Journal of Orthopaedic Surgery and Research

RESEARCH

Open Access

Pain mediator NGF improves chondrocyte extracellular matrix synthesis via PI3K/AKT pathway

Mengling Wang[1], Jie Lian[1], Maoqing Ye[2] and Bingchen An[1*]

Abstract

Objectives Nerve growth factor (NGF) is a key mediator in osteoarthritis pain signaling. Clinical studies revealed that anti-NGF antibodies are often accompanied by progressively worsening cartilage degeneration, although they exhibit significant analgesic effects. However, the relationship between NGF expression and cartilage destruction remains unclear. Our study aimed to investigate the effects of NGF on chondrocytes and to elucidate the underlying mechanisms involved.

Methods The ATDC5 cells were induced to differentiate into chondrocytes and stimulated with NGF at different concentrations (0.5–10 ng/mL). The cell counting kit-8 assay (CCK-8) was used to measure the effects of NGF on chondrocyte proliferation. Chondrocytes were subsequently stimulated with varying doses of NGF to identify the expression levels of the extracellular matrix. Chondrocytes were pretreated with GNF5837 (a tropomyosin receptor kinase A inhibitor) or LY294002 (a phosphoinositide 3-kinase inhibitor) before exposure to 5 ng/mL NGF to analyze associated signaling pathways. Western blotting and immunofluorescence staining were employed to analyze expression of related proteins.

Results Alcian blue, toluidine blue staining, and type II collagen immunofluorescence staining demonstrated that ATDC5 cells differentiated into functional chondrocytes after 14 days of chondrogenic induction. The CCK-8 assay confirmed that cell proliferation was unaffected. NGF (0.5–5 ng/mL) was found to enhance chondrocyte matrix synthesis in a dose-dependent fashion, particularly in the expression of aggrecan, type II collagen, Sox9, and through the activation of the PI3K/AKT signaling pathway. The highest promoting effects were exhibited at 5 ng/mL of NGF. Further analysis indicated that GNF5837 (TRKA inhibitor) or LY294002 (PI3K inhibitor) could reverse the protective effects of NGF on chondrocyte matrix synthesis.

Conclusion Our study identified a potentially beneficial role of NGF at concentrations of 0.5–5 ng/mL in chondrocytes, enhancing extracellular matrix synthesis, with significant involvement of the PI3K/AKT signaling pathway in this process.

Keywords Nerve growth factor, Chondrocytes, Anabolism, Osteoarthritis

*Correspondence:
Bingchen An
anbingchen@fudan.edu.cn
[1]Department of Rehabilitation, Huadong Hospital Affiliated to Fudan University, Shanghai 200040, China
[2]Department of Cardiology, Huadong Hospital Affiliated to Fudan University, Shanghai 200040, China

Introduction

Osteoarthritis (OA) is a degenerative disorder primarily characterized by progressive cartilage destruction, affecting nearly 300 million people globally [1]. Pain is the characteristic symptom of OA, which significantly affects daily life and leads to disability [2]. Nerve growth factor (NGF) is considered a key modulator in pain perception. Clinical trials involving individuals with OA demonstrated that anti-NGF antibodies significantly alleviated OA-associated pain, potentially offering a better alternative to non-steroidal anti-inflammatory drugs or opioid treatments. However, rapidly progressive osteoarthritis (RPOA), particularly the accelerated degeneration of cartilage, has been observed in a small subset of participants treated with NGF monoclonal antibodies [3]. This severe adverse effect was dose-dependent [4–6], significantly limiting the clinical use of NGF inhibitors. To date, the relationship between NGF expression and cartilage destruction remains unclear.

Chondrocytes, the only cell type of avascular cartilage, are wrapped in a matrix network of mostly type II collagen (Col-II) and aggrecan (ACAN) that they synthesize [7, 8]. Although NGF expression in healthy knee chondrocytes appears to be minimal, multiple studies have indicated that structurally damaged cartilage exhibits increased NGF expression at both the mRNA and protein levels [9–13]. Lannone et al. found that NGF levels gradually increased depending on the severity of osteoarthritic cartilage damage [9]. Mechanical injury, transforming growth factor-beta (TGF-β), and the inflammatory cytokine interleukin-1 beta (IL-1β) have been demonstrated to promote the expression of chondrogenic NGF in both human and animal cartilage [12, 14–16]. Furthermore, NGF receptors were highly expressed in OA cartilage [9, 17]. NGF and its receptors are closely linked to degenerated cartilage, suggesting that NGF signaling may play a crucial role in the pathophysiology of cartilaginous tissue.

Previous studies have indicated that NGF affects chondrocytes. Human chondrocytes co-cultured with 10 ng/mL NGF enhanced fibroblast growth factor 2 (FGF2) expression, resulting in angiogenesis [18]. Wei et al. found that 10 ng/mL NGF elevated the expression of acid-sensing ion channel 1a (ASIC1a), which subsequently triggered acid-induced chondrocyte apoptosis [19]. Jiang et al. observed that human OA cartilage exhibited increased sulfated glycosaminoglycan loss and cartilage matrix degradation after 14 days of co-culture with 10 ng/mL NGF [17]. In this study, we utilized ATDC5 chondrocytes to preliminarily examine how varying concentrations of NGF (0.5, 2.5, 5, and 10 ng/mL) affect chondrocyte proliferation and matrix synthesis, along with the underlying molecular mechanisms.

NGF has two receptors: the high-affinity tropomyosin-receptor kinase A (TRKA) and the p75 neurotrophin receptor (p75NTR). Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling is a classic regulatory pathway and can be activated by NGF binding to TRKA. The PI3K/AKT pathway plays a crucial role in the physiological effects of NGF and chondrogenic differentiation [20–23]. Yu et al. revealed that NGF-activated PI3K/AKT signaling induces angiogenesis in chondrocytes in vitro [18]. Furthermore, NGF-mediated activation of the PI3K/AKT pathway has been found to stimulate chondrogenic differentiation of mesenchymal stem cells [24]. Therefore, it would be valuable to investigate whether PI3K/AKT signaling is involved in regulating chondrocyte matrix components by NGF.

Materials and methods

Cell culture and chondrogenic differentiation

ATDC5 cells were sourced from Shanghai Shycbio Technology Co., Ltd. and maintained in Dulbecco's modified Eagle's medium/ Ham's F-12 Nutrient mixture (DMEM/F-12, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (LAISI, Shanghai, China). After reaching approximately 80% confluence, 1% insulin, transferrin, and selenite (ITS, Sigma, Germany) were added to the medium to induce the differentiation of ATDC5 cells at 37 °C in a humidified incubator with 5% CO_2 for 21 days. The differentiation medium was then replaced daily. The cells were passaged at a ratio of 1:3–1:4 using 0.25% trypsin-ethylenediaminetetraacetic acid solution (Thermo Fisher Scientific, USA) once they reached 90% confluence. The ATDC5 cells of 14 days of chondrogenic induction were used for the cell counting Kit-8 (CCK-8), western blotting, and immunofluorescence staining.

