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CircVCAN regulates the proliferation and apoptosis of osteoarthritis chondrocyte through NF-κB signaling pathway

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Abstract. – OBJECTIVE: Osteoarthritis is one of the chronic diseases with a high incidence. CircRNA is a circular non-coding RNA. Studies show that CircRNA is closely relevant to the pathogenesis of OA chondrocytes. However, the specific principle is still unclear.

PATIENTS AND METHODS: 38 patients with OA tissues and 38 patients with normal knee cartilage in our hospital were selected, re tively. The mRNA expression levels of Ci were measured by quantificational rea e polymerase chain reaction (qRT-PCR). Cell liferation was detected by the Cell Counting (CCK8). Cell cycle and apoptosis A chondr cytes were measured by flo ry. qRT PCR and western blot were d to ect PC-NA, p50, p52, p65 mRNA protein ression levels.

RESULTS: CircVCA as pres n OA tissues and OA idrocy ell prolifera-tion and PCNA ex sion level eased sig-nificantly after tion with si VCAN in OA-chondroc . H er, there w a signif-icant incre e on OA c rocytes after trans-fection w LV-CircVCA mpared with the si-NC p, the apoptosis of OA chondro-cytes s signi ntly increased after transfec-tio si-C CAN. The proportion of G0/G1 phas cycle s significantly reduced and th portion phase was significant-reas n th ntrary, the apoptosis rate w gnific uced after transfection with LV- CVCAN. proportion of G0/G1 phase in th cycle was significantly increased and of S phase was significantly re-ced. The mRNA and protein levels of p50, p52 p65 were significantly increased after trans-n of LV-CircVCAN in OA-chondrocytes. Fu rmore, PDTC (NF-κB inhibitor) transfection can significantly reverse the effect of overexpression of CircVCAN on the proliferation and apoptosis of OA chondrocytes.

CONCLUSIONS: C AN is overexpressed in C es and cells. VCAN can affect the p eration and apoptosis of OA chondrocytes b locking the a ivation of the NF-κB signaling way. Thus, VCAN may be an important t molecule OA treatment.

Key Wo
Osteoarthritis, CircVCAN, NF-κB, Proliferation, osis.

Introduction

As the most common arthritis, osteoarthritis seriously affects the health and quality of life of middle-aged and elderly people[1,2]. The main clinical symptoms are joint pain, swelling, stiffness, deformity and dysfunction[3,4]. Osteoarthritis clinical symptoms are mainly manifested as joint pain, swelling, stiffness, deformity and dysfunction[5-7]. However, the pathogenesis of osteoarthritis has not been fully understood.

Circular RNA (circ-RNA) is a new type of non-coding RNA. It is a covalent closed-loop structure without 5′ to 3′ polarity but does not contain a polyadenylation tail[8-10]. CircRNA is closely related to the occurrence and development of OA chondrocyte proliferation and differentiation, inflammatory response, ECM degradation, and signaling pathways. In arthritis and normal cartilage, there are differences between the expression of 71 types of circRNA, sixteen of them including circRNA_10086, circRNA_10118 and cirRNA_101914 were up-regulated and fifty-five others were down-regulated[10]. They may play important roles in the development of cartilage dam-

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age and arthritis. The expression levels of CircSERPINE2 and ERG were significantly increased in OA. CircSERPINE2 served as a sponge of miR-1271-5p to regulate the expression levels of ERG in OA, thus promoting or alleviating the catabolism of ECM[11]. Wu et al[12] found that CircRNA hsa_circ_0005105 could upregulate the expression of NAMPT, which facilitates the matrix (ECM) degradation by sponging miR-26.

In the present study, we found that CircVCAN was significantly up-regulated in OA tissues and chondrocytes. CircVCAN could have an influence on the proliferation and apoptosis of OA chondrocytes by inhibiting the activation of NF-κB signaling pathway. Thus, CircVCAN may become an important target molecule for OA treatment.

Patients and Methods

Patients

Thirty-eight OA patients and Thirty-eight meniscal injury patients who were admitted to our hospital from May 2017 to September 2018 were selected. The diagnostic criteria for OA were from Guid... for the diagnosis of osteoarthritis (2007) in... 24 males and 14 females were selected, respe...ly, the average age of patients was 45.2 ± 8.7 y... old. The average age of meniscus injury patients w... 43.7 ± 9.8 years old, and 22 mal... 16 femal... were included. None of the ...nts ...diseases such as rheumatoid arthriti... eumato... arthritis, and secondary osteoarth... All pa... the informed consent ...re ...e car...ge tissue was removed ...ng the o...on, the tissue samples were div...to the same ...nd stored in liquid nitrog...

