



# Transforming Growth Factor $\beta$ 1/Smad4 Signaling Affects Osteoclast Differentiation via Regulation of miR-155 Expression

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Transforming growth factor  $\beta$ 1 (TGF $\beta$ 1)/Smad4 signaling plays a pivotal role in maintenance of the dynamic balance between bone formation and resorption. The microRNA miR-155 has been reported to exert a significant role in the differentiation of macrophage and dendritic cells. The goal of this study was to determine whether miR-155 regulates osteoclast differentiation through TGF $\beta$ 1/Smad4 signaling. Here, we present that TGF $\beta$ 1 elevated miR-155 levels during osteoclast differentiation through the stimulation of M-CSF and RANKL. Additionally, we found that silencing Smad4 attenuated the upregulation of miR-155 induced by TGF $\beta$ 1. The results of luciferase reporter experiments and CHIP assays demonstrated that TGF $\beta$ 1 promoted the binding of Smad4 to the miR-155 promoter at a site located in 454 bp from the transcription start site *in vivo*, further verifying that miR-155 is a transcriptional target of the TGF $\beta$ 1/Smad4 pathway. Subsequently, TRAP staining and qRT-PCR analysis revealed that silencing Smad4 impaired the TGF $\beta$ 1-mediated inhibition on osteoclast differentiation. Finally, we found that miR-155 may target SOCS1 and MITF to suppress osteoclast differentiation. Taken together, we provide the first evidence that TGF $\beta$ 1/Smad4 signaling affects osteoclast differentiation by regulation of miR-155 expression and the use of miR-155 as a potential therapeutic target for osteoclast-related diseases shows great promise.

**Keywords:** miR-155, osteoclast differentiation, Smad4, TGF $\beta$ 1

## INTRODUCTION

The balance between bone production and resorption is mediated by osteoblasts and osteoclasts, respectively, and is essential for bone homeostasis (Takayanagi, 2007). Excessive bone resorption mediated by osteoclasts is often linked to bone loss-related diseases, including osteoporosis and rheumatoid arthritis (Goldring and Gravallesse, 2000). However, the mechanism of osteoclast differentiation has not yet been clearly established. Osteoclasts are multinucleated cells (MNCs) that differentiated from mononuclear precursors of the macrophage/monocyte-lineage (Kikuta and Ishii, 2013; Teitelbaum, 2000). The differentiation of osteoclast precursors into mature osteoclasts can now be achieved *in vitro* using purified macrophages in the presence of two critical cytokines derived from osteoblasts, macrophage colony stimulating factor (M-CSF) and the receptor activator of nuclear factor (NF)- $\kappa$ B (RANK) ligand (RANKL) (Kikuta and Ishii, 2013; Nakashima et al., 2011; Teitelbaum, 2000). M-CSF is essential for the survival and differentiation of osteoclast progenitors by binding its receptor c-Fms (Teitelbaum, 2000; Udagawa et al., 1990). Additionally, this cytokine is

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required for the terminal differentiation into mature osteoclasts (Tanaka et al., 1993). The interaction of RANKL with its receptor RANK specifically regulates the process of osteoclast formation from mononuclear precursors (Baud'huin et al., 2007). Additionally, RANKL also stimulates multiple signaling cascades, including mitogen-activated protein kinase (MAPK), NF- $\kappa$ B, serine/threonine protein kinase Akt, and Src pathways (Boyle et al., 2003).

Transforming growth factor  $\beta$  (TGF $\beta$ ) is involved in various cellular processes, including cell proliferation, differentiation, angiogenesis, apoptosis, motility, and death (Massague, 1998). TGF $\beta$ 1 is the most abundant isoform of factors in the TGF $\beta$  superfamily, which include three isoforms (TGF $\beta$ 1,  $\beta$ 2, and  $\beta$ 3), the activins, the nodals, and the bone morphogenetic proteins (BMPs) (Janssens et al., 2005; Massague, 1998). TGF $\beta$ 1 plays a pivotal role in the bone environment, affecting function of both osteoblasts and osteoclasts, thereby helping to maintain the dynamic balance between bone formation and resorption (Janssens et al., 2005). TGF $\beta$  exerts its function by binding to its type II serine/threonine kinase receptor (TbRII), resulting in the activation of type I receptor (TbRI), which then propagates signals via intracellular signaling mediators including the Smad transcription factors (Engel et al., 1998; Massague and Wotton, 2000). As pivotal mediators in the TGF $\beta$  signaling pathway, Smads can be divided into three subfamilies: receptor-regulated (R-Smad, Smad1, 2, 3, 5, 8), common-mediator (Co-Smad and Smad4), and inhibitory Smads (I-Smads, Smad6, 7) based on their structural and functional differences (Massague and Wotton, 2000). Smad2 and Smad3 mediate signaling by TGF $\beta$  and activin, and Smad1 and presumably Smad5, Smad8, and Smad9 can be modified through BMP exposure (Engel et al., 1998). Smad6 and Smad7 have been identified as inhibitors of these signaling pathways (Anumanthan et al., 2005). Of note, Smad4 plays a central role in signal transmission of TGF- $\beta$  signaling (Schwarte-Waldhoff and Schmiegel, 2002; Yang and Yang, 2010).

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that can play a key regulatory role in posttranscriptional repression by targeting mRNAs (Bartel, 2004). Recent studies have demonstrated that a few miRNAs contribute to the regulation of osteoclast differentiation (Xia et al., 2011). miR-155, a specific miRNA that plays a role in various physiological and pathological processes, is processed from the B-cell integration cluster (BIC) gene (now designated as the miR-155 host gene) on chromosome 21 in humans and chromosome 16 in mouse (Elton et al., 2013). It plays critical roles in processes such as hematopoietic lineage differentiation, immunity, inflammation, viral infections, cancer, cardiovascular disease, and Down syndrome (Elton et al., 2013). Additionally, miR-155 has been implicated in the commitment of monocyte progenitors to macrophage differentiation and the differentiation of dendritic cells (Mann et al., 2010; Mashima, 2015; O'Connell et al., 2012). However, whether miR-155 regulates osteoclast differentiation through TGF $\beta$ 1/Smad4 signaling or via another signaling pathway remains unclear.

A previous study reported that TGF $\beta$ 1 induced osteoclast apoptosis, suggesting an important role in osteoclast prolif-

eration (Houde et al., 2009). Smad4 has also been reported to be necessary for osteoclast differentiation (Huang, 2014). From this, we asked if the TGF $\beta$ 1/Smad4 signaling pathway may have a potential effect on osteoclast differentiation. There was also evidence demonstrating that miR-155 could be regulated by the TGF $\beta$ /Smad pathway (Kong et al., 2008). Given this evidence, we hypothesized that TGF $\beta$ 1/Smad4 signaling can regulate osteoclast differentiation via regulation of the expression of miR-155. In this study, we analyzed the expression of miR-155 in the presence of TGF $\beta$ 1 during osteoclastogenesis. We also evaluated the role and specificity of Smad4 in TGF $\beta$ 1-mediated miR-155 regulation. Moreover, we investigated the effect of Smad4 on TGF $\beta$ 1-mediated regulation of osteoclast differentiation. Finally, we discussed the potential mechanism of miR-155 in osteoclast differentiation regulation.

## MATERIALS AND METHODS

### Preparation and differentiation of bone marrow-derived macrophages

C57BL/6 mice (6-8 weeks old) were obtained from the Laboratory Animal Center of Shanghai Institutes for Biological Science. In brief, mice were sacrificed by cervical dislocation under anesthesia with 3% pentobarbital sodium. After separation of the femurs and tibias and cutting off both ends of the bones, the bone marrow was rinsed out with cold PBS containing 2% FBS using a 25-gauge syringe. The erythrocytes were then lysed after sieving and centrifugation. All animal experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

For the preparation of bone marrow-derived macrophages (BMMs), bone marrow cells were maintained overnight in  $\alpha$ -MEM containing 10% FBS in a humidified incubator at 37°C with 5% CO<sub>2</sub>. The following day, non-adherent cells were cultured in the presence of M-CSF (50 ng/ml). The 3 days later, the adherent cells served as osteoclast precursors. For osteoclast differentiation, BMMs were maintained in 24-well plates (4  $\times$  10<sup>4</sup> cells/well) in  $\alpha$ -MEM with stimulation of M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 6 days.

### Tartrate-resistant acid phosphatase (TRAP) staining

To identify osteoclasts, control BMMs or infected BMMs treated with or without TGF $\beta$ 1 in the presence of M-CSF and RANKL were fixed with 4% formaldehyde, washed with PBS, and stained for TRAP activity using a leukocyte acid phosphatase kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. TRAP-positive (pink to purple) multinucleated cells with more than three nuclei were counted as osteoclasts.

### Real time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from BMMs using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA was then reverse transcribed into cDNAs using the iScript cDNA synthesis kit (Bio-rad). The cDNA templates were amplified by qRT-PCR using SYBR Premix Dimmer Eraser kit (TaKaRa). The relative expression levels of miR-155, pri-miR-

155, pre-miR-155, Smad4, Smad2, Smad3, DC-STAMP, NFATc1, Ctsk, SOCS1, and MITF were calculated by the  $2^{-\Delta\Delta Ct}$  method and normalized to the internal control GAPDH. The primer sequences used are listed as follows: GAPDH: 5'-TGCACCACCACTGCTTA-3' (forward) and 5'-GATGCAGGGATGATGTT C-3' (reverse). miR-155: 5'-ATGCTTAATGCTAATTGTGAT-3' (forward) and 5'-GTGCAGGGTCCGAGGT-3' (reverse). Smad4: 5'-GTGACGTTGGGTCAGGTGC-3' (forward) and 5'-TATGACAGCGTCGCCAGGT-3' (reverse). Smad2: 5'-AAACCCTTCCACTATCAGA-3' (forward) and 5'-CACTACTTAGGCAC TCA-3' (reverse). Smad3: 5'-AGCACACAATAACTTGGACC-3' (forward) and 5'-TAAGACACACTGGAACAGCGGATG-3' (reverse). DC-STAMP: 5'-CAGACTCCCAAATGCTGGAT-3' (forward) and 5'-CTGTGGAGGAACCTAAGCG-3' (reverse). Ctsk: 5'-AGGGAAGCAAGCACTGGATA-3' (forward) and 5'-GCTGCTGGAATCACATCTT-3' (reverse). NFATc1: 5'-TCATCTCTGC CAACA CAAA-3' (forward) and 5'-TCACCTGGTGTCTTC CTC-3' (reverse). SOCS1: 5'-CTGCGCTTCTATTGGGGAC-3' (forward) and 5'-AAAAGGC-AGTCGAAGGTCTCG-3' (reverse). MITF: 5'-ACTTCCCTTATCCCATCCACC-3' (forward) and 5'-TGAGATCCAGATTGTCGTACA-3' (reverse).

#### Small-interfering RNA (siRNA) transfection

The siRNAs were synthesized by GenePharma (China). The sequences of the specific siRNAs are as follows (Zhang et al., 2012b), and a scramble siRNA was used as the negative control (si-NC): 5'-UUCUCCGAACGUGUCACGUTT-3' (forward) and 5'-ACGUGACACGUUCCGAGAATT-3' (reverse). Smad4 siRNA#1: 5'-GCCAGGUUAUCCUGAAUATT-3' (forward) and 5'-UAUUCAGGAUAACCUAGGCTT-3' (reverse). Smad4 siRNA#2: 5'-GGUGGAGAGAGUGAAACAUTT-3' (forward) and 5'-AUGUUUCACUCUCCACCTT-3' (reverse). Smad2 siRNA: 5'-CAGAACTCCGCCTCTGGA-3' (forward) and 5'-TCCAGAGGCGGAAGTT-3' (reverse). Smad3 siRNA: 5'-AACCTGAAGATCTTCAACAACCTGTCTC-3' (forward) and 5'-AAGTTGTTGAAGATCTTCAAGCCTGTCTC-3' (reverse). BMMs were transfected with these siRNAs using the Lipofectamine 2000™ (Invitrogen, USA) reagent and the silencing efficiency was determined by qRT-PCR.

#### Luciferase reporter assay

miR-155 is found within the BIC gene (miR-155 host gene) on chromosome 21 in humans and chromosome 16 in mouse. The genomic structure of human BIC consists of three exons, and exon 3 encodes miR-155. Sequence analysis of this region of the mouse genomic DNA revealed two putative Smad response elements (CAGAC and CTGTCTGT) located at -542bp and -454bp from the transcription start site, which are conserved in human miR-155 promoter (Kong et al., 2008). Therefore, we cloned a mouse promoter 1.0 kb upstream from the transcriptional start site of the BIC (pri-miR-155) gene into the pGL3-basic luciferase (Luc) vector. Two luciferase reporter constructs, pGL3-miR-155-(542)-Luc and pGL3-miR-155-(454)-Luc, were created by cloning a mouse miR-155 promoter containing one of the Smad4-binding sites, either located at -542 bp or -454 bp relative to the start site in the pGL3-basic vector (Promega). Before performing the luciferase reporter assay, the BMMs were transfected with pGL3-miR-155-(542)-Luc or pGL3-

miR-155-(454)-Luc, co-transfected with or without the Smad4-siRNA, and then treated with or without TGFβ1 for 36 h incubation. Luciferase reporter assays were then performed using the Luciferase Assay System (Promega). The activity of the experimental constructs were normalized to β-galactosidase activity.

#### Chromatin immunoprecipitation (ChIP) assay

BMMs were harvested to 70-80% confluence and treated with or without TGFβ1 at 5ng/mL for 24 h. ChIP assays were conducted by using a Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacturer's protocol. Antibodies against Smad4 or IgG were obtained from Cell Signaling Technology. After the removal of the protein and RNA, the DNA was purified and subjected to PCR. The sequences of primers specific for the Smad4-binding sites in the BIC/pri-miR-155 promoter are as follows: 5'-CCAAAGG AACTACTGGAGGA-3' and 5'-CCCACAGGTCCTAGGCAAT-3'. PCR-amplified products were then resolved by 1.5% agarose gel electrophoresis and visualized by BioImage Analyzer (BAS2000, Fuji Film, Japan).

#### Retroviral gene transduction in BMMs

To overexpress miR-155 or knockdown the endogenous miR-155 in BMMs, respectively, retroviral vectors were constructed carrying the primary miR-155 sequence or the miR-155 sponge sequence. Briefly, to generate the pBabe-155 retrovirus, about 300 bp of the primary miR-155 sequence was amplified and cloned into the pBabe retroviral vector and then packaged into retrovirus by transfection into Phoenix A cells. To generate the pBabe-155s retrovirus, seven repeats of the miR-155 sponge sequence were synthesized and cloned into the pBabe retroviral vector and packaged into retrovirus by transfection into Phoenix A cells. BMMs were incubated with the pBabe, pBabe-155, or pBabe-155s viral supernatants for 8 h in the presence of M-CSF (50 ng/ml) and polybrene (6 μg/ml). Then, the viral supernatant was removed and BMMs were further incubated for another 6 days in the presence of M-CSF (50 ng/ml) and RANKL (100 ng/ml). The sequences of primers for the miR-155-over-expressing vector were 5'-TCTACCTTGCCCTCAGATTCATAAT-3' (forward) and 5'-TGACTCTGGACTGTGCATCCTCCC-3' (reverse). The miR-155 sponge sequence was 5'-ACCCCTATGG TATTAGCATTAA-3' (Zhang et al., 2012a).

#### Western blot

The proteins were isolated from BMMs transfected with si-NC or si-Smad4 in lysis buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, 0.5% deoxycholate, 10 mM sodium pyrophosphate, 0.1% SDS, 5 mM EDTA). Subsequently, equal amounts of proteins were separated by 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% fat-free milk, primary antibodies against Smad4, Smad2, Smad3, SOCS1, and MITF (Cell signaling, USA) were added, followed by incubation with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG. GAPDH was used as the loading control. The protein was detected with an enhanced chemiluminescence kit (Applygen Technologies, China) and the band intensity

was quantified with Image-Pro Plus 6.0 software.

### Statistical analysis

All statistical analyses were performed using SPSS 16.0 (SPSS Inc., USA). The data were analyzed using Student's *t*-test between the two groups. The data are presented as the mean ± SD. All experiments were repeated at least three times. A value of *P* < 0.05 was considered statistically significant.

## RESULTS

### TGFβ1 induces miR-155 during osteoclast differentiation

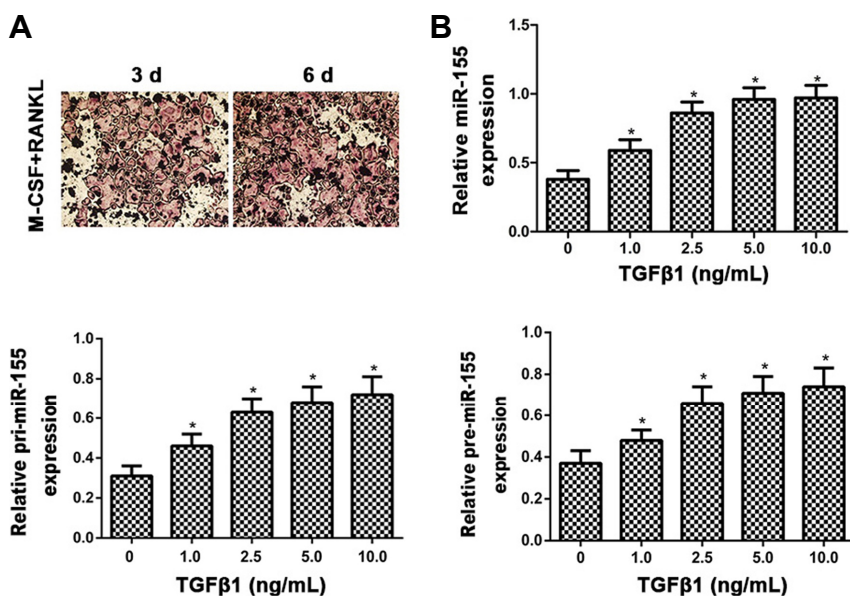
For osteoclast differentiation, BMMs were cultured in the presence of M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 6 days. TRAP staining analysis showed that after stimulation for 3 days, the BMMs differentiated into TRAP-positive osteoclasts. The irregular cells increased in number and in volume, and the MNCs developed. After stimulation for 6 d, the MNCs grew significantly in number (Fig. 1A). To explore the potential role of TGFβ1 on miR-155 during osteoclast differentiation from stimulation of M-CSF and RANKL, BMMs were treated with TGFβ1 at increasing concentrations (1.0, 2.5, 5.0, and 10 ng/ml) and the level of miR-155 expression was examined by qRT-PCR. As shown in Fig. 1B, TGFβ1 treatment elevated the expression of miR-155 in a concentration-dependent manner compared with the control group (0 ng/ml of TGFβ1). Next, we investigated the relative levels of precursor miR-155 (pre-miR-155) and primary transcripts of miR-155 (pri-miR-155) in the presence of TGFβ1. Results showed that TGFβ1 treatment also elevated the expressions of pre-miR-155 and pri-miR-155 in a concentration-dependent manner (Fig. 1B), which were consistent with the observed miR-155 expression. These results supported the transcriptional regulation of miR-155 by TGFβ1. However, when the concentration of TGFβ1 reached

10 ng/ml, the expression levels of miR-155, pri-miR-155, and pre-miR-155 were only slightly higher than those treated with 5 ng/ml TGFβ1. As a result, 5.0 ng/ml of TGFβ1 treatment for BMMs was selected for use in subsequent experiments.

### Silencing Smad4 impairs the TGFβ1-induced upregulation of miR-155

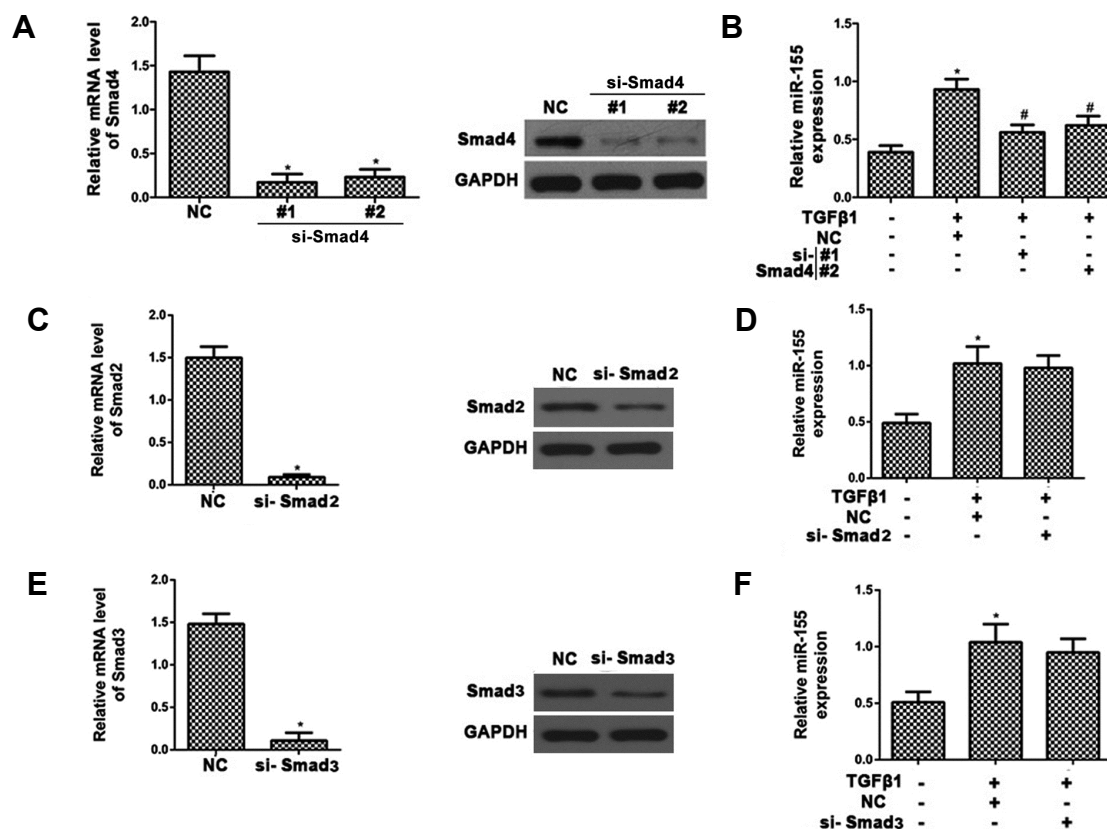
Considering that Smad2, Smad3, and Smad4 are all involved in signal transmission of TGF-β signaling, we examined whether these three Smads mediated TGFβ1-induced upregulation of miR-155. First, we evaluated the potential role of Smad4 on TGFβ1-mediated induction of miR-155. BMMs were separately transfected with si-Smad4#1, si-Smad4#2 and si-NC. Results of both qRT-PCR and Western blot showed that the relative mRNA and protein expression levels of Smad4 were significantly decreased (Fig. 2A). M-CSF and RANKL were then used to stimulate BMMs to differentiate into osteoclasts. Next, the control BMMs or transfected BMMs were treated with or without TGFβ1, and the relative miR-155 expression was detected by qRT-PCR. As shown in Fig. 2C, the relative expression of miR-155 in BMMs transfected with si-NC, si-Smad4#1, or si-Smad4#2 followed by treatment with 5 ng/ml TGFβ1 was higher than the level in the control BMMs, suggesting that TGFβ1 led to the upregulation of miR-155 in BMMs. We also found that the relative expression of miR-155 in BMMs transfected with si-Smad4#1 or si-Smad4#2 was lower than that in BMMs transfected with si-NC. These results showed that silencing Smad4 attenuated the stimulating effect of TGFβ1 on the expression level of miR-155.

To evaluate Smad4 specificity in TGFβ1-mediated miR-155, we also evaluated the effect of silencing Smad2 or Smad3 on the expression of miR-155 in the presence of TGFβ1. The transfection efficiency of silencing Smad2 or Smad3 was determined by both qRT-PCR and Western blot (Figs. 2C and



**Fig. 1. TGFβ1 induces miR-155 in BMMs stimulated by M-CSF and RANKL to differentiate into osteoclasts.** (A) BMMs were stimulated by M-CSF and RANKL for 3 d or 6 d to differentiate into osteoclasts. TRAP-positive multinucleated cells were counted as osteoclasts. (B) BMMs were treated with TGFβ1 at various concentrations (0, 1.0, 2.5, 5.0, and 10 ng/ml) and the relative expression levels of miR-155, pri-miR-155, and pre-miR-155 were examined by qRT-PCR. \**P* < 0.05 vs. the control group (0 ng/ml TGFβ1).





