Gallium ions promote osteoinduction of human and mouse osteoblasts via the TRPM7/Akt signaling pathway

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Abstract. Gallium (Ga) ions have been widely utilized for biomedical applications; however, their role in osteoblast regulation is not completely understood. The aim of the present study was to investigate the potential effect of Ga ions on osteoinduction in two osteoblast cell lines and to explore the underlying mechanisms. Human hFOB1.19 and mouse MC3T3-E1 osteoblasts were treated with Ga nitride (GaN) at different concentrations, and the degree of osteoinduction was assessed. Ga ion treatment was found to increase alkaline phosphatase activity and accelerate calcium nodule formation, as assessed using ALP activity assay and Alizarin red S staining. Moreover, upregulated expression levels of osteogenic proteins in osteoblasts were identified using western blotting and reverse transcription-quantitative PCR. Western blotting was also performed to demonstrate that the biological action of Ga ions was closely associated with the transient receptor potential melastatin 7/Akt signaling pathway. Furthermore, it was found that Ga ions did not cause osteoblast apoptosis, as indicated via flow cytometry, but promoted osteoclast proliferation, migration or invasion. The present study investigated the potential role of Ga ions in regulating osteoinduction of osteoblasts, thereby providing a promising strategy for the treatment of osteoporosis.

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Introduction

Gallium (Ga) is a semi-metallic element that has been explored for a variety of biomedical applications, including disease diagnosis and treatment of metabolic bone diseases, different types of cancer and inflammatory diseases (1). The applications of the element are primarily attributed to its unique characteristics and functionalities, which include decreasing accelerated bone mineral resorption and lowering associated elevated plasma calcium levels (2,3). In particular, the discovery of the antihypercalcemic effect of Ga, which results from the inhibition of bone resorption rather than the increment of urinary calcium excretion, attracted considerable research interest in exploring its bone-related activity (4,5). The antiresorptive ability of Ga also contributes to the suppression of osteolysis and bone pain associated with multiple myeloma and bone metastases (6), as well as the effective treatment of Pagets bone disease (7). Previous studies using *in vitro* bone fragment models or *in vivo* implant rodent models have demonstrated that Ga ions often display a pronounced affinity for bone surfaces and, thus, affect both bone cells and tissues (8-10). For example, Verron *et al* (10) reported that Ga ions can inhibit the differentiation and resorbing activity of osteoclasts in a dose-dependent manner, without affecting osteoblast viability and proliferation. Therefore, the aforementioned studies suggest the therapeutic potential of Ga for bone-related diseases; however, further investigation is required.

Osteoporosis is a common bone disorder characterized by reduced bone mass, impaired bone strength and altered bone architecture, which often leads to increased skeletal fragility and high-stakes fractures (11-13). Osteoporosis is primarily caused by several factors, such as increasing age, estrogen or androgen deficiency, rheumatoid arthritis, low body mass index and excessive use of certain drugs (14). The disorder is characterized by an unbalanced increase in bone turnover, with bone resorption exceeding bone formation, which may be attributed to the increased number of osteoclasts or excessive osteoclast activity that induce osteolysis (15-17). In addition to hyperactive osteolysis, decreased osteogenesis is another pathological characteristic observed in patients with osteoporosis (18). Therefore, an approach involving both inhibition of excessive osteoclast osteolysis and simultaneous promotion of osteoblast osteogenesis may serve as an optimal strategy for

patients with severe bone loss (19). However, the development of effective drugs to simultaneously inhibit osteoclastogenesis while promoting osteoblastogenesis for the treatment of osteoporosis remains a challenge.

The primary purpose of the present study was to investigate the effect of Ga ions on osteoinduction of osteoblasts and to explore the underlying biological mechanisms. The osteogenic differentiation of two osteoblast cell lines after treatment with Ga nitride (GaN) at the optimized concentration was evaluated by measuring alkaline phosphatase (ALP) activity, calcium nodule formation and the expression levels of osteogenesis-related proteins. The effect of Ga ions on osteoblast migration and invasion was also investigated. The results demonstrated that Ga ions effectively promoted osteogenic differentiation, and osteoblast migration and invasion. By investigating the underlying molecular mechanisms, the results suggested that transient receptor potential melastatin 7 (TRPM7) was involved in Ga ion-mediated regulation of osteoblast osteoinduction.

Materials and methods

Cell culture. A human osteoblast cell line hFOB1.19 and mouse MC3T3-E1 pre-osteoblast cells were purchased from American Type Culture Collection. hFOB1.19 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. MC3T3-E1 cells were cultured in Minimum Essential Medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine. All cells were cultured at 37˚C with 5% CO_2 in a humidified incubator. The cell culture medium was replaced every 2 days. Mouse MC3T3-E1 cells are pre-osteoblasts and were cultured in osteoinduction medium (50 mg/ml β-glycerophosphate and 10 mM L-ascorbic acid).

Design of TRPM7 siRNAs and transfection. TRPM7 siRNA and negative control siRNA were purchased from Santa Cruz Biotechnology, Inc. Cells (5x10⁵ cells/well) into 6-well were transfected with 50 pmol/ml siRNA using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturers instructions. The siRNA sequences are listed in Table I. Following transfection for 72 or 96 h, cells were used for subsequent experiments. In total, three siRNA specific to TRPM7 and a non-specific siRNA specific to TRPM7 were obtained from GenaPharma (Shanghai, China). The three siRNA specific to TRPM7 were compared with TRPM7 non-specific siRNA, and it was found that TRPM7 siRNA2 had the strongest knockout effect.

Antibodies and western blot analysis. Western blotting was performed as previously described (20). The following primary antibodies were used: Anti-GAPDH (Abcam; cat. no. ab181602; 1:1,000), anti-Osterix (Abcam; cat. no. ab22552; 1:1,000), anti-osteocalcin (OCN; Abcam; cat. no. ab93876; 1:1,000), anti-matrix metallopeptidase (MMP)2 (Abcam; cat. no. ab97779; 1:1,000), anti-MMP9 (Abcam; cat. no. ab58803; 1:500), anti-Akt (Cell Signaling Technology, Inc.; cat. no. 4691; 1:1,000), anti-phosphorylated

(p)-Akt (Cell Signaling Technology, Inc.; cat. no. 4060; 1:1,000), anti-Runt-related transcription factor 2 (Runx2; Cell Signaling Technology, Inc.; cat. no. 12556; 1:1,000), anti-Na/K ATPase (Cell Signaling Technology, Inc.; cat. no. 3010; 1:1,000), anti-osteopontin (OPN; Santa Cruz Biotechnology, Inc.; cat. no. SC-21742; 1:1,000) and anti-TRPM7 (Affinity Biosciences; cat. no. DF7513; 1:1,000). Western blot images were obtained using a Molecular Imager® (Bio-Rad Laboratories, Inc.). Protein expression levels were measured via densitometry analysis (ImageJ, v 1.8.0_112; National Institutes of Health) with GAPDH as the loading control.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturers protocol. Total RNA was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (cat. no. RR036Q; Takara Bio, Inc.). Subsequently, qPCR was performed using a StepOne Plus Real-Time PCR Detection system (7500ABI; Applied Biosystems; Thermo Fisher Scientific, Inc.) and TB GreenTM Premix Ex Taq™ II (Tli RNaseH Plus; cat. no. RR820Q; Takara Bio, Inc.). PCR cycling conditions were a follows: One cycle at 50˚C for 2 min and one cycle at 95˚C for 10 min, a two-step PAC procedure was used consisting of 15 sec at 95˚C and 1 min at 60˚C for 45 cycles. The primers used for qPCR are presented in Table II. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (21).

