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Original Article

Alendronate stimulates osteoblast differentiation through PKA-STAT3 and STAT1 in an osteoporosis rat model

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Abstract: Alendronate is the most common used for the treatment of osteoporosis. However, the underlying pathological molecular mechanisms of Alendronate-mediated osteoblast differentiation are not clearly explained. In this study, the Alendronate-mediated signal pathway in osteoblast was examined in an osteoporosis rat model. PKA-STAT3 and activator of transcription 1 pathway (STAT1) signaling pathway was studied in osteoblast after treatment with Alendronate *in vitro*. Cell viability assay, cell differentiation assay, gene silencing, and Western blot techniques were used to analyze the effects of Alendronate on osteoclasts and osteoblasts activity and PKA-STAT3 and STAT1 signaling pathway. Results showed that Alendronate significantly inhibited the activity of osteoporotic osteoclasts and increased the viability and activity of osteoblasts compared to control. Alendronate increased expression and phosphorylation levels of PKA, STAT3, and STAT1 in osteoclasts. Alendronate also enhanced osteoblast differentiation, up-regulated expression levels of alkaline phosphatase and osteocalcin. Knockdown of PKA or transcription 1 abolished Alendronate-increased the viability and activity of osteoblasts. In conclusion, these results suggest that Alendronate can regulate osteoblast differentiation through up-regulation of PKA-STAT3 and STAT1 pathway.

Keywords: Alendronate, osteoporosis, osteoblast, PKA, STAT3, STAT1

Introduction

Osteoporosis is a disease characterized by low bone density and bone strength, which can increase the risk of fractures [1]. The clinical consequences of osteoporosis are fractures in the upper extremity, hip and even spine, which may result in loss of function and independence, impairment quality of life [2]. The World Health Organization has identified osteoporosis as one of the leading health problems in the Western world [3]. Various treatments for osteoporosis have proposed in a large number of reports [4-7]. Currently, the levels of osteo-associated hormones can be detected in patients with osteoporosis [8-10]. Additionally, comprehensive treatments of anti-restorative agents in preventing new non-vertebral fractures in patients with osteoporosis have been investigated in preclinical and clinical investigation [11].

Bisphosphonates have been widely used to prevent and treat osteoporosis since the introduction of alendronate in 1995 [12-14]. In recent years, Alendronate is an efficient drug for fracture prevention in women with osteoporosis [15]. Patients who received the Alendronate therapy increased bone mineral density in elderly postmenopausal women with established osteoporosis [16]. Alendronate therapy increased bone turnover markers in androgen deprivation therapy-related osteoporosis in a prospective randomized multicenter international study [17]. Effects of Alendronate (70 mg) in a short-term treatment with once-a-week medication have been clinically investigated in women with postmenopausal osteoporosis determined by bone turnover markers [18]. These data suggest that Alendronate treatment is beneficial for the treatment of patients with postmenopausal osteoporosis.

The importance of STAT1 gene expression in monocytes has been reported and outcomes have found that the progression of osteoporosis could be regulated by expression and phos-

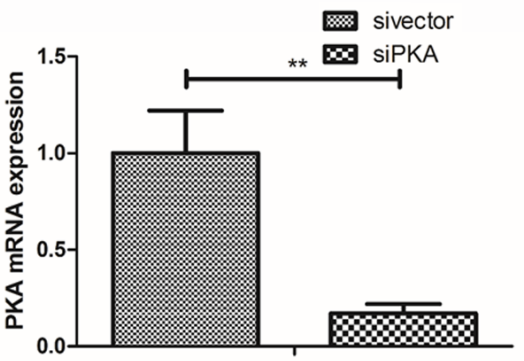

Figure 1. Expression of PKA in osteoblast after transfection with siPKA. **P<0.01 vs. siRNA vector.

phorylation levels of STAT1 [19]. STAT1 Signaling pathway is strongly activated by IFN-β in the pathogenesis and progression of osteoporosis and previous results contribute to well understand pathological signaling pathways of osteoporosis [20]. A study also found that pathway blocking cAMP-dependent protein kinase A (PKA) pathway exhibited its antagonistic roles for caffeine-induced osteoporosis [21]. Alendronate also promoted osteoblast differentiation and bone formation through interferon-beta/signal transducer and activator of transcription 1 pathway in ovariectomy-induced osteoporosis [22]. These findings suggest that PKA-STAT3 and activator of transcription 1 pathway may be associated with Alendronate-mediated anti-osteoporosis therapy.

