

Role of p53 in promoting BMP9-induced osteogenic differentiation of mesenchymal stem cells through TGF- β 1

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Received August 11, 2022; Accepted February 24, 2023

DOI: 10.3892/etm.2023.11947

Abstract. Known as a tumour suppressor gene, p53 also plays a key role in controlling the differentiation of mesenchymal stem cells (MSCs). Bone morphogenetic protein 9 (BMP9) has been identified as a potent factor in inducing osteogenic differentiation of MSCs, but its relationship with p53 remains unclear. The present study revealed that TP53 was expressed at higher levels in MSCs from patients with osteoporosis and was associated with the top 10 core central genes found in the current osteoporosis genetic screen. p53 was expressed in C2C12, C3H10T1/2, 3T3-L1, MEFs, and MG-63 cell lines, and could be upregulated by BMP9, as measured by western blotting and reverse-transcription quantitative PCR (RT-qPCR). Furthermore, overexpression of p53 increased the mRNA and protein levels of osteogenic marker Runx2 and osteopontin, as evaluated by western blotting and RT-qPCR in BMP9-induced MSCs, whereas the p53 inhibitor pifithrin (PFT)- α attenuated these effects. The same trend was found in alkaline phosphatase activities and matrix mineralization, as measured by alkaline phosphatase staining and alizarin red S staining. Moreover, p53 overexpression reduced adipo-differentiation markers of PPAR γ and lipid droplet formation, as measured by western blotting, RT-qPCR and oil red O staining, respectively, whereas PFT- α facilitated adipo-differentiation in MSCs. In addition, p53 promoted TGF- β 1 expression and inhibition of TGF- β 1 by LY364947 partially attenuated the effects of p53 on promoting BMP9-induced MSC osteo-differentiation and inhibiting adipo-differentiation. The inhibitory effect of PFT- α on osteogenic markers and the promoting effect on adipo-genic markers can be reversed when combined with TGF- β 1. TGF- β 1 may enhance the promotion of osteo-differentiation of MSCs by p53 through inhibition of adipo-differentiation.

Collectively, by promoting BMP9-induced MSCs bone differentiation and inhibiting adipose differentiation, p53 may be a novel therapeutic target for bone-related diseases.

Introduction

Osteoporosis is considered to be one of the most common metabolic bone diseases. It is generally believed that the prevalence of osteoporosis in patients increases with age. The incidence of osteoporotic fractures will increase due to the aged tendency of population (1). Women are usually more susceptible to osteoporosis after menopause due to hormonal changes and have a higher incidence than men, but men have a higher mortality rate from fracture-related disease (2,3). Although there is significant interindividual variation in polygenic diseases, we can identify many osteoporosis susceptibility genes from genetic studies. Effective promotion of bone regeneration can significantly improve the cure rate of osteoporotic (4). Moreover, in orthopedic clinical treatment, bone tissue engineering technology with mesenchymal stem cells (MSCs) as the main raw material, with access and *in vitro* expansion, can provide a solution for clinical treatment of orthopedic diseases such as osteoporosis (5,6).

Bone Morphogenetic Proteins (BMPs) are members of the Transforming Growth Factor Beta (TGF- β), of which BMP9 has a higher osteogenesis potential and better clinical application prospects (7,8). However, while promoting MSCs osteogenic differentiation, BMP9 also promotes their adipogenic differentiation (9). Finding effective targets to both promote BMP9-induced osteogenic differentiation of MSCs and suppress their adipogenic differentiation is the key to address tissue bone engineering for the treatment of bone diseases such as osteoporosis.

The role of p53 as an oncogene in cancer has been studied in depth, TP53 mutation leads to the occurrence of cancer (10). Under normal conditions, p53 is inactivated, and would get activated in response to extra- or intra-cellular stress or impaired function (11). Moreover, exquisite regulation of p53 functions such as post-translational modifications is also critical for cell fate decision (12). Numerous studies demonstrated that p53 is a key cell proliferation regulator, while its function goes far beyond this. The active p53 regulates the expression of related target genes and participates in regulation of

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Key words: p53, bone remodeling, mesenchymal stem cells, osteogenic differentiation, TGF- β 1 signaling

DNA damage repair, cell cycle, apoptosis, and metabolic (13). However, p53 in development, stem cell differentiation, and non-tumor diseases still need further investigation. In the process of tumor treatment, radiotherapy and chemotherapy have negative effects on bone, and bone metastasis of cancer itself will also destroy osteogenesis. Some data suggested that p53 act as a sequence-specific transcription factor can be an important regulator in cell differentiation (14-16). The role of p53 in bone formation remains controversial. Some studies found that p53 deficiency results in osteogenesis inhibition (17,18), whereas other studies demonstrated that p53 is a negative regulator of osteoblast differentiation and skeletal development (19,20). Either view affirms the involvement of p53 in the regulation of MSCs osteogenic differentiation.

The present study systematically analyzed the effect of p53 on MSCs differentiation by detecting MSCs osteogenic and adipogenic differentiation markers at different stages, and preliminarily explored the molecular mechanisms related to TGF- β 1. Our finding provides a new experimental basis for promoting the clinical development and application of p53 and BMP9 in improving the therapeutic of bone diseases.

Materials and methods

Reagents. p53 inhibitor Pifithrin- α (PFT α) HBr (Cat# S2929), and TGF- β 1 inhibitor LY364947 (Cat# S2805) was purchased from Selleck Chem.

Cell culture and transfection. C2C12, C3H10T1/2, 3T3-L1 and MG-63 cells originally obtained from ATCC and mouse embryonic fibroblasts (MEFs) extracted from a pregnant NIH mouse were generously provided by Professor Tongchuan He (Medical Center of the University of Chicago). All cells were immortalized and can be sub-cultured in medium of Dulbecco's modified Eagle medium (Saimike, Cat#SMK200.01, China) containing 10% fetal bovine serum (Cat#SMK110.01, Saimike, China). The recombinant adenoviruses tagged with green fluorescent protein (GFP) were designated as AdBMP9, Adp53, AdTGF- β 1, while expressing GFP only were used as vehicle control. All recombinant adenoviruses were generously provided by Professor Tongchuan He, which were generated previously using the AdEasy system. MSCs were seeded in cell culture plates, and polybrene (5 μ g/ml, HY-112735, MCE) was added to give the final concentration of the culture medium when the cells were adherent (approximately 3 h). Then the recombinant adenoviruses were added and cultured at 37°C, 5% CO₂. Eight hours after transfection was recorded as a starting point. The virus titer of AdBMP9+, AdBMP9++, and AdBMP9+++ were MOI 10, MOI 15, MOI 20, respectively. Transfections with AdBMP9, Adp53 and AdTGF- β 1 with MOI 15 were selected in the experiment.

