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Original Article

GAB2 inhibits chondrocyte apoptosis through PI3K-AKT signaling in osteoarthritis

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Abstract: Cartilage degeneration is considered the main pathologic feature of osteoarthritis (OA). Cumulative evidence indicates that chondrocyte apoptosis is associated with cartilage degradation. However, the underlying molecular mechanism of chondrocyte apoptosis remains unclear. Growth factor receptor-bound protein 2 (GAB2), an adaptor protein, belongs to the Gab family and is involved in various biologic processes. Here, we explored the role of GAB2 in the pathogenesis of osteoarthritis (OA). GAB2 expression was markedly increased in OA articular cartilage. GAB2 expression was also increased in an in vitro model of TNFα-induced apoptosis. GAB2 depletion by siRNA promoted expression of the apoptosis markers, PARP and caspase-3, and increased the number of apoptotic cells, indicating that GAB2 might have an anti-apoptotic effect in chondrocytes. Moreover, GAB2 knockdown inhibited AKT phosphorylation, increased BAX expression, and decreased BCL2 expression, which indicated that GAB2 regulates chondrocyte apoptosis through PI3K-AKT signaling. Taken together, our study indicates that GAB2 plays a vital role in chondrocyte apoptosis and provides a new therapeutic target for OA.

Keywords: GAB2, osteoarthritis, apoptosis, PI3K-AKT

Introduction

Osteoarthritis (OA) is characterized by the failure of chondrocytes to maintain a balance between articular matrix synthesis and degradation, which causes the progressive destruction of articular cartilage. However, the exact pathogenesis of OA has not yet been elucidated. Apoptosis, or programmed cell death, has a close relationship with OA progression [1]. Chondrocytes are an important component of cartilage and play a vital role in cartilage repair, regeneration, homeostasis, and structural integrity. Cartilage destruction in OA appears to be a result of chondrocyte apoptosis [2, 3]. Chondrocyte apoptosis is regulated by cytokines and a series of signaling pathways (involving PARP, caspase 3, BAX, and BCL2) [4, 5]. However, successful treatment of osteoarthritis requires knowledge of the proteins and molecular mechanisms involved in the pathologic process of chondrocyte apoptosis.

Growth factor receptor-bound protein 2 (GAB2), an adaptor protein, belongs to the Gab family [6]. This protein contains signaling molecule binding sites, such as for SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) and phosphatidylinositol-3 kinase (PI3K) [7], which act downstream of several membrane receptors for cytokines, antigens, hormones, and growth factors to regulate multiple signaling pathways [8]. The interaction between GAB2 and PI3K regulates the PI3K-AKT signaling pathway [9]. Previous studies have demonstrated a vital role of PI3K-AKT signaling in the progression of chondrocyte apoptosis in OA [10, 11]. These findings strongly indicate that GAB2 is involved in chondrocyte apoptosis in OA.

Therefore, we hypothesized that GAB2 plays a vital role in OA progression through PI3K-AKT signaling. In this study, we explored the role of GAB2 in the pathogenesis of OA. We explored the correlation between GAB2 expression and chondrocyte apoptosis. Furthermore, we analyzed its potential association with PI3K/AKT activation during OA progression. This study provides a better understanding of the role of GAB2 in OA development.

Materials and methods

Collection of human cartilage tissue

Cartilage was obtained from patients with OA (n = 8) who underwent total knee replacement surgery. Control group cartilage samples (n = 6) were obtained from severe lower extremity trauma patients with no history of OA who underwent above-knee amputation. All cartilage tissue was derived from the knee femoral condyle. The mean age of the control group was 40 ± 5 years, and that of the OA group was 50 ± 6 years. The study was approved by the Medical Ethics Committee of the Affiliated Hospital of Nantong University. All procedures involving human subjects were performed in accordance with the Helsinki Declaration of 1975, as revised in 2000, and written informed consent was obtained from all participants.

Histology and histomorphometry

All tissue samples were fixed, decalcified, paraffin-embedded, and sectioned at a thickness of 4 μm. Sections of cartilage were then stained with Safranin O and scored according to the Osteoarthritis Research Society International (OARSI) score for cartilage histopathology grade assessment [12]. OARSI score 0-1 was classified as normal cartilage. Vertical fissure, erosion, exfoliation, and cartilage loss with an OARSI score ≥ 4 was classified as OA cartilage [13].

