RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.071

ScienceAsia 48 (2022): **423–428**

FOXP1 promotes osteoblast differentiation via regulation of TGF-β/ALK-5 pathway

Min Zhang[a], **Tao Ma**[a], **Bin Hu**[a], **Weiwei Xiang**[b,*]

[a] Department of Hand and Foot, The First Affiliated Hospital of Wannan Medical College (Yijishan Hospital), Wuhu, Anhui 241000 China

[b] Department of Orthopedics, The First Affiliated Hospital of Gannan Medical University, Ganzhou, Jiangxi 341000 China

[*]Corresponding author, e-mail: xiangweiwei0926@163.com

Received 29 Sep 2021, Accepted 25 Jan 2021
Available online 16 Apr 2022

ABSTRACT: Osteoblast differentiation plays a crucial role in the development of skeleton. The purpose of this study was to investigate the role of FOXP1 in osteoblast differentiation. Expressions of FOXP1, ALP, OSX, and OCN were upregulated in MC3T3-E1 cells during osteoblast differentiation. Downregulation of FOXP1 inhibited the expressions of ALP, OSX, and OCN. ALP activity was reduced by inhibition of FOXP1. Osteoblast mineralization was suppressed by knockdown of FOXP1. The knockdown of FOXP1 also downregulated the expressions of TGF-β, ALK-5, and Smad4 and inhibited the phosphorylation of Smad2/3. Repression of ALK-5 attenuated the effects of FOXP1 on MC3T3-E1 cells during osteoblast differentiation. In summary, data of this study demonstrated that upregulation of FOXP1 enhanced osteoblast differentiation through enhancing activation of TGF-β/ALK-5 pathway.

KEYWORDS: FOXP1, osteoblast differentiation, TGF-β, ALK-5

INTRODUCTION

Skeleton component cells include chondrocytes, osteoblasts, adipocytes, myoblasts, tendon cells, fibroblasts, and osteocytes, which play indispensable roles in the development and biological functions of skeleton [1]. Among these cells, osteoblasts are one of the key components in bone formation secreting extracellular matrix proteins, providing structural support to the skeleton together with calcium hydroxyapatite [2]. Osteoblast differentiation is essential to produce bone matrix during bone modeling and remodeling [3]. The mature osteoblast formation experienced three distinct stages: osteoprogenitor, pre-osteoblast, and osteoblast [2].

Differentiation of osteoblasts is mainly controlled by bone morphogenic proteins (BMPs) and wingless-related integration site (WNT) pathways [4]. The first step of osteoblast differentiation is the activation of master osteogenic transcriptional factors, such as osterix (OSX) and runt-related transcription factor 2 (RUNX2), promoting osteoprogenitors differentiated into pre-osteoblasts [4]. Early osteogenic genes, including alkaline phosphatase (ALP) and collagen1α1 chain (COL1A1), were transcribed and expressed throughout osteoblasts' life [4]. Mature osteoblasts expressed osteopontin (OPN), bone sialoprotein II (BSP II), and osteocalcin (OCN), which are common osteoblast markers [5]. Finally the osteoblasts were undergone apoptosis and became bone lining cells or osteocytes [4].

Forkhead box protein 1 (FOXP1) is a transcription factor belonging to Forkhead-box (FOX) gene family [6]. It has a broad range of physiological functions in cell differentiantion. For example, FOXP1 repressed brown/beige adipocyte differentiation and thermogenesis through directly inhibiting the transcription of β3-adrenergic receptor in adipocytes [7]. FOXP1 played a beneficial effect in the differentiation of embryonic neural stem cells through transcriptional repression of Notch-ligand Jagged1 during corticogenesis [8]. In the episode of osteoporosis, FOXP1 enhanced osteogenic differentiation *in vitro* via circFOXP1/miR-33a-5p/FOXP1 networks, promoting bone regeneration [9]. FOXP1 attenuated mesenchymal stem cell senescence through directly repressed transcription of p16INK4K during skeletal aging [10]. However, no studies reported the mechanism of FOXP1 in regulating osteoblast differentiation. Transforming growth factor-beta (TGF-β) was a mediator involved in the regulation of osteoblast differentiation through β-catenin signaling in human mesenchymal stem cells [11]. Several studies have revealed the interaction between FOXP1 and TGF-β in cardiac remodeling and collagen synthesis [12, 13]. But there have been no reports about the relationship between FOXP1 and TGF-β pathway during osteoblast differentiation. The purpose of this study was to investigate the role of FOXP1 in osteoblast differentiation and the modulation of TGF-β/activating receptor-like kinase 5 (ALK-5) pathway during osteoblast differentiation.