ALP activity assay. hFOB1.19 and MC3T3-E1 cells (5x10⁴ cells/well) were seeded in 24-well plates and cultured overnight at 37˚C for cell attachment. The cell culture medium in each well was carefully removed, and fresh osteogenic induction medium (DMEM-F12; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 50 mg/ml β-glycerophosphate, 10 mM L-ascorbic acid and different concentrations (10^{-7}-10^{-2} M) of GaN (Shanghai Aladdin Bio-Chem Technology Co., Ltd.) was added to each well. Following culture for 7 days at 37˚C, cells were washed twice with PBS, scraped off the plates and collected into tubes. ALP activity was measured using the Sensolyte® pNPP Alkaline Phosphatase Assay kit (AnaSpec) according to the manufacturers instructions. Briefly, 980 μl reaction buffer was added into a new tube, and 960 μl reaction buffer was added into the test tube and enzyme control tube. Subsequently, 20 μl pNPP solution (0.67 M) was added to each tube and equilibrated at 37˚C. Then, 20 μl sample was added into the test tube and 20 μl diluted ALP solution added into the enzyme control tube. The mixture was immediately mixed and the absorbance of each tube was measured at a wavelength of 405 nm using an enzyme standard instrument (Thermo Fisher Scientific, Inc.) within 5 min to obtain the maximum linear rate of the test (ΔA405 nm/min).

Cell proliferation assay. MC3T3-E1 and hFOB1.19 cells were seeded (5x10³ cells/well) into 96-well plates and cultured for 24 h at 37˚C with 5% CO_2 in a humidified incubator. MC3T3-E1 cells were treated with GaN (10^{-4} M) and TRPM7 siRNA or LY294002 (APExBIO Technology; 10 μM) for 0, 7 or 14 days at 37˚C. hFOB1.19 cells were treated with GaN (10^{-4} M) and TRPM7 siRNA or LY294002 (10 μM; APExBIO Technology) for 0, 3 or 7 days at 37˚C, and without cell pretreatment, effective

Table I. Sequences of TRPM7 siRNA used for hFOB1.19 and MC3T3-E1 cell transfection.

A, hFOB1.19

siRNA	Sequence (5'→3')
TRPM7 siRNA1	F: GCGCUUUCCUUAUCCACUUTT
	R: AAUGGAUAAGGAAAGCGCTT
TRPM7 siRNA2	F: CCAUAUCCCACAAUCUCAATT
	R: UUGAGAUUGUGGGAUAUGGTT
TRPM7 siRNA3	F: GUCUUGCCAUGAAAUACUCUU
	R: GAGUAUUUCAUGGCAAGACUU
Control siRNA	F: UUCUCCGAACGUGUCACGUTT
	R: ACGUGACACGUUCGGAGAATT

B, MC3T3-E1

siRNA	Sequence (5'→3')
TRPM7 siRNA1	F: GCGCUUUCCUUAUCCACUUTT
	R: AAUGGAUAAGGAAAGCGCTT
TRPM7 siRNA2	F: CCAUAUCCCACAAUCUCAATT
	R: UUGAGAUUGUGGGAUAUGGTT
TRPM7 siRNA4	F: GGUGUUCCCAGAAAGGCAATT
	R: UUGCCUUUCUGGGAACACCTT
Control siRNA	F: UUCUCCGAACGUGUCACGUTT
	R: ACGUGACACGUUCGGAGAATT

siRNA, small interfering RNA; TRPM7, transient receptor potential melastatin 7; F, forward; R, reverse.

Table II. Primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'→3')
Mus Runx2	F: GACTGTGGTTACCGTCATGGC
	R: ACTTGGTTTTTCATAACAGCGGA
Mus Osterix	F: CCACCAAGAATTACTCAGAACCC
	R: AAGGACCGACTCACTCAGTCT
Mus OCN (Bglap2)	F: CGCCTACAAACGCATCTACG
	R: CAGAGAGAGAGGACAGGGAGGA
Mus OPN	F: GAGCGAGGATTCTGTGGA
	R: TCGACTGTAGGGACGATTG
Homo MMP2	F: TGACTTTCTTGGATCGGGTCG
	R: AAGCACCACATCAGATGACTG
Homo MMP9	F: TGTACCGCTATGGTTACACTCG
	R: GGCAGGGACAGTTGCTTCT
Homo Runx2	F: TGGTTACTGTCATGGCGGGTA
	R: TCTCAGATCGTTGAACCTTGCTA
Homo VEGF	F: GAGGAGCAGTTACGGTCTGTG
	R: TCCTTTCCTTAGCTGACACTTGT
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA

Runx2, Runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin; MMP, matrix metallopeptidase; VEGF, vascular endothelial growth factor.

concentrations of TRPM7 siRNA and LY294002 were always maintained in the medium. Subsequently, cells were washed with PBS and 20 μl Cell Counting Kit-8 (CCK-8) solution (MCE, Inc.) according to the manufacturers instructions in fresh cell culture medium was added to each well. Following incubation at 37°C for 4 h, the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader, which was used to calculate cell proliferation.

Cell apoptosis analysis. hFOB1.19 and MC3T3-E1 cells were seeded (2x10^5 cells/well) into 6-well cell culture plates and cultured at 37°C with 5% CO_2 overnight for attachment. Following cells were treated with GaN (10^-4 M), cell apoptosis was assessed using the Annexin V-FITC/PI kit (Haigene, Inc.; http://www.haigene.cn/) according to the manufacturers instructions. Briefly, cells were collected and suspended in 500 μl buffer. Subsequently, cells were incubated with 5 μl Annexin V-FITC and 5 μl propidium iodide solution at room temperature for 15 min. Apoptotic cells were measured via flow cytometry (FACSCanto™ II; BD FACSCanto™ System Software Full v 3.0, BD Biosciences), and early and late apoptosis were assessed.

Alizarin red S staining. To demonstrate the effect of Ga ions on the ability of osteoblasts to produce calcium nodules, Alizarin red S staining was performed. MC3T3-E1 cells cultured (2x10^5 cells/well) in 6-well cell culture plates were treated with GaN (10^-4 M) and TRPM7 siRNA or LY294002 (10 μM) in osteogenic induction medium for 14 days at 37°C (22). Subsequently, cells were fixed with 4% paraformaldehyde at room temperature for 15 min and washed three times with PBS. Cells were incubated in Alizarin Red S solution at room temperature for 5 min, followed by washing with distilled water. Stained cells were dried at 60°C, mounted in xylene for 5 min and observed using a light microscope (magnification, x400).

Cell migration analysis. Cell migration was assessed using Transwell 24-well cell culture plates with an 8-μm pore polycarbonate membrane insert. Briefly, 0.2 ml hFOB1.19 cells (3x10^5 cells/ml) suspended in serum-free DMEM were seeded into the upper chambers and 0.8 ml DMEM supplemented with 10% FBS was seeded into the lower chambers. Cells were treated with GaN (10^-4 M) and TRPM7 siRNA or LY294002 (10 μM) for 24 h at 37°C. Migratory cells were fixed with 70% ice-cold ethanol for 1 h at 4°C, stained with 5% crystal violet solution for 20 min at room temperature and counted using a light microscope (magnification, x400).

Cell invasion analysis. Cell invasion was assessed using Transwell 24-well cell culture plates that were precooled at -20°C overnight. Matrigel® was melted at 4°C and diluted to a final concentration of 1 mg/ml with serum-free medium on ice. The upper chambers were precoated with diluted Matrigel® for 4-5 h at 37°C. hFOB1.19 cells (2.5x10^5 cells/ml) in serum-free

DMEM/F12 (200 μl) containing GaN (10^{-4} M) and TRPM7 siRNA or LY294002 (10 μM) were seeded into the upper chambers. DMEM (800 μl) supplemented with 10% FBS was seeded into the lower chambers. After incubation for 24 h at 37°C, invading cells were fixed with 70% ice-cold ethanol for 1 h at 4°C, stained with 5% crystal violet solution for 20 min at room temperature and counted using a light microscope (magnification, x400).

