

# GATA4 regulates osteoblastic differentiation and bone remodeling via p38-mediated signaling

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**Abstract** Osteoblasts play a major role in bone remodeling and are regulated by transcription factors. GATA4, a zinc finger transcription factor from the GATA family, has an unclear role in osteoblast differentiation. In this study, the role of GATA4 in osteoblast differentiation was studied both in vitro and in vivo by GATA4 knockdown. GATA4 expression increased during osteoblast differentiation. GATA4 knockdown in osteoblast precursor cells reduced alkaline phosphatase activity and decreased the formation of calcified nodule in an osteogenic-induced cell culture system. In vivo, micro-CT showed that local injection of lentivirus-delivered GATA4 shRNA caused reduced new bone formation during tooth movement. Histological analyses such as total collagen and Goldner's trichrome staining confirmed these results. In vivo immunohistochemical analysis showed reduced expression of osterix (OSX), osteopontin (OPN), and osteocalcin (OCN) in the shGATA4 group ( $P < 0.05$ ). Consistently, both western blotting and quantitative reverse-transcription PCR proved that expression of osteogenesis-related genes, including OSX, OPN, and OCN, was significantly repressed in the shGATA4 group in vitro ( $P < 0.01$ ). For further analysis of the pathways involved in this process, we examined the MAPK signaling pathway, and found knockdown of GATA4, downregulated p38 signaling pathways ( $P < 0.01$ ).

Collectively, these results imply GATA4 is a regulator of osteoblastic differentiation via the p38 signaling pathways.

**Keywords** GATA4 · Osteoblast differentiation · Tooth movement model · Lentiviral · MAPK

## Introduction

Physiologically, the bone remodeling is a complex and a regulated process. The two type of cells involved in bone remodeling: osteoclasts that modulate bone resorption and are derived from hematopoietic cells and osteoblasts that mediate new bone formation and are derived from mesenchymal cells (Pittenger et al. 1999; Shoji et al. 2010; Tripu-wabhrut et al. 2013; Yokoya et al. 1997). Balance between osteoclasts and osteoblasts is vital for bone homeostasis, whereas an imbalance may lead to bone diseases such as osteoporosis, hyperostosis, and periodontal disease (Teitelbaum 2000).

Proliferation, differentiation, and maturation of osteoblasts are critical events in bone formation and remodeling. Osteoblast differentiation is a complex process, consisting of maturation of preosteoblasts into matrix-secreting mature osteoblasts (Pittenger 2008; Rodriguez-Lozano et al. 2011). Osteoblasts synthesize and release various osteoblast-specific marker proteins such as alkaline phosphatase (ALP), osterix (OSX), osteopontin (OPN), and osteocalcin (OCN), which not only function as structural support, but also play a role in osteoblast maturation and function with regard to cell interaction, matrix deposition, and mineralization (de Oliveira et al. 2003; Wu et al. 2015). Therefore, differentiation of osteoblasts is controlled by orchestration of diverse transcription factors.

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GATA4, a transcription factor, and a member of the GATA zinc finger family, proves to be a cardiac-specific transcriptional activator. Previous studies have pointed out that GATA4 is not only necessary for stabilization of post-natal cardiac function and avoiding stress-induced heart failure but also controls pathological cardiac hypertrophy, which may give rise to serious cardiac diseases. In addition to its important role in cardiac pathology, GATA4 has also been reported to regulate transcription in the gut, lungs, and ovaries (Mazaud et al. 2007; Molkentin et al. 1998; Viger et al. 2008). Mitogen-activated protein kinases (MAPKs) cascades are evolutionarily conserved and are the fundamental mechanism for cellular responses to a broad range of extracellular signals, especially many of the extracellular ligands related to osteoblasts, such as PTH, BMPs, noncanonical WNTs, FGFs, and TNF (Suzuki et al. 2002; Xiao et al. 2000; Zhou et al. 2006). Previous studies have shown that GATA4 is a vital transcription factor that induces/regulates the hypertrophy partly by receiving signals from MAPKs in the heart (van Berlo et al. 2011). Nevertheless, the synergistic effect of GATA4 and MAPK in osteoblast differentiation is still unclear.

In this study, the role of GATA4 in the regulation of the osteoblast differentiation and bone formation process was examined. The study showed that knockdown of GATA4 in osteoblast precursor cells and in mouse tooth movement (TM) model inhibited osteoblast differentiation and decreased expression of bone-related genes both in vitro and in vivo. Additionally, the study showed that p38 signaling mediates these processes under the influence of GATA4. These results imply GATA4 is a vital regulator of osteoblast differentiation, which may provide a new understanding to osteogenesis and lead to better treatment alternatives for bone diseases.

## Materials and methods

### Experimental animals

Mice (C57BL/6J) were housed in the Nanjing Medical University animal facility and had free access to mouse water and chow. All animals were handled in compliance with protocols approved by the Experimental Animal Care and Use Committee of Nanjing Medical University (permit no. 2015-03-40).

### Osteoblast precursor cells isolation and culture

Primary osteoblast precursor cells were acquired from calvarial bones of 3-day-old mice. Initially, calvarial bones were separated and placed in phosphate-buffered saline (PBS) to remove the surrounding soft tissues. Calvarial

pieces were then digested with 0.25% trypsin–EDTA for 10 min, then 0.2% collagenase in Hank's solution for 30 min, and, finally, with 0.2% collagenase in Hank's solution for 60 min, all at 37 °C. Following the third digestion, cells were collected and resuspended in complete medium with Minimum Essential Medium Alpha ( $\alpha$ -MEM, Gibco, Invitrogen, Grand Island, USA), 15% fetal bovine serum (FBS, Invitrogen), 1% penicillin–streptomycin, and cultured at 37 °C with 5% CO<sub>2</sub> (Zhong et al. 2016).

