

Original Article

CCAR2 reduces the number of osteoclasts by controlling osteoclast apoptosis

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Abstract

Estrogen-associated signals regulating osteoclast apoptosis control osteoclast numbers. Enhancing anti-apoptotic activity during M-CSF and RANKL stimulation, which promotes osteoclast differentiation, increases the number of osteoclasts. Cell cycle and apoptosis regulator 2 (CCAR2) significantly modulates cancer cell survival and apoptosis by interacting with estrogen receptor α (ER α). Because CCAR2 regulates cell fate through ER α , we hypothesize that CCAR2 may inhibit the formation of bone-resorbing osteoclasts. We screen a public database of inbred mouse strain information to identify correlations between *Ccar2* mRNA expression in bone and bone mineral density (BMD). We find that *Ccar2* expression correlates positively with femoral and spine BMD. In addition, an increased abundance of osteoclasts is observed in aged mice, but the expression of CCAR2 in osteoclasts is lower in old mice than in young mice. To identify the role of CCAR2 in osteoclasts, we generate CCAR2-knockout (KO) RAW 264.7 cells using CRISPR-Cas9. The CCAR2-KO cells exhibit increased osteoclast numbers, reduced intracellular ROS levels, and decreased apoptosis during M-CSF- and RANKL-induced osteoclastogenesis. Mechanistically, CCAR2 binds to ER α , altering its nuclear translocation and increasing apoptotic transcriptional activity. These findings suggest that CCAR2 inhibits osteoclast numbers by increasing ER α -mediated apoptosis.

Key words CCAR2, osteoclast, apoptosis, estrogen receptor α

Introduction

Bone loss advances with increasing age in both men and women [1]. Bone loss causes deterioration in the bone micro-architecture, increasing the risk of fragility fractures, which are among the most common causes of disability in elderly people and hasten mortality [2]. Age-related bone loss results from increased bone resorption and decreased bone formation [3]. This unbalanced remodeling is caused by factors extrinsic and intrinsic to osteoclasts that increase the number of osteoclasts over time. Extrinsic factors include increased levels of receptor activator of nuclear factor kappa-B (NF- κ B) ligand (RANKL) from osteocytes [4] and pro-inflammatory secretome- or senescence-associated secretory phenotype factors that originate from B cells, T cells, and osteoblastic lineage cells [5].

The intrinsic factors include changes in age-related proteins and osteoclast differentiation factors through epigenetic alteration or reprogramming with age [6,7]. However, the exact molecular mechanism behind the age-related increase in osteoclast numbers is still unclear.

The lifespan of osteoclasts, which is regulated by survival and apoptosis, is the key to bone remodeling [8]. Signaling through macrophage colony-stimulating factor (M-CSF) and RANKL is involved in the survival of osteoclasts [9], whereas both an intrinsic pathway (mitochondrial apoptotic pathway) [10] and extrinsic death receptor pathway (Fas/FasL system) [11,12] control the apoptosis of osteoclasts. In many pathologic bone diseases, such as postmenopausal osteoporosis, rheumatoid arthritis, multiple mye-

loma, and Paget's disease, osteoclasts undergo abnormal apoptosis, resulting in changes in osteoclast lifespan and bone resorption [13]. Based on that etiology, many studies have reported osteoclast apoptosis as a pharmacological mechanism of action for existing osteoporosis drugs, such as estrogen, tamoxifen, bisphosphonate, and osteoprotegerin [14–16].

Cell cycle and apoptosis regulator 2 (CCAR2) has recently been found to play a novel and important apoptotic role by interacting with transcription factors, chromatin remodeling enzymes, and nuclear receptors [17,18]. In particular, CCAR2 regulates both the tumor necrosis factor α (TNF α)-mediated extrinsic apoptotic pathway and the p53-dependent intrinsic apoptotic pathway. The amino-terminally truncated CCAR2 produced during TNF α -mediated apoptosis causes mitochondrial dysfunction [19], and CCAR2 promotes p53-mediated apoptosis by inhibiting the deacetylation function of SIRT1 [20,21] and stabilizing p53 by competitively binding with the ubiquitin E3 ligase [22]. Furthermore, mass spectrometry-based screens have shown that CCAR2 interacts directly with estrogen receptor α (ER α) in estrogen-dependent [23] and estrogen-independent manners [24], thereby modulating cancer cell survival and apoptosis. Pulling these independent lines of evidence together, we hypothesized that CCAR2 plays a role in osteoclast survival and apoptosis that affects bone homeostasis.

Materials and Methods

Detection of *Ccar2* coexpressed genes and relevant molecular characteristics

Bone femur microarray data (UCLA GSE27483 BXD bone femur ILM Mouse WG-6 v1) and bone mineral density (BMD) data from a Hybrid Mouse Diversity Panel (HMDDP) from the Jackson Laboratory [25] were analyzed to find correlations with *Ccar2* transcript expression. All raw data are publicly available at the National Center for Biotechnology Information Gene Expression Omnibus (GSE27483), GeneNetwork (www.genenetwork.org), and the Jackson Laboratory (<http://phenome.jax.org>).

Animal experiments

Three Male C57BL/6 mice aged 12 weeks and 20 months were purchased from Jackson Laboratory (Bar Harbor, USA). Subsequently, bone marrow samples were isolated for either protein expression analysis or histological evaluation. Briefly, after removal of the femurs and tibias from mice, both ends were cut, and bone marrow cells were extracted by passing alpha MEM (15140122; Gibco, Grand Island, USA) through the bones using a syringe. The cell suspension was then passed through a 70- μ m mesh filter screen, red blood cells were removed using ACK lysis buffer (A1049201; Gibco), and the cells were placed in a culture dish. The following day, the supernatant was collected, and CD11b positive selection was performed using CD11b MicroBeads (130-097-142; Miltenyi Biotec, San Diego, USA).

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee of Chonbuk National University (permit No: CBNU 2020-061).

Cell culture and transient transfection

Murine macrophage RAW 264.7 cells (TIB-71; ATCC, Manassas, USA) were incubated in alpha MEM (15140122; Gibco) with 10% FBS (16000044; Gibco) and 1% penicillin/streptomycin (15140122;

Gibco) at 37°C under 5% CO₂ and humidified air. To assess promoter activity, FasL and ERE promoter luciferase plasmids (CS184901; Promega, Madison, USA) were each introduced into HEK293T cells (CRL-3216; ATCC). For exogenous protein expression, HEK293T cells were transfected with Flag-CCAR2 (HG19022-CF; Sino Biological, Beijing, China) or HA-ER α (215093; Addgene, Watertown, USA) plasmid using Lipofectamine 3000 reagent (3000001; Invitrogen, Carlsbad, USA). For electroporation, primary bone marrow cells or RAW 264.7 cells were used following 2 days of M-CSF (315-02; PeproTech, London, UK)-induced differentiation. Briefly, primary bone marrow cells or RAW 264.7 cells were resuspended in 100 μ L of Nucleofector Solution (MIR 50117; Mirus Bio, Madison, USA) and mixed with 2 μ g of shCCAR2 (expressed under H1 promoter, sc-72274-SH; Santa Cruz Biotechnology, Dallas, USA), control shRNA (expressed under H1 promoter, sc-108060; Santa Cruz Biotechnology), or CCAR2 plasmid. Electroporation was performed using the Amaxa Nucleofector II device (Lonza, Cologne, Germany) with program Y-001. Cells were immediately transferred into pre-warmed medium.

