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RESEARCH ARTICLE

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Alpinetin alleviates osteoporosis by promoting osteogenic differentiation in BMSCs by triggering autophagy via PKA/mTOR/ULK1 signaling

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Abstract

Osteoporosis, a systemic bone disease that is characterized by a reduction in bone mass and destruction of bone microstructure, is becoming a serious problem worldwide. Bone marrow mesenchymal stem cells (BMSCs) can differentiate into bone-forming osteoblasts, and play an important role in maintaining homeostasis of bone metabolism, thus being a potential therapeutic target for osteoporosis. Although the phytochemical alpinetin (APT) has been reported to possess a variety of pharmacological activities, it is still unclear whether APT can influence the osteogenic differentiation of on BMSCs and if it can improve osteoporosis. In this study, we found that APT treatment was able to enhance osteogenic differentiation levels of human BMSCs in vitro and mouse ones in vivo as revealed by multiple osteogenic markers including increased alkaline phosphatase activity and osteocalcin expression. Mechanistically, the protein kinase A (PKA)/mTOR/ULK1 signaling was involved in the action of APT to enhance the osteogenic differentiation of BMSCs. In addition, oral administration of APT significantly mitigated the bone loss in a dexamethasone-induced mouse model of osteoporosis through strengthening PKA signaling and autophagy. Altogether, these data demonstrate that APT promotes osteogenic differentiation in BMSCs by augmenting the PKA/mTOR/ULK1 autophagy signaling, highlighting its potential therapeutic application for treating osteoporotic diseases.

KEYWORDS
alpinetin, autophagy, bone marrow mesenchymal stem cells, osteogenic differentiation, osteoporosis, protein kinase A

1 | INTRODUCTION

Nowadays, with the development of society, osteoporosis is increasingly becoming a serious problem worldwide. It is manifested by increased fragility of bone and risk of fracture even in the case of slight trauma or no trauma (van den Bergh, van Geel, & Geusens, 2012; Yang et al., 2020). Owing to declines in bone density and bone quality, the patients are susceptibility to fracture, back pain, body length shortening, bone pain, and even the occurrence of systemic metabolic diseases with weakened respiratory function, which

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brings heavy psychological pressure and economic burdens to patients and their families (Diem et al., 2017; Ensrud & Crandall, 2017; Rachner, Khosla, & Hofbauer, 2011). Currently, there are many chemical drugs that are commonly used to treat osteoporosis, such as estrogen, selective estrogen receptor modulators, calcitonin, bisphosphonates, and parathyroid hormones (Cotts & Cifu, 2018). After treatment, the overall survival rate of patients improves, but these drugs may cause breast cancer, cardiovascular and cerebrovascular diseases, and other adverse reactions, and the cost is quite expensive, which poses heavy economic burdens to the families of patients (Lorentzon, 2019; Valenti, Dalle Carbonare, & Mottes, 2016). Novel therapeutic agents are therefore urgently needed to be developed for the treatment of osteoporosis.

It is worth noting that the imbalance of bone synthesis and osteolysis is the main reason for primary osteoporosis (Lewiecki, 2011). Bone marrow mesenchymal stem cells (BMSCs) are a type of regenerative pluripotent stem cells and the most important cells in normal bone growth and metabolism; these cells can differentiate into cartilage, bone, and adipose tissue (Ohata & Ozono, 2014; Xu, De Veirman, De Becker, Vanderkerken, & Van Riet, 2018; Zheng, Sui, Qiu, Hu, & Jin, 2020). Under the appropriate stimulation of microenvironmental regulatory factors, BMSCs can gradually differentiate into preosteoblast, immature osteoblasts, mature osteoblasts, and lastly osteocytes. This osteogenic differentiation process is regulated by delicate signaling molecules, abnormal expression of which could lead to bone metabolism disorder (Anthony & Link, 2014). In particular, osteoblast differentiation plays an important role in maintaining the homeostasis of the bone (Xiao et al., 2016). It has been reported that the abnormal differentiation of endogenous BMSCs leads to the occurrence and progression of osteoporosis (Qi et al., 2017); (Li, Ayoub, et al., 2019). These results suggest that BMSC-based therapy represents a promising strategy for the treatment of osteoporosis.

Flavonoids are natural compounds widely found in plants and have a variety of biological activities. In recent years, through in vitro cell experiments and in vivo animal studies, researchers have confirmed that flavonoids exhibit significant antiosteoporosis effects by regulating the differentiation of MSCs (Rodríguez-Merchán, 2021; Zhang et al., 2022), thus representing potential alternative agents for the treatment of osteoporosis. Alpinetin (APT) is a natural flavonoid compound isolated from the dry and near mature seeds of Alpinia fragrans that possess various pharmacological activities such as antiinflammatory, analgesic, antioxidative, and antitumor effects (Liu et al., 2019; Lv et al., 2018; Zhao et al., 2022); Although this flavonoid has been used to treat acute gastritis, colitis, hypertension, hyperlipidemia, and inflammation-related diseases (Liang et al., 2018; Lv et al., 2018; Wang et al., 2016), it remains unclear whether APT can influence the osteogenic differentiation process of BMSCs and ameliorate osteoporosis. It is therefore of interest to explore the role of APT in influencing MSC osteogenic differentiation and osteoporosis, laying a research foundation for its future application in the treatment of bone defect diseases.

Autophagy is the main degradation system in eukaryotes, which can transfer damaged organelles or other cytoplasmic components into lysosomes for degradation after processing, and its metabolic products can be recycled by cells (Galluzzi & Green, 2019; Kim & Lee, 2014). Recently, many studies have shown that autophagy has an important role in maintaining the stability and balance of the bone system. Autophagy can drive the osteogenic differentiation of human mesenchymal stem cells (Vidoni et al., 2019). Defects in autophagy in osteoblasts can lead to bone loss in mice (Kroemer, 2015). It is worth noting that the osteoporotic symptoms of ovariectomized (OVX) mice can be effectively mitigated by activating autophagy in mesenchymal stem cells (Liang et al., 2019). Under such circumstances, activating autophagy in BMSCs may have important physiological effects as an alternative avenue to prevent and/or alleviate osteoporosis.

In this study, we aimed to investigate whether APT had any influence on osteogenic differentiation in vitro and osteoporosis in vivo. Our results showed that APT was able to significantly promote the osteogenic differentiation of BMSCs, which was mediated by enhancing the protein kinase A (PKA) signaling pathway and thus culminating in augmented autophagy. Furthermore, oral administration of APT markedly attenuated osteoporotic symptoms in a mouse model of osteoporosis. Our results highlight that APT can strengthen the osteogenic differentiation of BMSCs, thus representing a potential therapeutic agent against osteoporosis.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

APT ($C_{16}H_{14}O_4$, molecular weight: 270.28); (≥98% purity) was obtained from Shanghai Yuanye Biotechnology Company (B21121-500 mg, China). Dulbecco's modified Eagle's medium (DMEM, A3021002) with high glucose and penicillin–streptomycin solution (15140122) were obtained from Gibco (United States). Stem Cell MesenCult Expansion Kit (Mouse) was purchased from Stem Cell (05513-1Kit, Canada). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Material Company (11011-8611, China). EDTA-decalcifying fluid was purchased from BOSTER (AR107, China). Trypsin was obtained from Biosharp (BL501A, China). DMSO was purchased from MP (219605580, United States). Cell Counting Kit (CCK8, C0038-500T), H89 (S1643-100mg), BCIP/NBT alkaline phosphatase (ALP) color development kit (C3206), 5× SDS–PAGE protein loading buffer (P0286-2 ml), SDS–PAGE gel preparation kits (P0012A), and antibodies against GAPDH (1:5000, AF5009) and p-(Ser/Thr) PKA substrate (1:1000, AF1942) were purchased from Beyotime Biotechnology (Haimen, China). The ALP assay kit was purchased from Nanjing Jiancheng Biotech (A059-2-2-96T, China). Alizarin Red S was purchased from Solarbio (G8550, China). Antibodies against Runx2 (12556S), active β-catenin (2009S), β-catenin (8480S), p-Akt (4060S), Akt (4691S), p-p38 (ab178867), p38 (ab170099), p-ERK (4370S), ERK (4695S), p-JNK (4668S), JNK (9252S), p-mTOR (2971S), mTOR (2972S), p-ULK1 (5869T), p-Smad (8828S), Smad (8685S), and p62 (8025S) were purchased from Cell Signaling Technology (1:1000, United States). Antibodies against osteocalcin (OCN, 1:500, ab93876), osteopontin (OPN,

1:500, ab214050), LC3B (1:1000, ab192890), and BMP2 (ab284387) were purchased from Abcam (England). Horse-radish peroxidase (HRP)-conjugated horse anti-mouse IgG (A-1101) and HRP-conjugated goat-anti-rabbit IgG (A-21428) were purchased from Thermo Fisher (United States). Beclin-1 (1:1000, sc-48341) was purchased from Santa Cruz (United States). APT was dissolved in DMSO at a concentration of 100 mM and stored at −20°C, then diluted to the appropriate concentration with osteogenic differentiation medium for cell experiments, and diluted with 2% Tween-80 in PBS for animal experiments.

2.2 | Isolation and culture of BMSCs

This study was approved by the Ethics Committee of the Eighth Affiliated Hospital of Sun Yat-sen University and conformed to the Ethical Guidelines of the Declaration of Helsinki. In this study, 15 volunteers who passed the physical examination were selected and signed the informed consent. The inclusion criteria of the volunteers were that they had no family hereditary or contagious history, and there were no abnormalities in the hepatitis B and C, Epstein–Barr virus and cytomegalovirus virus DNA quantification and sexually transmitted diseases including HIV and Syphilis. BMSCs were isolated and cultured as described previously (Li, Wang, et al., 2019; Li et al., 2018; Xie et al., 2018). BMSCs from one individual were cultured as one sample. In brief, 20–30 ml bone marrow was extracted with a sterile syringe from donors and left standing at room temperature for 30 min, and then the marrow supernatant was collected by pipette into a sterile 50 ml centrifuge tube. BMSCs were isolated and purified by density gradient centrifugation at 500× g for 15 min at room temperature, then the cells were re-suspended in a culture medium (DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) and differentiated for 48 h at 37°C in a humidified incubator with 5% CO_2, after which the culture medium was changed to dislodge the unattached cells. Then, the culture medium was changed every 3 days. When the cell density reached 85–95%, the BMSCs were rinsed with 15 ml of sterile PBS three times and digested with 0.25% trypsin including 0.53 mM ethylenediaminetetraacetic acid (EDTA), then the cells were transferred into a new flask as passage 1. BMSCs were extended to passages 3–5 for subsequent experiments. In our study, all cell experiments were repeated thrice with three different BMSC donors.

