




ZNRF3 Regulates Collagen-Induced Arthritis Through NF- κ B and Wnt Pathways

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Abstract— Although the E3 ubiquitin ligase Zinc and ring finger 3 (ZNRF3) negatively regulates the Wnt signaling pathway, its function in rheumatoid arthritis (RA) is elusive. Here, the effects and the mechanism of ZNRF3 on a mouse model of collagen-induced arthritis (CIA) and human fibroblast-like synoviocytes (FLS) obtained from RA patients were determined. Our results showed that ZNRF3 was highly expressed in tissues and FLSs compared to trauma patients. Lentivirus-mediated silencing of ZNRF3 induced apoptosis decreased cell viability and significantly attenuated inflammation in RA-FLSs *via* tumor necrosis- α (TNF- α). Additionally, silencing of ZNRF3 reduced knee joint damage and also decreased the level of TNF- α , IL-1 β , and IL-6 in the CIA mouse model. These effects were mediated by the crosstalk between Wnt and NF- κ B pathways in RA-FLS.

KEY WORDS: rheumatoid arthritis; synovial fibroblasts; ZNRF3; collagen-induced arthritis; Wnt/ β -catenin pathway; NF- κ B pathway.

INTRODUCTION

Rheumatoid arthritis (RA) belongs to a group of chronic autoimmune conditions associated with invasive arthritis. Unlike healthy individuals, the synovium of RA

patients is characterized by bone destruction, pannus formation, synovial inflammation, and joint cartilage erosion. Fibroblast-like synoviocytes (FLSs) are vital components of the synovium that contribute to the development of RA. RA-FLS can proliferate and secrete various immunoregulatory factors, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs) [1–4].

In recent years, Wnt signaling pathways have been found to be associated with the activation of RA-FLS [5, 6]. Therefore, inhibition of the Wnt pathway may effectively treat RA.

The transmembrane E3 ubiquitin ligase Zinc and ring finger 3 (ZNRF3) is a member of Goliath and Godzilla families which inhibits the Wnt/ β -catenin signaling cascades [7, 8]. The activation of Wnt signaling triggers the production of β -catenin which then translocates to the nucleus where it interacts with transcription factors of the

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TCF family leading to the expression of ZNRF3. When ZNRF3 translocates into the plasma membrane, it recognizes the Wnt receptor FZD through DVL. FZD is then ubiquitinated and degraded, resulting in the inhibition of the Wnt/ β -catenin signaling. In the extramembrane region, ZNRF3 forms a complex with R-spondin and LGR4/5 and induce the ubiquitination and degradation of itself [7, 9]. In this study, we speculated that ZNRF3 may regulate the synovitis of RA *via* the Wnt pathway.

In this study, we examined the expression of ZNRF3 in the synovial tissues and FLSs, and the effect of the lentivirus-mediated silencing of ZNRF3 on apoptosis, proliferation, and inflammatory response in RA-FLS. The mechanisms of these effects were explored using a mouse model of arthritis.

MATERIALS AND PROTOCOLS

Patients Enrollment and Synovial Tissue Preparation

Synovial tissues were extracted from 10 patients with RA (3 men and 7 women; 49–68 years old) and 15 patients with trauma (3 women and 12 men; 21–32 years old). Synovial tissues of patients with trauma were used as normal controls. The American College of Rheumatology (ACR) criteria for RA were used to select patients [10]. Patients with trauma were diagnosed as having soft tissue injuries such as ligaments during exercise. Patients with other immune and musculoskeletal disorders were excluded. RA patients were treated with total knee replacement surgery. Arthroscopic examination was performed to examine patients with knee joint trauma. This study was approved by the Ethics Committee of the Shanghai Changhai Hospital.

Immunohistochemical

Synovial tissues were fixed in 4% paraformaldehyde for 48 h and then analyzed by immunohistochemistry using previously described protocols [11]. The scores were analyzed as reported in a previous study (0 = 0–9%, 1 = 10–24%, 2 = 25–49%, 3 = 50–74%, and 4 = 75–100%) [12].

Cell Isolation and Culture

FLS were isolated from the synovial tissues by enzymatic digestion [13]. The cells were cultured in a DMEM/F12 solution supplemented with 100 U/mL penicillin, 10% fetal bovine serum, and 100 μ g/mL streptomycin (FBS; Gibco, Carlsbad, CA, USA) at 37 °C, 5% CO₂ in

a humidified incubator. Thereafter, they were harvested at 4–6 passages for use in experiments.

Immunofluorescence

The distribution of ZNRF3 in Trauma-FLSs and RA-FLSs samples was assessed as described previously [14]. The cells were stained and then imaged with a fluorescence microscope (Olympus, Tokyo, Japan).

Cell Infection

Cells were added into 6-well plates at a density of 1×10^6 per well and incubated for 24 h. Next, a fresh medium containing lentivirus and 8 μ g/mL Polybrene (Heyuan, