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Melatonin induces RAW264.7 cell apoptosis via the BMAL1/ROS/MAPK-p38 pathway to improve postmenopausal osteoporosis

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Aims

Currently, the effect of drug treatment for osteoporosis is relatively poor, and the side effects are numerous and serious. Melatonin is a potential drug to improve bone mass in postmenopausal women. Unfortunately, the mechanism by which melatonin improves bone metabolism remains unclear. The aim of this study was to further investigate the potential mechanism of melatonin in the treatment of osteoporosis.

Methods

The effects of melatonin on mitochondrial apoptosis protein, bmal1 gene, and related pathway proteins of RAW264.7 (mouse mononuclear macrophage leukaemia cells) were analyzed by western blot. Cell Counting Kit-8 was used to evaluate the effect of melatonin on cell viability. Flow cytometry was used to evaluate the effect of melatonin on the apoptosis of RAW264.7 cells and mitochondrial membrane potential. A reactive oxygen species (ROS) detection kit was used to evaluate the level of ROS in osteoclast precursors. We used bmal1-small interfering RNAs (siRNAs) to downregulate the *Bmal1* gene. We established a postmenopausal mouse model and verified the effect of melatonin on the bone mass of postmenopausal osteoporosis in mice via micro-CT. *Bmal1* lentiviral activation particles were used to establish an in vitro model of overexpression of the bmal1 gene.

Results

Melatonin promoted apoptosis of RAW264.7 cells and increased the expression of BMAL1 to inhibit the activation of ROS and phosphorylation of mitogen-activated protein kinase (MAPK)-p38. Silencing the bmal1 gene weakened the above effects of melatonin. After that, we used dehydrocorydaline (DHC) to enhance the activation of MAPK-p38, and the effects of melatonin on reducing ROS levels and promoting apoptosis of RAW264.7 cells were also blocked. Then, we constructed a mouse model of postmenopausal osteoporosis and administered melatonin. The results showed that melatonin improves bone loss in ovariectomized mice. Finally, we established a model of overexpression of the bmal1 gene, and these results suggest that the bmal1 gene can regulate ROS activity and change the level of the MAPK-p38 signalling pathway.

Conclusion

Our study confirmed that melatonin promotes the apoptosis of RAW264.7 cells through BMAL1/ROS/MAPK-p38, and revealed the therapeutic effect and mechanism of melatonin in postmenopausal osteoporosis. This finding enriches BMAL1 as a potential target for the treatment of osteoporosis and the pathogenesis of postmenopausal osteoporosis.

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Article focus

- The effects of drug treatment for osteoporosis are relatively poor, and the side effects are numerous and serious.
- Melatonin is a potential drug to improve bone mass in postmenopausal women.

- In our experiment, we studied the molecular mechanism by which melatonin inhibits RAW264.7 in mouse mononuclear macrophage leukaemia cells.

Key messages

- Melatonin regulates RAW264.7 cell apoptosis via *Bmal1* gene.
- Upregulated *Bmal1* can scavenge reactive oxygen species (ROS) and inhibit MAPK-p38 to regulate RAW264.7 cell apoptosis.
- Melatonin has therapeutic effects on postmenopausal osteoporosis in animal test subjects.

Strengths and limitations

- Our study confirmed that melatonin promotes the apoptosis of RAW264.7 cells through BMAL1/ROS/MAPK-p38, thus revealing the therapeutic effect and mechanism of melatonin in postmenopausal osteoporosis. This finding improves the quality of BMAL1 as a potential target for the treatment of osteoporosis and the pathogenesis of postmenopausal osteoporosis.
- Our research is mainly done at the cellular level. Therefore, our research group will further clarify the role of *Bmal1*, an important target, in postmenopausal osteoporosis through multi-omics analysis in subsequent projects.

Introduction

Postmenopausal osteoporosis is a systemic bone disease characterized by decreased bone mass and microstructural changes in bone tissue,[1] leading to bone brittleness and an increased risk of fracture.[2] Fractures caused by osteoporosis are becoming increasingly common in females over the age of 55 years, leading to a large number of fracture-related diseases and increased mortality and medical costs.[3-7] The pathogenesis of osteoporosis involves complex signalling pathway regulation and protein modification; however, little is currently known about this process. Osteoporosis is mainly caused by osteoclast-mediated bone resorption.[8] Traditional drugs for treating osteoporosis by inhibiting osteoclasts have achieved remarkable clinical effects. However, these drugs, include bisphosphonates, calcitonin, selective oestrogen receptor modulators (SERMs), and RANKL inhibitors, risk worrisome side-effects including jaw necrosis, severe musculoskeletal pain, oesophageal or breast cancer, and renal failure.[9,10] Furthermore, the side effect of these drugs and the lack of clear evidence to support their long-term efficacy lead to poor patient compliance.[11] Therefore, the development of safe and effective treatment options is crucial. Natural substances provide new avenues for the treatment of osteoporosis.

The endogenous hormone melatonin (N-acetyl-5-methoxytryptamine) has long been recognized for its regulation of circadian rhythms,[12] and it is the strongest antioxidant in the body.[13,14] Melatonin was not toxic to animals over a wide dose range.[15] For mice, the acute toxicity (LD50) of exogenous melatonin is as high as 3,200 mg/day. Melatonin is mainly secreted by the pineal gland but can also be synthesized by peripheral tissues and organs, including the skin, thymus, spleen, liver, bone marrow, and lymphocytes.[16-19] Since melatonin exhibits not only endocrine but also autocrine and paracrine effects,[20] it may influence ageing, tumour growth, and bone physiology.[21] It acts as the biological clock for all peripheral tissues, including bones. It synchronizes bone metabolism with the circadian cycle, and maintains the rhythmic homeostasis of the bone microenvironment.[22] The role of melatonin in developing postmenopausal osteoporosis has been reported in a recent study.[23] It described a direct effect on bone remodelling by promoting osteogenesis and suppressing osteoclastogenesis, regulating the biological rhythm of bone tissue, attenuating the damage induced by oxidative stress and inflammation on osteoblasts, and preventing osteolysis from reactive oxygen species (ROS) and inflammatory factors. The purpose of the present study was to determine the role of melatonin in the treatment of postmenopausal osteoporosis.

There is increasing evidence that the rhythm gene *Bmal1* is essential for hard tissue development, including bone, cartilage, and teeth. Loss of BMAL1 in animals inhibits bone and cartilage development and results in abnormal bone mass.[24] In mesenchymal cells, BMAL1 deficiency inhibits osteoblast and chondrocyte differentiation.[25] Silencing of *Baml1* gene also promotes osteoclast differentiation and increases bone resorption.[24] However, the relationship between BMAL1 and osteoclast progenitor cell apoptosis has not been reported.

Maintenance of rhythmic homeostasis is of great help in alleviating oxidative stress. Oxidative stress is a pathological state of the body, in which ROS cause excessive oxidation, which leads to ageing and disease.[26] It is also an important regulator of osteoclast precursors.[27,28] One clinical study found that the bodies of postmenopausal women with osteoporosis are highly oxidized.[29] This phenomenon can be explained by the fact that oestrogen is an antioxidant, so improving oxidation is an important means of reversing bone loss in postmenopausal women. Exploring whether melatonin inhibits oxidative stress by affecting the expression of BMAL1 can improve the mechanism of melatonin against osteoporosis, and provide new theoretical support for BMAL1 as an anti-osteoporosis target.

Therefore, we decided to explore the hypothesis that melatonin affects osteoclast apoptosis through the BMAL1/ROS/P38 axis. The roles of rhythm genes and oxidative damage in the development of postmenopausal osteoporosis were evaluated, and the results provided a potential target for the treatment of osteoporosis. The direct effect of melatonin on RAW264.7 cells and the therapeutic effect of melatonin on a postmenopausal osteoporosis animal model were also revealed. These findings may help us to understand the pathogenesis of postmenopausal osteoporosis and propose new therapeutic targets.

Methods

Reagents and cell culture. The reagents and chemicals used in this study are listed below. RAW264.7 cells (mouse mononuclear macrophage leukaemia cells) were purchased from the Academy of Sciences Cell Bank. Melatonin was purchased from Sigma-Aldrich (USA) and Merck KGaA (Germany). Antibodies against Bax (1:2,000; cat. no. ab182733), Bcl-2 (1:2,000; cat. no. ab182858), Cleaved-Caspase-3 (1:5,000; cat. no. ab214430), Caspase-3 (1:2,000; cat. no. ab184787), Cytochrome-C (1:5,000; cat. no. ab133504), BMAL1 (1:1,000; cat. no. ab230822), β Actin (1:5,000; cat. no. ab6276), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2,500; cat. no. ab9485), Cathepsin K (1:1,000; cat. no. ab187647), NFAT2 (1:1,000; cat. no. ab25916), Trap (1:1,000; cat. no. ab191406), p38 α/MAPK14 (1:1,000; cat. no. ab182453), and phosphorylated (p-)P38 (1:1,000; cat. no. ab195049) were obtained from Abcam (USA). Dehydrocorydaline (DHC) (Cat. HY-N0674A) was purchased from MedChemExpress (USA).

RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cytiva, USA) supplemented with 10% fetal bovine serum (Cytiva), 100 U/ml streptomycin sulfate, and 100 mg/ml penicillin. Cells were grown in a humidified incubator with 5% CO_2 at 37°C. The culture medium was replenished daily. Melatonin was added to the medium at a concentration gradient of 0.01, 0.1, 0.25, and 0.5 mM.

Cell Counting Kit-8 assay. Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Japan) was used to detect cell viability after treatment with different concentrations of melatonin. This reagent is an indicator of redox reactions. In the presence of the electron carrier 1-methoxy-PMS, dehydrogenase in living cells can catalyze the production of formazan dye from tetrazolium salt WST-8, and the production of formazan dye has a linear relationship with the number of living cells.

Apoptosis assay by flow cytometry. Various concentrations of melatonin (0.01, 0.1, 0.25, and 0.5 mM) were added to six-well plates seeded with 1.0×10^6 RAW264.7 cells incubated at 37°C for six hours. The cells were then harvested and resuspended in binding buffer and stained with FITC-Annexin V/propidium iodide for 15 minutes using an Annexin V-FITC kit (Beyotime Institute of Biotechnology, China) in the dark at room temperature. Apoptosis was detected with a FACScan flow cytometer (BD Biosciences, USA) and analyzed with CytExpert 2.3 (Beckman Coulter, USA).

Western blot analysis. To isolate the proteins, the culture solution was decanted from the treated cells and phosphate-buffered saline (PBS) was used to wash the cells three times. Then, RAW264.7 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing phenylmethanesulfonyl fluoride and phosphatase inhibitor cocktail, followed by centrifugation at 4°C at 12,000× g for 30 minutes. Quantitative analysis of protein concentration was performed using a bicinchoninic acid (BCA) assay kit, and each sample was loaded at a concentration of 3 μg/μl in RIPA and loading buffer.

Then, proteins were resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes with proteins of various molecular weights were immersed in blocking buffer for 1.5 hours. After the membranes were washed with 1% tris-buffered saline with 0.1% Tween 20 detergent (TBST), they were incubated with a primary antibody at 4°C overnight and a secondary antibody at 4°C the next day. After the membranes were thoroughly washed, the protein bands were coated with luminescent solution and visualized using a chemiluminescence (ECL) system (UVP, USA). The protein level was normalized to that of β-actin (molecular weight of 43 kDa). Finally, ImageJ software (National Institutes of Health, USA) was used to calculate the optical density and relative protein expression levels.

Detection of mitochondrial membrane potential using JC-1. A Mitochondrial Membrane Potential Assay kit with JC-1 (Beyotime Institute of Biotechnology, China) was used to evaluate the mitochondrial membrane potential of the RAW264.7 cells after the aforementioned treatments. The cells were suspended in JC-1 staining working solution for 30 minutes in a humidified incubator at 37°C and then washed with JC-1 buffer solution three times. Relative changes were detected by flow cytometry (CytoFLEX System B3-R3-V3) and analyzed with CytExpert 2.3 (both Beckman Coulter). Absolute changes were detected using a multifunctional microplate reader. JC-1 fluorescence was measured at excitation/emission wavelengths of 585/590 nm (red) and 510/527 nm (green). The red/green ratios were calculated to indicate the mitochondrial membrane potential.

Detection of cellular ROS using a reactive oxygen species detection kit. Detection of ROS in RAW264.7 cells occurred using the fluorescent probe diacetyldichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology). DCFH-DA itself has no fluorescence and can freely pass through the cell membrane. After entering the cell, it can be hydrolyzed by intracellular esterase to generate DCFH. DCFH, on the other hand, cannot penetrate the cell membrane, making it easy for probes to be loaded into cells. Intracellular ROS can oxidize non-fluorescent DCFH to generate fluorescent DCF. Detection of DCF fluorescence can reveal the level of intracellular ROS.

RAW264.7 cells were seeded into six-well plates at 1×10^6 cells and incubated for 24 hours. DCFH-DA was diluted 1:1,000 in serum-free medium to a final concentration of 10 μmol/l. The cell culture medium was removed, and 1 ml of diluted DCFH-DA was added to fully cover the cells. The six-well plate was incubated in a 37°C cell incubator for 20 minutes. Cells were washed three times with serum-free cell culture medium to sufficiently remove DCFH-DA that did not enter the cells. Then, the cells were directly observed with a laser confocal microscope.

Cell transfection. Bmal1-small interfering RNAs (siRNAs) were designed and synthesized by Santa Cruz Biotechnology. *Bmal1*-siRNA is a pool of three target-specific 19-25 nt siRNAs designed to knock down gene expression. In the six-well tissue culture plate, 1×10^5 cells were inoculated in each well, and 2 ml normal growth medium without antibiotics was added. The cells were incubated at 37°C in the incubator until the cells reached 60% to 80% confluence. Then, according to the manufacturer's instructions (Santa Cruz Biotechnology, USA), the cells were used in the experiment on the third day of transfection.

Bmal1 lentiviral activation particles (m): sc-419206-LAC was designed and synthesized by Santa Cruz Biotechnology (USA), which contain the following synergistic activation mediator (SAM) activation elements: a deactivated Cas9 (dCas9) nuclease (D10A and N863A) fused to the transactivation domain VP64, an MS2-p65-HSF1 fusion protein, and a target-specific 20 nt guide RNA. Then, according to the manufacturer's instructions, the cells were used in the experiment after four days of transfection.

Animal experiment. A total of 21 C57BL/6 J female mice (eight weeks, 20 to 25 g) were obtained and housed in the Department of Laboratory Animal Science. The laboratory environment was maintained at a temperature of 20°C to 26°C, 40% to 70% relative humidity, ≤ 14 mg/m^3 ammonia concentration, ≤ 60 dB noise, and a 12-hour alternating light/dark cycle with ad libitum access to food and drinking water. All animals were adapted to the environment for two weeks before the experiments. The mice were randomly divided into three groups (n = 7/group); two groups (ovariectomized (OVX)) were selected for ovariectomy treatment, and the remaining group underwent a sham surgery (the epidermis and peritoneum were cut and then sutured). The mice in the OVX groups were subjected to bilateral ovariectomy under 1.4% to 1.5% isoflurane inhalation anaesthesia with oxygen. Melatonin was directly dissolved in 0.9% normal saline, and mice in one of the OVX groups (OVX+ Met) were injected with melatonin (30 mg/kg/day) intragastrically starting three days after surgery. The mice in the OVX and sham groups were treated with normal saline in a similar manner. After treatment for eight weeks, all mice were killed via exsanguination under isoflurane anaesthesia. Bilateral femora and tibiae were harvested for imaging and protein extraction. All animal experiments were performed according to laboratory and animal welfare guidelines, and were approved by the Animal Ethics Committee.

Statistical analysis. The experimental data were means and standard deviations (SDs) by using GraphPad Prism 8 (USA) and SPSS 22.0 (USA). Independent-samples *t*-tests and one-way analysis of variance (ANOVA) were used for statistical analysis of three replicate experiments by SPSS. A p-value < 0.05 was considered statistically significant.

Results

Melatonin promotes apoptosis of RAW264.7 cells. We first performed a CCK-8 cell viability assay to investigate the viability of RAW264.7 cells after melatonin treatment at different concentrations (0.01, 0.1, 0.3, and 0.5 mM), and the results showed that 0.3 mM and 0.5 mM melatonin had inhibitory effects on mouse mononuclear macrophage leukaemia cells (Figure 1a). We further detected the mitochondrial membrane potential in RAW264.7 cells with JC-1 dye. The results showed that different concentrations of melatonin could lead to a decrease in mitochondrial membrane potential (Figure 1b), among which 0.3 mM melatonin had the most obvious effect (Figure 1c). We continued to perform flow cytometry detection (Figure 1d), and 0.1, 0.3, and 0.5 mM melatonin could induce RAW264.7 cell apoptosis, of which 0.3 mM melatonin worked best (Figure 1e). Finally, western blot analysis of proteins involved in the mitochondrial apoptosis pathway showed that the levels of Bax, cleaved-caspase-3, and cytochrome c increased with increasing melatonin concentrations, while the levels of Bcl-2 and caspase-3 decreased. Proportionally, Bax/Bcl-2 and cleaved-caspase-3/caspase-3 increased (Figure 1f). These western blotting results showed that with the increase in melatonin concentration, the apoptosis of RAW264.7 cells became increasingly obvious, and the apoptosis-inducing effects of 0.3 and 0.5 mM melatonin were similar (Figure 1g). Combined analysis of JC-1 and flow cytometry results showed that 0.3 mM melatonin was the most suitable melatonin concentration to promote apoptosis of RAW264.7 cells without cytotoxicity. Therefore, this concentration of melatonin was used in subsequent experiments.

Melatonin induces apoptosis of RAW264.7 cells by upregulating BMAL1 expression. We observed a direct effect on BMAL1 after melatonin treatment of cells, and the results indicated that melatonin upregulated the transcription and expression of BMAL1 (Figures 2e and 2f) to further determine the role of BMAL1 in melatonin-promoted apoptosis of RAW264.7 cells. The *bmal1* gene was knocked down by transfection of the cells with *bmal1*-siRNA (Figures 2e and 2f). According to the results of polymerase chain reaction (PCR) and western blot, the transfection efficiency is about 78%. Subsequent experimental data showed that targeted knockdown of the bmal1 gene attenuated the melatonin-induced decrease in mitochondrial membrane potential and apoptosis in melatonin-treated cells (Figure 2d). Markers of apoptosis were detected by western blotting (Figures 2e and 2g). In melatonin-treated cells, bmal1 knockdown attenuated the levels of Bax, cleaved-caspase-3, and cytochrome c, and Bcl-2 and caspase-3 levels increased. The aforementioned results show that melatonin promotes the apoptosis of RAW264.7 cells by upregulating the expression of BMAL1.

Melatonin increased the expression of BMAL1 to inhibit the activation of ROS and phosphorylation of MAPK-p38. ROS play an important role in the transmission of cellular