Alcian blue and toluidine blue staining

The ATDC5 cells on days 0, 7, 14, and 21 of induced differentiation were seeded at a density of 2.5×10^6 cells/mL per 6-cm dish and left overnight. Subsequently, cells were incubated with 4% paraformaldehyde (MKBio, Shanghai, China) for 20 min at room temperature. The cells were then rinsed with phosphate-buffered saline (PBS) and blotted. Alcian blue staining (Abcam, UK) was applied for 30 min, and toluidine blue (Bioss, Beijing, China) for 5 min. After staining, an equal volume of distilled water was added for an additional 15 min. The cells were then washed three times with PBS for 5 min each to remove non-specific staining and subsequently photographed under a light microscope. The staining intensity of Alcian blue and toluidine blue was quantified using ImageJ software.

Cell proliferation assay

The effect of NGF on chondrocyte proliferation was measured using the CCK-8 assay (Abcam, Cambridge, UK). Chondrocytes were seeded in a 96-well plate at a density of 8×10^3 cells/mL and incubated for 12 h. The old medium was then discarded, and the cells were incubated in a fresh medium with NGF (ProteinTech, Shanghai, China) at 0–10 ng/mL for 24–48 h. Subsequently, 10 μL of the CCK-8 solution was added to a 96-well plate. After incubation at 37 °C for 2 h, the optical density (OD) values at a wavelength of 460 nm were read by a microplate reader (Thermo Fisher Scientific, Inc.).

Western blotting

Total cell proteins were extracted from chondrocytes using radioimmunoprecipitation assay lysis buffer on ice. The bicinchoninic acid protein assay kit (Beyotime, Shanghai, China) was used to measure the protein concentration of whole cell lysates. Proteins from different samples were loaded onto 8–12% gels of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore Corporation, Billerica, MA, USA). After blocking with 10 mL of 5% non-fat milk at room temperature for 2 h, the membranes were incubated overnight with anti-Col-II (1:500, AF0135, Affinity, OH, USA), anti-Sox9 (1:500, AF6330, Affinity), anti-ACAN (1:1000, DF7561, Affinity), anti-p-PI3K (1:1000, ab278545, Abcam), anti-PI3K (1:1000, cst4292, Cell Signaling Technology), and anti-β-actin (1:1000, cst4970, Cell Signaling Technology) antibodies on a rocker. Subsequently, primary antibodies were removed, washed, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at 1:5000 dilution for 2 h at room temperature. Finally, the reactive protein bands were developed with the ECL reagent.

Immunofluorescence

Chondrocytes grown on coverslips were rinsed three times with PBS before being fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were permeabilized with 0.5% Triton X-100 for 20 min and blocked with goat serum for 30 min. Subsequently, the slides were incubated with primary antibodies against Col-II (1:50; AF1035, Affinity) and ACAN (1:50; DF7561, Affinity) overnight at 4 °C. On the second day, the samples were incubated with the corresponding secondary antibodies for 1 h at 37 °C. After being washed with PBS, the coverslips were labeled with DAPI for 5 min to visualize the nuclei and finally imaged with a fluorescence microscope (Olympus Inc. Tokyo, Japan).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (version 9, GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± standard deviation (SD) of three independent experiments. Student's *t*- test was performed between the two groups. For experiments with more than two groups, a one-way analysis of variance followed by Tukey's post-hoc test was conducted. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

Results

Chondrocytes identification and effects of NGF on chondrocyte proliferation

ATDC5 cells were cultured in a chondrogenic differentiation medium for three weeks. Alcian blue and toluidine blue staining were conducted to assess the synthesis of sulfated proteoglycans, demonstrating successful chondrogenesis. The results revealed that the accumulation of proteoglycans in ATDC5 cells gradually increased from day 0, reached its highest point on day 14 ($P = 0.0135$, Alcian blue staining; $P < 0.0001$, Toluidine blue staining, Fig. 1A-C), and significantly decreased on day 21 ($P = 0.430$, Alcian blue staining; $P = 0.0244$, Toluidine blue staining, Fig. 1A-C). Morphological analysis of ATDC5 cells revealed that they initially appeared as long spindles or polygons (day 0) but transformed into closely rounded or pavement-like shapes by day 14 (Fig. 1D). On day 14 of ITS induction, ATDC5 cells quickly proliferated, condensed, and converged, ultimately forming cartilage-like nodule structures indicative of chondrogenesis (Fig. 1E) [25]. ATDC5 cells, after 14 days of induction, displayed increased levels of Col-II compared with undifferentiated cells ($P = 0.0012$, Fig. 1F-G). After 14 days of culture with ITS, ATDC5 chondrocytes, which displayed characteristics of mature chondrocytes, were selected for subsequent exploration. The CCK8 assay demonstrated that NGF at concentrations of 0.5–10 ng/mL was not cytotoxic towards ATDC5 chondrocytes. Furthermore, the proliferation rate of these cells was not affected between 24 and 48 h (Fig. 1H).

Effects of NGF on extracellular matrix-related proteins of chondrocytes

Chondrocytes were subjected to overnight starvation prior to being treated with varying doses of NGF (0–10 ng/mL) for 24 h. As shown in Fig. 2A-D, the NGF (0.5, 2.5 and 5 ng/mL) upregulated ACAN, Col-II, and Sox9 in a dose-dependent manner, indicative of its promoted anabolism effect. The 5 ng/mL concentration of NGF demonstrated the most pronounced promoting effects ($P = 0.0011$, 0.0001 and 0.0002, respectively, Fig. 2A-D) and was, therefore, chosen for further experiments.