qRT-PCR

The ...RNA from OA t...s and OA chondroc... were p...ured by TRIzol reagent (Invitrog... arlsb...CA, USA) according to the manufactu...ruction...ne synthesis of cDNA ...circ...nd m...A was detected by reverse t...ription ...KaRa, Otsu, Shiga, Japan). SY... Green ...Kit (TaKaRa, Otsu, Shiga, Japa...sed to measure the quantification of Circ...A ... The levels of mRNA and circRNA ...pression were normalized by GAPDH and ... All primer sequences were synthesized and p... by Genery (Guangzhou, China). The results were processed by the 2-ΔΔCt methods. The primers used in our study were as follows: CircVCAN F 5'-GTATAGGTGGAACAGTCTTAA-3', R 5'-TTATATTCCTTCTTTAGAGTTTGG-3'. PCNA F 5'-GACACATACCGCTGCGATCG-3', R 5'-TCACCACAGCATCTCCAATAT-3'. β-actin F 5'-TGCAGCGACTAAGCA...-3', R 5'-TCACCAGCACGA AGGAC... F 5'-CAGGTCCACTGTCTGCC...-3', ... 5'-GGAAGGATGT CTCCAC...-3'. P52 F 5'-TCCATTTGTTCCTCCTG...-3', R 5'-GGTGGCCC TTGAC...TCT... P65 F 5'-GACCTGGAGCA...GATTAG... 5'-CACTGT CACCTG...AGCA...A-3'. ... DH F 5'-ACCA...TCCA...CATCAC-3', R 5'-TCCACCA...T...TGTA-3', U6 F 5'-CGCTT...CAC...ATATA..., R 5'-AAATATG...ACGCTTC...A...

Western blot

The cells were co...d and the total protein was...y extracted ...h after transfection. B...kit (Pierce, Rockford, IL, USA) was used to ...sure the prot...n concentration. 40 μg of the ...in was adde... each well of the sodium do...ulphate-p...crylamide gel electrophoresis (SD...GE) ...Millipore, Billerica, MA, USA). Experi...onditions are set to 110 V electrophoresis, 250 mA current was transferred to the ...ylidene difluoride (PVDF) membrane and ...med milk powder was blocked at 37°C for 1 hour. PCNA, p50, p52, p65, β-catenin (Abcam, Cambridge, UK) and β-actin primary antibody were added and incubated for overnight at 4°C condition. TBST was washed for 3 × 10 min, secondary antibody was incubated for 1 h at 37°C, then TBST was washed for 3 × 30 mins, ECL was used for development. Quantity one software was used to analyze protein band gray value. β-actin was used as internal reference and calculation of expression levels was relative expression.

CCK-8 Assay

The cells were taken in logarithmic growth phase, 3 × 10^5 cells / well were inoculated in 6-well plates. Next, the cells were trypsinized and collected after transfection for 24 h and washed with PBS to prepare cell suspensions; pre-chilled 70% ethanol was added, and cells were fixed overnight at 4°C. After twice washing with PBS, the supernatant was discarded. Cell proliferation was assessed by a CCK-8 (CCK-8, CK04, Dojindo Molecular Technologies, Kumamoto, Japan). PI working solution was added, and the cells were incubated for 30 minutes in the dark at room temperature. Then the cell cycle was detected by flow cytometry. Cells were washed twice with pre-

chilled PBS, then 10 µl Annexin V-FITC and 5 µl propidium iodide staining solution were added. Cells were mixed gently and incubated for 20 min at room temperature in the dark.

Cell Apoptosis Assay

The apoptosis of OA chondrocytes was measured by PE Annexin V apoptosis detection kits (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instruction. CellQuest analysis software was used to analyze date of results by (BectonDickinson, Brea, CA, USA). All experiments were performed in triplicate.

