

Article

Retinol Binding Protein 4 Promotes Chondrocyte and Osteoclast Differentiation

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Simple Summary

This study investigated whether the metabolic protein RBP4 contributes to inflammation and joint tissue changes linked to osteoarthritis, particularly in people with type 2 diabetes. Laboratory experiments showed that inflammation and high glucose conditions increased RBP4 levels in cartilage-related cells along with markers of tissue breakdown. Inhibiting RBP4 reduced inflammatory responses and changes associated with cartilage and bone remodeling. These findings suggest that RBP4 could be a future target for preventing or slowing osteoarthritis, especially in people with diabetes.

Abstract

Retinol-binding protein 4 (RBP4), an adipokine secreted by adipose tissues, has been implicated in metabolic inflammation and insulin resistance. Type 2 diabetes (T2D) is a recognized risk factor for osteoarthritis, with both conditions characterized by chronic low-grade inflammation, suggesting potential links between metabolic disorder and joint degeneration. This study aimed to investigate whether inflammatory and metabolic stresses regulate RBP4 expression and function in joint-related cells. Murine immature chondrocyte cells (iMACs) and the mouse AT805 teratocarcinoma cell line, clone 5, that differentiates into chondrogenic cells (ATDC5), were used as in vitro models for chondrocyte cells. *Rbp4* mRNA expression increased during differentiation of iMACs, with 3.6- and 2.2-fold elevations observed on days 7 and 14, respectively ($p < 0.01$ vs. undifferentiated controls). Inflammatory stimulation with interleukin-6 (IL-6) significantly increased *Rbp4* mRNA expression in ATDC5 cells ($p < 0.05$ vs. vehicle), along with elevated expression of catabolic and inflammatory mediators, including monocyte chemoattractant protein-1 (*Mcp1*), cyclooxygenase-2 (*Cox2*), and matrix metalloproteinase-3 (*Mmp3*) ($p < 0.05$ vs. vehicle). Pharmacological inhibition of RBP4 using fenretinide (FEN) attenuated chondrogenic differentiation marker expression, reduced glycosaminoglycan synthesis during chondrogenic differentiation, and mitigated high-glucose-induced catabolic responses, as indicated by reduced *Mcp2* ($p = 0.04$) and *Mmp13* ($p = 0.01$) expression in ATDC5 cells treated with FEN compared with cells treated with the vehicle under high-glucose conditions. Furthermore, in RAW 264.7 cells, a murine macrophage cell line commonly used as an in vitro model for osteoclastogenesis, FEN significantly reduced the expression of osteoclast differentiation markers, dendritic cell-specific transmembrane protein (*DC-Stamp*), nuclear factor of activated T-cells, cytoplasmic 1 (*Nf-atc1*), cathepsin k (*Cath.k*), and tartrate-resistant acid phosphatase (*Trap*) under osteoclastogenic conditions ($p < 0.01$ vs. vehicle). Collectively, these findings suggest that RBP4 functions as a metabolic-inflammatory mediator influencing both cartilage and



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bone-remodeling processes. This study reveals a previously unrecognized role of RBP4 in regulating osteoclast-associated pathways. Targeting RBP4 may, therefore, represent a promising therapeutic strategy for delaying or preventing osteoarthritis progression, particularly in metabolically compromised conditions.

Keywords: retinol-binding protein 4; osteoclast; chondrocyte; fenretinide

1. Introduction

Osteoarthritis (OA), a degenerative joint disease, is characterized by progressive cartilage degeneration and pathological changes in subchondral bone remodeling [1,2]. OA pathogenesis is heterogeneous and influenced by multiple factors, including the local joint microenvironment, lifestyle, environmental exposure, sex, and genetic background [3–8]. Accumulating evidence indicates that chronic low-grade inflammation associated with aging, joint injury, and metabolic disorders such as obesity and type 2 diabetes (T2D) plays a critical role in OA development and progression by disrupting cartilage and bone homeostasis [9–12]. These inflammatory conditions can alter articular chondrocyte fate and osteochondral bone formation at multiple levels. Current pharmaceutical therapies are largely limited to symptomatic relief, primarily through systemic administration of anti-inflammatory agents affecting the immune system. Severe side effects have been documented in some patients [13,14]. Consequently, a better understanding of the molecular pathways that drive cartilage degeneration, chondrocyte hypertrophy, and aberrant osteochondral remodeling is urgently needed to develop targeted and disease-modifying therapies for OA.

Among molecular mediators implicated in OA pathogenesis, increasing attention has been directed toward adipokines, which link metabolic dysfunction and inflammation to joint degeneration. Retinol-binding protein 4 (RBP4) is an adipokine primarily known for transporting retinol—an active form of vitamin A—to target tissues via binding to receptors such as those stimulated by retinoic acid 6. Beyond its classical transport function, RBP4 is an inflammatory mediator that can activate toll-like receptors and promote pro-inflammatory signaling [15]. Elevated expression of *Rbp4* in OA chondrocytes cultured in vitro, and circulating RBP4 levels are correlated with expression of *Mmp3* and other adipokines implicated in cartilage degradation [16].

Despite growing evidence linking RBP4 to OA-associated inflammation, the local effect of RBP4 on joint-resident cell populations has not been fully elucidated. While chondrocyte dysfunction is central to OA pathogenesis, abnormal osteoclast activity and subchondral bone remodeling are increasingly being recognized as critical contributors to disease initiation and progression. However, to date, the role of RBP4 in regulating osteoclast differentiation and function has not been investigated.

In this study, we examined the effects of inflammatory and metabolic stress on *Rbp4* expression in chondrocytes and assessed the functional role of RBP4 in regulating both chondrocyte and osteoclast differentiation. By addressing this previously unexplored axis, our work aims to elucidate a novel metabolic–inflammatory mechanism linking cartilage degeneration and bone remodeling in OA.