In this study, a possible mechanism mediated by Alendronate was investigated in osteoblast in rat with osteoporosis. Osteoblast differentiation was analyzed after treatment with Alendronate. PKA-STAT3 and STAT1 pathway signaling pathway was also investigated in osteoblast after treatment with Alendronate.

Materials and methods

Cells culture

Osteoclasts and osteoblasts were isolated from osteoporosis rat model as described previously [23]. Osteoclasts and osteoblasts were grown in RPMI-1640 culture medium (Gibco, Life Technologies, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO_2. Cells were treated with STAT1 inhibitor (1 mg/ml, A4ZVS7, S UniProtKB) for 6 hours at 37°C for further analysis.

Cells viability assay

Osteoclasts (2×10^3 cells/well) or osteoblasts (2×10^3 cells/well) were seeded in 96-well plates and cultured at 37°C for 12 hours. Cells were then treated with 10 µl of MTT (5 mg/ml, Sigma-Aldrich) for 3 hours at 37°C. After incubation, purple formazan crystals were dissolved using isopropanol (15 µl, isopropanol). The absorbance was recorded on a microplate reader (Multiskan FC, THERMO SCIENTIFIC) at a wavelength of 570 nm. Cells viability was determined by percent of cell viability calculated as the ratio between mean absorbance of three samples and mean absorbance of controls.

Gene silencing

PKA silencing was performed by using lentiviral vectors expressing specific siRNA-PKA (siPKA, 50 nM; sense, 5'-UUACGGUUCCUAUAUAACGdTdT-3'; antisense, 5'-CGUUAUAUAGGAACCGUAAdTdT-3'; OriGene Technologies, Inc. MD, USA), (50 nM; sense, 5'-UUACGGUUCCUAUAUAACGdTdT-3'; antisense, 5'-CGUUAUAUAGGAACCGUAAdTdT-3'; OriGene Technologies, Inc. MD, USA) scramble (control) siRNA-vector (50 nM; sense, 5'-AAUCCGCUGUCGGCUGGA AdTdT-3'; antisense, 5'-UUCCAGCCGACAGCGGAUUdTdT-3'; OriGene Technologies, Inc. MD, USA, USA). Experiments were performed by using Amaxa Electroporation System (Amaxa Inc, Germany) according to the manufacturer's protocol. After 48 hours, siRNA Transfection efficiency was assessed by Real Time-PCR using PKA gene specific primer probes (**Figure 1**).

Western blot

Osteoblasts (1×10^7) were lysed in RIPA buffer (Thermo Scientific) and homogenized at 4°C for 10 minutes. Protein concentration was measured by a BCA protein assay kit (Thermo Scientific, Pittsburgh PA, USA). A total of 10 µg protein was electrophoresed on 12% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, USA). The membranes were incubated in blocking buffer (5% BSA) prior to incubation with primary antibodies: PKA (1:1,000, ab75991, Abcam), Abcam, STAT1 (1:1,200, ab31369, Abcam), pSTAT1 (1:1,000, ab30645, Abcam), STAT3 (1:1,200, ab68153, Abcam), pSTAT3 (1:500, ab76315, Abcam), alkaline phosphatase (ALP) (1:1,000, ab95462, Abc-