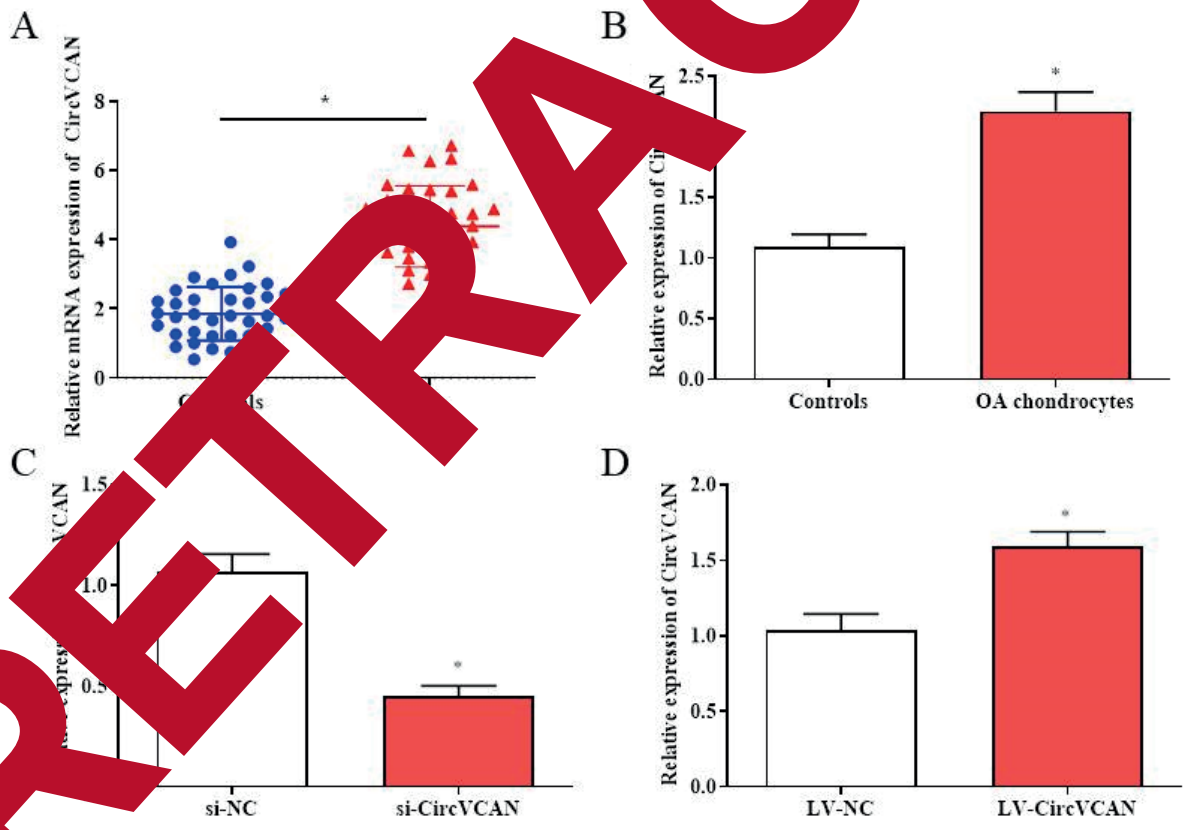
Statistical Analysis

All data were analyzed statistically using SPSS 22.0 statistical software (SPSS Inc., IBM, Armonk, NY, USA), which expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

CircVCAN Was Highly Expressed in OA Tissues and OA Chondrocytes

We examined the expression level CAN in OA tissues and OA chond tes. The results showed that the expre level of CircVCAN in cartilage tissue o patients was significantly higher th that o ilage tissue in meniscus injur ients (Fig Similarly, the expres level CircV in OA chondrocyte s sign antly higher than that of chon oc meniscus injury (Figure 1B) en C ondro s were transfected w i-CircVC h ative expression le ircVCAN w gnificantly reduced (ure On the contrary, the relative expression leve ircVCAN was significant ased in OA drocytes transfected h LV-CircVCAN (F gure 1D).



1. CircVCAN was highly expressed in OA tissues and OA chondrocytes**. A,** The expression levels of CircVCAN were det d by qRT-PCR in OA tissues and meniscus damaged tissues. **B,** CircVCAN expression was measured by qRT-PCR in OA chondrocytes and chondrocytes with meniscus injury. **C,** CircVCAN relative expression was measured by qRT-PCR after transfection with si-CircVCAN in OA chondrocytes. **D,** qRT-PCR was used to detect the relative expression of CircVCAN after transfection with LV-CircVCAN in OA chondrocytes. The data were expressed as mean ± SD.*$p<0.05$.

Low Expression of CircVCAN Inhibited the Proliferation of OA Chondrocytes

In order to further explore the effect of CircVCAN on the proliferation of OA chondrocytes, OA chondrocytes were transfected with si-CircVCAN and LV-CircVCAN, respectively. PCNA mRNA and protein expression levels were detected by qRT-PCR and Western blot after three days of culture. The results showed that the expression levels of PCNA mRNA and protein in OA chondrocytes were significantly lower than the si-NC group after transfection with si-CircVCAN (Figure 2A-2C). Conversely, the expression levels of PCNA mRNA and protein in OA chondrocytes were significantly higher than the si-NC group after transfection with LV-CircVCAN (Figure 2D-2F).

The Expression of CircVCAN Had a Great Influence on Cell Cycle of OA Chondrocytes

The effect of CircVCAN expression levels on the cell cycle was further explored, flow cytometry was used to detect the cell content at different stages in the cell cycle. The results showed that the proportion of cells in the G0/G1 phase [illegible] cell cycle increased significantly, while t[illegible] portion of cells in the S phase decreased s[illegible]i-cantly when OA chondrocytes were transfe[illegible] with si-CircVCAN (Figure 3A-3C). In contra[illegible] when OA chondrocytes were transfected with LV-CircVCAN, the proportion of cells in the G0/G1 phase of the cell cycle decreased significantly, while the proportion of cells in the S ph[illegible] increased significantly (Figure 3D-3F).

Downregulation of CircVCA[illegible]omoted Apoptosis of OA Chondrocyte[illegible]

To further explore the eff[illegible]of Cir[illegible]N expression level on apoptosi[illegible]OA chond[illegible] flow cytometry was us[illegible] detec[illegible]he apop rate after transfecti[illegible]ith si[illegible]cVCAN and LV-CircVCAN, res[illegible] results[illegible]owed that the apoptosi[illegible]te of [illegible]hondro[illegible]s after transfection w[illegible]i-CircVCA[illegible]nificantly increased c[illegible] with the g[illegible] transfected with si-N[illegible]igur[illegible]4C). In contrast, the apoptosis rate of OA cho[illegible]ytes after transfection wit[illegible]rcVCAN w[illegible]gnificantly reduced c[illegible]ared to the group t[illegible]nsfected with si-NC ([illegible]ure 4D-4F).

C[illegible]VCAN Ha[illegible] Great Effect on NF-κB Pa[illegible] in [illegible] Chondrocytes

The [illegible] circVCAN on NF-κB pathway in OA chondrocytes was further explored. The [illegible] and protein expression levels of mole-[illegible]ated to NF-κB pathway were measured by qRT-PCR and Western blot. When OA chon-

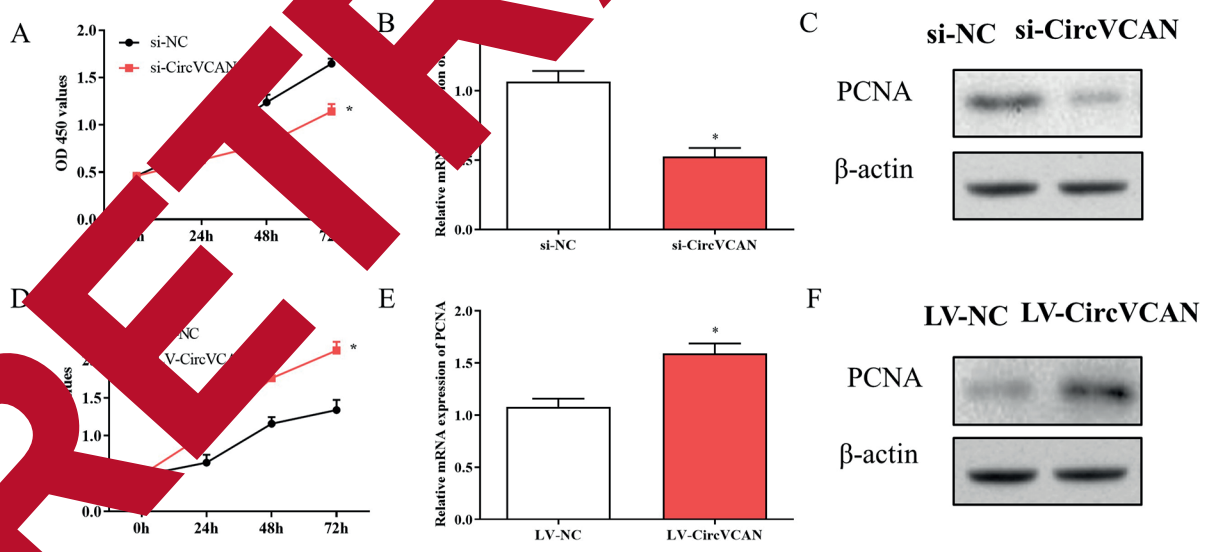


Figure 2. Low expression of CircVCAN inhibits the proliferation of OA chondrocytes. **A-B.** CCK-8 assay was used to detect the [illegible]liferation of OA chondrocytes after transfection with si-CircVCAN. **C.** PCNA and β-actin protein expression levels were measured by Western blotting after transfection with si-CircVCAN. **D-E.** The proliferation of OA chondrocytes was detected by CCK-8 assay after transfection with LV-CircVCAN. **F.** PCNA and β-actin protein expression levels were measured by Western blotting after transfection with LV-CircVCAN.