2. Materials and Methods

2.1. Ethics Statement

The Institutional Biosafety Committee (IBC) at the Veterans Affairs Loma Linda Healthcare System (VALLHS) approved this study, and it was conducted in accordance with

relevant guidelines and regulations. All animals were housed and maintained within the Veterinary Medical Unit of the Jerry L. Pettis Memorial VA Medical Center. All procedures with animals were approved by the Institutional Animal Care and Use Committee at the VA Loma Linda Health Care System, USA (ACORP# 0007-1213 and 0005-1442). All work was performed and reported in accordance with ARRIVE guidelines (PMID: 20613859).

2.2. Animals

3–4-day old C57BL/6J (B6) mice were used in this study. Animals were housed in a standard animal room with food and water ad libitum under controlled conditions of temperature (21 ± 2 °C), and humidity (45–75%), under a 12-hour (h)light/12 h dark lighting cycle. The mice were monitored by professional technicians and a licensed veterinarian. For euthanasia, animals were exposed to CO₂ prior to cervical dislocation, which is approved by the panel of Euthanasia of the American Veterinary Medical Association.

2.3. RBP4 Inhibitors and Recombinant Proteins

4-hydroxyphenylretinamide (fenretinide; FEN; cat#390900, lot#4110027) and A1120 (2-[[[4-[2-(trifluoromethyl)phenyl]-1-piperidinyl]carbonyl]amino]-benzoic acid; cat#HY-107633, batch#84777) were purchased from Sigma (Millipore/Sigma, Burlington, MA, USA). Both inhibitors were reconstituted in dimethyl sulfoxide (DMSO) and stored at -20 °C. Working concentrations were prepared via dilution in a culture medium immediately before use. Recombinant mouse interleukin-6 (IL-6; cat#406, lot#BE116061; R&D Systems, Minneapolis, MN, USA) was reconstituted in phosphate-buffered saline (PBS) at a stock concentration of 5 µg/mL and diluted to a final concentration of 10 ng/mL in culture medium. Recombinant mouse receptor activator of nuclear factor κB ligand (RANKL; cat#462, lot#CWA 1815112; R&D Systems (R&D Systems/Bio-Techne, Minneapolis, MN, USA) was reconstituted in PBS at a stock concentration of 100 µg/mL and stored at -80 °C.

2.4. Cell Culture

2.4.1. Immature Articular Chondrocytes (iMACs)

iMACs were isolated from the knees of 3–4-day-old C57BL/6J (B6) mice, as previously described [17]. Cells isolated from knee joints, collected from six litters, were used in this study. The cells were plated at a density of 4×10^5 cells per well in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (DMEM/F-12; Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA). The cells were maintained in a humidified incubator at 37 °C with 5% CO₂. After 24 h, the medium was replaced with DMEM/F-12 containing 2% FBS (a control growth medium). The serum was reduced to minimize the presence of exogenous factors or extracellular vesicles found in FBS that might interfere with experimental outcomes or analysis. The cells were treated in a differentiation medium (DM) comprising growth media supplemented with 50 µg/mL ascorbic acid (AA), 10 µM β-glycerophosphate (BGP), and insulin–transferrin–selenium (ITS; 1 mg/mL insulin, 0.55 mg/mL transferrin, and 0.67 µg/mL selenium; Thermo Fisher Scientific, Riverside, CA, USA) for 7 or 14 days to induce chondrocyte differentiation. The culture medium was changed every 2–3 days. ITS promotes chondrocyte differentiation in the presence of FBS—a source of retinol—while AA enhances this effect. When using the RBP4 inhibitor, the DM was supplemented with either DMSO (vehicle) or FEN at the indicated concentrations.

2.4.2. ATDC5 Chondrogenic Cell Line

ATDC5 chondrogenic cells (Sigma-Aldrich, St. Louis, MO, USA) were maintained in a growth medium consisting of DMEM/F-12 (1:1) supplemented with 5% FBS and

penicillin/streptomycin. For different treatments, cells were seeded at a density of 6×10^3 cells per well in 12-well plates. Upon reaching confluence, the medium was replaced with either a growth medium containing normal glucose (5.5 mM; control) or a growth medium supplemented with glucose at a final concentration of 10 g/L (HG), in the presence of vehicle or FEN. The cultures were maintained for 5 days, and the medium was replaced every 2 days. The ATDC5 cells were treated for 5 days with 10 ng/mL recombinant IL-6 under differentiation conditions to assess the involvement of RBP4 in inflammation. The ATDC5 cells in the differentiation media were treated with vehicle (DMSO), FEN, or A1120 at the indicated concentrations to investigate the role of RBP4 in chondrocyte differentiation.

2.4.3. Murine RAW 264.7 Cell Line

The murine RAW 264.7 monocyte/macrophage cell line (ATCC, Manassas, VA, USA), a well-recognized in vitro model for studies on osteoclast development and functions [18], was used in this study. RAW cells were cultured in α -modified Eagle's medium (α -MEM; cat#12571-063, Gibco BRL, Carlsbad, CA, USA) supplemented with 10% FBS and penicillin/streptomycin (growth medium) at 37 °C in 5% CO₂. Upon reaching approximately 80% confluence, the cells were scraped and replated at a density of 5×10^4 cells per well in 6-well plates and allowed to adhere overnight. Osteoclast differentiation was induced in a growth medium supplemented with 50 ng/mL receptor activator of nuclear factor κ B ligand (RANKL; R&D Systems, Minneapolis, MN, USA). Cells cultured in the absence of RANKL served as undifferentiated controls. The cells differentiated in the presence of DMSO (vehicle) or FEN at the indicated concentrations. The experiments were performed in duplicate, with consistent findings. A single representative dataset is shown.

2.5. RNA Extraction and Quantitative Real-Time PCR (qPCR)

At the indicated time points, total RNA was extracted using an EZ-10 Total RNA Mini-Preps Kit (cat#BS88583; BioBasic, Markham, ON, Canada). cDNA was synthesized from 0.5 μ g of total RNA using an M-MLV reverse transcriptase kit (cat#M1708, lot# 642009; Promega, Madison, WI, USA). Quantitative PCR was performed using pre-designed primers (Integrated DNA Technologies, San Diego, CA, USA) and a SYBR Green real-time PCR kit (cat#RM21203, lot#9625030731WA; Abclonal, Woburn, MA, USA) on a ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific, Riverside, CA, USA).

Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. ΔC_t values were calculated by subtracting the C_t of the internal control gene from the C_t of the target gene for each sample. $\Delta\Delta C_t$ values were obtained by subtracting the average ΔC_t of the control group from the ΔC_t of each treated sample. The fold change was calculated based on $2^{-\Delta\Delta C_t}$. Data normalization was performed using both peptidylprolyl isomerase A (Ppia) and 18S rRNA as internal controls. Only genes displaying consistent changes in expression after normalization with both reference genes are presented and discussed.

2.6. Chondrocyte Proliferation Assay

The potential effects of RBP4 inhibitors on cell proliferation were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cat# M-2128, lot#41H5008; Sigma, St. Louis, MO, USA). ATDC5 cells were seeded in 96-well plates at a density of 3×10^4 cells per well in media without serum and treated with DMSO (vehicle), A1120 (0.1, 1, and 10 μ M), or FEN (1, 2.5, 5, and 10 μ M) for 24 h. MTT was added to a final concentration of 1 mg/mL, and the cells were incubated for 3 h at 37 °C. Formazan crystals were dissolved using acidic isopropanol, and the absorbance was measured at 570 nm using a microplate reader (Synergy 2, BioTek, Winooski, VT, USA) to quantify viable cells.

2.7. Proteoglycan Synthesis Assay

Glycosaminoglycan (GAG) synthesis was assessed using the Alcian blue staining method, as previously described [19]. Following treatment, the cells were washed with PBS, fixed in 95% methanol for 20 min at room temperature, and stained overnight with 0.1% Alcian blue in 0.1 N HCl. After rinsing with distilled water, the Alcian blue-stained cultures were extracted with 6 M of guanidine-HCl for 6 h at room temperature. The optical density was measured at 630 nm using spectrophotometry.

2.8. Statistical Analysis

The data are presented as mean $\pm$ standard deviation (SD). In vitro experiments using primary chondrocytes were performed using cells isolated from 3 to 4-day-old mice collected from six litters, each having 4 to 6 pups, and statistical significance was determined using two-tailed Student's *t*-tests. One-way ANOVA, followed by post hoc Tukey HSD tests to adjust for multiple comparisons, was conducted for all the other assays. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

Given the emerging evidence of the role of retinoid signaling in chondrocyte differentiation [20], we first examined *Rbp4* expression during chondrogenesis using murine iMACs isolated from neonatal mice. *Rbp4* mRNA expression was increased by 3.6- and 2.5-fold (both $p < 0.05$) compared with control cells in growth media, respectively, at days 7 and 14, while that of the retinoid receptor *Rxrg* was elevated at day 14 only (2.10-fold; $p < 0.05$; Figure 1). As expected, the expression levels of chondrocyte differentiation markers, including *Mmp3* (3.2-fold; $p < 0.005$), *Mmp13* (2.1-fold; $p < 0.05$), *Col10* (2.3-fold; $p < 0.05$), and Aggrecan (*Acan*; 2.1-fold; $p < 0.05$) were significantly upregulated on day 14. This suggests that *Rbp4* expression is dynamically regulated during chondrocyte differentiation and is associated with the acquisition of a hypertrophic phenotype.

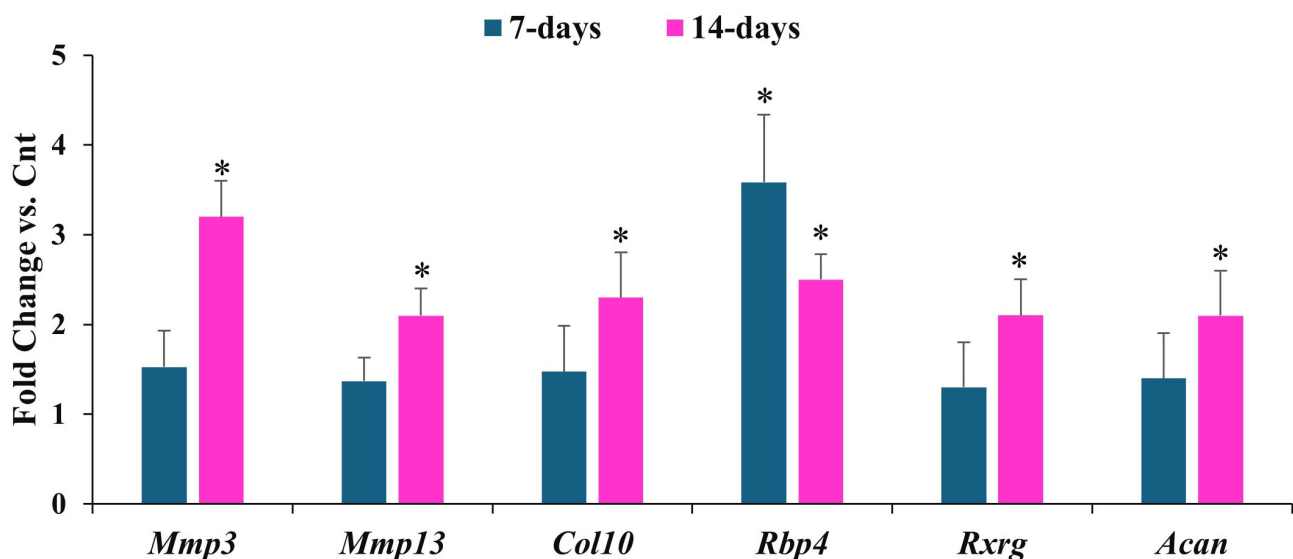


Figure 1. Changes in expression levels of *Rbp4* and chondrocyte differentiation markers during in vitro differentiation of primary articular chondrocytes. Murine iMACs cultured in DM and in growth media (control) for 7 and 14 days. Cells were harvested, and RNA was extracted and reverse-transcribed. qPCR was performed using pre-designated primers. Fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method. * $p < 0.05$ vs. control (Cnt); $n = 6$.