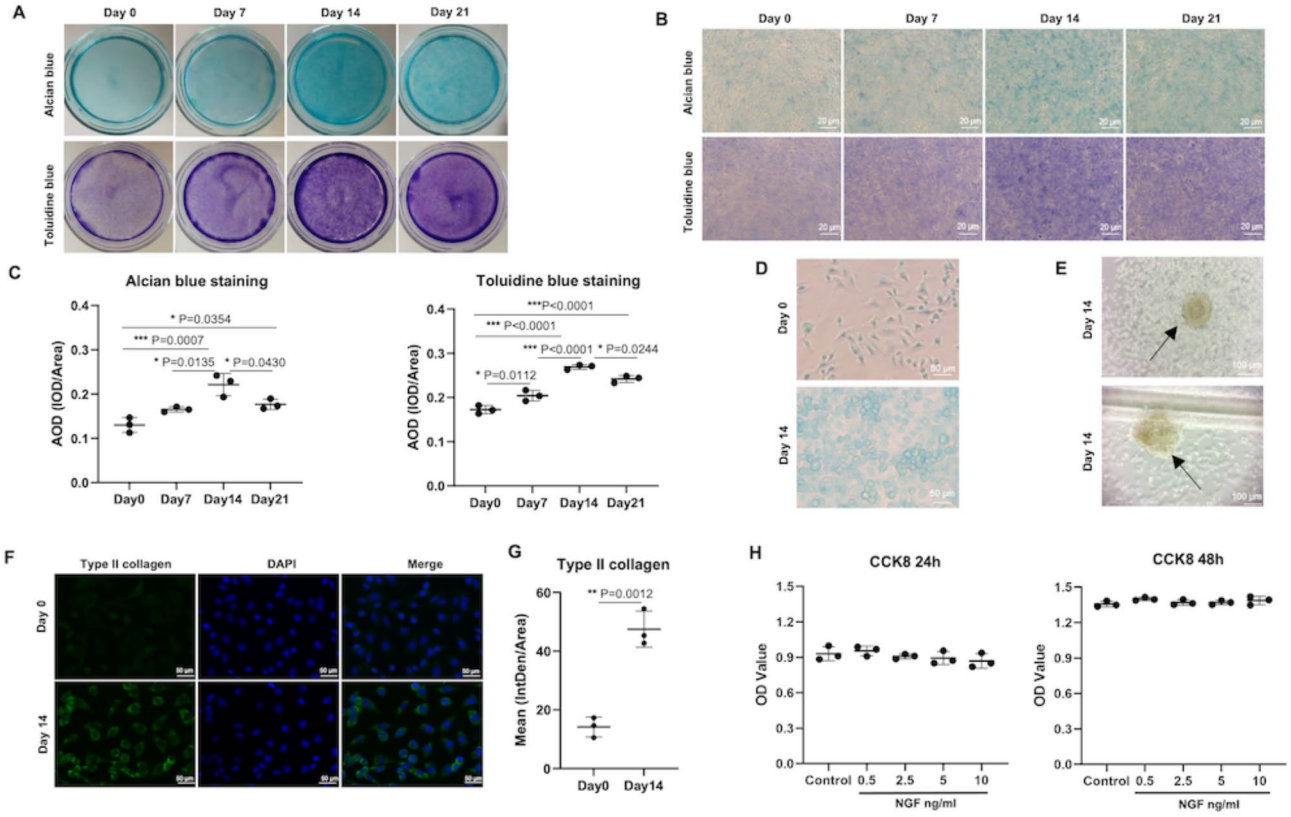

Fig. 1 Chondrocytes identification and the effects of NGF on chondrocyte proliferation (**A**–**H**). Alcian blue staining and toluidine blue staining were performed to visualize the secretion of proteoglycans on days 0, 7, 14, and 21. (**A**) Observed on culture plates of alcian blue and toluidine blue staining. (**B**) Observed under the light microscope of alcian blue and toluidine blue staining. Scale bars = 20 μm. (**C**) Quantification of (**B**). (**D**) Morphological analysis of chondrocytes induced differentiation at day 0 and day 14. Scale bar = 50 μm. (**E**) Arrowhead cartilage nodules. Scale bars = 100 μm. (**F**–**G**) Chondrocytes were identified by the immunocytochemistry staining of Col-II. (**F**) Representative images of immunofluorescence staining. Green represents Col-II (fluorescein isothiocyanate-conjugated). Blue indicates the nucleus using 4′,6-diamidino-2- phenylindole staining. Scale bar = 50 μm. (**G**) Quantitative analysis of Col-II expression. (**H**) Chondrocytes were stimulated with NGF at 0, 0.5, 2.5, 5, and 10 ng/mL for 24 or 48 h by CCK-8 assay. Data are presented as mean ± SD from three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

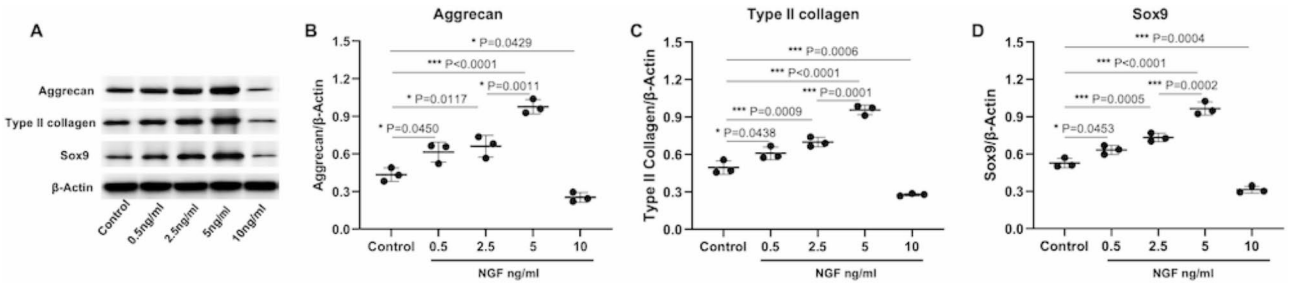

Fig. 2 Effects of NGF on extracellular matrix-related proteins of chondrocytes. Chondrocytes were subjected to starvation in a serum-free medium overnight and subsequently stimulated with NGF at varying concentrations (0.5, 2.5, 5, and 10 ng/mL) for 24 h. (**A**) Representative images of Western blotting. (**B**–**D**) Quantitative analysis of the expression levels of ACAN (**B**), Col-II (**C**), and Sox9 (**D**). All data are presented as mean ± SD from three experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$

In contrast, 10 ng/mL NGF inhibited the synthesis ($P = 0.0429$, 0.0006, and 0.0004, respectively, Fig. 2A-D).

NGF facilitated matrix synthesis via TRKA in chondrocytes

To further explore the functional role of TRKA in NGF-induced matrix synthesis, chondrocytes were stimulated with 5 ng/mL NGF in the presence or absence of a TRKA inhibitor (GNF5837). Western blot analysis revealed that GNF5837 significantly reversed the upregulation of ACAN, Col-II, and Sox9 proteins compared to the 5 ng/mL NGF group ($P = 0.0002$, 0.0005, and 0.0004, respectively, Fig. 3A-D). Consistent with these findings,

