TRX1 mediates melatonin-induced osteogenic differentiation and the inhibition of osteoporosis

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Research Article

Keywords: osteoporosis, melatonin, thioredoxin1, osteogenic differentiation

Posted Date: February 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1300940/v1

Abstract

Osteoporosis is a common systemic bone disorder characterized by bone loss and an increased risk of fracture. Melatonin has powerful antioxidant and anti-aging functions and has been proposed as a treatment for osteoporosis; however, the therapeutic effect of this agent needs further validation and the mechanisms remain to be explored. In this study, we observed that melatonin markedly stimulated osteogenic differentiation *in vitro* and alleviated osteoporosis symptoms in mice. After melatonin treatment, thioredoxin1 (TRX1) was upregulated under both in vivo and in vitro conditions, while its expression notably decreased in osteoporotic mice, thereby suggesting this gene possibly mediated the therapeutic effect of melatonin on osteoporosis. Moreover, the following overexpression or silencing of TRX1 proved its ability to induce osteogenesis of bone marrow mesenchymal stem cells (BMSCs). Furthermore, osteogenic differentiation and osteoporosis after melatonin pretreatment was attenuated both in BMSCs and in mice with TRX1 knockdown. These findings confirm that TRX1 is potentially associated with the beneficial effects of melatonin and that the use of melatonin may reduce osteoporosis. Overall, melatonin treatment induced expression of TRX1, and the up-regulated gene enhanced osteogenic differentiation and eventually reduced bone loss in osteoporosis. The results indicate that TRX1 may be a target of osteoporosis therapy.

Introduction

Osteoporosis is one of the most common systemic bone disorders and is hallmarked by a decrease in bone strength and an increased risk of fracture [1]. Osteoporosis reportedly affected roughly 18.3% of the world's population, and is a massive medical burden in the world [2, 3]. The pathogenesis of osteoporosis mainly originates from the breakdown of bone homeostasis between bone formation (osteoblasts) and bone resorption (osteoclasts) due to the decline of osteoblasts or abnormal activation of osteoclasts [4–6]. Current agents treating osteoporosis are anti-bone resorption drugs inhibiting osteoclast function and anabolic agents that induce osteoblastic bone formation [7, 8], but they are expensive and have complex side effects [9]. Thus, novel therapies need to be developed to treat osteoporosis.

Bone marrow mesenchymal stem cells (BMSCs), with the potential to self-renew and to differentiate into a variety of cells such as osteoblasts, adipocytes, and chondrocytes, play a key role in maintaining bone homeostasis [10, 11]. Therefore, enhancing BMSCs osteogenic differentiation is crucial for the treatment of osteoporosis. Melatonin (N-acetyl-5-methoxy-tryptamine) has been reported as a bone metabolic regulator that maintain bone metabolism homeostasis and prevents osteoporosis [12–14]. Melatonin treatment completely reversed the bone structural degeneration of ovariectomy-induced osteoporosis in mouse models by enhancing bone formation [13, 15]. Thus, melatonin may be a promising treatment for osteoporosis, but the therapeutic effect of melatonin requires confirmation and the mechanism needs to be further explored.

This study explored the effect of melatonin on BMSCs osteogenic differentiation and bone metabolism in osteoporotic mice. Thioredoxin1 (TRX1) was observed to be upregulated after melatonin treatment; this

gene was found to promote BMSCs osteogenesis differentiation. Finally, we verified that TRX-mediated promotion of bone by melatonin involved osteogenic differentiation and the prevention of in vivo osteoporosis. The findings suggest the use of melatonin as an alternative treatment for osteoporosis.

Materials And Methods

Cell culture

The human BMSCs from Procell Life Science & Technology Co. Ltd (Wuhan, China) were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (F12/DMEM; Hyclone, Utah, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, USA). Bone marrow mesenchymal stem cells induced osteogenesis medium (Cyagen, Suzhou, China) was used to induce BMSCs osteogenic differentiation.

Alkaline phosphatase measurement and Alizarin Red S staining

The level of alkaline phosphatase (ALP) and osteogenic differentiation of human BMSCs was evaluated by ALP Determination Kit (Szybio, Wuhan, China) and Alizarin Red S reagent (Cyagen, Suzhou, China), respectively. BMSCs were washed with phosphate buffer solution three times and immobilized with paraformaldehyde for 30 min at room temperature. Later, paraformaldehyde was discarded and phosphate buffer solution was added for cleaning BMSCs again. BMSCs were stained by Alizarin Red S for 5 min, and then the images were captured under an inverted microscope (Olympus, Beijing, China).

iTRAQ quantitative proteomics analysis

The cells were fully lysed by cell lysate for 5 min, and cold acetone containing 10 mM dithiothreitol (DTT) was added. Next, centrifuge at 4°C, 13000 g for 20 min, the precipitates were collected and mixed with 800 μl cold acetone at 56°C to break the disulfide bond of the protein. Subsequently, centrifugation at 12000r/min for 20 min at 4 °C, the precipitate was dissolved with 100 μl TEAB dissolution buffer and then stored at -80 °C. Protein concentration was determined by the Bradford method. The protein was digested by trypsin, and 0.1% FA was added to acidify the digested protein. Peptides were purified on Strata-X C18 columns. Following activation and washing, these peptides were eluted and labeled.