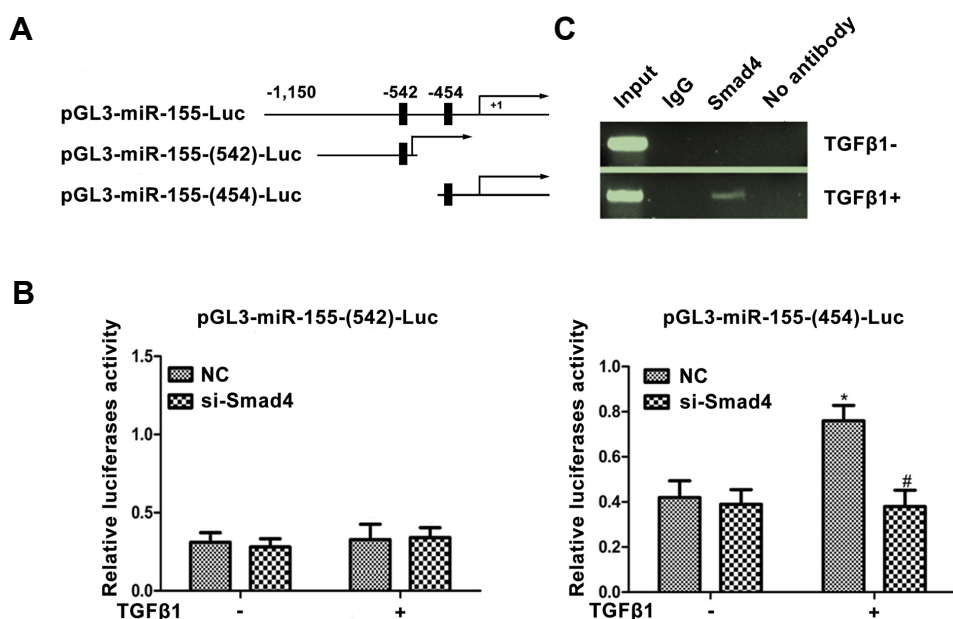
**Fig. 2. Silencing Smad4 impairs TGFβ1-induced upregulation of miR-155.** The relative mRNA and protein levels of (A) Smad4, (C) Smad2, and (E) Smad3 after transfection with negative control siRNA (si-NC), si-Smad4 (#1 and #2), si-Smad2, and si-Smad3 were examined by qRT-PCR and Western blot, respectively. GAPDH served as the loading control. M-CSF and RANKL were used to stimulate BMMs to differentiate into osteoclasts. The relative expression of miR-155 in control BMMs or BMMs transfected with si-NC, (B) si-Smad4 (#1 and #2), (D) si-Smad2, and (F) si-Smad3 respectively prior to TGFβ1 treatment was detected by qRT-PCR. \* $P < 0.05$  vs. the control group. # $P < 0.05$  vs. TGFβ1+si-NC group.

2E). Silencing either Smad2 or Smad3 had little effect on the TGFβ1-mediated induction of miR-155 (Figs. 2D and 2F). Taken together with the above effects of silencing Smad4, these results illustrated a strong and specific role for Smad4, but not Smad2 or Smad3, in the stimulation of miR-155 mediated by TGFβ1.

### miR-155 is a transcriptional target of the TGFβ1/Smad4 pathway

To verify whether miR-155 is directly regulated by the TGFβ1/Smad4 pathway, a luciferase reporter assay and a CHIP assay were performed. There are two putative Smad binding sites located -542 bp and -454 bp upstream of the transcriptional start site in the miR-155 promoter region, so two luciferase reporter constructs were generated in which one of the two sites was mutated, pGL3-miR-155-(542)-Luc and pGL3-miR-155-(454)-Luc (Fig. 3A). BMMs were transfected with si-Smad4#1, si-Smad4#2, and si-NC. Then, control and Smad4-knockdown cells were transfected with pGL3-miR-155-(542)-Luc or pGL3-miR-155-(454)-Luc, followed by treatment with or without TGFβ1. Next, cells were subjected to the luciferase reporter assay. There was no ob-

vious alteration in the activity of the miR-155 promoter in the Smad4-knockdown BMMs compared with the activity in control BMMs without stimulation of TGFβ1. However, the activity of miR-155 promoter in BMMs transfected with pGL3-miR-155-(454)-Luc was significantly enhanced by TGFβ1. The knockdown of Smad4 obviously impaired the TGFβ1-stimulated enhancement of miR-155 promoter activity. However, this effect mediated by TGFβ1 was not observed in BMMs transfected with the pGL3-miR-155-(542)-Luc construct (Fig. 3B). These results suggested that the Smad4 binding site at -454 bp was responsible for TGFβ1-stimulated miR-155 promoter activity. To verify this, a ChIP assay was performed to investigate whether Smad4 could bind to the 454 bp in the miR-155 promoter sequence following TGFβ1 treatment *in vivo*. BMMs were treated with or without TGFβ1 for 4 h. DNA fragments from anti-Smad4 immunoprecipitates were analyzed by PCR using primers specific for the Smad binding region that contained the -454 bp region that showed activity in the luciferase reporter assay. The results indicated that Smad4 bound to the miR-155 promoter following the stimulation of TGFβ1 (Fig. 3C). Taken together, our results indicated that miR-155 is a tran-



**Fig. 3. miR-155 is a transcriptional target of the TGFβ1/Smad4 pathway.** (A). Diagram displays the mouse miR-155 promoter containing two putative Smad4-binding sites located at -542 bp and -454 bp relative to the transcription start site. A luciferase reporter construct pGL3-miR-155-(542)-Luc was created by cloning individual Smad4-binding site deletion mutants (deletion of -454 bp) into the pGL3 plasmid. pGL3-miR-155-(454)-Luc was created by cloning individual Smad4-binding site deletion mutants (deletion of -542 bp) into the pGL3 plasmid. (B). BMMs were transfected with si-NC, si-Smad4#1, or si-Smad4#2. Next, the control and Smad4-knockdown cells were transfected with pGL3-miR-155-(542)-Luc or pGL3-miR-155-(454)-Luc, followed by treatment with or without 5 ng/ml TGFβ1. After incubation for 36 h, BMMs were subjected to the luciferase reporter assay. The relative luciferase activities of si-Smad4#1 and si-Smad4#2 groups were averaged. (C). ChIP analysis was performed in BMMs treated with or without TGFβ1. PCR was performed with DNA fragments from anti-Smad4 immunoprecipitates. IgG and anti-actin antibody served as negative controls. \* $P < 0.05$  vs. the control group. # $P < 0.05$  vs. TGFβ1+si-NC group.

scriptional target of the TGFβ1/Smad4 pathway.