### Flow cytometric analysis

Osteoblast precursor cells from the second passage were used to characterize stem cell surface markers by flow cytometric (FCM) analysis. These osteoblast precursor cells were harvested by 0.25% trypsin without EDTA, then washed with PBS 3 times, and resuspended. Cell suspensions ( $2 \times 10^6$  cells) were gained and incubated for 20 min at 4 °C in the dark with CD29-APC, CD34-FITC, CD45-PE, and CD105-PE antibodies (BD Biosciences, San Jose, USA). After staining, cells were washed twice in PBS and then analyzed with flow cytometry (Ge et al. 2016; Yu et al. 2012).

### Osteogenic differentiation

Cells were plated in 6-well plates at a density of  $1.5 \times 10^5$  cells/well with osteogenic differentiation medium including 10% FBS, 10 mM  $\beta$ -glycerolphosphate (Sigma, St. Louis, USA), 10 nM dexamethasone (Sigma), and 50 mg/mL ascorbate phosphate (Sigma).

### Lentiviral transfection

Knockdown of GATA4 in osteoblast precursor cells was conducted by lentiviral transduction of short-hairpin RNAs (shRNAs; GenePharma, Shanghai, China). The target sequence of shRNA with a green fluorescent protein (GFP) tag to knockdown mouse GATA4 (shGATA4) was 5'-CCA AGCAGGACTCTTGAA-3'. A scrambled sequence that was also tagged with GFP (5'-TTCTCCGAACGTGTC ACGT-3') was taken as the negative control (shCTRL). The multiplicity of infection (MOI) was estimated basing on a published protocol (Maranga et al. 2004). Then, recombinant lentivirus was applied to infect osteoblast precursor cells with an MOI of 50 in a medium supplemented with 5  $\mu$ g/mL Polybrene (GenePharma), the medium was switched to a normal medium 24 h after transfection. After 3-day infection, fluorescence microscopy (Leica Microsystems Inc, Ontario, Canada) was chosen to measure the efficiency of lentiviral infection, and the knockdown efficiency of GATA4 was assessed by Western blotting after both 3-day and 14-day infection. To perform the mineralization

induction experiment, the medium was switched to an osteogenic differentiation medium 24 h after transfection, with medium replacement every 3 days.

### Cell counting kit-8 assay

Osteoblast precursor cells that were transfected with shGATA4 or shCTRL lentivirus were cultured in 96-well plates at a density of  $1.0 \times 10^3$  cells/well in  $\alpha$ -MEM with 10% FBS 24 h after transfection. For four subsequent days, a cell counting kit-8 (CCK8) solution (Dojindo, Tokyo, Japan) was added to each well, cultured for 30 min at 37 °C according to the manufacturer's instructions, and then, the cell proliferation rate was quantified by a microplate reader at 450 nm.

### Alkaline phosphatase (ALP) staining

Cells transfected with shCTRL or shGATA4 were seeded at a density of  $5 \times 10^4$  cells/well in 12-well plates in osteogenic medium and ALP staining was conducted 5 days after inducing ossification with the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol.

### Alizarin Red S (ARS) staining

ARS staining was undertaken 2 weeks after inducing ossification with 2% alizarin red (Beyotime Institute of Biotechnology) according to the manufacturer's instructions to visualize mineralized nodules. Micrographs of these mineralized nodules were captured prior to dissolution of these nodules in each well by addition of 600  $\mu$ L 10% cetylpyridinium chloride (CPC) at room temperature for 30 min. Then, 100  $\mu$ L of the supernatant was transferred into 96-well plates and an automatic microplate reader was employed to measure absorbance at 562 nm.

### Tooth movement model protocol

Induction of tooth movement (TM) was done as described in previous studies (Guo et al. 2016; Taddei et al. 2012). In brief, an orthodontic appliance consisting of a  $0.25 \times 0.76$  mm Ni–Ti sentalloy closed coil spring (Grikin, Beijing, China) was applied to ligate the region between the upper incisors and upper left first molar using a dental adhesive resin (3M Unitek, Monrovia, USA), delivering a mesial direction force of 0.35 N. After 14 days of force application, the mice were sacrificed. Eight-week-old male mice were randomly divided into control ( $n=5$ ) and experimental ( $n=5$ ) groups.

### Lentivirus injection

Mice in the experimental group received 5  $\mu$ L concentrated lentiviral supernatant (shGATA4;  $1 \times 10^9$  TU/mL) injected under the buccal periosteum of the left upper first molar in the TM model on days 3–10 after induction of tooth movement, whereas control group mice received the same dose of lentivirus delivering a nonspecific sequence (shCTRL).

### Micro-CT analysis

To analyze the extent of bone formation, we separated the maxilla free from soft tissue, then fixed it in 70% ethanol overnight at 4 °C, and scanned it at a resolution of 9  $\mu$ m/pixel with a micro-CT system (Skyscan 1176, Kontich, Belgium) at 50 kV and 456  $\mu$ A. NRecon v1.6 and CTAn v1.13.8.1 were used to reconstruct and analyze the images (Zhou et al. 2016). The area of alveolar bone between the mesial and distal roots of the first maxillary molar in the TM model was defined as the region of interest (ROI). Six continuous images from this ROI were used for 3-dimensional reconstruction and analysis. Quantitative parameters analyzed were: trabecular bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th). The scan analysis operator was blinded to the treatments linked to each sample.

