

Atorvastatin Promotes Bone Formation in Aged apoE^{-/-} Mice through the Sirt1–Runx2 Axis

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Abstract

Background

Statins are the most widely used drugs in elderly patients, the most common clinical application of statins is in aged hyperlipemia patients. There are few studies on the effects and mechanisms of statins on bone in elderly mice with hyperlipemia. The study is to examine the effects of atorvastatin on bone phenotypes and metabolism in aged apolipoprotein E-deficient (apoE^{-/-}) mice, and the possible mechanisms involved in these changes.

Methods

Twenty-four 60-week-old apoE^{-/-} mice were randomly allocated to two groups. Twelve mice were orally gavaged with atorvastatin (10 mg/kg body weight/day) for 12 weeks; the others served as the control group. Bone mass and skeletal microarchitecture were determined using micro-CT. Bone metabolism was assessed by serum analyses, qRT-PCR, and Western blot. Bone marrow-derived mesenchymal stem cells (BMSCs) from apoE^{-/-} mice were differentiated into osteoblasts and treated with atorvastatin and Sirt1 inhibitor EX-527.

Results

The results showed that long-term administration of atorvastatin increases bone mass and improves bone microarchitecture in trabecular bone but not in cortical bone. Furthermore, the serum bone formation marker osteocalcin (OCN) was ameliorated by atorvastatin, whereas the bone resorption marker tartrate-resistant acid phosphatase 5b (Trap5b) did not appear to be impacted by atorvastatin treatment. The mRNA expression of Sirt1, runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and OCN in bone tissue were increased after atorvastatin administration. Western blot showed same trend in Sirt1 and Runx2. The in vitro study showed that when BMSCs from apoE^{-/-} mice were pretreated with EX527, the higher expression of Runx2, ALP and OCN activated by atorvastatin decreased significantly or showed no difference compared with the control. The protein expression of Runx2 showed same trend.

Conclusions

Accordingly, the current study validates the hypothesis that atorvastatin can increase bone mass and promote osteogenesis in aged apoE^{-/-} mice by regulating the Sirt1–Runx2 axis.

Background

Osteoporosis (OP) and atherosclerosis (AS) are public health problems that accompany the aging process, and have numerous epidemiological links with and economic consequences for the elderly population. Clinical studies have demonstrated that cardiovascular diseases are associated with reduced bone mineral density (BMD) and increased bone fractures [1–4]. These two age-related diseases may be sustained by similar risk factors, for example, oxidative stress, inflammation, free radicals, lipid metabolism, and estrogen deficiency, as well as common pathological mechanisms and molecular mediators, such as the Wnt pathway, receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), bone morphogenic protein (BMP), and Sirt1 [5–8].

Additional evidence suggests that a link between AS and OP may be related to their common response to drugs. For example, bisphosphonates, raloxifene, and statins may be effective in treating both AS and OP, which also suggests a common pathophysiological basis [7, 9, 10]. Among these drugs, statins are the most widely used drugs in elderly patients with atherosclerosis. Besides its anti-atherosclerotic, anti-inflammatory, and antioxidative stress effects, *in vitro* studies have shown that statins can stimulate osteoblast differentiation [11–13] as well as inhibit osteoclast function [14, 15] through various signaling pathways. However, the effects of statins on osteoporosis and fracture prevention in clinical and animal experiments remain controversial [9, 16–18]. Moreover, most previous studies focused on the effects of statins on ovariectomized (OVX) or fracture models [19–21]. However, the most common clinical application of statins is in aged hyperlipemia patients [22, 23]. There are few studies on the effects and mechanisms of statins on bone in elderly mice with hyperlipemia.

Sirt1, a highly conserved NAD⁺-dependent histone deacetylase, is a vital protein and target for pathways involved in regulation of lifespan and aging-related diseases [24]. Sirt1 has been shown to be atheroprotective in apoE^{-/-} mice [25, 26]. Recent studies have shown that Sirt1 also plays an important role in the pathogenesis of osteoporosis, as well as in mesenchymal stem cell differentiation [27, 28]. Our previous study found that Sirt1 is involved in decreased bone formation in aged apolipoprotein E-deficient mice. Long-term treatment with oxidized low-density lipoprotein (ox-LDL) decreased the expression of Sirt1 in BMSCs [29]. On the other hand, resveratrol (an agonist of Sirt1) was recently found to mediate the modulation of Sirt1-Runx2, promoting the osteogenic differentiation of mesenchymal stem cells, due to its effect on Runx2 deacetylation [30]. Statins have demonstrated antioxidative effects on atherosclerosis through the inhibition of Sirt1 expression [31, 32].

Thus, we first investigated the effects of oral atorvastatin on bone mass and bone formation in aged apoE^{-/-} mice. We then further hypothesized that statins may regulate osteoblast differentiation by targeting the Sirt1–Runx2 axis in an aged hyperlipemia mouse model. To test this hypothesis, the effects of atorvastatin on Sirt1 expression in bone and the role of Sirt1-Runx2 in the expression of BMSCs from apoE^{-/-} mice were also evaluated.

Materials And Methods

Animal Experiments

Sixty-week-old apoE^{-/-} mice with a C57Bl/6J background were purchased from Nanjing Biomedical Research Institute (Nanjing University, Nanjing, China) and fed with regular rodent chow (containing 20.5% protein, 4.68% fat, 1.23% calcium, and 0.91% phosphorus). Twenty-four mice were randomly allocated to two groups. Twelve mice (apoE^{-/-} + AT) were orally gavaged with 10 mg kg⁻¹ day⁻¹ atorvastatin (Pfizer, New York, USA) dissolved in DMSO (dimethyl sulfoxide) for 12 weeks. The other mice served as the apoE^{-/-} group and received an equivalent amount of DMSO as placebo. The mice were maintained in groups under a strict 12 h light/dark cycle in a temperature-controlled environment (25 °C) with free access to food and water. All animal experiments were conducted with the approval of the Committee on Animal Resources at Huadong Hospital, Fudan University.

Microcomputed Tomography (Micro-CT) Analysis

The femurs collected from apoE^{-/-} mice at 72 weeks of age were harvested, fixed, and scanned by the SkyScan-1176 micro-CT instrument (Bruker, Kontich, Belgium). For cortical bone, measurements were performed on an 0.5 mm region of the mid-diaphysis of the femur. For trabecular bone, micro-CT evaluation was performed on a 1 mm region of the metaphyseal spongiosa on the distal femur. The regions were located 0.5 mm above the growth plate. NR Econ software, version 1.6 was used for the three-dimensional (3D) reconstruction and viewing of images [29].

