Original Article

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Fibroblast growth factor-2 inhibits mineralization of osteoblast-like Saos-2 cells by inhibiting the functioning of matrix vesicles

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Summary **Fibroblast growth factor-2 (FGF2) inhibits osteoblast mineralization, but the mechanism by which it does so is not fully understood. Matrix vesicles (MVs) play an essential role in the initiation of mineralization, so the current study examined the effect of FGF2 on the functioning of MVs to investigate this mechanism. This study found that FGF2 significantly inhibited differentiation and mineralization of osteoblast-like Saos-2 cells, as indicated by down-regulation of mRNA expression of the osteogenic master regulator runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and collagen 1 alpha 1 (Colla1), and by decreasing the formation of bone nodules. MVs were isolated from Saos-2 cells cultured in osteogenic medium supplemented with and without FGF2 and their presence was verified using electron microscopy and Western blotting. FGF2 markedly reduced the ALP activity of and in vitro mineralization by MVs. These findings suggest that FGF2 inhibits osteoblast mineralization by limiting the capacity of MVs.**

Keywords: Fibroblast growth factor-2, mineralization, matrix vesicles, Saos-2 cells

1. Introduction

Osteoblasts produce and secrete a variety of growth factors such as fibroblast growth factor-2 (FGF2), transforming growth factor β (TGFβ), insulin-like growth factor-1 (IGF1), platelet-derived growth factor (PDGF), and prostaglandin E2 (PGE2) that regulate osteoblast proliferation and differentiation in an autocrine and paracrine manner (*1*). FGF2 has been reported to play an important role in bone and callus formation (*2,3*). Disruption of the *FGF2* gene in mice leads to decreased bone mass and decreased bone formation (*4*). However, transgenic mice overexpressing FGF2 were found to have decreased osteoblast differentiation and impaired bone formation (*5*). *In vitro*, continuous treatment with a high concentration of exogenous FGF2 inhibits expression of osteogenic marker gene collagen type 1 (Col1), it reduces alkaline phosphatase (ALP) activity, and it decreases matrix mineralization in osteoblasts (*6-8*). Although FGF2 is reported to act by regulating expression of various genes involved in osteoblast proliferation and differentiation and activating signal transduction pathways including extracellular signal-regulated kinase (ERK) (*9,10*), the mechanism by which FGF2 inhibits mineralization is still understood poorly. Its effect on extracellular components of osteoblasts has not been explored.

Matrix vesicles (MVs) are secreted by mature osteoblasts into the extracellular region. These small vesicles have a diameter ranging from 30 to 400 nm and they contain abundant phospholipid and proteins. MVs play an important role in the initial stage of bone mineralization by promoting the deposition of hydroxyapatite (HA) crystals (*11,12*). MVs manage mineral nucleation, they regulate the inorganic phosphate (Pi)/inorganic pyrophosphate (PPi) ratio in the intra- and extra-cellular fluid, and they control calcium ion and Pi homeostasis (*13*).

Therefore, MVs are presumably involved in FGF2 inhibiting osteoblast mineralization. The current study used osteoblast-like Saos-2 cells to observe the effect

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of FGF2 on expression of osteogenic factors and mineralization. MVs were isolated and their presence was then verified using electron microscopy and biomarker detection. Assays of the ALP activity of and mineralization by MVs were also performed in order to determine the effect of FGF2 on the capacity of MVs and to provide a better understanding of the mechanism by which FGF2 inhibits osteoblast mineralization.

2. Materials and Methods

2.1. *Cell culture and treatment*

Human osteoblast-like Saos-2 cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in McCoy's 5A (Gibco, Carlsbad, CA, USA) supplemented with 15% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA) with 1% penicillin-streptomycin (Beyotime, Haimen, China) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. To induce mineralization, subconfluent Saos-2 cells were cultured in osteogenic medium supplemented with 7.5 mM β-glycerophosphate (β-GP) (Sigma, MO, USA) and 50 μg/mL ascorbic acid (AA) (Sigma, St. Louis, MO, USA). To study the effect of FGF2 treatment, human recombinant FGF2 (Peprotech, Rocky Hill, NJ, USA) was added to a final concentration of 50 ng/mL in osteogenic medium (containing β-GP and AA) (*7*). Media were replaced every three days with the same type of fresh media.