### Silencing Smad4 impaired the TGFβ1-mediated inhibition on osteoclast differentiation

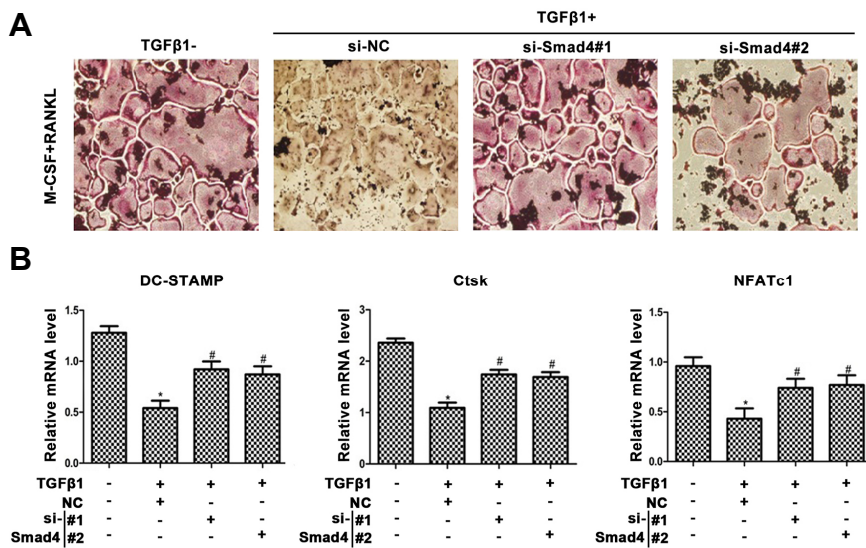
To investigate the effect of TGFβ1 and Smad4 on osteoclast differentiation in response to stimulation of M-CSF and RANKL, BMMs were separately transfected with si-NC, si-Smad4#1, and si-Smad4#2, then treated with 5 ng/ml of TGFβ1. TRAP staining demonstrated that MNCs in BMMs treated with TGFβ1 decreased in number and in volume compared to those in BMMs without TGFβ1 treatment, suggesting that TGFβ1 treatment inhibited osteoclast differentiation in BMMs. Additionally, MNCs in BMMs transfected with si-Smad4#1 and si-Smad4#2 increased in number and in volume compared to BMMs transfected with si-NC, all followed by TGFβ1 treatment, suggesting that silencing Smad4 impaired the inhibition of osteoclast differentiation that was mediated by TGFβ1 (Fig. 4A).

Next, qRT-PCR was performed to examine the relative expression levels of osteoclast differentiation marker genes DC-STAMP (dendritic cell-specific transmembrane protein), Ctsk (Cathepsin K), and NFATc1 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1). BMMs were separately transfected with si-NC, si-Smad4#1, and si-Smad4#2, and then treated with 5 ng/ml of TGFβ1. Data showed that

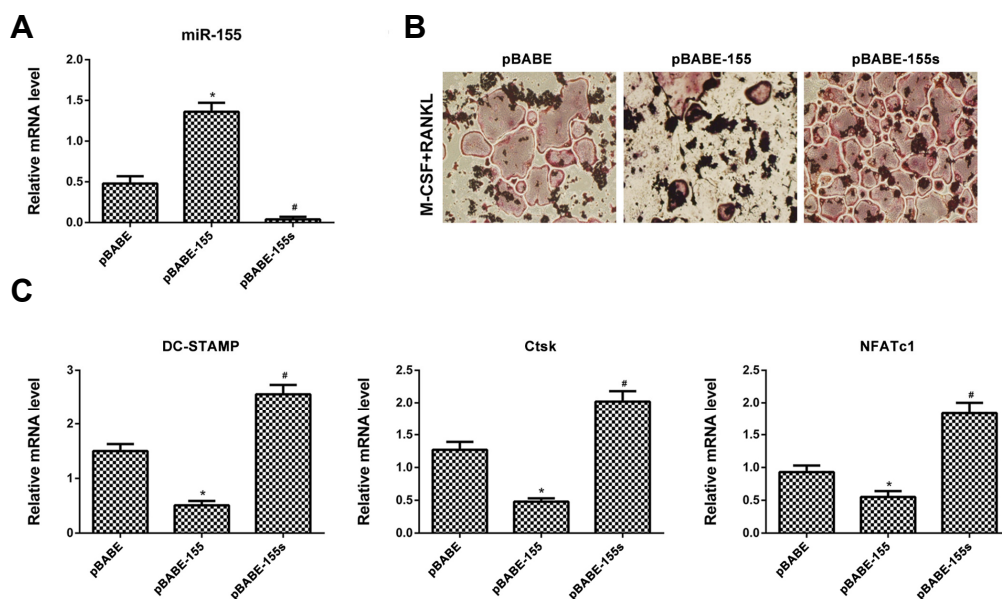
the relative mRNA expression level of DC-STAMP in BMMs without TGFβ1 treatment was higher than that in BMMs treated with TGFβ1, suggesting that TGFβ1 downregulated the relative expression level of DC-STAMP. Furthermore, the relative mRNA expression level of DC-STAMP in Smad4-knockdown BMMs treated with TGFβ1 was higher than that in BMMs transfected with si-NC followed by TGFβ1 treatment, which implied that silencing Smad4 may impair the TGFβ1-mediated downregulation of DC-STAMP. Similar results were also observed in the relative mRNA expression levels of Ctsk and NFATc1 (Fig. 4B). Taken together, our results showed that TGFβ1 inhibited osteoclast differentiation, and this effect was impaired by silencing Smad4.