### Histological analysis

Maxillary halves from the operated side of each animal were separated and fixed in 4% paraformaldehyde (PFA) for 24 h, decalcified with 10% EDTA (pH 7.4) for 3 weeks at 4 °C, embedded in paraffin, and cut into 5  $\mu$ m-thick sections. The sections were stained with total collagen and Goldner's trichrome, and stained sections were observed using light microscopy (Leica Microsystems Inc.) and photographed using Leica imaging software at  $\times 200$  the original magnification (Chen et al. 2014; Ma et al. 2013). Histological analysis of bone parameters were scaled by consecutively analyzing the images with Image J, as reported previously (Egan et al. 2012).

### Immunofluorescence staining

Tissue sections of alveolar bone were stained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime Institute of Biotechnology) at 1:1000 in PBS and then observed with fluorescence microscopy (Leica Microsystems Inc.) and

analyzed using Leica imaging software at  $\times 400$  the original magnification.

### Immunohistochemistry

Antigens from stained sections were retrieved by boiling in sodium citrate buffer solution and, then, endogenous peroxidase activity was quenched with 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). After blocking with 1% goat serum for 30 min at  $37^\circ\text{C}$ , sections were incubated with primary antibodies overnight at  $4^\circ\text{C}$  (Sun et al. 2016). The following primary antibodies were employed: anti-GATA4, anti-osterix, anti-osteopontin, and anti-osteocalcin (ab84593, ab22552, ab63856, and ab93876, respectively, Abcam, Cambridge, UK). Antibodies were detected with goat anti-rabbit immunoglobulin G (IgG) and visualized by developing with a diaminobenzidine (DAB) substrate–chromogen system (Maxim, Fuzhou, China). Sections were observed at  $\times 400$  the original magnification. The percentage of positive cells compared to the total osteoblasts at the bone boundary or positive areas in each sample were counted using Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, USA) (Yu et al. 2014).

### Western blotting

Total proteins obtained through cell lysis buffer, resolved by SDS–PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes. Methods of western blotting protocol used in this study were as described by Li et al. (2015). Antibodies against GATA4, OSX, OPN, and OCN (Abcam), and antibodies against p-p38, p38, p-ERK, ERK (Cell Signaling Technology, Boston, USA) and antibody against GAPDH (Bioworld, Nanjing, China) were used as primary antibodies. Semi-quantitative measurements were carried out using Image J.

### Quantitative reverse-transcription PCR

Total RNA was isolated with a RNA isolation kit (Takara, Dalian, China) and a quantitative RT-PCR was carried out as described by Guo et al. (2016). Primers included: GAPDH 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse); OSX 5'-GCTGGGTAGAGGAAGGTC-3' (forward) and 5'-CAAAGAGAGAAGAACTGAGGAG-3' (reverse); OPN 5'-ACCATGCAGAGAGCGAGGATT-3' (forward) and 5'-GGGACATCGACTGTAGGGACG-3' (reverse); and OCN 5'-ATCTTTCTGCTCACTCTGCT-3' (forward) and 5'-CTACCTTATTGCCCTCCTG-3' (reverse).

### Statistical analysis

All values are displayed as mean  $\pm$  SD. Experiments were conducted at least three times separately. Statistical analysis was conducted using Student's *t* test (SPSS 19.0), and *P* values  $< 0.05$  were regarded as indicative of statistical significance.

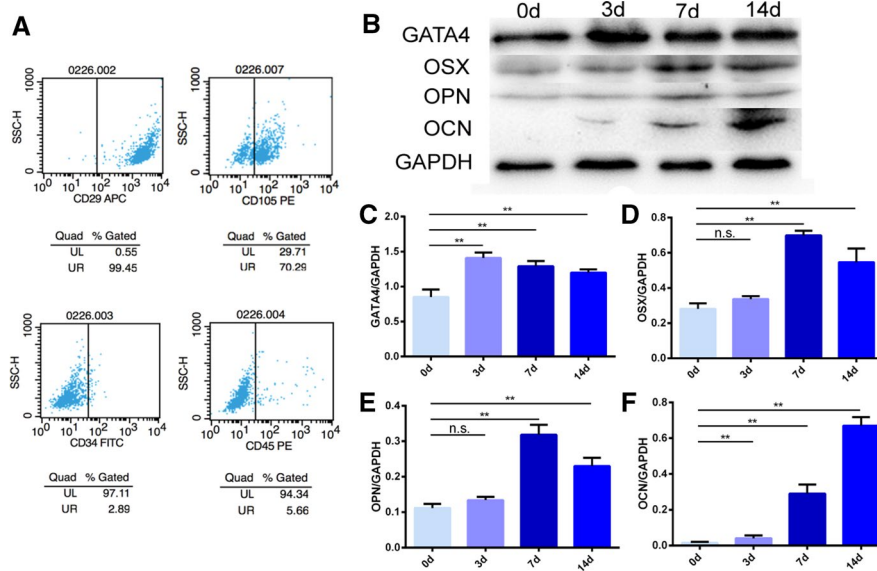
## Results

### High-level expression of GATA4 in mouse osteoblast precursor cells

Osteoblast precursor cells from the second passage were characterized by FCM analysis. As displayed in Fig. 1a, osteoblast precursor cells showed positive expression of mesenchymal stem cell surface markers CD29 and CD105, whereas expression of hematopoietic stem cell markers CD34 and CD45 were absent, indicating that osteoblast precursor cells may be a stem cells population, which makes them suitable for studying osteoblast differentiation. To further determine the association between GATA4 and osteogenic differentiation, osteoblast precursor cells were cultured in osteogenic medium for 0, 3, 7, and 14 days. Then, western blotting was carried out to examine the time-dependent expression mode of GATA4 and osteoblast-specific marker proteins such as OSX, OPN, and OCN in mouse osteoblast precursor cells. As shown in Fig. 1b–f, expression of GATA4 and osteoblast-specific marker proteins transiently increased during osteoblast differentiation, implying GATA4 may be crucial for the regulation of osteoblast differentiation.

