Increased level of Complement C3 in patients with osteoporosis and enhanced osteogenic ability in osteoblasts with Complement component 3 knockdown

Kang Liu (liukang1982@163.com)
The Second Affiliated Hospital of Zhejiang Chinese Medical University
Yang Yu
Zhejiang Chinese Medical University
Minghao Tong
Zhejiang Chinese Medical University
Binbin Tang
The Second Affiliated Hospital of Zhejiang Chinese Medical University
Xiaolin Shi
The Second Affiliated Hospital of Zhejiang Chinese Medical University

Research Article

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Abstract

Objective(s):Complement C3 has the potential to impact bone homeostasis. The purpose of this study is to determine the difference in serum Complement C3 levels between osteoporosis patients and non-osteoporosis patients, as well as how Complement C3 silencing impacts and works on osteoblast osteogenesis.

Materials and Methods: 58 people who underwent dual energy X-ray bone mineral densit(BMD) measurement in The Second Affiliated Hospital of Zhejiang Chinese Medical University from June to September 2021 were analyzed retrospectively. According to the classification of BMD t value, the subjects were divided into 31 cases in osteoporosis group and 27 cases in non- osteoporosis group. Complement C3 levels were recorded. and compared between the two groups. Then, the Complement C3 knockdown lentivirus was used to transfect Raji. The Raji-osteoblast line MG63 co-culture system was constructed after the stable strain was screened. CCK-8 and flow cytometry were used to assess the proliferation and apoptosis of MG63, alkaline phosphatase (ALP) activity was used to evaluate the osteogenic ability of MG63, and RT-PCR was used to assess the expression level of osteoprotegerin（OPG） of MG63.

Results: Complement C3 levels in osteoporosis patients' serum are elevated，the proliferation level and osteogenic ability of MG63 were improved in Complement C3 knockdown Raji-osteoblast MG63 co-culture system. Furthermore, OPG expression in MG63 was enhanced**.**

Conclusion:To sum it up, the level of Complement C3 in the serum of osteoporosis patients is elevated.Knocking down Complement C3 boost osteoblast osteogenic ability, which may be achieved by controlling OPG / RANKL / RANK system.

1. Introduction

Bone homeostasis refers to the dynamic equilibrium between the quantity and activity of osteoblasts and osteoclasts, and osteoporosis occurs when bone homeostasis is imbalanced [1, 2]. Numerous studies in recent years have confirmed the linkage between the complement system and bone homeostasis [3, 4]. Complement component 3 (C3) is a crucial link between osteoblasts and osteoclasts, and it plays an essential part in bone homeostasis. C3a promotes osteoclast formation [5, 6], and its complement receptor C3aR is strongly upregulated during the differentiation of human MSCs into mature osteoblasts [5, 7]. C3 deficiency is associated with systemic lupus erythematosus, which often presents with a higher risk of osteoporosis [8, 9]. C3 also influences the growth and differentiation of bone cells, and it seems to play specific functions in growth plates during bone development. C3 is mostly expressed in the growth plate's resting and proliferative zones[10]. In rats, the epiphyseal growth plate thickens due to a lack of C3, which means cartilage ossification is delayed[11]. C3 and C3a act as two-way signals: osteoblast-derived C3 affects osteoclasts, and osteoclast-derived C3a stimulates osteoblast differentiation [6]. Whether the level of Complement C3 changed in patients with osteoporosis and the mechanism underpinning C3's

influence on bone homeostasis, however, have yet to be discovered. The goal of this research was to identify the expression level of Complement C3 in the serum of patients with osteoporosis and non-osteoporosis as well as investigate the particular mechanism which Complement C3 affects osteoblast bone growth by measuring the proliferation, apoptosis, alkaline phosphatase (ALP) activity, and osteoprotegerin (OPG) expression in MG63 cells using a Raji-MG36 co-culture system with C3 knockdown.