Biochemical and Bone Markers Assays of Serum

Serums from two groups' mice were analyzed for OCN by enzyme-linked immunosorbent assays (ELISAs) using a Mouse Osteocalcin EIA Kit (Immutopics International, San Clemente, CA, USA) and for TRAP5b using MouseTRAP™ [TRAcP5b] ELISA (Immunodiagnostic Systems Limited, London, England) Serum total cholesterol (TC) was measured by a Hitachi 917 autoanalyzer (Roche, Meylan, France), and ox-LDL was measured by a mouse ox-LDL ELISA kit (Uscn Life Science & Technology, Wuhan, China) [29].

Cell Culture

BMSCs were obtained from the femurs and tibias of apoE^{-/-} mice (male, 18 weeks old). The second generation of cells was seeded at 1 × 10⁵ cells/well in a six-well culture plate and cultured in L-DMEM containing 10% fetal bovine serum.

(1) BMSCs were cultured with atorvastatin at different concentrations (0, 10, 100, 1000 nmol/L) at 6 h for qRT-PCR and at 3 days for Western blot. (2) BMSCs were cultured with EX-527 (Sigma, USA) at a concentration of 10 μM for 2 h; then, the medium was changed to atorvastatin (100 nmol/L) at 6 h for qRT-PCR and at 3 days for Western blot.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissues and cultured BMSCs. Real time PCR was conducted as previously described [29]. The primers were obtained from SBSgene (www.sbsgene.com) with the following primer sequences: Sirt1, 5'-GCA GGT TGC AGG AAT CCA A-3' and 5'-GGC AAG ATG CTG TTG CAA A-3' (63 bp,

XM_006514342.1); Runt-related transcription factor 2 (Runx2), 5'-TGT TCT CTG ATC GCC TCA GTG-3' and 5'-CCT GGG ATC TGT AAT CTG ACT CT-3' (146 bp, XM_006523545.1); ALP, 5'-CAC GCG ATG CAA CAC CAC TCA GG-3' and 5'-GCA TGT CCC CGG GCT CAA AGA-3' (479 bp, XM_006538500.1); osteocalcin (OCN), 5'-ACC CTG GCT GCG CTC TGT CTC T-3' and 5'-GAT GCG TTT GTA GGC GGT CTT CA-3' (240 bp, NM_007541.3); and glyceroldehyde - 3-phosphate dehydrogenase (GAPDH), 5'-AGC CTC GTC CCG TAG ACA-3' and 5'-CTC GCT CCT GGA AGA TGG-3' (255 bp, NM_008084.3).

Western Blot Analysis

All samples were collected, and the Western blot was conducted as previously described [29]. The primary antibodies used were rabbit anti-Sirt1 (1:2000; Proteintech, Rosemont, USA), goat anti-Runx2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-GAPDH (1:10,000; Kangcheng, Shanghai, China). The protein bands were quantitatively analyzed using an image analysis system (QuantityOne software; Bio-Rad, Hercules, CA, USA).

Results

Atorvastatin Increases Trabecular Bone Volume and bone formation in Aged apoE^{-/-} mice

To determine the effects of atorvastatin on bone phenotype in aged apoE^{-/-} mice, we first performed micro-CT reconstruction on the distal metaphyseal region of the femurs (Fig. 1). Relative to the apoE^{-/-} (control) group, femurs from apoE^{-/-} + AT (atorvastatin) mice exhibited marked increase in trabecular BMD (+22.57%, $p < 0.01$), trabecular bone volume (BV/TV, +26.93%, $p < 0.05$), trabecular thickness (Tb.Th, +19.25%, $p < 0.01$), trabecular number (Tb.N, +31.24%, $p < 0.05$), and decreased in trabecular spacing (Tb.sp, -17.87%, $p < 0.05$) (Fig. 1a,b) after adjustment of body weight. No significant changes in the cortical parameters of femurs were observed between the apoE^{-/-} + AT and apoE^{-/-} (control) groups (Fig. 1c,d).

Atorvastatin improved the balance of the bone turnover in Aged apoE^{-/-} mice

To further understand the changes of the bone turnover in aged apoE^{-/-} mice after treatment of atorvastatin, the level of serum TRAP5b and OCN were also measured between two groups. The level of serum OCN significantly increased (+66.2%, ** $p < 0.01$, Fig. 2d) in aged apoE^{-/-} mice treated with atorvastatin accompanied by the decrease of serum total cholesterol (TC, -51.6%, ** $p < 0.01$, Fig. 2b) and oxidized low-density lipoprotein (ox-LDL, -48.4%, ** $p < 0.01$, Fig. 2c). There were no remarkable changes in body weight and serum tartrate resistant acid phosphatase 5b (TRAP5b) between two groups. (Fig. 2a,e).

Atorvastatin Increased Sirt1 Expression in the Bone Tissue of Aged apoE^{-/-} Mice

To further analyze the mechanisms of the increase in bone mass and bone formation in aged apoE^{-/-} mice after atorvastatin treatment, we measured Sirt1 and Runx2 expression in the bone tissue of aged apoE^{-/-} + AT and apoE^{-/-} mice (Fig. 3). When aged apoE^{-/-} mice were treated with atorvastatin (10 mg kg⁻¹ day⁻¹) for 12 weeks, the mRNA expression of Sirt1 in bone tissue increased by 82.1% compared with the apoE^{-/-} group ($p < 0.05$) (Fig. 3a). Similarly, the expression of mRNA in Runx2, ALP, and OCN was significantly higher in apoE^{-/-} + AT mice after normalization (+ 72.6%, + 68.6%, and + 82.8%, respectively, $p < 0.05$) (Fig. 3a). Western blot analysis showed that the levels of Sirt1 and Runx2 increased by 65.8% and 105.6% in apoE^{-/-} + AT group, respectively to apoE^{-/-} group ($p < 0.05$) (Fig. 3b-d).

Atorvastatin Increases the Expression of Sirt1 and Runx2 in BMSCs from apoE^{-/-} Mice In Vitro

To comprehensively determine the mechanism for atorvastatin-induced osteogenesis in apoE^{-/-} mice, we investigated the effect of atorvastatin on the expression of Sirt1 and Runx2 in BMSCs in vitro. We treated BMSCs from 18-week-old apoE^{-/-} mice exposed to different concentrations of atorvastatin (0, 10, 100, 1000 nmol/L). The results showed that the mRNA and protein expression of Sirt1 and Runx2 increased significantly with the concentration of atorvastatin increased, reaching the top at the concentration of 100 nmol/L ($p < 0.05$ and $p < 0.01$) (Fig. 4a-d). The mRNA expression of ALP and OCN in BMSCs showed same trend ($p < 0.01$) (Fig. 4a).