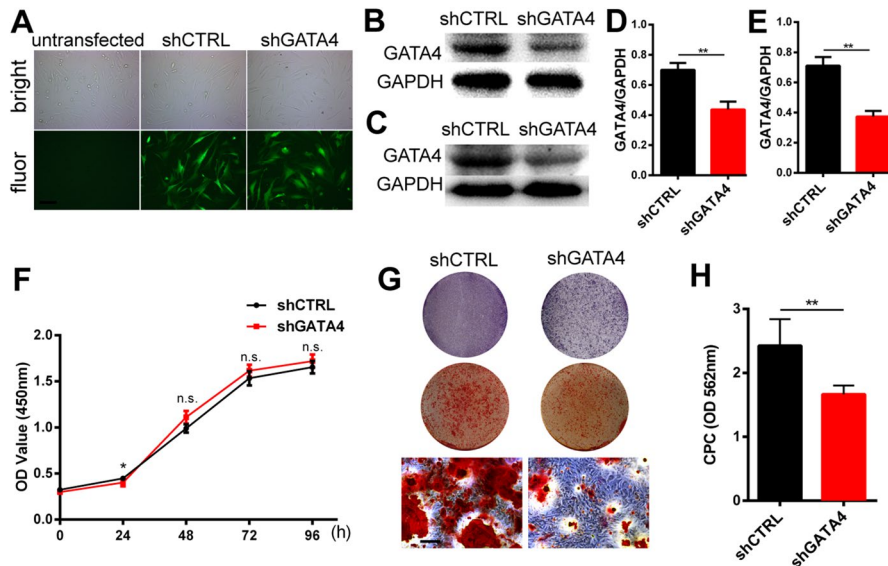
### GATA4 knockdown inhibits osteoblast differentiation

To investigate the possible role of GATA4 in osteoblast differentiation, we knocked down the expression of endogenous GATA4 by lentivirus-mediated infection with a specific shRNA. Seventy-two hours after osteoblast precursor cells were transfected by lentiviral particles (MOI 50) in the presence of Polybrene, distinct green fluorescence expression was observed on fluorescence microscopy (Fig. 2a). Transfection efficiency was  $> 80\%$ . To confirm the effect of transfection of the shGATA4 lentiviral vector on GATA4 knockdown in osteoblast precursor cells, protein extraction was done on days 3 and 14 after transfection in normal media, followed by western blotting to quantify protein expression levels of GATA4 in osteoblast precursor cells. Western blotting analysis indicated that GATA4 expression was efficiently and stably knocked down in the experimental group, compared to the control group (Fig. 2b–e). To determine the effect of GATA4 on proliferative ability of



**Fig. 1** Characterization of osteoblast precursor cells and GATA4 expression in osteoblast precursor cells in mice. **a** Surface markers of osteoblast precursor cells were identified by flow cytometric analysis. Osteoblast precursor cells were stained with stem cell markers (CD29 and CD105) and hematopoietic markers (CD34 and CD45). **b** Western blotting results confirmed that mineralization-induced osteoblast

precursor cells at all stages had higher GATA4, OSX, OPN and OCN expression than undifferentiated cells. **c–f** Western blotting bands from **b** were quantitatively calculated as the ratio of GATA4, OSX, OPN and OCN to GAPDH. \* $P < 0.05$ , \*\* $P < 0.01$ . All data are shown as mean  $\pm$  SD



**Fig. 2** GATA4 knockdown reduced osteogenic differentiation of osteoblast precursor cells. **a** Transfection efficiency of osteoblast precursor cells was observed under fluorescence microscopy 72 h after lentiviral infection. The *up* row of the panel shows the bright field of the cells. Transfected osteoblast precursor cells displayed green fluorescence (*low*). Scale bar 50  $\mu$ m. **b**, **c** GATA4 expression was tested by western blotting at 3 and 14 days after transfection, respectively. **d**, **e** Western blotting bands from **b** and **c** were quantitatively calculated

as the ratio of GATA4 to GAPDH, respectively. **f** Cell proliferation of osteoblast precursor cells was analyzed at 0–4 days following transfection with lentivirus by a cell counting kit-8 (CCK-8) assay. **g** 5 days after mineralization, ALP staining was performed and observed with a scanner. Alizarin Red S staining was conducted 14 days after mineralization and then observed with a scanner and microscope. Scale bar 50  $\mu$ m. **h** Calcium semiquantitative test was performed. \* $P < 0.05$ , \*\* $P < 0.01$ . All data are shown as mean  $\pm$  SD