Western blot. Cells were lysed in RIPA buffer (MilliporeSigma, Cat# R0278) and heated to denature the protein. An equal amount of protein was loaded onto 4-10% polyacrylamide gel and transferred to a 0.45- μ m PVDF membrane (Thermo Fisher Scientific, Inc., Cat# IPVH00010), then blocked with 5% (w/v) bovine serum albumin. The membranes were incubated with the primary antibodies at 1:1,000 dilution, respectively [rabbit anti-p53 (Affinity Biosciences Ltd., Cat#

AF0879), anti-p-p53 (p53-18) (Santa Cruz Biotechnology, Inc., Cat# sc-13580), mouse anti-GAPDH (ProteinTech Group Inc., Cat# 60004-1-Ig), mouse anti-RUNX2 (Santa Cruz Biotechnology, Inc., Cat# sc-390351), mouse anti-OPN (Santa Cruz Biotechnology, Inc., Cat# sc-21742), rabbit anti-PPAR γ (Affinity Biosciences Ltd., Cat# AF6284) in primary antibody diluent (Beyotime Institute of Biotechnology, Cat# P0256)]. The membranes were washed in TBST and probed with secondary antibodies, then washed in TBST for three times and scanned using the CLiNX Scan Image system (CLiNX Science Instruments).

Reverse transcription-quantitative PCR (RT-qPCR). Cells were lysed in TRIzol (Invitrogen, Cat # 15596026), and the total RNA was extracted. After measuring the concentration with Nanodrop™ One (Thermo Fisher Scientific, Inc.), the reverse transcription reaction was performed (Takara Bio, Inc., Cat# RR037A). RT-qPCR was performed with the above cDNA using primers (Table I) and SYBR-Green qPCR mix (Bimake, Cat# b21202).

Alkaline phosphatase (ALP) staining. ALP activity was detected by a BCIP/NCT ALP assay kit (Beyotime Institute of Biotechnology, Cat# C3206). Cells were seeded in well plates with factors and stained on the day 5 and 7 protected from light, scanned and captured using a microscope (Olympus Corporation).

Alizarin red S (AZR) staining. Calcium mineralization was detected using an AZR assay kit (Saimike, Cat# SV0019). Cells treated with factors were cultured in well plates, and osteogenic medium was replaced 2 days later with DMEM supplemented with 5% FBS, dexamethasone (10 nM), glycerol-2-phosphate (10 mM), and vitamin C (50 μ g/ml). On day 21, the plates were stained, scanned, captured using a microscope (Olympus Corporation) and quantified using ImageJ software.

Oil red O (ORO) staining. Cells treated with factors were fixed and stained using an ORO assay kit (Saimike, Cat# SR0007). On day 10, the plates were stained, scanned, captured using a microscope (Olympus, Japan) and quantified using ImageJ software.

Statistical analysis. Datasets presented in this study can be found in the GEO datasets (GSE35959_RAW). All data were expressed in the form of means $\pm$ standard deviation (SD) analyzed using GraphPad prism 8.0 software. The data in each group are obtained from three independent experiments of biological replicates at least. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference. Adobe Illustrator 5 software was used to generate the figures.

Results

Expression of p53 induced in progenitor cells. Transcriptome sequencing of MSCs from osteoporotic and non-osteoporotic patient groups showed that TP53 was expressed at higher levels in MSCs from osteoporotic patients Fig. 1A. The results

Table I. Primer sequences.

Gene	Species	Forward, 5'-3'	Reverse, 5'-3'
p53	Mouse	AGAGACCGCCGTACAGAAGA	CTGTAGCATGGGCATCCTTT
Runx2	Mouse	GCCGGAATGATGAGAACTA	GGACCGTCCACTGTCACCTT
OPN	Mouse	TGCACCCAGATCCTATAGCC	CTCCATCGTCATCATCATCG
PPAR γ	Mouse	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT
β -actin	Mouse	TGCTGACAGGATGCAGAAGG	CGGACTCATCGTACTCCTGC
TP53	Human	GGCCCACTTCACCGTACTAA	GTGGTTTCAAGGCCAGATGT
β -actin	Human	CCACCATGTACCCTGGCATT	CGGACTCGTCATACTCCTGC