Construction of the CCAR2-knockout (KO) cell line using the CRISPR-Cas9 gene-editing system

We generated CCAR2-deficient RAW 264.7 cells using CRISPR-Cas9-mediated gene knockout. RAW 264.7 cells were transfected with control (a single scrambled gRNA sequence, sc-418922; Santa Cruz Biotechnology) or *Ccar2* CRISPR-Cas9 plasmids (gRNA sequences consisting of three oligo pools: 5'-CCCAAAGTAGTCATGCAAGC-3'; 5'-TCTTCTGCATGTAGCAGCCC-3'; 5'-CGGATCAACCCGCTTCCAGG-3'; sc-403056; Santa Cruz Biotechnology) in accordance with the manufacturer's instructions. After 24 h, RAW 264.7 cells expressing green fluorescent protein (GFP) were sorted using a FACS Aria III (BD, Franklin Lakes, USA) and maintained in complete medium (alpha MEM) with 10% FBS and 1% penicillin/streptomycin. Western blot analysis confirmed that the CRISPR-Cas9 *Ccar2* plasmid induced *Ccar2* KO.

Histology

After decalcification, mouse femurs were processed by embedding in paraffin blocks and subsequently sectioned for downstream histological procedures. Immunohistochemical analysis was conducted on 5- μ m-thick sections of femoral bone tissue. Antigen retrieval was performed by boiling femur bone tissue samples in a microwave oven in pH 6.0 antigen retrieval solution (S1700; DAKO, Glostrup, Denmark) for 20 min. Anti-DBC1 (CCAR2, 5693; Cell Signaling, Beverly, USA) was used as the primary antibody. To evaluate osteoclast formation, bone tissue sections underwent tartrate-resistant acid phosphatase (TRAP) staining using a standardized detection kit (CS0740; Sigma-Aldrich, St Louis, USA), following the procedures recommended by the manufacturer. Cells identified as TRAP-positive and containing more than three nuclei were considered mature osteoclasts and were manually counted under a conventional light microscope (Leica, Wetzlar, Germany) for quantitative assessment.

Subcellular fractionation, western blotting, and co-immunoprecipitation

Nuclear and cytoplasmic extracts were isolated using a subcellular protein fractionation kit (78833; Thermo Fisher Scientific, Rockford, USA) according to the manufacturer's protocol. The transfected

cells were harvested and lysed to analyze the expression levels of various proteins, including ER α , FasL (sc-542, sc-19681; Santa Cruz Biotechnology), CCAR2, cleaved caspase-8, cleaved caspase-3 (5693, 9496, and 9661; Cell Signaling), HA (11583816001; Roche Diagnostics, Mannheim, Germany), FLAG (F7425; Sigma-Aldrich), Lamin B1 (AP6001; Bioworld Technology, St Louis Park, USA), and GAPDH (AP0066; Bioworld Technology). To further examine the interactions among these proteins, co-immunoprecipitation (Co-IP) assays were carried out. Briefly, total protein lysate (500 μ g) was incubated with Pierce[™] Protein G Agarose (20399; Thermo Fisher Scientific) conjugated with either anti-FLAG or anti-HA antibodies. The immunoprecipitated complexes were then analyzed to detect the expression of FLAG- or HA-tagged proteins.

Osteoclastic differentiation

RAW 264.7 cells were cultured in the presence of M-CSF (10 ng/mL, 315-02; PeproTech) and RANKL (70 ng/mL, 315-11; PeproTech) for 3 days or 5 days. After fixation, TRAP staining was used to determine osteoclast formation.

Osteogenic Differentiation

Mouse preosteoblasts (MC3T3-E1, CRL-2593; ATCC) were used to evaluate osteogenic differentiation. Osteogenic differentiation was induced in osteogenic medium [DMEM (11965092; Gibco), 10% FBS, 1% penicillin/streptomycin, 10 mM β -glycerophosphate (G9422; Sigma-Aldrich), 100 nM dexamethasone (D4902; Sigma-Aldrich), and 50 nM L-ascorbic acid-2-phosphate (A8960; Sigma-Aldrich)] for 4 d or 7 d. Cells were fixed for 10 min using 4% paraformaldehyde (158127; Sigma-Aldrich). For alkaline phosphatase (ALP) staining, cells cultured for 4 d were treated with the BCIP/NBT kit (K4151; APEXBio, Houston, USA). The absorbance (OD) was measured at 405 nm using the alkaline phosphatase assay kit (ab83369; Abcam, Milpitas, USA) to quantify the results. Cells cultured for 7 days for Alizarin Red S (ARS) staining were treated with 40 mM ARS (A5533; Sigma-Aldrich) at pH 4.0. Then, 10% pivaloyl chloride (T72605; Sigma-Aldrich) was added, and the reaction product was quantified by measuring the absorbance at 560 nm.

Staining for apoptosis

Apoptotic cells were detected using an APOPercentage apoptosis assay kit (A1000; Biocolor, Belfast, Northern Ireland) on day 5 after the induction of osteoclastogenesis. To quantify apoptotic osteoclasts, the stained cells were incubated in the supplied Dye-Release Reagent. The contents of each dish were transferred to a 96-well plate, and the absorbance at 550 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, USA).

Measurement of the oxygen consumption rate (OCR)

To evaluate mitochondrial function, both RAW 264.7 and CCAR2-KO RAW 264.7 cells were induced to differentiate into osteoclasts. After differentiation, the cells were plated into Seahorse XF24 microplates (Agilent Technologies, Santa Clara, USA) at a density of 1×10^5 cells per well. The OCR was then measured using the XF24 extracellular flux analyzer (Agilent Technologies) in accordance with the manufacturer's instructions provided in the XF Cell Mito Stress Test Kit (Agilent Technologies). Briefly, respiration is measured under basal conditions, and in response to ATP synthase inhibitor oligomycin (1 μ M), followed by the addition of the ionophore 4-(trifluoromethoxy) phenylhydrazine (FCCP; 1 μ M) to

induce maximal mitochondrial respiration. Finally, respiration is stopped by adding the mitochondrial complex I inhibitors Rotenone and Antimycin A (0.5 μ M). The Wave software platform was applied for data analysis.