METHODS

Cell culture and transfection

Osteoblast medium (OM) was freshly prepared using 90% MEM α, nucleosides (Gibco, USA), 10% fetal bovine serum (Invitrogen, USA), 1%

Antibiotic-Antimycotic (100×, Gibco), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 50 μg/ml ascorbate (Sigma-Aldrich, USA), 5 mM β-glycerophosphate (Solarbio, China), and 1000 μg/ml bone morphogenic protein 2 (BMP2, Gibco). Preosteoblastic cell line MC3T3-E1 cells (Cobier, China) were cultured in freshly prepared OM at 37 °C with 5% CO_2.

Silence RNA of FOXP1 (siFOXP1) and its scramble RNA (siNC), siALK-5 and its negative controls were provided by Ribo Biotechnology Co., Ltd (Ribobio, China). FOXP1 plasmid was constructed by Guangzhou Yunzhou Science and Technology Co., Ltd (Yunzhou, China). Cell transfection was performed using Lipofectamine LTX with Plus Reagent (Thermo Fisher, USA) following the manufacturer's instruction.

Alizarin red staining

Osteoblast mineralization was detected using alizarin red staining. After treatment, MC3T3-E1 cells were fixed using 4% Paraformaldehyde Fix Solution (Beyotime, China) for 10 min at room temperature and then stained using Alizarin Red S Staining Kit (Beyotime) for 30 min at room temperature. Osteoblast mineralization was observed and photographed under an inverted microscope (Keyence, China).

Measurement of ALP activity

Activity of ALP was measured using Alkaline Phosphatase Activity Detection Kit (Yeasen, China) following the manufacturer's protocols.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extraction of RNA was achieved using TRIzol RNA Isolation Reagents (Thermo Fisher). Reverse transcription was performed using iscript cDNA synthesis kit (Bio-Rad, USA). qRT-PCR was performed using Script™ reverse transcription supermix for RT-qPCR (Bio-Rad). The relative RNA expression was calculated using $2^{-\Delta\Delta CT}$ method. The primary sequences (Genscript, USA) used in this study were: FOXP1 forward 5′-GGTCTGAGACAAAAAGTAACGGA-3′ and reverse 5′-CGCACTCTAGTAAGTGGTTGC-3′; and β-actin forward 5′-GTCTGCCTTGGTAGTGGATAATG-3′ and reverse 5′-TCGAGGACGCCCTATCATGG-3′.

Western blotting

Protein extraction was done using BugBuster® 10X Protein Extraction Reagent (Merck KGaA, Germany). Protein concentration was measured using Bicinchoninic Acid Kit for Protein Determination (Merck KGaA). Total proteins (50 μg) were separated by electrophoresis using Criterion XT precast gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Thermo Fisher), which were then blocked by Every-Blot Blocking Buffer (Bio-Rad) for 1 h at room temperature and incubated with proper primary antibodies at 4 °C overnight. Secondary antibodies were used to probe the proteins linked to primary antibodies. The proteins bands were developed using Ultra High Sensitivity ECL Substrate Kit (Abcam, UK) through Chemi-Doc MP Imaging System (Bio-Rad). Primary antibodies (Abcam) used in this study were: FOXP1 (ab134055, 1:1000 dilution); ALP (ab67228, 1:800 dilution); OSX (ab229285, 1:500 dilution); OCN (ab133612, 1:1500 dilution); TGF-β (ab92486, 1:2000 dilution); ALK-5 (ab31013, 1:1000 dilution); p-Smad2/3 (ab272332, 1:1500 dilution); Smad2/3 (ab207447, 1:500 dilution); Smad4 (ab40759, 1:1000 dilution); and β-actin (ab8227, 1:5000 dilution).