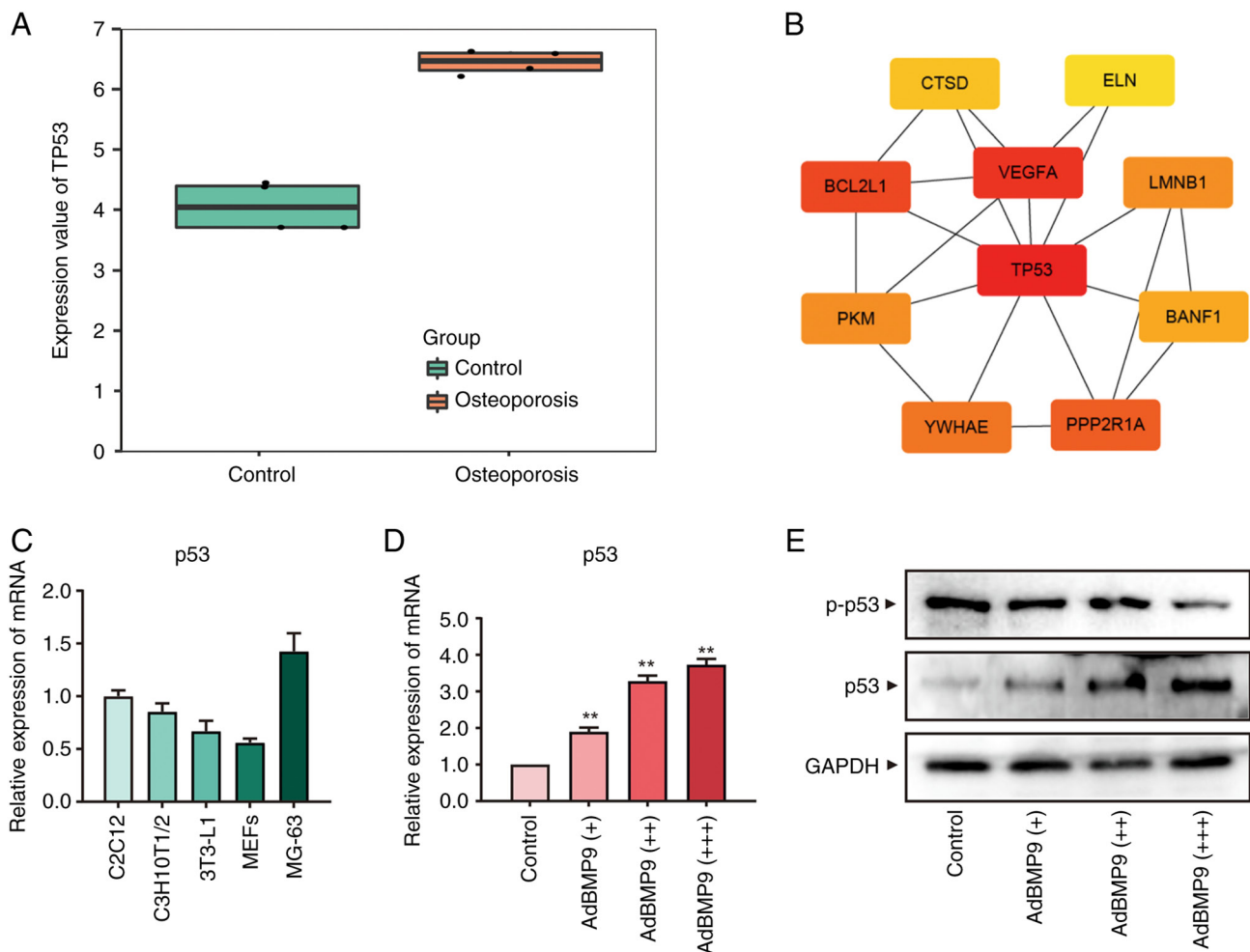


Figure 1. Expression of p53 in bone tissues and MSCs. (A) TP53 expression in bone tissues of patients with osteoporosis vs. normal patients (n=4). Data from the GEO dataset (GSE35959_RAW). (B) Bioinformatics analysis of core genes in transcriptome sequencing data of osteoporosis models. (C) The endogenous mRNA expression in C2C12, C3H10T1/2, 3T3-L1, MEFs, and MG-63 cells at 48 h. (D) RT-qPCR to detect the mRNA expression of p53 in AdBMP9 treated C3H10T1/2 cells. '+' indicates the titer of AdBMP9 at 48 h. (E) Western blot analysis of p53 and p-p53 in AdBMP9 treated C3H10T1/2 cells. '+' indicates the titer of BMP9 adenovirus at 48 h. **P<0.01 vs. Control. MEFs, mouse embryonic fibroblasts; RT-qPCR, reverse transcription-quantitative PCR.

of differential expression gene analysis showed that TP53 had a high correlation among the top 10 core central genes found in this model gene screening (Fig. 1B). Bioinformatics results suggested a high correlation between TP53 and osteoporosis. To examine the effect of p53 on MSCs differentiation at the cellular level, we examined the expression of p53 in several MSCs, including C2C12, C3H10T1/2, 3T3-L1, MEFs, and MG-63 cells. We found that p53 were expressed in all of the

above MSCs with different degrees of differentiation (Fig. 1C). Herein, progenitor cell line C3H10T1/2 cells were selected, which are less differentiated, have relatively high p53 expression and are widely used for the present study (21). Given the strong ability of BMP9 to promote the osteo-differentiation of MSCs, this experiment was conducted on the basis of BMP9 (22). MSCs were treated with different titers of BMP9 (Fig. S1A) to detect the gene expression and protein level of

p53 and phosphorylation of p53. As shown in the Fig. 1D, mRNA expression and protein level of p53 were significantly increased in MSCs at 24 and 48 h with increasing BMP9 titers, whereas phosphorylation of p53 were decreased (Fig. 1E). These results suggested that p53 may work with BMP9 to regulate the osteogenic potential of MSCs.

Effects of a p53-specific inhibitor on osteogenic and adipogenic markers induced by BMP9 in MSCs. The possible relationship between BMP9-induced osteoblast differentiation and p53 were analyzed. Recombinant adenoviral vector Adp53 for p53 was constructed and validated firstly (Figs. 2A and S1B). MSCs were sequentially tested for markers of osteogenic/lipogenic differentiation at different stages. Runt-related transcription factor 2 (Runx2), an early marker of MSCs osteogenic differentiation (23), can be upregulated by a variety of osteogenic factors. The induction of Runx2 by BMP9 was increased by overexpression of p53 (Fig. 2B). Alkaline phosphatase (ALP) is a stable and sensitive intermediate marker of osteogenic differentiation in MSCs (24). On day 5 and 7, ALP activity in BMP9-induced MSCs were promoted by Adp53 (Fig. 2C). Osteopontin (OPN) is an essential bone matrix protein closely associated with bone formation and development (25). Western blotting on day 9 and 11 showed that Adp53 increased the protein expression level of OPN in BMP9-induced MSCs (Fig. 2D). Alizarin Red S staining (26) showed that p53 significantly increased calcium salt deposition (Fig. 2E). The above results suggested that p53 contributes to the osteogenic differentiation of MSCs induced by BMP9. Nevertheless, BMP9 promotes osteogenic differentiation in MSCs while also inducing lipogenic differentiation.

The increased intracellular lipid accumulation that occurs during normal physiological differentiation leads to activation of p53, which inhibits lipogenesis by repressing the key lipogenic transcription factor PPAR γ to maintain homeostasis *in vivo* (27). PPAR γ were used as an early lipogenic marker to observe the effect of p53 on adipogenic differentiation in MSCs (28). Both RT-qPCR as well as western blotting results demonstrated that p53 promotes the mRNA and protein levels of the early-stage adipogenic marker PPAR γ (Fig. 2F and G). Moreover, the induction of later-stage adipogenic marker droplet formation of BMP9-induced MSCs measured by oil red O staining (29) were significantly inhibited by p53 (Fig. 2H). Taken together, p53 in promoting BMP9-induced MSCs osteogenic differentiation can be accompanied by inhibition of their adipogenesis.

Effects of a p53-specific inhibitor on osteogenic and adipogenic markers induced by BMP9 in MSCs. To further investigate the effect of p53 in BMP9-induced MSCs differentiation, we used PFT- α , a selective inhibitor of p53 (30), to perform assays from the opposite perspective. RT-qPCR and western blotting showed that Runx2, an early marker of osteogenic differentiation, were suppressed by PFT- α (15 μ M) in BMP9-induced MSCs (Fig. 3A and B). ALP staining further confirmed that ALP activity of MSCs was reduced by PFT- α significantly (Fig. 3C). Osteopontin (OPN), an advanced marker closely related to bone formation, also showed the suppression role of PFT- α (Fig. 3D). In addition, MSCs were cultured in osteogenic induction medium to evaluate the matrix mineralization

of osteoblasts. As shown in Fig. 3E, the formation of alizarin red-positive nodules treatment with PFT- α significantly were restrained. Taken together, these findings indicated that PFT- α reduced the osteogenic potential of BMP9 in MSCs. In terms of MSCs adipogenic differentiation, both early-stage marker PPAR γ expression (Fig. 3F and G) and later-stage marker oil red O staining (Fig. 3H) showed that the formation of BMP9-induced markers of MSCs adipogenic differentiation were facilitated by PFT- α . Collectively, these data suggested that silencing p53 disrupts the bone-lipid homeostasis in MSCs and shifts them towards adipogenesis.