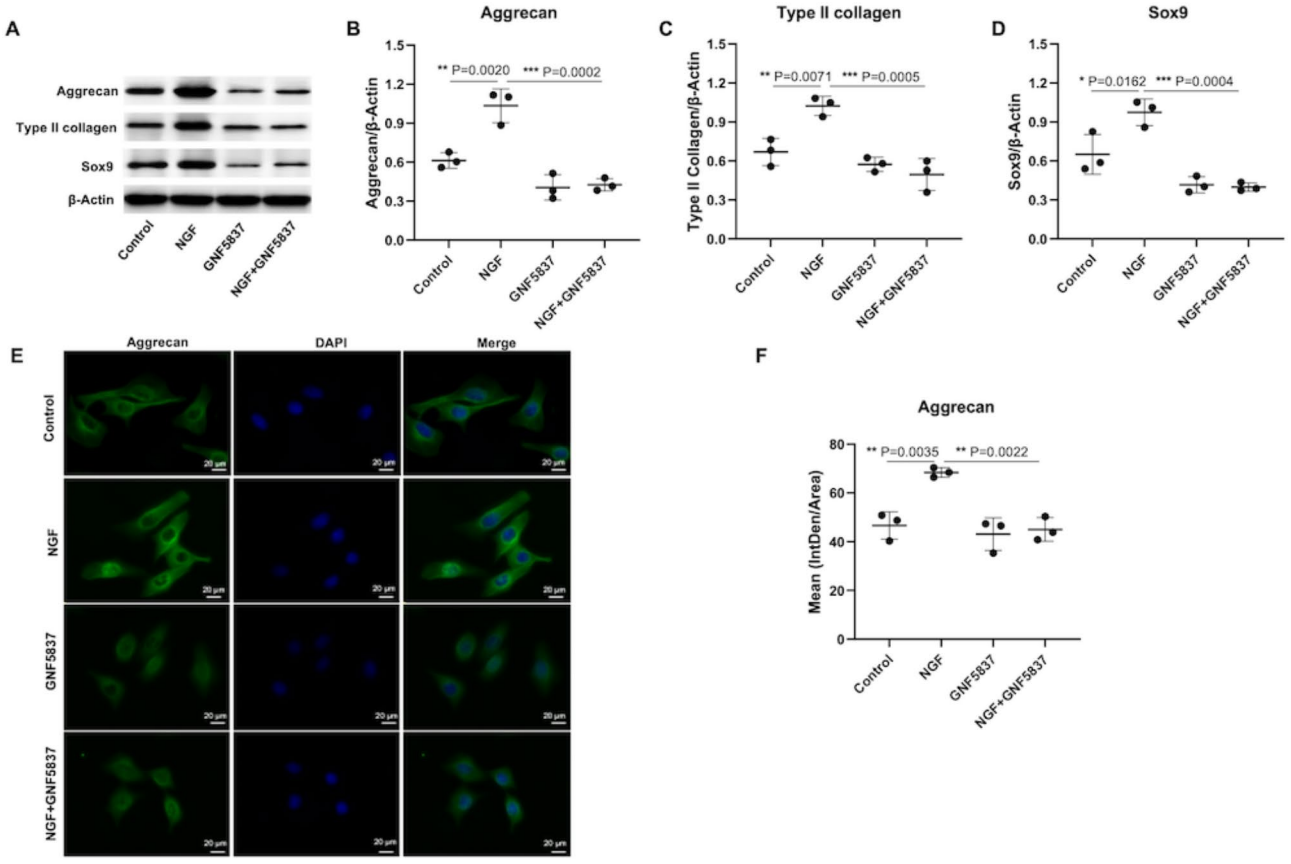

Fig. 3 NGF facilitated matrix synthesis via TRKA in chondrocytes. The expressions of ACAN, Col-II, and Sox9 in the control, NGF, GNF5837, and NGF + GNF5837 groups were analyzed by Western blotting and immunofluorescence. (**A**) Representative images of Western blotting. (**B**–**D**) The expression levels of ACAN (**B**), Col-II (**C**), and Sox9 (**D**). (**E**) Representative images of immunofluorescence. Scale bar = 20 μm. Green represents ACAN (fluorescein isothiocyanate-conjugated). Blue indicates the nucleus using 4′,6-diamidino-2-phenylindole staining. (**F**) Quantitative analysis of the expression levels of ACAN. All data are presented as mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

immunofluorescence results showed that when NGF was combined with GNF5837 in chondrocytes, the NGF-induced increase in ACAN expression was reversed ($P = 0.0022$, Fig. 3E-F). These results suggested that chondrocyte anabolic metabolism was significantly mediated by NGF interaction with its high-affinity TRKA receptor.

NGF regulated PI3K/AKT signaling in chondrocytes

To investigate whether NGF could activate PI3K/AKT signaling in chondrocytes, we exposed these cells to NGF at 0.5–5 ng/mL for 2 h. The results demonstrated that PI3K/AKT signaling activity increased with increasing NGF concentrations (0.5, 2.5, and 5 ng/mL) ($P = 0.0493$, 0.0031 and 0.0001, respectively; Fig. 4A-B). The chondrocytes were then pretreated with GNF5837 (1 μmol/mL), a TRKA inhibitor, or LY294002 (2 μmol/mL), a specific PI3K/AKT inhibitor, respectively, followed by treatment with or without NGF (5 ng/mL) for 2 h. As anticipated, GNF5837 or LY294002 suppressed the activation ($P < 0.0001$ and $P < 0.0001$, respectively; Fig. 4C-D), demonstrating minimal phosphorylation of PI3K. Our results demonstrated that NGF could bind to its high-affinity receptor TRKA, subsequently activating downstream PI3K/AKT signaling in chondrocytes.

PI3K/AKT signaling was involved in NGF-mediated matrix synthesis

To explore the link between PI3K/AKT signaling and NGF-stimulated matrix production, chondrocytes were incubated with 5 ng/mL NGF with or without the PI3K inhibitor LY294002. After 24 h of stimulation, western blot results indicated that NGF increased the expression of ACAN, Col-II, and Sox9 ($P = 0.0073$, 0.0005, and 0.0112, respectively, Fig. 5A-D), which was significantly reversed by LY294002 ($P = 0.0269$, 0.0044, and 0.0490, respectively; Fig. 5A-D). Immunofluorescence results also showed a significant downregulation of ACAN expression in the NGF + LY294002 group compared to the NGF group ($P = 0.0002$, Fig. 5E-F). These findings revealed that PI3K/AKT signaling exhibited significant effects on NGF-mediated ECM-related protein synthesis.

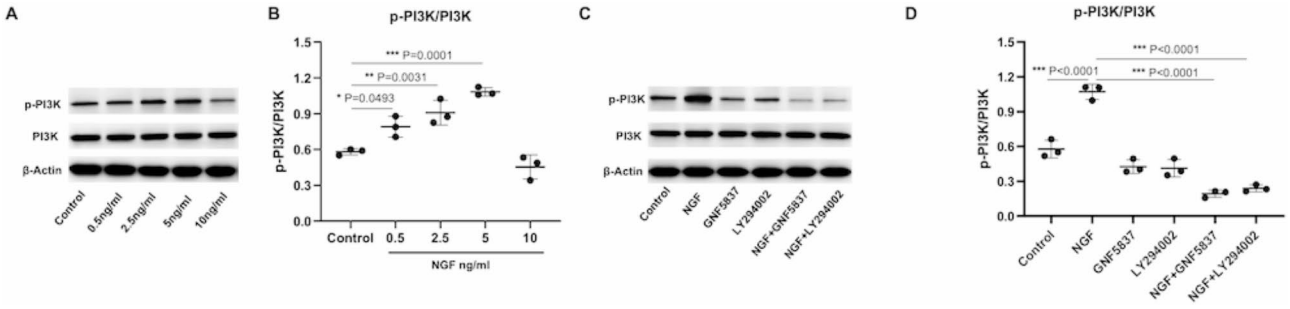

Fig. 4 NGF regulated PI3K/AKT signaling in chondrocytes. (**A**–**B**) The expressions of p-PI3K and PI3K in chondrocytes treated with NGF at concentrations of (0.5, 2.5, 5, 10 ng/mL) for 2 h. (**A**) Representative images of Western blotting. (**B**) Quantitative analysis of the expression levels of p-PI3K/PI3K. (**C**–**D**) Chondrocytes were pretreated with GNF5837 (1 μmol/mL) or LY294002 (2 μmol/mL) for 1 h, followed by treatment with or without NGF (5 ng/mL) for 2 h. (**C**) Representative images of Western blotting. (**D**) Quantitative analysis of the expression levels of p-PI3K/PI3K. All data are presented as mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