Statistical analysis

SPSS 17.0 (SPSS, USA) was used to perform the statistical analysis of this study. All data were demonstrated as mean Âś standard deviation. Significant differences were analyzed using student t-test (between two groups) and one-way analysis of variance followed by Tukey's test (among more than two groups). $p < 0.05$ indicated statistical significance.

RESULTS

FOXP1 was upregulated in osteoblast

FOXP1 expression was gradually increased as time went by (Fig. 1). Expression of ALP, OSX, and OCN did not show any significant change on Day 3 but significantly upregulated on Day 6 and Day 9 in MC3T3-E1 cells. Thus, these data demonstrated that FOXP1 was significantly upregulated in osteoblast.

Knockdown of FOXP1 prevented osteoblast differentiation

Expressions of FOXP1 mRNA and protein were downregulated in MC3T3-E1cells transfected with siFOXP1 (Fig. 2A,B). Upregulation of ALP, OSX, and OCN during osteoblast differentiation was suppressed by suppression of FOXP1 (Fig. 2C). Elevation of ALP activity was reduced by downregulation of FOXP1 (Fig. 2D). The number of alizarin red-positive cells was increased during osteoblast differentiation (Fig. 2E). These data suggested that knockdown of FOXP1 prevented osteoblast differentiation.

FOXP1 enhanced the activation of TGF-β/ALK-5 pathway in osteoblast

Knockdown of FOXP1 reduced the expressions of TGF-β and ALK-5 in MC3T3-E1 cells (Fig. 3A). Phosphorylation of Smad2/3 and expression of Smad4 were inhibited in MC3T3-E1cells transfected with siFOXP1 (Fig. 3B). Therefore, inhibition of FOXP1 prevented the signal transduction of TGF-β/ALK-5 pathway in MC3T3-E1 cells.