Effects of LY364947 and/or p53 on BMP9-induced differentiation in MSCs. TGF- β 1 has bidirectional regulation of osteogenesis and adipogenesis and a shared downstream target Smad signaling with BMP9 (31). Our previous study showed that TGF- β 1 selective inhibitor LY364947 could suppress the BMP9 induced MSCs osteo-differentiation (32). ALP staining showed that ALP activity induced by BMP9 in MSCs could be enhanced by Adp53, however, the TGF- β 1 selective inhibitor LY364947 (33) practically eliminated this effect (Fig. 4A). PCR results showed that the effect of BMP9 on increasing mRNA level of OPN were induced by Adp53, whereas reduced by LY364947 (Fig. 4B). Although inhibition of TGF- β 1 may reverse the promotion of BMP9 osteogenic potential by p53, it is not clear whether the specific manner of the effect is related to adipose differentiation. PCR analysis of PPAR γ expression showed that LY364947 attenuated the inhibitory effect of p53 on BMP9-induced adipogenic differentiation (Fig. 4C). Moreover, Oil red O staining showed that p53 inhibited the formation of lipid droplets in BMP9-induced differentiation of MSCs, which could be partially reversed by LY264947 (Fig. 4D). The above results demonstrated that the regulatory actions of p53 on the osteogenic potential of BMP9 in MSCs were associated with the level of TGF- β 1.

Effects of TGF- β 1 and/or PFT- α on BMP9-induced differentiation in MSCs. On the contrary, ALP activity induced by BMP9 in MSCs can be reduced by p53 inhibitor PFT- α , and exogenous TGF- β 1 can partially reverse this effect (Figs. 5A and S1C). In addition, BMP9-induced OPN mRNA levels could be reduced by PFT- α , to the extent that this effect was in part reversed by TGF- β 1 overexpression (Fig. 5B). In terms of lipogenic differentiation, PFT- α promotes BMP9-induced mRNA expression of PPAR γ , whereas attenuated by exogenous TGF- β 1 (Fig. 5C). The oil red O staining showed the same trend in lipid droplet formation (Fig. 5D). The above results evidenced that TGF- β 1 may mediate the regulation of BMP9-induced MSCs adipogenic differentiation by p53.

Effects of p53 and BMP9 on regulating the expression of TGF- β 1. The role of TGF- β 1 on p53 in MSCs osteogenic differentiation were confirmed. However, the exact relationship between them needs to be further clarified. PCR showed that BMP9 promotes TGF- β 1 mRNA expression, exogenous p53 promotes the upregulation of TGF- β 1 by BMP9 (Fig. 6A), while inhibition of p53 suppresses the effect of BMP9 on TGF- β 1 mRNA levels (Fig. 6B). Results above further suggested that TGF- β 1 is involved in regulating MSCs differentiation engaged by p53.