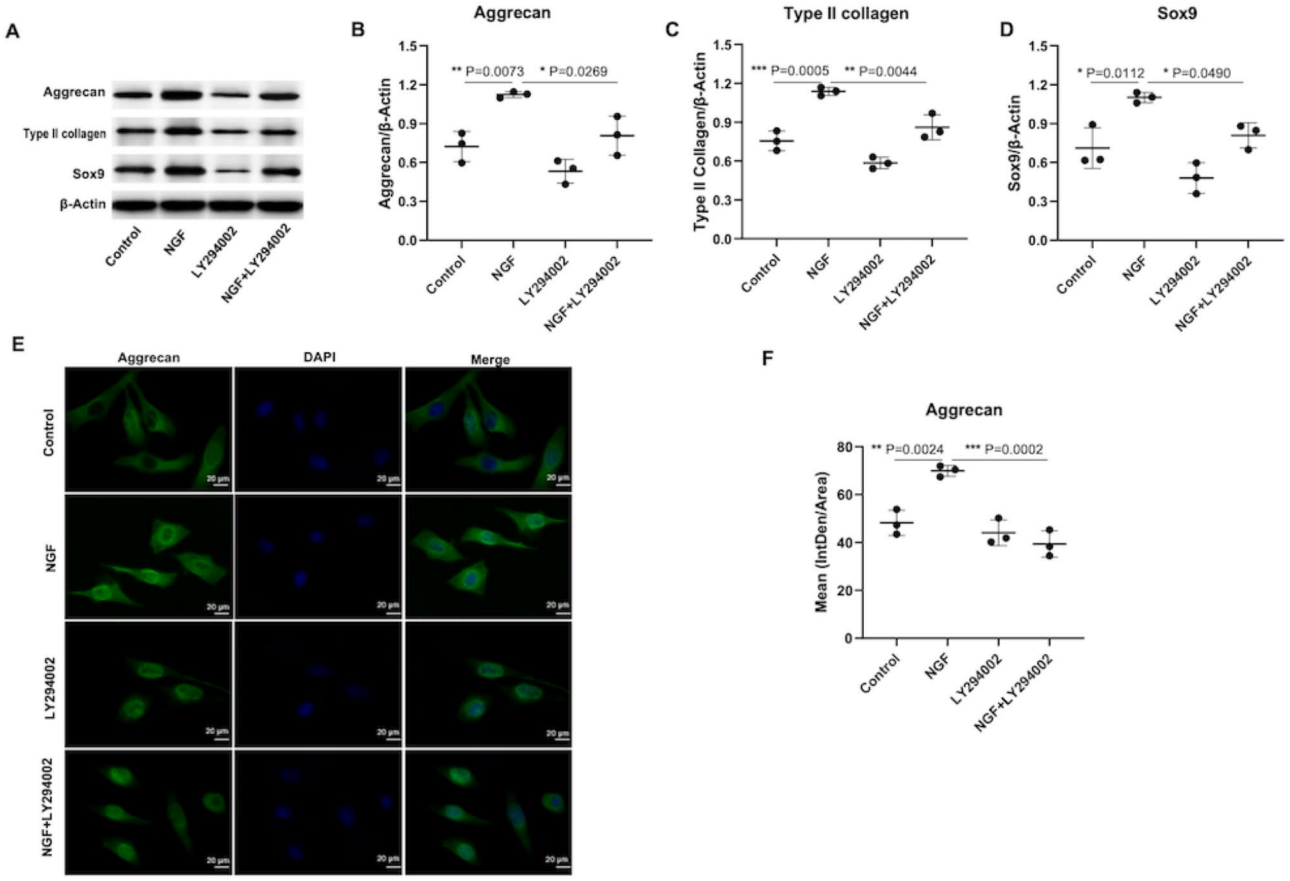

Fig. 5 PI3K/AKT signaling was involved in NGF-mediated matrix synthesis. The expressions of ACAN, Col-II, and Sox9 in the control, NGF, LY294002, NGF + LY294002 groups were analyzed by Western blotting and immunofluorescence. (**A**) Representative images of Western blotting. (**B**–**D**) Quantitative analysis of the expression levels of ACAN (**B**), Col-II (**C**), and Sox9 (**D**). (**E**) Representative images of immunofluorescence. Scale bar = 20 μm. Blue indicates the nucleus using 4′,6-diamidino-2-phenylindole staining. Green represents ACAN (fluorescein isothiocyanate-conjugated). (**F**) Quantitative analysis of the expression levels of ACAN. All data are presented as mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Discussion

Earlier studies primarily focused on the analgesic efficacy and mechanism of NGF inhibition, whereas the mechanism of cartilage damage linked to anti-NGF treatment remains unclear. Our study investigated the effects of NGF on ATDC5 chondrocyte proliferation and anabolism. We found that NGF had no obvious effect on chondrocyte proliferation. NGF concentrations ranging from 0.5 to 5 ng/mL facilitated matrix secretion by chondrocytes in a dose-dependent manner, and the PI3K/AKT

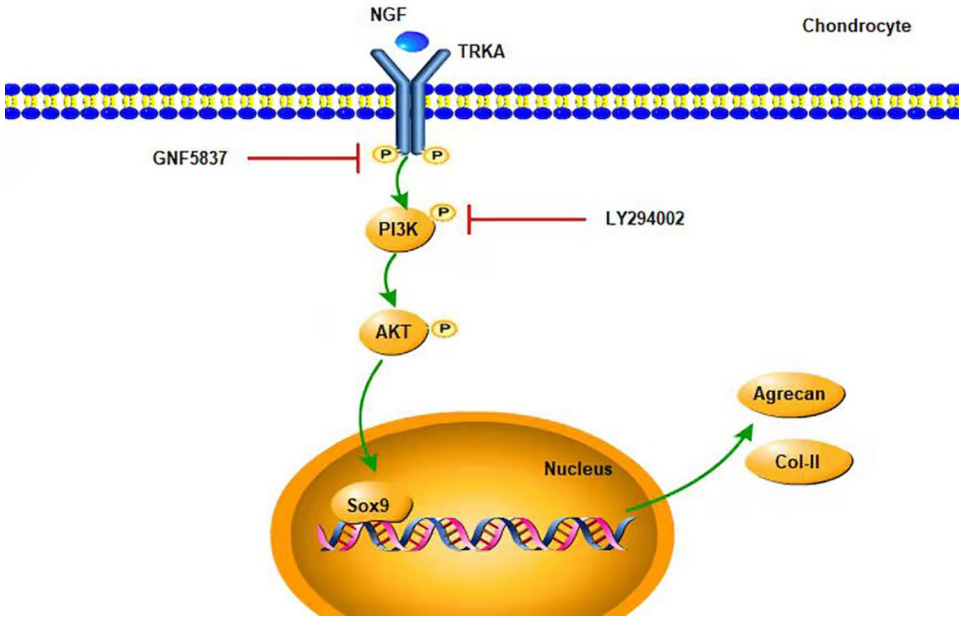

Fig. 6 Schematic of the proposed mechanism by which NGF stimulated the synthesis of matrix in chondrocytes. NGF and its high-affinity receptor TRKA interaction activated PI3K/AKT pathway, ulteriorly led to Sox9 expression. Sox9 further activated ECM-related proteins Col-II and ACAN. Additionally, GNF5837 and LY294002 reversed this process

pathway was involved in this process (Fig. 6). These results suggest a potentially beneficial role of NGF at 0.5–5 ng/mL of chondrocytes.

The ATDC5 cell line, derived from mouse embryonic carcinoma, is a well-established and widely recognized in vitro model for studying chondrocyte biology. It retains chondroprogenitor characteristics and exhibits efficient chondrogenic differentiation in vitro [26–28]. Previous studies have demonstrated that supplementing the culture medium with 1% ITS induced ATDC5 cells to differentiate into chondrocytes and expressed characteristic cartilage matrix components, such as ACAN and Col-II [29]. In this study, alcian blue and toluidine blue staining, along with Col-II immunofluorescence, demonstrated that ATDC5 cells stably and abundantly expressed chondrocyte ECM on the 14th day of differentiation induced by 1% ITS. Morphological observation and the formation of cartilage nodules further confirmed the successful induction of ATDC5 cells into functional chondrocytes. NGF at concentrations of 0.5–5 ng/mL promoted ACAN, Col-II, and Sox9 expression in a dose-response manner, especially at 5 ng/mL. Chondrocytes express the transcription factor Sox9, which plays a crucial role in extracellular matrix formation and in maintaining the chondrocyte phenotype [30–32]. Increased Sox9 expression facilitates the repair of cartilage degradation [33]. Conversely, Sox9 misexpression in cartilage-induced vascular invasion, resulting in aggravation of OA [34]. Sox9, together with Sox5 and Sox6, plays a pivotal role in regulating the gene expression of ACAN and Col-II, thereby enhancing the synthesis of cartilage extracellular matrix [35]. Sox5 and Sox6 can form a transcriptional complex in vivo, binding to Sox9 recognition sites on enhancers. This interaction stabilizes Sox9-DNA binding and significantly amplifies Sox9's transcriptional activity [36]. In our study, the upregulation of ACAN and Col-II expression was consistent with the increase of Sox9 in NGF groups compared with the control.

Further studies have demonstrated that TrkA recognition and subsequent activation of the downstream PI3K/AKT pathway are closely linked to NGF-mediated extracellular matrix synthesis. The PI3K/AKT pathway is associated with chondrocyte differentiation, proliferation, survival, and ECM synthesis [20–22]. Increased Sox9 expression facilitates the repair of cartilage degradation [33]. In our study, the levels of p-PI3K/PI3K and matrix protein expression displayed a similar trend in chondrocytes incubated with NGF. Our findings were substantiated by the observation that TRKA or PI3K inhibitors suppressed the phosphorylation of the PI3K signaling pathway, which consequently reduced the expression of Sox9, ACAN, and Col-II. Sox9, a key regulator of cartilage-specific gene expression, is modulated by the PI3K/AKT signaling pathway. Consistent with current findings, the expression of specific proteins of chondrogenesis is suppressed by blocking the PI3K/AKT pathway and enhanced by its activation [22, 23]. In chondrocytes, the transcription factor cAMP Response Element-Binding Protein (CREB) interacts with Sox9 to synergistically promote chondrocyte repair and ECM synthesis. Activated

CREB binds to the promoter region of Sox9, enhancing its transcriptional activity [37]. The PI3K/Akt pathway regulates the activation of the transcription factor CREB. Sun et al. [38] reported that melatonin inhibited VEGF-induced chondrocyte degeneration by activating the AKT/CREB/Sox9 pathway. As an essential transcriptional coactivator, p300 is recruited along with Sox9 to the promoter regions of target genes, enhancing transcriptional activity [39]. Cheng et al. [20] demonstrated that PI3K signaling promoted the transactivation activity of p300 in nucleus pulposus cells, further enhancing Sox9 activity and regulating the expression of Col-II and ACAN. However, the precise mechanisms by which NGF-induced PI3K/AKT pathway regulates Sox9 expression in chondrocytes remain to be further explored. The ERK/MAPK pathway, another critical signaling cascade activated through the binding of NGF to its TrkA receptor, is closely associated with chondrogenesis. Lu et al. [24] demonstrated that NGF activated the ERK/MAPK pathway, promoting chondrogenic differentiation and enhancing the expression of Sox9, Col-II, and ACAN. Therefore, it is crucial for our future research to investigate whether the ERK/MAPK pathway contributes to the anabolic metabolism of chondrocytes and whether it serves an alternative or complementary role.

NGF—as an important mediator of nerve pain transmission—has been extensively studied as a novel analgesic strategy for osteoarthritis. Phase III clinical trials of NGF monoclonal antibodies suggested the dose-dependent relationship between rapidly progressive osteoarthritis and anti-NGF tanezumab (2.5–10 mg) or fasinumab (3–9 mg) [4, 5, 40]. Similar side effects were also observed in a CRISPR/Cas9-based NGF ablation mouse experiment [41]. However, the role of NGF in cartilage remains unclear. In addition to being a pain factor, NGF has exhibited a therapeutic role in healing damaged tissues, except for nerve tissues, such as corneal ulcers, skin ulcers and macular degeneration [42–45]. Matsuda et al. found that injured skin tissues rapidly released a significant amount of NGF, which accelerated wound healing in normal and diabetic mice [46]. Interestingly, previous studies demonstrated that degenerative cartilage secreted NGF and produced more NGF in response to inflammation and mechanical stimulation [9–14]. Elevated NGF, also found in the OA synovium, synovial fluid, subchondral bone, and blood [47–50], could be a potential source of cartilaginous NGF. Huang et al. found that NGF upregulation slowed chondrocyte hypertrophic differentiation, protecting against OA progression [51]. NGF derived from mouse and snake venom induced chondrogenesis of mesenchymal stem cells and maintained the chondrocyte phenotype [24, 52, 53]. Our study focused on the effect of NGF on the anabolism of chondrocytes and demonstrated dose-dependent stimulation of ECM production in ATDC5 chondrocytes. This result was similar to the findings of Tan and colleagues, who suggested that NGF isolated from venom of Chinese cobra effectively promoted anabolism of human chondrocytes in vitro [54].

Our study initially explored 10ng/mL NGF inhibited chondrocyte anabolism and the PI3K/AKT pathway was inhibited. Jiang et al. found that 10ng/ml NGF treatment of OA cartilage explants resulted in higher MMPs and increased cartilage matrix loss [17]. They suggested that NGF may act through cartilage stem/progenitor cells, whereas there was a lack of researches on chondrocytes. At higher concentrations, NGF may exert its effects not only through the TrkA receptor but also via its low-affinity receptor, p75NTR. The p75NTR receptor is frequently associated with negative regulatory effects, such as apoptosis, degeneration, or pro-inflammatory responses [55, 56]. Activation of p75NTR under elevated NGF concentrations may be a key factor inhibiting ECM synthesis, further exacerbating cartilage degradation. This is a complex mechanism that requires further investigation to fully understand. Future therapeutic strategies should avoid complete inhibition of NGF and focus on optimizing treatment dosages or combining NGF antagonists with chondroprotective agents to balance its roles in pain modulation and cartilage preservation. Further research is required to elucidate the dose-dependent mechanisms of NGF to enhance its safety and efficacy as a therapeutic target for osteoarthritis.

