

Original Article

***GATA1* Knockdown Attenuates Osteoclast Differentiation and Subchondral Bone Loss in Experimental Osteoarthritis Through an *SKP2*-Associated Mechanism**

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Background: Osteoclast-mediated subchondral bone remodeling contributes to osteoarthritis (OA), but the role of GATA binding protein 1 (*GATA1*) remains unclear. This study examined whether *GATA1* is associated with osteoclast differentiation and S-phase kinase-associated protein 2 (*SKP2*) regulation in experimental OA. **Methods:** OA was induced by anterior cruciate ligament transection in rats. Bulk subchondral bone mRNA sequencing, intra-articular *GATA1* knockdown, micro-computed tomography, histology, and tartrate-resistant acid phosphatase (TRAP) staining were combined with nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) induced differentiation of RAW264.7 cells, gene-expression analyses, NFATc1 localization, Filamentous actin (F-actin)-ring assessment, an *SKP2* promoter-reporter assay, and *SKP2* rescue. **Results:** *GATA1* expression was elevated in bulk OA subchondral bone. *GATA1* knockdown was associated with higher bone mineral density and bone volume and fewer TRAP-positive osteoclasts *in vivo*. In RAW264.7 cells, *GATA1* knockdown reduced TRAP-positive multinucleated cells, osteoclast-associated gene expression, NFATc1 nuclear localization, and F-actin-ring formation. *GATA1* overexpression increased *SKP2* promoter-reporter activity, whereas *SKP2* overexpression partially reversed the effects of *GATA1* knockdown. **Conclusions:** The findings support an *SKP2*-associated role for *GATA1* in osteoclast differentiation and subchondral bone remodeling. Direct *GATA1* binding to endogenous *SKP2*, cell-resolved localization *in vivo*, and validation in primary cells, human OA, and reciprocal epistasis models remain necessary.

Keywords: Osteoarthritis, GATA binding protein 1, osteoclast differentiation, subchondral bone remodeling, S-phase kinase-associated protein 2.

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Introduction

Osteoarthritis (OA) is a chronic degenerative disorder driven by factors such as joint overuse, trauma, and inflammation. It is characterized pathologically by cartilage degeneration, synovial hyperplasia, osteophyte formation, and joint space narrowing, leading to pain and reduced mobility in patients, thereby impacting their quality of life [1]. Surveys reveal that from 1990 to 2019, the number of OA patients increased by 48%, becoming one of the major global public health issues [2]. Currently, clinical management primarily focuses on anti-inflammatory and analgesic therapy, with no targeted effective medications available. Joint replacement surgery remains the only effective therapy for end-stage patients. However, the limited lifespan of artificial joints, coupled with the increasing prevalence of OA among younger individuals and the rising number of joint replacement surgeries, has significantly exacerbated

the healthcare burden. Consequently, a deep understanding of the biological mechanisms underlying the progression of OA is crucial for the identification of novel drug targets for its prevention and treatment.

In the past, studies on the pathogenesis and drug targets of OA primarily focused on delaying cartilage degeneration and mitigating synovial inflammation [3,4]. It is noteworthy that studies on the pathological mechanisms of subchondral bone have become a focus of attention in recent years. As tissues that provide support and nutrition to cartilage, microstructural alterations in subchondral bones are a pivotal aspect in the research of OA [5,6]. Subchondral bone remodeling varies with the stage of OA: early OA is characterized by increased remodeling and bone loss, whereas late-stage OA shows reduced bone resorption with a relative bias toward bone formation [7]. Studies demonstrated that drugs repressing osteoclast activity, such as bisphosphonates and cathepsin K inhibitor MIV711, can sup-

press excessive subchondral bone resorption in early OA [8,9]. The osteoclast prostaglandin E receptor 4 (EP4) receptor antagonist HL-43 has been demonstrated to alleviate OA progression by inhibiting osteoclast formation and H-type angiogenesis [10].

GATA binding protein 1 (*GATA1*) is a member of the GATA transcription factor family and a key factor in hematopoietic system development [11]. It has been reported that *GATA1* promotes the progression of airway inflammation in asthma [12]. Knockout of *GATA1* promotes M2 macrophage polarization and alleviates preeclampsia symptoms [13]. Notably, a study reported that the absence of *GATA1* in mice led to elevated levels of osteoprotegerin (*OPG*) (osteoclast inhibitory factor), which alleviated the loss of trabecular bone caused by *OPG* deficiency [14]. Downregulation of optineurin (*OPTN*) in rheumatoid arthritis synovial fibroblasts increased receptor activator of nuclear factor kappa-B ligand (*RANKL*) expression and promoted osteoclast differentiation, whereas *OPG* mRNA remained unchanged; *GATA1* expression showed a nonsignificant upward trend [15]. Additionally, *GATA1* is bound by the long non-coding RNA X-inactive specific transcript (*XIST*), contributing to the promotion of synovial injury and inflammation in rats, thereby driving rheumatoid arthritis [16]. In human synovial tissues, *GATA1* also exacerbates rheumatoid arthritis by transcriptionally activating the nitric oxide synthase 2 (*NOS2*) signaling pathway [17]. Transcriptome sequencing revealed that *GATA1* was upregulated in subchondral bone plate tissues from OA rats compared to controls ($\log_2$ fold change = 2.32, $p < 0.05$). Hence, we hypothesize that *GATA1* may influence OA progression by affecting osteoclast-mediated bone resorption.