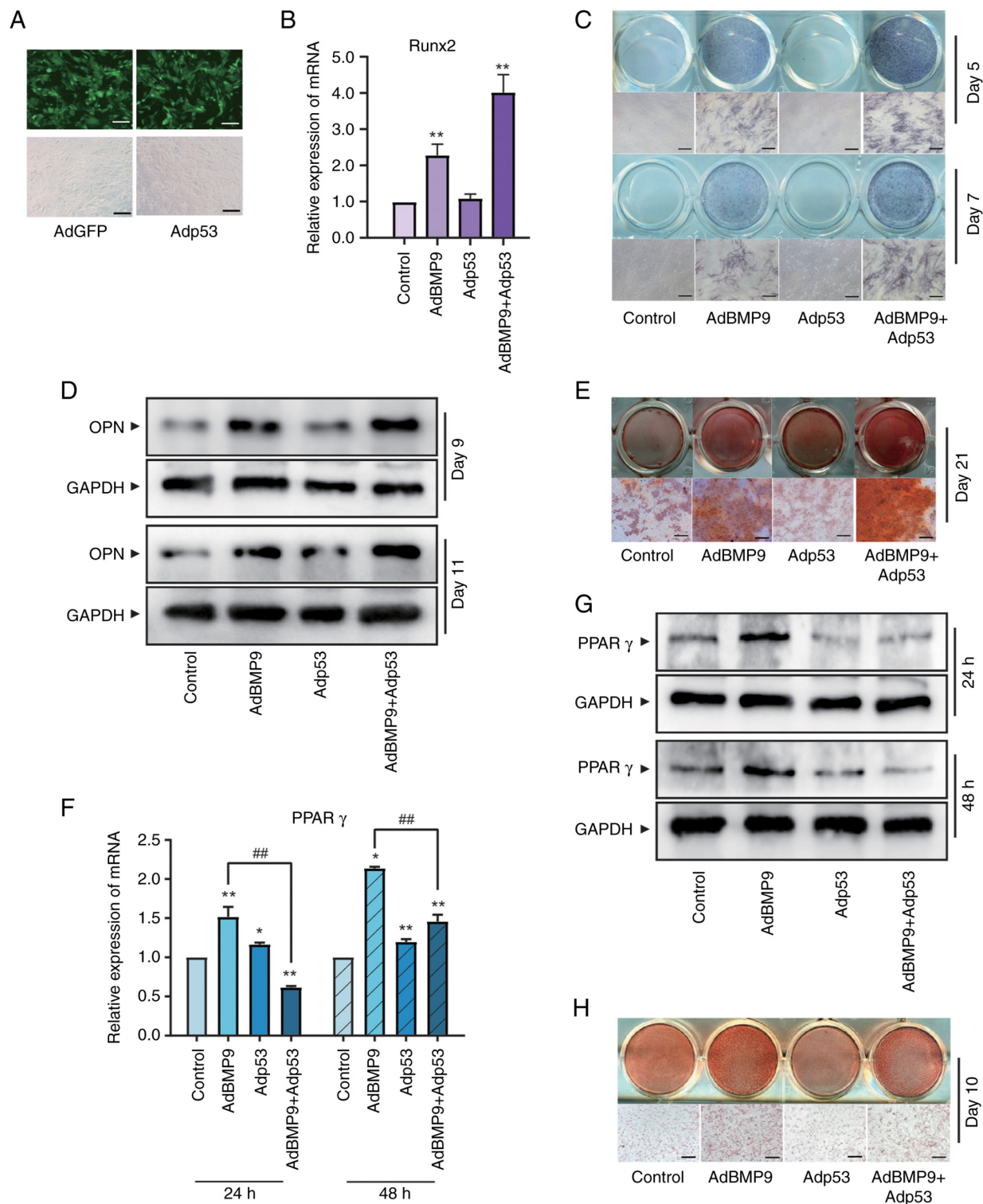


Figure 2. Effect of the p53 on osteogenic differentiation and adipogenesis differentiation in C3H10T1/2 cells. (A) C3H10T1/2 cells transfected by Adp53 or AdGFP at 48 h. (B) RT-qPCR of Runx2 in C3H10T1/2 cells with/without Adp53 at 48 h. '+' indicates the titer of Adp53. (C) ALP activity in C3H10T1/2 cells with/without Adp53 at D 5 and D 7. Scale bar, 100 μ m. (D) Western blot of OPN in C3H10T1/2 cells with/without Adp53 at D 9 and D 11. (E) Alizarin red S staining in C3H10T1/2 cells with/without Adp53 at D 21. Scale bar, 100 μ m. (F) RT-qPCR of PPAR γ in C3H10T1/2 cells with/without Adp53 at 24 and 48 h. (G) Western blot of PPAR γ in C3H10T1/2 cells with/without Adp53 at 24 and 48 h. (H) Oil red O staining for lipid droplet in C3H10T1/2 cells with/without Adp53 at D 10. Scale bar, 50 μ m. * P <0.05 and ** P <0.01 vs. Control. ## P <0.01 vs. AdBMP9. RT-qPCR, reverse transcription-quantitative PCR; ALP, alkaline phosphatase; OPN, osteopontin.

Discussion

Bones are a living dynamic tissue constantly regenerating themselves to stay healthy. In certain pathological conditions,

such as osteoporosis, insufficient bone mass could seriously affect the quality of survival of patients and even induce other diseases (34). Promotion of MSCs osteo-differentiation is a potential therapeutic breakthrough for the clinical treatment

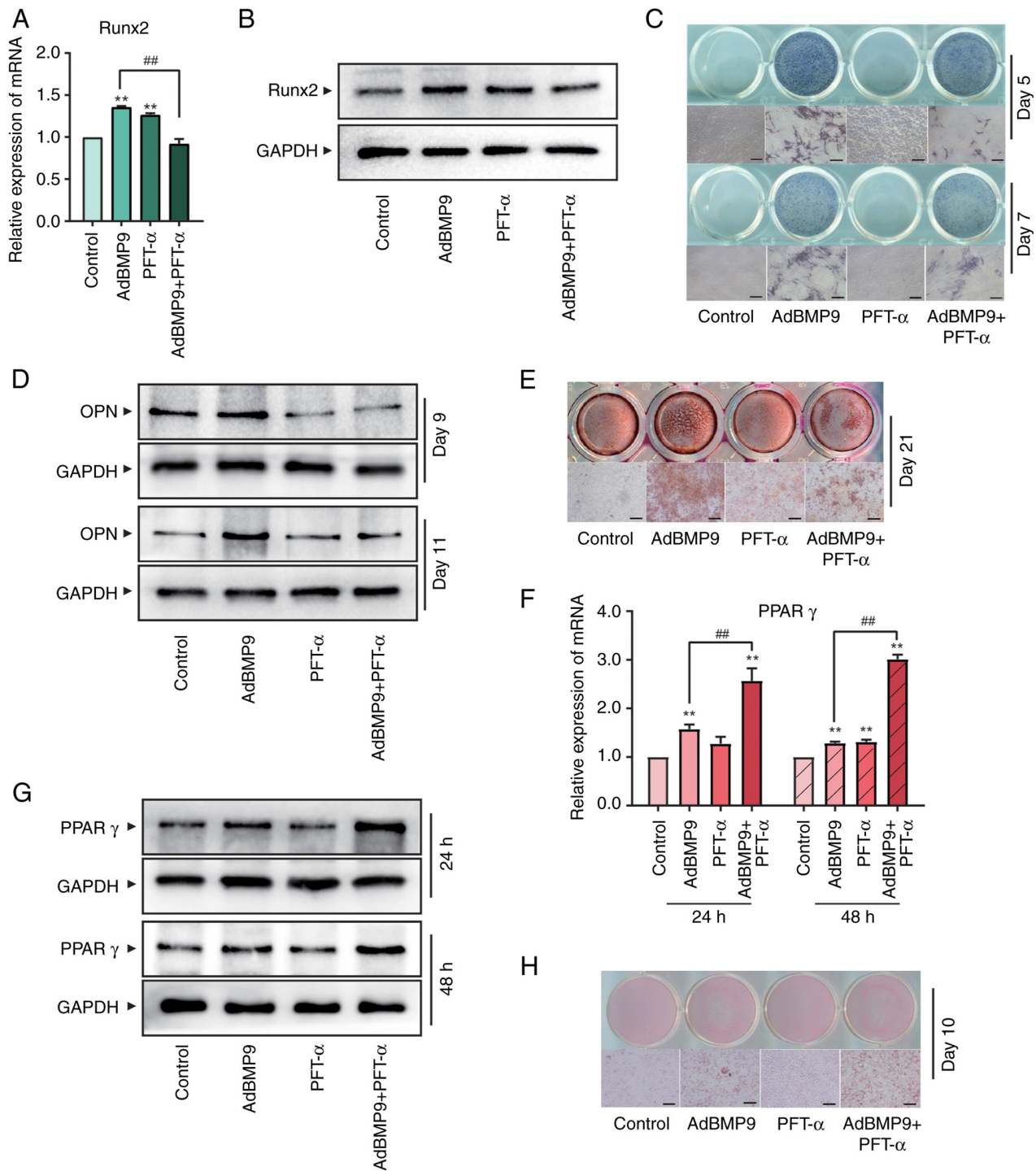


Figure 3. Effect of PFT- α on osteogenesis and adipogenesis differentiation in C3H10T1/2 cells. (A) RT-qPCR and (B) western blot of Runx2 in C3H10T1/2 cells with/without PFT- α (15 μ M) at 48 h. (C) ALP activity in C3H10T1/2 cells with/without PFT- α (15 μ M) at 48 h. (D) Western blot of OPN in C3H10T1/2 cells with/without PFT- α (15 μ M) at D 9 and D 11. (E) Alizarin red S staining in C3H10T1/2 cells with/without PFT- α (15 μ M) at D 21. Scale bar, 100 μ m. (F) RT-qPCR of PPAR γ in C3H10T1/2 cells with/without PFT- α (15 μ M) at 24 and 48 h. (G) Western blot of PPAR γ in C3H10T1/2 cells with/without PFT- α (15 μ M) at 48 h. (H) Oil red O staining in C3H10T1/2 cells with/without PFT- α (15 μ M) at D 10. Scale bar, 50 μ m. ** P <0.01 vs. Control. ## P <0.01 vs. AdBMP9. PFT- α , pifithrin- α ; RT-qPCR, reverse transcription-quantitative PCR; ALP, alkaline phosphatase; OPN, osteopontin.

of osteoporosis. Exploring the molecular mechanisms that enhance the ability of MSCs to osteodifferentiate could provide therapeutic options for clinical bone diseases (35). In the present study, we systematically investigated the role of p53 in MSCs differentiation induced by BMP9 and the underlying molecular mechanisms, expecting to provide new ideas for the treatment of bone diseases.