2.2. *Analysis of mineralization*

The mineralization of Saos-2 cells was determined in 12-well plates using Alizarin red S staining during osteogenic induction (3, 6, and 9 days). The cells were fixed with 4% paraformaldehyde for 10 min after they were washed with PBS, and then they were stained with 0.5% (w/v) alizarin red S solution for 1 h. Dye was thoroughly washed from wells using PBS. For quantitative analysis, the cells were incubated in 10% (w/v) cetylpyridium chloride at 37°C for 1 h, and then the optical density of the supernatant was measured at 562 nm.

2.3. *RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR)*

RNA was isolated from 12-well plates using Trizol reagent (Gibco, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA was then used for cDNA synthesis with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After the RT reaction, cDNA was used as the template for RT-qPCR of ALP, runt-related transcription factor 2 (Runx2), and collagen 1 a1 (Colla1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. RT-qPCR was performed using a SYBR Green qPCR Kit (Toyobo, Osaka, Japan) in a real-time PCR detection system with a LightCycler 480 thermocycler (Roche Applied Science, Mannheim, Germany) with gene-specific primers: 5'-CCG TGG CAA CTC TAT CTT GG-3' and 5'-GCC ATA CAG GAT GGC AGT GA-3' for ALP, 5'-AGC AAG GTT CAA CGA TCT GAG AT-3' and 5'-TTT GTG AAG ACG GTT ATG GTC AA-3' for Runx2, 5'-CCC TGG AAA GAA TGG AGA TGA T-3' and 5'-ACTGAA ACC TCT GTG TCC CTT CA-3' for Col1a1, and 5'-CAC CAT CTT CCA GGA GC-3' and 5'-AGT GGA CTC CAC GAC GTA-3' for GAPDH.

2.4. *Isolation of MVs*

After induction for 9 days, Saos-2 cells were washed twice with Hank's balanced salt mixture (Solabio, Shanghai, China) and then digested with 1 mg/mL collagenase Type IA (Sigma, St. Louis, MO, USA) at 37°C for 3 h. The supernatant was collected by centrifugation at 3,000 g for 30 min and concentrated through a 100K Amicon Ultra filter (Millipore Corporation, Billerica, MA, USA) to about 1 mL. The concentrated supernatant was mixed with Exoquick™ (System Biosciences Inc, Carlsbad, CA, USA). The mixture was refrigerated at 4°C overnight and centrifuged at 3,000 g for 30 min to collect MVs that were located at the bottom of the tubes (*13*).

2.5. *Transmission electron microscopy of MVs*

Freshly collected MVs were centrifuged at 8,000 g for 30 min to yield pellets that were then fixed with 2.5% glutaraldehyde at 4°C for 2 h and incubated in 1% osmium tetroxide phosphate buffer solution for 1 h in turn. The samples were dehydrated in a graded ethanol series with acetone before they were embedded in epoxy resin. Seventy-five-nm-thick semithin sections were prepared and mounted on copper grids and stained with a uranyl acetate solution and lead citrate solution to enhance the contrast. An H800 transmission electron microscope (TEM) (Hitachi Electronic Instruments, Japan) was used to obtain electron micrographs.

2.6. *Western blotting*

MV pellets were lysed with radio-immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) on ice for 60 min and then centrifuged at 14,000 g for 15 min at 4°C. The amount of MV lysate protein was determined using a Bradford assay (BioRad Laboratories, Carlsbad, CA, USA). Equal amounts (25 μg) of MV protein from each sample were fractioned on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to

PVDF membranes (Millipore Corporation, Billerica, MA, USA). After blocking, the membrane was incubated with anti-CD63 and CD9 antibodies (1:1,000) (Carlsbad, CA, USA) at 4°C overnight. Primary antibodies were immunostained with goat anti-rabbit IgG peroxidase-conjugated secondary antibodies. Blots were developed with enhanced chemiluminescence (ECL) (Millipore Corporation, Billerica, MA, USA) and exposed to X-ray film.