ELISA. Levels of vascular endothelial growth factor (VEGF) protein in media of hFOB1.19 cells were measured using ELISA (Gibco; Thermo Fisher Scientific, Inc.; cat. no. KHG0111), according to the manufacturers instructions.

Statistical analysis. All experiments were performed in triplicate (n=3). Data are presented as the mean ± standard deviation. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukeys post hoc test. Statistical analyses were performed using SPSS statistical software (version 21.0; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Ga ions enhance osteoblast ALP activity. Metal elements have been reported to affect the intracellular ALP activity of osteoblast cells in a concentration-dependent manner (23-25). Therefore, the present study investigated the effect of different Ga ion concentrations on osteoblast ALP activity. Following treatment with GaN (10^{-7}-10^{-2} M) in osteogenic induction medium for 7 days, mouse MC3T3-E1 osteoblasts were examined using an ALP activity assay. Compared with control cells, intracellular ALP activity was significantly increased in cells cultured in osteogenic induction medium. In addition, the ALP activity of GaN-treated cells cultured in osteogenic induction medium increased with increasing GaN concentration, reaching a maximum level of ALP activity at 10^{-4} M (Fig. 1A). At GaN concentrations above 10^{-4} M, ALP activity decreased compared with the 10^{-4} M GaN group. The effect of Ga ions on the ALP activity of human hFOB1.19 osteoblasts was also investigated by treating cells with different concentrations of GaN (10^{-7}-10^{-2} M) for 7 days. Similarly, the ALP activity of hFOB1.19 cells increased with increasing GaN concentrations, reaching a maximum level of ALP activity at 10^{-4} M. Furthermore, at GaN concentrations above 10^{-4} M, ALP activity decreased compared with the 10^{-4} M GaN group (Fig. 1B). The results suggested that Ga ions affect osteoblast ALP activity in a concentration-dependent manner. Based on the aforementioned results, 10^{-4} M GaN was selected for subsequent experiments, as it was identified as the concentration that induced the highest levels of ALP activity.

Effect of Ga ions on the Akt signaling pathway in osteoblasts. Based on the key roles of the Akt signaling pathway in regulating ALP activity (26,27), the influence of Ga ions on the Akt signaling pathway in osteoblasts was investigated. MC3T3-E1 osteoblasts were cultured in osteogenic induction medium with or without GaN (10^{-4} M) for 0, 7 or 14 days. Subsequently, Akt activation and phosphorylation were assessed via western blotting. The results indicated that the

levels of p-Akt expression in MC3T3-E1 cells cultured in osteogenic induction medium were markedly higher compared with control cells after 7 and 14 days (Fig. 2A). Furthermore, the expression levels of p-Akt were increased in GaN-treated cells cultured in osteogenic induction medium compared with untreated cells cultured in osteogenic induction medium after 7 and 14 days, particularly after 14 days of incubation. The expression of p-Akt increased in MC3T3-E1 cells in the osteoinduction group in a time-dependent manner compared with osteoinduction + GaN group (Fig. 2A), and as well as in hFOB1.19 cells cultured in osteogenic induction medium in a time-dependent manner compared with control cells (Fig. 2B). The results indicated that GaN may activate the Akt signaling pathway in osteoblasts.

Construction and screening of TRPM7 siRNA. It has been reported that TRPM7 expression is associated with osteoblast ALP activity and osteogenesis following stimulation with magnesium ions (22). Therefore, it was hypothesized that TRPM7 may also serve a pivotal role in Ga ion-mediated regulation of osteoinduction in osteoblasts. To test the hypothesis, four different TRPM7-specific siRNAs were designed and constructed. MC3T3-E1 and hFOB1.19 cells were transfected with TRPM7 siRNAs for 72 h. Subsequently, the membrane and total protein expression levels of TRPM7 were assessed via western blotting. Compared with the control and control siRNA groups, TRPM7 siRNA1, TRPM7 siRNA2 and TRPM7 siRNA3 notably decreased TRPM7 protein expression levels via siRNA-mediated knockdown in MC3T3-E1 cells (Fig. 3A and B). TRPM7 siRNA2 decreased TRPM7 protein expression to the lowest level in MC3T3 cells among the different TRPM7 siRNAs, and thus was selected for subsequent experiments. hFOB1.19 cells were transfected with TRPM7 siRNA1, TRPM7 siRNA2 or TRPM7 siRNA4. The results indicated that all three TRPM7 siRNAs decreased TRPM7 expression levels compared with the control and control siRNA groups. However, TRPM7 siRNA4 reduced TRPM7 protein expression to the lowest level among the different TRPM7 siRNAs in hFOB1.19 cells, so TRPM7 siRNA4 was selected for subsequent experiments (Fig. 3C and D). The results indicated the transfection efficiency of the TRPM7 siRNAs in the two osteoblast cell lines.

Ga ions promote ALP activity and calcium nodule formation by activating the TRPM7/Akt signaling pathway. To investigate whether Ga ion-mediated stimulation of ALP activity was associated with the TRPM7/Akt signaling pathway, osteoblasts were treated with TRPM7 siRNA or an Akt inhibitor (LY294002) to assess the effect on ALP activity. In MC3T3-E1 cells, ALP activity was significantly increased in GaN-treated cells cultured in osteogenic induction medium compared with cells cultured in osteogenic induction medium alone (Fig. 4A). However, GaN-induced ALP activity was significantly decreased by TRMP7 siRNA transfection or LY294002 treatment in MC3T3-E1 cells. For example, MC3T3-E1 cell ALP activity was 3- and 2.6-fold lower in TRPM7 siRNA-transfected and LY294002-treated cells compared with the osteoinduction + GaN group, respectively. Moreover, MC3T3-E1 cell ALP activity in the osteoinduction + GaN + control siRNA group was similar to the osteoinduction + GaN group. A
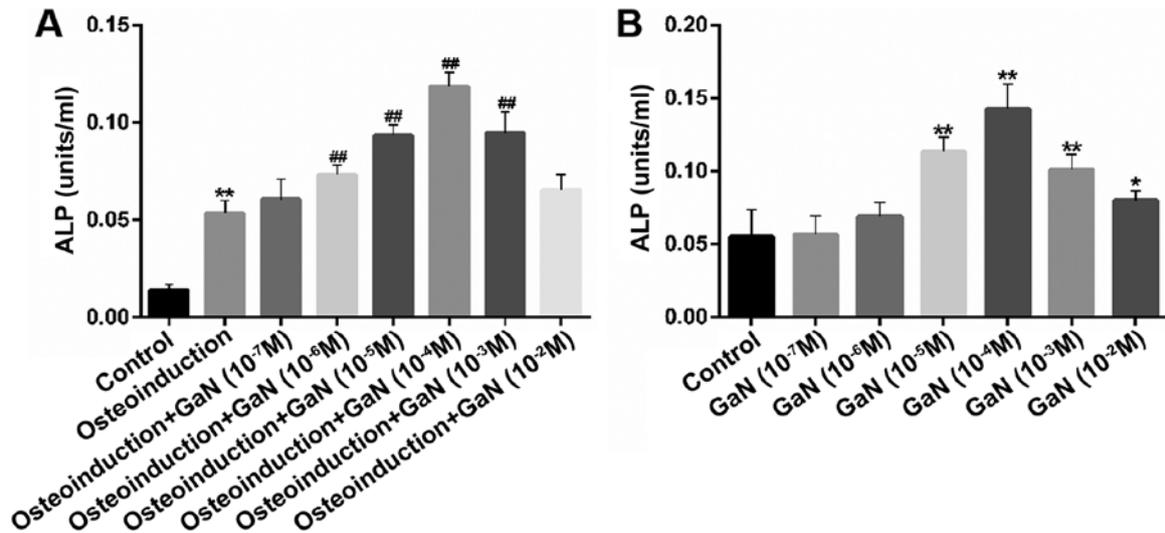
Figure 1. Effect of different Ga ion concentrations on MC3T3-E1 and hFOB1.19 cell ALP activity. ALP activity of (A) MC3T3-E1 and (B) hFOB1.19 cells following treatment with different concentrations of GaN for 7 days. *P<0.05 and **P<0.01 vs. control; ##P<0.01 vs. osteoinduction. Ga, gallium; ALP, alkaline phosphatase; GaN, gallium nitride.
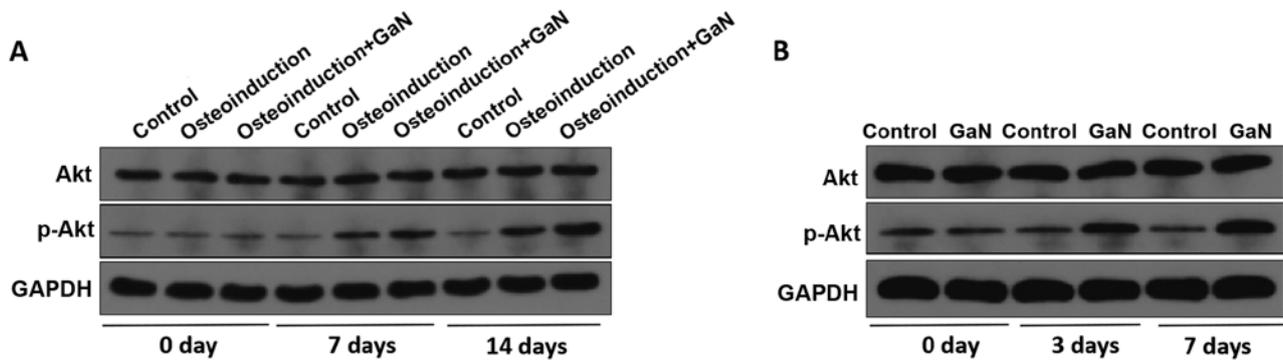