S-phase kinase-associated protein 2 (*SKP2*) is an F-box protein that contributes to proteasomal degradation and has been implicated in tumor biology [18,19]. Previous studies also suggest that *SKP2* participates in OA progression and osteoclastogenesis [20,21]. A Joint Project on the Collection and Analysis of Promoters of Eukaryotic Genes (JASPAR) database analysis predicted putative *GATA1*-binding motifs in the *SKP2* promoter. Accordingly, this study evaluated the role of *GATA1* in experimental OA and examined whether *GATA1*-dependent osteoclast differentiation is associated with regulation of *SKP2* expression and *SKP2* promoter-reporter activity.

Materials and Methods

Animal Experiment Protocol

Twelve-week-old male Wistar rats (350 ± 20 g) were supplied by Changsheng Biotechnology Co., Ltd. Rats were maintained in ventilated cages under specific pathogen-free conditions and housed under a 12 h light/12 h dark cycle at 22 ± 1 °C and 45–55% humidity, with adequate food and water. Taking into account the modeling success rate (95.4%), a total of 18 rats were randomly divided into Sham and OA groups for mRNA sequencing

analysis. After one-week acclimatization, OA models were constructed via anterior cruciate ligament (ACL) transection surgery, as previously described [22]. In brief, the right knee of the rats was exposed via a medial parapatellar incision. The patella was dislocated posteriorly, and the ACL was cut while the knee was held in maximum flexion. Rats in the sham surgery group underwent the same surgery procedure without ACL transection. Rats that died following surgery were excluded from subsequent experiments. The same number of rats were initially included in each group. Predefined exclusion criteria were as follows: death due to anesthesia complications or massive hemorrhage during surgery, death due to infection within 72 h postoperatively, and failure to meet the OA modeling criteria upon postoperative assessment. Animals that did not meet the criteria were excluded, and the remaining valid samples from each group were included in the statistical analysis. Subsequently, a total of 46 rats were randomly divided into four groups—Sham ($n = 10$), OA ($n = 12$), OA + Lv-shNC ($n = 12$), and OA + Lv-shGATA1 ($n = 12$)—for subsequent evaluation of the effects of *GATA1* on OA. Three experimental animals succumbed to the experimental procedures. Rats were euthanized 8 weeks post-modeling, and subchondral bone plate tissues were collected.

mRNA Sequencing

Total RNA was isolated from subchondral bone plate samples from Sham and OA rats. Complementary DNA libraries were constructed and subjected to high-throughput mRNA sequencing. Differentially expressed genes (DEGs) were defined by $|\log_2$ fold change| > 1 and $p < 0.05$. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were used to characterize enriched biological processes and pathways. GeneCards was queried using the term “transcription factor”; entries were ranked by the GeneCards relevance score, and the top 100 genes were intersected with the DEG list to prioritize candidate transcription factors.

Micro-Computed Tomography (micro-CT) Scanning

At eight weeks after model induction, three rats were randomly selected from each group for exploratory micro-CT analysis. Right knee specimens were scanned using a PerkinElmer micro-CT system (PerkinElmer, Shanghai, China). Bone mineral density (BMD) and bone volume fraction/total volume (BV/TV) of the subchondral bone plate were quantified. The small imaging subset limits the precision and generalizability of this endpoint. The investigators performing histological quantification and micro-CT analysis were blinded to treatment groups.

Lentiviral Construct Packaging

To knock down *GATA1* expression, shRNA targeting *GATA1* (shGATA1) was synthesized. Subsequently, the shRNA was packaged into lentiviral vectors,

Table 1. Primer sequences.

Name	Sequences	Primer length	Tm	Product length
Rat <i>GATA1</i> F	CGCTCTACCCTGCCTCAA	18	56.7	119
Rat <i>GATA1</i> R	CAGCCGCTCCGTCTTCA	17	58.1	
Rat <i>SKP2</i> F	AAGGCTAAAGAGCAAAGG	18	49.8	145
Rat <i>SKP2</i> R	CTCAGGGAGGCACAGAC	17	49.8	
Mus <i>GATA1</i> F	CTTTCCCAGTCCTTCTTCT	20	52.8	252
Mus <i>GATA1</i> R	CTGACAATCATTCGCTTCTT	20	52.7	
Mus <i>CTSK</i> F	AGTTTCTGCTGCTACCC	17	46.9	114
Mus <i>CTSK</i> R	CATCCACCTTGCTGTTAT	18	47.8	
Mus <i>NEATc1</i> F	ACACGGTTACTTGGAGAATGA	21	54.7	132
Mus <i>NEATc1</i> R	TGGCTGGTGGTGGAGAC	17	54	
Mus <i>MMP9</i> F	AGCACGGCAACGGAGAAG	18	59.2	108
Mus <i>MMP9</i> R	CGCACCAGCGGTAACCAT	18	59.8	
Mus <i>SKP2</i> F	AAACGAGTCAAGGGCAAAG	19	55.2	142
Mus <i>SKP2</i> R	GGAGGCACAGACAGGAAAA	19	55.4	
Rat β -actin F	TGGCACCACACTTCTACAATGAGC	25	63.7	106
Rat β -actin R	GGGTCATCTTTTCACGGTTGG	21	59.5	
Mus β -actin F	GGCTGTATTCCCCTCCATCG	20	59.96	154
Mus β -actin R	CCAGTTGGTAAACATGCCATGT	22	59.44	

which were then injected into the joint cavities of rats. The shRNA sequences are listed below: shGATA1-1ccgGCGAATGATTGTCAGCAAACGttcaagagaCGTTTGCTGACAATCATTCGCTtttt; shGATA1-2ccgGCGGAGATCCGGTATGCAATGttcaagaga CATTGCATACCGGATCTCCGCTtttt; shNC: ccgTTCTCCGAACGTGTCACGTttcaagagaACGTGACACGTTTCGAGAAAtttt.