2.3 | Osteogenic differentiation induction

BMSCs were cultured in 12-well plates at 0.5×10^5 cells/well in complete DMEM (DMEM contained 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C for 24 h as described above (Xie et al., 2018). The culture medium was subsequently changed to osteogenic differentiation medium (OM) containing DMEM (glucose: 1,000 mg/L), 10%FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 μM dexamethasone (DXMS), 10 mM β-glycerol phosphate, and 50 μM ascorbic acid for 0–14 days. The medium was replaced every 3 days. The differentiation of osteoblasts was identified by Alizarin Red S (ARS) and ALP as described below.

2.4 | Cell treatments

To measure the influence of APT on the proliferation of BMSCs, cells were cultured in a basal culture medium containing different concentrations of APT (0, 10, 20, and 40 μM), and cell viability was measured on days 0, 3, 7, and 14, respectively. Meanwhile, to assess the influence of APT on the osteogenic differentiation of BMSCs, cells were cultured in an osteogenic differentiation medium with graded concentrations of APT (0, 10, 20, and 40 μM). To explore the relationship between APT on PKA signaling, the PKA-specific inhibitor H89 was added to the osteogenic differentiation medium in accordance with the experimental design scheme.

To investigate the effect of autophagy on the osteogenic differentiation of BMSCs, BMSCs were treated with an appropriate concentration of 3-MA (an autophagy inhibitor). Then, the osteogenic differentiation of BMSCs was examined.

2.5 | Cell viability assay

CCK-8 kit was used to examine cell viability according to the manufacturer's instructions. In brief, BMSCs were cultured in 96-well plates (3,000 cells/well) and then subjected to different treatments. For the assay, BMSCs were incubated for 30 min with a culture medium supplement with 10% CCK-8 reagent at room temperature and the optical density (OD) value at 570 nm was recorded using a spectrophotometer. Cell viability (%) = [OD (APT)-OD (blank)]/[OD (control)-OD (blank)] × 100%.

2.6 | Small interfering RNA

The small interfering RNA (siRNA); (CCAGAAGCAUAACAUUCAA) duplexes that target human PKA and negative control (NC) siRNA were designed and synthesized by RiboBio (Guangzhou, China). Lipofectamine RNAiMAX (ThermoFisher/Invitrogen) was transfected according to the manufacturer's instructions. In brief, before transfection, BMSCs were planted on 12-well plates at a ratio of 30–50% overnight and transfected by PKA siRNA (20 nM) or NC siRNA (20 nM) the next day. The cells were cultured in a DMEM medium containing 10% FBS for 48 h, followed by stimulation with 40 μM APT, which was diluted by an osteogenic differentiation medium. The differentiation of osteoblasts was identified by ARS and ALP as described below.

2.7 | ARS staining

ARS staining was used to detect calcium deposition in cells as previously described (Li et al., 2020), which was used to evaluate

osteogenic mineralization after 14 days. In brief, cells were washed with PBS three times for removing the medium, then fixed with 4% paraformaldehyde for 30 min. After that, BMSCs were washed three times, then stained with 1% ARS (G8550, Solarbio, China) for 15 min at room temperature. The cells were washed three times again to remove unspecific staining and then visually observed, and photos were taken using a microscope. To measure spectrophotometric absorbance, we used 10% cetylpyridinium chloride monohydrate (C0732, Sigma–Aldrich, United States) to destain the cells for 1 h at room temperature. Then, the liquid was transformed into the 96-well plate, and the spectrophotometric absorbance was evaluated at 562 nm using a spectrophotometer.

2.8 | ALP staining and activity assay

ALP is one of the reliable markers of osteoblast maturation and mineralization and it expresses as a common glycoprotein on the cell membrane that can hydrolyze phospholipids and provide a material basis for bone formation (Vaisman, McCarthy, & Cortizo, 2005).

After 7 days of culture in an osteogenic differentiation medium containing APT, the cells were washed with PBS and subsequently fixed for 15 min with formaldehyde solution. Then, the cells were stained using a BCIP/NBT ALP kit according to the manufacturer's instructions. The cells were observed and photographed with an optical microscope.

To examine ALP activity, we used ALP activity kits to analyze the cells, and the process was performed according to a previously described protocol (Tang et al., 2019). In brief, BMSCs were lysed using RIPA buffer including protease and phosphatase inhibitors (Beyotime Biotechnology, China) and then centrifuged at 4000× g for 30 min at 4°C, and the supernatants were incubated using by reaction buffer for 15 min at 37°C. After the termination solution was added, the absorbance was measured at 405 nm. The total protein concentration was determined by Pierce bicinchoninic acid (BCA) protein detection kit (23227, Thermo Fisher Scientific). The results were normalized to the total intracellular protein level, and the results are expressed as units per gram of protein.

2.9 | Immunoblotting

Immunoblotting was performed as previously described (Xie et al., 2016). Briefly, cells were washed with cold PBS and lysed in RIPA buffer (CoWin Biosciences, China) supplemented with 1% protease inhibitors (CoWin Biosciences, China) and 1% phosphatase inhibitors (CoWin Biosciences, China) on ice, after which the cells were lysed by ultrasonication. Then, lysates were acquired by centrifugation at 4000× g at 4°C for 30 min. A Pierce BCA protein assay kit was used to determine the total protein concentration. Then, equal amounts of proteins were separated by SDS–PAGE, followed by electrotransfer to polyvinylidene difluoride membranes (IPVH00010-3 Mx37.5 cm, Merck Millipore, United States). The membranes were blocked and incubated with the indicated primary antibodies at 4°C overnight, followed by incubation with the HRP-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (1:1,000). Bands were revealed by chemiluminescent reagents (Merck Millipore, United States), and the densitometry of each band was quantified by UVP ChemStudio 815 (Analytikjena, Germany).

2.10 | Immunofluorescence microscopy of BMSCs and femur bone sections

Immunofluorescence analysis was performed as previously described (Li, Wang, et al., 2019). Briefly, BMSCs were seeded on sterile glass coverslips (0.4×10^5 cells/glass) and cultured in complete DMEM at 37°C overnight. The cells received different treatments according to the experimental design. After microcomputed tomography (micro-CT) analysis, a blade was used to remove excess muscle tissues from the surface of the femurs. The femurs were then decalcified on the 37°C constant temperature shaker with 10% EDTA (pH = 7.4) for 10 days and the solution was changed twice in 48 h. Then the bones were cleaned with sterile PBS three times, embedded in paraffin, and sectioned (5-mm thickness). Paraffin sections were dewaxed to water and cleaned with xylene and gradient alcohol (100%, 95%, 85%, and 75%) for 5 min, incubated with antigen repair solution (pH 9.0 EDTA) for 1 h, and then immersed in distilled water for 3 min. Immunofluorescence staining was performed as described previously with minor modification (Li, Wang, et al., 2019). Briefly, after fixation and permeabilization and blocking, the cells and femur bone were blocked for 1 h at 37°C and then incubated with the indicated primary antibodies (1:300) overnight at 4°C, followed by staining with anti-rabbit IgG (1:500, Cell Signaling Technology, 4413) and anti-mouse IgG (1:500, Cell Signaling Technology, 4408) secondary antibodies for 1 h at room temperature. After washing with TBST three times, nuclei were stained with 50 μl 4′, 6-diamidino-2-phenylindole (DAPI, 1:500, Beyotime Biotechnology, P0131) for 10 min in the dark. Then, coverslips were installed on glass slides with fade-proof mounting medium. Finally, the samples were observed under a laser scanning confocal microscope at wavelengths of 488 nm (green, OCN), 555 nm (red, p-PKA, LC3B), and 405 nm (blue, DAPI), and images were obtained using an LSM 5 Exciter confocal imaging system (Carl Zeiss). Immunofluorescence staining was quantitatively analyzed by Image-Pro Plus software.

2.11 | Autophagosome formation assay

The autophagosome formation assay was performed as described previously (Li et al., 2016), which was performed according to the manufacturer's instructions (D676, DOJINDO, China). In brief, BMSCs (5×10^3 cells) were plated in confocal dishes in complete DMEM at 37°C overnight. On the second day, BMSCs were washed three times with DMEM. And then appropriate DAP Green Working Solution was added to the cells, and the cells were cultured at 37°C for 30 min.

Appropriate treatment of APT After discarding the supernatant and wash cells twice with DMEM. After 24 h, we used Leica TCS Sp8 confocal laser scanning microscope (Leica, Germany) to observe the intracellular autophagy of BMSCs. The images were captured under a 40× oil objective lens.

2.12 | Experimental mice

Male C57BL/6 mice (12 weeks of age, 23–25 g; 18 months of age; 30–35 g) were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). The mice were kept at standard temperature and humidity conditions, kept on a 12-hr light/dark cycle, and had free access to food and water. All animal experiments were carried out according to the guidelines for the care and use of animals approved by the Committee on the Ethics of Animal Experiments of Sun Yat-sen University.

2.13 | Osteoporosis mouse model construction and treatment

The murine osteoporosis model was established according to a previously described protocol (Li et al., 2020). In brief, the C57BL/6 mice were injected into hind leg muscle with DXMS at the dose of 2 mg/kg three times a week and then were sacrificed after 8 weeks. The symptoms of the mice, like the decreased number of bone trabecula, increased bone space, and adipocytes were taken as the criteria for judging the success of the model of the osteoporosis mouse model. Thirty C57BL/6 mice were randomly divided into six groups (6 mice/group) as the normal mice, the glucocorticoid-induced osteoporosis (GIOP) mice, the GIOP mice intragastrically administered with APT (GIOP+APT, 40 mg/kg/d), GIOP mice intraperitoneally injected with 3-MA (5 mg/kg, three times a week for 8 weeks), GIOP mice intraperitoneally injected with H89 (1 mg/kg); (Lee et al., 2019), three times a week for 8 weeks and GIOP mice intraperitoneally injected with parathyroid hormone (PTH); (30 μg/kg/d); (Zhang et al., 2020), dissolved in a vehicle of 0.2% bovine serum albumin and 0.1% 1 mN hydrochloric acid in 0.9% saline to form a solution of 10 μg/ml for delivery, Bachem California, Inc., Torrance, CA. The dose of APT used in this study was based on previous studies (Huo et al., 2012; Lv et al., 2018; Zhang et al., 2020). After 8 weeks, the femurs and serum were acquired for micro-CT, staining studies, and Western blot analysis, respectively. The groups were blind to the observers.

2.14 | Enzyme-linked immunosorbent assay (ELISA)

Serum levels of OCN and ALP were measured in accordance with the manufacturer's instructions, and the detailed method was essential as previously described (Li et al., 2020). After the last administration, the blood was collected by the orbital blood sampling method and incubated at 4°C overnight. Then, the whole blood was centrifuged at 800× g for 10 min at 4°C, and the serum was separated. Finally, the serum levels of OCN and ALP were determined by ELISA at 450 nm.