Apoptosis signal detection

In accordance with the manufacturer's protocol, TUNEL kit (G7360; Promega) was employed to detect apoptotic cells in osteoclasts derived from both RAW 264.7 cells and CCAR2-KO RAW 264.7 cells. Hematoxylin (MHS80; Sigma-Aldrich) was used for nuclear counterstaining. To assess intracellular reactive oxygen species (ROS) levels, differentiated osteoclasts were treated with H₂DCFDA (D399; Life Technologies, Eugene, USA), followed by staining of nuclei with Hoechst dye (HY-15559; MedChemExpress, Monmouth Junction, USA). The stained cells were visualized and images were captured using an APX100 microscope (Olympus, Nagano, Japan). The proportion of stained cells was analyzed using ImageJ (v1.53a) across six randomly selected fields, with results expressed relative to total cell counts.

Flow cytometry

Apoptosis was measured using an apoptosis detection kit (556547; Invitrogen) by flow cytometry analysis based on FITC-conjugated annexin V and propidium iodide staining. Cells were suspended in 100 μ L of binding buffer at a concentration of 1×10^6 cell/mL. Five microliters of annexin V-FITC and 5 μ L of propidium iodide were added to the suspended cells, and the cells were incubated in the dark at 37°C for 15 min. Data were acquired with a FACS Aria III (BD) installed in the Center for University-wide Research Facilities (CURF) at Jeonbuk National University. The analysis was performed using FlowJo software (Version 10.0.7; Tree Star, San Carlos, USA).

Immunofluorescence staining

Paraffin-embedded mouse bone tissue sections were deparaffinized and rehydrated, followed by antigen retrieval. The sections were then incubated overnight at 4°C with appropriately diluted CCAR2 Rabbit polyAb (1:100, 5693; Cell Signaling), Cathepsin K mouse mAb (1:100, sc-48353; Santa Cruz Biochemicals). After washing, the samples were incubated for 1 h at room temperature with appropriate Alexa Fluor 488-conjugated anti-rabbit IgG (1:500, A-1008; Invitrogen) or Alexa Fluor 594-conjugated anti-mouse IgG (1:500, A-11032; Invitrogen) secondary antibody. Finally, the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:200, D3571; Invitrogen) for 5 min to visualize nuclear DNA. The stained bone tissue sections were visualized and images were captured using an APX100 fluorescence microscope (Olympus).

The cells (1×10^4) were seeded per well on Millicell EZ glass slides (PEZGS0896; Sigma-Aldrich), and transfected HEK293T cells were fixed in 4% paraformaldehyde (158127; Sigma-Aldrich). Subsequently, cells were permeabilized using 0.3% Triton X-100 (648463; Sigma-Aldrich) for 10 min at room temperature in the dark. After blocking, cells were incubated overnight at 4°C with appropriately diluted HA mouse mAb (1:100, 11583816001; Roche Diagnostics), FLAG Rabbit polyAb (1:100, F7425; Sigma-Aldrich). After washing, the samples were incubated for 1 h at room temperature with appropriate Alexa Fluor 488-conjugated anti-rabbit IgG (1:500, A-1008; Invitrogen) or Alexa Fluor 594-conjugated anti-mouse IgG (1:500, A-11032; Invitrogen) secondary

antibody. Finally, the nuclei were counterstained with DAPI (1:200), for 5 min to visualize nuclear DNA. The stained cells were visualized and captured using an APX100 fluorescence microscope (Olympus).

Luciferase assay

HEK293T cells were transiently co-transfected with FasL-luc, ER α , and CCAR2 expression plasmids using Lipofectamine 3000 to assess reporter gene activity. Following incubation, the cells were lysed using passive lysis buffer (E1941; Promega). Promoter activity was quantified by measuring luciferase signals with beetle luciferin substrate and a GLOMAX luminometer (Promega). All reagents for luciferase detection were obtained from Promega.

Molecular docking analysis

We obtained the protein structures of ER α and CCAR2 from the UniProt database and then processed them using PyMOL (Version 2.1; Schrödinger, New York, USA) and uploaded them to the HDock SERVER website (<http://hdock.phys.hust.edu.cn/>) for protein-protein docking. We selected the combination with the highest docking score and confidence score for simulating the docking site and used PyMOL for the visual analysis.

Statistical analysis

Quantitative results were reported as the mean $\pm$ standard error of the mean (SEM) to represent data variability. For pairwise group comparisons, statistical significance was assessed using the

unpaired two-tailed Student's *t* test. Graphs and tables were generated using GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, USA). A threshold of $P < 0.05$ was adopted to determine statistical relevance.

Results

Ccar2 in bone correlates positively with BMD in a panel of inbred mouse strains

To determine whether CCAR2 is associated with apoptosis in bone, we first assessed the mRNA expression of *Ccar2* in the femoral bones of 92 inbred mouse strains (UCLA GSE27483 BXD bone femur ILM Mouse WG-6 v1). Then, we observed how the expression transcripts correlates with the apoptotic pathway. The mRNA expression levels related to the apoptotic pathway (*Apaf1*, *FasL*, *Bid*, and *Bax*) correlated positively with *Ccar2* mRNA (Figure 1A–D). Conversely, mRNA levels related to the anti-apoptotic pathway (*Bcl2*, *Hspa1b*, and *Bag3*) correlated negatively with *Ccar2* mRNA (Figure 1E–G). Furthermore, we observed that the *Ccar2* mRNA level correlated positively with femur and lumbar spine BMD (<http://phenome.jax.org>, Dataset: HMDPpheno1) [25] (Figure 1H,I). These results indicate that *Ccar2* expression in bone is closely associated with apoptotic signaling and is essential for maintaining bone homeostasis.