Immunohistochemistry

Cartilage sections were dewaxed, washed, and blocked. Sections were then incubated with a primary antibodies against GAB2 (anti-rabbit, 1:500; Abcam), at 4°C overnight, followed by a biotinylated secondary antibody for 2 h. After washing with PBS, the sections were incubated with DAB as a chromogenic substrate and observed under an optical microscope (Leica, Wetzlar, Germany).

Quantitative real-time PCR

Total RNA was isolated from articular cartilage tissues. The primer sequences for RT-PCR were: GAB2 forward primer, 5'-GTGGGGGATCTGAATGTTTTTATG-3' and reverse primer 5'-GCCCCAGGGTAGAATGAAACG-3'; GAPDH forward primer 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse primer 5'-GAAGATGGTGATGGGATTTC-3'. The PCR amplification included pre-denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s, melting curve increasing from 60°C to 95°C by 0.3°C per 15 s. Relative gene expression was detected by the $2^{-\Delta\Delta Ct}$ method.

Cell culture and stimulation

SW1353 cells were purchased from the Chinese Academy of Sciences Cell Bank and cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. For stimulation experiments, cells were stimulated with 20 ng/ml TNFα for 6, 12, 24, 36, or 48 h and then harvested in lysis buffer for western blot analysis. siRNAs for GAB2 were purchased from Guangzhou RiboBio: 5'-CCATCACTTTGACTCACTT-3' (siRNA-001), 5'-GATGCAGGCCTGACCTTTA-3' (siRNA-002) and 5'-ACAACTATGTGCCCATGAA-3' (siRNA-003). The control scrambled siRNA was 5'-GGCUCUAGAAAAGCCUAUGCdTdT-3' and the negative control sequence (siGab2-NC) was 3'-dTdTCCGAGAUCUUUUCGGAUACG-5'. After siRNA transfection, cells were cultivated in serum-free DMEM at 37°C for 6 h and then in DMEM supplemented with 10% FBS for 48 h. Cells were then stimulated for 30 min with TNFα (20 ng/ml) and then harvested.

Western blot analysis

Protein samples were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies against GAB2 (anti-rabbit, 1:1000; Abcam), cleaved-caspase 3 (anti-rabbit, 1:1000; Abcam), cleaved-PARP (anti-rabbit, 1:1000; Abcam), AKT (anti-rabbit, 1:1000; Abcam), p-AKT (anti-rabbit, 1:1000; Abcam), BAX (anti-rabbit, 1:500; Abcam), BCL2 (anti-mouse, 1:500; Santa Cruz) or GAPDH (anti-mouse, 1:5000 Santa Cruz) antibodies at 4°C overnight. Membranes were then incubated with appropriate secondary antibodies for 2 h at room temperature.

Fluorescent immunocytochemistry

After SW1353 cells were stimulated with TNFα, the cells were fixed in 4% formaldehyde and treated with 0.1% Triton X-100. The cells were then treated with 10% BSA at 4°C for 2 h and then incubated with primary antibodies against GAB2 (anti-mouse, 1:100; Cell Signaling), and cleaved-caspase 3 (anti-rabbit, 1:1000; Ab-

cam) at 4°C overnight. The cells were then washed with PBS and then incubated with a secondary antibody for 2 h at room temperature.

Flow cytometry with annexin V/PI staining

Apoptosis was detected as previously described [14]. Briefly, SW1353 cells were digested with 0.1% EDTA-free trypsin and washed with PBS and then resuspended in cold binding buffer at a density of 10^6 cells per ml. After incubation for 15 minutes in a dark room at room temperature, the stained cells were detected by flow cytometry (BD FACSAria II).

Statistical analysis

All statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL) and data are expressed as the mean ± SEM. Comparisons between measurements at two different times were performed using the paired t-test if the data were normally distributed. Otherwise, the Wilcoxon rank sum test was conducted. P values < 0.05 were considered significant.