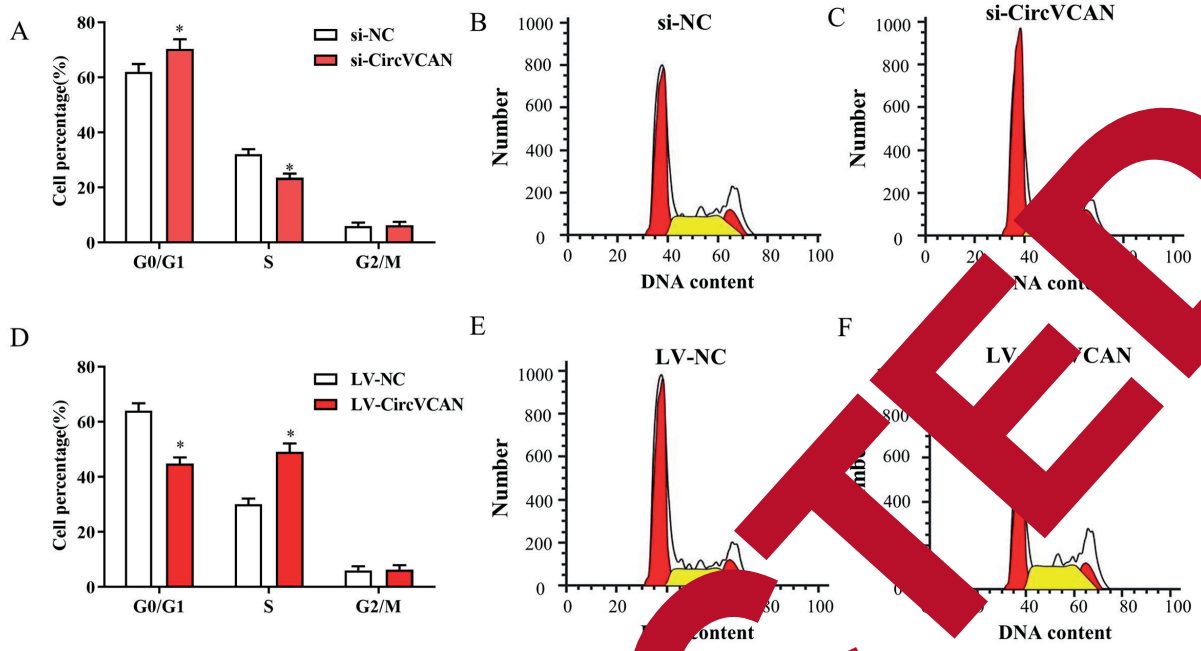


Figure 3. The expression of CircVCAN has a great influence on c ycle of OA cho cytes. **A-C.** The proportion of cells at different stages was detected by flow cytometry after transfectio si-CircVCA **-F.** The proportion of cells at different stages was detected by flow cytometry after transfection with LV CAN.

drocytes were transfected with si-CircVCA he VCAN group (Figure 6D and 6H). Furrelative expression levels of molecules relat , the apoptosis rate of OA chondrocytes NF-κB pathway such as p50, p52 and p65 w transfected with LV-CircVCAN and PDTC was significantly reduced (Figure 5A C). On t higher than transfection with LV-CircVCAN contrary, when OA chondroc ansfect- alone (Figure 6I and 6L).
ed with LV-CircVCAN, t elative pression
levels of NF-κB pathwa ed m
as p50, p52 and p65 s d
(Figure 5B and 5D

CircVCAN H t Effect o Proliferati nd A osis of OA Chondr e After Bl g NF-κB Signal Pathway

To ther e re the effect of blocking the NF signal pathway on the proliferation and apopt A chon ytes, we examined the iferat nd a osis of OA chondrocytes a ransf th LV-NC, LV-CircVCAN, LV cVCAN d PDTC. The results showed th els of PCNA mRNA and protein in OA di transfected with LV-CircVCAN and DTC were lower than those in LV-CircVCAN p alone (Figure 6A and 6C). At the same t he proportion of G0/G1 phase cells transfected with LV-CircVCAN and PDTC was higher than that of LV-CircVCAN group, while S phase proportion cells was lower than transfection with

Discussion

Osteoarthritis is the most common orthopedic disease, which not only affects the limb movement of patients, but also severely causes paralysis and even death[1,2]. Articular chondrocyte destruction, extracellular matrix degradation, and synthetic disorders are important factors in the occurrence of osteoarthritis[3,13]. However, its specific pathogenesis is unknown. CircRNA is a kind of non-coding circular RNA. More and more studies show that CircRNA is closely involved in the occurrence and development of osteoarthritis[10-12,14,15]. For example, has_circ_0005105 can promote the degradation of cartilage ECM by up-regulating the target gene of miR-26 NAMPT, thereby regulating the inflammatory response of OA chondrocytes[12]. Has_Circ_0045714 can up-regulate the expression of proteoglycan and type II collagen, thereby promoting the proliferation of OA chondrocytes[10].