The peptides were labeled according to the ITRAQ-8 standard Kit instructions (SCIEX, Massachusetts, USA) and mixed. Next, these labeled peptides were segregated via the Ultimate 3000 HPLC system (Thermo DINOEX, USA). Subsequently, the peptide samples were dissolved in buffer A (2% acetonitrile, 0.1% formic acid, 98% H_2O) and analyzed by an Eksigent nanoLC-MS/MS (Triple TOF 5600 plus) system. The peptide solution was added to a C18 capture column (100 μm × 20 mm; 5 μm) and eluted at 300 nl/min on a C18 analytical column (75 μm × 150 mm; 3 μm) with a 90-min gradient. The two mobile phases are buffer A and buffer B (98% acetonitrile, 0.1% formic acid, 2% H_2O).

Cell transfections and infection

When BMSCs in the logarithmic growth period were inoculated in 6-well plates and grew to 70% density, the supernatant was replaced with F12/DMEM medium containing different plasmids and siRNA without serum for transfection. According to the manufacturer's instructions, TRX1 overexpression pcDNA3.1 plasmid (OE-TRX1) and negative control plasmids (OE-NC) were transfected into BMSCs by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), TRX1 siRNA (si-TRX1), and negative control siRNA (si-NC) were transfected by lipofectamine RNAiMAX (Invitrogen, USA). Subsequently, these BMSCs were cultured for subsequent experiments. These plasmids and siRNAs were obtained from Genecreate Biological Co. Ltd (Wuhan, China). The sequences were shown as follows: si-TRX1：GCUGCAGGUGAUAAACUUGUAUTT; si-NC：UUCUCCGAACGUGUCACGUTT.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary deoxyribose nucleic acid (cDNA) was synthesized by using a reverse transcription kit (Toyobo, Osaka, Japan). Quantitative polymerase chain reaction (qPCR) was performed in an ABI Prism 7300HT Real-Time PCR amplification apparatus. The qPCR amplification procedure was as follows: Hot-Start DNA Polymerase activation at 95 ℃ for 1 min; denaturation at 95°C for 15s and extension at 60°C for 30s, with 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the reference gene. The results were analyzed using DataAssist™ v3.0 software (ABI) with the $2^{-\Delta\Delta Ct}$ method. The primer sequences for TRX1 are as follows: Forward: GCCTTGCAAAATGATCAACC, Reverse: ACCCACCTTTTGTCCCTTCT.

Western blot

Briefly, BMSCs were digested in a 1.5 ml EP tube with trypsin. Then, total proteins were extracted using a lysis buffer. The protein concentrations were estimated by the bicinchoninic acid method. Proteins were electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated with primary antibodies and secondary antibodies. Subsequently, the membranes were exposed after adding enhanced chemiluminescence plus reagent. All reagents on western blot were from Aspen Biotechnology Co. Ltd (Wuhan, China).

Animal and model establishment

Female balb/C mice (10 weeks), from the Department of Experimental Animals, Kunming Medical University, were reared under standard indoor conditions (temperature 22.1 ℃, humidity 55.5%). Animal experiments strictly followed the animal ethics guidelines of China's National Health and Medical Research Commission and were approved by the Ethics Committee of Kunming Medical University. These mice were randomly divided into three groups (8 mice in each group): sham-operated group (sham group), osteoporosis model group (OP group), melatonin treatment group (OP+melatonin group), negative control group (OP+melatonin+sh-NC group), and sh-TRX1 group (OP+melatonin+sh-TRX1 group. All mice were anesthetized with 5% pentobarbital (10 mg/kg). Mice were fixed in the prone position and disinfected with 75% alcohol after skin preparation. The back skin and peritoneum of mice were cut to

expose the ovaries. After ovariectomy, the back skin was sutured by ligation with absorbable sutures. Anesthesia, fixation, and incision selection in the sham group were the same as those in the OP group, however, the ovaries were preserved and only the surrounding fat was removed. The melatonin group was given daily melatonin intragastric therapy (10 mg/kg) after ovariectomy. Mice in sh-NC and sh-TRX1 groups were injected 1.5×10^9 PFU/ml adenovirus containing sh-NC and sh-TRX1 through the tail vein. Later, these mice in the five groups were put into the cage and fed for 3 months.

Micro-CT analysis

After euthanasia, femurs in each group were fixed in formalin saline. Then Hiscan XM Micro CT with a 10 μm resolution was utilized to perform CT detection. The X-Ray tube settings of 80 kV and 100 uA were applied. The exposure time was set to 50 ms, and the scanning angle interval was 0.5. Hiscan Reconstruction software (Version 3.0) was used to reconstruct the image, and further evaluate bone mineral density (BMD), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp).

Statistical analysis

Prism 8.0 (GraphPad Software, San Diego, CA) was used for statistical analyses. All data were expressed as the mean ± SD, and a one-way analysis of variance (ANOVA) was used to compare data between different groups. $P<0.05$ indicated that the difference was statistically significant.