We tested the effects of RBP4 inhibitors, FEN and A1120, on the ATDC5 chondrogenic cell line to examine whether locally expressed RBP4 influenced chondrocyte proliferation, survival, and differentiation. ATDC5 cell proliferation, as determined using MTT assay, was not significantly affected by treatment with FEN (1, 2.5, 5, and 10 μ M) or A1120 (0.1, 1, and 10 μ M) compared with vehicle-treated controls (Figure 2), as evidenced by the absence of significant changes in optical density (OD) values (Figure 2; $p < 0.05$ vs. vehicle).

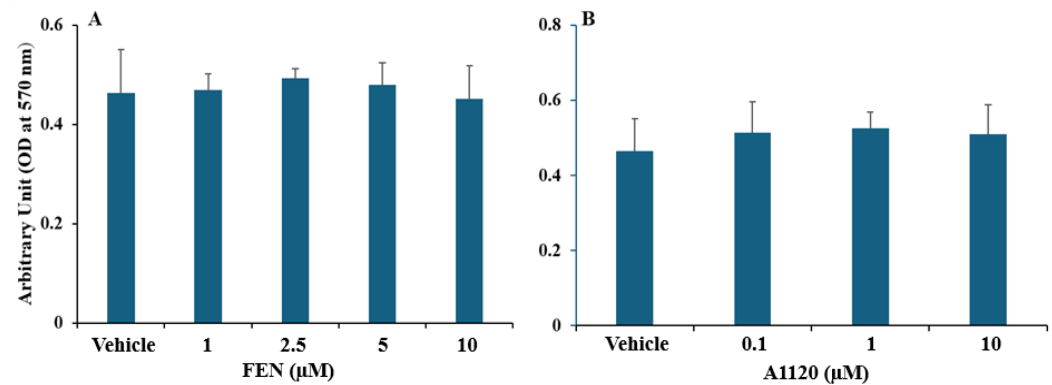


Figure 2. Effects of FEN and A1120 on ATDC5 proliferation measured using MTT assay. Cells were incubated in media without serum for 24 h in the presence of a vehicle (DMSO), different concentrations (1, 2.5, 5, and 10 μ M) of FEN (A), or 0.1, 1, and 10 μ M doses of A1120 (B). After MTT incubation for 3 h, the formazan precipitate was dissolved by adding acidic isopropanol before measuring the absorbance at 570 nm. $n = 6$; * $p < 0.05$ vs. vehicle.

ATDC5 cells were cultured under hyperglycemic (HG) or inflammatory conditions to assess the contribution of RBP4 to metabolic and inflammatory responses (Figure 3). HG exposure significantly increased the expressions of *Mmp13* (2.60-fold vs. Cnt) and *Mcp2* (*Ccl8*; 3.42-fold vs. Cnt), whereas treatment with FEN significantly reduced the expressions of *Mmp13* and *Ccl8* ($p > 0.05$ vs. Cnt; $p < 0.05$ vs. vehicle; Figure 3A).

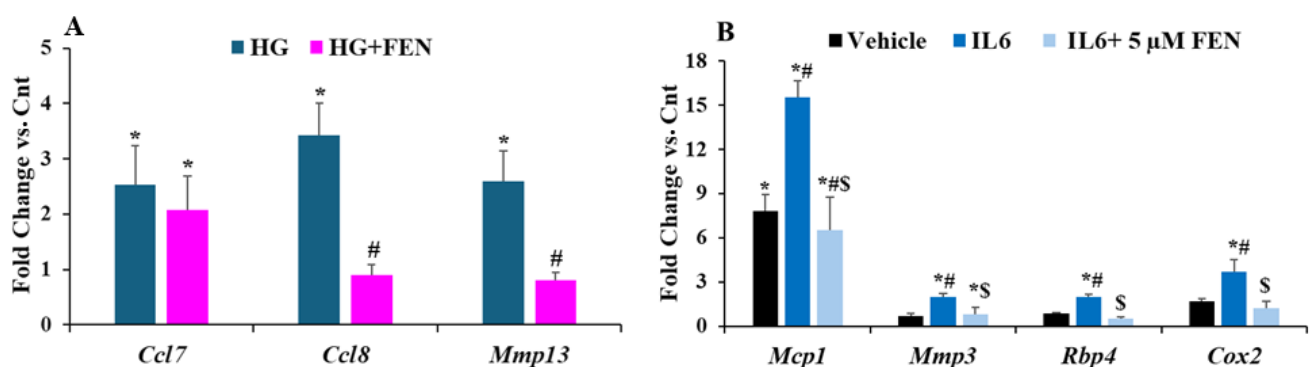


Figure 3. FEN reverses the catabolic effect of hyperglycemia and inflammation in chondrocytes. ATDC5 cells were cultured in growth media with 5% FBS (Cnt). The media was supplemented with 10 g/L glucose (HG) in the presence or absence of 5 μ M FEN (A). Cells were switched to growth media with 2% FBS (Cnt), or DM containing a vehicle and 10 ng/mL IL-6, or IL-6 and 5 μ M FEN (B). The cells were treated for 5 days. Data are expressed as averages of fold changes in all samples from each treatment compared with Cnt (growth media) and presented as mean $\pm$ SD. $n = 4$ –6. * $p < 0.05$ vs. Cnt, # $p < 0.05$ vs. HG (A), or # $p < 0.05$ vs. DM with vehicle (B), and \$ $p < 0.05$ vs. DM with IL6 (B).