2. Materials And Methods

2.1. Subjects

From June to September 2021, the patients who were admitted to our hospital for bone mineral density(BMD) tests were collected. Exclusion criteria: (1)Diabetes, Cushing syndrome, Thyroid or parathyroid dysfunction, osteomalacia, rheumatoid arthritis, multiple myeloma, osteoarthropathy, osteogenesis imperfection, and other disorders that disrupt bone or calcium metabolism are all examples of disorders that disrupt bone or calcium metabolism; (2)Have taken estrogen, steroid hormones, calcitonin, parathyroid hormone, bisphosphate, fluoride, vitamin D, anticonvulsant, diuretic, or other bone-related medicines in the last 6 months; After removing the influencing factors that have a significant impact on the results of a bone mineral density test, 58 cases were selected for retrospective analysis and divided into osteoporosis group (OS, n = 31) and non osteoporosis group (NOS, n = 27) according to the diagnostic criteria of dual energy X-ray bone mineral density (DXA) of Chinese osteoporosis (2018). The ethics review committee of The Second Affiliated Hospital of Zhejiang Chinese Medical University[2019-KL-007-01] has accepted this research design. This study is exempt from requiring written consent because it is the gathering or investigation of previously archived data, records, or diagnostic specimens, and these are open-access resources.

2.2. BMD measures

The lumbar spine and right hip BMD were assessed by DXA scanner(General Electric Company). The lumbar spine measurement range includes two to four vertebral bodies, the femoral neck, and the femoral trochanter. All lumbar imaging pictures were reviewed independently by two competent radiologists to determine the presence of a vertebral fracture in each case. If there is a disagreement, it must be resolved through conversation.

2.3. Complement C3 level measures

All individuals had 5ml of fasting venous blood collected, which was kept at room temperature for 0.5 hours before centrifuging at 3000 G at the speed of relative centrifugal force for 10 minutes, serum separation, and computer detection within 2 hours. According to the manufacturer's instructions, ELISA was used to measure the protein expression levels of Complement C3.

2.4. Cell culture

Raji and MG63 cells (ATCC, Shanghai, China) were grown at 37 ℃ in 5% CO_2 in DMEM (Gibco) supplied with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). The 293A cell line (ATCC, Shanghai, China) was used to construct a lentivirus containing shC3.

2.5. Construction of recombinant lentivirus

The following sequences were used in C3 siRNA:

Forward:5’-GATCCCCAAGACACCCAAATTCTTCTCGAGAAGAATTTGGGTGTCTTGGTTTTTA-3’

Reverse:5’-AGCTTAAAAACCAAGACACCCAAATTCTTCTCGAGAAGAATTTGGGTGTCTTGGG-3’

Both the forward and reverse sequences were inserted into PLKO.1 (Addgen, Shanghai). Lentiviruses were made by utilizing Lipofectamine 2000(Invitrogen) to triple transfect 90 percent confluent 293A cells with pLKO.1-shC3, psPAX2, and pMD2G (Addgen. Shanghai). After transfection, the medium was replaced with full media for 4–6 hours, and the viral solution was collected after 72 hours. For lentivirus transfection, Raji cells were planted in 24-well plates and divided into three groups, each with 3 wells: blank control, empty vector, and recombinant lentivirus interference.The recombinant lentivirus interference group was infected with C3-shRNA [shC3 (forward)/(reverse)], the empty vector group was infected with control shRNA (shCon), and the blank control group was added PBS in equal amount to other groups for 48 h in serum-free medium.Subsequently, the medium was replaced with regular DMEM containing 10% fetal bovine serum, and the cells were cultivated for 96 hours at room temperature in 5% CO2. The C3 primer in the vector was sent to Shanghai Meiji Biological Co, Ltd. for sequencing. The sequencing results were compared with the original sequence, and there were no base deletions, mutations, and shifts, indicating that the vector had been successfully constructed.

2.6. Co-culture of Raji and MG63 cells *in vitro*

MG63 cells in the logarithmic growth phase were digested with trypsin (Solarbio, Beijing, T1300-100) and prepared as cell suspension after counting under the microscope. Three groups of cells were separated from the cell suspension, with 300 μl for each group in a 24-well culture plate, inoculated in 3 wells per group, 1×10^4cells / well, and co-cultured with transfected Raji cells at 37 ℃.