2.7. *Analysis of the ALP activity of MVs*

P-nitrophenyl phosphate (p-NPP) (Sigma, St. Louis, MO, USA), as substrate, was used to determine the ALP activity of MVs. MV pellets were lysed with an appropriate volume of lysis buffer containing 25 mM Tris-HCl (pH 7.4) and 0.5% Triton X-100. One microliter of MV lysate was incubated with 100 µL p-nitrophenyl phosphate. The reaction was stopped by addition of 50 µL NaOH (3 M) and absorbance was measured at 405 nm. ALP activity was normalized to the protein content of MVs.

2.8. *In vitro biomineralization by MVs*

Calcium precipitation by MVs was assayed in synthetic cartilage lymph (SCL) (100 mM NaCl, 12.7 mM KCl, 0.57 mM $MgCl_2$, 1.83 mM $NaHCO_3$, 0.57 mM Na_2SO_4, 3.42 mM NaH_2PO_4, 2 mM $CaCl_2$, 5.55 mM D-glucose, 63.5 mM sucrose, and 16.5 mM TES, pH = 7.5). An appropriate volume of fresh MVs was added to the aforementioned SCL buffer and the mixture was incubated for 12 h at 37°C. After centrifugation at 8,800 g for 15 min, the calcium phosphate mineral complex was washed twice with water. The precipitant was dissolved in 0.1 M HCl for 3 h. The calcium content of the supernatant was determined using a Calcium Assay Kit (Bioassay Systems, Carlsbad, CA, USA) and was normalized to total protein content.

2.9. *Statistical analysis*

For quantitative data, results are expressed as the mean ± S.D. To determine the differences between groups, an unpaired Student's *t*-test was performed, with $p < 0.05$ being considered statistically significant.

3. Results and Discussion

3.1. *The effect of FGF2 on mineralization of Saos-2 cells*

To evaluate calcium deposition in the Saos-2 cell matrix, alizarin red staining was performed and staining was quantified using a colorimetric analysis based on solubilization of the red matrix precipitate with cetylpyridinium chloride after 3, 6, and 9 days of osteogenic induction. Obvious mineralized nodules were noted after 3 days of induction and increased in a time-dependent manner, becoming extensive after 6 and 9 days (Figure 1A). Cetylpyridinium chloride analysis revealed significantly decreased mineralization in Saos-2 cells treated with FGF2 after 3, 6, and 9 days of induction compared to that in cells cultured in osteogenic medium (Figure 1B). This finding is consistent with those of previous studies (*14,15*).

3.2. *The effect of FGF2 on the expression of osteogenic marker genes in Saos-2 cells*

Osteoblast differentiation and mineralization requires the expression of Runx2, ALP, and Colla1. Runx2, which is a crucial transcriptional factor and expressed in the earliest stage of osteogenic differentiation, regulates the expression of major bone matrix protein genes and osteoblast differentiation and function (*16*). ALP is an early marker of osteogenic differentiation, it hydrolyzes pyrophosphate, and it provides inorganic phosphate to promote mineralization (*17*). Colla1 also plays an important role in osteogenesis by laying the foundation for bone matrix mineralization (*18*).