Real Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from tissues or cells using TRIpure lysis (BioTeke Corporation, Beijing, China; catalog no. RP1001). Subsequently, cDNA synthesis was performed using a reverse transcription kit (YoungGen, Yunnan, China; catalog no. RT203) according to the manufacturer's recommended protocol. PCR reactions were conducted employing SYBR GREEN (Solarbio, Beijing, China; catalog no. SR4110), 2 × Fast Taq plus PCR Master Mix (Biosharp, Anhui, China; catalog no. BL1014), cDNA, upstream and downstream primers on a quantitative fluorescence analyzer (Pangaea 3; AperiBio, Suzhou, China). Primer sequences utilized during amplification are listed in Table 1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western-Blot

Whole protein extracts derived from tissues or cells were obtained utilizing Cell lysis buffer for Western and immunoprecipitation (IP) (P0013; beyotime, Shanghai, China). Following quantification with a bicinchoninic acid (BCA) assay kit (P0011; beyotime, China), equal amounts of protein were subjected to electrophoresis for separation and subsequently transferred onto polyvinylidene fluoride

(PVDF) membranes. After blocking the separated proteins for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies against GATA1 (1:5000; Proteintech, Wuhan, China; catalog no. P023) and α -tubulin (1:1000; Santa cruz, TX, USA; catalog no. sc-69971). Subsequently, incubation with secondary antibodies at room temperature for 1 h was carried out, and protein bands were visualized through enhanced chemiluminescence substrate (Beyotime, Shanghai, China). The secondary antibodies employed were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000; A0208; Beyotime, Shanghai, China) and HRP-conjugated goat anti-mouse IgG (1:5000; A0216; Beyotime, Shanghai, China).

Hematoxylin and Eosin (H&E) Staining

To assess pathological changes in the subchondral bone plate tissues, H&E staining was carried out. Briefly, the collected subchondral bone plate tissue samples underwent fixation, dehydration, and paraffin embedding before being sectioned into 5 μ m-thick slices. Rehydrated slices were subjected to staining, involving 5 min of hematoxylin staining followed by 3 min of eosin staining. Images were captured under a microscope (40×; BX53; OLYMPUS, Olympus Corporation, Tokyo, Japan) following dehydration and sealing.

Immunohistochemistry (IHC)

Tissue slices of subchondral bone embedded in paraffin were subjected to dehydration in a series of increasing concentrations of alcohol, followed by dewaxing in xylene. Slices were subjected to 10-minute heat treatment in sodium citrate buffer to retrieve antigens. Subsequently, slices were exposed to 3% hydrogen peroxide for 15 min

to block endogenous peroxidase activity, following by 15-minute incubation with 1% bovine serum albumin (BSA) solution at room temperature to prevent non-specific antigen binding. Primary and secondary antibodies were then applied sequentially for incubation. Primary antibodies: RANK antibody (1:100; Zenbio, Chengdu, China; catalog no. 251971), RANKL antibody (1:100; Zenbio, Chengdu, China; catalog no. 340892) and SKP2 antibody (1:100; Zenbio, Chengdu, China; catalog no. R382104), incubated overnight at 4 °C. Secondary antibody: HRP-conjugated goat anti-rabbit IgG (1:200; Sangon, Shanghai, China; catalog no. D110058), incubated at room temperature for 1 h. Visualization was performed employing Diaminobenzidine (DAB), followed by counterstaining with hematoxylin. Images were obtained under a microscope (400×; BX53; OLYMPUS, Japan).

Tartrate-Resistant Acid Phosphatase (TRAP) Staining

TRAP staining was conducted to examine mature osteoclasts. For tissue samples, subchondral bone plate tissues were sectioned into 5-μm paraffin sections. After decalcification, slices were stained utilizing a TRAP staining kit (Servicebio, Wuhan, China; catalog no. G1050-50T) according to the manufacturer's instructions. For cell samples, cells are initially fixed with pre-chilled TRAP fixative, then incubated with TRAP incubation buffer at 37 °C for 45–60 min. Finally, cells are counterstained with hematoxylin for 2–3 min, and mature osteoclasts were observed under a microscope (100× and 200×; BX53, OLYMPUS, Japan).

Cell Culture and Cell Transfection

RAW264.7 cells were cultured in Minimum Essential Medium (MEM) (Saiwei, Suzhou, China; catalog no. G4511) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biology, Huzhou, China; catalog no. 11011-8611) at 37 °C in a 5% CO₂ incubator. The cells were harvested from Cellverse Bioscience Technology Co., Ltd. Cells were incubated for five days in α-MEM (Baisha, China; catalog no. BL1920A) medium containing 50 ng/mL RANKL (Sigma-Aldrich, St. Louis, MO, USA; catalog no. R0525) to induce osteoclast formation. To explore the role of *GATA1* in osteoclast differentiation, sh*GATA1* or shNC was transfected into cells for 48 h prior to inducing osteoclast differentiation.