The expression of CCAR2 by osteoclasts is attenuated in old mice

To evaluate the role of CCAR2 in osteoclasts during aging, CD11b+

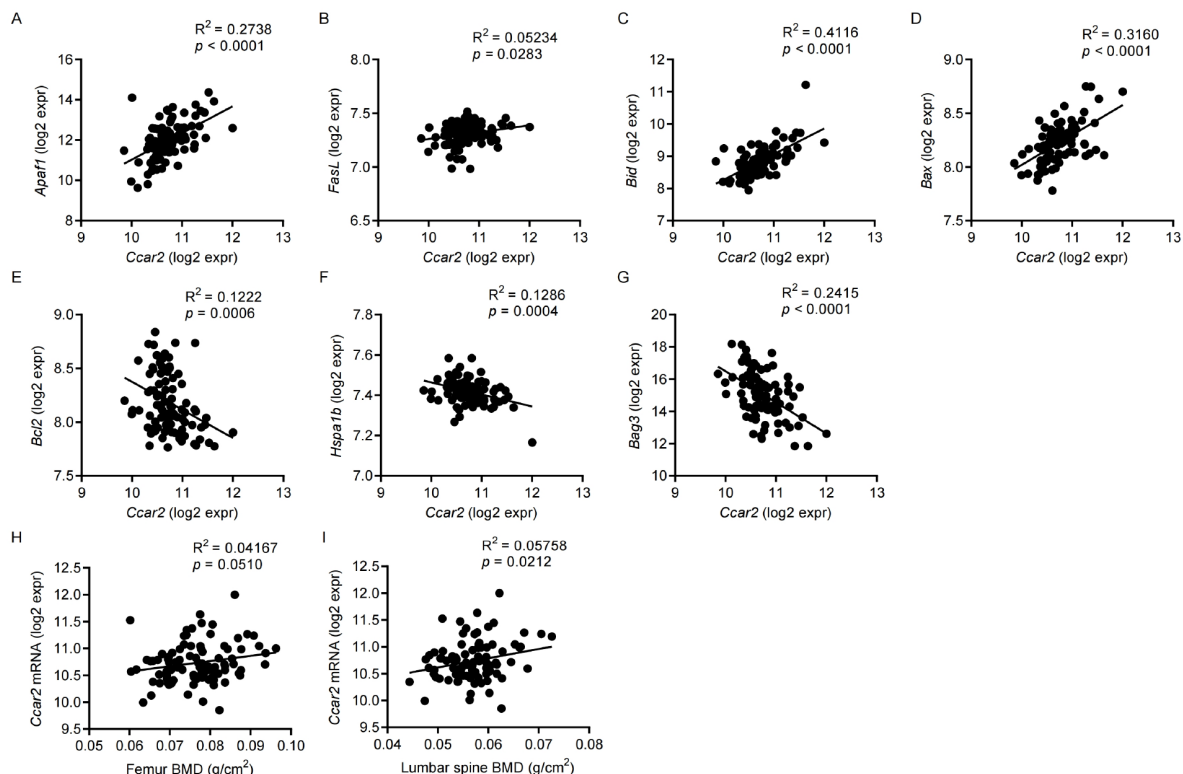


Figure 1. The relationship between *Ccar2* and transcripts involved in the apoptotic pathway and bone mineral density (BMD) in mice (A–G) Correlation between *Ccar2* transcript expression and apoptosis-related transcript expression or antiapoptosis-related transcript expression in 92 inbred mouse strains ($n = 92$). (H,I) Correlation between *Ccar2* transcript expression and BMD in 92 mouse strains ($n = 92$). The Pearson correlation coefficients and P values between *Ccar2* transcript expression and the variables were obtained using GraphPad Prism software and are indicated in the upper right corner of each graph.

cells were isolated from the bone marrow of young (3-month-old) and aged (20-month-old) mice and subsequently differentiated into osteoclasts to examine CCAR2 expression. When we analyzed protein lysates after treatment with osteoclast differentiation for three days, the expression of CCAR2 was markedly attenuated in osteoclasts from old mice (Figure 2A). Moreover, when osteoclast differentiation was induced through M-CSF and RANKL, the number of TRAP-positive osteoclasts in CD11b⁺ cells extracted from old mice was much higher than that in CD11b⁺ cells extracted from young mice (Figure 2B). Histologically, CCAR2 was strongly expressed in cells surrounding the primary spongiosa of young mice, but that expression was not observed in old mice (Figure 2C). In contrast, TRAP-positive cells were significantly increased in old mice compared with young mice (Figure 2D). In addition, the expression of cathepsin K, which is a marker for osteoclasts, and CCAR2 double-positive cells in young mice was significantly higher than that in old mice (Figure 2E). These results suggest that the role of CCAR2 in osteoclasts is associated with aging.

Downregulation of CCAR2 increases osteoclastogenesis

We next created *CCAR2*-KO RAW 264.7 cells using the CRISPR-Cas9 technique to identify the role of CCAR2 in osteoclastogenesis. Control RAW 264.7 cells exhibited strong CCAR2 expression after the induction of osteoclastogenesis, whereas the *CCAR2*-KO cell

clones lost their CCAR2 expression (Figure 3A). Because the ER α -FasL axis plays a pivotal role in osteoclast apoptosis [11,12] and CCAR2 directly interacts with ER α to regulate apoptosis [23,24], we evaluated ER α -FasL-mediated apoptosis signaling upon stimulation with osteoclast differentiation in control and *CCAR2*-KO cells. We assessed the activation of ER α -FasL-mediated apoptotic signaling pathways in both control and *CCAR2*-KO cells following stimulation with M-CSF and RANKL. Interestingly, the expression of ER α was observed to increase without a difference between the two groups upon osteoclastogenesis; however, under *CCAR2* knockout conditions, the expression of FasL and its downstream apoptotic molecules was significantly reduced (Figure 3A). The transcription of *FasL* was also reduced in *CCAR2*-KO cells, along with protein changes (Figure 3B). As in the old mice, *CCAR2*-KO significantly increased the number of osteoclasts upon osteoclast differentiation (Figure 3C). In addition, to validate the role of CCAR2 in osteoclasts, we isolated primary bone marrow cells from the femur and tibia, knocked down *CCAR2* using electroporation, and differentiated them into osteoclasts. Similar to the *CCAR2*-KO condition in RAW 264.7 cells, the number of osteoclasts significantly increased in the *CCAR2*-KO group compared to the empty vector group (Supplementary Figure S1). However, overexpression of CCAR2 did not significantly increase ER α -FasL-mediated apoptosis signaling compared with the control group, and the number of osteoclasts did not