2.7. RNA extraction and real-time PCR (RT-PCR)

Total RNA was extracted with Invitrogen's Trizol reagent, and cDNA was made following the manufacturer's instructions. The primer sequences for the test were as follows:

C3, forward: 5'-CACCAGCAGACCGTAACCATC-3'; reverse: 5'-GCAGCCTTGACTTCCACTTCC-3'

OPG, forward: 5'-ATCAGTTGGTGGGAATGAAG-3'; reverse: 5'-GGGATGACACAGAAGATAGTAG-3'

GAPDH, forward: 5'-GGAGTCTACTGGCGTCTTCAC-3'; reverse: 5'- ATGAGCCCTTCCACGATGC – 3'

The amplification conditions were: initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of denaturation at 95 ℃ for 15 sec and annealing extension at 60 ℃ for 15 sec. SYBR green dye (Invitrogen) was used to measure mRNA levels, and the comparative Ct method was used to calculate relative mRNA levels. GAPDH was used to standardize all reactions. The relative differences between PCR findings were calculated using Comparative Ct.

2.8. Western blotting analysis

The cells were lysed until completely cracked in RIPA lysate containing protease and phosphatase inhibitors (BYL40825, JRDUN, Shanghai, China), then ,centrifuged at 12000 rpm for 10 minutes at 4 ℃. The total protein concentration in the supernatant was determined using a BCA protein analysis kit(Beyotime, Shanghai, China). SDS-PAGE was used to separate the same amount of protein (25g) and transfer it to PVDF membranes (Bio-Rad). The membrane was blocked for 1 hour with 4% skimmed milk before being incubated overnight at 4 ℃ with the primary antibodies C3 (1:2000, Abcam), and GAPDH (1:2000, Abcam). The membrane was incubated in 37 ℃ for 1 hour with HRP-conjugated antibodies (Wuhan Boster Bioengineering Co, Ltd.). and then the enhanced ECL chemiluminescence reagent (Millipore) and GelDoc imaging system (Millipore) were used to quantify immunoreactive bands.

2.9. MG63 cell viability

After the corresponding incubation times (3 h, 6 h, 12 h, 24 h, 48 h, 72 h), Cell Counting Kit-8 (CCK-8) and serum-free essential basic medium were mixed in a 1:10 volume ratio, each test well received 300 ul of mixture, which was then incubated for 1 hour at 37 ℃ in a 5 percent CO_2 incubator. Absorbance at 450 nm was used to determine cell viability.

2.10. MG63 cell apoptosis analysis

To explore cell apoptosis,annexin V fluorescein isothiocyanate (FITC)-conjugated and propidium iodide were used to label the MG63 cells.The cells were then examined using a FACSCalibur flow cytometer (BD Biosciences).

2.11. ALP activity assay

Following the manufacturer's instructions, an ALP assay kit(Sigma) was used to detect ALP activity. In each well of a 24-well plate, cells were mixed with the substrate buffer. The reaction was terminated with 0.2 M sodium hydroxide after 15 minutes of incubation at 37 ℃. A microplate spectrophotometer was used to test sample absorbance at 405 nm.Following the manufacturer's directions, the amounts of proteins in cell lysates were measured using a BCA protein assay kit. Enzyme activity was measured in units per gram of protein. At pH 9.8 at 37 ℃, the release of 1 nmol p-nitrophenol per minute was defined as one unit of enzyme activity.

2.12 Statistical analysis

The data were all given as means ± standard deviation (mean ± SD). An independent sample t-test was used to compare the mean of the two groups of measurement data. One-way ANOVA with post hoc Tukey's multiple comparison tests was used to assess differences between data from three groups. SPSS 20.0 was used for the analyses (IBM). Statistical significance was assigned to values with $p < 0.05$.

3. Results

3.1. Complement C3 levels in osteoporosis patients' blood are abnormally high.

The level of Complement C3 expression in the serum of osteoporosis and non-osteoporosis patients was determined using an ELISA test. The findings of an ELISA test revealed that Complement C3 level rose in the serum of osteoporosis patients(FIg.1).

3.2. Lentivirus-mediated knockdown of C3 inhibited its expression

To determine whether C3 affects the osteogenic ability of MG63 cells, lentivirus-mediated shRNAs targeting C3 (Complement C3(F)/(R)) were used to suppress its expression in the Raji-MG63 co-culture system. RT-PCR analysis showed that, compared with the blank control or empty vector groups, the mRNA levels of C3 were significantly lowered in the recombinant lentivirus interference group in the Raji-MG63 co-culture system(Fig. 2A). Western blot analysis confirmed C3 knockdown(Fig. 2B and C). These findings suggested that C3 expression in Raji-MG63 Co-Culture System might be suppressed by recombinant lentivirus-mediated shRNA.