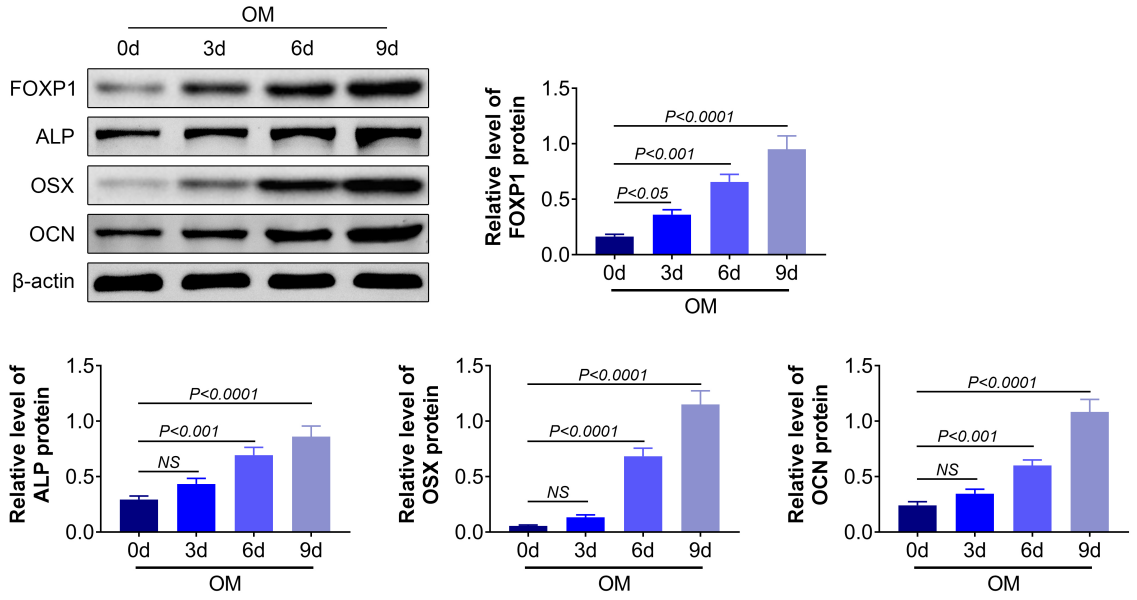

Fig. 1 FOXP1 was upregulated in osteoblast. Expressions of FOXP1, ALP, OSX, and OCN were gradually increased as time went by. OM: Osteoblast Medium. Each experiment was repeated three times.

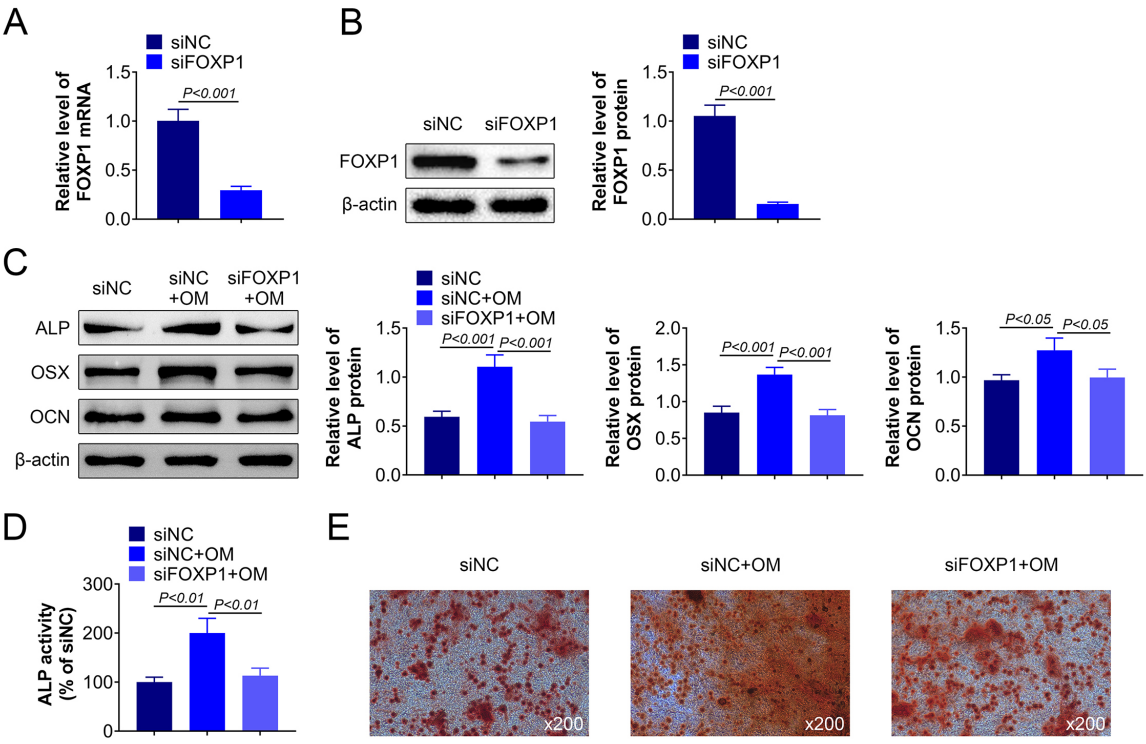

Fig. 2 Knockdown of FOXP1 prevented osteoblast differentiation: (A), Expression of FOXP1 mRNA was downregulated by siFOXP1; (B), Expression of FOXP1 protein was downregulated by siFOXP1; (C), Knockdown of FOXP1 downregulated the expression of ALP, OSX, and OCN; (D), Knockdown of FOXP1 reduced ALP activity; (E), Knockdown of FOXP1 reduced the number of alizarin red-positive cells. siFOXP1: silence RNA of FOXP1; siNC: scramble RNA of siFOXP1. Each experiment was repeated three times.