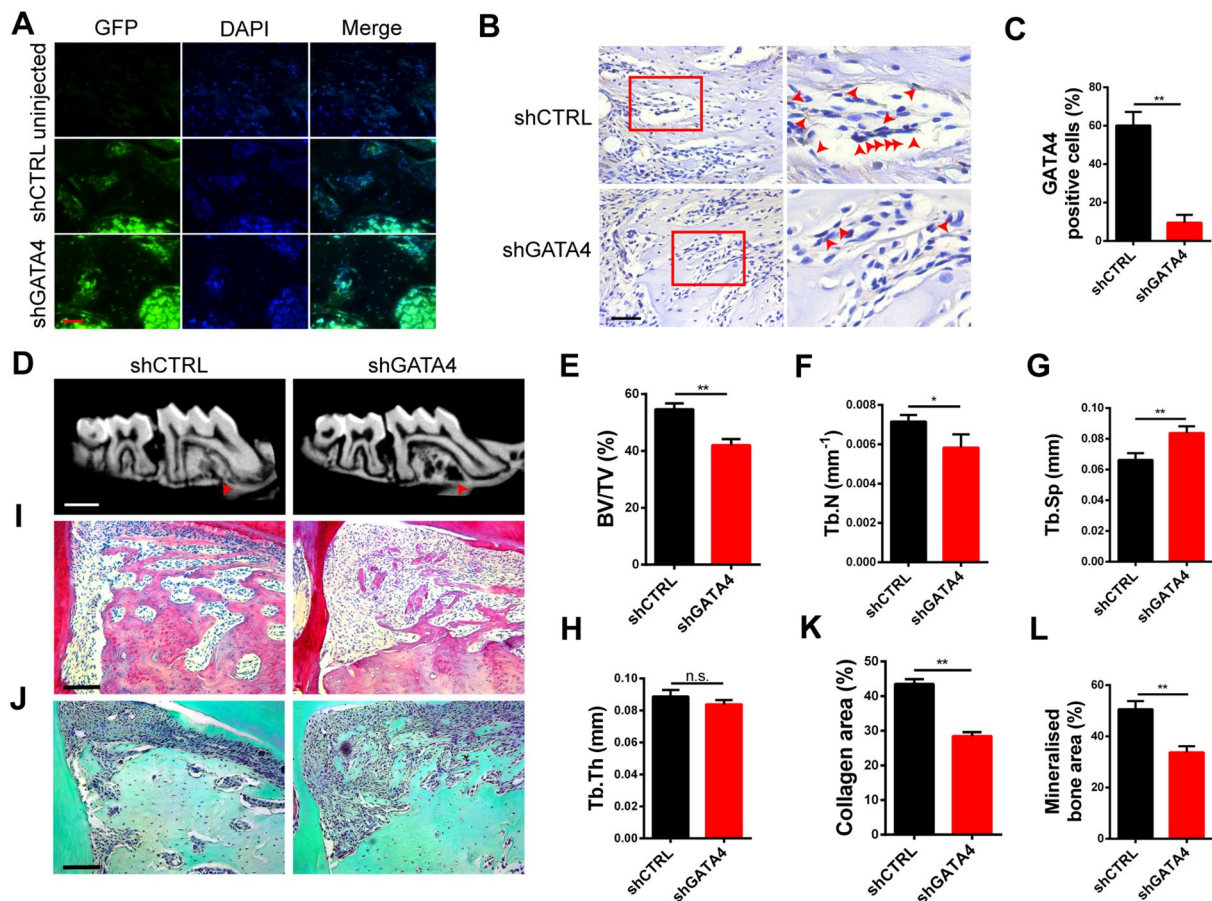
osteoblast precursor cells, we quantified cell number with a CCK-8 kit. No significant differences were observed in the numbers of cells, indicating that GATA4 did not affect the proliferative ability of osteoblast precursor cells (Fig. 2f).

When osteoblast precursor cells were cultured with mineral medium for 5 days, ALP activity was examined by ALP kit, the shGATA4 transfected cells presented weaker ALP activity compared to the shCTRL group. In addition, ARS staining was conducted for 14 days after osteogenic induction, and it presented that the density of calcium nodes observably decreased in shGATA4 group (Fig. 2g). Meanwhile, the units of calcium reduced significantly, as indicated by the calcium semiquantitative test in GATA4 knocked-down osteoblast precursor cells (Fig. 2h). All of

these findings suggest that GATA4 may be an essential factor for osteoblast differentiation.

### Local silencing of GATA4 decreases bone formation during TM

To examine the role of GATA4 in bone formation in vivo, a TM animal model was chosen, and alveolar bone remodeling in shCTRL- and shGATA4-injected mice was observed. The selected shRNA vectors contained a GFP tag; thus, cells infected with the lentivirus would express GFP. Indeed, strong GFP expression was seen in experimental and control mice, whereas the groups not injected, displayed only background fluorescence, indicating that



**Fig. 3** shRNA-mediated knockdown of GATA4 in vivo and decreased bone formation in shGATA4-injected mice. **a** Double-labeled fluorescent immunostaining of GFP (green, marker of transduction) in the first column, 4',6-diamidino-2-phenylindole (DAPI)-stained cell nuclei (blue) in the second column, and merged images of the first and second column (the third column) in alveolar bone at day 14 after the operation for inducing tooth movement. Scale bar 50  $\mu$ m. **b** GATA4-expressing cells determined by anti-GATA4 immunohistochemistry of alveolar bone at day 14 after TM. Scale bar 50  $\mu$ m. **c** Percentages of GATA4-positive cells in experimental and control groups at day 14 after TM. **d** Micro-CT sagittal view images of alveo-

lar bone at day 14 after TM. Red arrows indicate altered bone formation of alveolar bone. Scale bar 500  $\mu$ m. **e–h** Quantitative analysis of bone volume over total volume (BV/TV), number (Tb.N), trabecular separation (Tb.Sp), and thickness (Tb.Th) of alveolar bone by micro-CT. **i** Representative photographs of alveolar sections stained histochemically for total collagen. Scale bar 100  $\mu$ m. **j** Tissue sections were stained by Goldner's trichrome staining. Mineralized matrix was stained green. Scale bar 100  $\mu$ m. **k, l** Collagen area and mineralized bone area from sections of two different stains compared to total area, respectively. \* $P < 0.05$ , \*\* $P < 0.01$ . All data are shown as mean  $\pm$  SD. (Color figure online)