2.15 | Isolation and culture of murine BMSCs

Mice were euthanized by cervical dislocation. The epiphysis of the femur and tibia were incised in the sterile ultra-clean workbench, and the sterile medium was sucked by 5 ml syringe to flush the shaft cavity until the shaft became white. After that, the medium containing cells was collected by an aseptic centrifuge tube and centrifuged at 1,600× g for 10 min. The supernatant was dislodged, and the precipitate was mixed with a specific culture medium (05513, Stem Cell, Canada) for murine BMSCs containing 10% FBS, penicillin, and streptomycin. BMSCs were cultured at 37°C supplemented with 5% CO_2. After cell density attained 85–95%, they were digested by trypsin and then passaged for three passages.

2.16 | Flow cytometric analysis of human and murine BMSCs

After one passage, the cells were examined by flow cytometry analysis. The cells were incubated with specific primary antibodies against the following proteins: human BMSCs: CD14 (555397), CD34 (555821), CD45 (560973), CD73 (561254), CD90 (561970), CD105 (560839), HLA-DR (562304) and murine BMSCs: CD29 (561796), CD34 (551387), CD44 (560452), CD45 (561018), and Sca-1(561021); (all the above flow antibodies were purchased from BD) at 4°C for 15 min and then washed with PBS once followed by fixation with 1% formaldehyde. FACSCalibur flow cytometer (BD) and its software were used to analyze the samples and relevant results.

2.17 | Micro-CT analysis

To estimate the bone structure of the femur between different groups, femurs were acquired after sacrifice and placed in 4% paraformaldehyde for 2 days. After that, we used micro-CT to scanned and evaluated relevant bone parameters. In every step of the 360 rotational steps, the effective pixel size of the image was 8.82 lm, the voltage was 80 kV, the current was 500 lA, and the exposure time was 1,500 ms. We used Inveon Research Workplace (Siemens) to calculate bone volume/total volume (BV/TV), trabecular number, trabecular thickness, trabecular spacing, and cortical bone parameters in line with guidelines developed by the American Association for Bone and Mineral Research. The volume of interest (VOI) of the trabecular bone included 100 slices, starting 50 slices up from the growth plate section of the distal femur. And for cortical bone analysis, 100 slices were selected as the VOI. Beyond that, 5 mm thick sections of the femur were taken from different groups, and hematoxylin and eosin (H&E) staining was used to evaluate the bone loss of mice.

2.18 | H&E staining and TRAP staining

The distal femurs were embedded in paraffin and sliced into 5 μm thick sections for H&E staining and TRAP staining. H&E staining was performed to observe pathological changes in mice with osteoporosis, which was performed according to the manufacturer's instructions (AR1180, Boster, China). TRAP staining was performed to detect the number of osteoclasts, which was performed according to the manufacturer's instructions (BP088, Biossc, China). Images of TRAP-positive cells were captured using a microscope. The numbers of TRAP-positive cells in each image were measured by Image-Pro Plus software.

2.19 | Statistical analysis

Experiments were executed three times independently, and one representative experiment is exhibited. The data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, United States). One-way analysis of variance followed by Tukey's post hoc test and unpaired Student's *t*-test were used to analyze the statistical significance among multiple groups and between two groups, respectively. A $p < .05$ was considered statistically significant. The requirements for best practices in natural products pharmacological research have been taken into account (Heinrich et al., 2018; Izzo et al., 2020).

3 | RESULTS

3.1 | APT promoted osteogenic differentiation in BMSCs

We initially assayed the influences of APT (for chemical structure see Figure 1a) on osteogenic differentiation of human BMSCs in vitro. BMSCs were prepared and characterized by their phenotypes and their differentiation potential. Flow cytometry analysis showed that the isolated human BMSCs were negative for CD14, CD45, HLA-DR, and CD34 while being positive for CD73, CD90, and CD105 (Figure S1a). In addition, these cells were able to differentiate into chondroblasts, osteoblasts (as revealed by ARS staining for calcium deposition), and adipocytes, respectively, under appropriate conditions (Figure S1b). Thus, the isolated human BMSCs displayed the right phenotypes and pluripotent differentiation ability thus meeting the International Society for Stem Cell Research standard (Dominici et al., 2006) and used for the following experiments.

We subsequently determined to investigate the potential cytotoxicity of APT on human BMSCs using the CCK-8 assay. As shown in Figure S1c, APT was not cytotoxic to the cells at the tested concentrations (10, 20, and 40 μM) for 3, 7, and 14 days of incubation but instead could slightly enhance the proliferation of the cells at the two higher concentrations (20 and 40 μM). Thus, these concentrations were used for most of the following experiments. Next, we evaluated the effects of APT on the osteogenic differentiation levels of BMSCs by assaying osteogenic markers including ALP activity and ARS staining for calcium deposition. Under osteogenic differentiation conditions, BMSCs were induced to differentiate into osteogenic osteoblasts as revealed by increased ALP activity and ARS staining (indicative of calcium deposition). Notably, APT dose-dependently enhanced the osteogenic differentiation levels in human BMSCs as revealed by increased ALP activity in treated cells compared with untreated ones after 7 days of differentiation (Figure 1b,c). Consistent with this, as compared to untreated control, APT also dose-dependently increased the levels of calcium deposition (as shown by ARS staining) in cells after 14 days of differentiation (Figure 1b,c). Besides, Western blot analysis showed that APT was able to dose-dependently increased the protein levels of Runx2, OCN, and OPN in the differentiated cells (Figure 1d,e), indicating enhanced osteogenic differentiation. In addition, APT could also dose-dependently promote osteogenic differentiation of BMSCs derived from aged mice (Figure S2). Together, these results indicate that APT can obviously promote the osteogenic differentiation of BMSCs.

3.2 | APT activated the PKA signaling pathway in BMSCs during osteogenesis

Having found that APT can promote osteogenic differentiation from BMSCs, we next explored the underlying mechanism by which APT promotes the osteogenic differentiation of BMSCs. Considering that several critical, such as mitogen-activated protein kinases and PKA signaling, have been shown to play crucial roles in osteogenic differentiation of BMSCs (Anthony & Link, 2014; Chen et al., 2016; Hayrapetyan, Jansen, & van den Beucken, 2015), we focused on evaluating those signaling pathways in this study using Western blotting. Interestingly, the results showed that APT markedly increased the expression levels of p-PKA but had minimal effects on the expression levels of p-JNK, p-AKT, p-p38, p-β-catenin, p-ERK, BMP2, or p-Smad (Figure 2a,b), indicating that APT had activated PKA signaling but not the other signaling pathways. In line with these observations, immunofluorescence microscopy revealed that PKA signaling was activated by APT during the osteogenic differentiation of BMSCs (Figure 2c,d). To further support the role of PKA signaling in mediating the action of APT, we blocked PKA signaling either by its inhibitor H89 or by siRNA knockdown of PKA. As expected, H89 significantly decreased the levels of ALP activity and ARS staining as well as Runx2, OCN, and OPN expression in APT-treated cells compared with APT alone (Figure 3a-c), indicating that blockade of PKA signaling antagonized APT-induced enhancement of osteogenic differentiation in BMSCs. Furthermore, genetical knockdown of PKA expression in BMSCs by siRNA (Figure 3d) also significantly attenuated the enhanced osteogenic differentiation in BMSCs by APT, as indicated by reduced ALP activity and ARS staining (Figure 3e). Taken together, these results indicate that APT enhances the osteogenic differentiation of human BMSCs by activating PKA signaling.

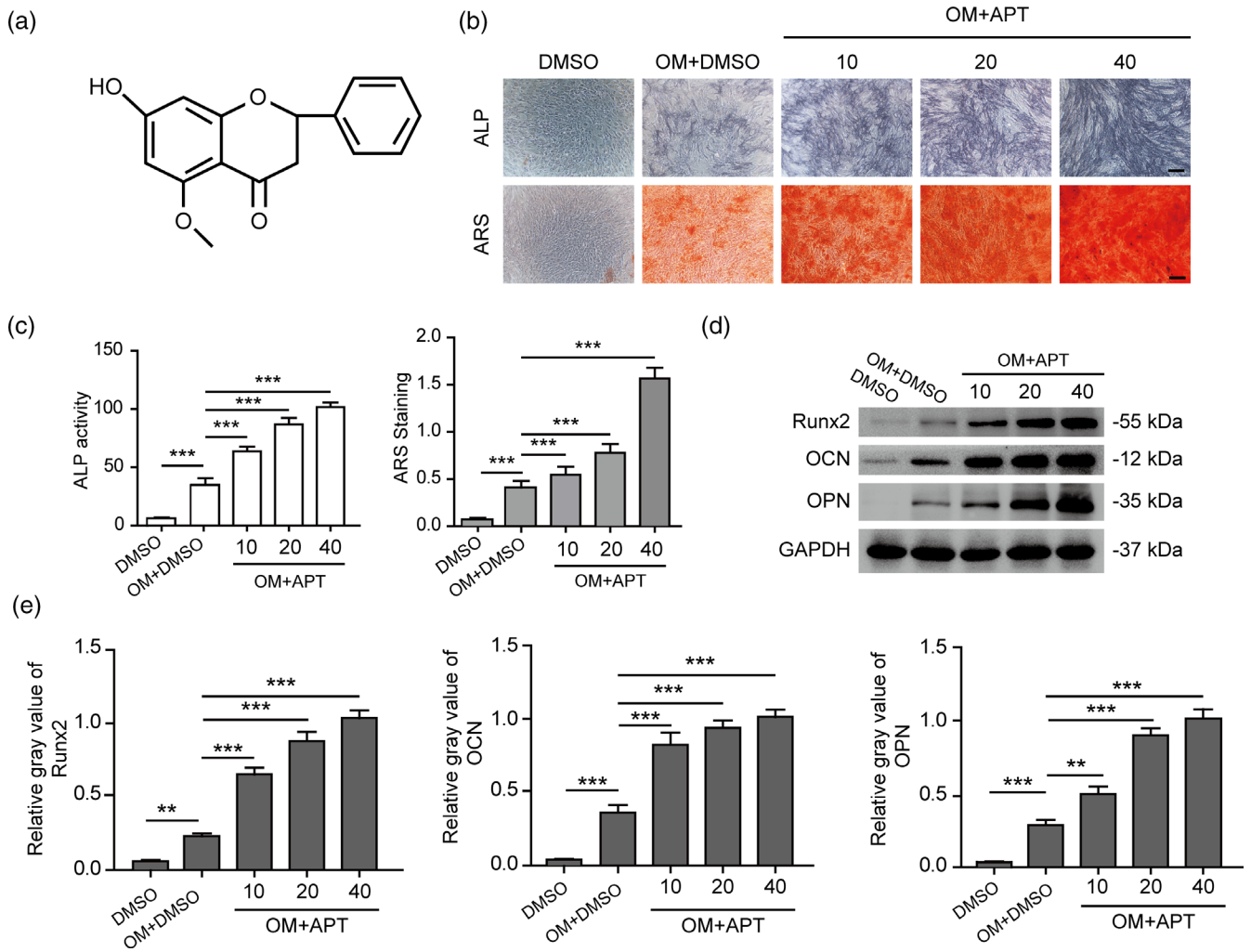

FIGURE 1 Chemical structure of alpinetin (APT) and its effect on the osteogenic differentiation of human Bone marrow mesenchymal stem cells (BMSCs). (a) The two-dimensional structure of APT. (b-c) BMSCs were incubated in an osteogenic medium containing APT (0, 10, 20, and 40 μM) for 7 and 14 days, and then alkaline phosphatase (ALP) activity detection and Alizarin red S (ARS) staining for calcium deposition were performed as described in "Materials and Methods." Scale bar, 100 μm. (d-e) Western blot analysis of BMSCs after osteogenic differentiation for 7 days indicated that APT increased the expression levels of the osteogenic protein markers Runx2, osteocalcin (OCN), and osteopontin (OPN); (d), and the relative gray values are shown in the histograms (e). The data are shown as the mean ± SD ($n = 3$). ***$p < .001$. OM, osteogenic differentiation medium. DMSO was used as the control group. OM + DMSO was adopted as the vehicle group