3.3. Knockdown of C3 promoted the proliferation of MG63 Cells

To investigate the proliferation of MG63 cells following C3 lentivirus transfection., CCK-8 assay was used to evaluate cell viability at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h. After 12 hours of co-culture, the proliferation of MG63 cells in the C3 knockdown group was considerably promoted than in the blank control or empty vector groups(Fig. 3A).

To further evaluate the influence of C3 on MG63 apoptosis, flow cytometry was employed to study cell cycle progression in the Raji-MG63 co-culture system. The percentage of apoptotic cells in the C3 knockdown group was much lower than in the control and empty vector groups, according to flow cytometry(Fig. 3B).

Then, to investigate the involvement of C3 in osteogenic differentiation, we measured ALP activity and found that the C3 knockdown group had higher ALP activity than the control and empty vector

groups(Fig. 4).

These results indicate that C3 knockdown promotes osteoblast proliferation, reduces osteoblast apoptosis, and enhances ALP activity.

3.4. Downregulation of C3 enhances OPG expression

In order to study the mechanism of C3 knockdown improving MG63 cell proliferation, reducing osteoblast apoptosis, and enhancing ALP activity, OPG expression was evaluated. RT-PCR analysis showed that OPG expression was elevated in the C3 knockdown groups(Fig. 5). These results suggest that C3 downregulation aided MG63 cells growth, possibly by modulating the OPG/RANKL/RANK system.

4. Discussion

C3 is the core of the complement system, which is the hub for the convergence of the classical, lectin, and alternative pathways in the complement activation pathway [12]. C3 has been linked to bone homeostasis in recent times, according to research. [1]. Sato et al. [13] showed that primary osteoblasts could produce C3, and that blocking C3 in bone marrow cell culture weakened osteoclast differentiation. The use of monoclonal antibodies to block C3 or the C3 receptor could significantly inhibit osteoclast differentiation of bone marrow mononuclear cells stimulated by 1,25-(OH)2-VitD3. In MM patients, C3a activates osteoclasts by modulating the PI3K/PDK1/SGK3 pathway [14]. MacKay et al. [15] found that C3 was revealed to be important in bone homeostasis after ovariectomy in mice. C3 deficiency resulted in less bone loss, improved trabecular microstructure, and potentially improved bone mechanical properties. However, this experiment did not directly reflect the effect of C3 on bone formation. The above experiments suggested that C3 could change bone homeostasis by affecting bone resorption, but its effect on bone formation and its mechanism were rarely explored.

Compared with previous studies, the present research investigated whether people with osteoporosis have a high level of Complement C3 in their blood and the role of C3 in promoting osteoblast bone formation. Firslyt, serum Complement C3 levels in osteoporosis and non-osteoporosis individuals were examined retrospectively, secondly, the shRNAs targeting the C3 gene were transduced into Raji cells by using lentivirus-mediated shRNAi. Then, the transfected Raji cells were co-cultured with the osteoblast line MG63.Silencing C3 increased the growth of MG63 cells and decreased their apoptosis, according to CCK-8 detection and flow cytometry.,suggesting that C3 may be a factor inhibiting osteoblast proliferation *in vitro*. Moreover, as an early marker of bone formation, ALP activity was measured to detect the role of C3 in osteogenic differentiation. The results confirmed that C3 deletion enhances osteoblast proliferation.

According to the theory that immune system cells govern bone formation and resorption, the combination of RANK and RANKL can boost osteoclast precursor cell differentiation and cause osteoclast maturation.OPG secreted by bone marrow stromal cells and OPG can competitively bind RANKL, as its N-terminal and the RANK structure have high similarity, blocking the combination of RANK and RANKL and

thereby inhibiting bone resorption [16]. OPG was detected in each group using RT-PCR.. The results revealed that OPG expression in the C3 silencing group was much higher than in the control group, implying that C3's effect on osteogenic ability could be done via modulating OPG levels and then altering the OPG / RANKL / RANK system.This research demonstrated that C3 silencing could enhance the bone formation ability of MG63 cells. MacKay et al. [15] also found that C3 was involved in bone homeostasis after ovariectomy in mice. The loss of C3 reduced bone loss, improved trabecular microstructure, and potentially improved bone mechanical properties. However, Matsuoka et al. [17] found that C3a showed osteoblast stimulating activity in mature osteoclast medium, and osteoclast conditional medium promoted osteoblast differentiation through C3aR signaling. The tibial metaphyseal bone loss of ddY mice treated with C3aR antagonist was greater than that of untreated mice. However, MacKay [15] believe that, in the study by Matsuoka et al. [17], the analysis of some experiments *in vivo* was limited to a single anatomical site, without mechanical testing. The role of C3 in osteoclasts or osteoblasts over time is also uncertain.