This present study has several limitations. First, we mainly explored NGF-induced chondrocyte anabolism. However, the effect of NGF on catabolism requires clarification. Second, our group only used the ATDC5 cell line for the in vitro experiments, without primary human OA chondrocytes. Studies indicated that TRKA expression was up-regulated in damaged knee chondrocytes in patients with OA, while it was absent in the cartilaginous tissues of the osteoarthritic lumbar facet joint [9, 13]. The TRKA was differentially regulated in OA chondrocytes, which may lead to different cellular behaviors. Finally, ATDC5 chondrocytes were plate-cultured, in contrast to real cartilage tissue, where chondrocytes are under a three-dimensional hypoxic condition with very limited regenerative capacity. To enhance the clinical relevance of our research, future studies will utilize human OA chondrocytes, along with advanced 3D cartilage models or in vivo models, to further validate these findings. Future research on NGF's role in osteoarthritic cartilage could provide valuable insights for the development of anti-NGF treatments that minimize adverse joint effects.

Conclusion

Our study identified a potentially beneficial role of NGF at concentrations of 0.5–5 ng/mL in chondrocytes, enhancing extracellular matrix synthesis, with significant involvement of the PI3K/AKT signaling pathway in this process.

Abbreviations

NGF	Nerve Growth Factor
ECM	Extracellular Matrix
Col-II	Type II Collagen
ACAN	Aggrecan
Sox9	Sex Determining Region Y box 9
CCK-8	Cell Counting Kit-8
TRKA	Tropomyosin-Receptor Kinase A
P75NTR	P75 Neurotrophin Receptor
p-PI3K	Phosphorylation-Phosphatidylinositol 3-Kinase
PI3K	Phosphatidylinositol 3-Kinase
AKT	Protein Kinase B

Acknowledgements
None.

Author contributions
Bingchen An conceived and designed the study. Mengling Wang conducted the experiments, completed data analysis and wrote the manuscript. Jie Lian and Maoqing Ye revised the manuscript. All authors reviewed and approved the final manuscript submitted for publication.

Funding
Science and Technology Commission of Shanghai Municipality (NO.16411964000 and NO.21Y11903100).

Data availability
The Data will be available on reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests
The authors declare no competing interests.

Received: 23 October 2024 / Accepted: 15 January 2025
Published online: 27 February 2025

References

1. Kolasinski SL, Neogi T, Hochberg MC, et al. 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, hip, and Knee. Arthritis Rheumatol. 2020;72(2):220–33.
2. Global regional. National incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the global burden of Disease Study 2017. Lancet. 2018;392(10159):1789–858.
3. Lane NE, Schnitzer TJ, Birbara CA, et al. Tanezumab for the treatment of pain from osteoarthritis of the knee. N Engl J Med. 2010;363(16):1521–31.
4. Hochberg MC. Serious joint-related adverse events in randomized controlled trials of anti-nerve growth factor monoclonal antibodies. Osteoarthritis Cartilage. 2015;23(Suppl 1):S18–21.
5. Lane NE, Corr M, Osteoarthritis. in 2016: Anti-NGF treatments for pain - two steps forward, one step back? Nat Rev Rheumatol. 2017;13(2): 76–78.
6. Karsdal MA, Verburg KM, West CR, et al. Serological biomarker profiles of rapidly progressive osteoarthritis in tanezumab-treated patients. Osteoarthritis Cartilage. 2019;27(3):484–92.
7. Watanabe H, de Caestecker MP, Yamada Y. Transcriptional cross-talk between smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. J Biol Chem. 2001;276(17):14466–73.
8. Lee AS, Ellman MB, Yan D, et al. A current review of molecular mechanisms regarding osteoarthritis and pain. Gene. 2013;527(2):440–7.
9. Iannone F, De Bari C, Dell'Accio F, et al. Increased expression of nerve growth factor (NGF) and high affinity NGF receptor (p140 TrkA) in human osteoarthritic chondrocytes. Rheumatology (Oxford). 2002;41(12):1413–8.
10. Sato T, Konomi K, Yamasaki S, et al. Comparative analysis of gene expression profiles in intact and damaged regions of human osteoarthritic cartilage. Arthritis Rheum. 2006;54(3):808–17.
11. Ramos YF, den Hollander W, Bovée JV, et al. Genes involved in the osteoarthritis process identified through genome wide expression analysis in articular cartilage; the RAAK study. PLoS ONE. 2014;9(7):e103056.
12. Driscoll C, Chanalaris A, Knights C, et al. Nociceptive sensitizers are regulated in damaged joint tissues, including articular cartilage, when Osteoarthritic mice Display Pain Behavior. Volume 68. Arthritis & rheumatology; 2016. pp. 857–67. (Hoboken, N.J.). 4.
13. Seidel MF, Netzer C, Chobaz V, et al. Localization of nerve growth factor expression to structurally damaged cartilaginous tissues in human lumbar Facet Joint Osteoarthritis. Front Immunol. 2022;13:783076.
14. Pecchi E, Priam S, Gosset M, et al. Induction of nerve growth factor expression and release by mechanical and inflammatory stimuli in chondrocytes: possible involvement in osteoarthritis pain. Arthritis Res Ther. 2014;16(1):R16.
15. Blaney Davidson EN, van Caam AP, Vitters EL, et al. TGF-β is a potent inducer of nerve growth factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain? Osteoarthr Cartil. 2015;23(3):478–86.
16. Shang X, Zhang L, Jin R, et al. Estrogen regulation of the expression of Pain factor NGF in rat chondrocytes. J pain Res. 2021;14:931–40.
17. Jiang Y, Hu C, Yu S, et al. Cartilage stem/progenitor cells are activated in osteoarthritis via interleukin-1β/nerve growth factor signaling. Arthritis Res Ther. 2015;17:327.
18. Yu X, Qi Y, Zhao T, et al. NGF increases FGF2 expression and promotes endothelial cell migration and tube formation through PI3K/Akt and ERK/MAPK pathways in human chondrocytes. Osteoarthritis Cartilage. 2019;27(3):526–34.
19. Wei X, Sun C, Zhou RP, et al. Nerve growth factor promotes ASIC1a expression via the NF-κB pathway and enhances acid-induced chondrocyte apoptosis. Int Immunopharmacol. 2020;82:106340.
20. Cheng CC, Uchiyama Y, Hiyama A, et al. PI3K/AKT regulates aggrecan gene expression by modulating Sox9 expression and activity in nucleus pulposus cells of the intervertebral disc. J Cell Physiol. 2009;221(3):668–76.
21. Hidaka K, Kanematsu T, Takeuchi H, et al. Involvement of the phosphoinositide 3-kinase/protein kinase B signaling pathway in insulin/IGF-I-induced chondrogenesis of the mouse embryonal carcinoma-derived cell line ATDC5. Int J Biochem Cell Biol. 2001;33(11):1094–103.
22. Fujita T, Fukuyama R, Enomoto H, et al. Dexamethasone inhibits insulin-induced chondrogenesis of ATDC5 cells by preventing PI3K-Akt signaling and DNA binding of Runx2. J Cell Biochem. 2004;93(2):374–83.
23. Kita K, Kimura T, Nakamura N, et al. PI3K/Akt signaling as a key regulatory pathway for chondrocyte terminal differentiation. Genes Cells. 2008;13(8):839–50.
24. Lu Z, Lei D, Jiang T, et al. Nerve growth factor from Chinese cobra venom stimulates chondrogenic differentiation of mesenchymal stem cells. Cell Death Dis. 2017;8(5):e2801.
25. Shukunami C, Ishizeki K, Atsumi T, et al. Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. J Bone Min Res. 1997;12(8):1174–88.
26. Atsumi T, Miwa Y, Kimata K, et al. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Differ Dev. 1990;30(2):109–16.
27. Yao Y, Wang Y. ATDC5: an excellent in vitro model cell line for skeletal development. J Cell Biochem. 2013;114(6):1223–9.
28. Yao Y, Zhang T, Chen H, et al. Enhanced chondrogenesis in a coculture system with genetically manipulated dedifferentiated chondrocytes and ATDC5 cells. Biotechnol Bioeng. 2020;117(10):3173–81.
29. Yao Y, Zhai Z, Wang Y. Evaluation of insulin medium or chondrogenic medium on proliferation and chondrogenesis of ATDC5 cells. Biomed Res Int. 2014;2014:569241.