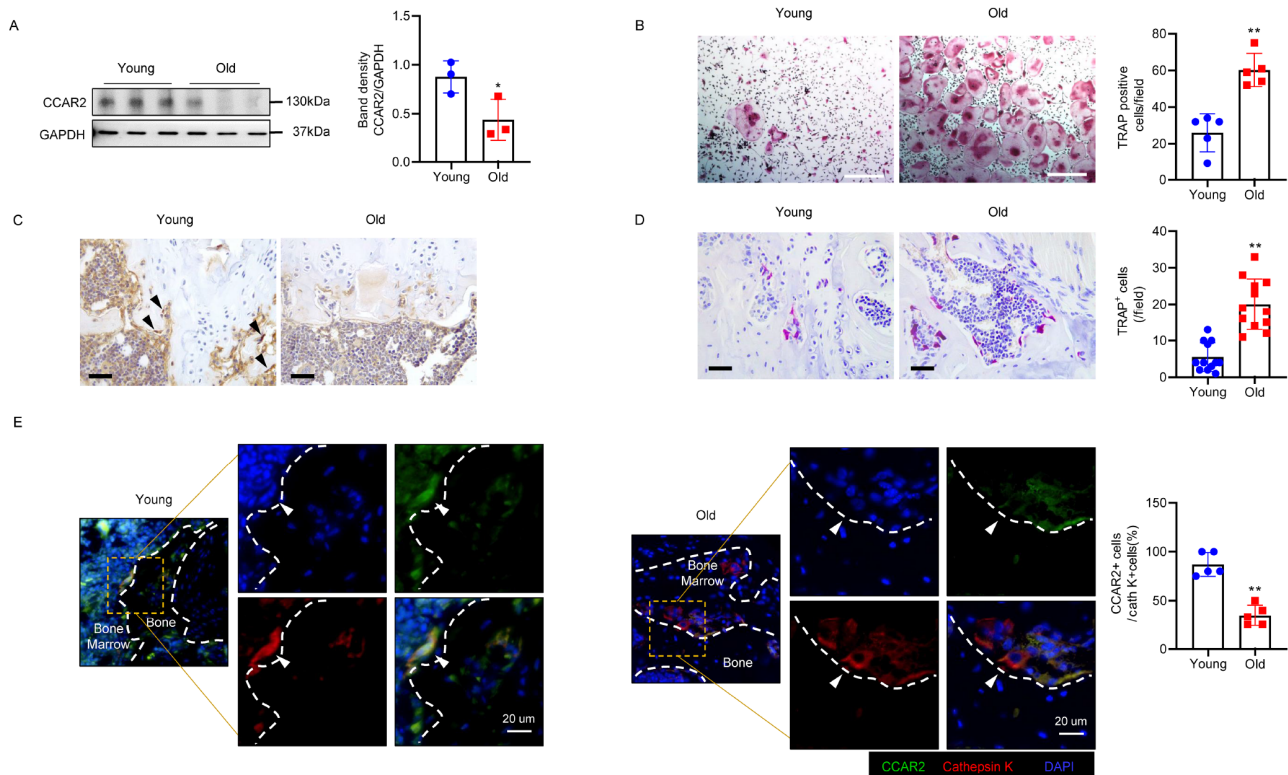


Figure 2. Decreasing expression of CCAR2 in osteoclasts from old mice (A) CD11b⁺ cells isolated from young (3-month-old) and aged (20-month-old) mice were subjected to osteoclast differentiation for three days, after which CCAR2 expression levels were assessed and quantified ($n = 3$). (B) TRAP staining was performed following osteoclast differentiation induction. TRAP-positive multinucleated cells were measured across five fields ($n = 5$) (scale bar = 200 μ m). (C) Representative immunohistochemical staining with the CCAR2 antibody (scale bar = 50 μ m). Arrowheads indicate CCAR2⁺ cells. (D) Representative TRAP staining (scale bar = 50 μ m). (E) Representative immunofluorescence staining in young and old bone tissue. Arrowheads indicate cathepsin K⁺ cells (scale bar = 20 μ m). The values are the mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ versus young mice.

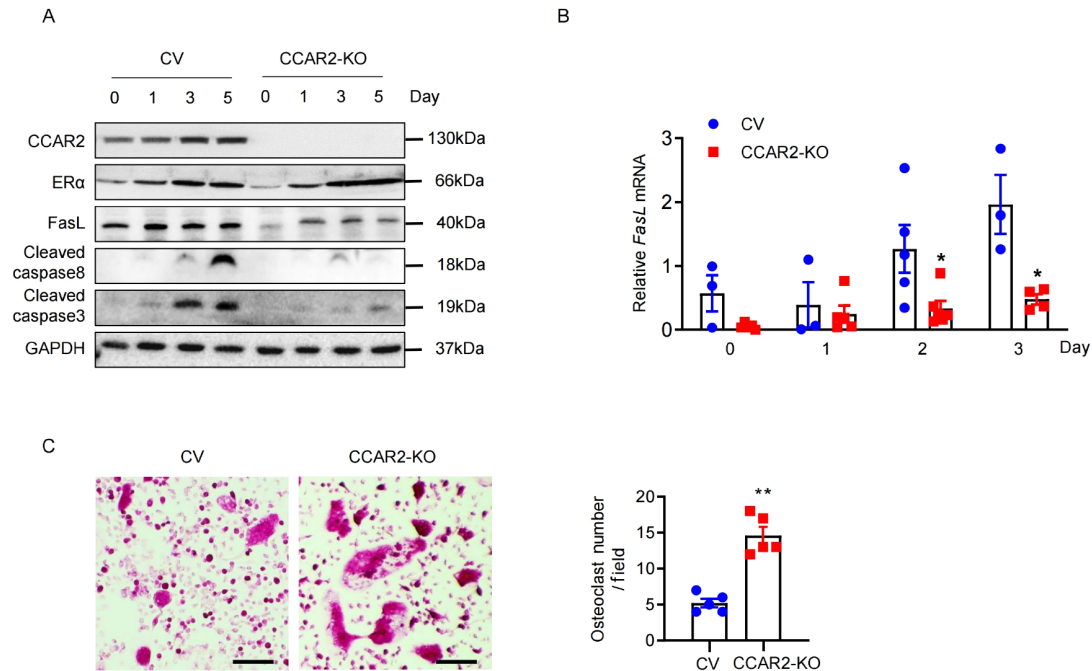


Figure 3. Increasing number of osteoclasts with loss of CCAR2 Control RAW 264.7 cells (treated with Cas9 vehicle) and *CCAR2*-KO RAW 264.7 cells were induced to undergo osteoclast differentiation for the indicated time periods. (A) Time-dependent expression of proteins involved in the extrinsic apoptotic pathway during osteoclast differentiation. (B) Time-dependent *FasL* mRNA expression ($n = 4-5$). (C) TRAP staining was performed on control and *CCAR2*-KO RAW 264.7 cells following 5 days of osteoclast differentiation, and TRAP-positive cells were quantified ($n = 5$) (scale bar = 40 μ m). * $P < 0.05$ and ** $P < 0.01$.

differ significantly (Supplementary Figure S2). These results suggested that the increased level of CCAR2 protein during osteoclast differentiation is sufficient to suppress osteoclast number. Meanwhile, to confirm the role of CCAR2 in osteoblasts, we analyzed public data (GSE169224) showing changes in the transcriptome of C3H10T1/2 cells, a well-characterized mouse embryonic mesenchymal stem cell (MSC) line, which was differentiated into osteoblasts for 3 days. No significant changes in the transcript levels of CCAR2 were observed before and after osteoblast differentiation (Supplementary Figure S3A). Additionally, we downregulated the expression of CCAR2 in preosteoblastic MC3T3-E1 cells (Supplementary Figure S3B) and confirmed mineralization after differentiation into osteoblasts. The reduction in CCAR2 did not significantly change ALP staining and alizarin red S staining compared to the control group (Supplementary Figure S3C,D). These results suggest that CCAR2 has a less significant effect on osteoblast differentiation.