Results

1. Melatonin could promote osteogenic differentiation and treat osteoporosis

To test the effect of melatonin on osteogenic differentiation *in vitro*, BMSCs osteogenic differentiation was measured. Alizarin Red S staining showed that melatonin treatment (100uM) promoted the osteogenic differentiation of BMSCs (Figure 1a). Western blot results indicated that the expression levels of osteogenic related proteins such as ALP, Collagen-I, and OCN increased in a dose-dependent manner after melatonin treatment (Figure 1b). As outlined above, these results documented that melatonin promoted osteogenic differentiation of BMSCs. Broadly speaking, enhancing the osteogenic potential of BMSCs is beneficial to osteoporosis treatment. To further investigate the effect of melatonin treatment on osteoporosis, the osteoporotic mice model was established by ovariectomy. Mice in the OP group had obvious bone loss compared to the sham mice as expected; treatment with melatonin markedly increased bone mass (Figure 1c). Meanwhile, melatonin prevented the decrease of ALP expression caused by ovariectomy (Figure 1d). These results suggested that melatonin enabled improved osteoporosis in mice.

2. Upregulated TRX1 may be involved in mediating osteogenesis regulation of melatonin

To find the potential target of melatonin enhancing effect on osteogenic differentiation, we identified the differentially expressed proteins in human BMSCs after melatonin treatment by proteomics analysis. A total of 3883 proteins was detected, among which we observed that the expression of 285 proteins was significantly altered by melatonin treatment based on fold change > 1.2 and $P < 0.05$, including 181 up-regulated, and 104 down-regulated ones (supplement Table 1). These significantly differentially expressed proteins, potentially involved in mediating melatonin regulation of osteogenesis, were visualized by hierarchical clustering (Figure 2a) and a volcano plot (Figure 2b). TRX1 reportedly inhibits oxidative stress and apoptosis [16]; both oxidative stress and apoptosis are closely related to osteoporosis [17], indicating that this gene could mediate the improvement of bone after melatonin treatment. As anticipated, TRX1 was observed to be prominently up-regulated in iTRAQ quantitative proteomics analysis. Combined qPCR assays and western blot results further supported the elevated expression of TRX1 in BMSCs after melatonin treatment (Figure 2c, d). Furthermore, TRX1 expression increased after melatonin treatment while its expression in the blood of osteoporosis mice was decreased compared with that of sham group mice (Figure 2e). Overall, these results showed that TRX1 could bridge the connection of melatonin treatment and improve osteogenesis; hence, it was therefore targeted for subsequent studies.

3. TRX1 promoted BMSCs osteogenic differentiation

To explore the efficacy of TRX1 in BMSCs osteogenic differentiation, the TRX1 overexpression plasmid and specific siRNAs targeting TRX1 were transfected into BMSCs for overexpression and silencing of TRX1 expression, respectively. qPCR and western blot results solidly confirmed plasmid-induced TRX1 overexpression and siRNA-induced TRX1 knockdown (Figure 3a, b). Subsequent experiments verified the positive role of TRX1 on improving BMSCs osteogenic differentiation. Based on the results of Alizarin Red S staining and ALP detection, TRX1 overexpression resulted in observably enhanced ALP content and osteogenic differentiation of BMSCs, while TRX1 knockdown decreased ALP content and osteogenic differentiation (Figure 3c, d). Furthermore, the expression of osteogenic associated proteins including ALP, collagen-Ⅰ, and OCN increased after TRX1 overexpression; on the contrary, the expression of these factors was decreased in TRX1-silenced cells (Figure 3e). These observations documented that TRX1 enabled BMSCs osteogenic differentiation.

4. TRX1 mediated promotion to osteogenesis and amelioration of osteoporosis symptoms after melatonin treatment

Based on the above experiments, we presumed that melatonin promoted osteogenic differentiation by elevating TRX1 expression. To verify this hypothesis, TRX1 was overexpressed and silenced by TRX1 overexpression plasmid and TRX1 siRNA during melatonin treatment, respectively. As shown in Figure 4a and 4b, melatonin promoted osteogenic differentiation and ALP production, and TRX1 knockdown reversed the effect of melatonin treatment while TRX1 overexpression enhanced the effect of melatonin. In addition, melatonin increased the expression of osteogenic differentiation proteins (including ALP, collagen-Ⅰ, and OCN) in BMSCs; silencing TRX1 reversed the melatonin-mediated effects of osteogenic

differentiation, by contrast, overexpression of TRX1 enhanced the melatonin-treated effect (Figure 4c). To the end, these results indicated that TRX1 mediated the osteogenic effect of melatonin on BMSCs.

qPCR and western blot results confirmed the knockdown effect of short-hairpin RNA targeting TRX1 (sh-TRX1) on TRX1 expression in mice (Figure 5a, b), and thus the expression of TRX1 was silenced. Next, micro-CT was performed to further validate the role of TRX1 in the melatonin promoting bone regeneration. Bone loss was obviously noticed in the OP group compared to the sham group; melatonin markedly promoted bone growth, but the therapeutic effect of melatonin was visibly reduced after silencing TRX1 (Figure 5c). In detail, the mice in the OP group showed notably decreased BMD, Tb.N and Tb.Th and increased Tb.Sp in contrast to the sham group (Figure 5d-g). Melatonin treatment elevated BMD, Tb.N, and Tb.Th and reduced Tb.Sp (vs. OP group), while the therapeutic effect typically weakened after treatment with melatonin when down-regulating TRX1 expression (Figure 5d-g). These combined data typically suggested that TRX1-mediated amelioration of the melatonin to osteoporosis symptoms.

Discussion

Recent reports have shown that melatonin functions in promoting osteogenic differentiation [18, 19]. This study provided new evidence of melatonin's anti-osteoporotic properties both *in vivo* and *in vitro*. To be specific, melatonin treatment reduced the expression of TRX1, and the upregulated TRX1 further enhanced BMSCs osteogenic differentiation. Moreover, TRX1 knockdown weakened the beneficial actions of melatonin in BMSCs osteogenic differentiation and osteoporosis in mice. These findings documented that melatonin promoted osteogenesis and ameliorated osteoporosis phenotypes by raising TRX1 expression.