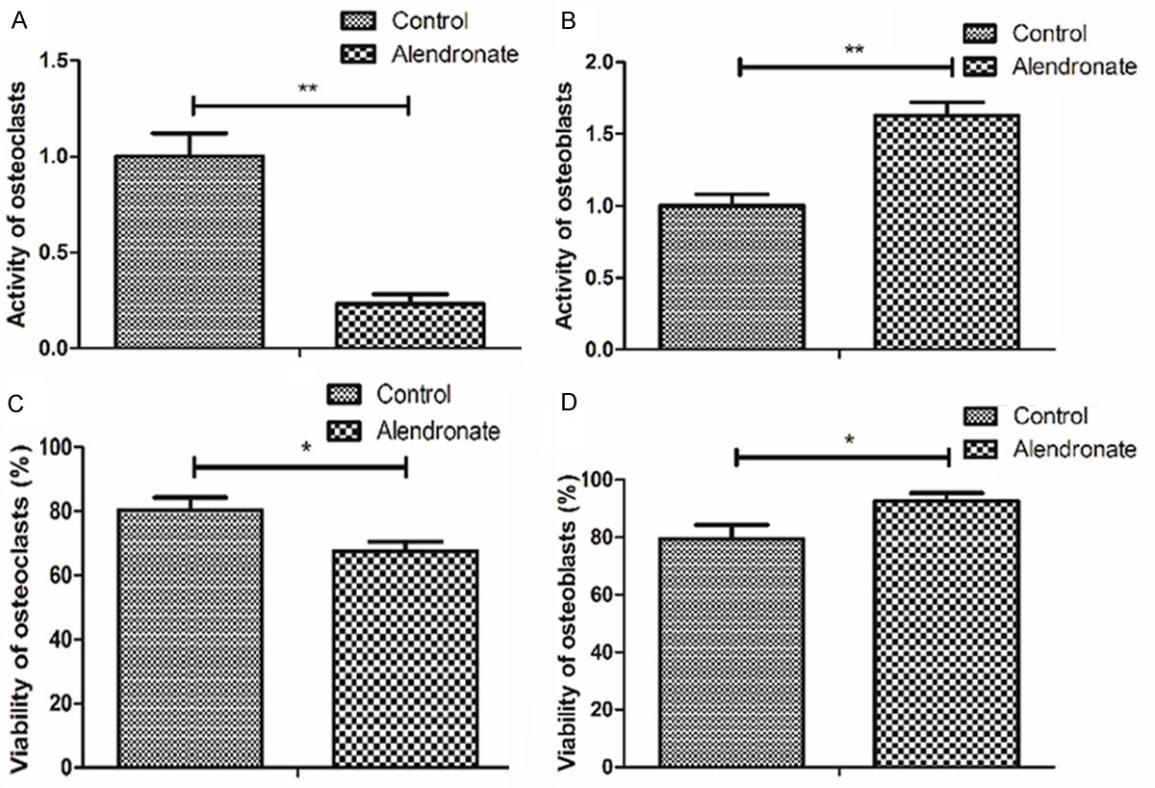

Figure 2. Alendronate regulates the activity of osteoporotic osteoclasts and osteoblasts (A, B) Effects of Alendronate on the activity of osteoporotic osteoclasts. (A) and osteoblasts (B) *in vitro*. (C, D) Effects of Alendronate on viability of osteoporotic osteoclasts (C) and osteoblasts (D). *P<0.05, **P<0.01 vs. control.

am), osteocalcin (1:1,000, ab93876, Abcam), GSH-Px (1:1,000, ab94733, Abcam), OPG (1:1000, ab73400, Abcam), and β-actin (1: 2,000, ab8226, Abcam) for 12 hours at 4°C. The membrane was washed three times in PBST and incubated with HRP-conjugated goat anti-rabbit IgG mAb (1:2000, PV-6001, ZSGB-BIO, Beijing, China) for 2 hours at 37°C. After three-time washing in PBST, membrane was developed using a chemiluminescence assay system (Roche) and exposed to Kodak exposure films. Densitometric quantification of the immunoblot data was performed by using the software of Quantity-One 1.0 (Bio-Rad).

Cells differentiation of osteoblasts

Osteoblasts (1×10^7) were cultured in RPMI-1640 culture medium with 10% FBS at 37°C for 12 hours. Cells were treated with Alendronate (2 mg/ml, Sigma-ALdrich) for 120 hours at 37°C. The cells were fixed in 4% paraformaldehyde for 30 minutes at 37°C and were stained with 2% Alizarin Red S (pH 7.2, Sigma-Aldrich) for 30 minutes at room temperature. The cells were captured under light microscopy (Zeiss Axioplan; Zeiss S.p.A., Milano, Italy).

Assessment of osteoclasts and osteoblasts activity

Osteoclasts (1×10^5 cells/well) or osteoblasts (1×10^5 cells/well) were plated on a 6-well plate, treated with Alendronate (2 mg/ml, Sigma-ALdrich) for 24 hours at 37°C. Cells were cultured until they reached 70% confluency. To measure the osteoclasts and osteoblasts activity, cells were washed twice with PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Osteoclasts and osteoblasts activity was assayed using p-nitrophenylphosphate as a substrate by the Alkaline Phosphatase Test (Beyotime Biotechnology, China).