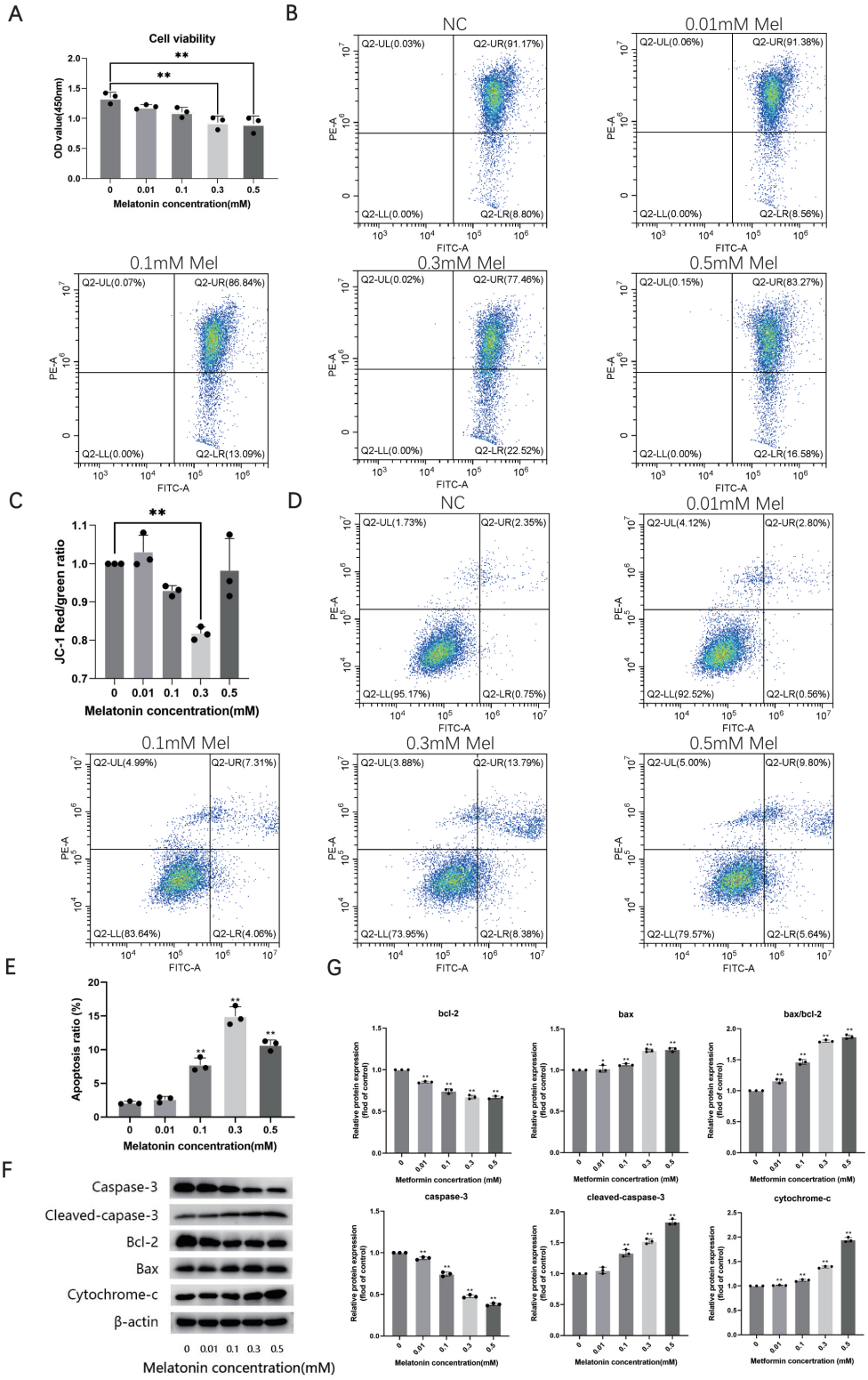

Fig. 1

Melatonin promotes apoptosis of RAW264.7 cells. a) Cell viability after treatment with different concentrations of melatonin (0.01 mM, 0.1 mM, 0.3 mM, and 0.5 mM). b) Qualitative changes in mitochondrial membrane potential were detected by flow cytometry after treatment with different concentrations of melatonin (0.01 mM, 0.1 mM, 0.3 mM, and 0.5 mM). c) Quantitative changes in mitochondrial membrane potential were detected using a full-wavelength multifunctional microplate reader. d) Representative flow cytometry plots. e) Apoptosis rates were detected by flow cytometry. f) Protein expression of Caspase-3, Cleaved-caspase-3, Bcl-2, Bax, Cytochrome-c, and β-actin indicated the effect of treatment with melatonin (0.01 mM, 0.1 mM, 0.3 mM, and 0.5 mM). g) Relative protein expression level of the proteins in f). Experiments were implemented in triplicate. Data are means and standard deviations. *$p < 0.05$, **$p < 0.01$ compared with control cells analyzed by using one-way analysis of variance. Mel, melatonin; NC, control group; OD, optical density.

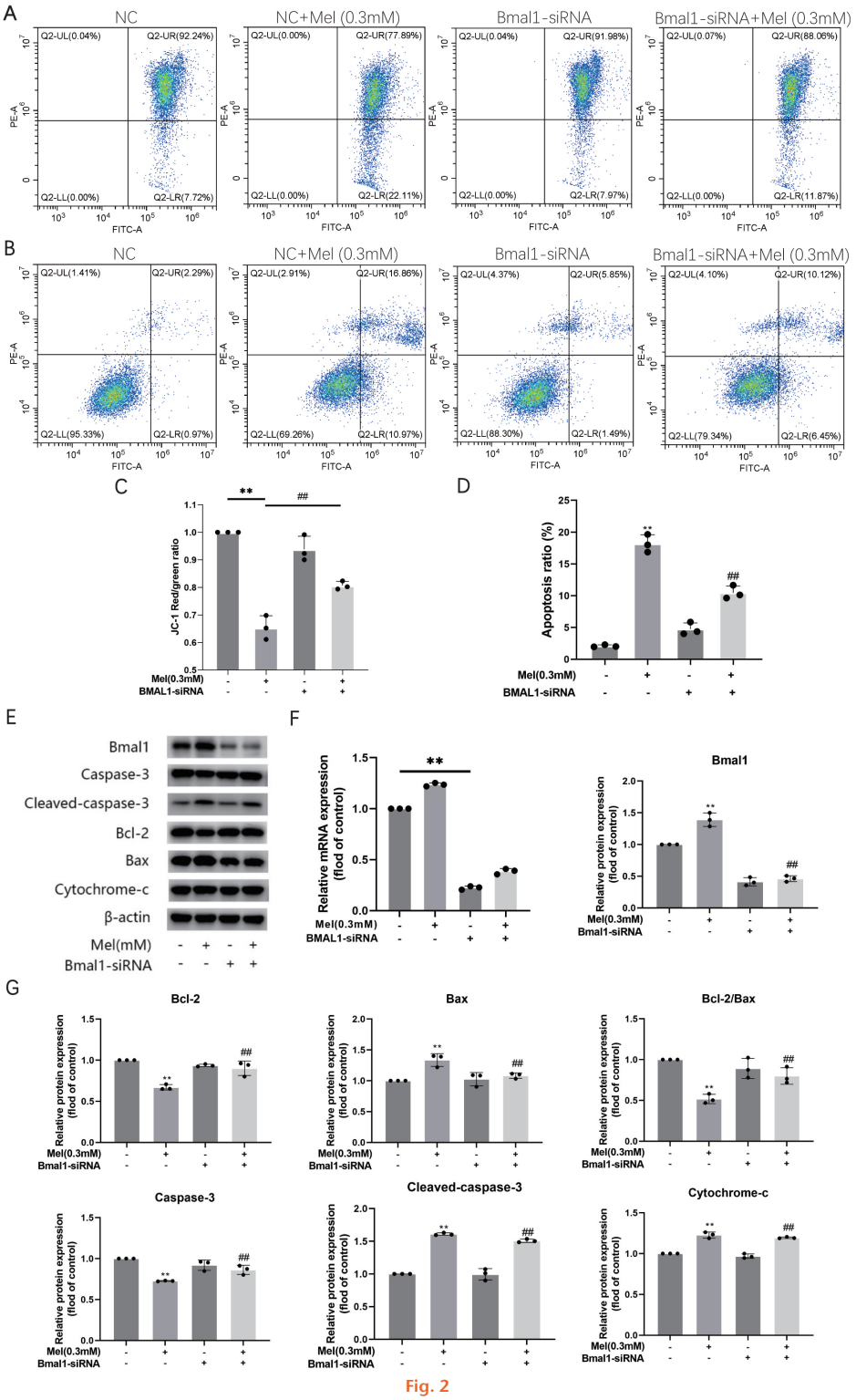

Fig. 2

Melatonin induces apoptosis of RAW264.7 cells by upregulating BMAL1 expression. a) Qualitative changes in mitochondrial membrane potential were detected by flow cytometry after treatment with 0.3 mM melatonin or BMAL1-small interfering RNA (siRNA). b) Representative flow cytometry plots after treatment with 0.3 mM melatonin or BMAL1-siRNA. c) Quantitative changes in mitochondrial membrane potential were detected using a full-wavelength multifunctional microplate reader. d) Apoptosis rates were detected by flow cytometry. e) The protein expression of Bmal1, Caspase-3, Cleaved-caspase-3, Bcl-2, Bax, Cytochrome-c, and β-actin indicated the effect of treatment with melatonin (0.3 mM) and BMAL1-siRNA. f) The mRNA transcription level of BMAL1 indicated the effect of melatonin. The transfection efficiency of *Bmal1* knockdown was detected at the messenger RNA (mRNA) level. The protein expression of BMAL1 indicated the effect of melatonin. The transfection efficiency of BMAL1 knockdown was detected at the protein level. g) Relative protein expression level of the protein in e). Experiments were implemented in triplicate. Data are means and standard deviations. *p < 0.05, **p < 0.01 compared with control cells and #p < 0.05, ##p < 0.01 compared with melatonin (0.3 mM) applied alone, analyzed by using one-way analysis of variance. Mel, melatonin.

signals and other physiological functions, and are crucial regulators of RAW264.7 cells. Mitogen-activated protein kinase (MAPK) family proteins are key signalling proteins, and MAPK-p38 is closely related to cell proliferation, differentiation, and apoptosis. We detected the level of ROS in RAW264.7 cells and western blotting of proteins related to the MAPK-p38 signalling pathway (Figures 3a and 3b). Compared with normal cells, the level of ROS in melatonin-treated cells decreased significantly. Compared with melatonin-treated cells, the level of ROS in BMAL1-targeted knockdown cells recovered. Compared with normal cells, melatonin treatment significantly decreased the phosphorylation of MAPK-p38 in cells. In contrast, the level of MAPK-p38 phosphorylation in BMAL1-targeted knockdown cells treated with melatonin was increased. These results suggest that melatonin can upregulate the expression of BMAL1 to inhibit the activity of ROS and the level of p38 phosphorylation in RAW264.7 cells.

Melatonin induced RAW264.7 cells apoptosis through MAPK-P38. In this part of the experiment, we explored whether the apoptosis-promoting effect of melatonin on RAW264.7 cells was mediated by activating the MAPK-p38 signalling pathway, and we hyperphosphorylated p38 protein by DHC. The results showed that the mitochondrial membrane potential increased with melatonin treatment after p38 hyperphosphorylation (Figures 4a and 4c), and the apoptosis rate of cells treated with melatonin after p38 hyperphosphorylation decreased significantly compared with melatonin treatment only (Figures 4b and 4d). Apoptotic proteins were detected by western blotting (Figure 4e). In the hyperphosphorylated cells treated with melatonin, the levels of Bax, cleaved-caspase-3, and cytochrome c decreased, while the levels of Bcl-2 and caspase-3 increased (Figure 4f). These results suggest that hyperphosphorylation of p38 signalling pathway proteins can reduce the apoptosis rate of RAW264.7 cells, and melatonin-induced RAW264.7 apoptosis is MAPK-p38 signalling pathway-dependent.

Melatonin improves bone loss in OVX mice. As shown in Figure 5a, the trabecular mass in the ovariectomized group was significantly lower than that in the sham-operated group, and melatonin attenuated this phenomenon. The results of the micro-CT scan showed that the bone mineral density (BMD),[30,31] trabecular number (Tb.N), trabecular thickness (Tb.Th), and bone volume/total volume (BV/TV) in the OVX group were lower than those in the sham-operated group, while the BMD, Tb.N, Tb.Th, and BV/TV in the OVX+ melatonin group were higher than those in the OVX group. In addition, trabecular separation (Tb.Sp) and bone surface/bone volume (BS/BV) in the OVX group were higher than those in the sham operation group and decreased after administration of melatonin (Figure 5b). We also detected an increase in *bmal1* gene expression, and a decrease of osteoclast marker, in the intragastric group (Figures 5c and 5d). All data show that bilateral ovariectomy leads to a decrease in bone mass in female mice, and intragastric administration of melatonin can prevent or even reverse this deterioration. In our postmenopausal mouse model, we first observed downregulation of *bmal1* gene expression in postmenopausal mouse bone tissue (Figure 6).

BMAL1 attenuates the activation of ROS and phosphorylation of p38. In previous studies, we found that *bmal1* gene-targeted knockdown cells were also different from normal cells in the experimental index. To further study the role of the *bmal1* gene, we established a model of overexpression of the *bmal1* gene (Figure 7a) and transfection efficiency is about 145% (Figure 7b). Compared with normal cells, the activity of ROS in cells overexpressing the *bmal1* gene was decreased, and the level of MAPK-p38 phosphorylation was increased (Figure 7e). These results suggest that the *bmal1* gene can regulate the activity of ROS and change the level of MAPK-p38 phosphorylation.

Discussion

At present, the treatment of osteoporosis focuses on the direct effect on osteoblasts and osteoclasts; for example, bisphosphonate inhibits bone resorption, and teriparatide promotes bone formation.[32,33] However, to date, the effects of these drugs are not ideal, especially drugs that inhibit bone resorption, which have serious side-effects.[34-36] The disruption of the dynamic balance of bone remodelling is the direct cause of osteoporosis, which is characterized by large bone resorption and bone formation. Therefore, the inhibition of bone resorption is the top priority in the treatment of osteoporosis. However, the current research is limited to the inhibition of osteoclast differentiation. To the best of our knowledge, we are the first to find a direct apoptosis-promoting effect of melatonin on RAW264.7 cells, explore the optimal concentration of melatonin to promote RAW264.7 cell apoptosis, and use this concentration for follow-up research. This study found that melatonin can change the expression of mitochondrial apoptotic proteins, reduce the mitochondrial membrane potential, and ultimately promote the apoptosis of RAW264.7 cells. Promoting the apoptosis of RAW264.7 cells (osteoclast precursor cells; mouse mononuclear macrophage leukaemia cells) can reduce the source of multinucleated osteoclasts, which is an effective strategy to prevent postmenopausal osteoporosis.

In our experiments, we observed that melatonin upregulated the expression of *Bmal1* in RAW264.7 cells. Clinical data show that the bmal1 gene is closely related to bone-related diseases, such as mandibular hypoplasia, osteoarthritis, and osteoporosis. Some studies have shown that the inactivation of BMAL1 in RAW264.7 cells can promote the expression of matrix metalloproteinase 13, and bone resorption.[37] It has also been found that the decrease in osteoclast inhibitory factor, also known as osteoprotegerin (OPG), levels leads to the enhancement of bone resorption after coculture of BMAL1-inactivated MC3T3-E1 cells and RAW264.7 cells.[38] Our study shows that melatonin can upregulate the expression of the *bmal1* gene in