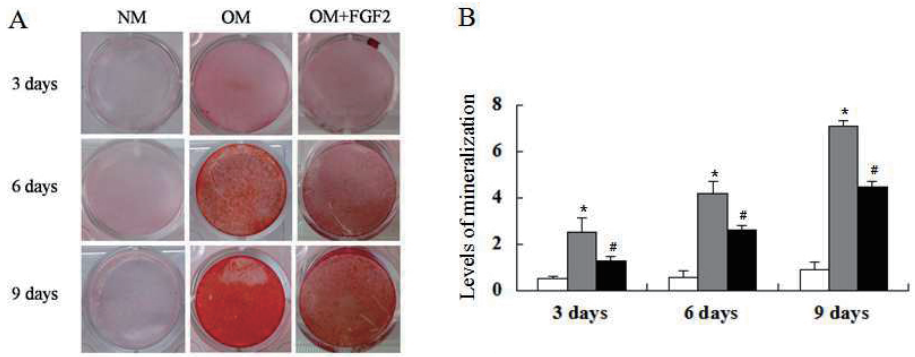

Figure 1. Effect of FGF2 on matrix mineralization of Saos-2 cells. (A) Saos-2 cells were incubated in normal medium (NM), osteogenic medium (OM) and OM supplemented with 50 ng/mL FGF2 for 3, 6, and 9 days. The mineralization of cells was detected using Alizarin Red S staining. **(B)** Cultured cells stained with Alizarin Red S were incubated in cetylpyridinium chloride and staining was quantified at 562 nm. Results are shown as the mean ± S.D., $n = 3$. * $p < 0.05$, *vs.* cells incubated in NM; # $p < 0.05$ *vs.* cells incubated in OM.

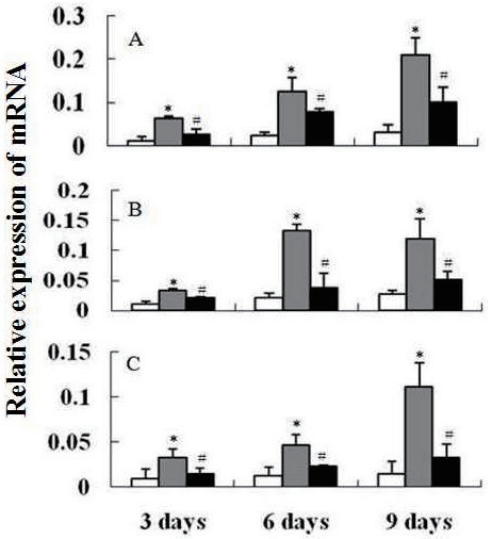

Figure 2. Effect of FGF2 on expression of osteoblast differentiation marker genes in Saos-2 cells. RT-qPCR was used to measure mRNA levels of ALP **(A)**, Runx2 **(B)**, and Collal **(C)** in Saos-2 cells incubated in NM (blank columns), OM (gray columns) and OM with 50 ng/mL FGF2 (black columns) for 3, 6, and 9 days. The expression was normalized to the expression of GAPDH. The experiments were repeated three times and obtained similar results. Results are the mean ± S.D., $n = 3$. * $p < 0.05$ *vs.* cells incubated in NM; # $p < 0.05$ vs. cells incubated in OM.

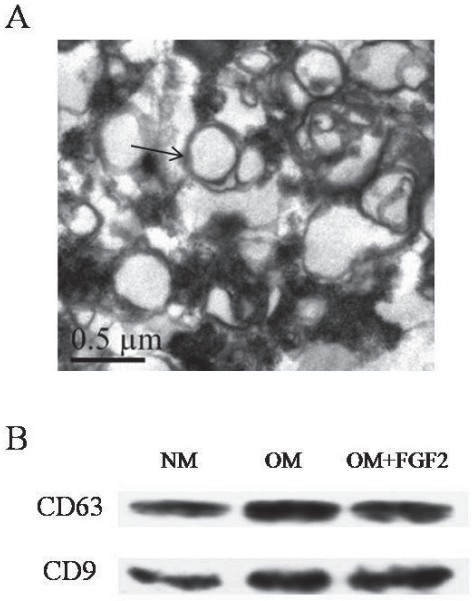

Figure 3. Verification of the presence of MVs. (A) In this TEM image of MVs isolated from Saos-2 cells, the black arrow shows MVs that were membrane-bounded bodies. **(B)** Western blots confirmed the presence of MVs and exosomal protein markers CD63 and CD9 in all of the MVs.

In the current study, levels of mRNA of 3 mineralization-related genes (*ALP*, *Runx2*, and *Col1a1*) were measured using RT-qPCR. Treatment with FGF2 markedly suppressed the expression of all 3 genes at different time points (Figure 2) in comparison to cells incubated in osteogenic medium, suggesting that FGF2 inhibits the osteogenic differentiation of Saos-2 cells.

3.3. *Isolation and characterization of MVs*

Exoquick™ reagents were used to successfully isolate MVs from Saos-2 cells after 9 days of induction, and the presence of MVs was verified using transmission electron microscopy and biomarker detection. As seen in Figure 3A, MVs were apparent as spherical membrane-bounded vesicle structures with a diameter ranging from 30 to 400 nm. Moreover, CD63 and CD9, which are biomarkers of MVs, were present in isolated MVs (Figure 3B).