In summary, the level of Complement C3 in the serum of osteoporosis patients was found to be elevated in this study, and after the establishment of the Raji-MG63 co-culture system, by detecting the proliferation and apoptosis of MG63 osteoblasts, ALP activity, and OPG expression, this study provides new information that silencing C3 could enhance the bone formation ability of osteoblast MG63 cells, which might be achieved by affecting the OPG / RANKL / RANK signaling pathway, but the specific mechanism needs further study.

5. Conclusion

To sum it up, Complement C3 level in the serum of osteoporosis patients is elevated and knocking down Complement C3 can boost osteoblast osteogenic ability, which may be achieved by controlling OPG / RANKL / rank system. More research into the specific mechanism is required.

Declarations

Acknowledgments

None.

Authors’ contributions

LK and XS designed the study. YY, TM, TB performed the experiments. YY, and TB analyzed data. YY and TM wrote the manuscript. All authors read and approved the final manuscript.

Founding

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request

Declarations

Ethics approval and consent to participate

The hospital's Institutional Review Board approved the current study

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Figures

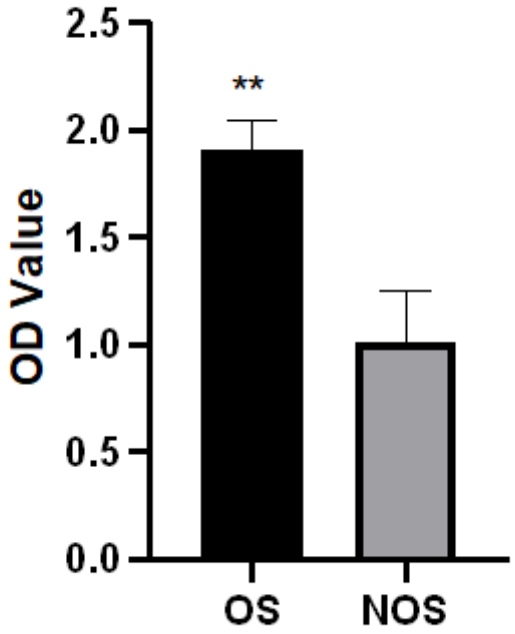

Figure 1

Complement C3 level rose in the serum of osteoporosis patients.

The assay indicating that Complement C3 level rose in the serum of osteoporosis patients. The data are presented as the means ± SD , **p<0.01.