3.3 | The PKA/mTOR/ULK1 autophagy signaling pathway was involved in APT-induced osteogenic differentiation in BMSCs

In view of the finding that autophagy is essential for osteogenic differentiation of BMSCs (Boya, Codogno, & Rodriguez-Muela, 2018; Liu et al., 2017), we next sought to explore the role of autophagy in APT-mediated enhancement of osteogenic differentiation in BMSCs. As shown in Figure 4a, immunofluorescence microscopy analysis showed that APT increased the expression of LC3B. Besides, APT also markedly promoted autophagosome formation during the osteogenic differentiation of BMSCs (Figure 4b). As autophagosome formation is reliant on the canonical autophagy pathway (Wu et al., 2020), we also examined whether APT affected the expression levels of autophagy-related proteins. Western blotting also showed that APT dose-dependently increased the ratios of LC3B-II to LC3B-I and beclin-1 levels while decreasing p62/SQSTM1 levels (Figure 4c), indicating increased autophagic fluxes activation during the accelerated osteogenic differentiation of BMSCs. Consistent with increased autophagy, mTOR signaling (the upstream signaling that inhibits autophagy) was suppressed while ULK1 signaling (the upstream signaling that initiates autophagy) was activated in response to different doses of APT (Figure 4c).

We further examined the role of autophagy in mediating the enhancing effects of APT on osteogenic differentiation. The autophagy inhibitor 3-MA was used to block autophagy signaling. As expected, 3-MA treatment was able to attenuate the enhanced osteogenic differentiation by APT (Figure 4d). Furthermore, we also explored whether the PKA inhibitor H89 affected the autophagy levels of BMSCs. Western blotting showed that H89 treatment

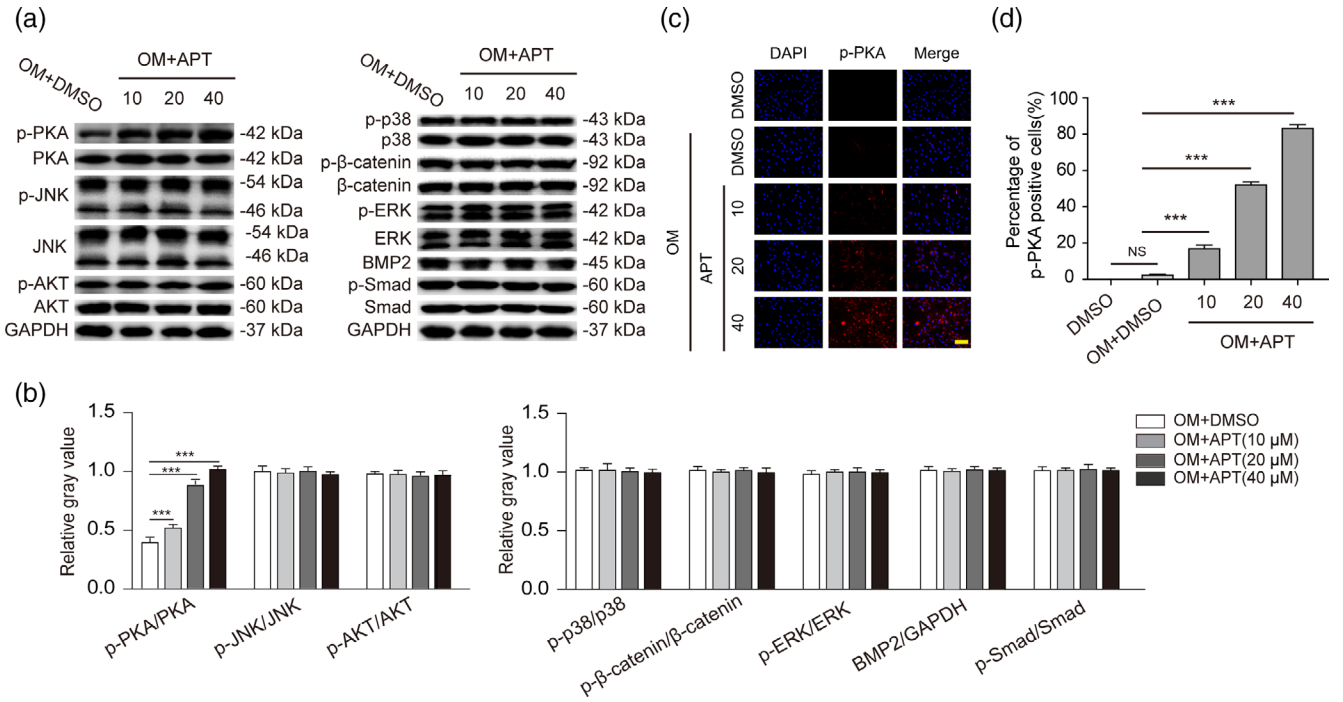

FIGURE 2 Alpinetin (APT) activated the protein kinase A (PKA) signaling pathway in human Bone marrow mesenchymal stem cells (BMSCs) during osteogenic differentiation. (a, b) Western blotting revealed that after 7 days of osteogenic differentiation, PKA signaling affected the osteogenic differentiation of BMSCs in the presence of APT (a), and the relative gray values are shown in the histograms (b). (c, d) Immunofluorescence microscopy showed that active p-PKA levels were increased after APT treatment. Scale bar, 50 μm. The data are shown as the mean ± SD ($n = 3$). ***$p < .001$. NS, not significant; OM, osteogenic differentiation medium

decreased the ratios of LC3B-II to LC3B-I while increasing the levels of p62/SQSTM1 and p-mTOR (Figure 4e,f), indicating that PKA signaling was required for APT to increase autophagic fluxes in BMSCs during osteogenic differentiation. Together, these results demonstrated that APT enhanced osteogenic differentiation in BMSCs by activating autophagy through the PKA/mTOR/ULK1 signaling.

3.4 | APT administration protected mice against GIOP

According to the aforementioned results that APT promoted osteogenic differentiation of BMSCs, we next explored whether APT administration had any preventive effects on osteoporosis in mice. The mouse model of osteoporosis was induced by DXMS and used in our study considering that this model is relevant to BMSCs as glucocorticoids can reduce the differentiation of BMSCs into osteoblasts (Han et al., 2019; Li et al., 2013). Mice were treated with DXMS (DXMS+PBS), oral administration of APT (DXMS+APT, 40 mg/kg body weight), PTH (DXMS+PTH, as the positive-drug control), or vehicle (NS., 2% Tween-80 in PBS), respectively (Figure 5a). We started with a preliminary study of the effective dose based on the literature (Lv et al., 2018; Wang et al., 2016; Zhang et al., 2020, Qiu et al., 2019). We chose the dose of 10, 20, and 40 mg/kg for preliminary experiments, and found that all three doses of APT had no toxic effect on mortality and body weight, and APT at the dose of 40 mg/kg could initially relieve the symptoms of osteoporosis in mice (Figure S3a,b). As shown in Figure 5c, DXMS-induced significant changes in femurs when compared with vehicle controls while APT or PTH treatment was able to alleviate such changes, as determined by micro-CT (Figure 5b) and H&E staining (Figure 5c). Quantitative analysis by micro-CT showed that the BV/TV, trabecular thickness, and trabecular numbers were increased in the APT-treated group (DXMS+APT) or PTH-treated group (DXMS+APT) while trabecular spacing being decreased when compared with those in the model control (DXMS+PBS); (Figure 5d). Furthermore, as shown in Figure 5f, compared with those in the vehicle control (NS.) group, the serum OCN levels in the OP group were markedly decreased, while ALP activity was significantly increased; and compared with those in the OP group (DXMS+PBS), the serum OCN levels in the OP+APT group (DXMS+APT) were increased, and serum ALP activity was decreased significantly. In addition, we also harvested BMSCs from mouse femurs of different groups. Flow cytometry showed that the murine BMSCs had the right phenotypes (Figure S1c). Passage 3 BMSCs were used to osteogenic differentiation induction for 7 or 14 days to evaluate their osteogenic differentiation levels. ALP analysis and the ARS staining results showed lower ALP activity and fewer mineralized nodules in BMSCs from GIOP mice (DXMS+PBS) than in those from control mice (NS. +PBS). However, APT treatment restored the osteogenic differentiation levels of BMSCs compared to those of