Similarly, treatment with recombinant IL-6 under differentiation conditions increased the expressions of *Rbp4* (2.3-fold; $p = 0.004$ vs. DM with vehicle), *Mcp1* (2.17-fold; $p = 0.04$ vs. DM with vehicle), *Mmp3* (2.45-fold; $p = 0.001$ vs. DM with vehicle), and cyclooxygenase-2

(*Cox2*; 2.2-fold; $p = 0.038$ vs. DM with vehicle). Treatment with FEN in DM significantly reduced the expression of all the genes tested (Figure 3B). Correlation analysis, comparing the changes in ΔCt values in response to different treatments, revealed a strong positive association between *Rbp4* and *Mmp3* expression ($R = +0.85$; $p < 0.001$), consistent with previous observations in OA cartilage [16]. However, no correlation was observed between *Rbp4* and *Mcp1* expression ($R = +0.12$; $p = 0.51$).

We next tested whether treating cells with the RBP4 inhibitor, FEN, affected the expression of chondrocyte differentiation markers in ATDC5 cells. Cells cultured in differentiation media, for 8 days, showed increased expression of *Acan* (4.5-fold; $p = 0.02$ vs. Cnt), alkaline phosphatase (*Alp*; 2.5-fold; $p = 0.005$ vs. Cnt), *Sox9* (1.95-fold; $p = 0.03$ vs. Cnt), and cyclooxygenase-2 (*Cox2*; 2.42-fold; $p = 0.04$ vs. Cnt) compared with control media (Figure 4A). However, treatment with 2.5 μM FEN, but not 0.25 μM FEN, significantly reduced the expression of *Acan*, *Alp*, and *Cox2*. This suggests a dose-dependent effect of FEN. However, neither 0.25 μM nor 2.5 μM FEN had any effect on *Sox9* expression (Figure 4A). ATDC5 cells were cultured in differentiation media in the presence of a vehicle or 5 and 10 μM FEN to evaluate the effect of FEN on GAG synthesis. Ten-day treatment with differentiation media significantly increased GAG synthesis, as evidenced by the increased optical density (OD) at 630 nm after guanidine extraction (Figure 4B), which was reduced by treatment with FEN in a dose-dependent manner.

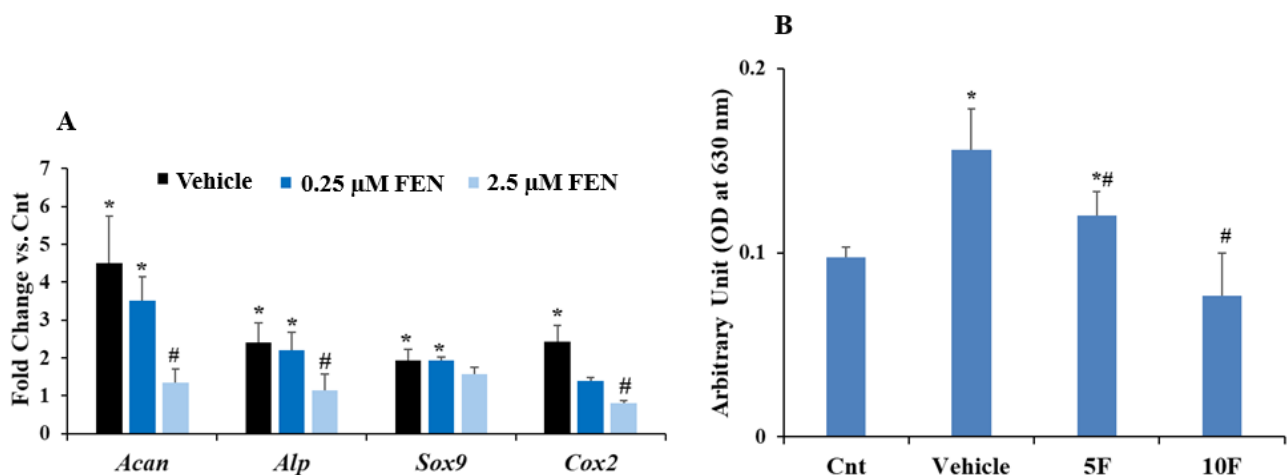


Figure 4. Treatment of chondrocyte precursors with FEN was associated with reduced expression of chondrocyte differentiation markers as well as reduced glycosaminoglycan (GAG) release in a dose-dependent manner. Murine chondrocyte progenitor cell line ATDC5 was treated with FEN at 0.25 and 2.5 μM in differentiating media (DM) for 8 days. Cells were then harvested, and RNA was extracted and reverse-transcribed. Then, quantitative PCR (qPCR) was performed using pre-designated primers. Fold change compared with growth media control was calculated based on the $2^{-\Delta\Delta\text{Ct}}$ method (A). ATDC5 cells were cultured in growth medium (Cnt) or in DM and treated for 10 days with vehicle and 5 (5FEN) and 10 μM (10FEN) FEN. Then, GAG was extracted with guanidine thiocyanate and measured at 630 nm (B). Data are presented as mean $\pm$ SD. * $p < 0.05$ vs. control (cells in growth medium); # $p < 0.05$ vs. vehicle-treated cells in DM. $n = 6$.

Mouse iMACs were cultured in the presence of 5 μM FEN or a vehicle for 14 days under differentiation conditions to confirm FEN's effect on chondrocyte differentiation. The expression of chondrogenic markers was then evaluated. As expected, the expression of aggrecan (*Acan*), type II collagen (*Col.2*), and type X collagen (*Col.10*) increased by 2.3-, 1.8-, and 5.5-fold, respectively, in DM compared with control undifferentiated cells (Figure 5). In contrast, expression of proteoglycan 4 (*Prg4*) was significantly reduced in

DM compared with the control (Cnt); however, co-treatment with 5 μ M FEN reversed this effect, confirming the influence of FEN on chondrocyte differentiation.