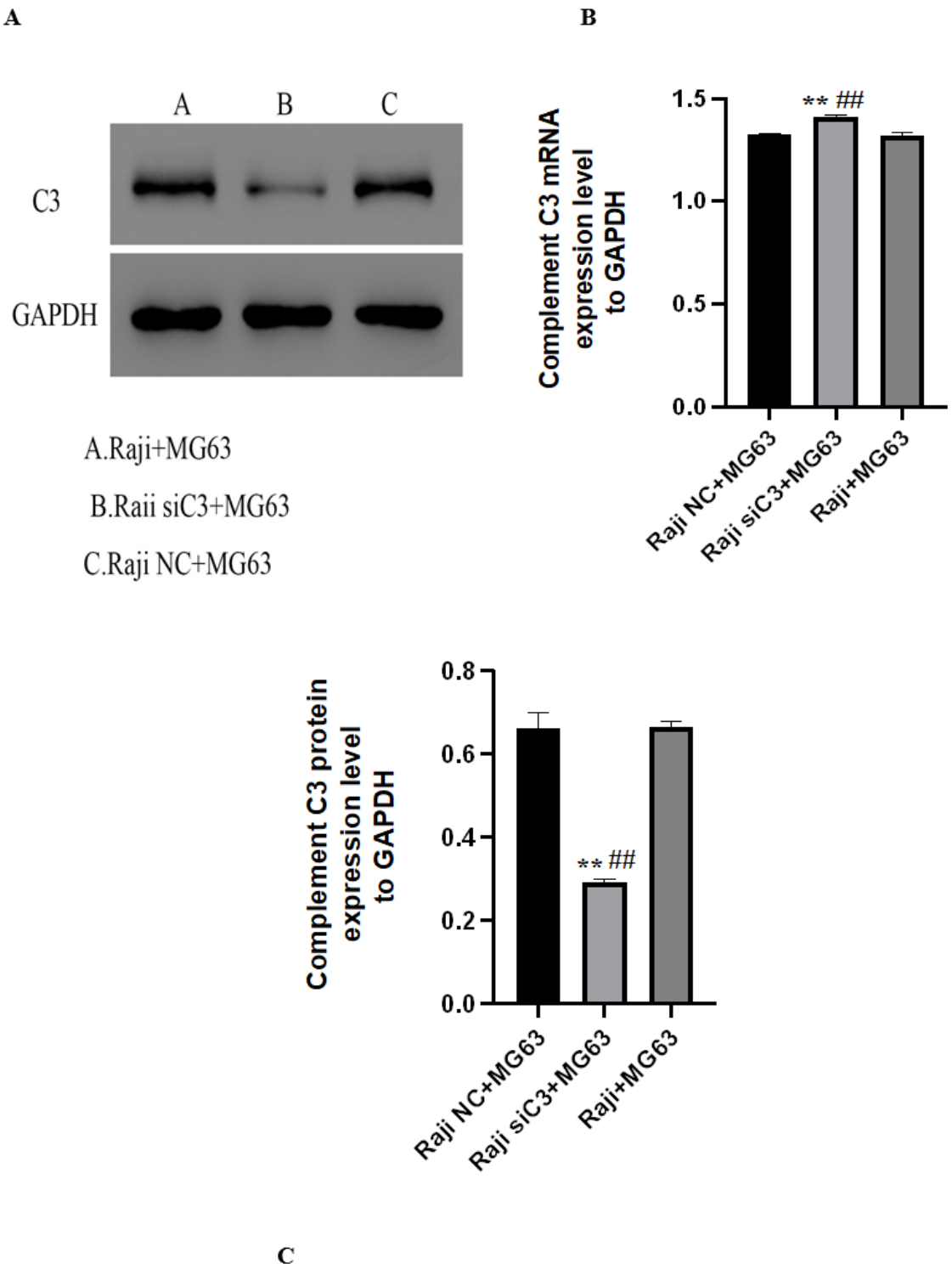

Figure 2

C3 is downregulated in the Raji-MG63 co-culture system

(A) Western blot analysis of C3 protein levels in co-culture systems with and without NC and a C3 inhibitor transfection.(B) C3 protein level quantification in co-culture system with and without transfection with NC and a C3 inhibitor. (C)RT-PCR analysis of C3 levels in co-culture systems with and without

transfection with NC and a C3 inhibitor. The data are presented as the means ± SD,**p<0.01 compared with the empty vector group. ##p<0.05 compared with the blank control group. One-way ANOVA followed by Tukey's post hoc test.

