluating their proliferative status by the BrdU-cell proliferation ELISA (upper panels) and MMP-3 protein expression by Western blots (upper row). Data are means ± S.D. of four independent experiments. ** $p < 0.01$ *vs*. control (day 0); ## $p <$ *vs*. control siRNA; † $p < 0.05$. Cell proliferation at 24 h after siRNA transfection was also evaluated by microscopy with a BrdU immunohistochemistry kit (lower panels). Nuclei of proliferating cells were stained dark brown. Scale bars = 100 μm.

3.4. *Poly(p)-induction of Wnt5a and Wnt5b mRNA and protein expression*

DPFCs were cultured in the presence of 0.1 mM Poly(P). Induction of Wnt5a and Wnt5b mRNA and protein was assessed by qPCR and Western blot analyses (Figures 4A and 4B), respectively. The mRNA and protein levels of both factors were increased by Poly(P). Bone-associated cells also express other Wnt proteins such as Wnt3a, Wnt6, Wnt10a, and Wnt11 (*41-43*). To assess whether the induction of Wnt5a and Wnt5b by Poly(P) is a specific response in DPFCs, we evaluated the expression of these other Wnt proteins following treatment with the same concentrations of Poly(P). However, there were no significant increases in the mRNA expression of these Wnts in response to Poly(P) (Figure 4C).

We examined whether the Wnt receptor, Lrp5 and Fzd9, was present in DPFCs, and whether its expression was influenced by Poly(P). Lrp5 and Fzd9 mRNA and protein were constitutively expressed in DPFCs, and their levels were elevated by Poly(P) treatment (0.1 mM; Figures 4D and 4E). However, several other Wnt5 receptors are also known, including Lpr6, Ror1, Ror2, Ryk, and Fzd2 (*44-46*). We therefore measured the mRNA and protein expression of these receptors in DPFCs. There was no evidence of Lpr6, Ror1, Ror2, Ryk, or Fzd2 expression in either unstimulated or Poly(P)-stimulated cells (Figure 4D). Conversely, Fzd9