Atorvastatin Promoted Osteogenesis in BMSCs from apoE^{-/-} Mice through Activation of Sirt1

Based on the preceding observations, we then searched for the mechanism underlying the effect of atorvastatin on Sirt1–Runx2 mediated osteogenesis. The mRNA expression of Runx2, ALP, and OCN in BMSCs increased significantly (+ 86.1%, + 111.7%, and + 258.9%, respectively, $p < 0.05$, $p < 0.01$) after atorvastatin (100 nmol/L) treatment. However, when the BMSCs were pretreated with EX527 (10 μ M), the atorvastatin-induced increase in the mRNA expression of Runx2, ALP, and OCN was abolished and even decreased significantly ($p < 0.05$) or showed no difference (Fig. 5a). The protein expression of Runx2 increased by 136.1% after atorvastatin treatment, when BMSCs were pretreated with EX527, the expression of Runx2 decreased by 18.9% after atorvastatin treatment compared with the control ($p < 0.05$) (Fig. 5b.c).

Discussion

Statins, the most commonly used lipid-lowering drugs, are routinely administered to treat hyperlipidemia in the elderly population. In the last two decades, evidence has shown that statins may be beneficial as a potential anti-osteoporosis drug for bone regeneration [11–13]. However, most previous studies focused on statins' effects on OVX animal or fracture animal models [19, 33–36]. The majority of related literatures showed an increase in BMD or promotion of osteogenic differentiation after statin treatment [35–39], while other studies detected a negative association between statin treatment and BMD and bone repair [40–42]. The role of statin in these animal models studies remains controversial.

To our best knowledge, there have been few studies on the effects of statins on bone in aged hyperlipidemia mouse models. apoE^{-/-} mice are characterized with severe hyperlipidemia and the spontaneous development of atherosclerosis [43]. In our previous results, aged apoE^{-/-} mice displayed low bone mass and decreased bone formation due to an ox-LDL effect on Sirt1 and Runx2 in BMSCs. Thus, in this study, we investigated the bone phenotypes and metabolism of aged apoE^{-/-} mice treated with oral atorvastatin and attempted to elucidate the possible mechanisms involved in these changes. Compared with the control group, after 3 months of oral treatment with therapeutic doses of atorvastatin, aged apoE^{-/-} mice demonstrated an increase in trabecular bone volume, but not cortical bone volume. Furthermore, serum bone OCN increased significantly in aged apoE^{-/-} mice after atorvastatin treatment. Serum osteocalcin levels were correlated with bone formation and osteoblast number. As a product of osteoblastic synthesis, osteocalcin has been used as a marker of bone formation. On the other hand, levels of the bone resorption marker, Trap5b, did not change significantly. These results indicate that atorvastatin could act as a bone anabolic agent to increase bone mass in aged apoE^{-/-} mice. Recently, a randomized controlled study focused on the effects of statins on BMD in elderly males with osteopenia and mild dyslipidemia [22]. Consistent with our findings, oral treatment with therapeutic doses of atorvastatin was associated with significantly higher values of total hip BMD in elderly men compared with the control group after a one year study period.

Several mechanisms of the actions of statins on bone have been demonstrated in previous studies. For example, statins induce osteoblastic differentiation by upregulating the MAPK/BMP-2 signaling pathway [44]. Statins inhibit osteoblastic apoptosis by the TGFβ/Smad3 signaling pathway [45]. In an aging population, more factors may be involved in. Sirt1 plays important roles in the progress of the age-related diseases atherosclerosis and osteoporosis [46–48]. Our previous study found low Sirt1 expression in the bone tissue of aged apoE^{-/-} mice compared with age-matched wild type mice [29]. Oxidized LDL is also an important contributor to the aging process. Ox-LDL levels gradually increase with age. The long-term accumulation of ox-LDL-induced Sirt1 decreases in bone marrow-derived mesenchymal stem cells. This may have contributed to the bone loss in aged apoE^{-/-} mice seen in our previous study [29]. Statins have been proven to have a preventive effect on aging through intervention with Sirt1 [49]. Statins could inhibit endothelial senescence and apoptosis through the upregulation of Sirt1 [50]. Long-term administration of atorvastatin to old rats inhibited the senescent phenotype through increased Sirt1 expression, improved endothelium-dependent relaxation, and ameliorated oxidative stress [51]. However, to our best knowledge, no study has reported the effects of statins on Sirt1 in bone. In the present study, after 3 months of

intervention with oral atorvastatin, the expression levels of Sirt1 and Runx2 were significantly higher than those of the control group. Runx2 is the most upstream transcription factor essential for osteoblast differentiation [52]. Studies have shown that Sirt1 mediates Runx2 deacetylation, promoting the osteogenic differentiation of mesenchymal stem cells. Resveratrol which is an agonist of Sirt1 promotes the osteogenesis of human mesenchymal stem cells by upregulating Runx2 gene expression via the Sirt1/Foxo3A axis. The activation of Sirt1, using resveratrol and isonicotinamide, was shown to stimulate osteoblastic differentiation in a dose-dependent manner by increasing the mRNA expression of Runx2, ALP, and OCN [53]. Based on these findings, we hypothesize that atorvastatin could promote osteogenesis in the BMSCs of apoE^{-/-} mice via enhancement of the Sirt1–Runx2 pathway [54]. To further confirm this hypothesis in more detail, we measured the expression levels of Sirt1 and Runx2 in BMSCs from apoE^{-/-} mice after stimulation with atorvastatin in vitro. qRT-PCR and Western blot analyses showed that atorvastatin enhanced Sirt1 and Runx2 expression in BMSCs from apoE^{-/-} mice, whereas atorvastatin-induced Sirt1 and Runx2 activity was totally abolished by pretreatment with the Sirt1 inhibitor EX527 (10 μM). The results showed that atorvastatin may promote bone formation in BMSCs from apoE^{-/-} mice via the Sirt1–Runx2 axis.

Conclusions

In this study, we investigated the bone phenotypes and metabolism of aged apoE^{-/-} mice treated orally with atorvastatin and attempted to elucidate possible mechanisms involved in these changes. Our results showed that long-term administration of atorvastatin increased trabecular bone mass and promoted bone formation in aged apoE^{-/-} mice. Our data also showed the mechanism of atorvastatin might promote the osteogenesis of BMSCs from apoE^{-/-} mice by upregulating the Sirt1–Runx2 axis.

Abbreviations

ALP	alkaline phosphatase
AT	atorvastatin
AS	atherosclerosis
BMSCs	bone marrow-derived mesenchymal stem cells
BMD	bone mineral density
BMP	bone morphogenic protein
BV/TV	bone volume volume
Ct.Ar	cortical area
Ct.Th	cortical thickness
DMSO	dimethyl sulfoxide
OCN	osteocalcin
OPG	osteoprotegerin
OVX	ovariectomized ox-LDL oxidized low-density lipoprotein
RANKL	receptor activator of nuclear factor kappa B ligand Runx2 runt-related transcription factor 2
Tb.Ar	total area
Tb.N	trabecular number
Tb.Sp	trabecular spacing
Tb.Th	trabecular thickness
TC	total cholesterol
Trap5b	tartrate-resistant acid phosphatase 5b

Declarations

Acknowledgments

Not applicable.

Author Contributions

Z.Y. and X.X. designed the study; X.X. and W.H. performed ELISA, RT-PCR, and Western blotting; Z.Q. and Z.L. contributed to the animal experiments; Z.W. performed the micro-CT analysis; X.X. and C.F. analyzed the data; W.H. wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

We declare that the materials described in the manuscript will be freely available to all scientists for noncommercial purpose.

Ethics approval and consent to participate

Ethics approval was provided by the Committee on Animal Resources at Huadong Hospital, Fudan University. Great efforts were made to minimize the animals and their pains.

Consent for publication

Not applicable.

Conflicts of interest

The authors declare no conflict of interest.

References

1. Ye C, Xu M, Wang S, Jiang S, Che X, Zhou X, He R. Decreased Bone Mineral Density Independent Predictor for the Development of Atherosclerosis: Systematic Review and Meta-Analysis. *PLoS ONE*. 2016;11:e0154740.
2. Lange V, Dörr M, Schminke U, Völz H, Nauck M, Wallaschofski H, Hannemann A. The Association between Bone Quality and Atherosclerosis: Results from Two Large Population-Based. *Int J Endocrinol* 2017; 3946569.
3. Ahmadi N, Mao SS, Hajsadeghi F, Arnold B, Kiramijyan S, Gao Y, Flores F, Azen S, Budoff M. The relation of low levels of bone mineral density with coronary artery calcium and mortality. *Osteoporos Int*. 2018;29:1609–16.
4. Tasić I, Popović MR, Stojanović S, Stamenković B, Kostić S, Popović D, Lazarević G, Bogdanović D, Stefanović V. Osteoporosis—A risk factor for cardiovascular diseases: A follow-up study. *Srp Arh Celok Lek*. 2015;143:28–34.
5. Simões Sato AY, Bub GL, Campos AH. BMP-2 and -4 produced by vascular smooth muscle cells from atherosclerotic lesions induce monocyte chemotaxis through direct BMPRII activation. *Atherosclerosis*. 2014;235:45–55.
6. Szekanec Z, Raterman HG, Pethő Z, Lems WF. Common mechanisms and holistic care in atherosclerosis and osteoporosis. *Arthritis Res Ther*. 2019;21:15–22.
7. Caffarelli C, Montagnani A, Nuti R, Gonnelli S. Bisphosphonates atherosclerosis and vascular calcification: Update and systematic review of clinical studies. *Clin Interv Aging*. 2017;12:1819–28.

8. Bartoli-Leonard F, Wilkinson FL, Langford-Smith AW, Alexander MY, Weston R. The Interplay of SIRT1 and Wnt Signaling in Vascular Calcification. *Front Cardiovasc Med.* 2018;5:183.
9. An T, Hao J, Sun S, Li R, Yang M, Cheng G, Zou M. Efficacy of statins for osteoporosis: Asystematic review and meta-analysis. *Osteoporos Int.* 2017;28:47–57.
10. Cetinkaya Demir B, Uyar Y, Ozbilgin K, Köse C. Effect of raloxifene and atorvastatin in atherosclerotic process in ovariectomized rats. *J Obstet Gynaecol Res.* 2013;39:229–36.
11. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. Stimulation of bone formation in vitro and in rodents by statins. *Science.* 1999;286:1946–9.
12. Chen PY, Sun JS, Tsuang YH, Chen MH, Weng PW, Lin FH. Simvastatin promotes osteoblast viability and differentiation via Ras/Smad/Erk/BMP-2 signaling pathway. *Nutr Res.* 2010;30:191–9.
13. Ghosh-Choudhury N, Mandal CC, Choudhury GG. Statin-induced Ras activation integrates the phosphatidylinositol 3-kinase signal to Akt and MAPK for bone morphogenetic protein-2 expression in osteoblast differentiation. *Biol Chem.* 2007;282:4983–93.
14. Lee WS, Lee EG, Sung MS, Choi YJ, Yoo WH. Atorvastatin inhibits osteoclast differentiation by suppressing NF-Kb and MAPK signaling during IL-1 β -induced osteoclastogenesis. *Korean J Intern Med.* 2018;33:397–406.
15. Dolci GS, Ballarini A, Gameiro GH, Onofrede Souza D, de Melo F, Fossati ACM. Atorvastatin inhibits osteoclastogenesis and arrest tooth movement. *Am J Orthod Dentofac Orthop.* 2018;153:872–82.
16. El-Nabarawi N, El-Wakd M, Salem M. Atorvastatin, a double weapon in osteoporosis treatment: An experimental and clinical study. *Drug Des Dev Ther.* 2017;2:1383–91.
17. Von Stechow D, Fish S, Yahalom D, Bab I, Chorev M, Müller R, Alexander JM. Does simvastatin stimulate bone formation in vivo? *BMC Musculoskelet Disord.* 2003;28:8.
18. Van Staa TP, Wegman S, De Vries F, Leufkens B, Cooper C. Use of statins and risk of fractures. *JAMA.* 2001;285:1850–5.
19. Zhou H, Xie Y, Baloch Z, Shi Q, Huo Q, Ma T. The effect of atorvastatin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (HMG-CoA), on the prevention of osteoporosis in ovariectomized rabbits. *J Bone Miner Metab.* 2017;35:245–54.
20. Ibrahim N, Khamis MF, Mod Yunoh MF, Abdullah S, Mohamed N, Shuid AN. Targeted delivery of lovastatin and tocotrienol to fracture site promotes fracture healing in osteoporosis model: Micro-computed tomography and biomechanical evaluation. *PLoS ONE.* 2014;9:e115595.
21. Oryan A, Kamali A, Moshiri A. Potential mechanisms and applications of statins on osteogenesis: Current modalities, conflicts and future directions. *J Control Release.* 2015;10:12–24.
22. Chen ZG, Cai HJ, Jin X, Lu JH, Wang J, Fang NY. Effects of atorvastatin on bone mineral density (BMD) and bone metabolism in elderly males with osteopenia and mild dyslipidemia: A 1-year randomized trial. *Arch Gerontol Geriatr.* 2014;59:515–21.
23. Bone HG, Kiel DP, Lindsay RS, Lewiecki EM, Bolognese MA, Leary ET, Lowe W, McClung MR. Effects of atorvastatin on bone in postmenopausal women with dyslipidemia: A double-blind, placebo-