lentivirus was transfected as expected (Fig. 3a). Then, in order to visualize and verify the knockdown efficiency of GATA4 *in vivo*, immunohistochemistry of sections of alveolar bone using the rabbit polyclonal anti-GATA4 antibody was performed. As expected, GATA4 was abundantly available in the alveolar tissue between the roots of the first maxillary molars of shCTRL-injected mice at day 14. Conversely, much lesser GATA4-labeled cells were detected in shGATA4-injected mice at day 14 (Fig. 3b, c). Micro-CT carried out to quantitatively observe bone constitution and analyze correlative parameters of alveolar bone showed that, in the TM model, shGATA4-injected mice exhibited remarkable reduction of alveolar bone within the roots of the first upper molar at day 14, whereas there was smaller influence on the alveolar bone in the control group (Fig. 3d). Furthermore, shGATA4-injected mice showed decreased BV/TV ( $P < 0.01$ ; Fig. 3e) and Tb.N ( $P < 0.05$ ; Fig. 3f) values, and increased Tb.Sp value ( $P < 0.01$ ; Fig. 3g). However, no significant difference of Tb.Th values was seen between experimental and control mice (Fig. 3h). Therefore, it can be speculated that GATA4 knockdown might play an inhibitory role in bone formation. To further explore our hypothesis, the sections were stained with total collagen or Goldner's trichrome to examine histological differences between control and experimental groups and to determine whether GATA4 knockdown may influence bone remodeling. The results showed that bone formation within the area between the roots of the first maxillary molar was decreased significantly in the shGATA4-injected group compared with controls (Fig. 3i–l), which was consistent with results from the micro-CT analysis.

### GATA4 knockdown inhibits osteoblast-specific gene expression

Local knockdown of GATA4 in mice resulted in decreased expression level of osteogenic markers such as OSX, OPN, and OCN in osteoblasts on the bone surface on day 14 after the TM operation when tested by immunohistochemistry (Fig. 4a–f). Both shGATA4- and shCTRL-transfected cells were cultured in mineralization medium for 14 days. Expression levels of selected representative osteogenic markers were analyzed by Western blotting and quantitative RT-PCR. As expected, the expression levels of GATA4, OSX, OPN, and OCN were also reduced in shGATA4-transfected cells (Fig. 4g–n), in concordance with *in vivo* results.

### GATA4 knockdown decreases p38 activation

Osteogenic differentiation is regulated by different signaling pathways including MAPK. To elucidate whether GATA4 is involved in MAPK signaling pathway, we

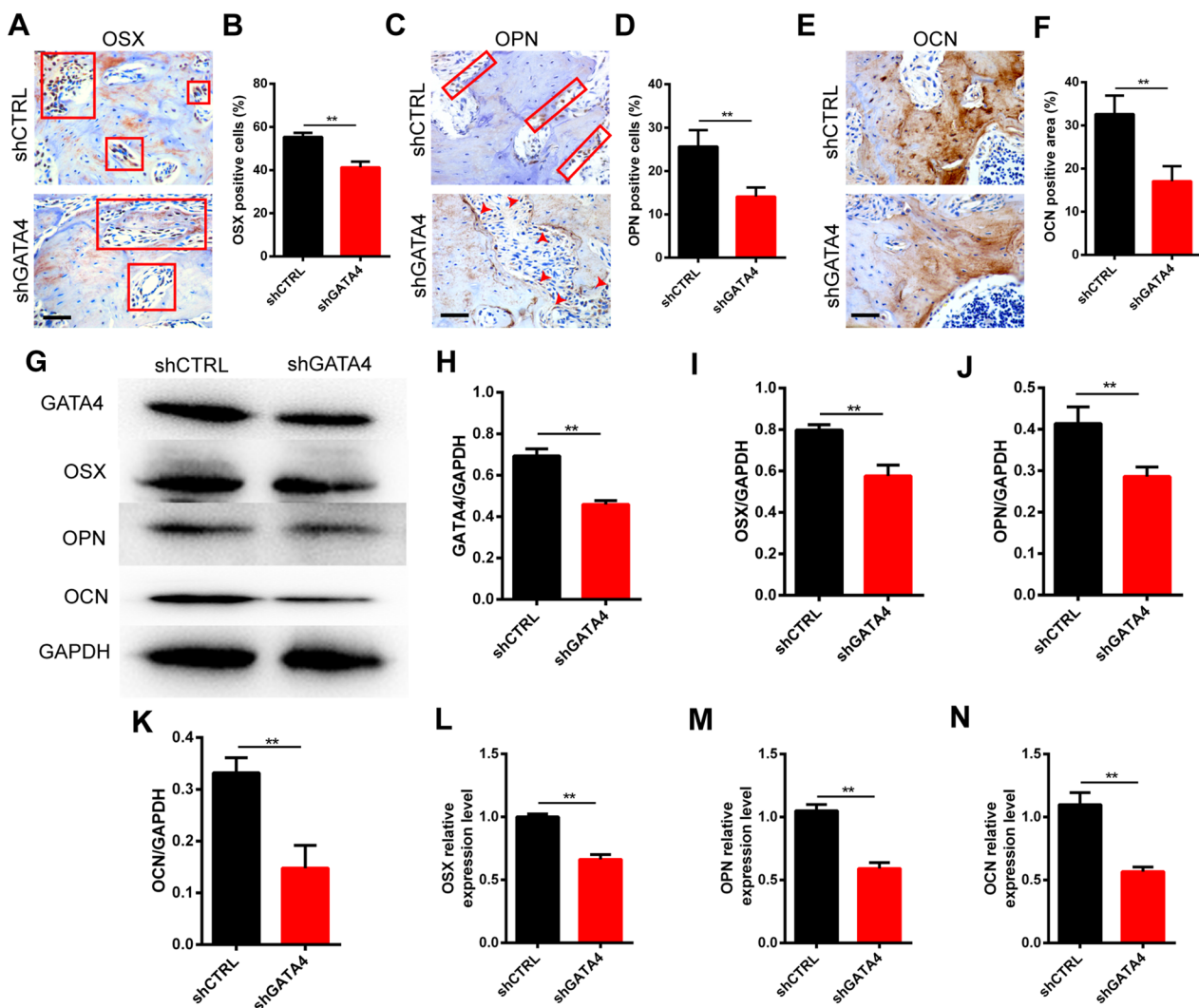
explored the reaction of the MAPK signaling pathway to osteogenic stimulation in control and shGATA4-transfected osteoblast precursor cells. The expression of phosphorylated p38 was markedly increased in control group undergoing osteogenic differentiation at day 7, whereas p38 phosphorylation in shGATA4-transfected cells were blunted compared to control group cells following induction of mineralization, although levels of phosphorylated ERK were unaffected (Fig. 5a–d). In addition, as shown in Fig. 5e–f, the expression of phosphorylated p38 transiently increased during osteoblast differentiation, indicating MAPK–p38 signaling may play a role in the regulation of osteoblast differentiation. Interestingly, the expression of GATA4 also showed an increasing tendency during osteoblast differentiation (Fig. 1b), consistent with the expression mode of phosphorylated p38, suggesting GATA4 may regulate osteoblastic differentiation and bone remodeling via p38-mediated signaling.