30. Ikegami D, Akiyama H, Suzuki A, et al. Sox9 sustains chondrocyte survival and hypertrophy in part through Pik3ca-Akt pathways. Development. 2011;138(8):1507–19.
31. Bi W, Deng JM, Zhang Z, et al. Sox9 is required for cartilage formation. Nat Genet. 1999;22(1):85–9.
32. Lefebvre V, Li P, de Crombrugghe. B a new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. Embo j. 1998;17(19):5718–33.
33. Hussain S, Sun M, Guo Y, et al. SFMBT2 positively regulates SOX9 and chondrocyte proliferation. Int J Mol Med. 2018;42(6):3503–12.
34. Hattori T, Müller C, Gebhard S, et al. SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. Development. 2010;137(6):901–11.
35. Yao B, Wang Q, Liu CF, et al. The SOX9 upstream region prone to chromosomal aberrations causing campomelic dysplasia contains multiple cartilage enhancers. Nucleic Acids Res. 2015;43(11):5394–408.
36. Liu CF, Lefebvre V. The transcription factors SOX9 and SOX5/SOX6 cooperate genome-wide through super-enhancers to drive chondrogenesis. Nucleic Acids Res. 2015;43(17):8183–203.
37. Piera-Velazquez S, Hawkins DF, Whitecavage MK, et al. Regulation of the human SOX9 promoter by Sp1 and CREB. Exp Cell Res. 2007;313(6):1069–79.
38. Sun X, Gu X, Li K et al. Melatonin promotes Antler Growth by accelerating MT1-Mediated mesenchymal cell differentiation and inhibiting VEGF-Induced degeneration of chondrocytes. Int J Mol Sci. 2022;23(2).
39. Furumatsu T, Tsuda M, Yoshida K, et al. Sox9 and p300 cooperatively regulate chromatin-mediated transcription. J Biol Chem. 2005;280(42):35203–8.
40. Schnitzer TJ, Ekman EF, Spierings EL, et al. Efficacy and safety of tanezumab monotherapy or combined with non-steroidal anti-inflammatory drugs in the treatment of knee or hip osteoarthritis pain. Ann Rheum Dis. 2015;74(6):1202–11.
41. Zhao L, Huang J, Fan Y, et al. Exploration of CRISPR/Cas9-based gene editing as therapy for osteoarthritis. Ann Rheum Dis. 2019;78(5):676–82.
42. Lambiase A, Rama P, Bonini S, et al. Topical treatment with nerve growth factor for corneal neurotrophic ulcers. N Engl J Med. 1998;338(17):1174–80.
43. Generini S, Tuveri MA, Matucci Cerinic M, et al. Topical application of nerve growth factor in human diabetic foot ulcers. A study of three cases. Exp Clin Endocrinol Diabetes. 2004;112(9):542–4.
44. Tuveri M, Generini S, Matucci-Cerinic M, et al. NGF, a useful tool in the treatment of chronic vasculitic ulcers in rheumatoid arthritis. Lancet. 2000;356(9243):1739–40.
45. Lambiase A, Coassin M, Tirassa P, et al. Nerve growth factor eye drops improve visual acuity and electrofunctional activity in age-related macular degeneration: a case report. Ann Ist Super Sanita. 2009;45(4):439–42.
46. Matsuda H, Koyama H, Sato H, et al. Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice. J Exp Med. 1998;187(3):297–306.
47. Takano S, Uchida K, Miyagi M, et al. Nerve growth factor regulation by TNF-α and IL-1β in synovial macrophages and fibroblasts in osteoarthritic mice. J Immunol Res. 2016;2016:5706359.
48. Montagnoli C, Tiribuzi R, Crispoltoni L, et al. β-NGF and β-NGF receptor upregulation in blood and synovial fluid in osteoarthritis. Biol Chem. 2017;398(9):1045–54.
49. Walsh DA, McWilliams DF, Turley MJ, et al. Angiogenesis and nerve growth factor at the osteochondral junction in rheumatoid arthritis and osteoarthritis. Rheumatology (Oxford). 2010;49(10):1852–61.
50. Aso K, Shahtaheri SM, Hill R, et al. Associations of symptomatic knee osteoarthritis with histopathologic features in subchondral bone. Arthritis Rheumatol. 2019;71(6):916–24.
51. Huang H, Shank G, Ma L, et al. Nerve growth factor induced after temporomandibular joint inflammation decelerates chondrocyte differentiation. Oral Dis. 2013;19(6):604–10.
52. Zhan X, Cai P, Lei D, et al. Comparative profiling of chondrogenic differentiation of mesenchymal stem cells (MSCs) driven by two different growth factors. Cell Biochem Funct. 2019;37(5):359–67.
53. Miao Z, Lu Z, Luo S, et al. Murine and Chinese cobra venom–derived nerve growth factor stimulate chondrogenic differentiation of BMSCs in vitro: a comparative study. Mol Med Rep. 2018;18(3):3341–9.
54. Tan J, Lu Z, Miao Z, et al. Effect of NGF from venom of Chinese Cobra (Naja Atra)on chondrocytes Proliferation and Metabolism in Vitro. J Cell Biochem. 2017;118(12):4308–16.
55. Lebrun-Julien F, Bertrand MJ, De Backer O, et al. ProNGF induces TNFalpha-dependent death of retinal ganglion cells through a p75NTR non-cell-autonomous signaling pathway. Proc Natl Acad Sci U S A. 2010;107(8):3817–22.
56. Farina L, Minnone G, Alivernini S, et al. Pro nerve growth factor and its receptor p75NTR activate inflammatory responses in synovial fibroblasts: a novel targetable mechanism in arthritis. Front Immunol. 2022;13:818630.

Publisher's note