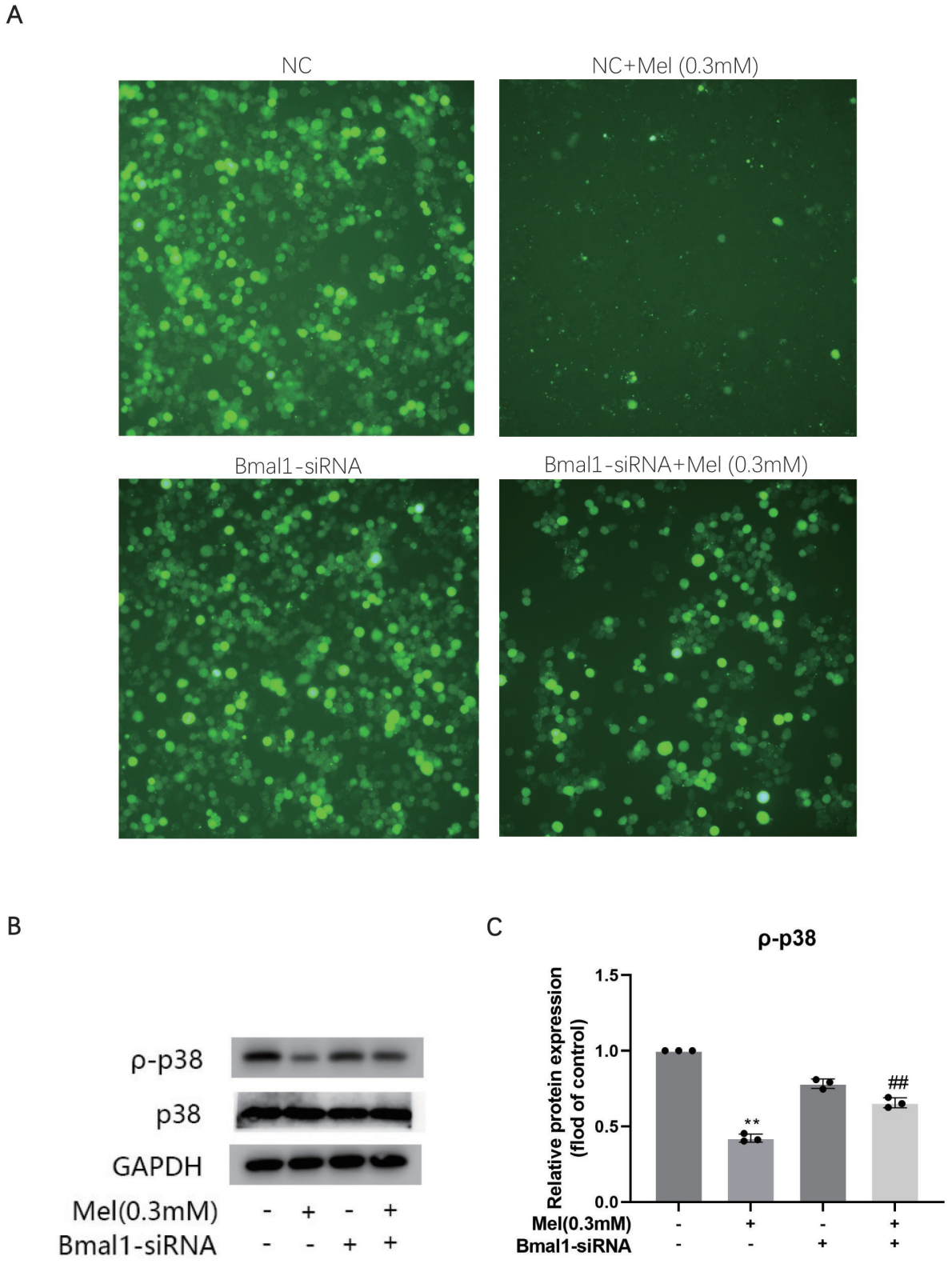

Fig. 3

Melatonin increased the expression of BMAL1 to inhibit the activation of reactive oxygen species (ROS) and phosphorylation of mitogen-activated protein kinase (MAPK)-p38. a) ROS were detected by a fluorescent probe after treatment with 0.3 mM melatonin or BMAL1-small interfering RNA (siRNA). b) Protein expression of phosphorylated p38, p38, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) indicated the effect of treatment with melatonin (0.3 mM) and BMAL1-siRNA. c) Relative protein expression levels of the proteins in b). Experiments were implemented in triplicate. Data are means and standard deviations, *p < 0.05, **p < 0.01 compared with control cells and #p < 0.05, ##p < 0.01 compared with melatonin (0.3 mM) applied alone analyzed by using one-way analysis of variance. Mel, melatonin.

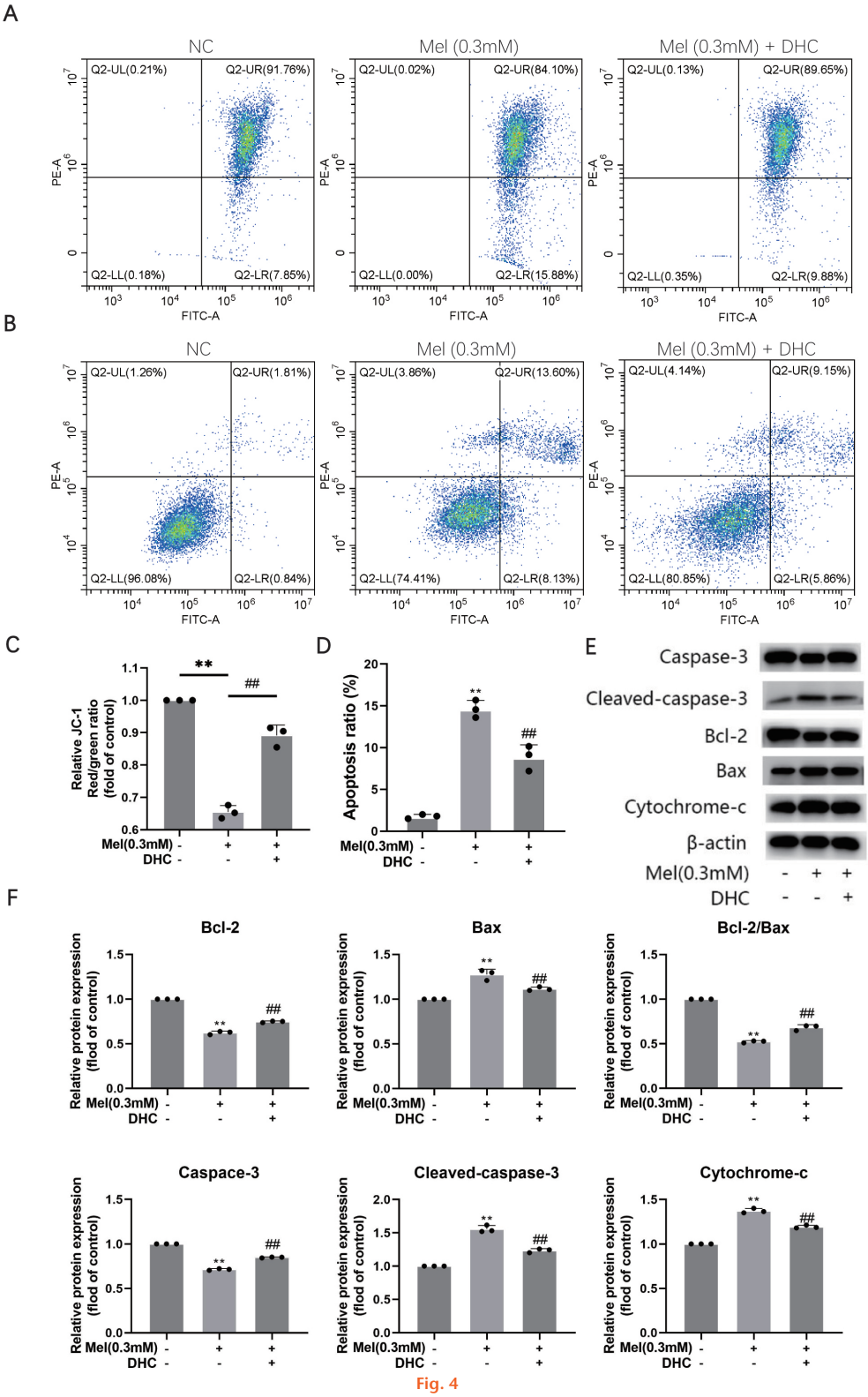

Fig. 4

Melatonin (Mel)-induced RAW264.7 apoptosis through mitogen-activated protein kinase (MAPK)-P38. a) Qualitative changes in mitochondrial membrane potential were detected by flow cytometry after treatment with 0.3 mM melatonin or DHC. b) Representative flow cytometry plots after treatment with 0.3 mM melatonin or DHC. c) Quantitative changes in mitochondrial membrane potential were detected using a full-wavelength multifunctional microplate reader. d) Apoptosis rates were detected by flow cytometry. e) The protein expression of Caspase-3, Cleaved-caspase-3, Bcl-2, Bax, Cytochrome-c, and β-actin indicated the effect of treatment with melatonin (0.3 mM) and DHC. f) Relative protein expression levels of the proteins in e). Experiments were implemented in triplicate. Data are means and standard deviations, *p < 0.05, **p < 0.01 compared with control cells and #p < 0.05, ##p < 0.01 compared with melatonin (0.3 mM) applied alone, analyzed by using one-way analysis of variance.