- controlled, dose-ranging trial. *J Clin Endocrinol Metab.* 2007;92:4671–7.
24. Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature.* 2001;410:227–30.
 25. Qian L, Ma L, Wu G, Yu Q, Lin H, Ying Q, Wen D, Gao C. G004, a synthetic sulfonylurea compound, exerts anti-atherosclerosis effects by targeting SIRT1 in ApoE^{-/-} mice. *Vascul Pharmacol.* 2017;89:49–57.
 26. Yang X, Wei J, He Y, Jing T, Li Y, Xiao Y, Wang B, Wang W, Zhang J, Lin R. SIRT1 inhibition promotes atherosclerosis through impaired autophagy. *Oncotarget.* 2017;8:51447–61.
 27. Bäckesjö CM, Li Y, Lindgren U, Haldosén LA. Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. *Cells Tissues Organs.* 2009;189:93–7.
 28. Zainabadi K. Drugs targeting SIRT1, a new generation of therapeutics for osteoporosis and other bone related disorders? *Pharmacol Res.* 2019;143:97–105.
 29. Hong W, Xu XY, Qiu ZH, Gao JJ, Wei ZY, Zhen L, Zhang X, Ye ZB. Sirt1 is involved in decreased bone formation in aged apolipoprotein E-deficient mice. *Act Pharmacol Sin.* 2015;36:1487–96.
 30. Zainabadi K, Liu CJ, Guarente L. SIRT1 is a positive regulator of the master osteoblast transcription factor, RUNX2. *PLoS ONE.* 2017;12:e0178520.
 31. Kilic U, Gok O, Elibol-Can B, Uysal O, Bacaksiz A. Efficacy of statins on sirtuin 1 and endothelial nitric oxide synthase expression: The role of sirtuin 1 gene variants in human coronary atherosclerosis. *Clin Exp Pharmacol Physiol.* 2015;42:321–30.
 32. Du G, Song Y, Zhang T, Ma L, Bian N, Chen X, Feng J, Chang Q, Li Z. Simvastatin attenuates TNF α induced apoptosis in endothelial progenitor cells via the upregulation of SIRT1. *Int J Mol Med.* 2014;34:177–82.
 33. Issa JP, Ingraci de Lucia C, Dos Santos Kotake BG, Gonçalves Gonzaga M, Tocchini de Figueiredo FA, Mizusaki Iyomasa D, Macedo AP, Ervolino E. The effect of simvastatin treatment on bone repair of femoral fracture in animal model. *Growth Factors.* 2015;33:139–48.
 34. Li X, Song QS, Wang JY, Leng HJ, Chen ZQ, Liu ZJ, Dang GT, Song CL. Simvastatin induces oestrogen receptor- α expression in bone, restores bone loss, and decreases ER α expression and uterine wet weight in ovariectomized rats. *J Bone Miner Metab.* 2011;29:396–403.
 35. Qadir F, Alam SM, Zehra T, Mehmood A, Siddiqi AQ. Role of Pitavastatin in Prevention of Osteopenic Changes in Ovariectomized Rats. *J Coll Physicians Surg Pak.* 2016;26:41–5.
 36. Wang W, Nyman JS, Moss HE, Gutierrez G, Mundy GR, Yang X, Elefteriou F. Local low-dose lovastatin delivery improves the bone-healing defect caused by Nf1 loss of function in osteoblasts. *J Bone Miner Res.* 2010;25:1658–67.
 37. Qiao LJ, Kang KL, Heo JS. Simvastatin promotes osteogenic differentiation of mouse embryonic stem cells via canonical Wnt/ β -catenin signaling. *Mol Cells.* 2011;32:437–44.
 38. Goes P, Lima AP, Melo IM, Rêgo RO, Lima V. Effect of Atorvastatin in radiographic density on alveolar bone loss in Wistar Rats. *Braz Dent J.* 2010;21:193–8.

39. Uyar Y, Baytur Y, Inceboz U, Demir BC, Gumuser G, Ozbilgin K. Comparative effects of risedronate, atorvastatin, estrogen and SERMs on bone mass and strength in ovariectomized rats. *Maturitas*. 2009;63:261–7.
40. Maritz FJ, Conradie MM, Hulley PA, Gopal R, Hough S. Effect of statins on bone mineral density and bone histomorphometry in rodents. *Arterioscler Thromb Vasc Biol*. 2001;21:1636–41.
41. Yao W, Farmer R, Cooper R, Chmielewski PA, Tian XY, Setterberg RB, Jee WS, Lundy MW. Simvastatin did not prevent nor restore ovariectomy-induced bone loss in adult rats. *J Musculoskelet Neuronal Interact*. 2006;6:277–83.
42. Oka S, Matsumoto T, Kubo S, Matsushita T, Sasaki H, Nishizawa Y, Matsuzaki T, Saito T, Nishida K, Tabata Y. Local administration of lowdose simvastatin-conjugated gelatin hydrogel for tendon-bone healing in anterior cruciate ligament reconstruction. *Tissue Eng*. 2013;19:1233–43.
43. Faziom S, Linton MF. Mouse models of hyperlipidemia and atherosclerosis. *Front Biosci*. 2001;6:515–25.
44. Ge C, Yang Q, Zhao G, Yu H, Kirkwood KL, Franceschi RT. Interactions between extracellular signal-regulated kinase 1/2 and p38 MAP kinase pathways in the control of RUNX2 phosphorylation and transcriptional activity. *J Bone Miner Res*. 2012;27:538–51.
45. Kaji H, Naito J, Inoue Y, Sowa H, Sugimoto T, Chihara K. Statin suppresses apoptosis in osteoblastic cells: Role of transforming growth factor–beta-Smad3 pathway. *Horm Metab Res*. 2008;40:746–51.
46. Guarente L. Sir2 links chromatin silencing, metabolism, and aging. *Genes Dev*. 2000;14:1021–6.
47. Kitada M, Ogura Y, Koya D. The protective role of Sirt1 in vascular tissue: Its relationship to vascular aging and atherosclerosis. *Aging*. 2016;810:2290–307.
48. Almeida M, Porter RM. Sirtuins and FoxOs in osteoporosis and osteoarthritis. *Bone*. 2019;121:284–92.
49. Hu HJ, Zhou SH, Liu QM. The magic and mystery of statins in aging: The potent preventive and therapeutic agent. *Int J Cardiol*. 2015;187:58–9.
50. Ota H, Eto M, Kano MR, Kahyo T, Setou M, Ogawa S, Iijima K, Akishita M, Ouchi Y. Induction of endothelial nitric oxide synthase, SIRT1, and catalase by statins inhibits endothelial senescence through the Akt pathway. *Arterioscler Thromb Vasc Biol*. 2010;30:2205–11.
51. Gong X, Ma Y, Ruan Y, Fu G, Wu S. Long-term atorvastatin improves age-related endothelial dysfunction by ameliorating oxidative stress and normalizing eNOS/iNOS imbalance in rat aorta. *Exp Gerontol*. 2014;52:9–17.
52. Komori T. Roles of Runx2 in Skeletal Development. *Adv Exp Med Biol*. 2017;962:83–93.
53. Shakibaei M, Shayan P, Busch F, Aldinger C, Buhrmann C, Lueders C, Mobasheri A. Resveratrol mediated modulation of Sirt-1/Runx2 promotes osteogenic differentiation of mesenchymal stem cells: Potential role of Runx2 deacetylation. *PLoS ONE*. 2012;7:e35712.
54. Tseng PC, Hou SM, Chen RJ, Peng HW, Hsieh CF, Kuo ML, Yen ML. Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the