Downregulation of CCAR2 attenuates apoptosis and reduces mitochondrial ROS production

Consistent with the decrease in *FasL* expression, the attenuation of apoptosis in *CCAR2*-KO cells was observed in the APOPercentage apoptosis and TUNEL staining tests (Figure 4A,B). To examine the role of CCAR2 in mitochondrial function during apoptosis, we used H2DCFDA and Hoechst staining to assess intracellular ROS production in early-stage osteoclasts. The results showed a significant reduction in intracellular ROS levels in the *CCAR2*-KO group (Figure 4B). Flow cytometry apoptosis analysis also showed reduced apoptosis of *CCAR2*-KO cells before osteoclast differentia-

tion and a much more significant difference between the apoptosis of control and *CCAR2*-KO cells after osteoclast differentiation (Figure 4C). Given the links between CCAR2 and reduced mitochondrial ROS generation and apoptosis, we measured the OCR in RAW 264.7 cells treated with M-CSF and RANKL to evaluate changes in mitochondrial function. Notably, *CCAR2*-KO cells exhibited a significant increase in maximal respiration (Figure 4D). However, as with osteoclast numbers in RAW 264.7 cells overexpressing CCAR2, CCAR2 overexpression did not result in significant changes in apoptosis, ROS, or OCR compared to the control group (Supplementary Figure S4).

CCAR2 translocates ERα to the nucleus and increases its transcriptional activity

Total ERα protein levels did not differ between the control and *CCAR2*-KO cells (Figure 3A). However, decreased expression of *FasL* and its downstream molecules and suppression of apoptosis were observed in *CCAR2*-KO cells, confirming that CCAR2 posttranslationally regulates ERα. As expected, in the luciferase reporter assay using ERE-Luc and *FasL*-Luc, overexpression of CCAR2 enhanced ERα transcriptional activity (Figure 5A,B). Since these results imply that CCAR2 can bind to ERα and enhance its transcriptional activity, we confirmed the degree of binding between the two proteins using the HDock server. The docking score of ERα (red) and CCAR2 (blue) on the HDock server was determined to be -257.33 kcal/mol, and the confidence score was 0.8954, indicating a high binding interaction of the two proteins (Figure 5C). Co-immunoprecipitation experiments confirmed a direct physical association between CCAR2 and ERα (Figure 5D).

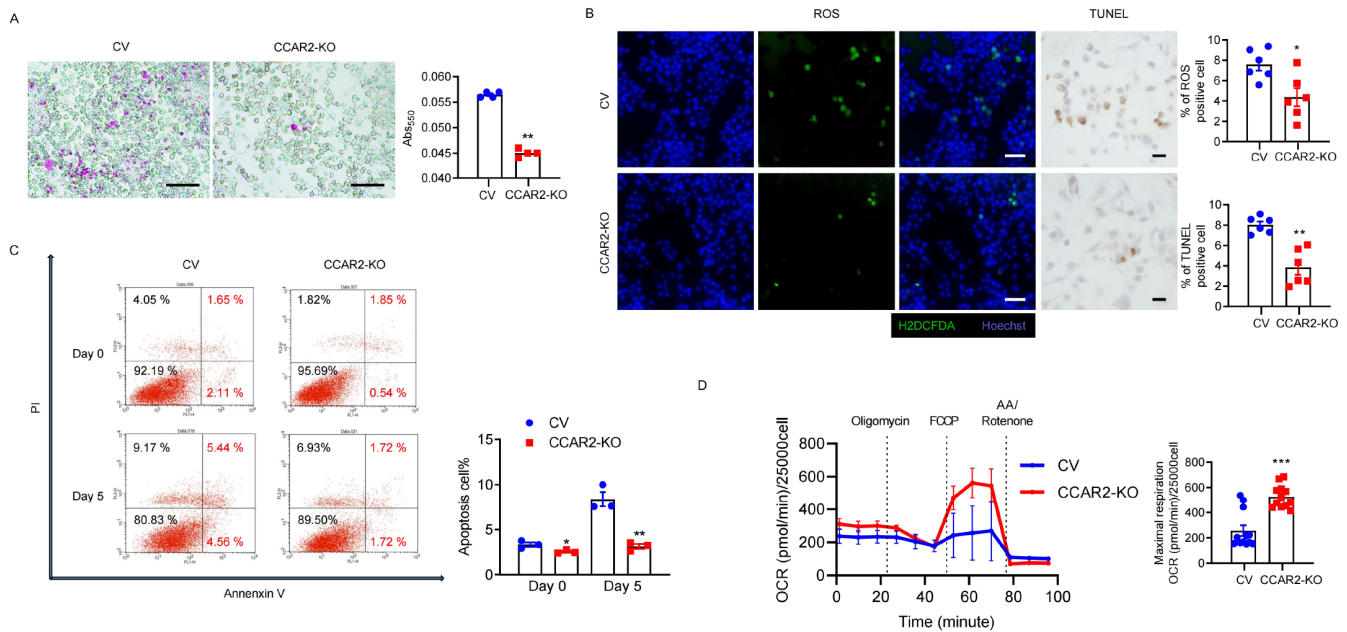


Figure 4. Attenuation of apoptosis with loss of CCAR2 (A) Apoptosis was assessed using an APOPercentage assay kit after treating cells with osteoclast differentiation for three days. Quantification of pink-stained apoptotic cells was performed by measuring absorbance at 550 nm. ($n = 4$) (scale bar = 100 μ m). (B) Apoptosis was assessed using a TUNEL kit. Cells were subjected to osteoclast differentiation for three days (scale bar = 40 μ m). ROS were measured using H2DCFDA (10 μ M) and Hoechst staining, and cells were imaged using an APX-100 microscope. The images were collected from six individual measurements of all the conditions ($n = 6$). Apoptotic cells appear dark brown and were counted in each field ($n = 6$). (C) Flow cytometry analysis performed with propidium iodide and annexin V staining. Control and CCAR2-KO cells were subjected to osteoclast differentiation for the indicated time periods. (D) The Seahorse Mito stress test was performed in control and CCAR2-KO RAW 264.7 cells after they were treated with osteoclast differentiation for three days. The oxygen consumption rate (OCR) and maximal respiration values are the mean $\pm$ SEM of 4 independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vector.

Because a difference in transcriptional activity was observed without a change in the total ER α protein level, it is possible to consider the movement of ER α from the cytosol to the nucleus. Therefore, we isolated cytoplasmic and nuclear proteins according to the presence or absence of CCAR2 and confirmed the change in ER α . In the absence of CCAR2 after osteoclast differentiation, ER α was observed more in the cytoplasm than in the control and decreased in the nucleus (Figure 5E,F). Conversely, when CCAR2 was overexpressed, ER α in the nucleus increased compared with that in the control (Figure 5G). Immunofluorescence staining revealed that, in the presence of CCAR2, ER α was fully translocated from the cytoplasm to the nucleus (Figure 5H). These results suggested that CCAR2 directly binds to ER α and increases its nuclear expression, thereby enhancing ER α 's apoptotic function.