Immunofluorescence (IF) Staining

After fixing cells with 4% paraformaldehyde for 15 min, they were permeabilized with 0.1% Triton X-100 and blocked with 1% BSA. The cells were incubated with diluted primary antibody at 4 °C, the following day, incubated with diluted secondary antibody at room temperature for 1 h prior to DAPI counterstaining. Fluorescent images were acquired using a fluorescence microscope (400×; BX53; OLYMPUS, Japan). Primary antibodies: *GATA1*

antibody (1:100; Proteintech, Wuhan, China), NFATc1 antibody (1:100; ABclonal, Wuhan, China) and Filamentous actin (F-actin) antibody (1:100; Zenbio, Chengdu, China). Secondary antibody: Alexa Fluor® 555-conjugated anti-rabbit IgG (1:200; CST, MA, USA) and Alexa Fluor® 555-conjugated anti-mouse IgG (1:200; CST, MA, USA).

Dual Luciferase Assay

Fragments of the *SKP2* promoter were cloned into the pGL3-Basic vector to generate a pGL3-*SKP2* promoter reporter. Human embryonic kidney 293T (HEK293T) cells were co-transfected with pGL3-Basic or the pGL3-*SKP2* promoter reporter and with a *GATA1* overexpression vector or empty vector. Luciferase activity was measured 48 h after transfection using a dual-luciferase reporter assay kit (Biosharp, Beijing, China; catalog no. BL555A). This reporter assay tests promoter activity under the transfection conditions but does not establish *GATA1* occupancy at the endogenous *SKP2* locus.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA). Two-group comparisons were performed using a two-sided Student's *t*-test. Multiple-group comparisons were performed using one-way analysis of variance followed by Tukey's or Holm–Sidak's multiple-comparisons test, as specified for the relevant analysis. Values are presented as mean ± standard deviation, and *p* < 0.05 was considered statistically significant. Exact *n* values are reported in the figure legends. For animal experiments, *n* denotes individual animals. Figs. 1–5 is created using GraphPad Prism 9.0.

Randomization, Exclusions, Replicates, and Blinding

Animals were randomly allocated as described above. Animals that died postoperatively were excluded from subsequent analyses. The micro-CT subset comprised three randomly selected rats per group. Investigators performing histological assessment and micro-CT acquisition or quantification were blinded to treatment group.

Results

*Bulk Transcriptomic Analysis Identifies *GATA1* as a Candidate Associated With OA Subchondral Bone*

To identify candidate regulators associated with OA, subchondral bone plate tissues from ACL-transected and Sham rats were analyzed by bulk mRNA sequencing (Fig. 1a). Principal component analysis separated the Sham and OA samples (Fig. 1b), and volcano plots and heatmaps summarized the DEGs (Fig. 1c,d). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses indicated enrichment in nuclear division, chromosome segregation, cell-cycle regulation, and phosphatidylinositol 3-kinase (PI3K)–protein kinase B (AKT) signaling pathway (Fig. 1e,f). Intersecting the DEGs with the 100 highest-

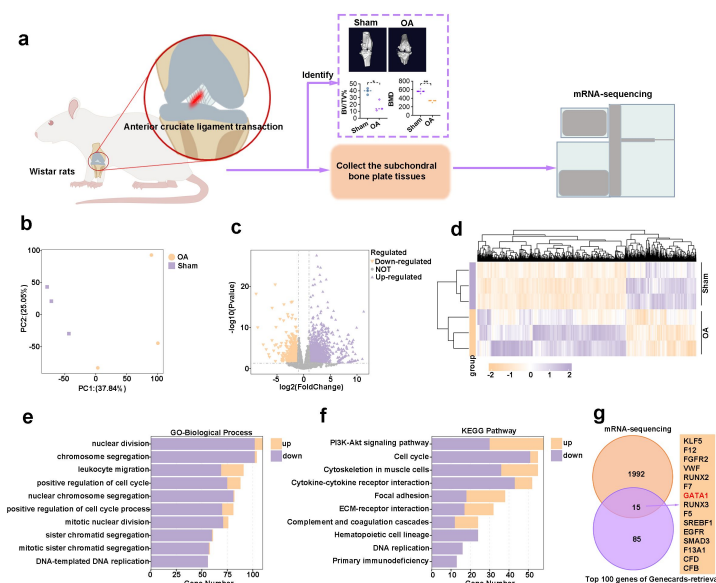


Fig. 1. Transcriptomic analysis in subchondral bone tissue of OA rats. (a) Workflow for the anterior cruciate ligament transection (ACLT) rat model and bulk subchondral bone mRNA sequencing. **(b)** Principal component analysis of Sham and OA samples. **(c,d)** Volcano plot and heatmap of DEGs. **(e,f)** The ten highest-ranked Gene Ontology biological-process and Kyoto Encyclopedia of Genes and Genomes pathway terms. **(g)** Intersection of DEGs with the 100 highest-ranked GeneCards entries retrieved using the query “transcription factor” and ranked by GeneCards relevance score.

ranked GeneCards entries retrieved using the query “transcription factor” identified *GATA1* among the candidate genes (Fig. 1g). Because these data were generated from bulk tissue, they do not identify the cellular source of *GATA1* expression.

GATA1 Knockdown is Associated With Improved Subchondral Bone Indices and Fewer Osteoclast-Related Phenotypes in Vivo

GATA1 expression was reduced after intra-articular delivery of Lv-sh*GATA1* (Fig. 2a,b). In the exploratory micro-CT subset, OA rats showed lower BMD and BV/TV than Sham rats, whereas the *GATA1*-knockdown group showed higher values than the corresponding OA control group (Fig. 2c–e). H&E staining qualitatively showed cartilage-surface irregularity and altered subchondral morphology after ACL transection, with less severe morphological changes in the *GATA1*-knockdown group (Fig. 2f). Representative immunohistochemical images qualitatively suggested lower RANK and RANKL staining after *GATA1* knockdown (Fig. 2g); no blinded integrated optical density (IOD) or positive-area quantification was performed, and these images are therefore not used as a quantitative endpoint. TRAP staining showed fewer TRAP-positive multinucleated cells after *GATA1* knockdown (Fig. 2h). These findings support an association between *GATA1* knockdown, subchondral bone microarchitecture, and osteoclast-related phenotypes in this rat model, but do not establish whole-joint disease modification.

GATA1 Knockdown Suppresses Osteoclast Differentiation and Markers of a Resorptive Phenotype in RAW264.7 Cells

To evaluate *GATA1* during osteoclast differentiation *in vitro*, *GATA1* was knocked down in RAW264.7 cells before RANKL exposure (Supplementary Fig. 1). *GATA1* IF increased after RANKL stimulation and was reduced by *GATA1* knockdown (Fig. 3a). *GATA1* knockdown reduced the number of TRAP-positive multinucleated cells and lowered Cathepsin K (*CTSK*), matrix metalloproteinase 9 (*MMP9*), and *NFATc1* mRNA expression (Fig. 3b,c). It also reduced *NFATc1* nuclear localization and F-actin-ring formation (Fig. 3d,e). These findings support reduced osteoclast differentiation and a less developed resorptive phenotype in RAW264.7 cells. Because no pit-formation or matrix-resorption assay was performed, the cellular experiments do not directly quantify bone resorption.

GATA1 is Associated With SKP2 Expression and Enhances SKP2 Promoter-Reporter Activity

GATA1 knockdown was associated with lower *SKP2* mRNA expression in subchondral bone plate tissue (Fig. 4a). Representative IHC qualitatively showed lower *SKP2* staining after *GATA1* knockdown, while the tissue real-time PCR result provides the quantitative comparison (Fig. 4a,b). *SKP2* mRNA was also lower in RANKL-treated RAW264.7 cells after *GATA1* knockdown (Fig. 4c). In the promoter-reporter assay, *GATA1* overexpression increased the activity of the pGL3-*SKP2* promoter reporter (Fig. 4d). These data indicate that *GATA1* can enhance *SKP2*

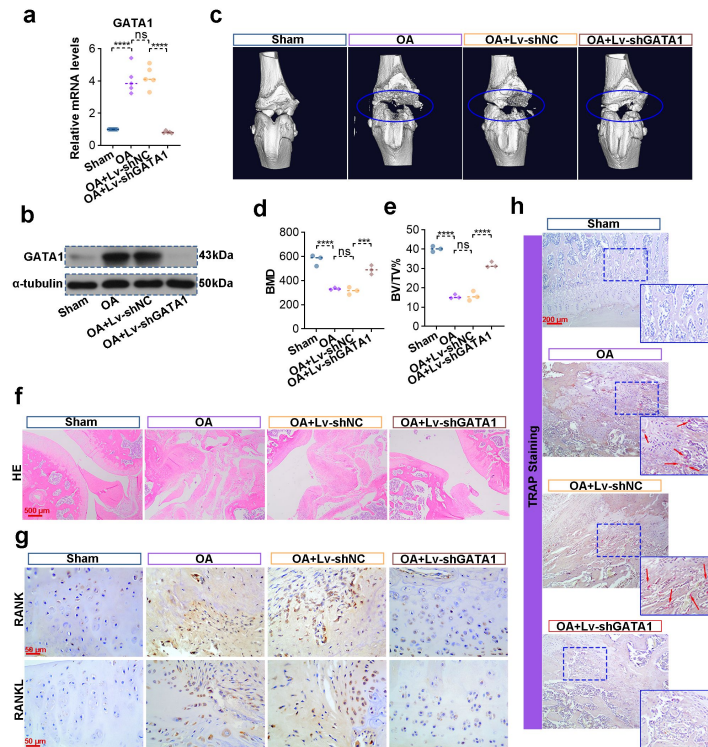


Fig. 2. The suppressive effect of *GATA1* knockdown on OA *in vivo*. (a) Relative mRNA levels of *GATA1* in the subchondral bone plate tissues from each group ($n = 5$). (b) Protein levels of *GATA1* in the subchondral bone plate tissues from each group. (c) Micro-CT images of the right knee bones in rats from each group. (d) BMD of the subchondral bone plate in rats of each group ($n = 3$). (e) Bone volume of the subchondral bone plate in rats of each group ($n = 3$). (f) H&E staining reveals morphological changes in the subchondral bone plate tissues of rats in each group. Scale bar, 500 μm . (g) IHC staining demonstrates the expression of RANK and RANKL in the subchondral bone plate tissues. Scale bar, 50 μm . (h) TRAP staining reveals the number of osteoclasts in the subchondral bone plate tissues. Scale bar, 200 μm . Values are presented as mean $\pm$ standard deviation. **** $p < 0.0001$.

promoter-reporter activity under the assay conditions and is associated with *SKP2* expression. They do not demonstrate direct *GATA1* binding to the endogenous *SKP2* promoter.

SKP2 Overexpression Partially Rescues the *GATA1*-Knockdown Phenotype

SKP2 was overexpressed in *GATA1*-knockdown RAW264.7 cells exposed to RANKL (Fig. 5a). *SKP2* overexpression partially restored the number of TRAP-positive multinucleated cells, *CTSK* and *MMP9* expression, and *NFATc1* nuclear localization relative to *GATA1* knockdown alone (Fig. 5b–d). Thus, the gain-of-function rescue supports the participation of *SKP2* in the *GATA1*-associated osteoclast phenotype. However, the absence of reciprocal *SKP2* loss-of-function during *GATA1* overexpression means that *SKP2* necessity has not been established.

Discussion

OA is characterized by abnormal remodeling of subchondral bone, and osteoclast activity contributes to subchondral structural change [23,24]. In this study, *GATA1* knockdown was associated with improved micro-CT in-

dices and fewer osteoclast-related phenotypes in an ACLT rat model, while RAW264.7 experiments supported a role for *GATA1* in RANKL-induced osteoclast differentiation. *SKP2* expression and promoter-reporter activity changed with *GATA1* manipulation, and *SKP2* overexpression produced a partial rescue. Collectively, these data support, but do not prove, an *SKP2*-associated mechanism linking *GATA1* to osteoclast differentiation and subchondral bone remodeling.

Subchondral bone is a component of the osteochondral unit and undergoes structural and functional changes during OA progression [25]. Consistent with previous ACLT studies [22,26], the OA group showed lower BV/TV and BMD. *GATA1* knockdown was associated with higher values of these indices. Because micro-CT was performed in only three animals per group, this endpoint is exploratory and should not be interpreted as a precise estimate of treatment effect.

Osteoclasts regulate bone resorption and contribute to subchondral bone homeostasis [27]. RANKL-RANK signaling drives osteoclast differentiation, while *CTSK*, *NFATc1*, *MMP9*, and F-actin rings mark distinct aspects

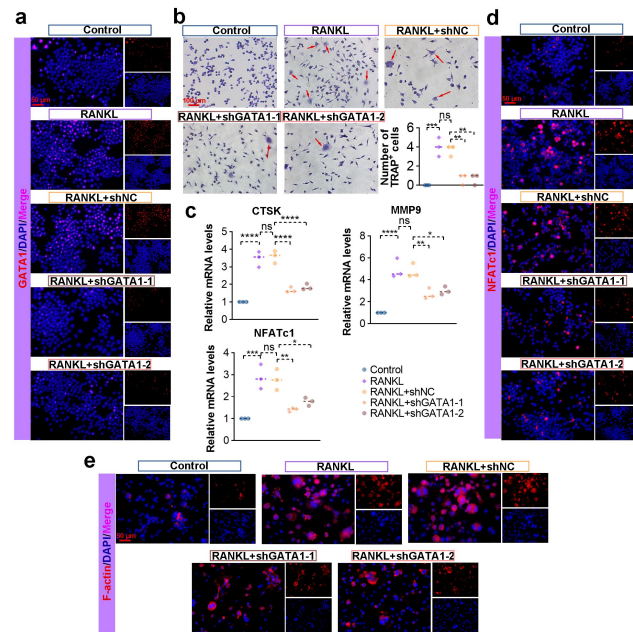


Fig. 3. *GATA1* knockdown suppresses osteoclast differentiation and markers of a resorptive phenotype in RAW264.7 cells. (a) IF staining shows the expression of GATA1 in cells of each group. Scale bar, 50 μ m. (b) TRAP staining reveals the number of osteoclasts in cells of each group (n = 3). Scale bar, 100 μ m. (c) Relative mRNA levels of *CTSK*, *MMP9* and *NFATc1* in cells of each group (n = 3). (d) IF staining displays the expression of NFATc1 in cells of each group. Scale bar, 50 μ m. (e) IF staining reveals the expression of F-actin in cells of each group. Scale bar, 50 μ m. Values are presented as mean $\pm$ standard deviation. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