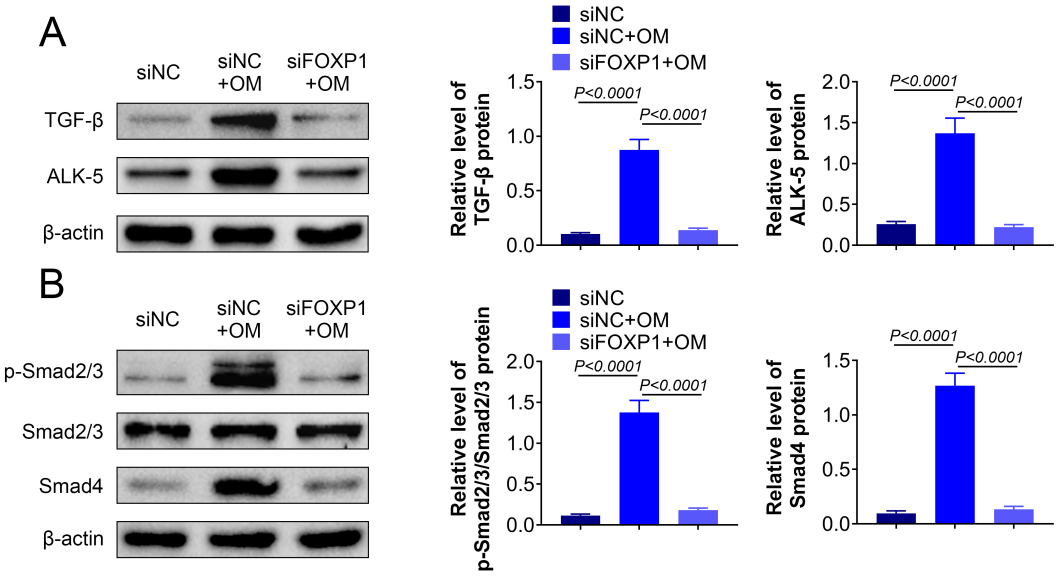

Fig. 3 FOXP1 enhanced the activation of TGF-β/ALK-5 pathway in osteoblast. Knockdown of FOXP1 downregulated the expressions of TGF-β, ALK-5, and Smad4 and inhibited the phosphorylation of Smad2/3. Each experiment was repeated three times.