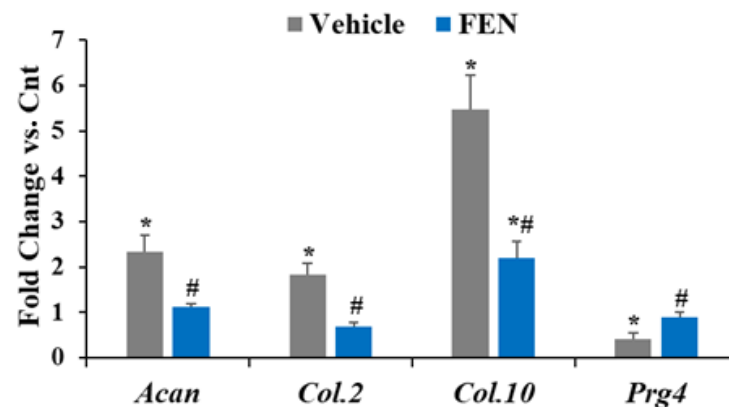


Figure 5. Expression of different chondrogenesis markers was abolished by FEN. iMACs were treated for 14 days with either vehicle or 5 μ M FEN in differentiation medium. Then, the cells were harvested, and mRNA expression changes were quantified using qPCR. Data are expressed as average fold changes in all samples from each treatment compared with Cnt (growth media) and presented as mean $\pm$ SD. n = 6. * $p < 0.05$ vs. Cnt; # $p < 0.05$.

Since RBP4 and FEN can affect both retinoid and non-retinoid pathways, we next tested the effect of a non-retinoid RBP4 antagonist, A1120, on chondrocyte differentiation. ATDC5 cells were cultured under DM in the presence of a vehicle or three different concentrations of A1120. The expression of *Acan*, *Alp*, *Sox9*, and *Cox2* increased by 2.96-, 1.8-, 2-, and 2.7-fold, respectively, in DM with the vehicle compared with the control growth media ($p < 0.05$). Meanwhile, blocking the function of RBP4 with 10 μ M A1120 in DM significantly reduced the expression of *Acan* and *Cox2* compared with the vehicle-treated DM ($p < 0.05$ vs. Cnt). However, A1120 did not affect the expression of *Alp* and *Sox9* (Figure 6).

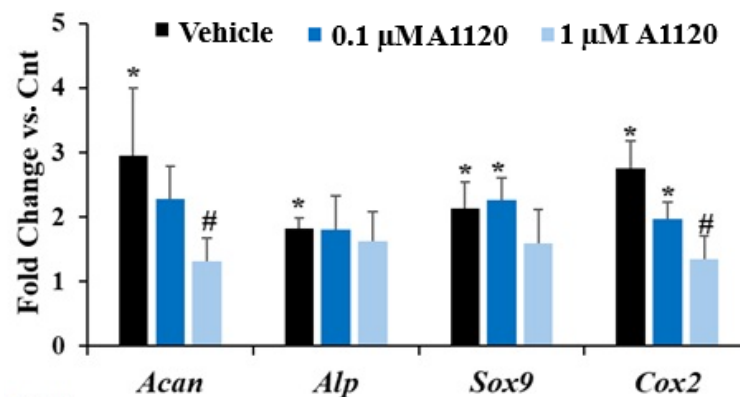


Figure 6. Effects of A1120, a non-retinol RBP4 antagonist, on the expression levels of chondrocyte differentiation markers in ATDC5 cells. Murine chondrocyte progenitor cell line ATDC5 was cultured in differentiation media and treated with vehicle or A1120 at 0.1 and 1 μ M for 8 days. Then, qPCR was performed using pre-designated primers. Fold changes were calculated based on $2^{-\Delta\Delta C_t}$. Data is presented as mean $\pm$ SD. * $p < 0.05$ vs. Cnt (cells in growth media); # $p < 0.05$ vs. vehicle-treated cells. n = 6.

Because abnormal osteoclast activity contributes to osteochondral remodeling in OA [21–23], we next examined whether RBP4 influenced osteoclast differentiation. We treated RAW264.7 cells with RANKL in the presence of a vehicle (DMSO) or FEN for seven days to induce osteoclast differentiation and to determine if *Rbp4* expression changed during osteoclast differentiation. As expected, RANKL treatment increased the expression

of *Nfatc1* (2.9-fold; $p = 0.001$ vs. Cnt), *Dc-stamp* (4.2-fold; $p = 0.001$ vs. Cnt), *Cath.k* (24.6-fold; $p < 0.001$ vs. Cnt), and *Trap* (39.7-fold; $p < 0.001$ vs. Cnt), which are all involved in cell migration, fusion, and osteoclast differentiation. While RANKL (vehicle) treatment only caused a modest increase in *Rbp4* expression, treatment with both 5 and 10 μ M FEN reduced the expression of osteoclast differentiation markers in a dose-dependent manner (Figure 7A, B). Compared with the control, co-treatment with 5 and 10 μ M FEN reduced the expression of *Nfatc1* to 1.2 and 1.3-fold, and the expression of *Dc-Stamp* to 0.53- and 0.35-fold, respectively. Similarly, the expression of *Cath.k* was reduced to 4.5- and 1.2-, and the expression of *Trap* to 10.6- and 8.5-fold, compared with the control when cells were co-treated with FEN. In addition, the expression of chemokine receptors known to be involved in osteoclast differentiation was evaluated after treatment with RANKL in the presence of the vehicle or FEN (Figure 7C). While the expression of both *Cxcr4* and *Cx3cr1* was reduced with RANKL treatment compared with the control undifferentiated cells (Figure 7C), *Ccr1* was significantly increased (9.4-fold; $p = 0.001$ vs. Cnt) after the seven-day treatment with RANKL. However, the addition of both 5 and 10 μ M FEN significantly reduced the expression of *Ccr1* compared with the vehicle ($p = 0.03$).

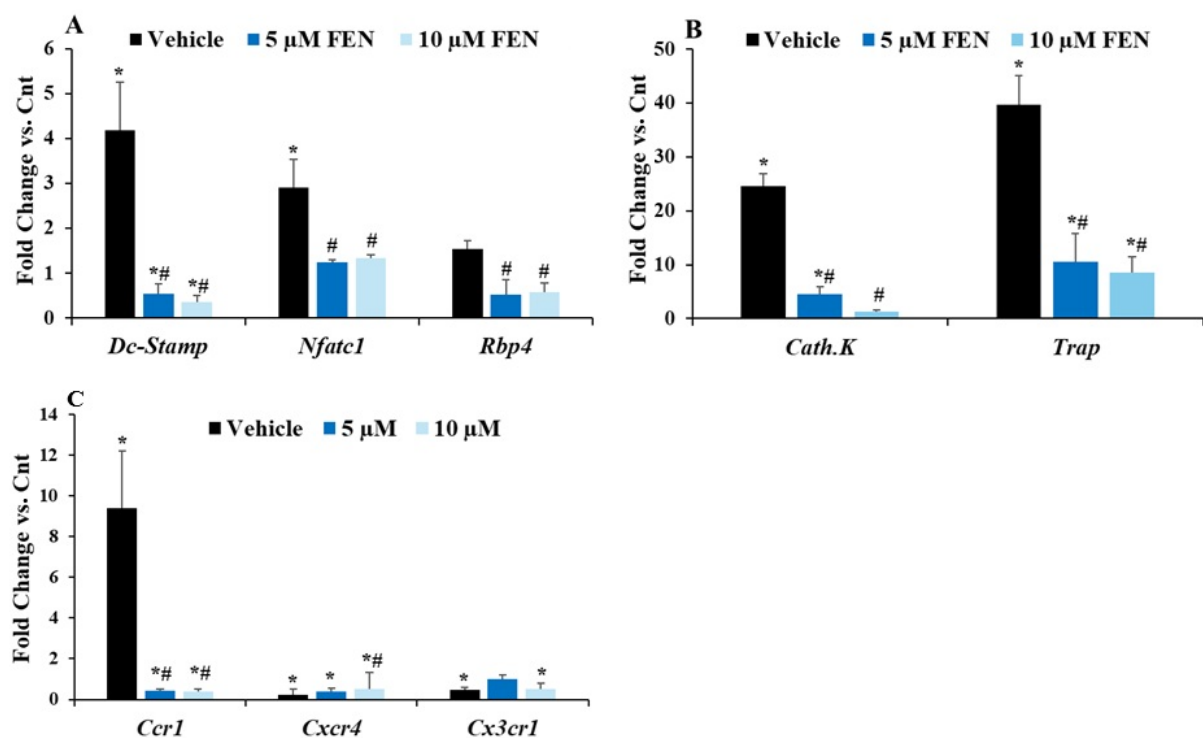


Figure 7. Treatment of osteoclast precursors with FEN reduces the expression of the major osteoclast differentiation markers in the presence of RANKL. RAW264.7 cells were induced to differentiate via treatment with 100 ng/mL RANKL in the presence of vehicle or FEN (5 or 10 μ M) for seven days. Cells were harvested, and mRNA expression of different genes was evaluated using qPCR (A–C). Data are expressed as average fold changes in all samples from each treatment versus control and are presented as mean $\pm$ SD. * $p < 0.05$ vs. Cnt, and # $p < 0.05$ vs. RANKL with vehicle-treated samples; $n = 6$.

4. Discussion

OA affects over 240 million individuals [24], and its increasing prevalence, combined with treatment-related adverse effects, represents a significant clinical and socioeconomic burden. Current pharmacological management of OA remains largely symptomatic and is frequently associated with significant adverse effects. Non-steroidal anti-inflammatory drugs (NSAIDs), commonly prescribed for pain relief, have been linked to gastrointestinal,

cardiovascular, and renal complications, particularly with long-term use [25]. Intra-articular corticosteroid injections may provide short-term symptom relief but have been associated with potential cartilage deterioration when repeatedly administered [26]. Similarly, while opioid analgesics are occasionally prescribed for severe OA pain, their use is limited by risks of dependence and systemic side effects [27]. These limitations highlight the need for safer disease-modifying approaches, underscoring the potential therapeutic relevance of the mechanisms explored in the present study.

In this study, we identified RBP4 as a metabolic–inflammatory mediator that regulated both chondrocyte and osteoclast differentiation, which are two central processes implicated in OA pathogenesis [23,28]. Using complementary *in vitro* models, we demonstrated the induction of *Rbp4* expression during chondrocyte differentiation and inflammatory stress; its pharmacological inhibition attenuated catabolic, inflammatory, and differentiation-associated gene expression. We provided the first evidence that RBP4 inhibition suppressed osteoclast differentiation, suggesting a previously unrecognized role for RBP4 in osteochondral remodeling.

Immature murine articular chondrocytes were induced to differentiate under defined chondrogenic conditions to explore the potential involvement of RBP4 in chondrocyte differentiation. Under these conditions, *Rbp4* mRNA expression was significantly increased relative to undifferentiated control cells. The changes in *Rbp4* expression during differentiation correlated with the expression of *Mmps*, which are established markers of chondrocyte hypertrophy and degradation. These observations are consistent with previously reported findings showing RBP4 localization in chondrocytes of developing mouse long bones [29] and reinforce the hypothesis that RBP4 contributes to chondrocyte hypertrophy and cartilage degeneration.