Melatonin, named for its ability to aggregate melanin particles in melanocytes, is an effective cell-protective agent mainly isolated from the pineal gland [20, 21]. In recent years, melatonin has attracted renewed attention due to its powerful lipophilic antioxidant and free radical scavenging effects and preventive and therapeutic functions for various diseases [22–24], especially for bone-related diseases [25, 26]. A considerable amount of evidence is available that melatonin promotes bone repair by increasing the number of bone regeneration, new blood vessel formation, and osteoblast-like cells [27, 28]. Herein, melatonin was found to accelerate BMSCs osteogenic differentiation and improve osteoporosis in mice. Therefore, melatonin appears to be a promising agent for the treatment of osteoporosis, and based on the present data some of the mechanisms involved have been clarified.

TRX1, a small multifunctional protein, is characterized for its remarkable antioxidant, anti-inflammatory, and anti-apoptotic effects [29, 30]. Thus, it is generally considered to be closely associated with the development of a variety of diseases [31–33]. In contrast to the sham mice, the expression of TRX1 was notably inhibited in OP mice (Figure 2e; Figure 5a). Moreover, TRX1 was observed to be prominently upregulated after treatment with melatonin under both *in vivo* and *in vitro* conditions. The upregulated TRX1 induced by melatonin pretreatment was possibly caused by the repressed expression of an endogenous TRX1 inhibitor [34]. The combined results suggest a key role of this gene in alleviating

osteoporosis after melatonin treatment. The next, positive impact of TRX1 on improving osteogenic differentiation of BMSCs was supported by TRX1 overexpression and silencing experiments. Further, TRX1 knockdown weakened the function of melatonin on accelerating BMSCs osteogenic differentiation and improving osteoporosis. Collectively, TRX1 is a key to melatonin improvements for osteogenesis and osteoporosis. Thus, TRX1 plays a crucial role in BMSCs osteogenic differentiation, and it is a promising therapeutic target as a treatment for osteoporosis. Melatonin is reported to alleviate bone loss in mice with retinoic acid-induced osteoporosis through the ERK/SMAD and NF-κB pathways [35]. Melatonin also suppressed activation of the NLRP3 inflammasome to ameliorate estrogen deficiency-induced osteoporosis mice [36]. Here, we identified TRX1 as a target of melatonin in inhibiting osteoporosis; this finding provides information regarding the mechanisms of actions of melaotnin as it relates to the treatment of osteoporosis.

In conclusion, melatonin not only effectively promoted osteogenic differentiation *in vitro*, but also relieved the symptoms of osteoporosis *in vivo*. After treatment with melatonin, TRX1 was prominently upregulated, and this gene was verified to induce BMSCs osteogenic differentiation. The rescue experiment further confirmed the indispensable role of TRX1 in melatonin therapy for osteoporosis through the manipulation of TRX1 expression. This study supports the proposal to treat osteoporosis with melatonin and provided a potential target, TRX1, to protect against osteoporosis.

Abbreviations

TRX1, thioredoxin1;

BMSCs, bone marrow mesenchymal stem cells;

ALP, alkaline phosphatase;

F12/DMEM, Dulbecco's modified Eagle's medium/Nutrient Mixture F-12;

BMD, bone mineral density;

Tb.N, trabecular number;

Tb.Th, trabecular thickness;

Tb.Sp, trabecular separation.

Declarations

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant numbers 31960136); Yunnan health training project of high-level talents (Grant numbers H-2017064); Yunnan Provincial Key

Laboratory of Digital Orthopedics; Yunnan Provincial Key Laboratory of Innovative application of characteristic Chinese Materia Medica.

Funding

This work was supported by National Natural Science Foundation of China (Grant numbers 31960136); Yunnan health training project of high-level talents (Grant numbers H-2017064); Yunnan Provincial Key Laboratory of Digital Orthopedics; Yunnan Provincial Key Laboratory of Innovative application of characteristic Chinese Materia Medica.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zhaowei Teng, Xiaochao Xu, Shaoxiong Zhang, Shuo Sun, Dake Zhao, and Lihong Jiang. The manuscript was written by Zhaowe Teng, Dake Zhao, and Russel J Reiter, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Kunming Medical University (No. kmmu2021462).

Consent to participate

Not applicable.

Consent to publish

All authors read the manuscript and agreed to submission.