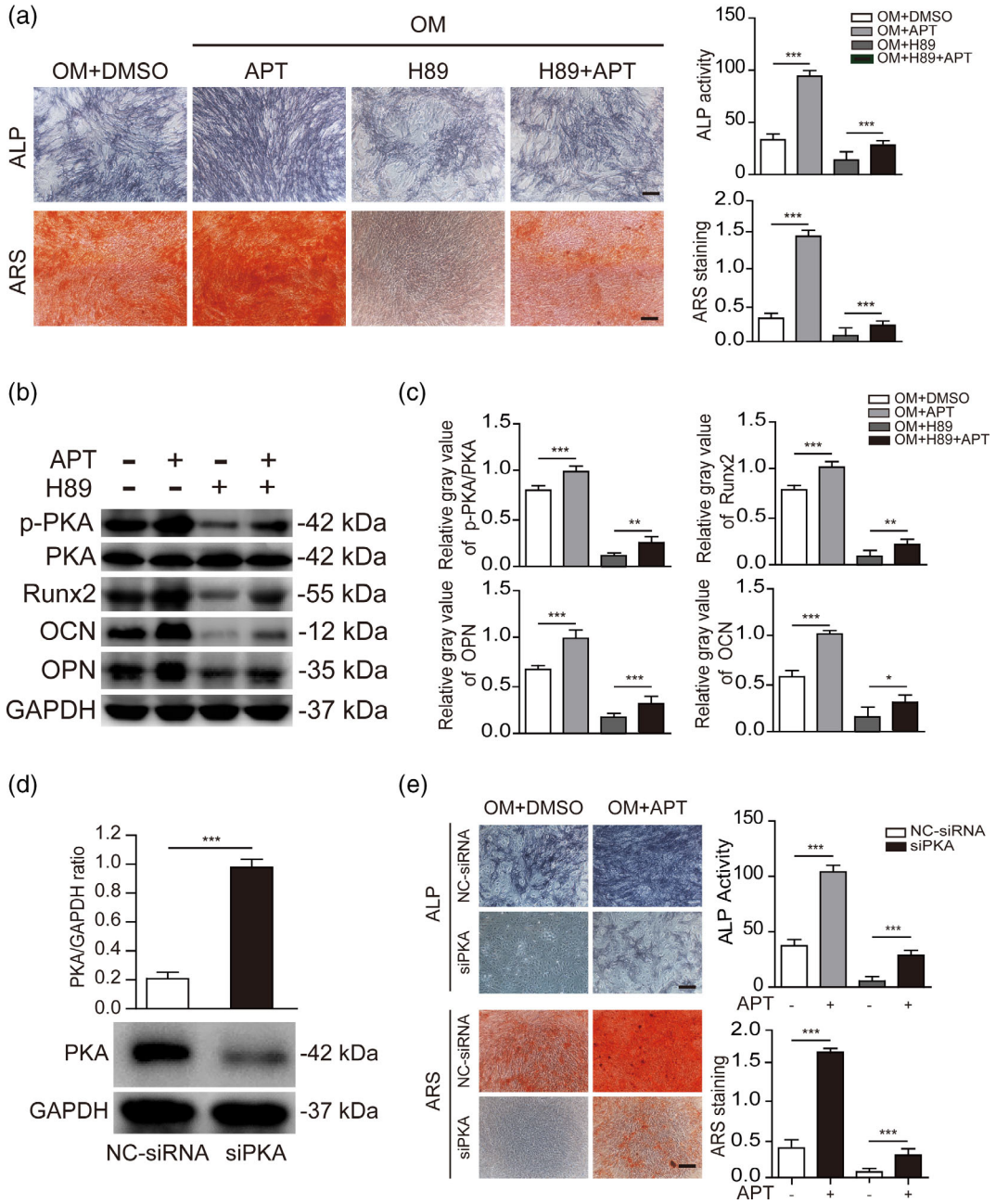

FIGURE 3 Inhibition of protein kinase A (PKA) signaling antagonized the enhancing effect of alpinetin (APT) on osteogenic differentiation in human Bone marrow mesenchymal stem cells (BMSCs). (a) BMSCs were pretreated with the PKA inhibitor H89 (10 μM) for 24 h and then incubated with APT (40 μM) for 7 or 14 days, respectively. Alkaline phosphatase (ALP) measurement and ARS staining were performed as described in "Materials and Methods." Scale bars, 100 μm. (b, c) Western blotting revealed that after 7 days of osteogenic differentiation, H89 inhibited PKA signaling and antagonized the enhancing effect of APT on protein hallmarks of osteogenic differentiation in BMSCs (b), and the relative gray values of those proteins are shown in the histograms (c). (d, e) PKA knockdown inhibited APT-induced augmentation of osteogenic differentiation. BMSCs were transfected with negative control (NC) siRNA or PKA-specific siRNA (siPKA) for 48 h. The cells were then stimulated with APT (40 μM) for 7 and 14 days, respectively. Cell lysates were analyzed for the efficiency of PKA knockdown by Western blotting (d). GAPDH was used as a loading control. Histograms showed the quantification of PKA levels relative to GAPDH. Osteogenic differentiation was evaluated by ALP staining and ARS staining (e). Scale bars, 100 μm. The data are shown as the mean ± SD ($n = 3$). *$p < .05$. **$p < .01$. ***$p < .001$. OM, osteogenic differentiation medium

GIOP mice (Figure 5e,g). It is worth noting that we also analyzed the effect of APT on cortical bone. The result showed that APT abrogated the cortical bone defect effect of DXMS (Figure S3e). Quantitative analysis showed that APT significantly alleviated DXMS-induced decrease in the cortical thickness and the increase in the cortical porosity of cortical bone (Figure S3f).

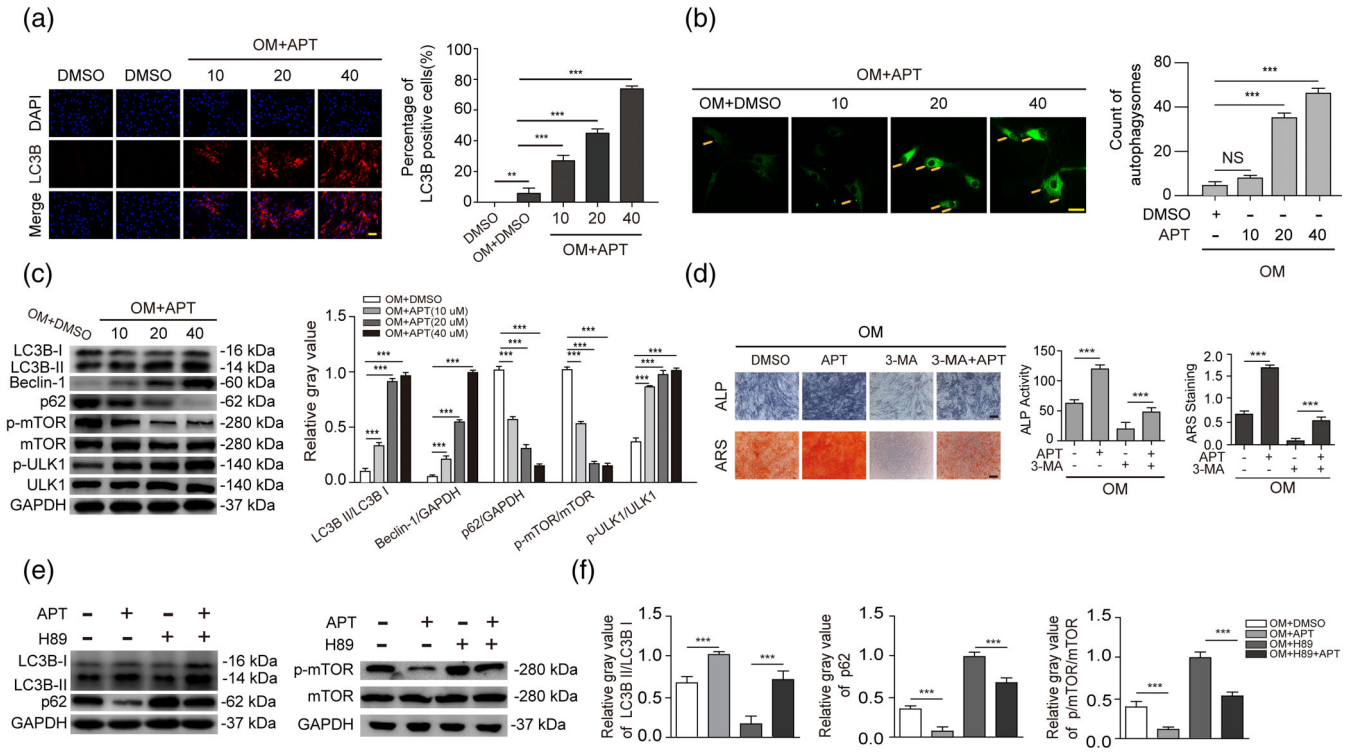

FIGURE 4 Alpinetin (APT) promoted osteogenic differentiation of human Bone marrow mesenchymal stem cells (BMSCs) by inducing autophagy via the protein kinase A (PKA) signaling pathway. (a) Immunofluorescence staining analysis of LC3B in BMSCs after 24 h of APT (0, 10, 20, and 40 μM) treatment. Scale bar, 50 μm. $^{**}p < .01$. $^{***}p < .001$. NS, not significant. (b) Immunofluorescence microscopy showing autophagosome formation in BMSCs treated with or without APT (0, 10, 20, and 40 μM) for 24 h; scale bar, 20 μm. (c) Western blot analysis of autophagy-related proteins in BMSCs after osteogenic differentiation for 7 days, indicating that APT notably increased the expression levels of the autophagy-related proteins LC3B, Beclin-1, and p-ULK1 while downregulating the expression levels of p62/SQSTM1 and p-mTOR. (d) BMSCs were pretreated with the autophagy inhibitor 3-MA (10 mM) for 24 h and then incubated with APT (40 μM) for 7 or 14 days, respectively. Alkaline phosphatase analysis and ARS staining were then performed. Scale bars, 100 μm. (e) Western blotting shows that APT-induced autophagy in BMSCs at 7 days of osteogenic differentiation was antagonized by the PKA inhibitor H89, the relative gray values are shown in the histograms (f). The data are shown as the mean ± SD ($n = 3$). $^{***}p < .001$. NS, not significant. OM, osteogenic differentiation medium

In view of the notions that osteoporosis is closely related to the imbalance of osteoblasts and osteoclasts, and that enhanced autophagy could induce osteoclast differentiation and ultimately lead to osteoporosis (Ng, Brigitte Patricia Ribet, & Pavlos, 2019), we also explored the influence of APT on osteoclast differentiation. The osteoclasts in sections of femurs were detected using a TRAP staining kit. The results showed that there was no significant difference in osteoclasts in mouse femurs of the GIOP and GIOP + APT groups (Figure S3c,d). Taken together, these results indicate that APT administration alleviated the severity of osteoporosis in mice likely by enhancing osteogenic differentiation of BMSCs.