3.4. *Effect of FGF2 on the ALP activity of and mineralization by MVs*

MVs are released by osteoblasts into the extracellular environment and serve as nucleation sites to accumulate calcium and Pi, thus stimulating the formation of HA crystals and initiating mineralization (*19*). One important role of MVs is to regulate the Pi/PPi ratio in extra-cellular fluid *via* their rich content of pyrophosphate/phosphate-regulating proteins, tissue-nonspecific ALP, ecto-nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), phosphatase orphan 1

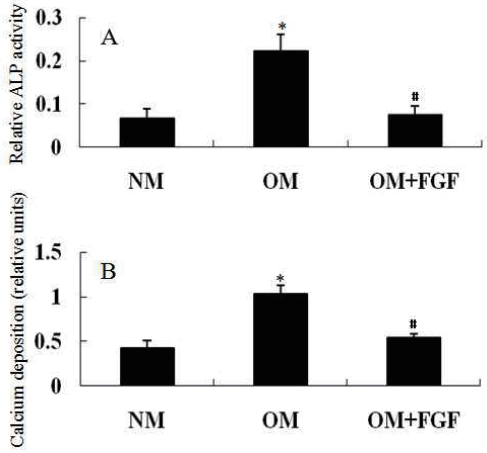

Figure 4. The effect of FGF2 on the capacity of MVs. (A) The effect of FGF2 on the ALP activity of MVs. Saos-2 cells were treated with FGF2 at 3-day intervals for a total of 9 days, resulting in MVs with decreased ALP activity compared to MVs isolated from OM. **(B)** The effect of FGF2 on MV mineralization. MVs from Saos-2 cells cultured in OM supplemented with FGF2 are capable of less calcium precipitation than MVs isolated from OM. Results are the mean ± S.D., $n = 3$. * $p < 0.05$ *vs.* cells incubated in NM; # $p < 0.05$ *vs.* cells incubated in OM.

(PHOSPHO1), and sodium-dependent Pi symporters (Pit1/2) (*20*). The current study found that Saos-2 cells treated with FGF2 yielded MVs with decreased ALP activity compared to untreated cells (Figure 4A). ALP associated with MVs may generate Pi by hydrolyzing pyrophosphate in the extracellular matrix (ECM). A decrease in the ALP activity of MVs may reduce hydrolysis of PPi and consequently fail to yield sufficient Pi to promote the growth of apatite crystals in the ECM. FGF2 reduced the ALP activity

of MVs, indicating this fibroblast growth factor's potential to prevent apatite growth at sites distant from osteoblasts. Furthermore, a biomineralization assay revealed a dramatic decrease in calcium precipitation by MVs derived from Saos-2 cells treated with FGF2 (Figure 4B). FGF2 may act on both the ALP activity of and calcium precipitation by MVs to decrease mineralization in the ECM of osteoblasts.

The regulatory effect of FGF2 on osteoblasts is mediated through the activation of the 4 FGF receptors (FGFRs) FGFR-1, FGFR-2, FGFR-3, and FGFR-4 (*21*). Enhanced FGF signaling caused by mutations in FGFRs is responsible for the aberrant mineralization phenotype of craniosynostosis syndromes (*22*). However, the pathogenesis of the craniosynostosis syndromes is still poorly understood. MVs are reportedly involved in several mineralization-related diseases such as atherosclerosis and osteoarthritis (*23*). In the current study, altered FGF signaling changed the functioning of MVs. This finding may help to elucidate the pathogenesis of FGF/FGFR-associated craniosynostosis syndromes.

In conclusion, this study has described a mechanism by which FGF2 regulates osteoblast mineralization. This study found that FGF2 significantly inhibited the differentiation and mineralization of Saos-2 cells. MVs yielded by Saos-2 cells treated with FGF2 had decreased ALP activity and limited capacity to precipitate calcium. These findings help to further understand the mechanism by which FGF2 inhibits osteoblast mineralization and they suggest that MVs may be involved in the pathogenesis of FGF/FGFR-related craniosynostosis syndromes.

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