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Figures

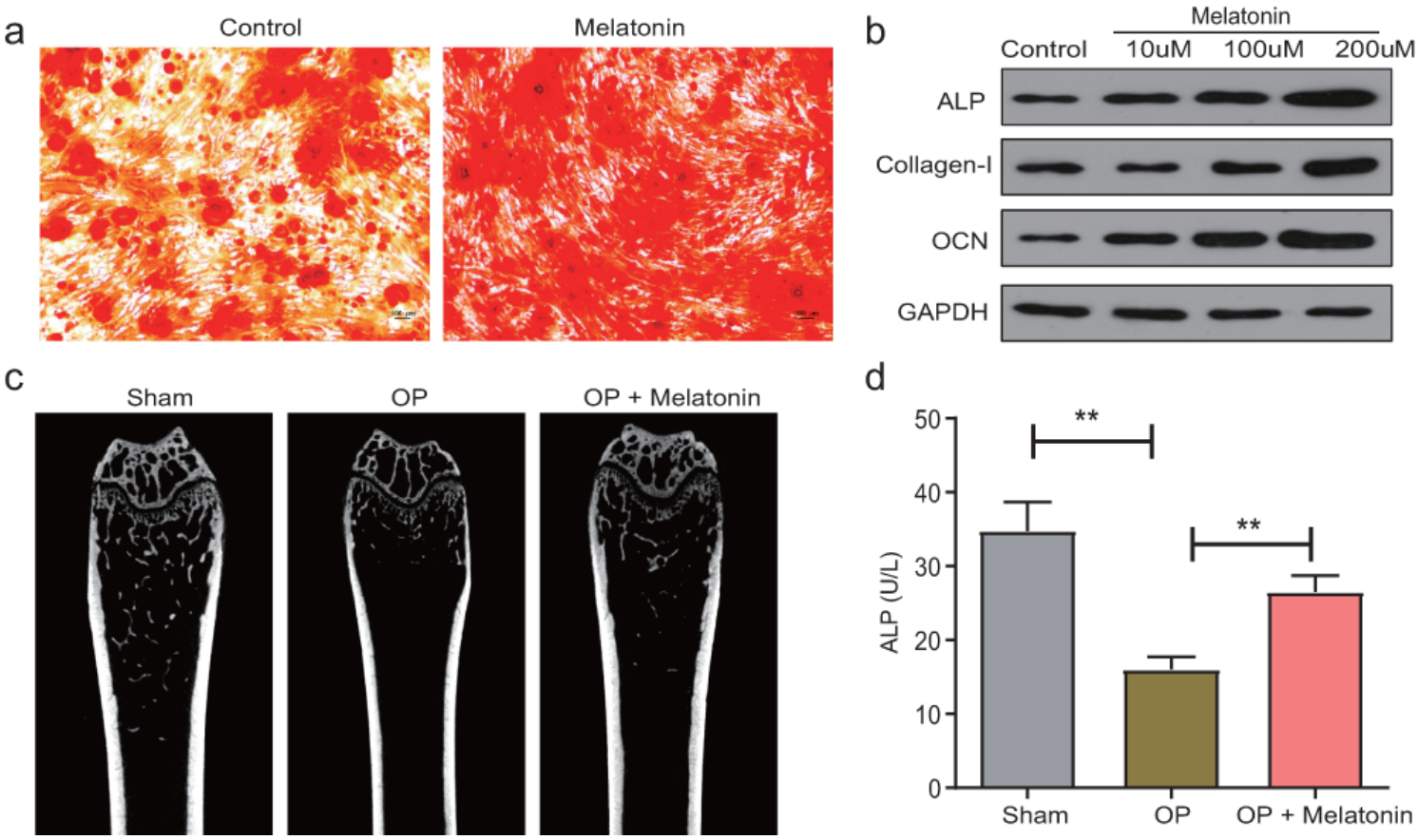

Figure 1

Melatonin promoted osteogenic differentiation and treated osteoporosis. a. Alizarin Red S staining of BMSCs after 21 days of incubation with BMSCs induced osteogenesis medium; b. Western blot results of ALP, Collagen-I, and OCN protein expression in BMSCs treated with melatonin at different concentrations; c. Longitudinal sectional micro-computed tomography images of left femur trabecular in mice; d. The detection of ALP in mice blood. *P<0.05, **P<0.01, ***P<0.001. TRX1, thioredoxin1. BMSCs, bone marrow mesenchymal stem cells.

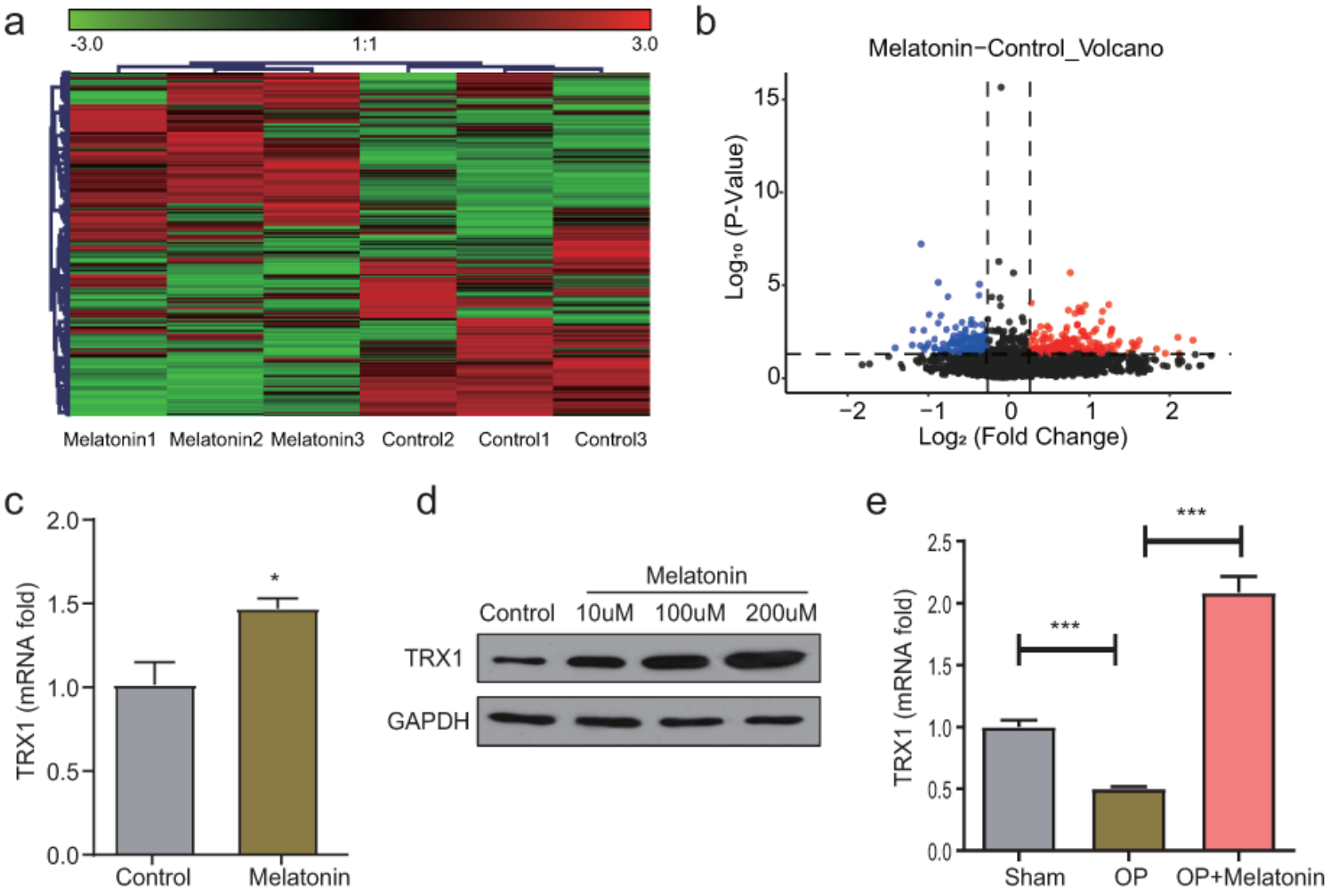

Figure 2

TRX1 was highly expressed after treatment with melatonin. a and b. The heatmap and volcano plot of proteomics analysis in BMSCs with or without melatonin treatment; red represents the protein with increased expression after melatonin treatment; Blue represents the protein with decreased expression after melatonin treatment; Black represents proteins with no significant difference. c and d. Quantitative polymerase chain reaction and western blot results of TRX1 in each group BMSCs; e. qPCR results of TRX1 in each group mice blood. *P<0.05, **P<0.01, ***P<0.001. TRX1, thioredoxin1; BMSCs, bone marrow mesenchymal stem cells.