BMP9 is the strongest osteogenic inducer of the BMPs family, which can bind to type II receptors and phosphorylate Smad proteins to regulate the expression of downstream transcription factors, thereby inducing osteogenic differentiation of MSCs, has been termed as a classical signaling (36). Moreover, other signaling factors such as COX-2, Wnt/ β -catenin, MAPK, etc. interact with BMP9 to regulate its expression (37,38). The

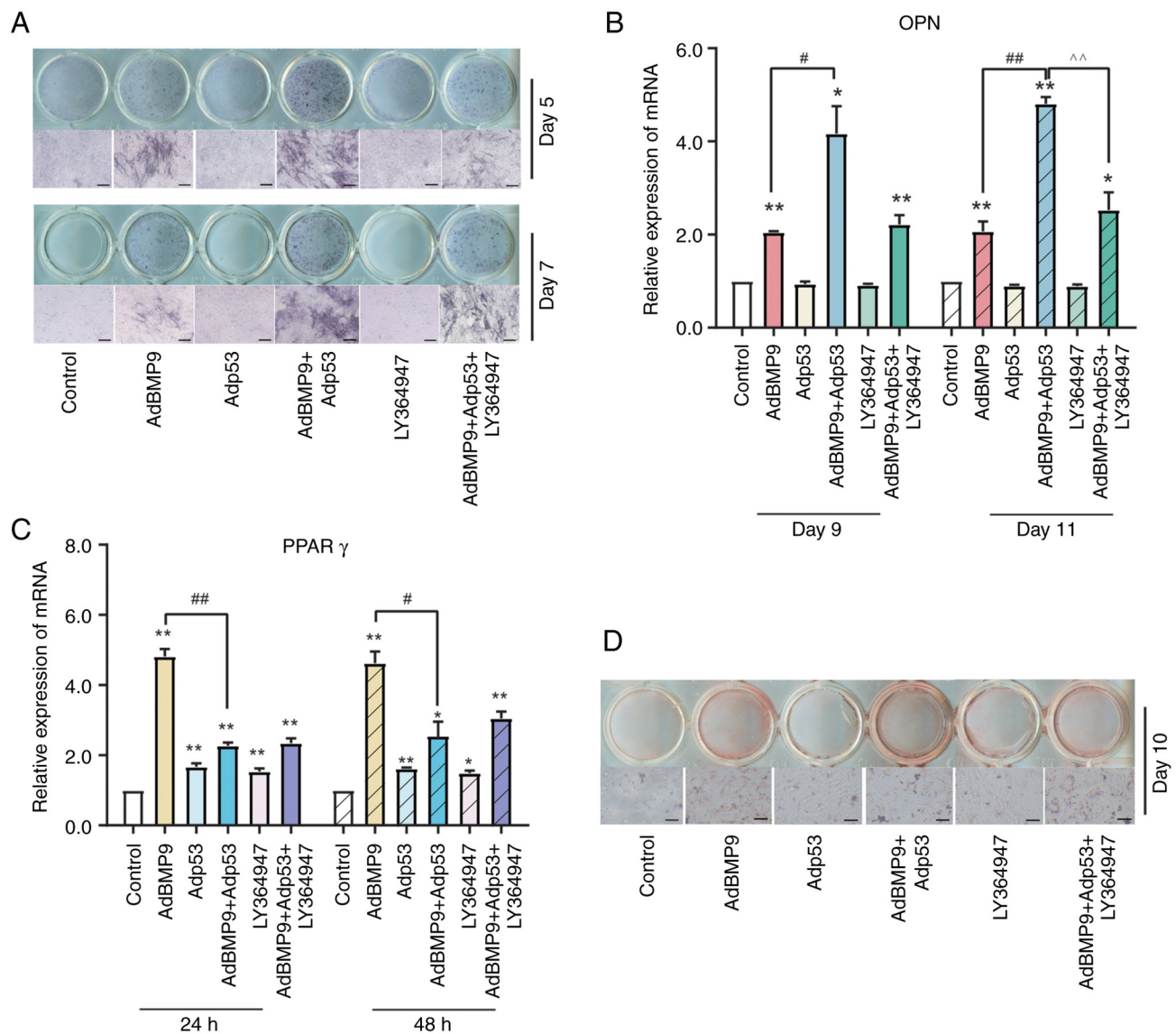


Figure 4. Effect of p53 and LY364947 on osteogenesis and adipogenesis differentiation homeostasis in C3H10T1/2 cells. (A) ALP activity in C3H10T1/2 cells with/without p53 and LY364947 (1 μ M) at D 5 and D 7 Scale bar, 100 μ m. (B) RT-qPCR of OPN expression in C3H10T1/2 cells with/without p53 and LY364947 (1 μ M) at D 9 and D 11. (C) RT-qPCR of PPAR γ in C3H10T1/2 cells with/without p53 and LY364947 (1 μ M) at 24 and 48 h. (D) Oil red O staining in C3H10T1/2 cells with/without p53 and LY364947 (1 μ M) at D10 Scale bar, 50 μ m. *P<0.05, and **P<0.01 vs. Control. #P<0.05 and ##P<0.01 vs. AdBMP9. ^^P<0.01 vs. AdBMP9 + Adp53. RT-qPCR, reverse transcription-quantitative PCR; ALP, alkaline phosphatase; OPN, osteopontin.

bioinformatics analysis of the current study revealed that p53 has a high expression in the osteoporosis model, therefore we speculate that p53 can possibly be a new regulator in the osteogenic differentiation process.

As an important oncogene, p53 contributes to the regulation of cell proliferation and DNA damage repair. Nevertheless, the regulatory role of p53 in the body goes far beyond these. p53 is also present in some newly discovered biological processes, such as ferroptosis (39,40). In terms of osteogenesis, several researchers have identified a negative regulatory effect of p53 on osteoblast differentiation, whereas some others suggested that mutations in p53 can increase osteoblast differentiation (41,42). The present study found that p53 was expressed in all MSCs tested and overexpression of p53 enhanced the potential of BMP9 induced osteogenic differentiation, while suppression of p53 decreased the expression level of early, intermediate, and late osteogenic markers, whereas p53 alone had no significant

effect on MSCs differentiation in this study. It has been shown in other studies that p53 knockout mice display more bone differentiation and bone erosion (19). PFT- α alone in the present study also resulted in upregulation of the Runx2 expression. However, PFT- α with BMP9 resulted in a trend of inhibition. These may be caused by different microenvironmental conditions. Taken together, our results support the notion that p53 may regulate BMP9 induced MSCs osteogenic differentiation.