Results

GAB2 expression is up-regulated in OA articular cartilage

Safranin-O staining was performed to evaluate the glycosaminoglycan content and pathologic changes in articular cartilage (**Figure 1A**). Less Safranin O staining and more roughening of the articular surface was observed in OA compared with control cartilage. The OARSI score of cartilage from patients with OA was higher than that of cartilage from individuals of the control group (**Figure 1B**). To examine whether GAB2 is involved in OA pathogenesis, we used immunohistochemistry to detect GAB2 levels in articular cartilage from OA patients. The number of GAB2-positive cells in OA cartilage was increased compared with that in normal cartilage (**Figure 1C**, **1D**). Quantitative real-time PCR analysis also showed significant up-regulation of GAB2 in articular OA cartilage compared to normal cartilage (**Figure 1E**).

GAB2 levels are enhanced after TNFα stimulation in vitro

Caspase-dependent chondrocyte apoptosis is significantly associated with OA pathogenesis [15]. In the present study, we established a chondrocyte apoptosis model of OA by TNFα induction in SW1353 cells [16]. Western blot analysis showed that GAB2 levels were upregulated in a time-dependent manner and reached a peak after TNFα stimulation for 36 h (**Figure 2A**, **2B**). We also investigated the expression levels of cleaved-caspase 3 and cleaved-PARP, which are general markers of apoptosis. Cleaved-caspase 3 and cleaved-PARP levels were significantly enhanced after TNFα stimulation (**Figure 2A**, **2B**). Next, we performed double immunofluorescent staining for GAB2 and cleaved-caspase 3. Colocalization of GAB2 and cleaved-caspase 3 was observed after TNF-α stimulation for 36 h (**Figure 2C**). These results indicated that GAB2 might be associated with chondrocyte apoptosis.

GAB2 knockdown promotes apoptosis after TNFα stimulation

To investigate the effect of GAB2 on chondrocyte apoptosis, we used GAB2-specific siRNA to knock down endogenous GAB2 in SW1353 cells. siGab2 knockdown efficiency of was assessed by western blot analysis and siGab2-002 significantly reduced the levels of GAB2 compared with the negative control (**Figure 3A**, **3B**). We then measured the levels of cleaved-caspase 3 and cleaved-PARP and their protein levels were increased after GAB2 knockdown (**Figure 3C**, **3D**). In addition, flow cytometry with annexin V/PI staining showed that siGab2 treatment also significantly increased apoptosis in TNFα stimulated cells (**Figure 3E**, **3F**). These data indicate that GAB2 might have a significant anti-apoptotic effect in chondrocytes.

GAB2 regulates apoptosis through PI3K-AKT signaling

Given the interaction between GAB2 and the PI3K-AKT signaling pathway, we tested whether GAB2 regulates apoptosis in SW1353 cells through PI3K-AKT signaling. We evaluated PI3K-AKT dependent AKT, p-AKT, pro-apoptotic protein, BAX, and anti-apoptotic protein, BCL2. Western blot analysis showed that p-AKT levels were decreased after TNFα stimulation, indicating inhibition of PI3K-AKT signaling. Moreover, p-AKT was also obviously inhibited by siGab2 treatment, while BAX expression was increased and BCL2 expression was reduced by siGab2 treatment (**Figure 4A**, **4B**). Taken together, our