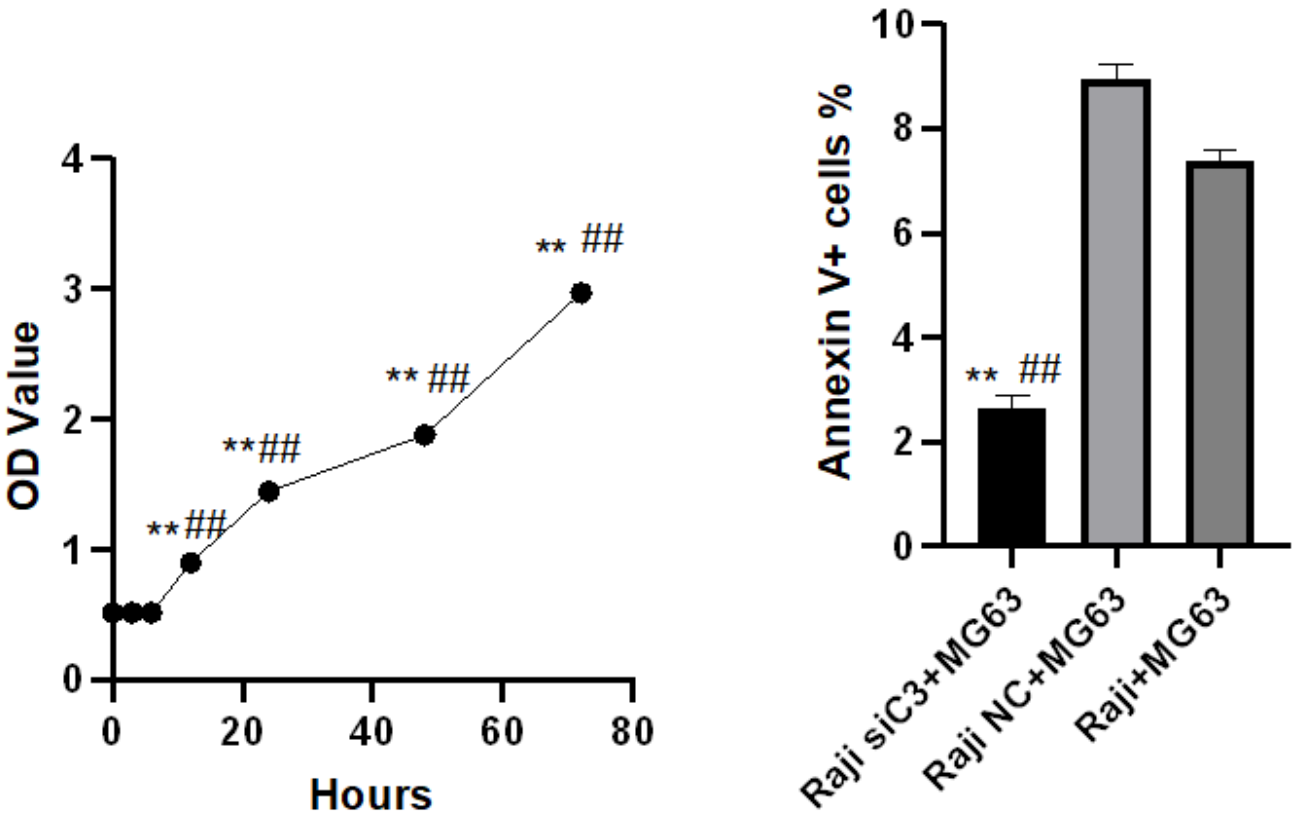

Figure 3

C3 knockdown enhanced the cellular activity of MG63

(A) The vitality of cells was determined using a CCK-8 kit at 0, 3, 6, 12, 24, 48, and 72 hours. (B) A FACS experiment was performed on the cells. Annexin V+ cells were thought to be undergoing apoptosis.The data are presented as the means ± SD, **p<0.01 compared with the empty vector group. ##p<0.05 compared with the blank control group. **p<0.01 compared with the empty vector group. ##p<0.05 compared with the blank control group.One-way ANOVA followed by Tukey's post hoc test.