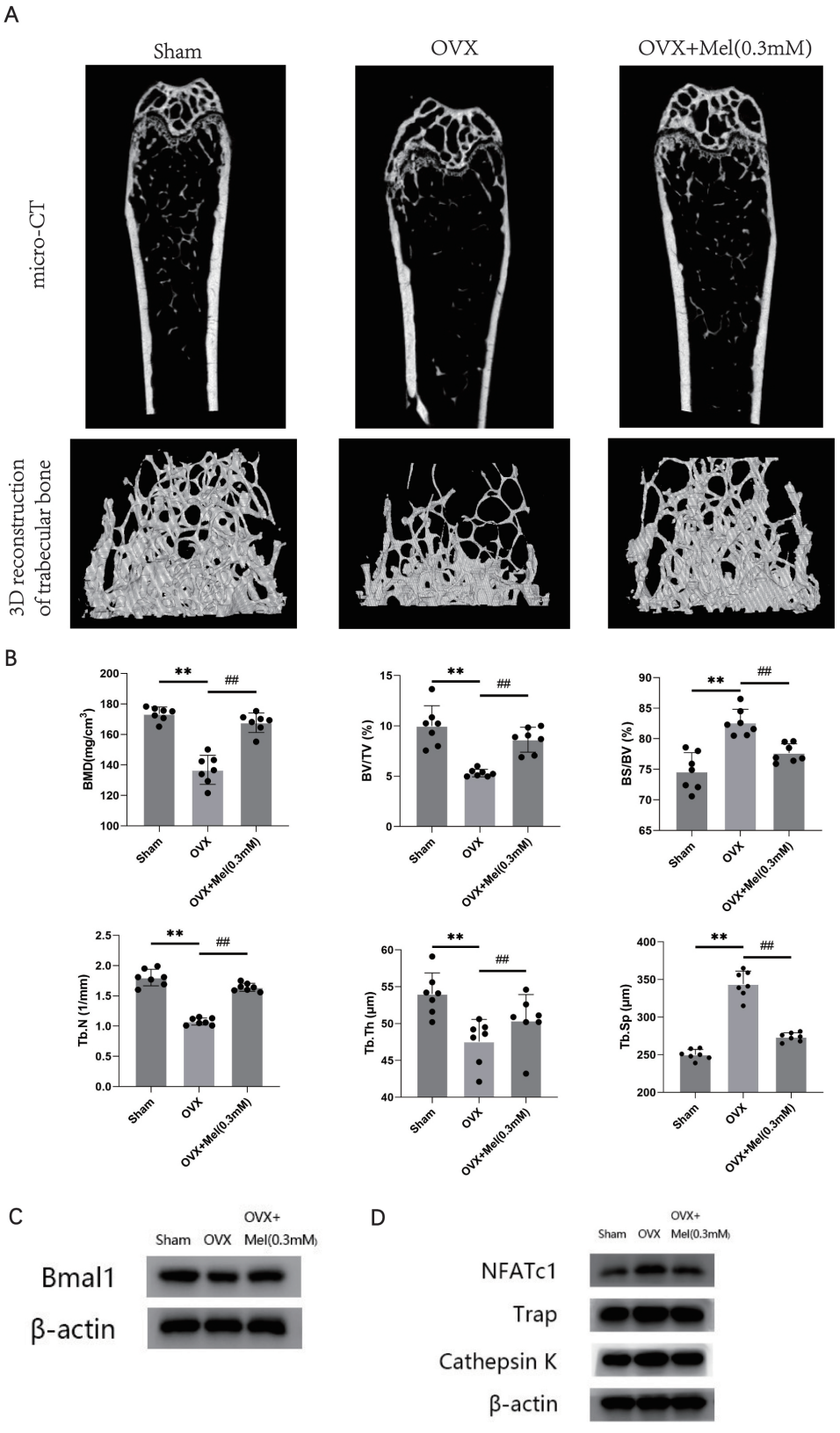

Fig. 5

Melatonin (Mel) improves bone loss in ovariectomized (OVX) mice. a) 2D and 3D reconstruction of the femur micro-CT images. b) Related parameters obtained from image analysis. c) Protein levels of the BMAL1 gene and β-actin in femur samples. d) Protein levels of the osteoclast markers in femur samples. Data are means and standard deviations, *p < 0.05, **p < 0.01 compared with sham and #p < 0.05, ##p < 0.01 compared with OVX, analyzed using one-way analysis of variance.

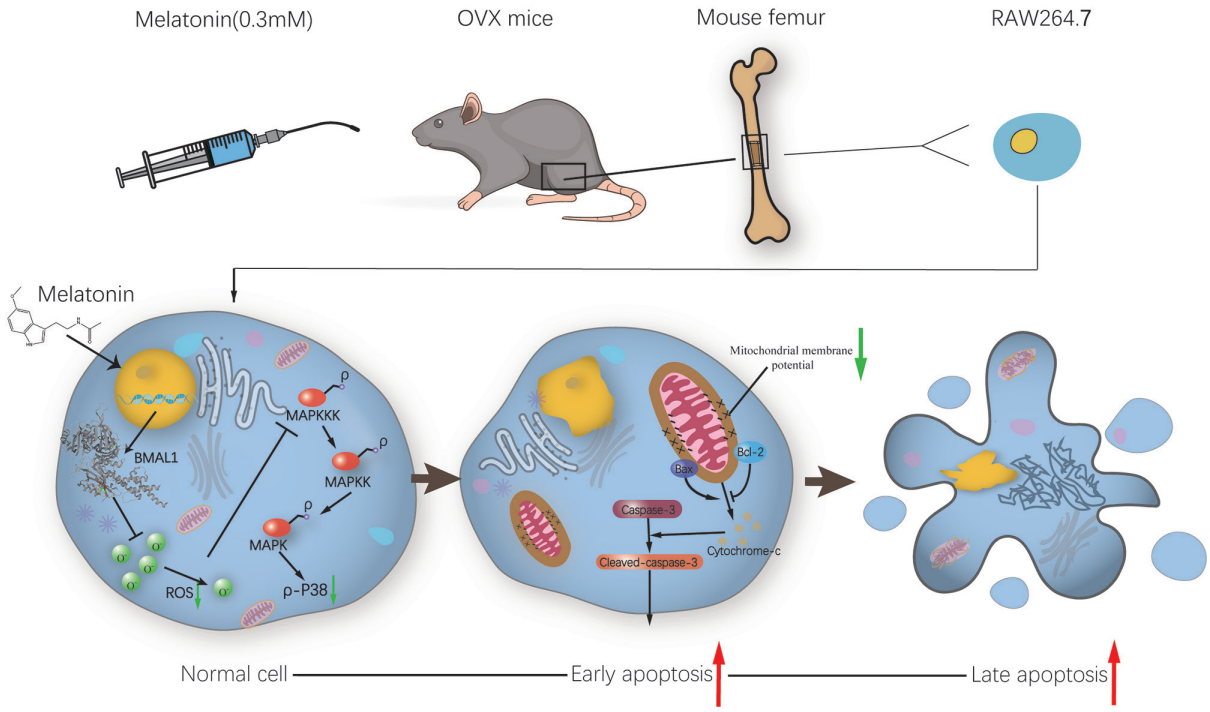

Fig. 6

Summarized figure of the signalling pathway. MAPK, mitogen-activated protein kinase; OVX, ovariectomized; ROS, reactive oxygen species.