Figures

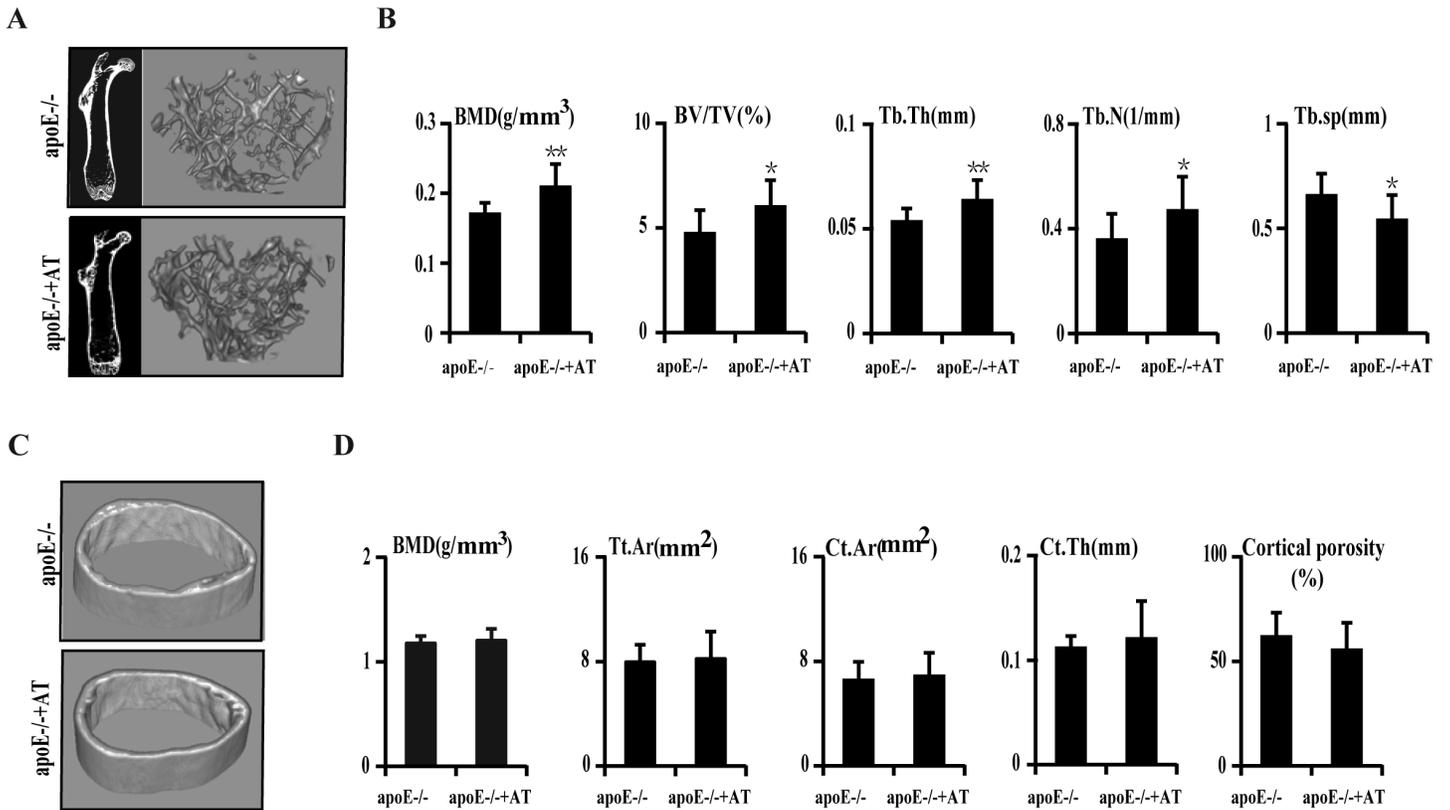


Figure 1

The effect of atorvastatin (10 mg/kg/day) treatment on bone volume in aged apoE^{-/-} mice, as determined by micro-CT. a Representative 3D micro-CT images showing regions of the trabecular bone in the distal femurs. b The graphs represent measurements of bo

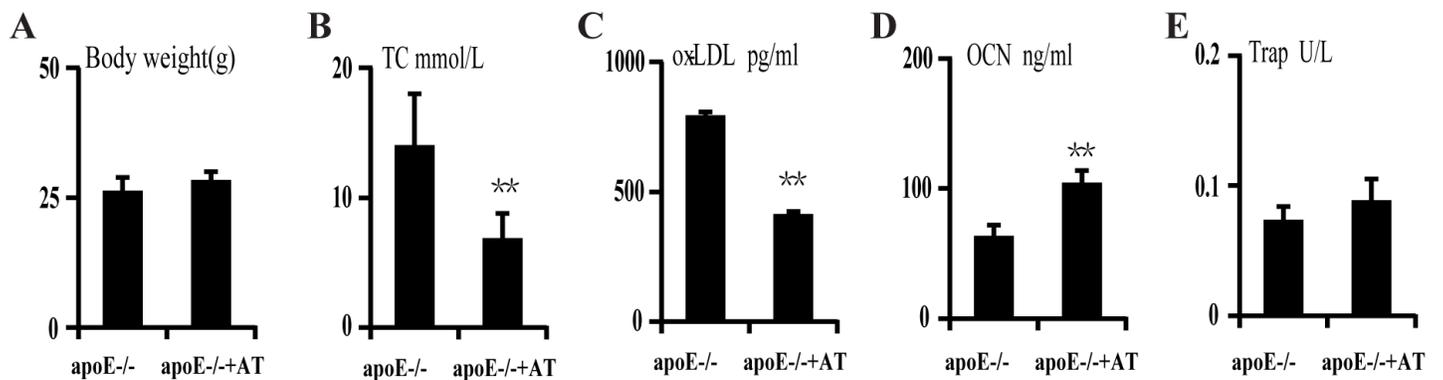


Figure 2

The effect of atorvastatin (10 mg/kg/d) treatment on bone turnover markers changes in aged apoE^{-/-} mice. a The body weight of aged apoE^{-/-} mice and apoE^{-/-} +AT mice. b The level of serum total

cholesterol (TC) in aged apoE^{-/-} mice and apoE^{-/-} +AT mice. c The level of oxidized low-density lipoprotein (ox-LDL) in aged apoE^{-/-} mice and apoE^{-/-} +AT mice. d The change of serum of osteocalcin (OCN) in two groups. e The change of serum of tartrate resistant acid phosphatase 5b in two groups. Statistical analysis was performed using two-way ANOVA followed by Dunnett's test. N=6 mice per group were examined. * p<0.05, ** p< 0.01,vs. apoE^{-/-} group.