Discussion

Apoptosis regulates osteoclast numbers during osteoclastogenesis and bone remodeling. Mechanistically, anti-osteoporotic drugs correct the imbalance of osteoclasts in osteoporosis by regulating osteoclast apoptosis [26]. CCAR2 is a critical regulator of cell death through mitochondrial dysregulation and the inhibition of SIRT1 activity [27]. However, the role of CCAR2 in osteoclast apoptosis has not yet been elucidated. In this study, CCAR2 was attenuated in osteoclasts from 20-month-old mice, and we showed that CCAR2 KO using CRISPR-Cas9 in RAW 264.7 cells caused an increase in the number of osteoclasts and the inhibition of apoptosis after osteoclast differentiation. These results are consistent with the significant correlations we found between *Ccar2* and other

apoptotic factors and BMD in transcripts from the femoral bones of a large number of inbred mouse strains. We demonstrated that CCAR2 regulation of apoptosis in osteoclasts occurs by direct binding between CCAR2 and ER α and the activation of FasL through cytoplasmic-to-nuclear ER α translocation (Figure 5G).

The expression of CCAR2 in the osteoclasts of old mice differed from that in the osteoclasts of young mice in response to the same amounts of osteoclast-inducing cytokines. This is thought to be a difference in the response of intrinsic factors to external stimuli, probably because aging *per se* induces changes in cells. Age-associated changes in the proteins of osteoclasts have been reported in relation to cellular senescence (p16) [28], factors associated with aging (SIRT6) [6], and osteoclast differentiation cytokine-binding receptors (*c-fms* and RANK) [29]. In these studies, when osteoclast differentiation was induced in bone marrow monocytes from 3-month-old and 20-month-old female mice using the same dose of M-CSF and RANKL, the number of osteoclasts from the old mice was significantly greater than that from the young mice. These results have been confirmed in human subjects, suggesting that the internal factors of osteoclasts are essential to progressive bone loss with advancing age in humans [6,30]. Several possible intrinsic factors in osteoclasts can be considered to regulate the number of osteoclasts at different ages. One possibility is epigenetic changes during aging. Various kinds of epigenetic alterations, such as histone posttranslational modifications, DNA methylation, protein acetylation, and altered noncoding RNA expression, occur during aging [31]. These changes lead to aberrant gene expression and genomic instability. According to a recent report [7], hypomethyla-

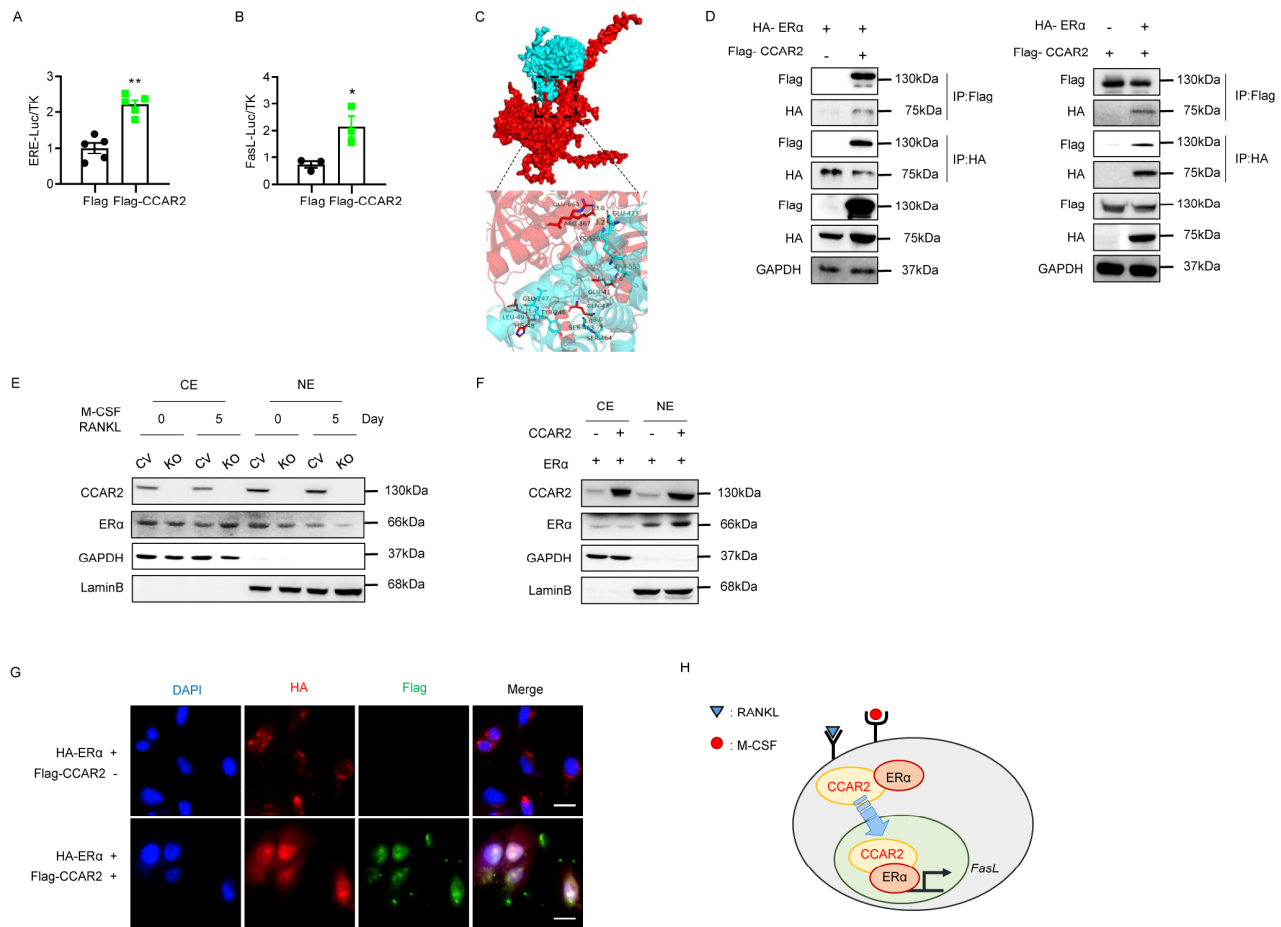


Figure 5. Translocation of ERα from the cytosol to the nucleus by CCAR2 (A,B) HEK293T cells were transfected with Flag or Flag-CCAR2 in the presence of ERα, and the ERE and FasL luciferase activities were measured ($n = 5$ and $n = 3$). (C) Molecular docking analysis of ERα (red) and CCAR2 (blue). (D) Co-IP revealed the interaction between ERα and CCAR2-interacting proteins. (E) Control and CCAR2-KO RAW 264.7 cells were subjected to osteoclast differentiation for the indicated time periods, and ERα protein levels in the cytosolic extract (CE) and nuclear extract (NE) were determined. (F) HEK293T cells were transfected with CCAR2 and ERα as indicated, and the ERα protein levels in the CE and NE were determined. (G) HEK293T cells underwent immunofluorescence staining after transfection with the indicated plasmids (scale bar = 20 μm). (H) Working model of the mechanism by which CCAR2 regulates ERα. * $P < 0.05$ and ** $P < 0.01$.