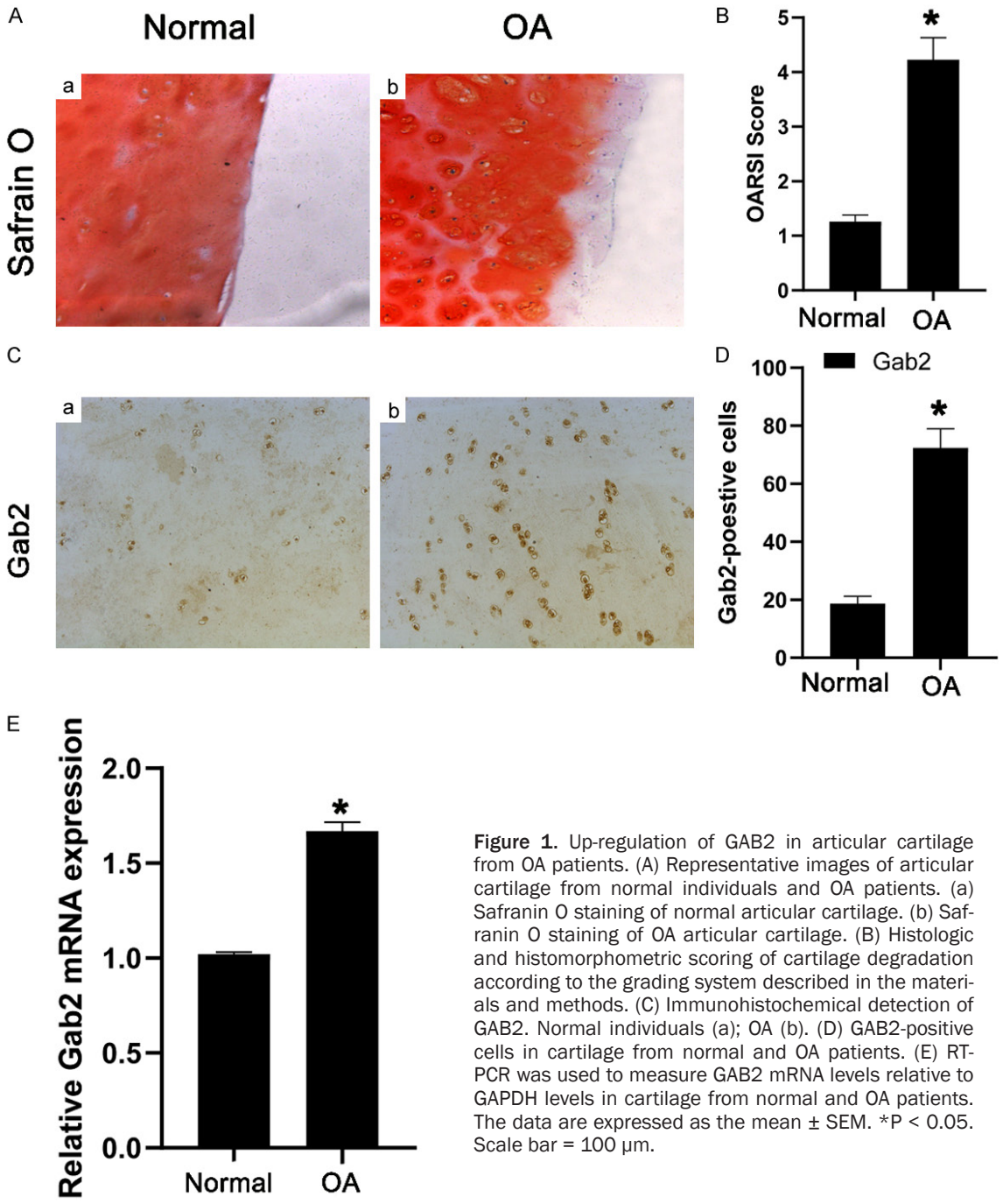

Figure 1. Up-regulation of GAB2 in articular cartilage from OA patients. (A) Representative images of articular cartilage from normal individuals and OA patients. (a) Safranin O staining of normal articular cartilage. (b) Safranin O staining of OA articular cartilage. (B) Histologic and histomorphometric scoring of cartilage degradation according to the grading system described in the materials and methods. (C) Immunohistochemical detection of GAB2. Normal individuals (a); OA (b). (D) GAB2-positive cells in cartilage from normal and OA patients. (E) RT-PCR was used to measure GAB2 mRNA levels relative to GAPDH levels in cartilage from normal and OA patients. The data are expressed as the mean ± SEM. *P < 0.05. Scale bar = 100 μm.

results indicate that GAB2 regulates apoptosis through PI3K-AKT signaling.

Discussion

Osteoarthritis is common in middle-aged and elderly populations, causing joint pain and dysfunction [17]. It is characterized by chondrocyte apoptosis and cartilage degeneration [18]. However, the specific molecular mechanism of chondrocyte apoptosis is still unknown. We hypothesized that OA could be slowed down or prevented by inhibiting chondrocyte apoptosis. First, we collected knee cartilage from patients without a history of osteoarthritis and from OA patients. The OARSI scores of the OA group were significantly higher than those of the normal group. Compared with normal articular car-