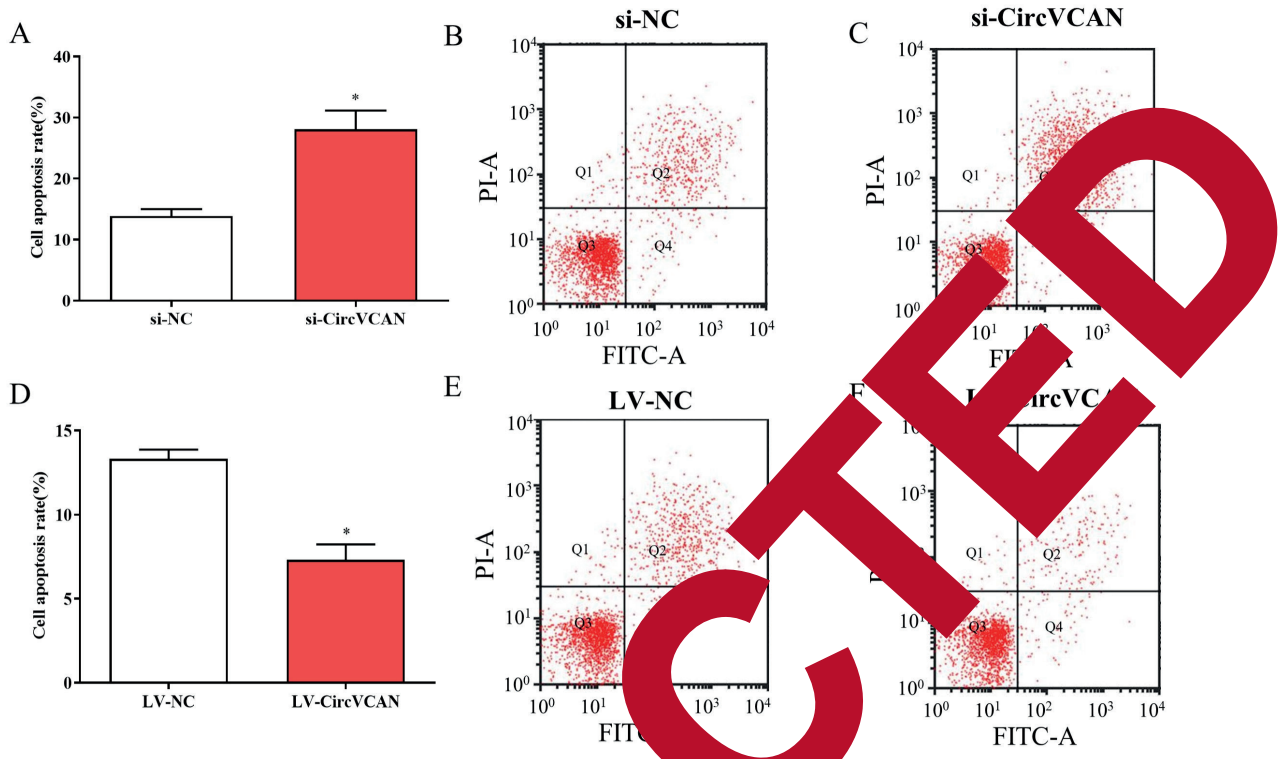


Figure 4. Downregulation of CircVCAN promoted [illegible] of OA chondrocytes. **A-C.** Flow cytometry was used to detect the apoptosis rates of OA chondrocytes after transfe[illegible]VCAN and si-NC. **D-F.** The apoptosis rates of OA chondrocytes were measured by flow cytometry after tra[illegible]ion w[illegible]CAN and LV-NC.