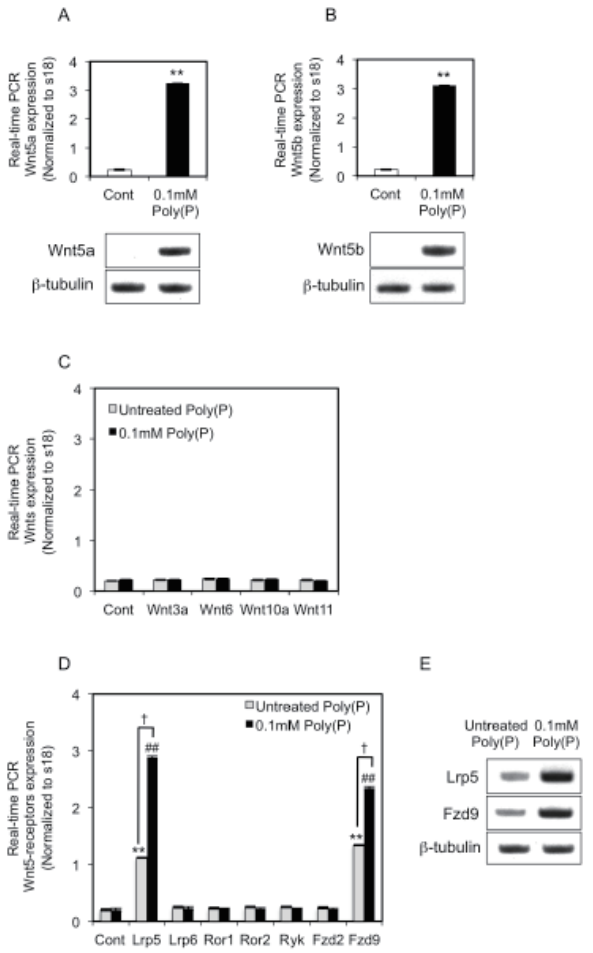

Figure 4. Poly(P)-induced expression of Wnt5a and Wnt5b mRNA and protein in DPFCs. DPFCs were incubated with Poly(P) (0 and 0.1 mM). **(A and B)** qPCR analysis of Wnt5a and Wnt5b mRNA expression relative to the control (S18 mRNA). Data are means ± S.D. of four independent experiments. ** $p < 0.01$. Western blot analysis of Wnt5a, Wnt5b, and β-tubulin protein levels following stimulation with Poly(P) (lower panels). Blots are representative of three independent experiments. **(C)** Expression of other Wnts in DPFCs. Cells were incubated with Poly(P) (0 and 0.1 mM) prior to qRT-PCR analysis of Wnt3a, Wnt6, Wnt10a, and Wnt11 mRNA expression compared to the control (S18 mRNA). Data are means ± S.D. of four independent experiments. **(D)** Expression of other Wnt5 receptors in DPFCs. Cells were incubated with Poly(P) (0 and 0.1 mM) prior to qPCR analysis of Lrp5, Lrp6, Ror1, Ror2, Ryk, Fzd2, and Fzd9 mRNA expression compared with the control (S18 mRNA). Data are means ± S.D. of four independent experiments. ** $p < 0.01$ *vs*. control (day 0); ## $p <$ *vs*. control siRNA; † $p < 0.05$. **(E)** Images below each panel show Western blot analysis of Lrp5, Fzd9, and β-tubulin protein expression. Images are representative of three independent experiments.

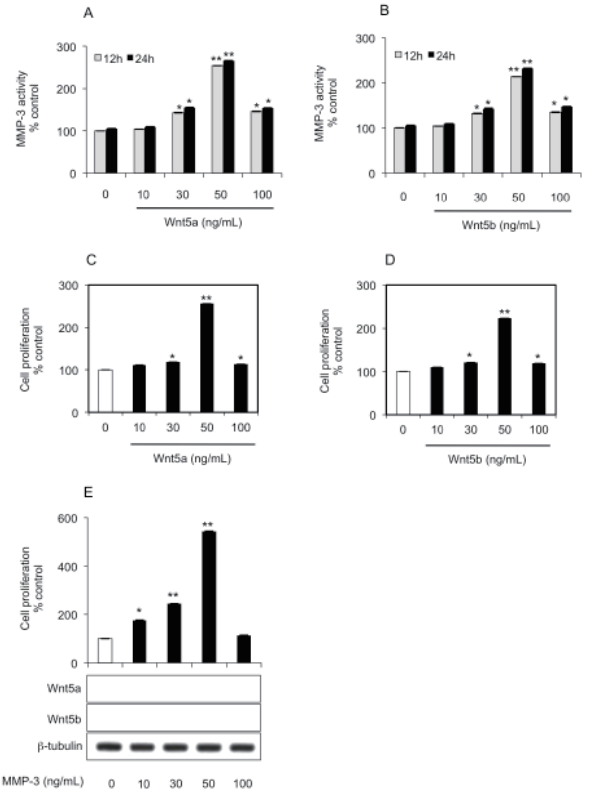

Figure 5. Effect of exogenous Wnt5a and Wnt5b on MMP-3 activity and cell proliferation in DPFCs. (A and B) Cells were incubated for 12 h (grey bars) and 24 h (black bars) with 0, 10, 30, 50, and 100 ng/mL exogenous Wnt5a or Wnt5b prior to analysis. Data are means ± S.D. of four independent experiments. * $p < 0.05$; ** $p < 0.01$. **(C and D)** Effect of exogenous Wnt5a and Wnt5b on cell proliferation. Cells were incubated with Wnt5a and Wnt5b (0, 10, 30, 50, and 100 ng/mL) for 24 h. Their proliferation status was then evaluated using the BrdU-cell proliferation ELISA (graphs). ELISA data are means ± S.D. of four independent experiments. * $p < 0.05$; ** $p < 0.01$. **(E)** Effects of exogenous MMP-3 on DPFC proliferation, and Wnt5a and Wnt5b protein expression. Cells were incubated in serum-free medium in the absence or presence of various concentrations of MMP-3 (0, 10, 30, 50, and 100 ng/mL) for 24 h before cell proliferation was evaluated using the BrdU-cell proliferation ELISA. Images below each panel show Western blot analysis of Wnt5a, Wnt5b, and β-tubulin protein expression. Images are representative of three independent experiments. Data are means ± S.D. of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ *vs*. control.

was constitutively expressed in unstimulated cells, and its mRNA and protein levels were both increased by Poly(P) (0.1 mM; Figures 4D and 4E).