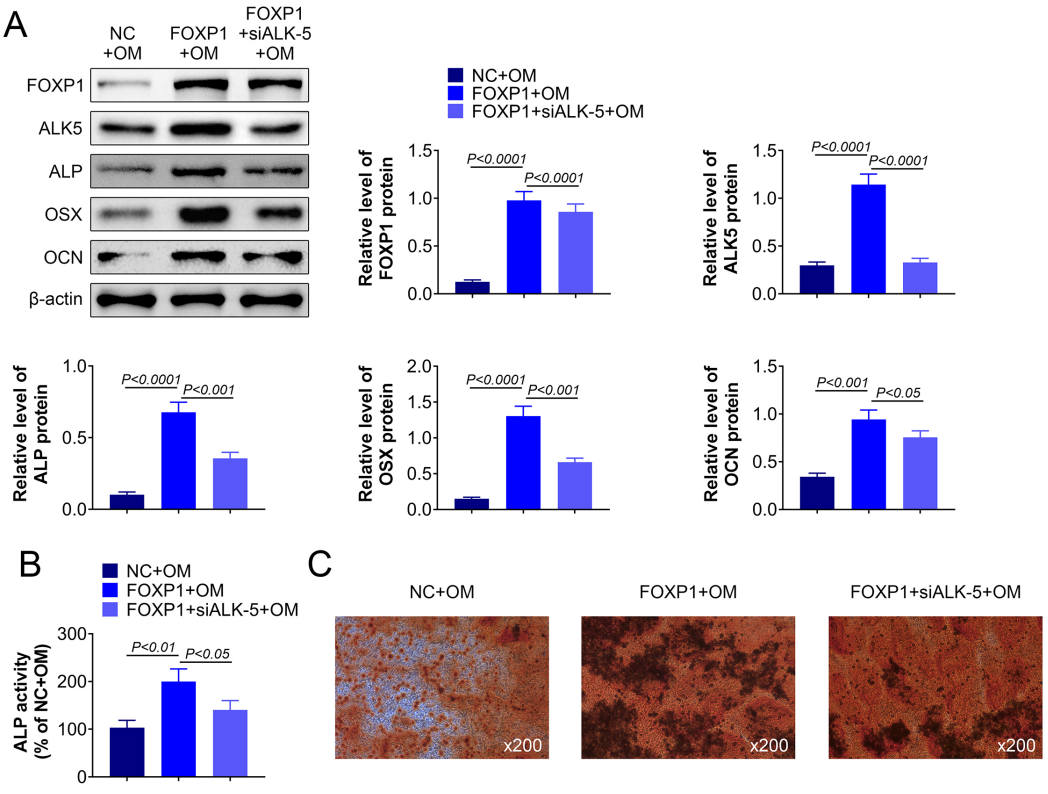

Fig. 4 FOXP1 promoted osteoblast differentiation through upregulation ALK-5. (A), Knockdown of ALK-5 reversed FOXP1-induced upregulation of ALP, OSX, and OCN; (B), Knockdown of ALK-5 reversed FOXP1-induced increment of ALP activity; (C), Knockdown of ALK-5 reversed FOXP1-induced increment of alizarin red-positive cells. Each experiment was repeated three times.

FOXP1 promoted osteoblast differentiation through upregulation ALK-5

The expressions of FOXP1, ALK5, ALP, OSX, and OCN were upregulated by overexpression of FOXP1 (Fig. 4A). Knockdown of ALK-5 attenuated FOXP1-induced upregulation of FOXP1, ALP, OSX, and OCN (Fig. 4A). Upregulation of FOXP1 increased ALP activity, while inhibition of ALK-5 reversed the increment of ALP activity induced by FOXP1 (Fig. 4B). The number of alizarin red-positive cells was elevated by upregulation of FOXP1, while this elevation was prevented by siALK-5 (Fig. 4C). These data indicated that FOXP1 promoted osteoblast differentiation through upregulation ALK-5.

DISCUSSION

Osteoblast differentiation is a key process of bone matrix production during bone modeling in the embryonic development and remodeling after bone injury [3]. Previous study has found that FOXP1 promoted osteosarcoma cell proliferation by downregulation of p21 and Rb [14]. In this study, osteoblast differentiation was observed after treating MC3T3-E1 cells with OM; and FOXP1 expression was upregulated during osteoblast differentiation. Knockdown of FOXP1 reduced the expressions of ALP, OSX, and OCN, and also inhibited osteoblast mineralization. Further experiments demonstrated that downregulation of FOXP1 suppressed TGF-β, ALK-5, and Smad4, and prevented phosphorylation of Smad2/3; while knockdown of ALK-5 attenuated the promotion of osteoblast differentiation induced by overexpression of FOXP1, which was consistent with a previous finding [15]. Therefore, overexpression of FOXP1 promoted osteoblast differentiation through enhancing the signal transduction of TGF-β/ALK-5 pathway.