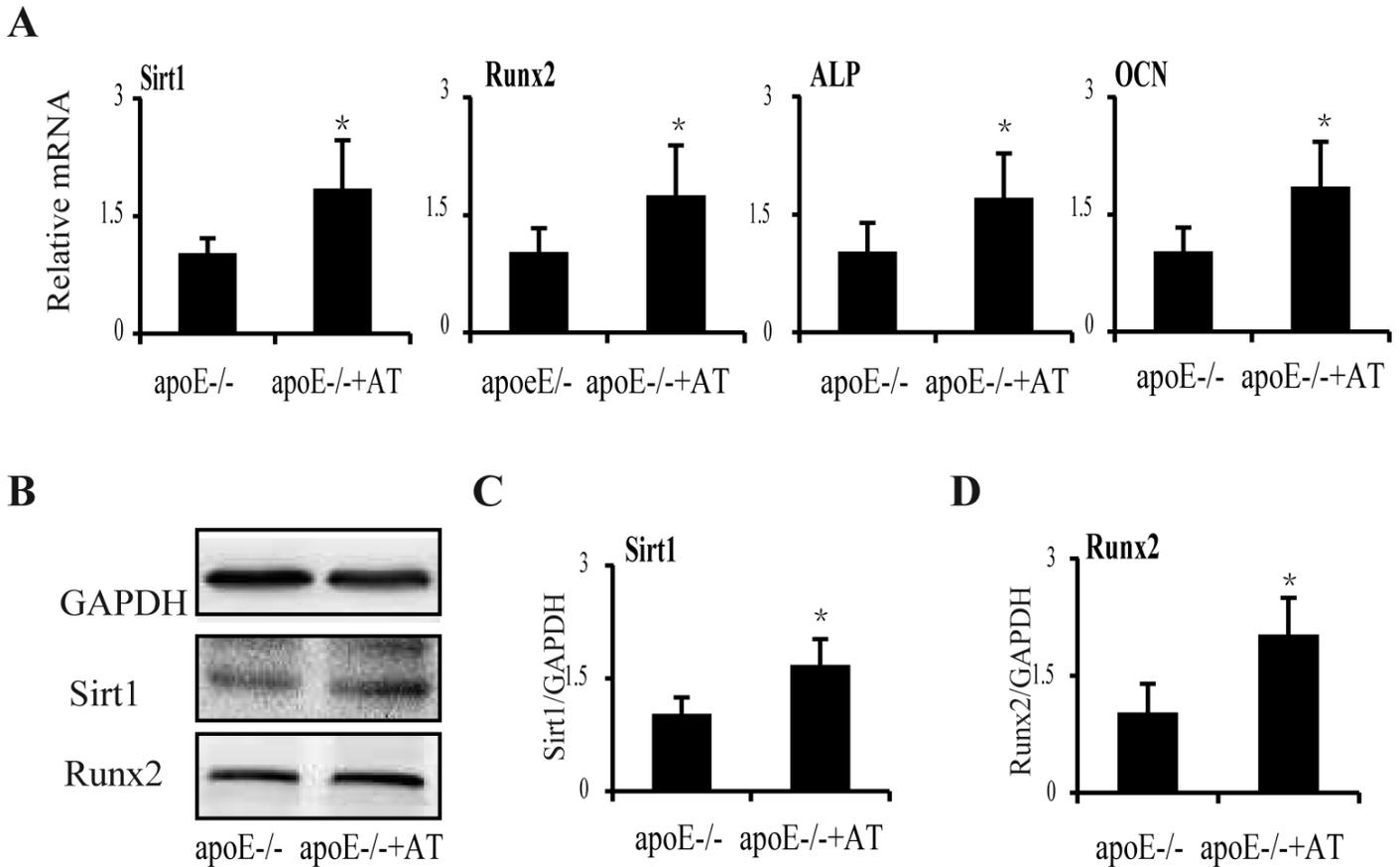


Figure 3

The gene and protein expressions of bone formation in bone tissue from aged apoE^{-/-} mice after atorvastatin treatment. a mRNA expression of Sirt1, Runx2, ALP, and OCN in whole femurs from apoE^{-/-} and apoE^{-/-} + AT groups. b-d Western blot showed levels of Sirt1 and Runx2 from whole femurs in apoE^{-/-} and apoE^{-/-} + AT groups. Statistical analysis was performed using two-way ANOVA followed by Dunnett's test. N = 3 mice per group were examined. * p< 0.05 vs. apoE^{-/-} group.