## Discussion

Osteoblast differentiation is essential for bone rebuilding, many transcription factors take part in the osteoblast differentiation process. GATA4, a transcription factor, has been reported to regulate transcription in many organs (Mazaud et al. 2007; Molkentin et al. 1998; Viger et al. 2008). However, the role of GATA4 in osteoblast differentiation is still unclear.

In the study, calvarial cells, which are considered osteoblast precursor cells, were employed for *in vitro* experiments and a TM *in vivo* model was established to observe the role of GATA4 in osteoblast differentiation. As GATA4 expression was up regulated during the osteoblast mineralization process as well as osteoblast-specific marker proteins such as OSX, OPN, and OCN, we proposed a hypothesis that GATA4 might be involved in osteoblastic differentiation. In order to confirm this hypothesis, we established GATA4 knockdown system to examine expression levels of osteogenesis-related genes, and found the expression of mineralization markers (OSX, OPN, and OCN) to be significantly decreased by GATA4 knockdown. Downregulation of GATA4 expression also weakened ALP activity, which is an early indicator of mineralization. Furthermore, findings from ARS staining and calcium semiquantitative analysis support the key role of GATA4 in osteoblastic differentiation.

In addition to the *in vitro* experiment, an *in vivo* experiment was conducted to explore the relation between GATA4 and osteoblast differentiation. Results of micro-CT and histological analysis showed clearly that bone formation in the experimental group was significantly decreased, compared to the control group. Meanwhile, expression of



**Fig. 4** Decreased expression of osteogenic markers in GATA4 knocked-down mice or cells compared with control group. **a, c, e** Immunohistochemically determined levels of representative osteogenic markers such as OSX, OPN, and OCN, were reduced in the alveolar bone on day 14 after the tooth movement operation in mice. Scale bar 50  $\mu$ m. **b, d** Percentages of OSX- or OPN-positive cells in experimental and control groups on day 14 after TM were calculated, respectively. **f** Percentage of OCN-positive area was calculated in experimental and control groups on day 14 after TM. **g**

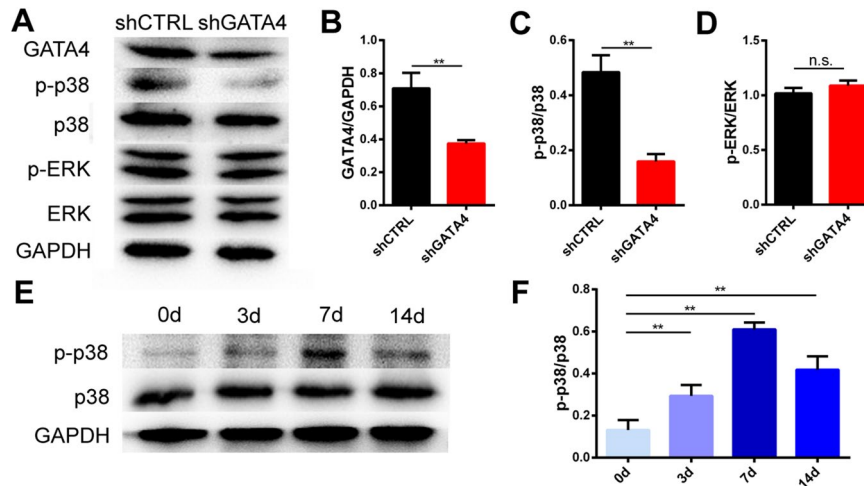
Protein expression levels of GATA4 and several osteogenic markers (OSX, OPN, and OCN) in shGATA4-transfected osteoblast precursor cells were reduced as compared with control cells after 14 days following mineralization induction. **h–k** Western blotting bands from **g** were quantitatively calculated as the ratio of GATA4, OSX, OPN, and OCN to GAPDH, respectively. **l–n** 14 days after mineralization, expression of osteogenesis-related genes (OSX, OPN, and OCN) were tested by RT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$ . All data are shown as mean  $\pm$  SD

mineralization-related markers such as OSX, OPN, and OCN was also reduced, in alveolar bone on day 14 after the TM operation. Our in vivo data is consistent with results from the in vitro experiment, further confirming our assumption that GATA4 plays a critical role in osteoblast differentiation.