3.5. *Effect of exogenous Wnt5a and Wnt5b on MMP-3 expression and cell proliferation*

We tested whether exogenous Wnt5a and Wnt5b enhanced MMP-3 expression in DPFCs. MMP-3 protein expression and activity, and cell proliferation were all slightly increased by both Wnt5a and Wnt5b at 30 ng/mL, and dramatically increased by these factors at 50 ng/mL ($p < 0.05$; Figures 5A-5D). However, the effects of Wnt5a and Wnt5b at 30 and 100 ng/mL were equivalent, indicating a significant decrease in the magnitude of the effects at this higher dose (Figures 5A-5D). In contrast, although exogenous MMP-3 increased cell proliferation of DPFCs, there were no significant increases in Wnt5a or Wnt5b protein expression in DPFCs (Figure 5E).

3.6. *Evaluation of the expression order during Poly(P)-induced cell proliferation by siRNA silencing*

Using several specific siRNAs, we examined the sequential order through which Wnt5a and MMP-

3 are expressed in DPFCs by Western blot analysis. Poly(P)-induced expression of MMP-3 was inhibited by Wnt5a and MMP-3 siRNAs (Figures 6A and 6B), whereas Poly(P)-induced Wnt5a expression was not inhibited by MMP-3 siRNA (Figure 6B). Thus, Poly(P)-induced MMP-3 was required for Wnt5a and MMP-3 induction. Taken together with the results shown in Figure 5, this signaling cascade appears to be Poly(P)→Wnt5a→MMP-3, which is intimately involved in the cell proliferation of DPFCs.

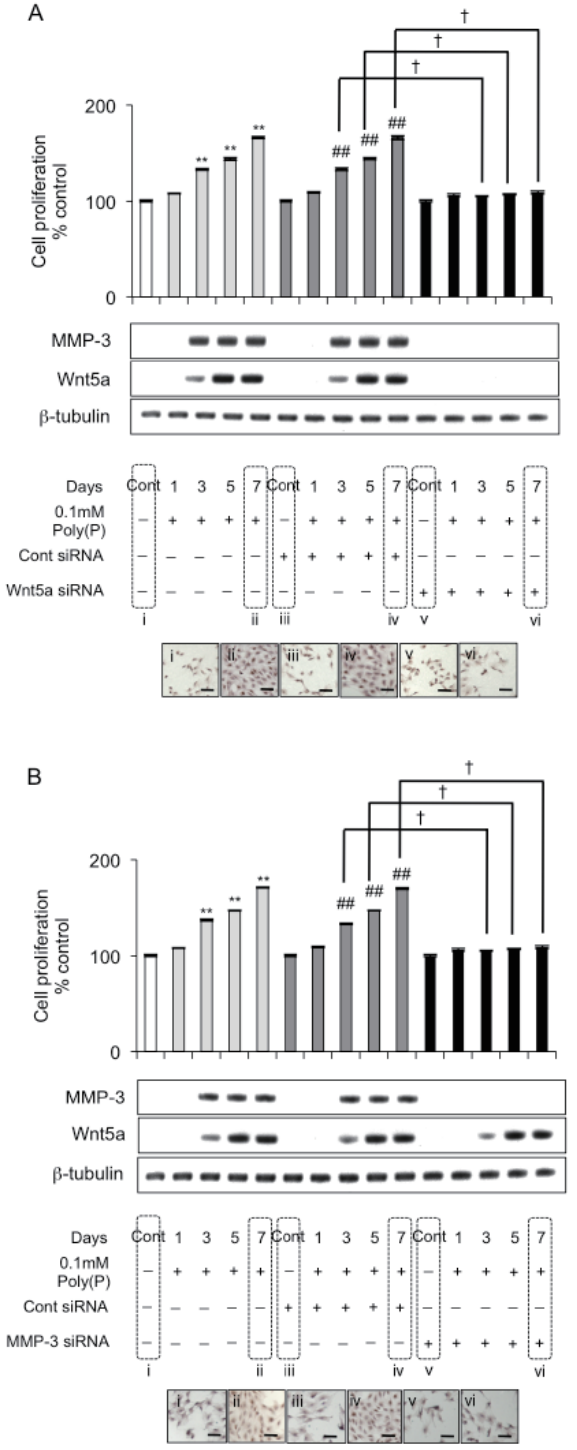

Figure 6. Evaluation of the expression order during Poly(P)-induced cell proliferation by gene silencing. (A and B) DPFCs were transfected with Wnt5a and MMP-3 siRNAs for 24 h before evaluating their proliferative status by the BrdU-cell proliferation ELISA (upper panels), and Wnt5a and MMP-3 protein expression by Western blots (upper row). Data are means ± S.D. of four independent experiments. ** $p < 0.01$ *vs*. control (day 0); ## $p < 0.01$ *vs*. control siRNA; † $p < 0.05$. Cell proliferation at 24 h after siRNA transfection was also evaluated by microscopy with the BrdU immunohistochemistry kit (lower panels). Nuclei of proliferating cells were stained dark brown. Scale bars = 100 μm.