3.5 | APT alleviated osteoporotic severity in GIOP mice by increasing PKA signaling and autophagy induction

Lastly, we sought to explore the in vivo mechanism underlying APT-mediated protection against osteoporosis in mice. Western blot was used to assay the levels of p-PKA, p-ULK1, and p-mTOR in mouse BMSCs derived from the vehicle (NS. +PBS), GIOP (DXMS+PBS), or GIOP+APT (DXMS+APT) groups. The results showed that the expression levels of p-PKA and p-ULK1 were reduced in BMSCs from GIOP mice but increased in those from GIOP+APT-treated mice compared with those from vehicle-treated BMSCs. In contrast, the expression level of p-mTOR was opposite to p-PKA and p-ULK1 (Figure 6a, b). The Western blotting result of p-PKA was further confirmed by immunofluorescence microscopy analysis of p-PKA in bones (Figure 6c,d), indicating increased PKA signaling in bones of APT-treated mice. Besides, immunofluorescence analysis showed LC3B accumulation in GIOP+APT bones as compared with GIOP bones (Figure 6e,f), suggesting increased autophagy in bones of APT-treated mice. To further verify the roles of PKA signaling and autophagy, we also used PKA inhibitor H89 or autophagy inhibitor 3-MA to treat GIOP mice (DXMS+H89 + APT, DXMS+3-MA + APT). Intriguingly, APT-induced alleviation of bone loss in GIOP mice was dramatically abrogated by H89 or by 3-MA, respectively (Figure 7a-e), further confirming that APT alleviated bone loss in GIOP mice mainly through

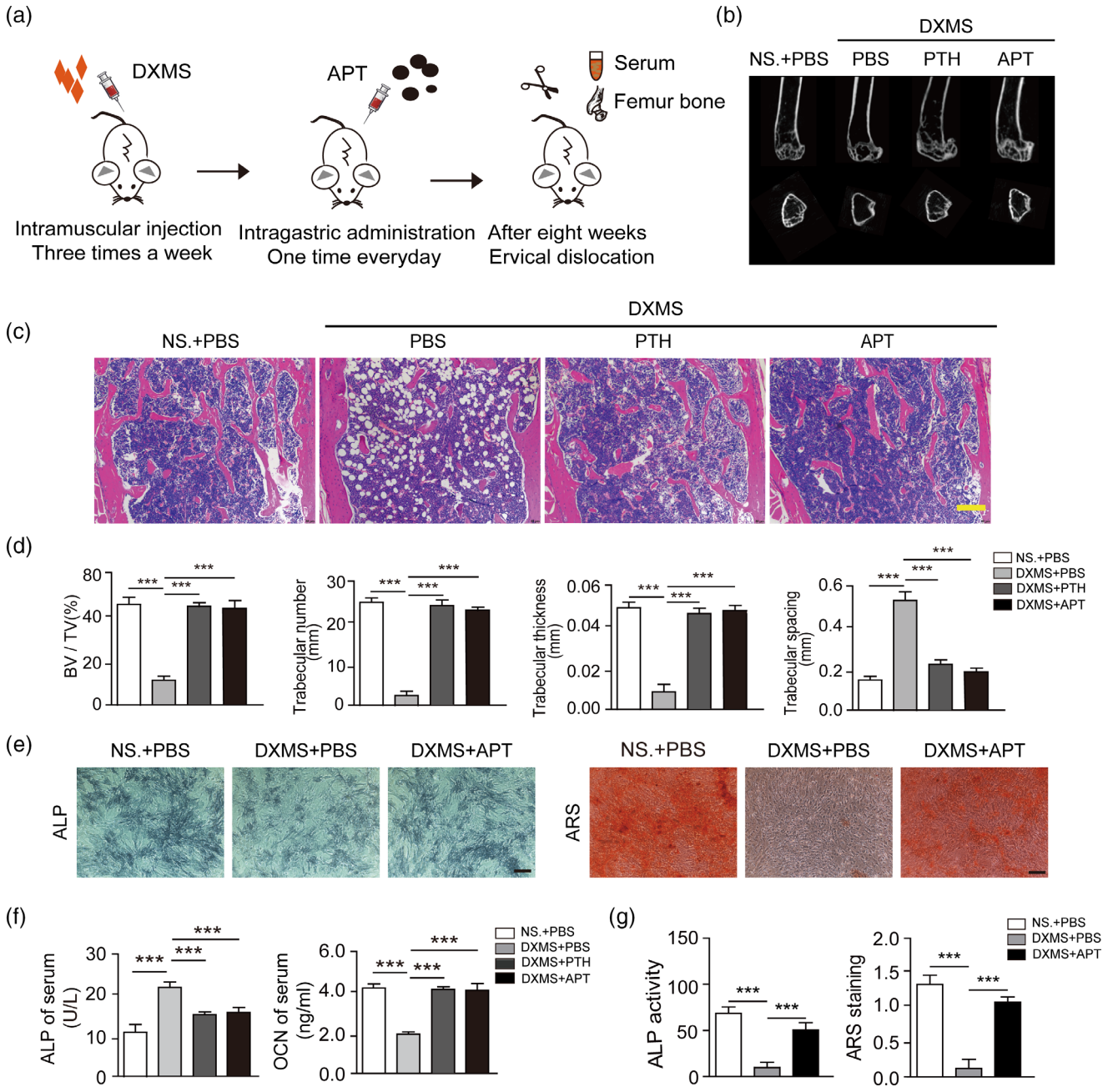

FIGURE 5 Alpinetin (APT) alleviated bone loss in DXMS-induced osteoporosis in mice. (a) Flowchart of GIOP animal experiments. The total period for the animal model is 60 days. APT was orally administered once a day. (b) Micro-CT analysis of femurs from mice treated with NS. +PBS mice ($n = 6$), DXMS+PBS mice ($n = 6$), DXMS+PTH mice ($n = 6$), or DXMS+APT-treated mice ($n = 6$), respectively. (c) H&E staining of the cancellous bone of terminal femurs of mice treated with NS. + PBS ($n = 6$), DXMS+PBS ($n = 6$), DXMS+PTH ($n = 6$) or DXMS+APT ($n = 6$), respectively. Scale bar, 500 μm. (d) BV/TV, trabecular thickness, trabecular number, and trabecular spacing analysis of femurs from mice treated with NS. + PBS ($n = 6$), DXMS+PBS ($n = 6$), DXMS+PTH ($n = 6$) or DXMS+APT ($n = 6$), respectively. (e, g) alkaline phosphatase (ALP) analysis and ARS staining were performed to detect the osteogenic differentiation of Bone marrow mesenchymal stem cells from mice treated with NS. + PBS ($n = 6$), DXMS+PBS ($n = 6$) or DXMS+APT ($n = 6$), respectively. Scale bar, 100 μ m. The data are shown as the mean ± SD ($n = 3$). (f) Effects of APT on ALP and OCN levels in serum of mice. $^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$. NS., normal saline. PBS, phosphate-buffered saline. DXMS, dexamethasone. PTH, parathyroid hormone, as the positive control

enhancing PKA signaling and autophagy induction. Altogether, these data indicate that APT was able to alleviate osteoporotic severity of GIOP in mice by regulating PKA/mTOR/ULK1 autophagy signaling (Figure 8).

4 | DISCUSSION

Osteoporosis is increasingly prevalent worldwide, but the treatment of osteoporosis is still limited (Berry, Shi, & Kiel, 2019). Currently,

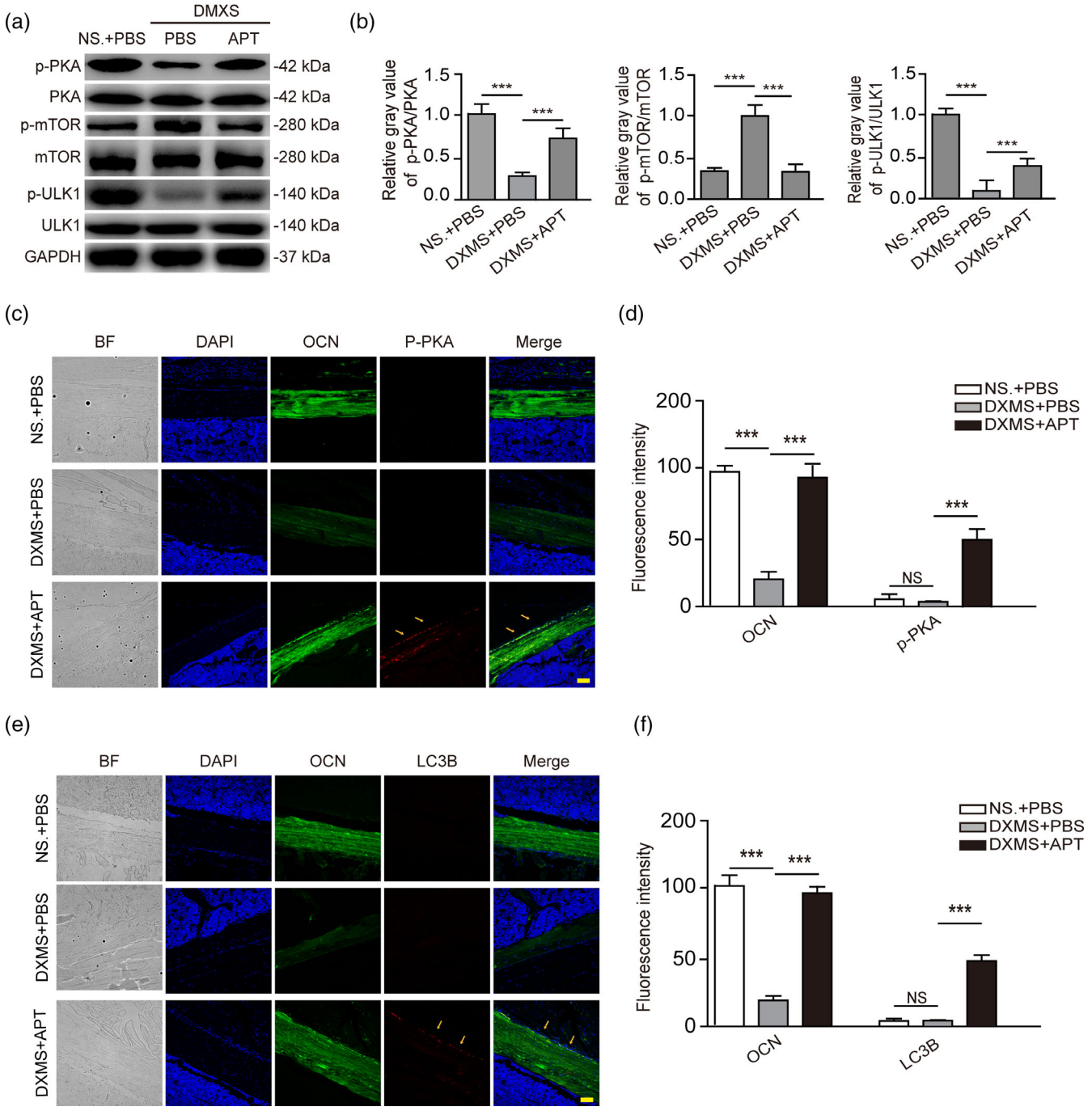

FIGURE 6 Protein kinase A (PKA) and autophagy signaling pathways were involved in Alpinetin (APT)-mediated alleviation of DXMS-induced osteoporosis. (a-b) Western blot analysis was used to measure the protein expression levels of p-PKA, p-mTOR, p-ULK1, and GAPDH in Bone marrow mesenchymal stem cells from mice treated with NS. + PBS, DXMS+PBS, or DXMS+APT, respectively. (c-f) Immunofluorescence staining of the cortical bone of terminal femurs of mice demonstrated that the expression of p-PKA and LC3B was enhanced in DXMS+APT bones compared with NS. + PBS and DXMS+PBS ones (yellow arrows). Osteocalcin (OCN) was also stained to reveal the bones. Scale bar: 50 μm. ***$p < .001$. NS, not significant. NS.: normal saline. PBS: phosphate buffered saline. DXMS: dexamethasone. BF: bright field

there are mainly three kinds of drugs to treat osteoporosis: inhibiting bone resorption with drugs, such as estrogen, calcitonin, and bisphosphonates; promoting bone formation with biological agents such as PTH and insulin-like growth factor 1 (IGF-1); and promoting bone mineralization calcium (Tanaka, 2017). However, there are limitations to these therapeutic drugs, which include lacking bone structural restoration as well as various side effects. An increasing number of studies have thus focused on the exploration of novel drug therapies that can block bone absorption, stimulate new bone formation, and restore bone density and strength (Cotts & Cifu, 2018; Kim et al., 2007;