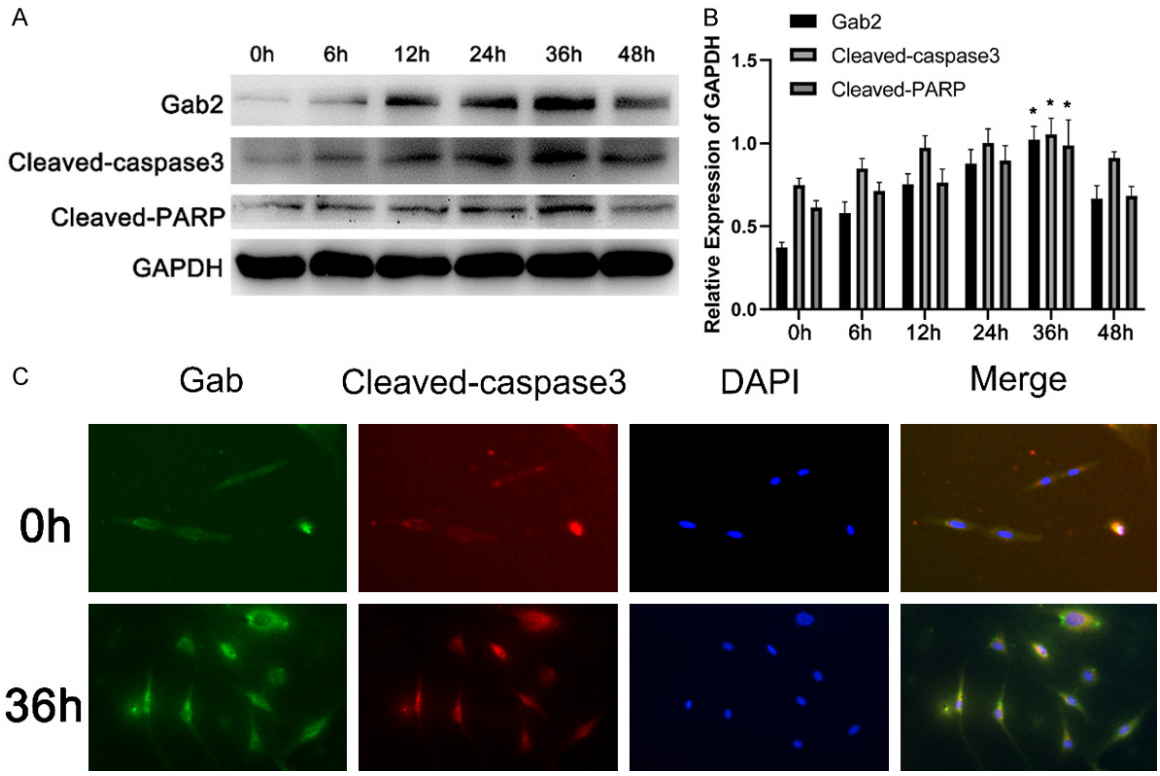

Figure 2. TNFα-induced GAB2 expression and apoptosis are increased in SW1353 cells. A. Western blot analysis showed GAB2 and apoptotic marker (cleaved caspase-3 and cleaved-PARP) protein levels in SW1353 cells following TNFα (20 ng/ml) stimulation. B. Bar chart showing the expression ratio of GAB2, cleaved caspase-3, and cleaved-PARP relative to GAPHD. C. Immunocytochemistry shows the co-localization of GAB2 (green) and cleaved caspase-3 (red) in TNFα-stimulated SW1353 cells (scale bar = 50 μm). The data are expressed as the mean ± SEM. *P < 0.05.

tilage, immunohistochemical staining and RT-PCR showed that GAB2 expression was significantly up-regulated in OA articular cartilage. This result provided preliminary evidence that GAB2 might participate in OA chondrocyte apoptosis.

We established an in vitro apoptosis model in SW1353 cells with TNFα stimulation [19]. Western blot analysis showed that GAB2 and cleaved-caspase 3/cleaved-PARP were up-regulated in a time-dependent manner. Furthermore, co-localization of GAB2/cleaved-caspase 3 was detected by double immunofluorescence staining. TNFα-induced cleaved-caspase 3/cleaved-PARP expression was increased after knockdown of GAB2 by siRNA. This indicated that chondrocyte apoptosis is negatively regulated by GAB2 by the caspase-dependent signaling pathway. Similarly, flow cytometry analysis showed that siGab2 treatment significantly increased TNFα-induced apoptosis in SW1353 cells. From these results, we speculated that GAB2 may play a key role in mitigating the progression of chondrocyte apoptosis in OA.