FEN, a synthetic derivative of all-trans-retinoic acid initially developed for anticancer therapy [30], was used to investigate the effects of RBP4 inhibition on both chondrocytes and osteoclasts. FEN acts as an atypical retinoid with both retinoid-dependent and retinoid-independent functions. A preliminary dose–response analysis was performed to assess FEN cytotoxicity over a broad concentration range (1–10 μ M), which did not reveal any adverse effects on cell viability. Based on these data, 5 and 10 μ M FEN were selected for functional assays assessing glycosaminoglycan (GAG) synthesis, where a dose-dependent effect was observed. We subsequently tested 0.25 and 2.5 μ M FEN to determine whether lower, submaximal concentrations could also modulate chondrocyte differentiation. This approach allowed us to evaluate biological effects at concentrations below those previously shown to influence matrix production.

In ATDC5 cells, treatment with FEN reduced the expression of *Acan*, *Alp*, and *Cox2* and decreased GAG synthesis. While *Acan* marks early-to-mid-stage chondrogenic differentiation, *Alp* is typically associated with late-stage mineralization. The early upregulation of *Alp* observed in this study may be attributable to the presence of BGP, which enhances phosphate availability and ALP activity [31]. The effect of FEN on chondrocyte differentiation was further confirmed in iMACs, where FEN significantly modulated key markers of cartilage hypertrophy. Specifically, *Col10* expression was reduced, whereas *Prg4* (lubricin) expression was increased following FEN treatment. Lubricin is a mucinous glycoprotein essential for articular cartilage lubrication and protection against chondrocyte apoptosis [30,32]. Accordingly, its upregulation suggests a protective role of FEN in maintaining cartilage homeostasis and joint integrity.

To explore the role of RBP4 in retinoid-independent signaling, we used A1120, a non-retinoid RBP4 antagonist that binds to RBP4 and prevents the interaction between RBP4 and its binding partner, transthyretin, consequently reducing retinol delivery [33]. Treatment with A1120 attenuated the expression of inflammatory and catabolic markers

in differentiating chondrocytes, supporting a retinoid-independent role for RBP4 in chondrocyte hypertrophy and inflammation. These findings align with the dual activity of RBP4 and confirm that its effects in OA are not solely mediated via canonical retinoid signaling pathways.

In this study, we established the link between RBP4, metabolic stress, and inflammation in chondrocytes. We demonstrated that FEN treatment could mitigate catabolic and inflammatory gene expression induced by hyperglycemic conditions. IL-6 stimulation increased the expression of *Cox2*, *Mmp3*, and *Mcp1*, whereas inhibition of RBP4 markedly reduced these responses. These findings suggest that RBP4 functions as an upstream mediator linking metabolic and inflammatory cues to catabolic signaling pathways relevant to OA. Furthermore, a positive correlation between *Rbp4* and *Mmp3* expression supported their roles in OA-associated inflammation and matrix degradation [34,35]. Given that MCP-1 recruits immune cells and exacerbates inflammation, especially in metabolic and post-traumatic settings, the interplay between RBP4, MMP3, and MCP-1 warrants further investigation.

In addition to its inflammatory role, OA is characterized by abnormal bone and cartilage resorption. Synovial macrophages contribute to osteophyte formation, fibrosis, and can differentiate into osteoclasts responsible for osteochondral degradation [36,37]. Targeting osteoclast activity could, therefore, provide therapeutic benefit by preserving joint integrity. Although previous research has linked RBP4 to systemic metabolic inflammation [34], its role in osteoclast biology has not been characterized. Here, we demonstrated that RBP4 pharmacologic inhibition using FEN significantly reduced RANKL-induced expression of osteoclastogenic transcription factors and markers, identifying RBP4 as a novel regulator of bone resorption in OA. These findings suggest that metabolic mediators such as RBP4 may directly influence osteochondral remodeling pathways.

While these findings highlight a novel role for RBP4 in OA pathogenesis, some limitations warrant further consideration. First, the reliance on immortalized cell lines requires follow-up validation in primary chondrocytes and osteoclast precursors, as well as in ex vivo joint tissue models. Second, although FEN is widely used as an RBP4 inhibitor, its specificity in blocking retinoid signaling needs to be confirmed in osteoclast precursors using genetic tools such as siRNA knockdown or conditional knockout models. Finally, in vivo studies using cell-type-specific deletion of *Rbp4* will be essential to establish its causal role in OA progression.

5. Conclusions

This study highlighted a novel role of RBP4 in osteoclastogenesis and identified RBP4 as a key link between metabolic dysfunction and joint degeneration. Considering the role of RBP4 in both metabolic and inflammatory pathways, an important consideration for its therapeutic targeting in OA is the potential impact on bone development and remodeling. Our findings indicate that inhibition of RBP4 reduces markers of chondrocyte hypertrophy and osteoclast differentiation, suggesting a role for RBP4 in pathological joint remodeling rather than in normal skeletal development. Importantly, therapeutic strategies aimed at RBP4 in OA would ideally rely on local, intra-articular delivery to limit systemic exposure and minimize potential effects on overall bone homeostasis. While our in vitro data support a modulatory role for RBP4 in cartilage and bone-associated pathways within the joint, the consequences of long-term or systemic RBP4 inhibition—particularly during growth or in non-diseased bone—remain to be determined. Future in vivo studies will be required to define the safety, spatial specificity, and translational potential of RBP4-targeted approaches in OA.

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Institutional Review Board Statement: The Institutional Biosafety Committee (IBC) at the Veterans Affairs Loma Linda Healthcare System (VALLHS) approved this study, and it was conducted in accordance with relevant guidelines and regulations. All animals were housed and maintained within the Veterinary Medical Unit of the Jerry L. Pettis Memorial VA Medical Center. All procedures were performed under humane conditions and approved by the Institutional Animal Care and Use Committee at the VA Loma Linda Health Care System, USA (ACORP# 0007-1213 (approval date: 16 August 2022) and 0005-1442 (approval date: 10 July 2024). All work was performed and reported in accordance with ARRIVE guidelines (PMID: 20613859).

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Data Availability Statement: The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

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