tion of the promoter region of the *TM7SF4* gene, which is the regulator of osteoclastogenesis, was observed in osteoclasts from elderly people, supporting this possibility. Additionally, many epigenetic changes associated with CCAR2 have been reported to affect cell proliferation and apoptosis. Acetylation at the N-terminus of CCAR2 has been reported to inhibit the expression of the Wnt downstream signals *MYC* and *MMP7* by interfering with the functions of the Wnt coactivator [32]. CCAR2 also inhibits the function of SIRT1, a deacetylase, thereby increasing the acetylation of p53 and FOXO, which participate in apoptosis [21]. Another possibility is that aging is associated with autophagy and oxidative stress in osteoclasts [33]. Research indicates that alterations in autophagy and consequent oxidative stress influence osteoclast activity as people age [33]. Selective osteoclast deletion of Atg7 or Atg5, which plays a role in the unique development of the autophagosomal membrane and fusion within cells, significantly reduced osteoclast differentiation and bone degradation induced by ovariectomy [34]. Interestingly, Iqbal *et al.* [35] pointed out that CCAR2 binds to SIRT1 and blocks the deacetylation function of SIRT1, thereby inhibiting the onset of autophagy. The evidence thus

suggests different potential mechanisms by which CCAR2 drives skeletal changes in aged mice and humans.

The integrated state of cell proliferation, differentiation, and apoptosis determines cell numbers. In the present work, we evaluated the function of CCAR2 in apoptosis and how it influences osteoclast numbers. The expression of CCAR2 gradually increased for 5 days during osteoclast induction by M-CSF and RANKL and increased in a pattern similar to that of apoptosis-related proteins. In the same study, we found that apoptosis was reduced in CCAR2-KO osteoclasts, and we confirmed this finding with various immunostaining and FACS analyses. Furthermore, CCAR2 exhibited a strong positive correlation with apoptosis-related transcripts, and an opposite pattern was noted for anti-apoptosis-related transcripts in inbred mouse strains. Last, our study demonstrated that CCAR2 interacts directly with ERα, a key modulator of osteoclast apoptosis, promoting its nuclear translocation and transcriptional activity, which are associated with apoptosis. Therefore, we anticipated CCAR2 to be more closely associated with osteoclast apoptosis than with the regulation of osteoclast differentiation. The relationship between CCAR2 and ERα adds

further evidence for CCAR2 regulation of osteoclast apoptosis. Previous studies have shown that CCAR2 interacts with ER α in both estrogen-dependent and estrogen-independent manners to regulate cell survival and apoptosis, particularly in cancer cells [36]. In CCAR2-KO osteoclasts, ER α was retained in the cytoplasm, impairing its ability to induce apoptosis via FasL. Conversely, CCAR2 overexpression enhanced ER α activity, as demonstrated by increased nuclear localization and transcriptional activation of FasL. This relationship underscores the critical role of CCAR2 as a co-regulator of estrogen receptor signaling in osteoclasts. The diminished expression of CCAR2 with aging likely exacerbates the effects of estrogen deficiency on osteoclast survival, contributing to increased bone resorption and reduced BMD. Targeting CCAR2 to enhance its expression or function could restore ER α -mediated apoptotic signaling in osteoclasts, offering a novel therapeutic strategy for age-related bone loss and osteoporosis.

ROS are crucial for promoting osteoclast differentiation [34]. It has been suggested that mitochondrial ROS accumulate in osteoclasts under hypoxic conditions, inducing osteoclast differentiation via mitochondrial respiratory stress signaling. The mitochondria-specific antioxidant mitoQ reversed this effect [37]. However, ROS behave differently in relation to osteoclast apoptosis. Bisphosphonates, an osteoporosis treatment agent known to induce osteoclast apoptosis, inhibit osteoclasts through a ROS-mediated apoptotic pathway [38]. Additionally, 4-hydroxyequilenin, which is a minor component of the estrogen replacement formulation, has been reported to form ROS in breast cancer cells via ER α and induce DNA damage and apoptosis, supporting the role of ROS in apoptosis [39]. Additionally, our results show a decrease in ROS in CCAR2-KO cells in an anti-apoptotic state, suggesting that reduced cellular ROS leads to diminished apoptosis.

In this study, we found that the number of osteoclasts in the endosteal region of the cortical bone of the femur increased in aged mice compared to young mice. Additionally, we observed lower expression of CCAR2 in osteoclasts in the cortical endosteal region of the femur of aged mice. This observation was made in a manner similar to the increased number of osteoclasts in the femoral cortex reported in aged mice in other studies and is thought to be involved in *de novo* osteonal remodeling leading to femoral cortical porosity [40]. However, osteoclast numbers and activity differ between cortical and cancellous bone in older age. Fewer osteoclasts were observed in the cancellous bone of the vertebral column of aged mice [41] and in the cancellous bone of the radius of aged patients [42]. This suggests that different mechanisms influence changes in osteoclasts with age in cortical and cancellous bone.

Although bone homeostasis is regulated by various cell types in the bone microenvironment within the basic multicellular unit (BMU), such as osteoclasts, osteoblasts, osteocytes, bone lining cells, osteomacs, vascular endothelial cells, and immune cells [43], this study has the limitation of focusing on ER α , which is regulated by CCAR2, which is confined to osteoclasts. Various studies have reported that ER α is closely related to the apoptosis of osteocytes, monoblastoid cells, and B cells [44–46]. However, the role of CCAR2 in cells that affect bone homeostasis, other than its role in osteoclasts identified in this study, has not yet been reported; thus, its role needs to be confirmed in the future.

In summary, our study identifies CCAR2 as a key regulator of osteoclast apoptosis through its interaction with ER α and facilitation

of FasL transcription. By promoting ER α nuclear translocation and activity, CCAR2 enhances pro-apoptotic signaling, thereby limiting osteoclast survival and bone resorption. The age-dependent decline in CCAR2 expression impairs this regulatory mechanism, contributing to increased osteoclast numbers and age-related bone loss. These findings provide new insights into the molecular interplay among CCAR2, ER α , and osteoclast apoptosis and suggest that CCAR2 could be a potential therapeutic target for preventing bone loss in aging and estrogen-deficient conditions.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

The datasets generated and/or analyzed during the current study are available at the NCBI GEO with accession numbers GSE27483 and GSE169224, and the data file will also be available on request at yjmoonos@jbnu.ac.kr.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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