4. Discussion

In our previous study, a cytokine cocktail induced expression of MMP-3 that regulated cell proliferation and suppressed apoptosis in mouse ES cell-derived odontoblast-like cells (*47*) and human skeletal muscle stem cell-derived odontoblast-like cells (*48*). Interleukin-1β and the cytokine cocktail also induced MMP-3-regulated cell proliferation and suppressed apoptosis in partially purified rat dental pulp cells (*18,19*). Here, we used a novel source of fibroblast-like cells derived from rat dental pulp cells to demonstrate that Poly(P) modulates MMP-3 in a manner similar to that in mouse and human cells treated with proinflammatory cytokines. Dental pulp tissue consists of fibroblasts, blood vessels, neuronal cells, and odontoblasts (*20,21*). Because this heterogeneous mix of cells would likely confound any assessment of the effects of Poly(P), we conducted our experiments using highly pure rat DPFCs as shown in Figure 1A. These cells provide an excellent *in vitro* model to study the pathophysiological mechanism(s) of wound healing following dental pulp injury (*18,19*).

Our study showed that Poly(P)-treated DPFCs are a novel *in vitro* dental pulp model of regeneration mediated by Wnt5. A low concentration of Poly(P) (0.1 mM) induced MMP-3 expression in DPFCs (Figure 2A), leading to enhanced cellular proliferation in a time-dependent manner (Figure 1B). In addition to the effect of MMP-3 on the proliferation of odontoblastic cells, the present findings suggest that targeting MMP-3 and Wnt5 genes in these fibroblasts may be a treatment modality for suppurative pulpitis because they are a predominate cell population in dental pulp.

The exact mechanism and molecular pathways underlying Poly(P)-induced MMP-3 up-regulation of DPFC proliferation remain unclear. A recent report from our laboratory has demonstrated that MMP-3 is associated with the cytokine-induced Wnt5 signaling pathway with Wnt5a capable of up-regulating MMP-3 in odontoblast-like cells (*49*). Because we demonstrated that Poly(P)-induced Wnt5 also resulted in induction of MMP-3 and increased cell proliferation in the current study, similar pathways are implicated in both cytokine- and Poly(P)-induced MMP-3 up-regulation in DPFCs. We found no basal expression of Wnt5 in DPFCs (Figures 4A and 4B), suggesting that the mechanism of Poly(P)-induced cell proliferation is clearly different from normal cell growth. In agreement with our previous report

(*49*), we found that only Lrp5 and Fzd9 were induced by Poly(P) in DPFCs. Because Lrp5 and Fzd9 form a complex (*50*), this complex may act as the Wnt5 receptor in DPFCs.

Because we found that Poly(P) induced MMP-3-regulated DPFC proliferation and induces odontoblast-like cell proliferation (*7*), the use of Poly(P) represents a potentially superior therapeutic approach for dental pulp injury. Recently, we established conditions for efficient conversion of human muscle stem cells to an odontoblast lineage (*48*). We will use these cells to revisit our previous and current studies on rat fibroblast-like cells to determine the extent to which our findings are relevant in the human system.

Poly(P) also enhanced differentiation of DPFCs into osteogenic cells (data not shown), although we were unable to determine precisely how many DPFCs had differentiated into osteogenic cells. However, phenotypic characterization based on calcification and the levels of alkaline phosphatase, osteocalcin, and osteopontin suggested that a large proportion of the DPFC population differentiated into osteogenic cells.

In summary, we have demonstrated that Wnt5 responds to Poly(P) by up-regulating MMP-3 expression *via* the Wnt signaling pathway in purified rat DPFCs. This pathway leads to increased cell proliferation of rat DPFCs. These results provide new insights into the role of Wnt5 in fibroblasts and may have relevance in our understanding and ability to improve wound healing following dental pulp injury.

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