Figure 2. Effect of Ga ions on the Akt signaling pathway in MC3T3-E1 and hFOB1.19 cells. (A) Akt and p-Akt expression levels in MC3T3-E1 cells following treatment with GaN (10^{-4} M) for 0, 7 or 14 days. (B) Akt and p-Akt expression levels in hFOB1.19 cells following treatment with GaN (10^{-4} M) for 0, 3 or 7 days. Ga, gallium; p, phosphorylated; GaN, gallium nitride.

similar pattern was observed in hFOB1.19 cells. hFOB1.19 cell ALP activity was significantly increased in GaN-treated cells compared with untreated cells. In addition, control siRNA transfection did not significantly alter the ALP activity of GaN-treated cells. By contrast, hFOB1.19 cell ALP activity was significantly decreased in the GaN + TRMP7 siRNA and GaN + LY294002 groups compared with the corresponding control groups (Fig. 4B). The results suggested that GaN promoted osteoblast ALP activity, whereas inhibition of the TRPM7/Akt signaling pathway inhibited osteoblast ALP activity.

Alizarin red staining was performed to assess calcium nodule formation, which is a hallmark of osteoinduction (23). The results suggested that osteogenic induction increased calcium nodule formation in MC3T3-E1 cells compared with control cells, which was further increased by GaN treatment. However, co-treatment with GaN and TRPM7 siRNA or LY294002 resulted in decreased calcium nodule formation compared with the osteoinduction + GaN group (Fig. 4C). The Alizarin red staining results were similar to the ALP activity results, suggesting that GaN

may increase MC3T3-E1 cell osteogenic differentiation, whereas TRPM7 siRNA and LY294002 may inhibit osteogenic differentiation.

Ga ions upregulate osteogenic-related protein expression by activating the TRPM7/Akt signaling pathway. It has been reported that osteogenic-related proteins are regulated during osteogenesis and may be used to evaluate the osteoinduction of osteoblasts (28). The expression levels of osteogenic-related proteins (Runx2, Osterix, OCN, OPN, Akt and p-Akt) in MC3T3-E1 cells were assessed via western blotting. Following osteogenic induction, the protein expression levels of Runx2, Osterix, p-Akt, OCN and OPN were markedly increased compared with control cells, and the addition of GaN treatment further increased the expression levels of these proteins. By contrast, TRPM7 siRNA transfection and LY294002 treatment notably decreased the expression levels of osteogenic-related proteins in GaN-treated cells cultured in osteogenic induction medium (Fig. 5A and B).

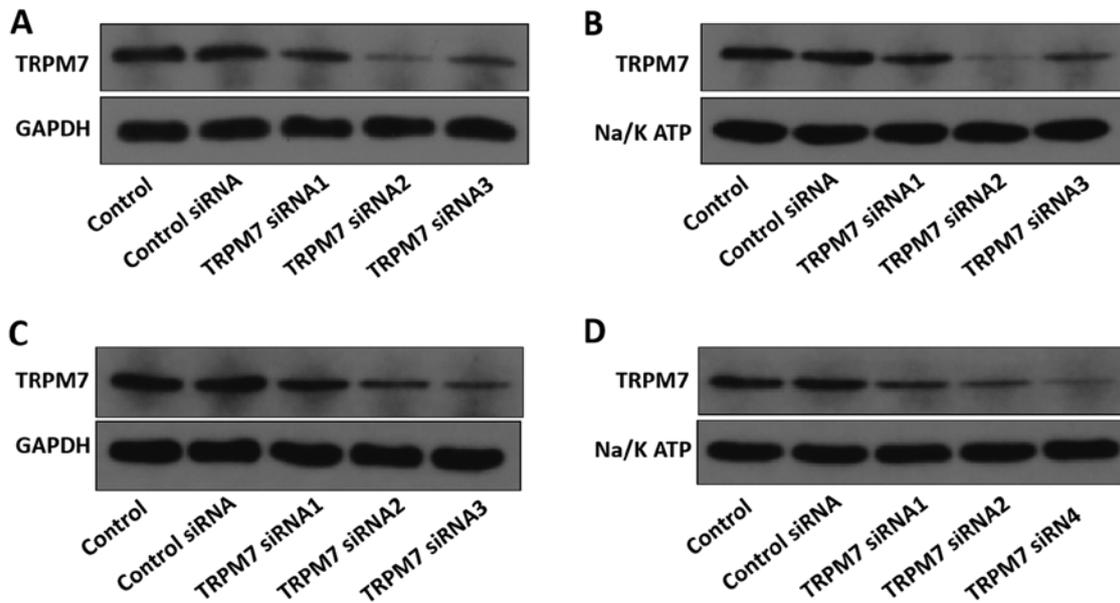The expression levels of osteogenic-related proteins (MMP2, MMP9, Runx2, Akt and p-Akt) in

Figure 3. Effect of TRPM7 siRNAs on TRPM7 protein expression in MC3T3-E1 and hFOB1.19 cells. (A) Total and (B) membrane TRPM7 protein expression levels in MC3T3 cells following transfection with TRPM7 siRNAs. (C) Total and (D) membrane TRPM7 protein expression levels in hFOB1.19 cells following transfection with TRPM7 siRNAs. TRPM7, transient receptor potential melastatin 7; siRNA, small interfering RNA.

hFOB1.19 cells were also evaluated. In GaN-treated and GaN-treated + control siRNA-transfected cells, the expression levels of MMP2, MMP9, Runx2 and p-Akt were markedly higher compared with control cells, which suggested that GaN upregulated the expression of osteogenic-related proteins (Fig. 5C and D). However, TRPM7 siRNA transfection and LY294002 treatment decreased the expression levels of osteogenic-related proteins in GaN-treated hFOB1.19 cells. In addition, the mRNA expression levels of osteogenic-related proteins in the two cell lines were measured via RT-qPCR, and the results were similar to the western blotting results (Fig. 5F and G).