RAW264.7 cells. We have also confirmed this in animal experiments. Interestingly, our study found that BMAL1 levels were decreased in the bone tissue of postmenopausal mice compared with the sham-operated group. Moreover, the *bmal1* gene plays an important role in this process. Very few studies have investigated the serious health effects of the disruption of circadian rhythms after menopause.[39,40] The sexual dimorphism of the rhythm raises concerns about health implications related to this after menopause.[39] It has been reported that the expression of the *bmal1* gene is reduced in the liver tissue of postmenopausal women,[40] but the changes in BMAL1 in various tissues are still unknown. We found for the first time that BMAL1 levels decreased in the bone tissue of postmenopausal mice. This may be the underlying mechanism of postmenopausal osteoporosis.

Subsequently, we studied the mechanism by which melatonin promotes the apoptosis of RAW264.7 cells. Oxidative stress is considered an important cause of ageing and organ damage.[41,42] Postmenopausal women have a significant decrease in antioxidant capacity and high levels of ROS. ROS are very important for cell physiological function and signal transmission,[43,44] especially for RAW264.7 cells, and the accumulation of ROS is very important.[45] Our study confirmed that melatonin can reduce ROS activity by upregulating the expression of the *bmal1* gene. Other studies have shown that the lack of BMAL1 can lead to early ageing and increased levels of ROS in mice,[46] which coincides with our results. The *bmal1* gene plays an important role in the circadian rhythm.[47] The analysis of several genes encoding major antioxidant enzymes in the data revealed the existence of rhythm-related elements, indicating that rhythm genes affect some genes that encode antioxidant enzymes. Catalase, superoxide dismutase, and glutathione peroxidase are the key regulators of redox reactions.[48] Therefore, we infer that by influencing different steps of the whole process, the CLOCK/BMAL1 transcription complex can regulate the whole reduction/oxidation chain, which may be an important reason why BMAL1 affects the activity of ROS. We confirmed this mechanism by overexpression of the *bmal1* gene. MAPK family proteins are key signalling proteins,[49] and MAPK-p38 is closely related to cell differentiation and apoptosis.[50,51] In eukaryotic cells, the MAPK signal cascade is the most conserved and well-studied signal transduction system. Classic MAPK cascade transduction is activated by a three-stage kinase cascade. It has been found that highly active ROS can increase the phosphorylation of MAPK-p38,[52] and activated p38 can further promote cell proliferation,[53] which is consistent with our results. Melatonin weakens MAPK-p38 phosphorylation and increases apoptosis.

In conclusion, due to the limited research on the pathogenesis of osteoporosis and the unsatisfactory efficacy of drugs for the treatment of osteoporosis, we studied the mechanism of melatonin in the treatment of osteoporosis through BMAL1/ROS/P38 (Figure 6), enriching the pathogenesis of osteoporosis and enriching the potential target BMAL1 for the treatment of osteoporosis, thus contributing to the design and development of drugs aimed at this target. The focus of future research will be further

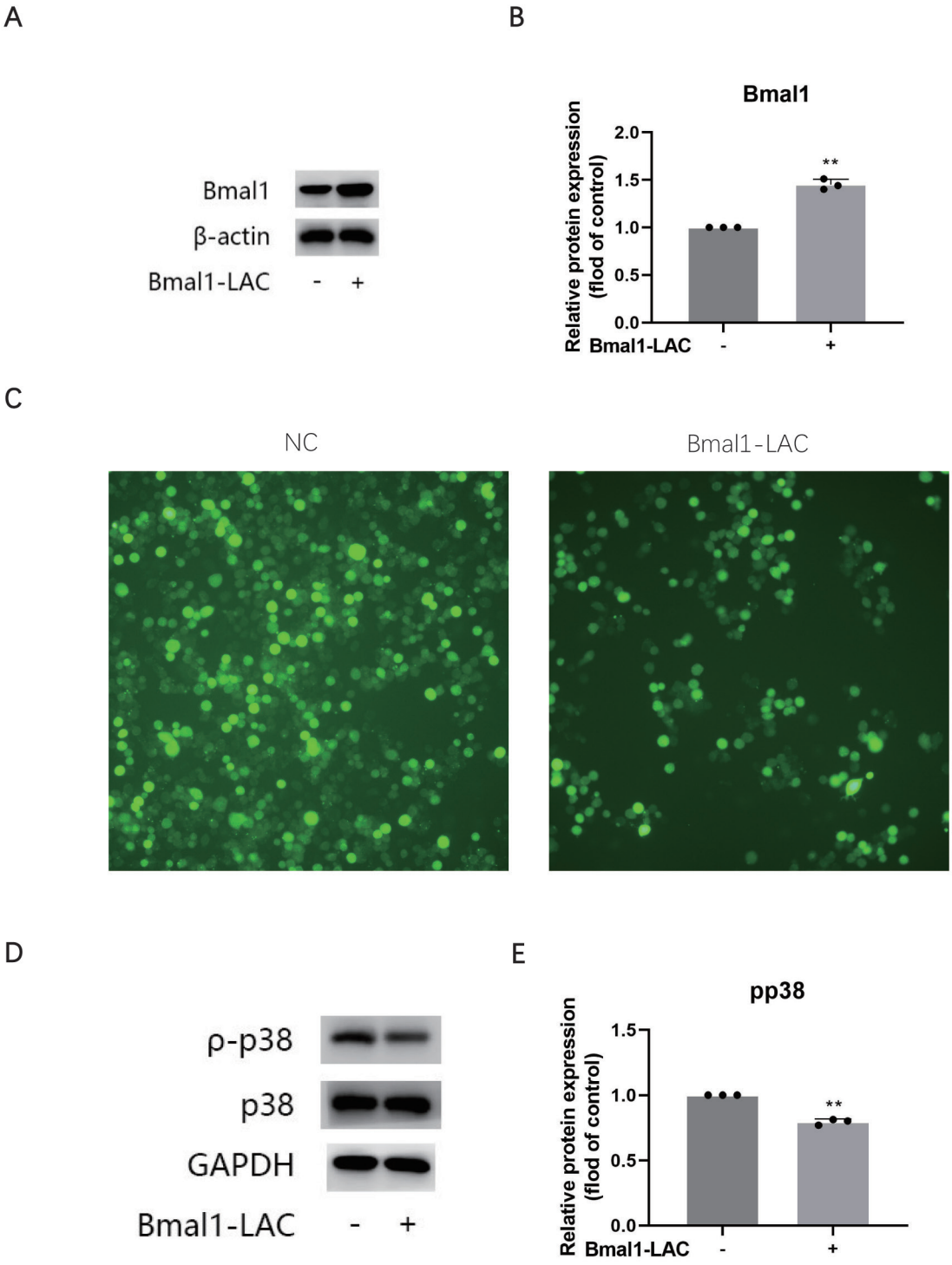

Fig. 7

BMAL1 attenuates the activation of reactive oxygen species (ROS) and phosphorylation of p38. a) Protein expression of BMAL1 and β-actin indicated the effect of BMAL1 lentiviral activation particles (BMAL1-LAC). b) Relative protein expression levels of the proteins in a). c) ROS were detected by a fluorescent probe after application with BMAL1-LAC. d) Protein expression of phosphorylated p38, p38, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) indicated the effect of application with BMAL1-LAC. e) Relative protein expression levels of the proteins in d). Experiments were implemented in triplicate. Data are means and standard deviations; *p < 0.05, **p < 0.01 compared with control cells analyzed by using one-way analysis of variance.

clarifying the role of the rhythmic regulation of oxidative damage in the development of osteoporosis, and the effect of melatonin on osteoclast precursor cells.

Supplementary material

ARRIVE checklist

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