As mentioned above, OSX, ALP, and OCN are key proteins originally expressed in osteoprogenitors, preosteoblasts, and osteoblasts, respectively [4], reflecting the whole process of osteoblast differentiation. Inhibition of FOXP1 repressed the expression of OSX, ALP, and OCN, indicating that osteoblast differentiation was prevented by downregulation of FOXP1. Osteoblast mineralization is an indispensable physiological process of bone formation to support body weight and movement [16]. High expression of ALP was commonly observed in mineralized cell, potentiating the formation of hard tissues including bone formation [17]. Stimulation of ALP activity could promote osteoblast differentiation, mineralization, and bone formation [18]. Knockdown of FOXP1 reduced the ALP activity and prevented osteoblast mineralization, thus inhibiting the process of bone formation. Taken together, FOXP1 is a regulator of osteoblast differentiation and mineralization, promoting bone formation.

TGF-β was considered as a mediator in regulating osteoblast differentiation and bone shape through BMP and WNT/β-catenin pathway [11, 19, 20]. It was reported that inhibition of TGF-β repressed proliferation, collagen synthesis, and inflammation factors in human embryonic lung fibroblasts [21]. During osteoblast differentiation, upregulation of TGF-β phosphorylated Smad2 and Smad3 and promoted interaction of Smad3 with Smad4, thus stabilizing β-catenin [11]. Results from the present study manifested that downregulation of FOXP1 reduced the expression of TGF-β and blocked the signal transduction of Smad signaling, indicating that TGF-β/Smad pathway medicated FOXP1-induced osteoblast differentiation. ALK5 was one of the type I TGF-β receptors which mediated signal transduction of TGF-β signaling to activate the Smad signaling, a key downstream pathway of TGF-β signaling [11]. A previous study found that ALK-5 took part in TGF-β1-induced bone turnover and osteoblastic differentiation [22]. In this study, expression of ALK-5 was downregulated by knockdown of FOXP1. Suppression of ALK-5 was found to reverse osteoblast differentiation and mineralization induced by overexpression of FOXP1. These results indicated the participation of ALK-5 in the regulation of FOXP1-induced osteoblast differentiation and mineralization through mediating TGF-β/Smad signal transduction. The finding, hence, provided a new mechanism and signaling pathway during osteoblast differentiation and mineralization.

In summary, data of this study demonstrated that FOXP1 was highly expressed during osteoblast differentiation. Knockdown of FOXP1 showed the transcriptional suppression of osteogenic proteins: OSX, ALP, and OCN. ALP activity and osteoblast mineralization were also repressed by the knockdown of FOXP1. Downregulation of FOXP1 suppressed the stimulation of TGF-β/ALK-5 pathway, and knockdown of ALK-5 attenuated the promotion of osteoblast differentiation induced by overexpression of FOXP1. Therefore, FOXP1 promoted osteoblast differentiation and mineralization through modulating TGF-β/ALK-5 pathway, providing a new mechanism and target in regulating bone formation.

Acknowledgements: This work was supported by the Youth Projects of Wannan Medical College. (Grant No. WK2019F01 and Grant No. WK2019ZF08).

REFERENCES

1. Komori T (2016) Cell death in chondrocytes, osteoblasts, and osteocytes. *Int J Mol Sci* **17**, ID 2045.
2. Salhotra A, Shah HN, Levi B, Longaker MT (2020) Mechanisms of bone development and repair. *Nat Rev Mol Cell Biol* **21**, 696–711.
3. Seeman E (2009) Bone modeling and remodeling. *Crit Rev Eukaryot Gene Expr* **19**, 219–233.
4. Ponzetti M, Rucci N (2021) Osteoblast differentiation and signaling: Established concepts and emerging topics. *Int J Mol Sci* **22**, ID 6651.