### miR-155 inhibits osteoclast differentiation

To explore the effect of miR-155 expression on osteoclast differentiation, we transduced BMMs with pBabe-155 to overexpress miR-155 and pBabe-155s to inhibit the function of endogenous miR-155, and then cultured these BMMs in the condition where osteoclastogenesis can occur. The transduction efficiency was high, as determined by qRT-PCR assays (Fig. 5A). TRAP staining revealed that MNCs in pBabe-155-transduced BMMs decreased in number and in volume. However, in pBabe-155s-transduced BMMs, the number of TRAP-positive cells increased (Fig. 5B). This sug-



**Fig. 4. Effects of TGFβ1/Smad4 signaling on osteoclast differentiation.** BMMs were stimulated by M-CSF and RANKL to differentiate into osteoclasts. (A). BMMs were separately transfected with si-NC, si-Smad4#1, or si-Smad4#2, and then treated with 5 ng/ml TGFβ1. TRAP-positive multinucleated cells were counted as osteoclasts. (B). The relative mRNA expression levels of osteoclast differentiation markers DC-STAMP, Ctsk, and NFATc1 in BMMs transfected with si-NC, si-Smad4#1 and si-Smad4#2 prior to 5 ng/ml TGFβ1 treatment were detected by qRT-PCR. \**P* < 0.05 vs. the control group. #*P* < 0.05 vs. TGFβ1+si-NC group.



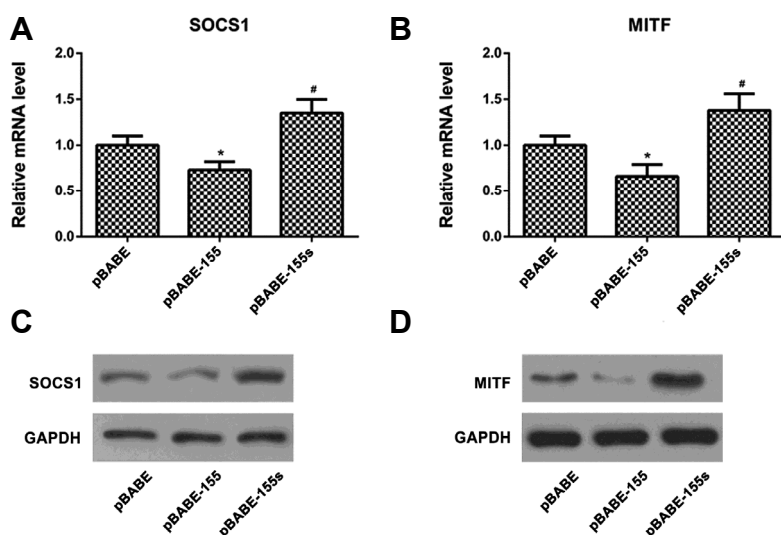
**Fig. 5. Effects of miR-155 on osteoclast differentiation.** (A). Relative mRNA levels of miR-155 in BMMs transduced with pBABE (as negative control), pBABE-155, and pBABE-155s retroviruses were analyzed by qRT-PCR. (B). The differentiation of BMMs transduced with pBABE, pBABE-155, and pBABE-155s retroviruses with stimulation by M-CSF and RANKL was analyzed by TRAP staining. TRAP-positive multinucleated cells were counted as osteoclasts. (C). qRT-PCR was performed to analyze the relative mRNA levels of osteoclast differentiation markers (DC-STAMP, Ctsk and NFATc1) in BMMs transduced with pBABE, pBABE-155, and pBABE-155s retroviruses. \**P* < 0.05 vs. pBABE group. #*P* < 0.05 vs. pBABE-155 group.

gested that overexpression of miR-155 inhibited osteoclast differentiation but inhibition of miR-155 facilitated it. Next, qRT-PCR was performed to analyze the relative expression levels of osteoclast differentiation marker genes DC-STAMP, Ctsk, and NFATc1. The data revealed that the relative DC-STAMP expression in pBABE-155-transduced BMMs was lower than that in control BMMs. However, pBABE-155s-transduced BMMs showed increased DC-STAMP expression. This indicated that miR-155 overexpression suppressed the

expression of DC-STAMP, but miR-155 inhibition promoted it. Similar results were observed in Ctsk and NFATc1 groups (Fig. 5C). Taken together, our results indicated that miR-155 inhibited osteoclast differentiation.

#### miR-155 suppressed SOCS1 and MITF expression during osteoclast differentiation

To investigate the mechanism of miR-155 in the regulation of osteoclast differentiation, we evaluated the effect of miR-



**Fig. 6. miR-155 suppresses SOCS1 and MITF expression during osteoclast differentiation.** (A). Relative mRNA and (C) protein levels of SOCS1 in BMMs transduced with pBABE (as the negative control), pBABE-155, and pBABE-155s retroviruses were analyzed by qRT-PCR and Western blot, respectively. (B) Relative mRNA and (D) protein levels of MITF in BMMs transduced with pBABE, pBABE-155, and pBABE-155s retroviruses were analyzed by qRT-PCR and Western blot, respectively. \* $P < 0.05$  vs. pBABE group. # $P < 0.05$  vs. pBABE-155 group. GAPDH served as the loading control.

155 on the expression of suppressor of cytokine signaling (SOCS1) and microphthalmia-associated transcription factor (MITF). SOCS1 and MITF are two essential regulators of osteoclastogenesis and are potential targets of miR-155 (Zhang et al., 2012a). We transduced BMMs with pBABE-155 and pBABE-155s retroviruses and cultured these cells in the osteoclastogenesis condition. qRT-PCR and Western blot analysis demonstrated that pBABE-155 inhibited the expression of SOCS1 and MITF, but pBABE-155s promoted their expression (Fig. 6). Thus, we hypothesized that miR-155 may suppress osteoclast differentiation, at least partly, by targeting SOCS1 and MITF.

## DISCUSSION

In this study, we provide the first evidence that TGF $\beta$ 1/Smad4 affects osteoclast differentiation by regulating miR-155 expression. First, qRT-PCR analysis showed that TGF $\beta$ 1 elevated miR-155 expression levels during osteoclast differentiation in response to the stimulation of M-CSF and RANKL. Next, we found that silencing Smad4 by transfection with Smad4 siRNA attenuated the upregulation of miR-155 induced by TGF $\beta$ 1. Moreover, luciferase reporter assay and CHIP assay were performed and further verified that miR-155 is a transcriptional target of the TGF $\beta$ 1/Smad4 pathway. Subsequently, TRAP staining and qRT-PCR analysis revealed that TGF $\beta$ 1 repressed osteoclast differentiation and this inhibition was impaired by silencing Smad4. Finally, we found that miR-155 may target SOCS1 and MITF to mediate its suppressive effect on osteoclast differentiation.