Furthermore, the intracellular expression of VEGF protein in hFOB1.19 cells was evaluated by performing an ELISA. Following treatment with GaN, the level of VEGF protein was significantly increased compared with control cells. Similar levels of VEGF expression were observed in the GaN + control siRNA and GaN groups (Fig. 5E). By contrast, VEGF protein levels were significantly decreased by TRPM7 siRNA transfection and LY294002 treatment in GaN-treated cells compared with the corresponding control groups.

The results suggested that GaN upregulated the expression levels of osteogenic-related proteins in human and mouse osteoblasts, thereby promoting osteoinduction. By investigating the underlying biological mechanisms, the results indicated that GaN-mediated osteoinduction was associated with the TRPM7/Akt signaling pathway, since TRPM7 knockdown and Akt inhibition markedly decreased the expression levels of osteogenic-related proteins in osteoblasts.

Effect of Ga ions on cell proliferation and apoptosis. To evaluate the application of Ga ions for biomedical treatment, the effect of Ga ions on cell proliferation and apoptosis was assessed. MC3T3-E1 cells were treated with GaN, TRPM7 siRNA or LY294002 for 0, 7 and 14 days. Subsequently, the

CCK-8 assay was performed to assess cell proliferation. After 7 and 14 days of culture, osteogenic induction significantly increased MC3T3-E1 cell proliferation compared with the control group. Following culture for 7 or 14 days, GaN treatment significantly increased MC3T3-E1 cell proliferation compared with the osteoinduction group (Fig. 6A). However, TRPM7 siRNA transfection and LY294002 treatment significantly inhibited cell proliferation in GaN-treated cells cultured in osteogenic induction medium compared with the corresponding control groups. Similar results were observed for hFOB1.19 cells. Following culture for 3 or 7 days, hFOB1.19 cell proliferation was significantly increased in GaN-treated cells compared with control cells (Fig. 6B). There was no significant difference in cell proliferation between the GaN and GaN + control siRNA group. However, TRPM7 siRNA transfection and LY294002 treatment significantly decreased GaN-treated cell proliferation compared with the corresponding control groups. The results indicated that GaN promoted osteoblast proliferation, whereas TRPM7 siRNA and LY294002 inhibited cell proliferation.

To further investigate the cytotoxicity of GaN, cell apoptosis was evaluated. MC3T3-E1 cells were treated with GaN, TRPM7 siRNA or LY294002 for 0, 7 or 14 days, and cell apoptosis was evaluated by performing flow cytometry. The results demonstrated that GaN-treated cells cultured in osteogenic induction medium displayed a similar level of apoptosis compared with untreated cells cultured in osteogenic induction medium, which indicated that GaN did not significantly alter MC3T3-E1 cell apoptosis (Figs. 6C and S1). In addition, GaN treatment did not significantly alter hFOB1.19 cell apoptosis compared with the control group (Figs. 6D and S2). However, on day 7, following TRPM7 siRNA transfection or LY294002 treatment, hFOB1.19 cell apoptosis was increased in GaN-treated cells, although the difference between the
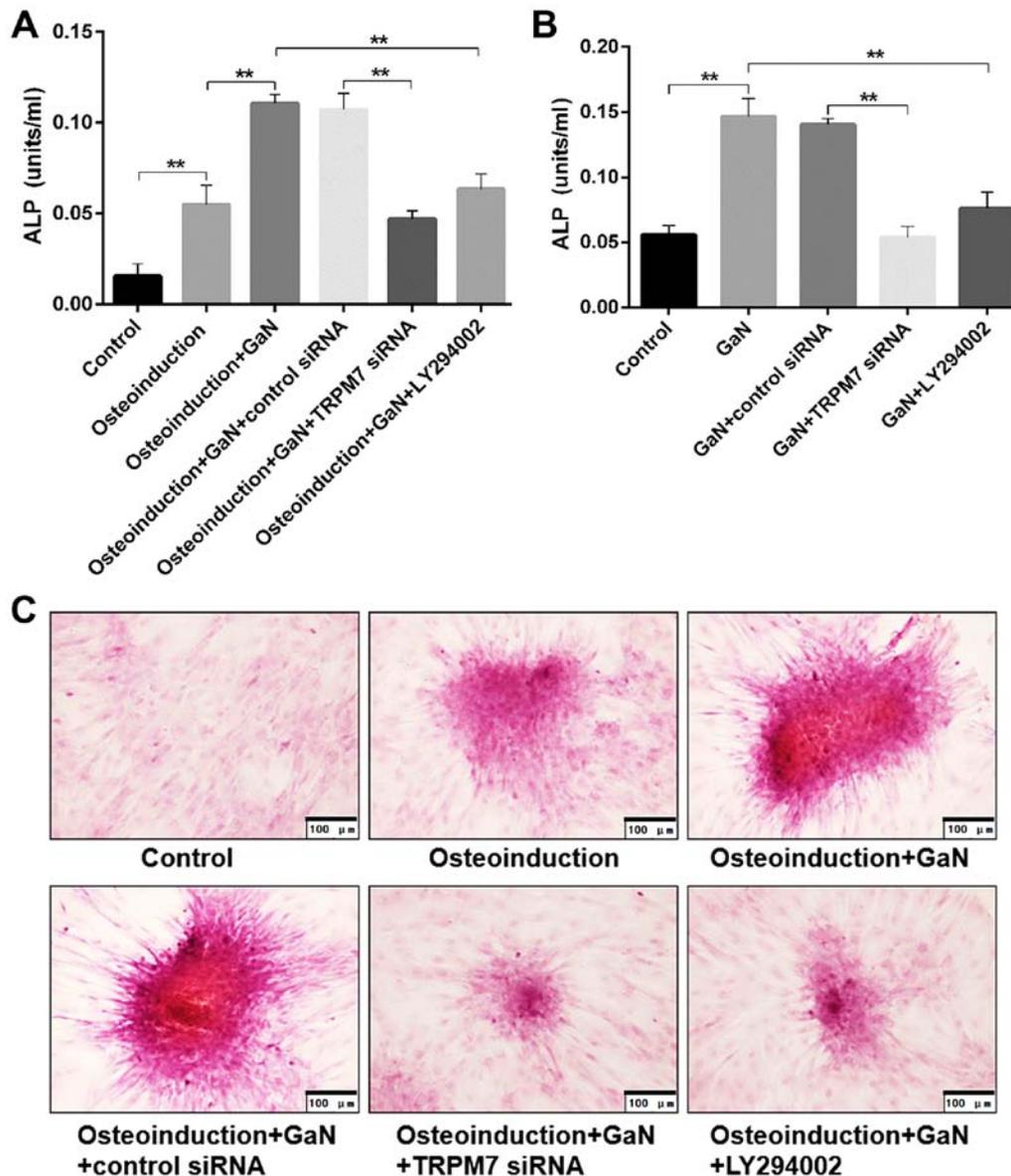
Figure 4. Effect of Ga ions on ALP activity and calcium nodule formation in MC3T3-E1 and hFOB1.19 cells. Cells were treated with GaN (10^{-4} M), with or without pre-treatment with TRPM7 siRNA (transfection for 96 h) or LY294002 (10 μM). ALP activity of (A) MC3T3-E1 and (B) hFOB1.19 cells. (C) Representative images of Alizarin Red S staining of MC3T3-E1 cells (scale bar, 100 μm). **$P<0.01$. Ga, gallium; ALP, alkaline phosphatase; siRNA, small interfering RNA; GaN, gallium nitride; TRPM7, transient receptor potential melastatin 7.

groups was not significant. The results further indicated the biocompatibility of Ga ions, providing support for the biomedical application of GaN.