Statistical analysis

All data were analyzed by SPSS 17.0 software (SPSS, Chicago, IL, USA). Data are presented as means ± SD. Significant differences between

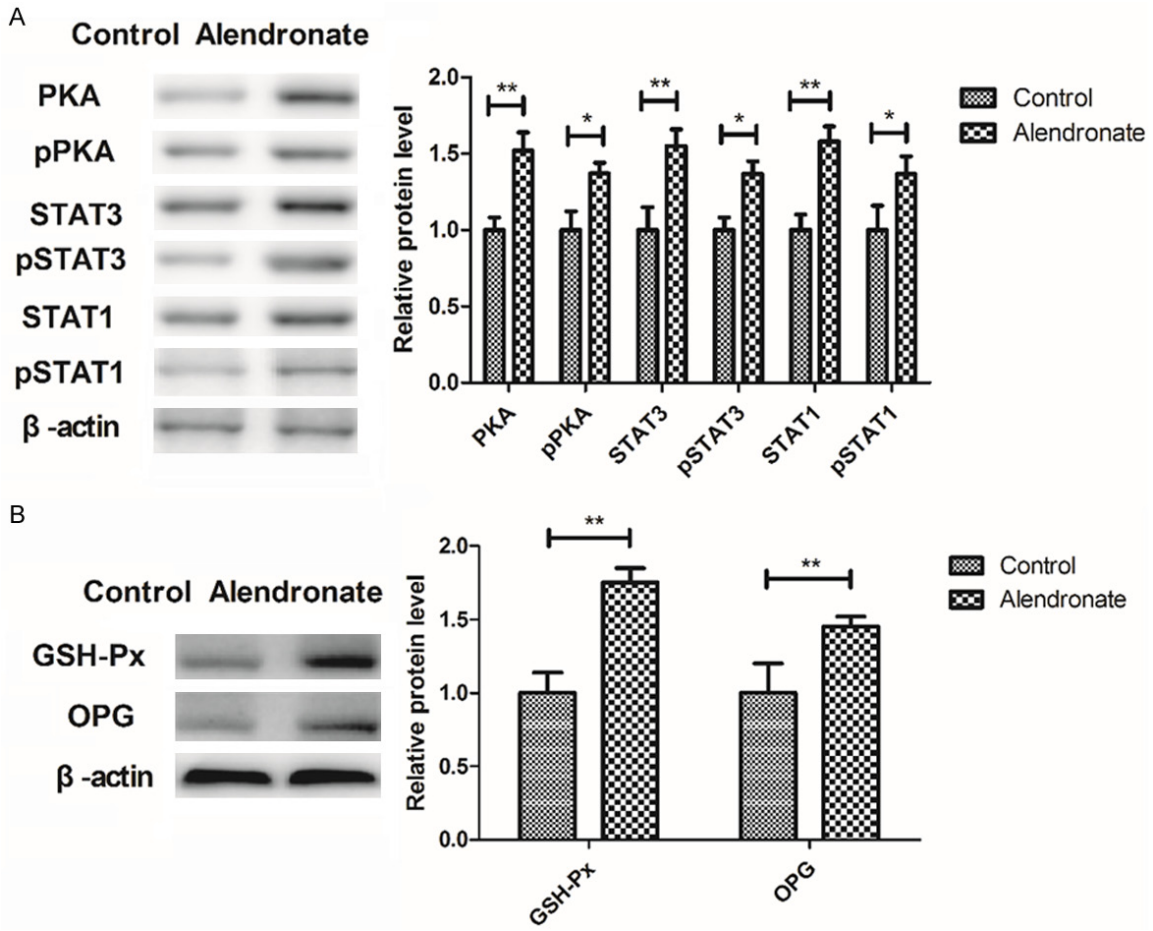

Figure 3. Alendronate down-regulates expression and phosphorylation levels of PKA, STAT3, and STAT1 in osteoclasts. A. Effects of Alendronate on expression and phosphorylation levels of PKA, STAT3 and STAT1 in osteoclasts. B. Effects of Alendronate on glutathione peroxidase (GSH-Px) and osteoprotegerin (OPG) expression in osteoclasts *in vitro*. *P<0.05, **P<0.01 vs. control.