The phenomenon of the bone-adipose imbalance can be seen in many diseases (43). In cases of heavy corticosteroid use, for example, bone formation decreases, while adipose tissue in the bone marrow increases (44). In the present study p53 was evidenced to modulate bone lipid homeostasis to influence MSCs differentiation. After infection with adenovirus overexpressing p53, microscopic observation revealed increased lipid droplet formation and increased expression of PPAR γ in C3H10T1/2 cells (27). These effects were attenuated

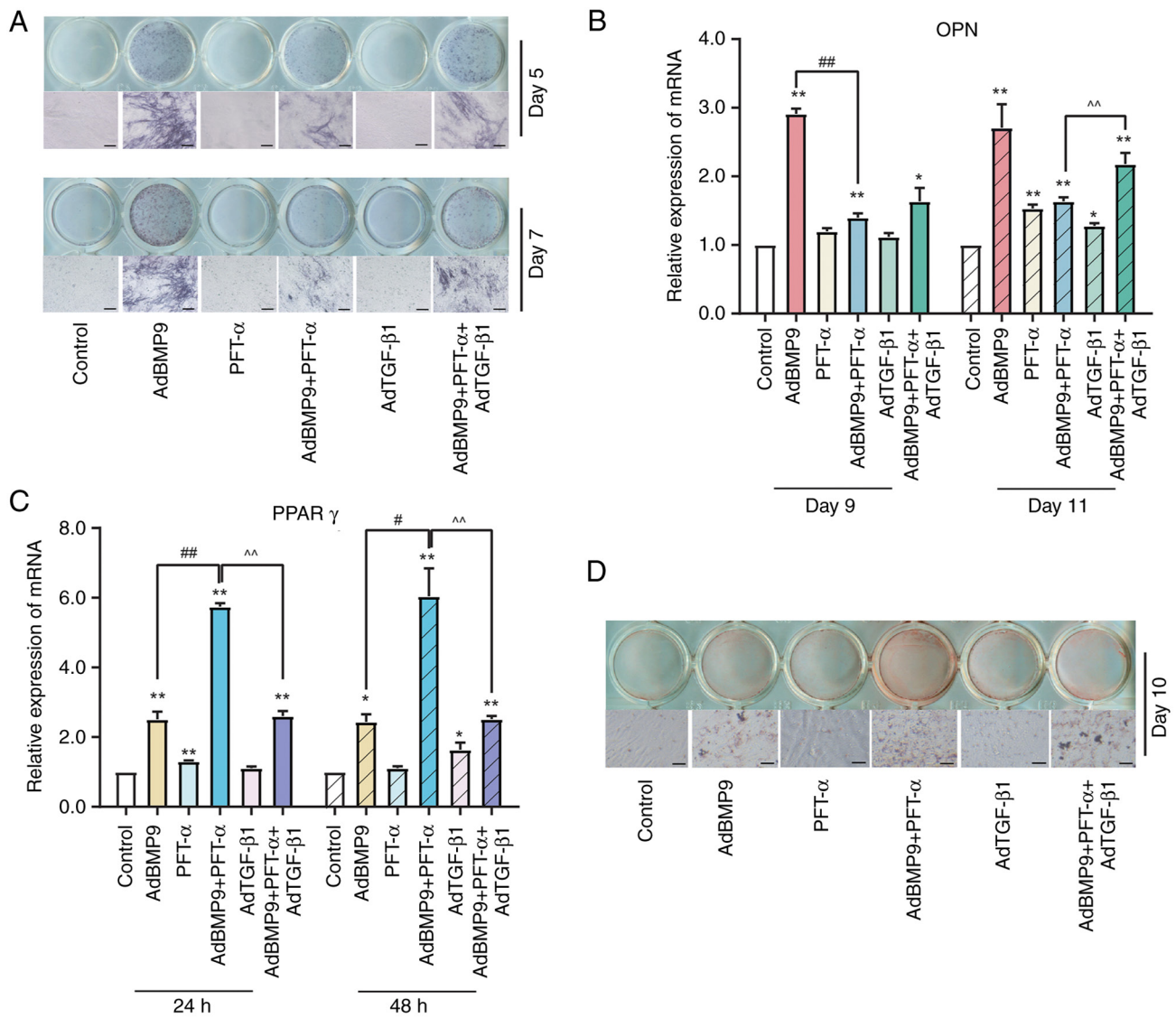


Figure 5. Effect of PFT- α and AdTGF- β 1 on osteogenesis and adipogenesis differentiation homeostasis in C3H10T1/2 cells. (A) ALP activity in C3H10T1/2 cells with/without PFT- α (15 μ M) and AdTGF- β 1 at D 5 and D 7. Scale bar, 100 μ m. (B) RT-qPCR of OPN expression in C3H10T1/2 cells with/without PFT- α (15 μ M) and AdTGF- β 1 at D 9 and D 11. (C) RT-qPCR of PPAR γ in C3H10T1/2 cells with/without PFT- α (15 μ M) and AdTGF- β 1 at 24 and 48 h. (D) Oil red O staining in C3H10T1/2 cells with/without PFT- α (15 μ M) and AdTGF- β 1 at D10. Scale bar, 50 μ m. * P <0.05, and ** P <0.01 vs. Control. # P <0.05 and ## P <0.01 vs. AdBMP9. ^^ P <0.01 vs. AdBMP9 + Adp53. PFT- α , pifithrin- α ; RT-qPCR, reverse transcription-quantitative PCR; ALP, alkaline phosphatase; OPN, osteopontin.

after treatment with p53 inhibitor, which revealed that p53 may facilitate BMP9-induced osteogenic differentiation by inhibiting lipogenic differentiation. Taken together, our study analyzed the effect of p53 on MSCs differentiation from the perspective of bone-lipid balance for the first time, which will provide a new perspective in bone marrow microenvironment on bone formation.