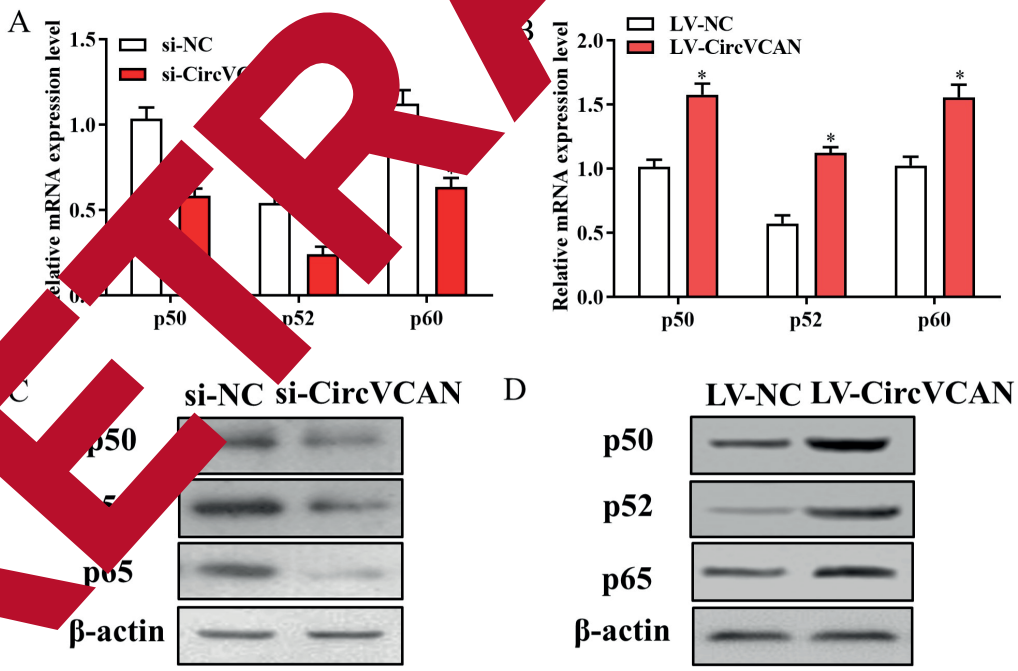


Figure 5. The expression of CircVCAN had a great effect on NF-κB pathway in OA chondrocytes**. A-B.** The relative expression levels of p50, p52 and p60 mRNA were measured by qRT-PCR after transfection with si-CircVCAN and LV-CircVCAN, respectively. **C-D.** The relative expression levels of p50, p52 and p60 protein were detected by Western blotting after transfection with si-CircVCAN and LV-CircVCAN, respectively.