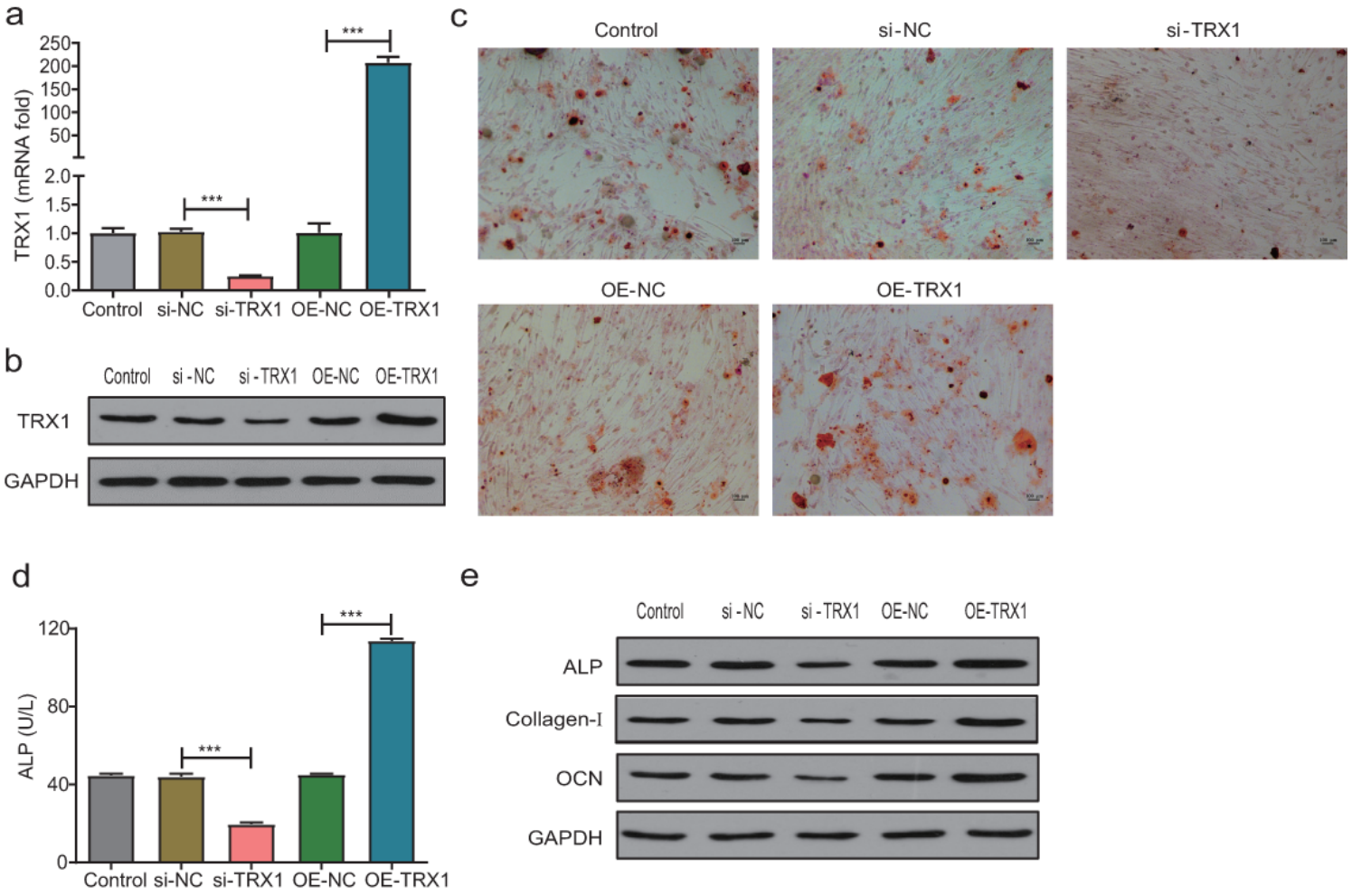

Figure 3

TRX1 promoted BMSCs osteogenic differentiation. a and b. Quantitative polymerase chain reaction and western blot results of TRX1 in BMSCs after transfection with si-TRX1 and TRX1 overexpression plasmids; c. Alizarin Red S staining of BMSCs after 14 days of incubation with induced osteogenesis medium; d. The detection of ALP in BMSCs; e. Western blot results of osteogenic related proteins (including ALP, Collagen-I, and OCN) in BMSCs. *P<0.05, **P<0.01, ***P<0.001. TRX1, thioredoxin1; NC, negative control; BMSCs, bone marrow mesenchymal stem cells.