5. Capulli M, Paone R, Rucci N (2014) Osteoblast and osteocyte: games without frontiers. *Arch Biochem Biophys* **561**, 3–12.
6. Katoh M, Katoh M (2004) Human FOX gene family (Review). *Int J Oncol* **25**, 1495–1500.
7. Liu P, Huang S, Ling S, Xu S, Wang F, Zhang W, Zhou R, He L, et al (2019) Foxp1 controls brown/beige adipocyte differentiation and thermogenesis through regulating β3-AR desensitization. *Nat Commun* **10**, ID 5070.
8. Braccioli L, Vervoort SJ, Adolfs Y, Heijnen CJ, Basak O, Pasterkamp RJ, Nijboer CH, Coffer PJ (2017) FOXP1 promotes embryonic neural stem cell differentiation by repressing Jagged1 expression. *Stem Cell Rep* **9**, 1530–1545.
9. Shen W, Sun B, Zhou C, Ming W, Zhang S, Wu X (2020) CircFOXP1/FOXP1 promotes osteogenic differentiation in adipose-derived mesenchymal stem cells and bone regeneration in osteoporosis via miR-33a-5p. *J Cell Mol Med* **24**, 12513–12524.
10. Li H, Liu P, Xu S, Li Y, Dekker JD, Li B, Fan Y, Zhang Z, et al (2017) FOXP1 controls mesenchymal stem cell commitment and senescence during skeletal aging. *J Clin Invest* **127**, 1241–1253.
11. Zhou S (2011) TGF-β regulates β-catenin signaling and osteoblast differentiation in human mesenchymal stem cells. *J Cell Biochem* **112**, 1651–1660.
12. Liu J, Zhuang T, Pi J, Chen X, Zhang Q, Li Y, Wang H, Shen Y, et al (2019) Endothelial forkhead box transcription factor P1 regulates pathological cardiac remodeling through transforming growth factor-β1-endothelin-1 signal pathway. *Circulation* **140**, 665–680.
13. Bot PT, Grundmann S, Goumans MJ, de Kleijn D, Moll F, de Boer O, van der Wal AC, van Soest A, et al (2011) Forkhead box protein P1 as a downstream target of transforming growth factor-β induces collagen synthesis and correlates with a more stable plaque phenotype. *Atherosclerosis* **218**, 33–43.
14. Li H, Han X, Yang S, Wang Y, Dong Y, Tang T (2021) FOXP1 drives osteosarcoma development by repressing P21 and RB transcription downstream of P53. *Oncogene* **40**, 2785–2802.
15. Zhao H, Zhou W, Yao Z, Wan Y, Cao J, Zhang L, Zhao J, Li H, et al (2015) Foxp1/2/4 regulate endochondral ossification as a suppresser complex. *Dev Biol* **398**, 242–254.
16. Murshed M (2018) Mechanism of bone mineralization. *Cold Spring Harb Perspect Med* **8**, a031229.
17. Vimalraj S (2020) Alkaline phosphatase: Structure, expression and its function in bone mineralization. *Gene* **754**, ID 144855.
18. Wang W, Olson D, Cheng B, Guo X, Wang K (2012) *Sanguis Draconis* resin stimulates osteoblast alkaline phosphatase activity and mineralization in MC3T3-E1 cells. *J Ethnopharmacol* **142**, 168–174.
19. Song NJ, Kwon SM, Kim S, Yoon HJ, Seo CR, Jang B, Chang SH, Ku JM, et al (2015) Sulfuretin induces osteoblast differentiation through activation of TGF-β signaling. *Mol Cell Biochem* **410**, 55–63.
20. Suzuki E, Ochiai-Shino H, Aoki H, Onodera S, Saito A, Saito A, Azuma T (2014) Akt activation is required for TGF-β1-induced osteoblast differentiation of MC3T3-E1 pre-osteoblasts. *PLoS One* **9**, e112566.
21. Lin S, Fu J, Wang C, Liu Q, Xu F, Yang Y (2020) Inhibition of transforming growth factor-β type I receptor signaling by RNA interference suppresses the proliferation, collagen synthesis, and inflammation factors in human embryonic lung fibroblasts. *ScienceAsia* **46**, 472–480.
22. Yang C, Wang Y, Xu H (2017) Fluoride regulate osteoblastic transforming growth factor-β1 signaling by mediating recycling of the type I receptor ALK5. *PLoS One* **12**, e0170674.