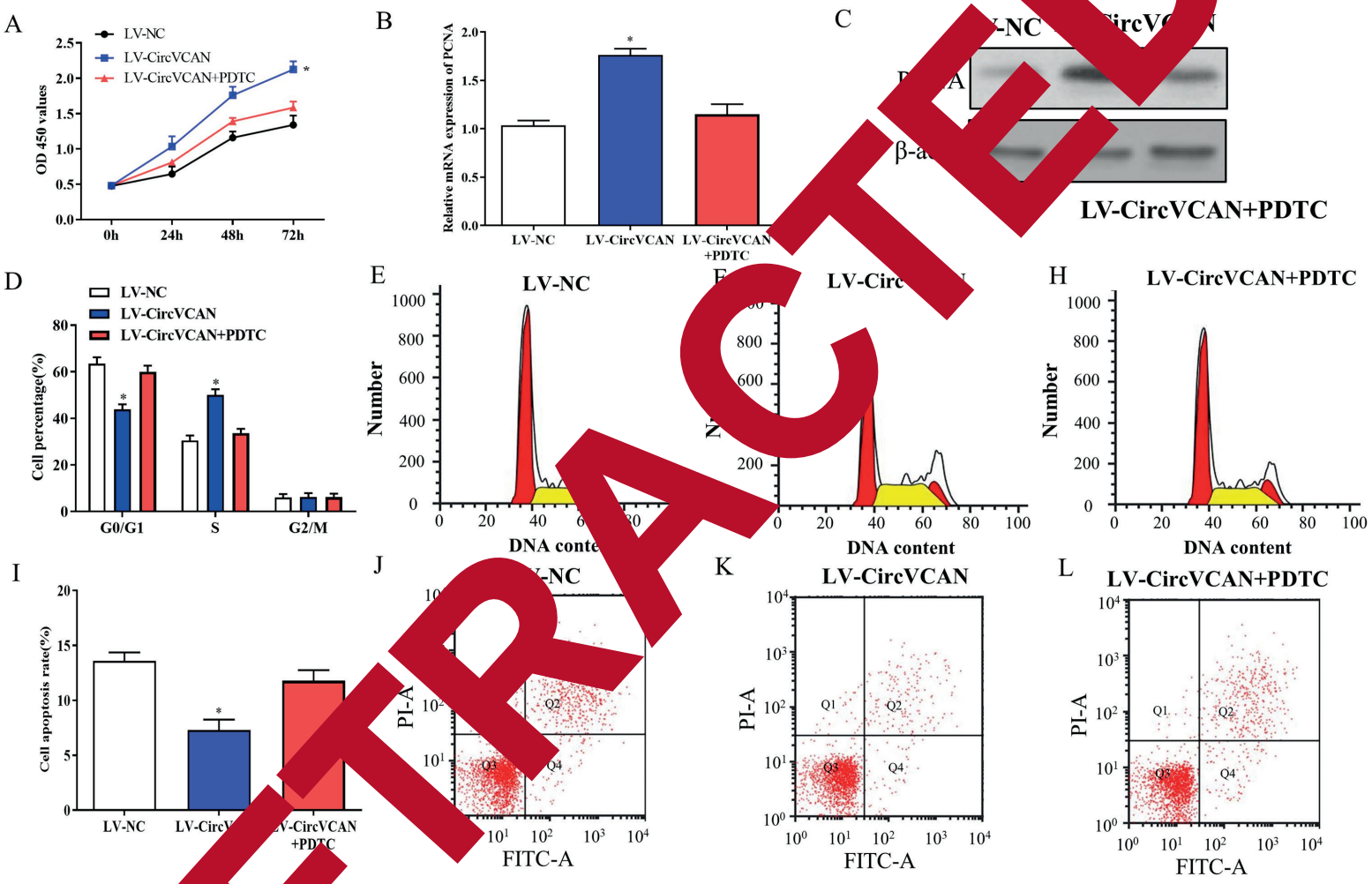


Figure 6. CircVCAN e[illegible]vels had [illegible]eat effect on OA chondrocyte proliferation and apoptosis after blocking NF-κB signaling pathway. **A-C.** PCNA mRNA and protei[illegible]lative [illegible]tion lev[illegible]ere measured by qRT-PCR and Western blotting after transfection with LV-NC, LV-CircVCAN, LV-CircVCAN and PDTC. **D-H.** C[illegible]as de[illegible]by[illegible] cytometry after transfection with LV-NC, LV-CircVCAN, LV-CircVCAN and PDTC. **I-L.** Apoptotic rates were measured by flo[illegible]omet[illegible]ter trai[illegible] with LV-NC, LV-CircVCAN, LV-CircVCAN and PDTC.

In our study, we found that the expression levels of CircVCAN were significantly higher in OA tissues and OA chondrocytes. When OA chondrocytes were transfected with si-CircVCAN, the relative expression level of CircVCAN was significantly reduced. However, the relative expression level of CircVCAN was significantly increased after transfection with LV-CircVCAN. This suggested that CircVCAN was abnormally expressed in OA chondrocytes, but the specific principle was not clear. Further research found that low expression of CircVCAN could effectively inhibit PCNA mRNA and protein expression levels in OA chondrocytes while high expression of CircVCAN could effectively promote PCNA mRNA and protein expression levels in OA chondrocytes. At the same time, low expression of CircVCAN could effectively increase the proportion of cells in the G0/G1 phase while reduce the proportion of cells in the S phase of the OA cell cycle. High expression could effectively reduce the proportion of cells in the G0/G1 phase of while increase the proportion of cells in the S phase of the OA cell cycle. The results further illustrated that CircVCAN had an important effect on the proliferation of OA ... drocytes. Low expression of CircVCAN in... cell proliferation and high expression pro... d cell proliferation. At the same time, the apo... sis rate of group transfected with si-CircVC... increased significantly while ... ransfecte... with LV-CircVCAN decrease... gni... tly. The... results indicated that Circ... N par... ated in the apoptosis process of ... chond... expression of CircVC... pr... optosis... d high expression inh... d apop...

NF-κB is a ... of protein ... ules that make up the t... c... n factor fa... y. It was stimulated ... activa... y pro-inflammatory cytokines, ... mokines, st... elated factors and ECM ... adation products. ... -κB molecules were ... uely i... ved in immunity, stress response, inflam... tory diseases, cell proliferation and c... The NF... signaling pathway was ... y mo... r pa... y in the process of osteo... is ca... gradation[16,17]. It promoted the sec... on of m... le degradation enzymes such as ... MMP-2, MMP-3, thus aggravating the ...ptos... cartilage of osteoarthritis chondrocytes and inflammatory response[18]. The expres... of IL-1β, IL-6, TNF-α and MMP were re... d... by inhibiting the NF-κB signaling pathway in the rat osteoarthritis model[19]. Tang et al[20] also found that modulation of the NF-κB signaling pathway could reduce IL-1β-induced ECM metabolic imbalance, proinflammatory cytokine production, cell viability and apoptosis. In this study, we revealed that low expression of CircVCAN could effectively inhibit the mRNA and ... in levels of molecules including p50, p5... related to NF-κB pathway. Conver... high ex... pression of CircVCAN could effe... y promote the mRNA and protein levels of 50, ... nd p65. This shows that CircVCAN i... volve... e ac... tivation of NF-κB pathwa... OA chond... Further research indi... that ... imulta... transfection with PD... (NF-κ... hibitor) a... LV-CircVCAN cou... e... verse t... ffect of LV-CircVCA... pro... on and ... optosis of OA chondr... . The resu... m... ated that CircVCAN ... proliferati... d apoptosis of OA ch... rocy... blocking the NF-κB signaling pathway.

...nclusions

... fir... e, we discovered the effect of CircVC... the phenotype of osteoarthritis through the NF-κB signaling pathway. CircV... as demonstrated to be highly expressed ... ssues and OA chondrocytes. Moreover, CircVCAN could regulate the proliferation and apoptosis of OA chondrocytes by blocking the activation of NF-κB signaling pathway. This study will provide new molecular mechanisms for CircRNA research in osteoarthritis and a new potential target for the treatment of osteoarthritis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgement

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