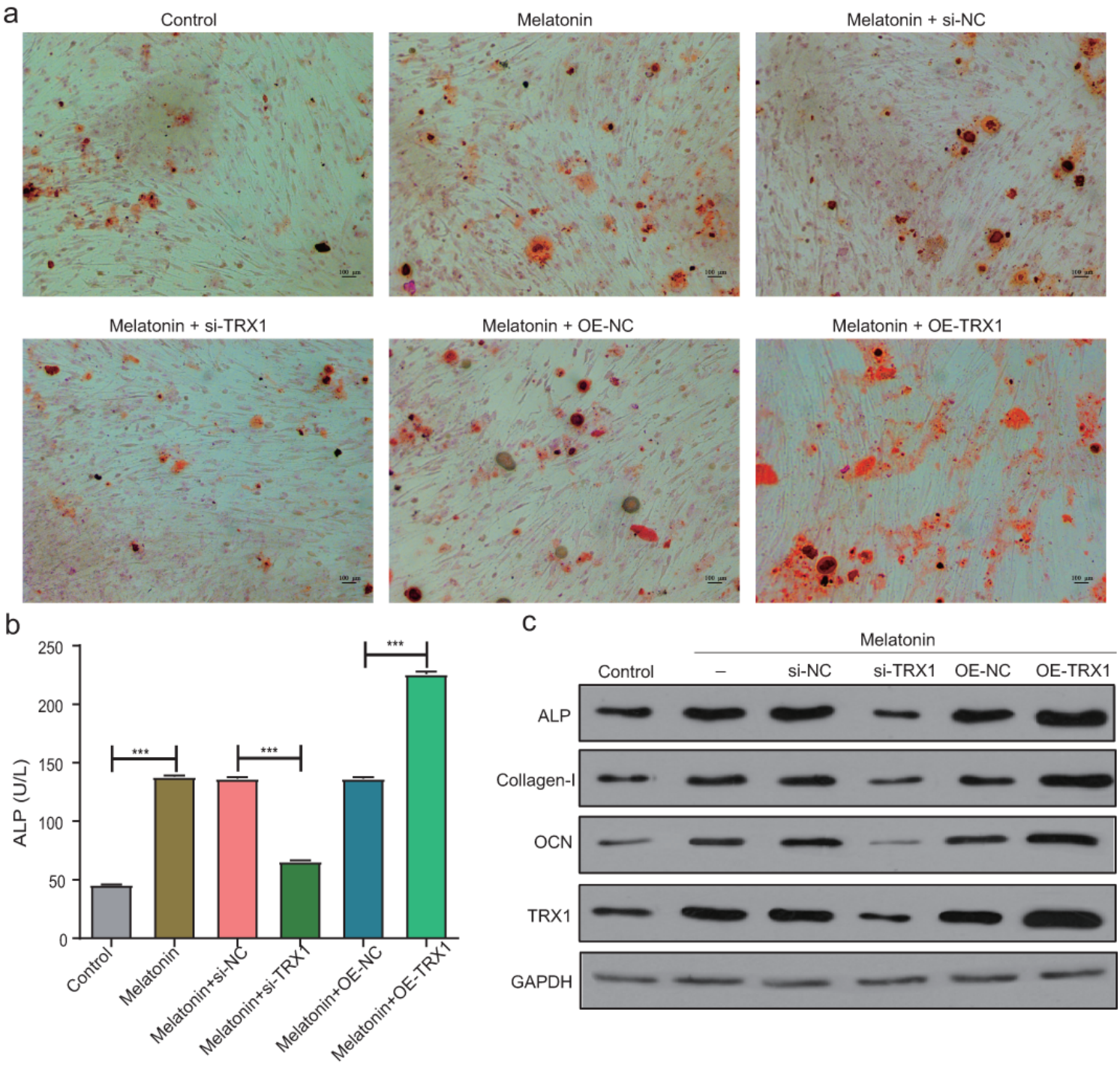

Figure 4

TRX1 mediated the osteogenic differentiation of melatonin *in vitro*. a. Alizarin Red S staining of BMSCs after 14 days of incubation with induced osteogenic medium. b. The ALP content detection. c. Western blot results of osteogenic related proteins (including ALP, Collagen-I, and OCN) in BMSCs. *P<0.05, **P<0.01, ***P<0.001. TRX1, thioredoxin1; NC, negative control; BMSCs, bone marrow mesenchymal stem cells.

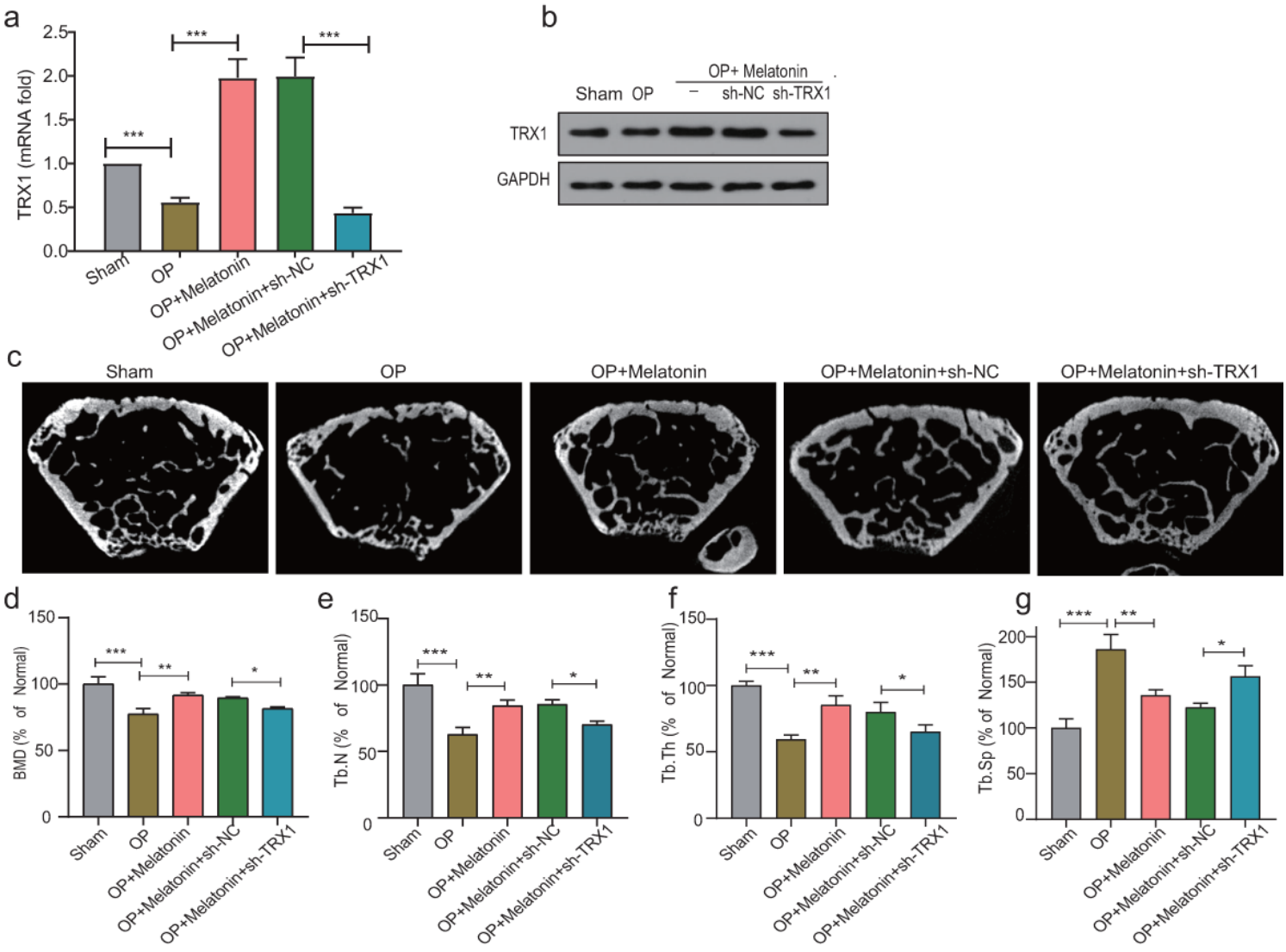

Figure 5

TRX1 mediated amelioration of the melatonin in osteoporosis phenotypes *in vivo*. a and b. Quantitative polymerase chain reaction and western blot results of TRX1 in mice blood; c. Transverse sectional micro-computed tomography images of left femur trabecular bone in mice. d-g. Quantitative analyses (including BMD, Tb.N, Tb.Th, and Tb.Sp) of trabecular bone microarchitecture in the left femora. *p < 0.05; **p < 0.01; ***p < 0.001. BMD, bone mineral density; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; TRX1, thioredoxin1; NC, negative control; OP, osteoporosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementTable1.xlsx