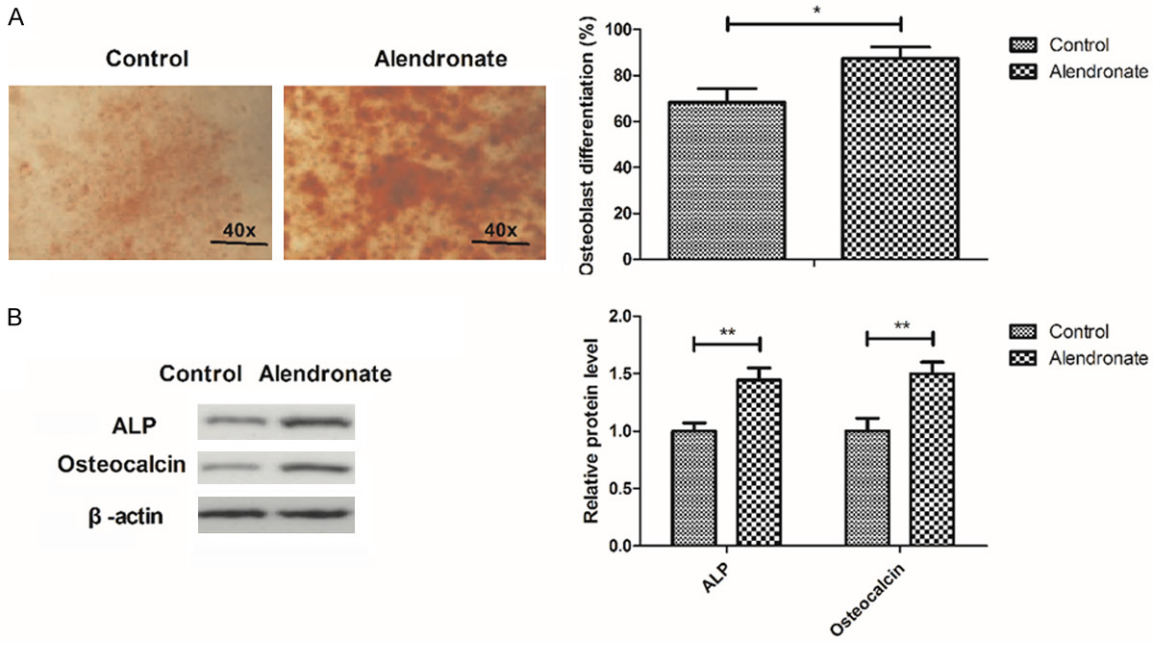

Figure 4. Alendronate enhances osteoblast differentiation and up-regulates expression levels of alkaline phosphatase and osteocalcin. A. Effects of Alendronate on osteoblast differentiation. B. Effects of Alendronate on alkaline phosphatase (ALP) and osteocalcin expression in osteoblast *in vitro*. *P<0.05, **P<0.01 vs. control.

two groups were analyzed by two-tail unpaired Student's *t*-test. Multiple groups differences were analyzed using one-way analysis of variance (ANOVA) followed Tukey HSD test. A *P*-value of <0.05 was considered to indicate a statistically significant.

Results

Alendronate inhibits the activity of osteoporotic osteoclasts and increased the viability and activity of osteoblasts

The effects of Alendronate on the activity of osteoporotic osteoclasts and osteoblasts were analyzed *in vitro*. Alendronate decreased the activity of osteoporotic osteoclasts and increased activity of osteoblasts (**Figure 2A**, **2B**). As shown in **Figure 2C**, **2D**, viability of osteoporotic osteoclasts was decreased and viability of osteoporotic osteoblasts was increased by Alendronate *in vitro*. These results indicate that Alendronate may be beneficial for the treatment of osteoporosis.

Alendronate increases expression and phosphorylation levels of PKA, STAT3 and STAT1 in osteoclasts

The changes of expression and phosphorylation levels of PKA, STAT3, and STAT1 were analyzed in osteoclasts. Results in **Figure 3A** demonstrated that Alendronate increased expression and phosphorylation levels of PKA, STAT3, and STAT1 in osteoclasts. As shown in **Figure 3B**, Alendronate effectively increased glutathione peroxidase (GSH-Px) and osteoprotegerin (OPG) expression in osteoclasts *in vitro*. These results indicated that Alendronate can up-regulated PKA, STAT3, and STAT1 expression and phosphorylation in osteoclasts.