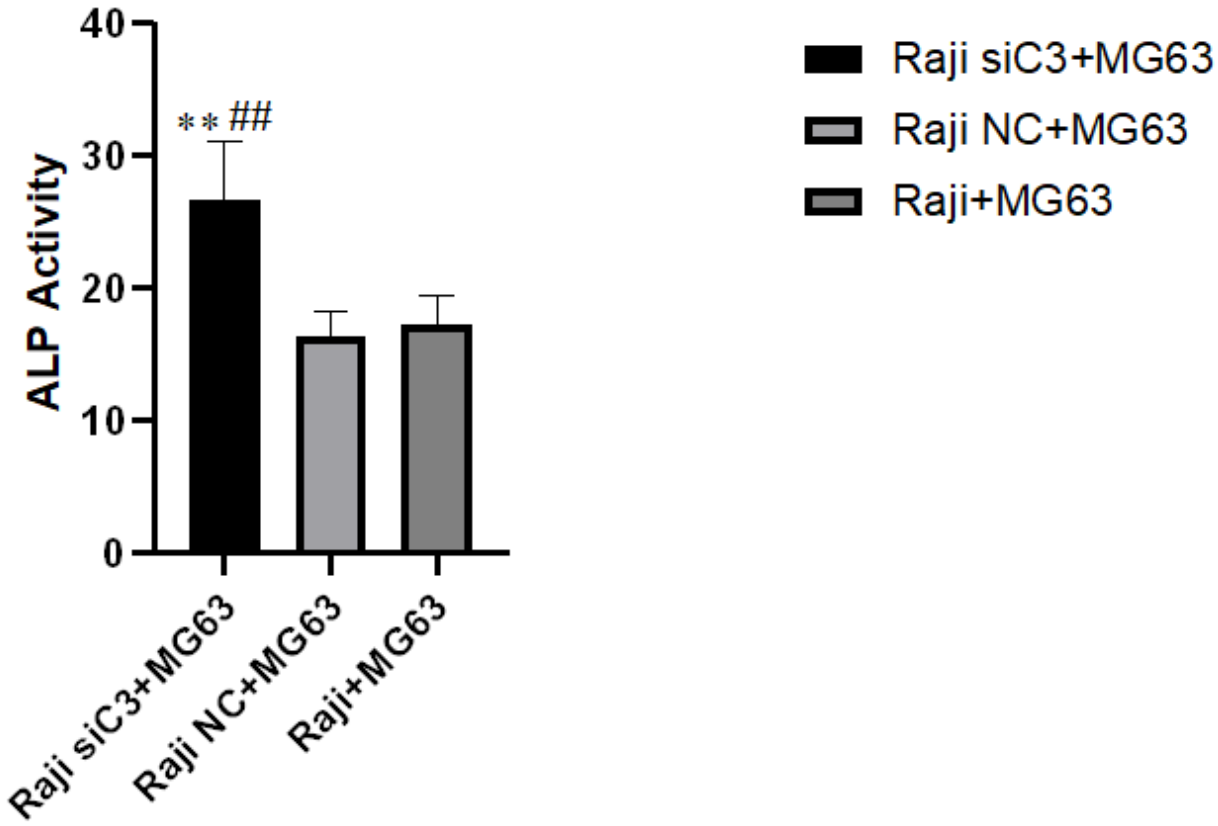

Figure 4

C3 knockdown enhanced the ALP activity of MG63

The assay indicating the *ALP* activity in the co-culture system with/without transfection with NC and a C3 inhibitor, **p<0.01 compared with the empty vector group. ##p<0.05 compared with the blank control group.

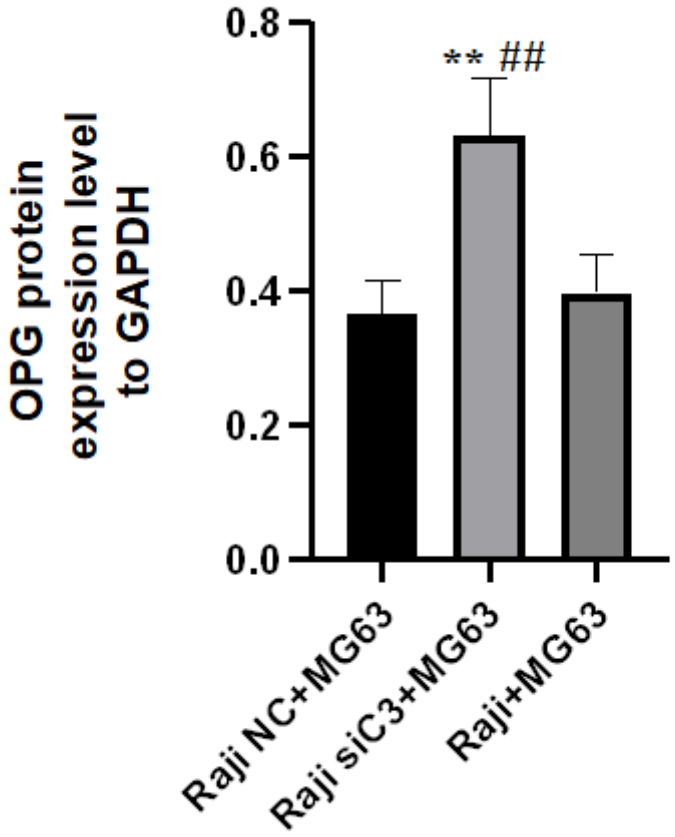

Figure 5

C3 knockdown downregulates OPG expression

RT-PCR analysis of OPG levels in co-culture systems with and without transfection with NC and a C3 inhibitor,**p<0.01 compared with the empty vector group. ##p<0.05 compared with the blank control group.