GAB2 is a member of the Grb2-associated binding protein (Gab) gene family, and is widely expressed in humans, especially in the brain, lung, heart, kidney, and ovary [20, 21]. GAB2 is involved in regulation of the PI3K-AKT signaling pathway and is closely related to the behavior of tumors [22, 23]. Gab proteins contain binding sites for multiple Src homology-2 (SH2) domain-containing proteins, such as SH2-containing protein tyrosine phosphatase 2 (SHP2) and the phosphatidylinositol 3-kinase (PI3K) regulatory subunit [24, 25]. AKT is a serine/threonine kinase that is important for survival signaling in vivo. AKT is downstream of PI3K and directly phosphorylates multiple transcription factors [26, 27]. Silencing GAB2 by siRNA enhanced the TNFα-induced expression of p-AKT, anti-apoptotic protein BCL2, and pro-apoptotic protein BAX. After inhibition of GAB2, therefore, p-AKT expression was decreased, the PI3K-AKT signaling pathway was inhibited, pro-apoptotic protein levels were increased, and anti-apoptotic protein levels were decreased, thereby promoting chondrocyte apoptosis.

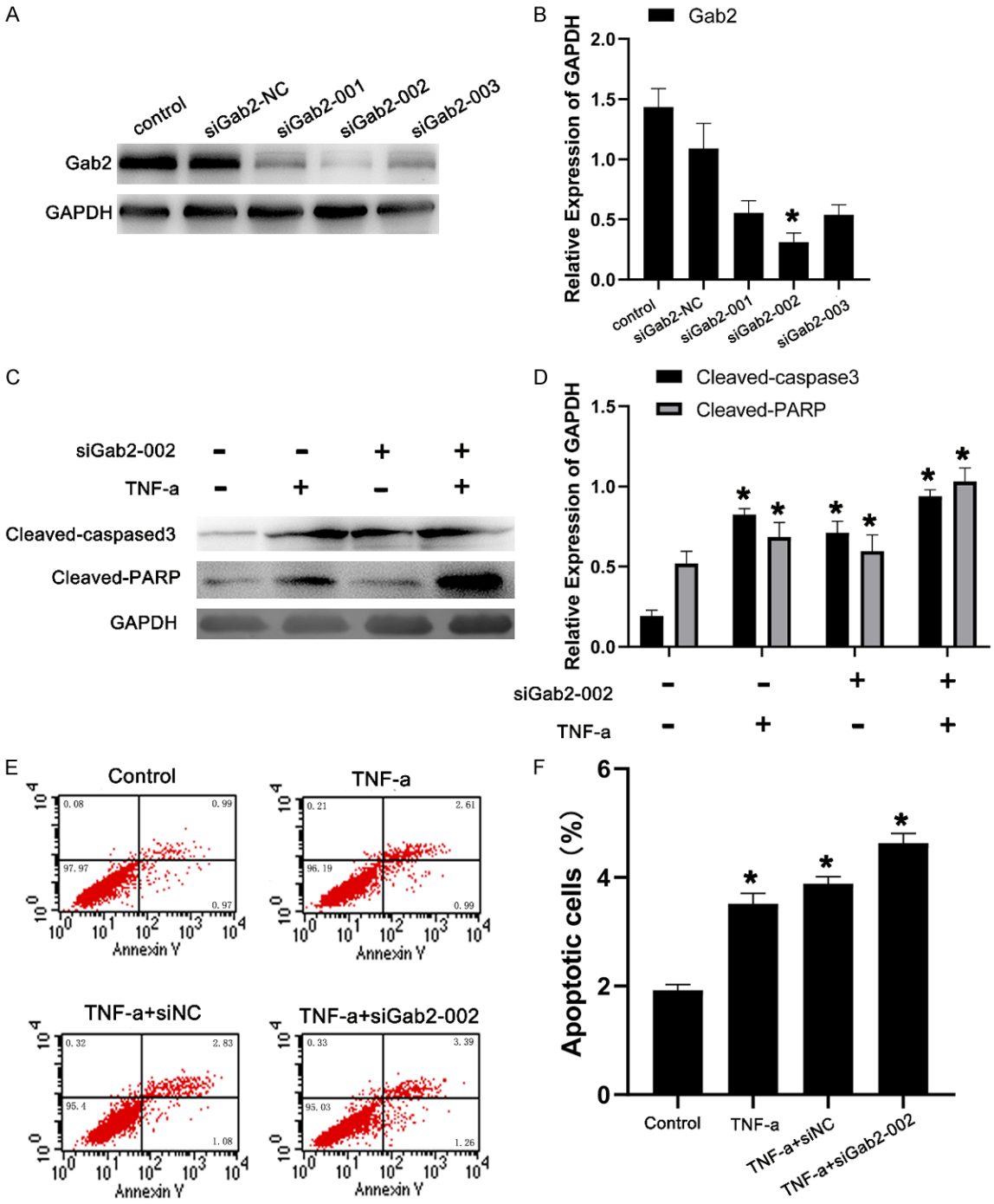

Figure 3. TNFα-induced apoptosis of SW1353 cells was increased after inhibiting GAB2 expression. A and B. The effect of silencing GAB2 in SW1353 cells was demonstrated by western blot analysis. C. siRNA knock down of GAB2 led to increased cleaved caspase 3/PARP