Alendronate enhances osteoblast differentiation and up-regulates expression levels of alkaline phosphatase and osteocalcin

The osteoblast differentiation was analyzed after treatment with Alendronate. Results showed that Alendronate increased osteoblast differentiation compared to control (**Figure 4A**). As shown in **Figure 4B**, Alendronate led to up-regulation of alkaline phosphatase (ALP) and osteocalcin in osteoblast *in vitro*. These data indicate that Alendronate is beneficial for the differentiation of osteoblast.

Alendronate stimulates osteoblast differentiation via PKA-STAT3 and activator of STAT1 pathway

Finally, the relationship between Alendronate and PKA-STAT3 and activator of transcription 1 pathway was investigated in osteoblast. Knockdown of PKA decreased STAT3 expression, but not affected STAT1 expression in osteoblasts (**Figure 5A**). Knockdown of PKA canceled Alendronate-increased the viability and activity of osteoblasts (**Figure 5B**, **5C**). As shown in **Figure 5D**, **5E**. STAT1 inhibitor (STAT1IR) abolished Alendronate-increased the viability and activity of osteoblasts. Results demonstrated that knockdown of PKA or STAT1 inhibitor abolished Alendronate-increased osteoblast differentiation (**Figure 5F**, **5G**). These results indicate that Alendronate may promote osteoblast differentiation via PKA-STAT3 and activator of STAT1 pathway.

Discussion

Previous reports have indicated that long-term Alendronate treatment could increase bone mineral density in postmenopausal osteoporosis patients [24-26]. Alendronate treatment in women with postmenopausal osteoporosis has been studied and results have showed that arterial stiffness was improved after monthly Alendronate treatment [27]. Ma et al. have reported that Alendronate promoted osteoblast differentiation and bone formation in ovariectomy-induced osteoporosis through interferon-beta/signal transducer and activator of transcription 1 pathway [22]. In this study, the effects of Alendronate on osteoblast differentiation were analyzed and the potential mechanism mediated by Alendronate in osteoblasts was explored. Findings in this study have indicated that Alendronate could regulate osteoblast differentiation through up-regulation of PKA-STAT3 and activator of transcription 1 pathway in rat with osteoporosis. The data may contribute to clinical treatments for the patients with osteoporosis.