Emerging evidence has highlighted the pivotal roles of osteoclasts, responsible for bone resorbing, not only in normal bone remodeling but in the skeletal osteopenia that occurs in diseases including osteoporosis, arthritis, periodontal disease and specific malignancies (Zhao et al., 2007). Various cytokines and transcription factors have been reported to play prominent roles in osteoclast differentiation and function, such as M-CSF, RANKL, Myc, Src, NF- $\kappa$ B, NFATc1, acti-

vator protein-1 (AP-1), and MITF (Zhao et al., 2007). A few genes such as TRAP, DC-STAMP, Ctsk, and NFATc1 that serve as osteoclast markers. TRAP is widely accepted as a differentiation marker for mature osteoclasts and as a cytochemical marker for osteoclast function (Huang, 2014). DC-STAMP, highly expressed in osteoclasts, is a putative seven-transmembrane protein that is essential for cell fusion and multinucleation in osteoclasts (Yagi et al., 2005). Ctsk, which localizes mainly to osteoclasts, has a strong proteolytic activity against bone matrix proteins (Goto et al., 2003). NFATc1 has been demonstrated to be involved in the regulation of osteoclast differentiation (Takayanagi et al., 2002). Our results showed that the relative mRNA levels of DC-STAMP, Ctsk, and NFATc1 were inhibited by the overexpression of miR-155, which was consistent with the impairment of TRAP staining activity in BMMs, suggesting the suppression of osteoclast differentiation.

Osteoclast differentiation is mainly regulated by three important signaling pathways, M-CSF signaling, RANKL-RANK signaling, and immunoreceptor tyrosine-based activation motif (ITAM)-dependent co-stimulatory signaling, which are activated by M-CSF, RANKL and ITAM, respectively (Ji et al., 2016). In addition to these three canonical signaling pathways, TGF $\beta$ 1 signaling plays a pivotal role in the dynamic bone environment, affecting the growth of both osteoblasts and osteoclasts in a manner that depends greatly on the cell differentiation stage, TGF $\beta$ 1 concentration, and other culture conditions, thereby helping to maintain the dynamic balance between bone formation and resorption (Janssens et al., 2005). For instance, TGF $\beta$ 1 was found to inhibit osteoblast differentiation and blockade of TGF $\beta$ 1 resulted in an increase of osteoblast numbers (Darcy et al., 2012). Another study reported that TGF $\beta$ 1 induced osteoclast apoptosis (Houde et al., 2009). Moreover, TGF $\beta$ 1 modulated the recruitment of osteoclast precursors to the bone environment, differentiation into mature osteoclasts, and bone resorption (Janssens et al., 2005). However, the precise role of TGF- $\beta$ 1 in osteoclastogenesis remains complex. Recent studies have



indicated that TGFβ1 promoted osteoclast formation at low concentrations (1-100 pg/ml), but inhibiting it at high concentrations (0.1-10 ng/ml) (Chenu et al., 1988; Hattersley and Chambers, 1991; Shinar and Rodan, 1990; Yamaguchi and Kishi, 1995). Our study found that 5 ng/ml TGFβ1 treatment inhibited osteoclast differentiation, which was impaired by silencing Smad4, suggesting that TGFβ1/Smad4 signaling affects osteoclast differentiation.

Cumulating evidence demonstrate that miRNAs play prominent roles in bone remodeling by regulating differentiation and function of osteoblasts and osteoclasts. Among these, several miRNAs are involved in osteoclast formation, differentiation, bone resorption, and apoptosis (Ji et al., 2016). Expression of specific miRNAs during osteoclastogenesis has been demonstrated to be downregulated or upregulated, for stimulatory or repressive effects on osteoclast differentiation. For example, miR-29 (Franceschetti et al., 2013), miR-31-5p (Mizoguchi et al., 2013), miR-183-5p (Ke et al., 2015), and miR-214 (Zhao et al., 2015) have been reported to be upregulated in BMMs and promote osteoclast formation. In contrast, miR-7b-5p (Dou et al., 2014), miR-34a-5p (Krzeszinski et al., 2014), miR-124-3p (Lee et al., 2013), and miR-218-5p (Qu et al., 2015) were downregulated in BMMs and inhibited osteoclast formation. However, the relationship between the expression pattern of miRNA and its role in osteoclastogenesis is complex. For instance, miR-26a-5p was elevated in BMMs but exerted an inhibitory effect on osteoclast formation, the opposite effect of the other upregulated miRNAs (Kim et al., 2015). Despite the abundant studies of miRNAs involved in osteoclast formation, there were few investigations of the role of miR-155 in osteoclastogenesis. Our study demonstrated that miR-155 is upregulated by TGFβ1, and exerts an inhibitory effect on osteoclast differentiation, which is consistent with the model that miR-155 overexpression may inhibit osteoclast differentiation (Kagiya and Nakamura, 2013).

SOCS1 and MITF were reported as potential targets of miR-155 (Androulidaki et al., 2009; Jiang et al., 2010; Lu et al., 2011; Mann et al., 2010). SOCS1 and MITF are induced in RANKL-treated BMMs and serve as positive regulators of osteoclast differentiation (Zhang et al., 2012a). SOCS1 facilitates osteoclast differentiation by suppressing (interferon-β) IFN-β downstream signaling (Hayashi et al., 2002) and MITF facilitates osteoclast differentiation by cooperation with NFATc1 (Feng et al., 2009). Of course, negative regulation of osteoclast differentiation is necessary to maintain proper osteoclast numbers and avoid osteoclast-related diseases. Our present study demonstrated that both TGFβ1 and its inducible miR-155 suppressed osteoclastogenesis. Furthermore, miR-155 inhibited expression of its two targets, SOCS1 and MITF. This data suggests a model that miR-155 can target SOCS1 and MITF to convey the suppressive effect of TGFβ1 on osteoclast differentiation.

In conclusion, TGFβ1/Smad4 signaling affects osteoclast differentiation by regulating miR-155 expression. Our findings reveal a suppressive role of miR-155 in osteoclast differentiation, suggesting great promise as a potential therapeutic target for osteoclast-related diseases.

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