ALP and OSX are early-stage markers of osteoblast differentiation while OPN and OCN are closely related to the late-stage osteoblast differentiation (Li et al. 2016; Yusa et al. 2016). ALP is an established marker of osteoblast activity and a functional marker of bone formation

(Mojarad et al. 2016). OSX plays a critical role in osteoblastic differentiation and is expressed in functional osteoblasts (Nakashima et al. 2002). OPN is an extracellular matrix protein expressed in many cell types especially osteoblasts, in which it plays multiple roles in diverse biological and pathological processes (Schlafer et al. 2012). OCN has close relations with bone maturation, which is identified as a late-stage marker of bone formation (Li et al. 2014a, b). However, the relationship between GATA4 and osteoblastic differentiation is still unknown. In this study, we demonstrated that GATA4 knockdown in osteoblast





**Fig. 5** GATA4 knockdown decreases p38 activation. **a** Levels of GATA4, p-p38, p38, p-ERK, and ERK were detected by western blotting 7 days after induction of mineralization. **b–d** Quantification of western blotting experiments was displayed with the mean level of GATA4 over GAPDH, p-p38 over p38 and p-ERK over ERK in control and experimental groups. **e** Western blotting results indicated

that mineralization-induced osteoblast precursor cells at all stages had higher phosphorylated p38 expression than undifferentiated cells. **f** Western blotting bands from **e** were quantitatively calculated as the ratio of p-p38 to p38. \* $P < 0.05$ , \*\* $P < 0.01$ . All data are shown as mean  $\pm$  SD

precursor cells significantly reduced the expression of both early and late stage osteogenic markers, implying the long-term effects of GATA4 on the differentiation of osteoblasts.

Recently, the role of GATA4 in osteoblast differentiation gained prominence when Miranda-Carboni et al. hypothesized that GATA4 is a possible E2 direct target in osteoblasts and could regulate osteoblast-specific genes by recruiting ER $\alpha$  to DNA in osteoblasts, Güemes et al. showed that GATA4, in osteoblasts, is essential for the survival and bone development, and GATA4 is a regulator of osteoblast commitment *via* E2-dependent and E2-independent pathways during early development (Miranda-Carboni et al. 2011; Güemes et al. 2014). However, Song et al. (2014) discovered that GATA4 may work to negatively modulate osteoblast differentiation by controlling Dlx5 binding ability to the runt-related transcription factor 2 (RUNX2) promoter. The signaling pathway involved in how GATA4 regulates osteoblast differentiation remains unclear. Increasing evidence suggests that MAPK pathways play a vital role in osteoblast differentiation. Some studies have proved ERK and p38 MAPKs are significant for early osteoblast differentiation and ERK or p38 inhibition suppresses osteoblast-specific gene expression (Suzuki et al. 2002; Xiao et al. 2000). Earlier literature suggested GATA4 as one of several p38 target proteins that may affect gonadal somatic cell development (Gierl et al. 2012). Overexpression of gelsolin could cause cardiac hypertrophy, which is due to the crosstalk of GATA4 and p38 signaling (Hu et al. 2014). Previous studies have also pointed out that the GATA4 protein has several possible MAPK phosphorylation sites within the N- and C-terminal domains, and p38

can directly interact with GATA4 within the N-terminal domain at several particular sites such as T100 and S105 (Charron et al. 2001). However, the specific interaction of GATA4 with MAPK signaling pathways during osteoblast differentiation has not been elucidated. In the study, incubating GATA4 knocked down cells in osteogenic-induced medium lead to decreased phosphorylation of p38, hence suggesting GATA4 downregulation may inhibit the sustained activation of p38, although levels of phosphorylated ERK were unaffected. In addition, the expression of phosphorylated p38 was upregulated during osteoblast differentiation, in agreement with the increase tendency of GATA4, indicating GATA4 may regulate osteoblastic differentiation and bone remodeling via p38-mediated signaling, and then loss-of-function of GATA4 in osteoblasts may influence osteoblast differentiation through downregulation of osteoblast-specific gene markers such as OSX, OPN, and OCN. Our study indicates that GATA4 can act as an activator in osteoblastic differentiation, consistent with results from Miranda-Carboni’s et al. (2011) and Güemes’ et al. (2014) study, and our data reveals that GATA4 plays a vital role in osteogenic differentiation and bone remodeling through p38-mediated signaling. Although MAPK activation often promotes cell proliferation, in the study downregulation of p-p38 in MAPK by GATA4 knockdown did not alter proliferation of osteoblasts without osteogenic induction. It is the first study elucidating a relationship between GATA4 and p38 signaling in osteoblast differentiation.

In summary, based on the presented results, it is reasonable to hypothesize that GATA4 may play a positive role in osteoblast differentiation, which may be affected through

p38-mediated signaling. These findings improve our understanding of the regulatory mechanism of bone formation and may help to provide novel therapeutic approaches through regulation of osteoblast differentiation to increase bone formation. Thus, GATA4 may be an effective target to treat bone-related disorders. Therefore, detailed mechanisms underlying GATA4 gene regulation should be further elucidated to facilitate the development of additional therapeutic approaches in various bone diseases such as osteoporosis, hyperostosis, and periodontal disease.

## Conclusion

The objective of this study was to evaluate the possible role of GATA4 in the differentiation of osteoblasts. Knockdown of GATA4 in calvarial cells and in the mouse TM model inhibited osteoblast differentiation, and decreased expression of bone-related genes both *in vitro* and *in vivo*, as these may be modulated via p38-mediated signaling. These results imply GATA4 is a vital regulator of osteoblast differentiation. Understanding the importance of the role of GATA4 in the differentiation of osteoblasts may facilitate the development of additional therapeutic approaches in various bone diseases.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest with the contents of this article.

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