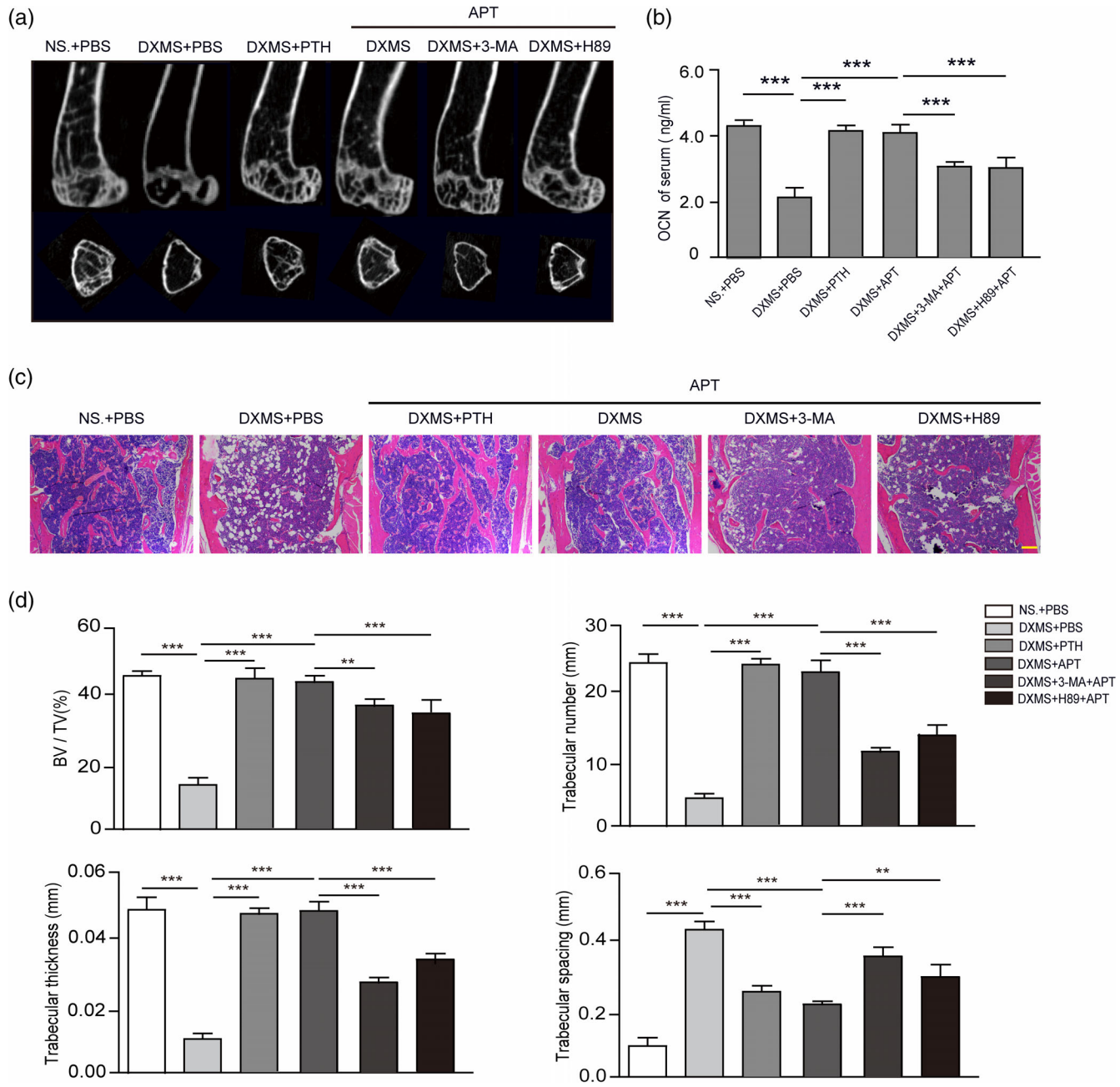

FIGURE 7 Alpinetin (APT)mediated alleviation of bone loss in osteoporosis mice alleviated the bone loss partially counteracted by protein kinase A (PKA) signal inhibitor H89 and autophagy signaling inhibitor 3-MA treated OP mice. Mice were respectively treated with NS. + PBS, DXMS+PBS, DXMS+PTH, DXMS+APT, DXMS+APT+H89, and DXMS+APT+3-MA, as described in "Materials and Methods." (a) Micro-CT analysis of the femurs of mice NS. +PBS mice, DXMS+PBS mice, DXMS+PTH mice, DXMS+APT mice, DXMS+APT+H89 mice, and DXMS+APT+3-MA treated mice. (b) Effects of APT on serum osteocalcin (OCN) levels in the serum of mice. (c) H&E staining of the terminal femurs of mice NS. +PBS mice, DXMS+PBS mice, DXMS+PTH mice, or DXMS+APT-treated mice. Scale bar, 500 μm. (d) BV/TV, trabecular thickness, trabecular number, and trabecular spacing analysis of femurs of mice for NS. +PBS mice, DXMS+PBS mice, DXMS+PTH mice, or DXMS+APT-treated mice. $^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$. NS: not significant. NS.: normal saline. PBS: phosphate buffer saline. DXMS: dexamethasone. PTH: parathyroid hormone, as the positive control. H89: PKA-specific inhibitor. 3-MA: autophagy-specific inhibitor

Reid, 2020). Recently, it has been shown that osteogenic differentiation is a promising target for the treatment of osteoporosis (Berry et al., 2019; Jiang, Zhang, Zhang, Lv, & Zhou, 2021). In this study, we found that the phytochemical APT was able to enhance osteogenic differentiation of human BMSCs in vitro and mouse BMSCs in vivo through activating the PKA signaling and thus induction of autophagy. Consistently, in our osteoporosis animal model, we used PTH as a positive control, and the results showed that APT significantly alleviated the symptoms of osteoporosis in GIOP mice, with no difference in the degree of relief compared with PTH. As a commonly used drug

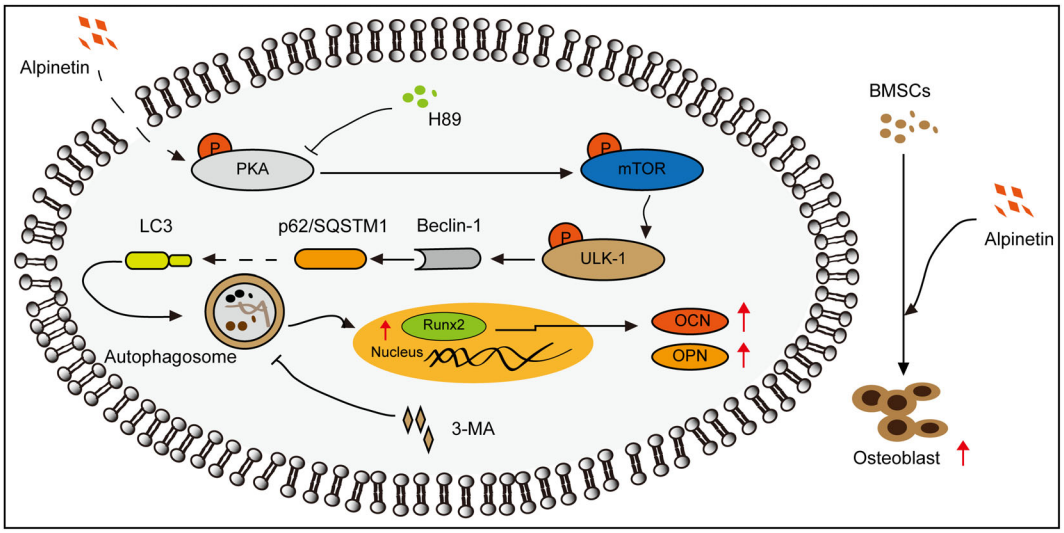

FIGURE 8 Schematic depicting the action mechanism for alpinetin (APT) to promote osteogenic differentiation in Bone marrow mesenchymal stem cells (BMSCs) via inducing autophagy. APT stimulates the formation of autophagosomes through activating the protein kinase A (PKA)/mTOR/ULK1 signaling pathway and thus accelerating the expression of osteogenic differentiation markers including Runx2, osteocalcin (OCN), and osteopontin (OPN). Moreover, both PKA inhibitor H89 and autophagy inhibitor 3-MA block the enhancing effects of APT. Collectively, our study reveals that APT promotes osteogenic differentiation in BMSCs and thereby ameliorates osteoporosis

for the treatment of osteoporosis, PTH mainly promotes bone formation and increases bone mass by increasing the number of osteoblasts and promoting the release of bone growth factors (Ding et al., 2018; Zweifler, Koh, Daignault-Newton, & McCauley, 2021). However, in recent years, many studies have found that excessive intake of PTH can enhance the activity of osteoclasts, promote bone absorption, and eventually lead to the side effects of bone calcium release into the blood (Esbrit, Herrera, Portal-Núñez, Nogués, & Díez-Pérez, 2016; Reid, Bristow, & Bolland, 2015; Wein & Kronenberg, 2018). As a traditional Chinese medicine component, APT has been widely used in the treatment of a variety of diseases without toxic side effects (Gao, Wang, He, Wang, & Yang, 2020; He et al., 2016; Zhang et al., 2020). In our study, APT significantly alleviated the osteoporotic severity in the mouse model of osteoporosis, which showed no toxic side effects on mortality and body weight at our text dose and did not significantly affect the activity of osteoclasts, highlighting its potential application as an alternative for treating osteoporosis through modulating osteogenic differentiation.

BMSCs are the most important source of osteoblasts and adipocytes in vivo and they can antagonize with each other the differentiation of osteoblasts and adipocytes (Li et al., 2018). The differentiation of BMSCs is regulated by a delicate array of molecules and signals (Chen et al., 2016; Pierce, Begun, Westendorf, & McGee-Lawrence, 2019), which involves many types of intercellular and intracellular signal transduction, such as signaling pathways, transcription factors, growth factors, and microRNAs, ultimately forming a complete negative feedback loop of bone metabolism regulation (Hankenson, Gagne, & Shaughnessy, 2015). Many studies have confirmed that the adipogenic and osteogenic differentiation of BMSCs are in a dynamic balance, and the dysregulation of this balance is closely related to obesity, osteoporosis, and other diseases. Consistent with this notion, an increasing number of small molecule drugs have been found to promote osteogenic differentiation of MSCs in vitro and in vivo, prevent osteoporosis in preclinical animal models, and augment the healing of maxillofacial bone defects and bone regeneration around implants to a certain extent (Zhang, Li, Laurent, & Ding, 2012). In line with these studies, our data indicated that the small-molecule flavonoid APT was able to promote osteogenic differentiation of BMSCs in vitro, which was revealed by increased expression of osteoblast markers such as ALP, Runx2, OCN, and OPN. Compared with bioactive growth factors and gene-related methods, natural small molecules, such as APT, provide an efficient, accurate, reversible, and cost-effective approach to osteogenic differentiation of BMSCs (Wang et al., 2015).