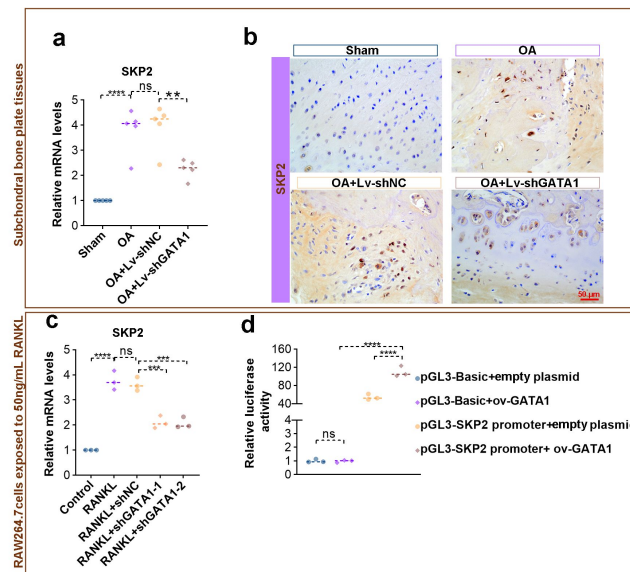


Fig. 4. *GATA1* is associated with *SKP2* expression and enhances *SKP2* promoter-reporter activity. (a) Relative mRNA levels of *SKP2* in the subchondral bone plate tissues from each group (n = 5). (b) IHC staining demonstrates the expression of *SKP2* in the subchondral bone plate tissues from each group. Scale bar, 50 μ m. (c) Relative mRNA levels of *SKP2* in cells of each group (n = 3). (d) Dual luciferase reporter assays are carried out with HEK293T cells co-transfected with pGL3-basic luciferase reporter containing *SKP2* promoter and the *GATA1* overexpression or empty vector (n = 3). Values are presented as mean $\pm$ standard deviation. ** p < 0.01; *** p < 0.001; **** p < 0.0001.

of osteoclast maturation and the resorptive phenotype [28– 34]. In RAW264.7 cells, *GATA1* knockdown reduced

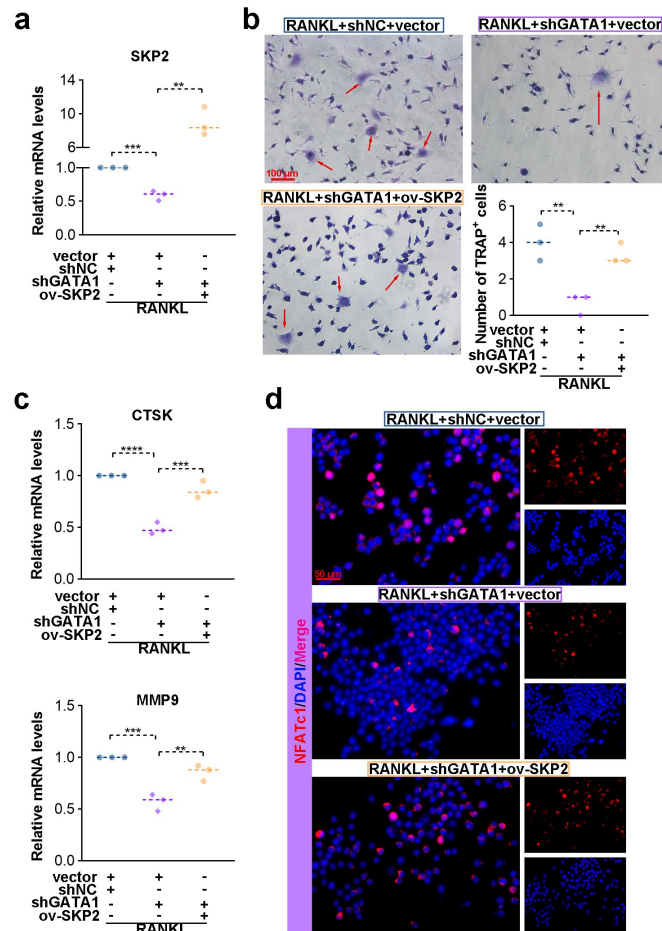


Fig. 5. *SKP2* overexpression partially rescues the *GATA1*-knockdown phenotype. (a) Relative mRNA levels of *SKP2* in cells of each group ($n = 3$). (b) TRAP staining reveals the number of osteoclasts in cells of each group ($n = 3$). Scale bar, 100 μ m. (c) Relative mRNA levels of CTSK and MMP9 in cells of each group ($n = 3$). (d) IF staining displays the expression of NFATc1 in cells of each group. Scale bar, 50 μ m. Values are presented as mean $\pm$ standard deviation. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

TRAP-positive multinucleated cells, osteoclast-associated gene expression, NFATc1 nuclear localization, and F-actin-ring formation. These assays support an effect on osteoclast differentiation and maturation, but a direct functional bone-resorption assay was not performed. The cellular conclusion is therefore limited to the RAW264.7 model and requires validation in primary bone marrow-derived macrophages.

GATA2 provides an important context for interpreting these findings. Classical developmental studies showed that GATA2 acts early in osteoclastogenesis by supporting the generation of osteoclast progenitors, whereas terminal differentiation depends on subsequent macrophage colony-stimulating factor (M-CSF) and RANKL signaling [35]. Later work placed GATA2 downstream of peroxisome proliferator-activated receptor gamma (PPAR γ) in osteoclast progenitor specification and showed that GATA2 depletion markedly suppresses RANKL-induced osteoclast formation [36]. Recent syntheses further emphasize the heterogeneity of osteoclast progenitors and the stage-specific

transcriptional programs that govern lineage commitment, differentiation, and bone resorption [37,38]. These observations suggest that different GATA-family members may act at distinct stages or in different cellular compartments of the osteoclast lineage. The present results implicate *GATA1* in RANKL-treated RAW264.7 cells and OA subchondral bone; however, they do not establish whether *GATA1* functions independently of, cooperatively with, or in a stage-specific relationship to GATA2. Comparative analyses of *GATA1* and *GATA2* in primary osteoclast progenitors are therefore warranted.