levels in TNFα-treated SW1353 cells. D. Quantification of the expression ratio of cleaved caspase-3/PARP relative to GAPHD. E. GAB2 inhibition increased the number of annexin V/PI-positive cells after TNFα stimulation. F. The bar graph shows analysis of apoptosis. The data are expressed as the mean ± SEM. *P < 0.05.

In conclusion, GAB2 is markedly upregulated in OA articular cartilage and GAB2 knockdown promotes apoptosis in TNFα-stimulated SW-1353 cells. Furthermore, GAB2 regulates chondrocyte apoptosis through PI3K-AKT signaling. This is the first report describing the role

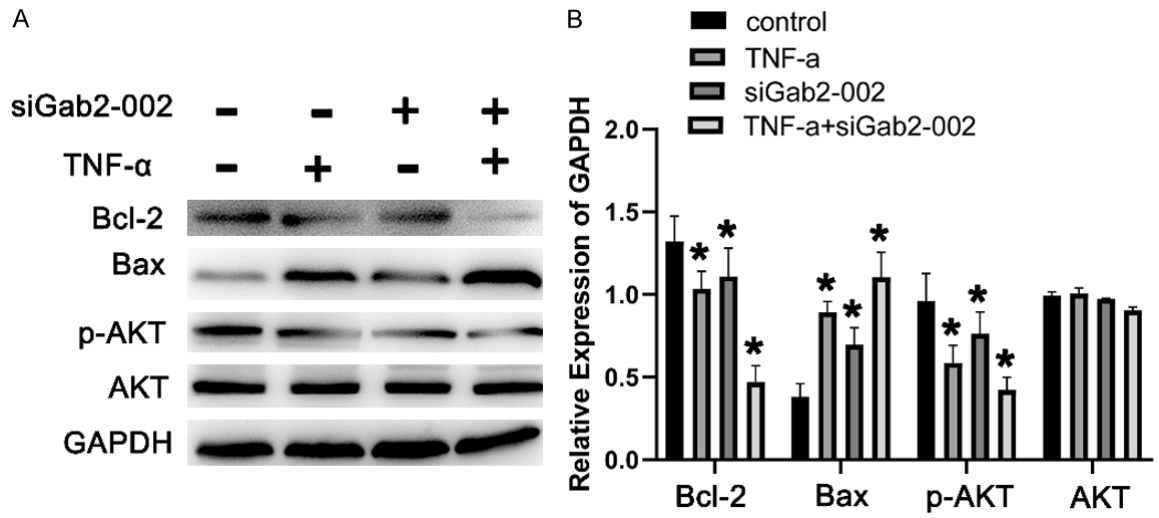

Figure 4. GAB2 regulates chondrocyte apoptosis through the PI3K-AKT signaling pathway. A. Western blotting was used to measure the protein levels of pro-apoptotic protein BAX, anti-apoptotic protein BCL2, AKT, and p-AKT after GAB2 inhibition and stimulation with TNFα. B. The bar chart illustrates quantification of the expression ratios of the above proteins relative to GAPDH. The data are expressed as the mean ± SEM. *P < 0.05.

of GAB2 in chondrocytes of OA patients, and indicates a potential new therapeutic target for OA. A limitation of our study is that other signaling pathways modulated by GAB2 that might affect chondrocyte apoptosis were not investigated.

Disclosure of conflict of interest

None.

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