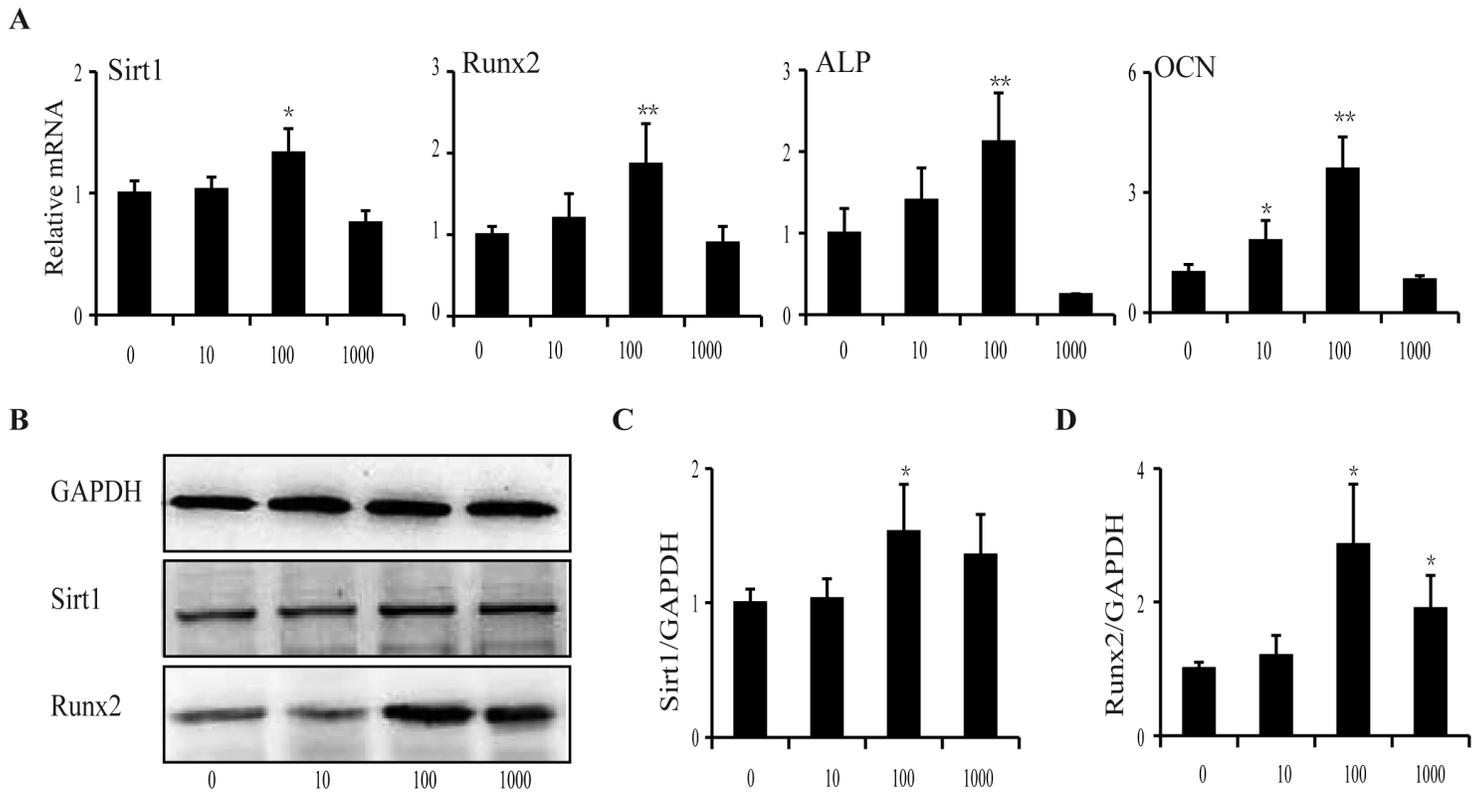


Figure 4

The expression of Sirt1, Runx2, ALP, and OCN in BMSCs from apoE^{-/-} mice treated with 10, 100, and 1000 nmol/L atorvastatin in vitro. a The mRNA expression of Sirt1, Runx2, ALP and OCN in BMSCs with different concentrations of atorvastatin. b–d The protein expression of Sirt1 and Runx2 in BMSCs with different concentrations of atorvastatin. The bar graphs represent mean \pm standard deviations; N = 3. * $p < 0.05$, ** $p < 0.01$ vs. apoE^{-/-} group.

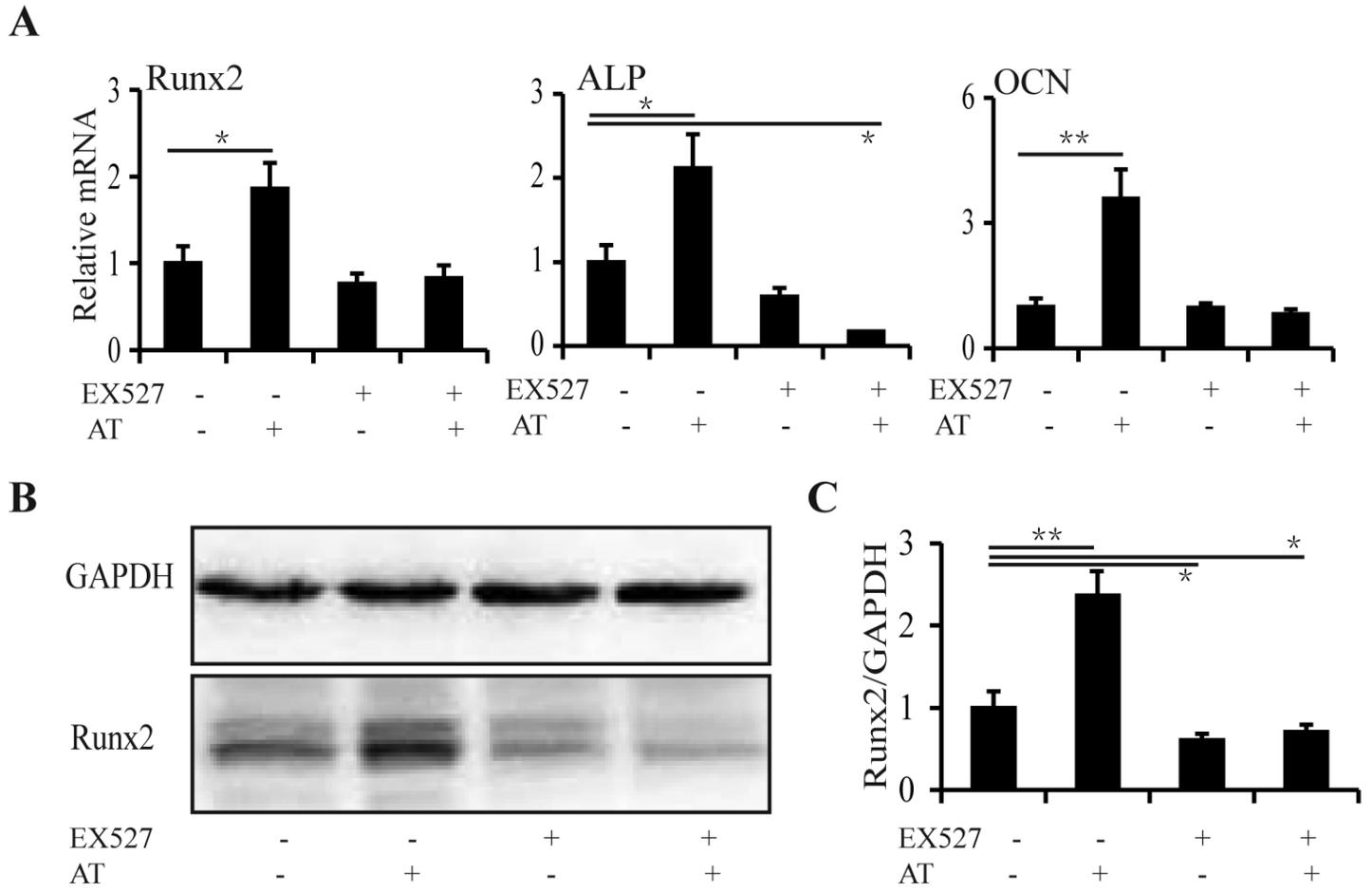


Figure 5

Sirt1-Runx2 is mainly involved in atorvastatin-induced osteogenesis in BMSCs from apoE^{-/-} mice. a The mRNA expression of Runx2, ALP and OCN in the presence of 100 nmol/L atorvastatin with or without of the treatment of EX527. b and c The protein expression of Runx2 in BMSCs from apoE^{-/-} mice in the presence of 100 nmol/L atorvastatin with or without the treatment of EX527. N = 3. * p < 0.05, ** p < 0.01 vs. apoE^{-/-} group.