Effect of Ga ions on cell migration. Osteoblast migration and invasion are physiological and pathological processes, which are crucial for maintaining the health and function of bone tissues (3,29,30); therefore, it is necessary to maintain and promote the biological activity of osteoblasts during the treatment of osteoporosis. The effect of Ga ions on the migratory ability of osteoblasts was investigated by performing crystal violet staining. Compared with control cells, the migratory ability of hFOB1.19 cells was significantly increased in GaN-treated cells. Control siRNA transfection did not alter the migratory ability of GaN-treated cells; however, TRPM7 siRNA transfection and LY294002

treatment significantly reduced the migratory ability of GaN-treated cells compared with the corresponding control groups (Fig. 7A and B).

Effect of Ga ions on cell invasion. The cell invasion ability of hFOB1.19 cells was evaluated by performing crystal violet staining and quantified by cell counting. The invasion ability of GaN-treated cells and GaN-treated + control siRNA-transfected cells was significantly increased compared with control cells. However, GaN-induced cell invasion was significantly inhibited by TRPM7 siRNA-transfection and LY294002 treatment compared with the corresponding control groups. Cell invasion was increased by 3-fold in GaN-treated and GaN-treated + control siRNA-transfected cells compared with control cells. By contrast, following TRPM7 siRNA transfection or LY294002 treatment, the number of invading
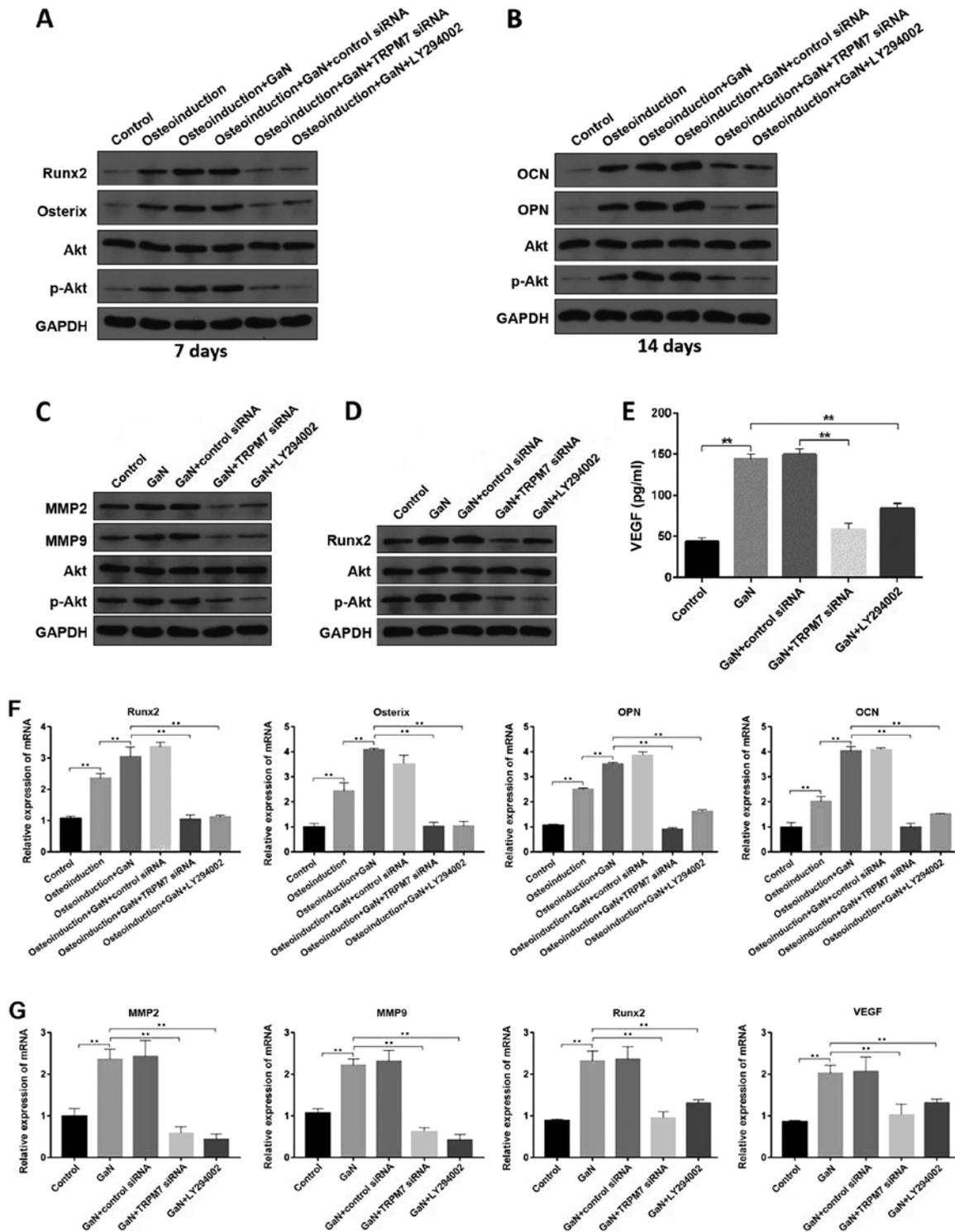
Figure 5. Effect of Ga ions on osteogenic-related protein expression in MC3T3-E1 and hFOB1.19 cells. Cells were treated with GaN (10^{-4} M), with or without pre-treatment with TRPM7 siRNA (transfection for 96 h) or LY294002 (10 μM). Protein expression levels of (A) Runx2, Osterix, Akt, p-Akt after 7 days, (B) OCN, OPN, Akt and p-Akt after 14 days in MC3T3-E1 cells. Protein expression levels of (C) MMP2, MMP9, Akt, p-Akt after 3 days, (D) Runx2, Akt and p-Akt after 7 days in hFOB1.19 cells. (E) Expression level of VEGF protein in hFOB1.19 cells. Akt and p-Akt were measured throughout the treatment process. (F) Relative mRNA expression levels of Runx2, Osterix, OPN and OCN in MC3T3-E1 cells. (G) Relative mRNA expression levels of MMP2, MMP9, Runx2 and VEGF in hFOB1.19 cells. **P<0.01. Ga, gallium; Runx2, Runt-related transcription factor 2; p, phosphorylated; OCN, osteocalcin; OPN, osteopontin; GaN, gallium nitride; siRNA, small interfering RNA; MMP, matrix metallopeptidase; VEGF, vascular endothelial growth factor; TRPM7, transient receptor potential melastatin 7.

cells was similar to the control group (Fig. 8A and B). The results suggested that GaN enhanced cell invasion, whereas cell invasion was inhibited in the absence of TRPM7 and Akt expression.