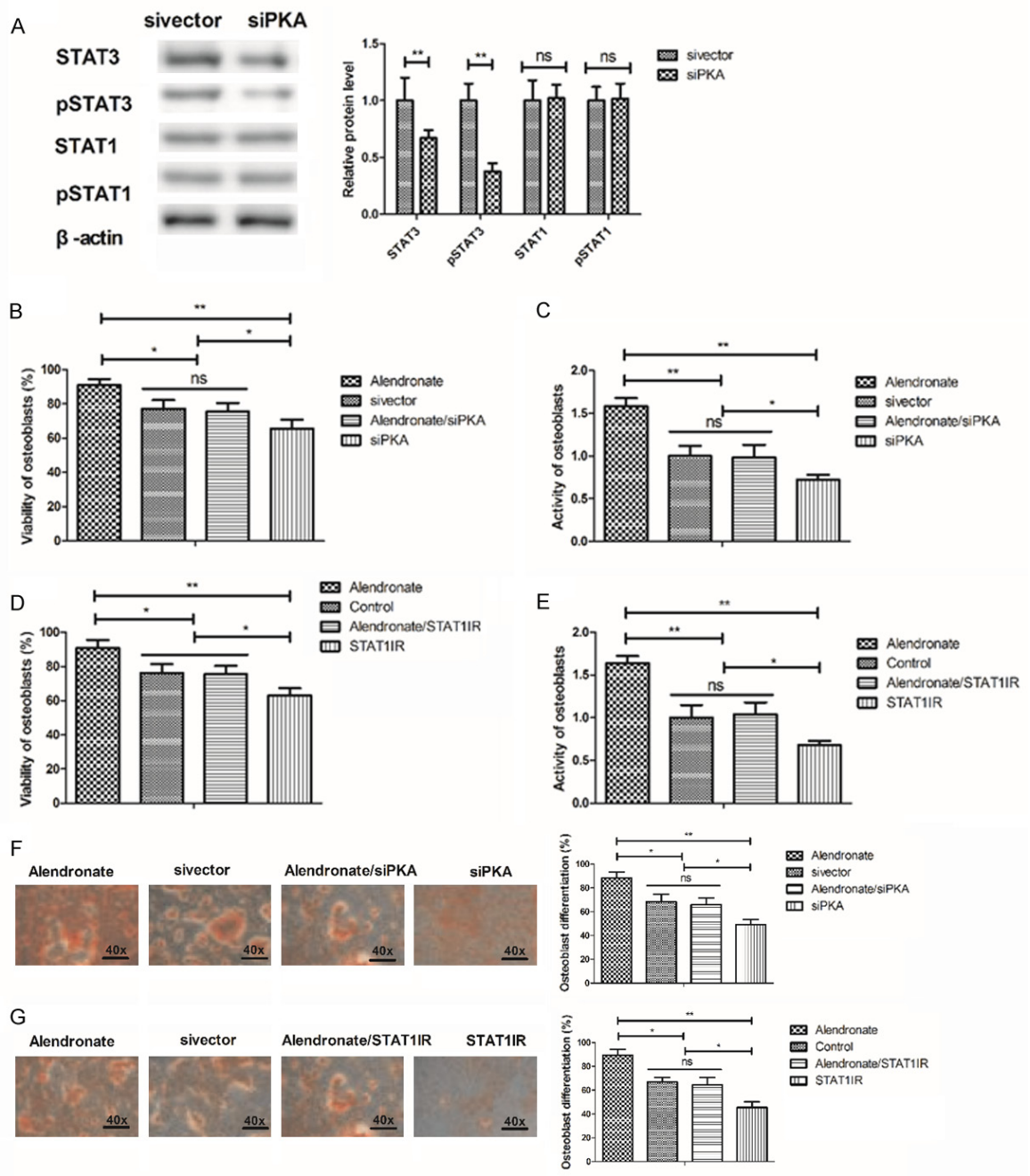

Figure 5. Alendronate stimulates osteoblast differentiation via PKA-STAT3 and activator of STAT1 pathway. (A) Effects of PKA knockdown on expression and phosphorylation of STAT3 and STAT1 in osteoblast. (B, C) Effects of PKA knockdown on Alendronate-increased the viability (B) and activity (C) of osteoblasts. (D, E) Effects of STAT1 inhibitor on Alendronate-increased the viability (D) and activity (E) of osteoblasts. (F, G) Effects of PKA knockdown (F) or STAT1 inhibitor (G) on Alendronate-increased osteoblast differentiation. *P<0.05, **P<0.01 vs. control.

Angiotensin II/Angiotensin II receptor blockade affects osteoporosis via the AT1/AT2-Mediated cAMP-dependent PKA Pathway [28]. Zhang et al. have showed that Osteoporosis with increased osteoclastogenesis in hematopoietic cell-specific STAT3-deficient mice [29]. In this study, Alendronate increased expression and phosphorylation level of PKA, STAT3, and transcription 1 in osteoblast. Study has indicated that the circulating level of OPG was inversely related to BMD and contributed to the development of osteoporosis in postmenopausal wo-

men [30]. Furthermore, STAT3 knockdown abolished Alendronate-increased PKA expression and phosphorylation level. A study demonstrated that Alendronate prevented bone-specific alkaline phosphatase reduction and reduced inflammatory infiltrate, without causing systemic alterations [31]. In addition, urinary osteocalcin is a useful marker for monitoring the effect of Alendronate therapy [32]. Data in this study confirmed the effects of Alendronate therapy on osteocalcin expression in osteoblast.

Alendronate is anti-resorptive drug in osteoporotic disease and Alendronate affects the osteoprotegerin/RANKL system in human osteoblast primary cultures from patients with osteoporosis [33]. A report has found that increasing osteoblast cell proliferation contributed to the treatment of osteoporosis [34]. In this study, Alendronate stimulated osteoblast differentiation and increased osteoblast viability and activity of osteoblasts. However, knockdown of PKA or transcription 1 inhibitor abolished Alendronate-increased osteoblast differentiation. However, whether clinical application of Alendronate could be beneficial for osteoblast differentiation in patients with osteoporosis needs further confirmation.

In conclusion, Alendronate may promote osteoblast differentiation through the PKA-STAT3 and activator of transcription 1 pathway, and Alendronate can increase osteoblast viability and activity of osteoblasts. Nevertheless, this conclusion was based on osteoblasts isolated form rat model of osteoporosis and Alendronate should be evaluated in future clinical trials.

Disclosure of conflict of interest

None.

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