The Smad dependent TGF- β and BMP signaling share common principles in bone remodeling and therapies (45,46). The combination of p53 and BMP9 could better enhance the expression level of TGF- β 1 than BMP9 alone. The TGF- β 1 mRNA level was suppressed even when BMP9 was overexpressed and inhibition of p53. Inhibition of TGF- β 1 with LY364947 impaired the osteogenic differentiation of BMP9-induced C3H10T1/2 cells by p53, and overexpression of TGF- β 1 partially restored the inhibitory effect of PFT- α on the osteogenic marker ALP, which was largely related to

the concentration of TGF- β 1. The same results were obtained for the intermediate and late-stage osteogenic marker OPN, suggesting that p53 influences all stages of osteogenic differentiation. To further corroborate this mechanism, we examined the levels of PPAR γ under the same treatment factors as well as oil red O staining, and the variation of these indicators strongly suggested their relevance. Our hypothesized molecular mechanism is shown in Fig. 6C. The expression of TGF- β 1 is promoted by p53, and TGF- β 1 may promote BMP9-induced osteo-differentiation and thus lipogenic differentiation by inhibiting p53. Thus, our data suggested that p53 may mediate the promotion of BMP9-induced osteogenic activity through upregulation of TGF- β 1. TGF- β 1 is a multifunctional protein that controls proliferation, differentiation, and other functions in many cell types. Clarifying the relationship between p53 and TGF- β 1 is beneficial to further study the effect of bone marrow microenvironment on bone formation, and provide

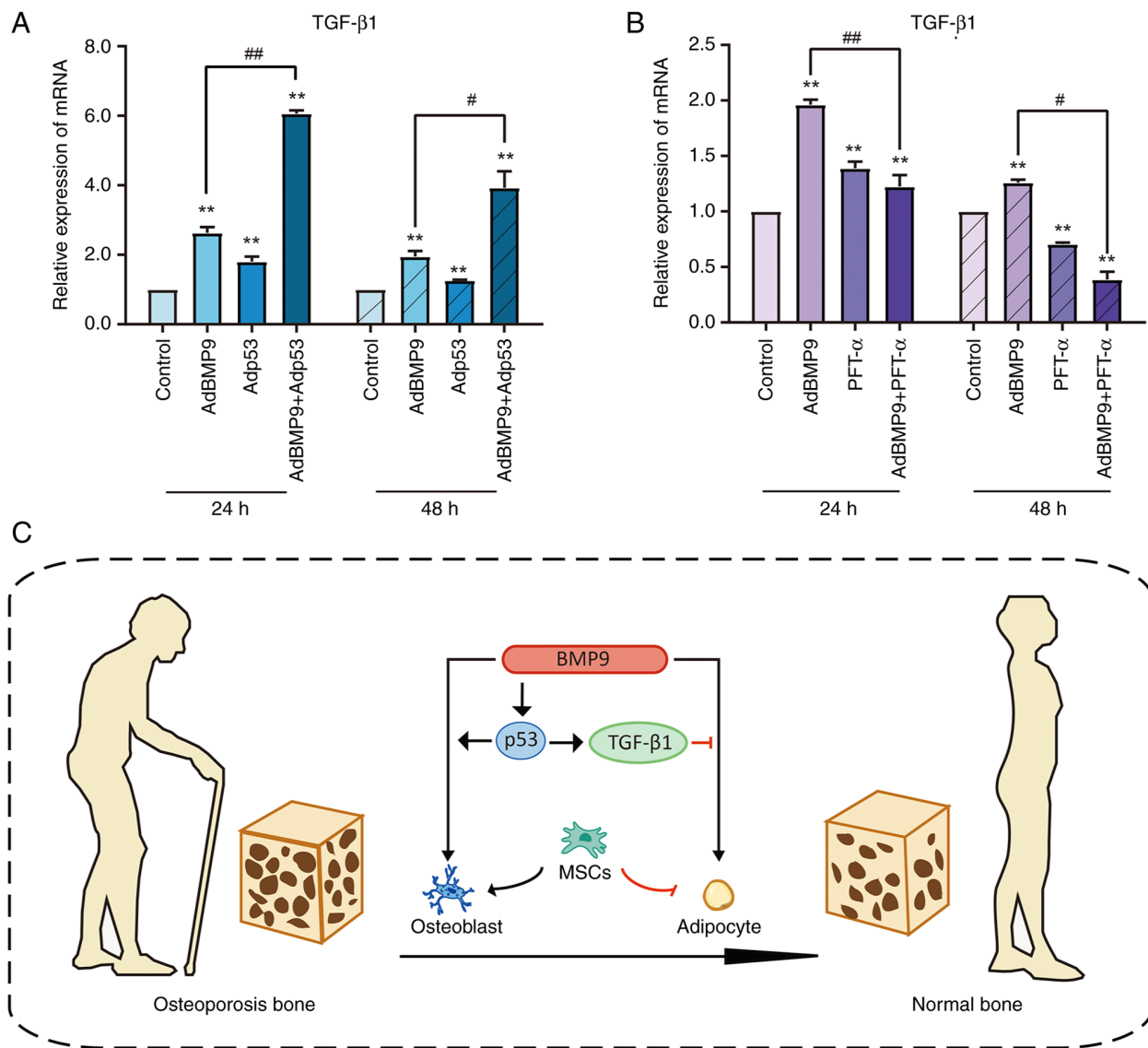


Figure 6. Molecular mechanism of the effect of TGF-β1 on p53 involved MSCs osteo-differentiation. RT-qPCR of TGF-β1 expression in C3H10T1/2 cells with/without (A) Adp53 and (B) PFT-α (15 μM) at 24 and 48 h. (C) Molecular mechanism diagrammatic drawing by which p53 regulates BMP9-induced MSCs differentiation via TGF-β1 in osteoporosis. **P<0.01 vs. Control. #P<0.05 and ##P<0.01 vs. AdBMP9. PFT-α, pifithrin-α; RT-qPCR, reverse transcription-quantitative PCR.

solutions for clinical treatment of bone diseases such as osteoporosis. Subsequent experiments are expected to perform more verification by conditional knockout of p53 mice, collection of more clinical specimens for sequencing and bioinformatics analysis to improve our study and beneficial to clinical.

In conclusion, the present study demonstrated that BMP9 can further induce osteogenic differentiation of MSCs by inhibiting lipogenic differentiation in a p53-dependent manner, and one of the critical mechanisms might be TGF-β1 signaling. Our findings provide another target for the treatment of osteoporosis. Furthermore, this work presents a compelling case for the further investigation of p53 as a crucial regulator for the osteogenic differentiation of MSCs.

Acknowledgements

The authors would like to thank Professor Tongchuan He (Molecular Oncology Laboratory, The University of Chicago

Medical Center, Chicago, Illinois, USA) for his generous provision of the MSCs [MEFS (47,48), C2C12, C3H10T1/2, 3T3-L1 and MG-63] and recombinant adenoviruses.

Funding

This work was supported by the National Key Research and Development Program of China (grant no. 2016YFC1000803) and the National Natural Science Foundation of China (NSFC, grant no. 81572226).

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GEO repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35959>. All other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XY, PL and BH confirm the authenticity of all the raw data. XY conceived the study, conducted the experiments, and wrote the manuscript. PL performed western blotting, RT-qPCR, and staining assays. YD performed bioinformatics assays. YY preformed the molecular cloning experiments. HL performed western blotting and RT-qPCR assays. BH conceived and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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