Discussion

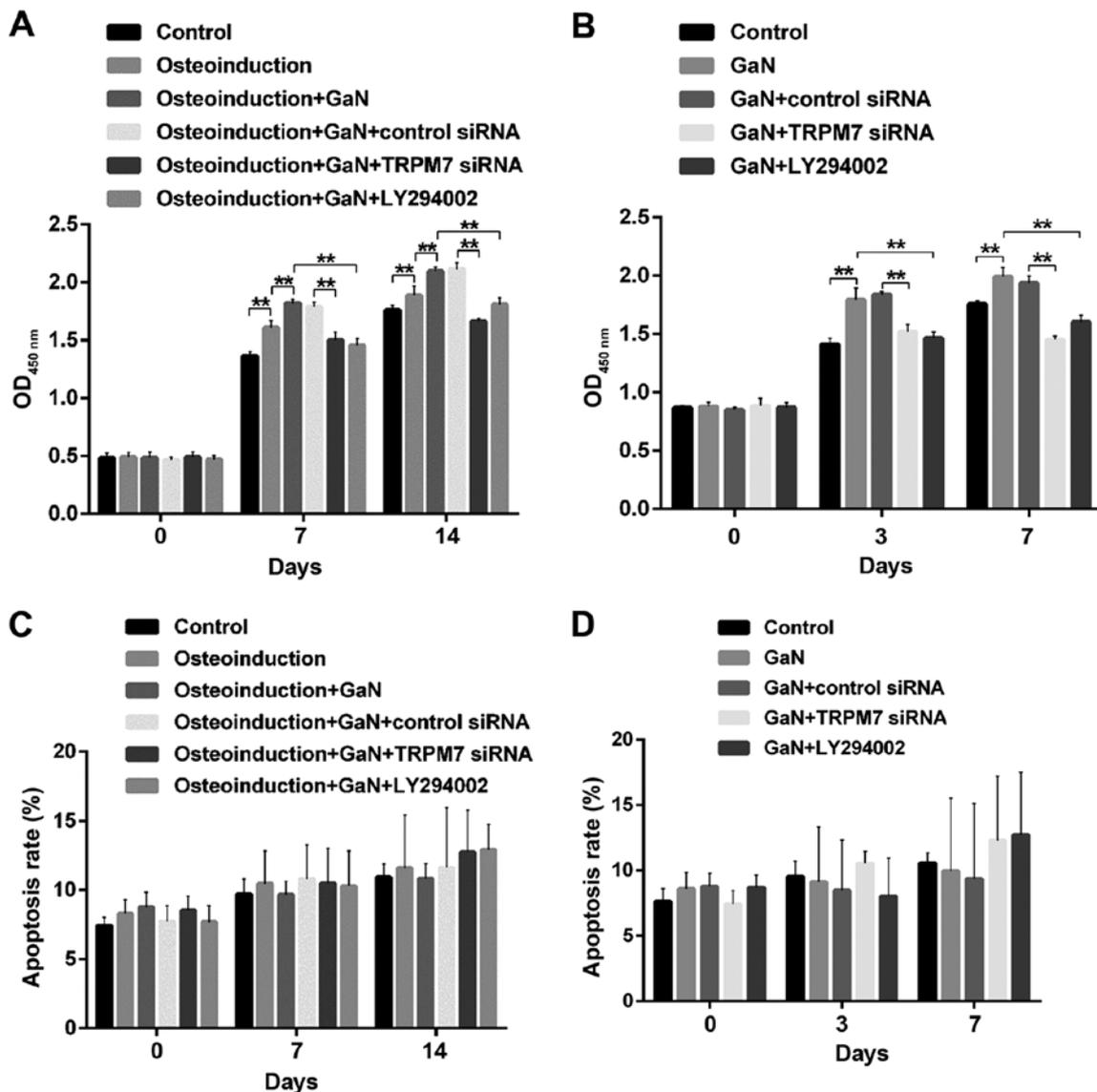Following the discovery of Ga compounds, they have been used for different biomedical applications; for example,

Figure 6. Effect of Ga ions on MC3T3-E1 and hFOB1.19 cell proliferation. MC3T3-E1 cells were treated with GaN (10^{-4} M), with or without pre-treatment with TRPM7 siRNA (transfection for 96 h) or LY294002 (10 μM) for 0, 7 or 14 days. hFOB1.19 cells were treated with GaN (10^{-4} M), with or without pre-treatment with siRNA (siRNA transfection for 96 h) or LY294002 (10 μM) for 0, 3 or 7 days. (A) MC3T3-E1 and (B) hFOB1.19 cell proliferation. (C) MC3T3-E1 and (D) hFOB1.19 cell apoptosis. **P<0.01. Ga, gallium; GaN, gallium nitride; siRNA, small interfering RNA; OD, optical density; TRPM7, transient receptor potential melastatin 7.

gallium nitrate was found to reduce blood calcium levels and decrease bone turnover in patients with hypercalcium and Pagets disease (29). Ga compounds often display a high affinity for proliferating biological tissues, such as solid tumors (30). In addition, Ga possesses antineoplastic properties, similar to the chemical element platinum (3). The properties of Ga have attracted increasing attention in the field of cancer treatment research. To date, considerable antitumor efficacy has been achieved in inhibiting the growth of malignant lymphoma (31), melanoma (32) and epithelial ovarian cancer (33). In addition, Ga has been reported to accumulate into inflamed and infected sites, manifesting anti-inflammatory and immunosuppressive properties in animal models of human diseases (34). For example, the subcutaneous administration of GaN suppressed the development of a rat model of adjuvant arthritis (35). Furthermore, the bone affinity and antiresorptive activity of Ga indicated its potential therapeutic value in suppressing

osteolysis and bone pain, which are associated with malignant bone tumors and bone metastases (6). However, the potential therapeutic applications of Ga in bone-related diseases have not been extensively investigated.

Osteoporosis is a common disease characterized by a systemic decrease in bone mass and disruption of micro-architecture. Due to an increased osteoclast population, or excessive osteoclast activity and decreased osteoblast activity in unhealthy bone sites, the treatment of osteoporosis may involve inhibition of osteoclast activity and/or promotion of osteoblast osteogenesis (19), However, few drugs with such actions are currently available. In a previous study, Ga ions inhibited the function and resorbing activity of osteoclasts in a dose-dependent manner (10); however, the effect of Ga ions on osteoblastogenesis was not investigated.

In the present study, Ga ions promoted the osteoinduction of both mouse and human osteoblasts, by enhancing ALP
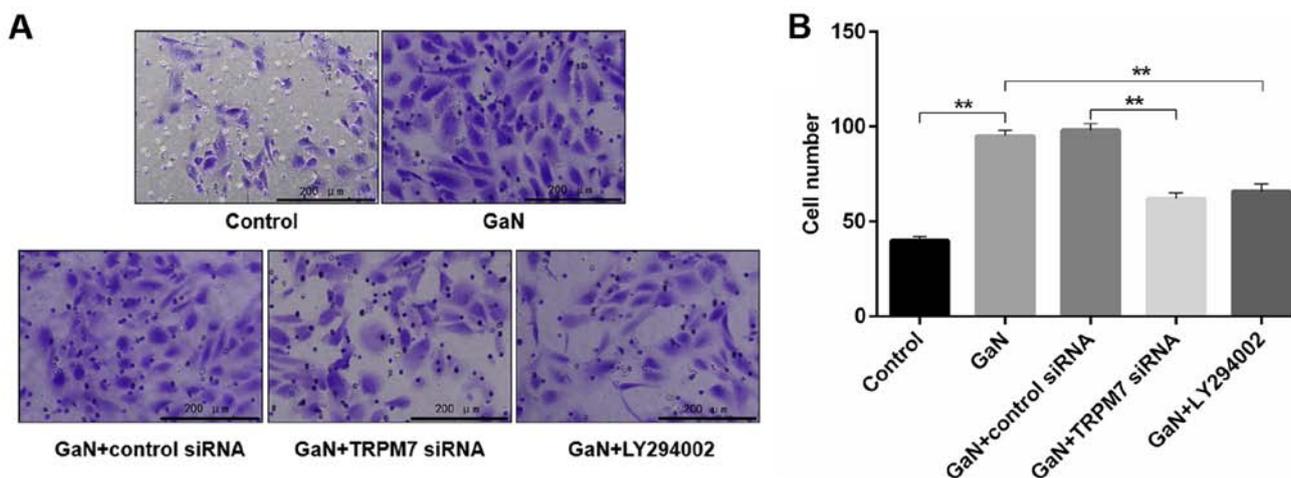
Figure 7. Effect of Ga ions on hFOB1.19 cell migration. hFOB1.19 cells were treated with GaN (10^{-4} M) for 24 h, with or without pre-treatment with TRPM7 siRNA (transfection for 96 h) or LY294002 (10 μM). (A) Representative images of the Transwell migration assay (scale bar, 200 μm). (B) Quantification of cell migration. **P<0.01. Ga, gallium; GaN, gallium nitride; siRNA, small interfering RNA; TRPM7, transient receptor potential melastatin 7.
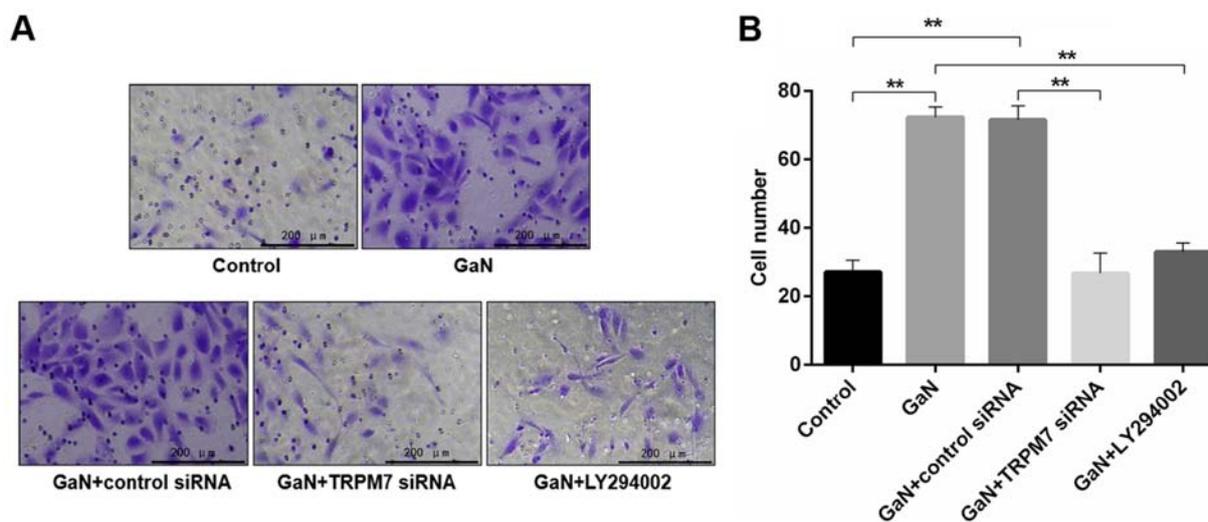