Mechanistically, we demonstrated the PKA/mTOR/ULK1 signaling was involved in the action of APT enhancing osteogenic differentiation of BMSCs. Accumulating evidence indicates that autophagy may be crucial for maintaining BMSCs, and autophagy induction can lead to the survival response of BMSCs to oxidative stress (Song, Song, & Tong, 2014). In addition, autophagy is involved in the maintenance of stem cell characteristics in umbilical cord mesenchymal stem cells (Hou et al., 2013). Autophagy contributes to the maintenance of bone mass, in part by sustaining the viability of osteoblasts (Yao et al., 2016). Consistently, another study has also revealed that AMP-activated protein kinase regulates osteogenic differentiation in human BMSCs through autophagy via inhibiting mTOR signaling at an early stage (Pantovic et al., 2013). Besides, it is known that the PKA/mTOR signaling plays a crucial role in regulating autophagic fluxes (Wu et al., 2018). One recent study also showed that the loss of autophagy in human-aged BMSCs is associated with impaired osteogenic

differentiation in the context of osteoporosis (Wan, Zhuo, Li, Zhao, & Jiang, 2017). In particular, it has been found that flavonoid-mediated autophagy induction leads to osteoblast differentiation and bone tissue formation (Kim et al., 2018). These studies thus identified a critical signaling pathway for autophagy activation in BMSCs, which may accelerate their osteogenic differentiation. In line with these studies, our data indicated that the natural flavonoid APT also exerted its osteogenic effects on BMSCs by regulating autophagy. Furthermore, PKA signaling played a critical role in mediating the effects of APT on the mTOR/ULK1 signaling upstream of autophagic induction in BMSCs. Thus, APT can enhance osteogenic differentiation in BMSCs by modulating the PKA/mTOR/ULK1 autophagy pathways.

Although previous studies and our data reveal a critical role of autophagy induction in promoting osteogenic differentiation, there are some studies showing that autophagy could negatively regulate osteogenic differentiation. One study showed that reducing the mTORC1 function in osteoblasts increased the levels of cellular autophagy, leading to a decrease in both matrix synthesis and mineralization capacity in osteoblasts. And another recent report revealed that leptin and 25(OH)D_3 could collectively stimulate osteogenic differentiation of rat mesenchymal stem cells by inhibiting autophagy (He et al., 2021). One explanation for this discrepancy may be due to different experimental conditions or different cellular models among those studies. Therefore, during cell differentiation, autophagy pathways are finely regulated to meet the metabolic requirements related to morphological and functional changes of BMSCs (Boya et al., 2018; Ma et al., 2018; T. Wang et al., 2019). Further investigations are thus needed to clarify the relationship between autophagy and BMSCs osteogenic differentiation.

As our in vitro data showed that APT enhanced osteogenic differentiation, it is of interest to study the effect of APT on an animal model of osteoporosis in vivo. The osteoporosis model should be of relevance to the osteogenic differentiation of BMSCs. DXMS is a typical synthetic glucocorticoid and can induce osteoporosis in mice. Notably, it has been shown that glucocorticoids reduce the differentiation of BMSCs into osteoblasts while inducing the differentiation of BMSCs into adipocytes (Li et al., 2013). In addition, glucocorticoids can reduce the maturity, lifespan, and function of osteoblasts, and ultimately lead to bone loss (Lane, 2019). A recent study has also confirmed that glucocorticoids play a key role in controlling osteogenic and adipogenic differentiation of BMSCs (Han et al., 2019). We therefore adopted the GIOP mouse model in this study considering that this model is relevant to osteogenic differentiation of BMSCs. To quantify the osteogenic marker expression following the stimulation of APT, measurements were performed at different stages of osteogenic differentiation. ALP and Runx2 are the primary regulators of osteogenic differentiation and modulate the expression of other bone markers aforementioned, which were detected during the early stages of osteogenic differentiation (Tao et al., 2016). And the expression of OCN and OPN are the prominent markers of osteoblast maturation, which were examined during the second week of osteogenic differentiation (Ching, Luddin, Rahman, & Ponnuraj, 2017; Tao et al., 2016). Thus, the promotion of osteogenic differentiation in BMSCs represents a potential therapeutic avenue to prevent osteoporosis. OCN and ALP of serum, bone volume/total volume (BV/TV), trabecular number, trabecular thickness, and trabecular spacing are important indexes to detect osteoporosis (Li, Wang, et al., 2019; Li et al., 2020). Osteoporosis will decrease the BV/TV, trabecular number, trabecular thickness, and increase trabecular spacing. In addition, analysis of cortical bone is an important indicator of pure anabolic effect in the process of osteoporosis (Song et al., 2020; Tripathi et al., 2022). Therefore, we tested these indicators in the GIOP mouse model.

Our results showed APT significantly reduced bone loss in GIOP mice concomitant with improved osteogenic differentiation of BMSCs. Such a preventive effect of APT against osteoporosis was mediated by augmenting PKA/autophagy signaling because it was suppressed by co-treatment with a PKA inhibitor or autophagy inhibitor (Figure 8). These results suggest that APT can confer protection against osteoporosis by modulating osteogenic differentiation of BMSCs in vivo. Yet how APT activates the PKA signaling remains unclear. One possible way is that APT acts on a receptor on the surface of the cell membrane to combine G protein, and then activates adenylate cyclase (cAMP), which ultimately activates the PKA signal (Qiu, Zhou, & Xiao, 2012). Further research is still needed to address this issue and to verify the effects of APT in other preclinical animal models of osteoporosis and in clinical studies.

It is important to point out that the activity of ALP was upregulated in the serum of GIOP mice and was decreased after APT treatment, which is consistent with some previous studies (Hozayen, El-Desouky, Soliman, Ahmed, & Khaliefa, 2016; Jing et al., 2019; Xu, Guan, Xu, Chen, & Sun, 2019). In glucocorticoid-induced osteoporosis, the enhanced bone turnover resulted in increased bone formation and bone resorption (Hozayen et al., 2016; Szulc, 2018). Therefore, the increase in serum ALP activity in GIOP mice may be related to the increase in GIOP bone transition status, which further indicates that DXMS ulteriorly inhibited the differentiation and mineralization of osteoblasts at a relatively higher level (Yoon et al., 2012). However, APT could reverse the abnormal elevation of ALP in the serum of GIOP mice. One possible explanation is that APT differentially regulated the release of ALP into serum and the local expression of ALP in bones under DXMS treatment, ultimately restoring bone formation in vivo. Further research is needed to clarify this issue.

In our in vitro experiments, we used human BMSCs to show the effects of APT on their osteogenic differentiation, thus highlighting the translational relevance of this study. Yet we did not systemically compare the responses to APT treatment of human BMSCs with mouse BMSCs. However, the differential capability of osteogenic differentiation of mouse BMSCs derived from APT-treated osteoporosis mice was observed in comparison with those from GIOP model mice, suggesting that APT had exerted similar effects on the mouse BMSCs in vivo.

There are still limitations in this study. One is that only preventive but not therapeutic administration of APT had been performed. The second limitation is that we used young, but not old human BMSCs to show the effects of APT on their osteogenic differentiation, which would weaken our conclusions to some extent. To further confirm our

conclusions, we extracted BMSCs from aged mice to confirm the osteogenic-promoting effect of APT. And the results showed that APT could also promote the osteogenic differentiation of BMSCs in aged mice (Figure S2). In the past, it was thought that osteoporosis only occurs in the elderly (Alonso-Bouzon & Duque, 2011; Sipos, Pietschmann, Rauner, Kerschan-Schindl, & Patsch, 2009), but recent studies have found that osteoporosis is not limited to the elderly but also has a high incidence in the young population (Briot & Roux, 2015; Cheng et al., 2021; Mäkitie et al., 2016). In future studies, more in-depth studies on the effects of APT on BMSCs derived from bone marrow in different age groups are warranted.

The third limitation of our study is that there are no further toxicological analyses of APT in mice and the effective dose is a single dose. Yet it seems that APT is non-toxic at the dose used in our study. In our animal experiments, APT was administered via oral route at a dose of 40 mg/kg body weight once per day, which is based on several previous studies and our preliminary experiment. A range of doses from 30 mg/kg body weight to 100 mg/kg body weight has been used in those studies, which showed no obvious toxicity to the experimental animals (Lv et al., 2018; Wang et al., 2016; Zhang et al., 2020). Another toxicity and metabolic study indicated that oral administration of APT at 40 mg/kg body weight daily did not result in death or significant changes (Qiu et al., 2019). Based on the above-mentioned studies, we chose the dose of 10, 20, and 40 mg/kg for preliminary experiments, and found that all three doses of APT had no toxic effect on mortality and body weight, and APT at the dose of 40 mg/kg could initially relieve the symptoms of osteoporosis in mice (Figure S3a,b). After determining the effective dose, we carried out a formal animal experiment. Our results revealed that the dose of 40 mg/kg of APT showed no significant toxic effects while indicating a relieving effect on osteoporosis in mice, providing an experimental basis for future clinical applications of APT.

In conclusion, our data indicate that the natural flavonoid APT can augment osteogenic differentiation of BMSCs and alleviate osteoporotic severity in mice likely by enhancing the PKA/mTOR/ULK1 autophagy pathway, thus highlighting APT as a potential therapeutic agent for the treatment of osteoporosis.

AUTHOR CONTRIBUTIONS

Chenying Zeng, Shan Wang, and Fenglei Chen: contributed to design experiments and perform in vitro studies. **Ziming Wang, Jinteng Li, and Zhongyu Xie:** conducted animal studies. **Mengjun Ma** and **Peng Wang:** analyzed the data; **Yanfeng Wu** and **Huiyong Shen:** wrote the paper.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions of this study are present in the paper. The raw data supporting the findings of this research are available from the corresponding author upon request.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Eighth Affiliated Hospital of Sun Yat-sen University (ZB-KYIRB-AF/SC-08/01.0, China) for the human BMSCs isolation and Sun Yat-sen University (SYSU-IACUC-2022-000450, China) for the mice experiment, and conformed to the Ethical Guidelines of the Declaration of Helsinki. And all the animal experiments were carried out in accordance with the Guidelines of the Animal Care and Use Committee.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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