The present rescue experiment indicates that increased *SKP2* can partially counter the effects of *GATA1* knockdown, but it does not establish *SKP2* as a necessary mediator. Previous work reported that *SKP2* promotes krüppel-like factor 11 (KLF11) degradation in chondrocytes [20]. It is therefore plausible that KLF11 or other *SKP2* substrates could contribute to osteoclast regulation downstream of the *GATA1*-*SKP2* association. This proposed *SKP2*-KLF11 route remains a hypothesis because neither KLF11 nor other

SKP2 substrates were tested in the present study.

Subchondral bone abnormalities are a recognized component of OA pathology. Magnetic resonance imaging (MRI)-detected subchondral bone marrow lesions (BMLs), historically described as bone marrow edema-like lesions, reflect heterogeneous histopathological changes, including fibrosis, necrosis, edema, bleeding, and abnormal trabecular remodeling [39]. BMLs are common in OA and are associated with pain, cartilage loss, and structural progression, supporting their potential value as imaging biomarkers of OA progression [39,40].

This study has several limitations. First, bulk subchondral bone sequencing cannot identify the cellular source of *GATA1*; co-localization with CTSK, acid phosphatase 5 (ACP5)/TRAP, RANK, or NFATc1, as well as RNAscope, single-cell, or spatial analyses, will be needed. Second, promoter-reporter activation does not demonstrate binding to the endogenous *SKP2* locus. Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) and mutation of predicted *GATA1* motifs are required. Third, *SKP2* overexpression produced only a partial rescue, and the reciprocal *GATA1*-overexpression/*SKP2*-knockdown experiment and *in vivo* *SKP2* rescue remain to be performed. Fourth, the cellular findings are derived from RAW264.7 cells and require validation in primary bone marrow-derived macrophages and human OA samples. Fifth, micro-CT used three animals per group, and the submitted records require author confirmation of blinding, replicate definitions, and postoperative exclusions. Sixth, no Safranin O/fast-green staining, osteoarthritis research society international (OARSI) cartilage score, blinded IHC morphometry, direct matrix-resorption assay, or synovitis assessment was performed. The findings therefore concern subchondral bone and osteoclast-related phenotypes and should not be interpreted as proof of whole-joint therapeutic efficacy. Finally, the *SKP2*-KLF11 route is speculative and requires direct experimental testing. These questions will be addressed in future cell-resolved, reciprocal epistasis, primary-cell, and *in vivo* studies.

Conclusions

In summary, *GATA1* expression was elevated in bulk subchondral bone from ACLT rats, and *GATA1* knockdown was associated with improved subchondral bone indices and reduced osteoclast-related phenotypes. In RAW264.7 cells, *GATA1* knockdown attenuated RANKL-induced osteoclast differentiation. *GATA1* manipulation altered *SKP2* expression and promoter-reporter activity, and *SKP2* overexpression partially rescued the *GATA1*-knockdown phenotype. These results support an *SKP2*-associated role for *GATA1* in experimental OA without establishing direct promoter binding, *SKP2* necessity, or therapeutic efficacy in humans.

List of Abbreviations

OA, osteoarthritis; *GATA1*, GATA binding protein 1; *SKP2*, S-phase kinase-associated protein 2; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; F-actin, Filamentous actin; EP4, prostaglandin E receptor 4; OPG, osteoprotegerin; OPTN, optineurin; RANKL, receptor activator of nuclear factor kappa-B ligand; XIST, X-inactive specific transcript; NOS2, nitric oxide synthase 2; JASPAR, A Joint Project on the Collection and Analysis of Promoters of Eukaryotic Genes; ACL, anterior cruciate ligament; DEGs, differentially expressed genes; micro-CT, micro-computed tomography; BMD, bone mineral density; BV/TV, bone volume/total volume; PCR, polymerase chain reaction; IP, immunoprecipitation; BCA, bicinchoninic acid; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; H&E, Hematoxylin and Eosin; IHC, immunohistochemistry; BSA, bovine serum albumin; IF, immunofluorescence; HEK293T, human embryonic kidney 293T; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; IOD, integrated optical density; ACLT, anterior cruciate ligament transection; CTSK, Cathepsin K; MMP9, matrix metalloproteinase 9; M-CSF, macrophage colony-stimulating factor; PPAR γ , peroxisome proliferator-activated receptor gamma; KLF11, krüppel-like factor 11; MRI, magnetic resonance imaging; BMLs, bone marrow lesions; ACP5, acid phosphatase 5; ChIP-qPCR, chromatin immunoprecipitation followed by quantitative PCR; OARSI, osteoarthritis research society international; MEM, Minimum Essential Medium.

Availability of Data and Materials

The raw mRNA sequencing data generated in this study must be deposited in a public repository. Other data supporting the findings are available from the corresponding author upon reasonable request, subject to applicable institutional requirements.

Author Contributions

YLD and YPZ conceived and designed the study, administered the project, executed all experiments, and wrote the article; YPZ and YJ contributed to the article revision; GXL, MMZ, and YZL assisted with the entire experimental process; YLD supervised the whole process; YLD and YPZ prepared the manuscript with inputs from all authors. All authors gave final approval of the version to be published. All authors participated fully in the work, took public responsibility for appropriate portions of the content, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or completeness of any part of the work were appropriately investigated and resolved.

Ethics Approval and Consent to Participate

The animal experimental protocol was reviewed and approved by the Laboratory Animal Welfare and Ethics Committee of Henan University of Traditional Chinese Medicine (approval no. IACUC-202604002). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Conflict of Interest

The authors declare that there are no conflicts of interests.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.22203/eCM.v059a15>.

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