Figure 8. Effect of Ga ions on hFOB1.19 cell invasion. hFOB1.19 cells were treated with GaN (10^{-4} M) for 24 h, with or without pre-treatment with TRPM7 siRNA (transfection for 96 h) or LY294002 (10 μM). (A) Representative images of the Transwell invasion assay (scale bar, 200 μm). (B) Quantification of cell invasion. **P<0.01. Ga, gallium; GaN, gallium nitride; siRNA, small interfering RNA; TRPM7, transient receptor potential melastatin 7.

activity, calcium nodule formation and osteogenic-related protein expression. Mouse MC3T3-E1 cells are pre-osteoblasts and it has been suggested that osteoinduction (50 mg/ml β-glycerophosphate and 10 mM L-ascorbic acid) can be used to promote the differentiation and maturation of the cells. By contrast, human hFOB1.19 cells are osteoblasts, so osteoinduction is not required (36). In the present study, Ga ions altered ALP activity in a concentration-dependent manner, increasing ALP activity to the highest level at a concentration of 10^{-4} M, which was considered as the optimal concentration for osteogenic differentiation of osteoblasts. However, a previous study reported that the optimal concentration of Ga ions for osteoclast inhibition was 100 mg/ml (10). The results suggested that osteoclast activity inhibition and promotion may not be achieved at the same dose. Controlled drug administration in a stepwise manner may serve as a viable strategy for effectively suppressing and promoting osteoclast activity for the treatment of osteoporosis.

Exploring the biological mechanisms underlying the positive effect of Ga ions on the osteoinduction of osteoblasts is important. TRPM7, a member of the transient receptor potential (TRP) protein family, is an ion channel protein with phosphate kinase activity, which is associated with cell proliferation and motility, and functions in various normal tissues (22,37-41). A previous study demonstrated that magnesium ions promoted osteoinduction of human osteoblasts via a TRPM7-related signaling pathway (22). Akt is a key signal transduction molecule in living systems, which can regulate cell proliferation, differentiation, apoptosis and glucose transport (42). Akt phosphorylation serves a key role in the osteogenesis of osteoblasts, as it can upregulate the Runx2 gene, which induces the expression of genes associated with bone repair and reconstruction, such as OCN, collagen I, OPN and ALP (43). Therefore, it was hypothesized that the TRPM7/Akt signaling pathway may also be involved in Ga ion-mediated osteoinduction in osteoblasts. Following

TRPM7 knockdown and inhibition of Akt activity, ALP activity, calcium nodule formation, osteogenic-related protein expression in osteoblasts was significantly decreased. The results indicated that Ga ions promoted the osteoinduction of osteoblasts via the TRPM7/Akt signaling pathway. TRPM7 is a channel protein for magnesium and calcium ions (22). The specific interaction between TRPM7 and Ga ions requires further investigation. In the present study, the two cell lines were cultured for different lengths of time due to different cell characteristics, such as growth rate and the time of cell passage. For instance, regarding hFOB1.19 cells, the suggested observation window for ALP activity and calcium nodule formation is 3-7 days; however, for MC3T3-E1 cells, the observation window of ALP activity and calcium nodule formation is 7-14 days. Therefore, the osteogenic characteristics of hFOB1.19 and MC3T3-E1 cells were observed at three different time points.

In addition to osteoinduction, osteoblast proliferation and apoptosis was also assessed. Ga ions promoted human and mouse osteoblast cell proliferation without causing cell apoptosis, which indicated that GaN may ensure sufficient numbers and activity of osteoblasts for bone repair. Osteoblast migration and recruitment are physiological and pathological processes that are crucial for maintaining bone health and normal function (44-46). In the present study, Ga ions promoted human osteoblast migration and invasion. The unique biological characteristics of Ga ions identified in the present study may justify their therapeutic application for osteoporosis.

The present study had several limitations. Firstly, Alizarin Red S staining was only performed in the MC3T3-E1 cell line. Therefore, Alizarin Red S staining in the hFOB1.19 cell line should be conducted in future studies. Secondly, the two cell lines were observed at different time points; however, within each cell line, the time points were identical for the control and treatment groups. Thirdly, the present study examined the expression of different proteins related to pre-osteoblasts and osteoblasts. Pre-osteoblasts (MC3T3-E1) were used to study the effects of Ga ions on the expression levels of Osterix, OCN and OPN, whereas osteoblasts (hFOB1.19) were used for studying the effects of Ga ions on the expression levels of MMP2, MMP9, p-Akt, Runx2 and VEGF. Finally, the migration and invasion assays were not performed for MC3T3-E1 cells as they are pre-osteoblasts; therefore, future studies should investigate the effects of Ga ions on MC3T3-E1 cell migration and invasion. In addition, future studies should verify the results of the present study using an additional cell line.

In summary, the present study investigated the effect of Ga ions on the osteoinduction of osteoblasts and explored the underlying biological mechanisms. Ga ions increased intracellular ALP activity in a concentration-dependent manner. At the optimal concentration, Ga ions increased ALP activity, accelerated the formation of calcium nodules and upregulated the expression levels of osteogenic-related proteins in osteoblasts. Ga-mediated biological actions were associated with the TRPM7/Akt signaling pathway and were suppressed by inhibiting TRPM7 and p-Akt expression. In addition, Ga ions did not induce cell apoptosis, but promoted osteoblast proliferation, migration and invasion. To the best of our knowledge, the present study was the first to investigate the effects of Ga ions in osteoinduction of osteoblasts. Therefore, the results of the present study may provide a novel therapeutic strategy for osteoporosis and other bone-related diseases.

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Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DZ and YW designed the study. GG made substantial contributions to conception and design, and final approval of the version to be published. MY and DC performed the experiments. YZ analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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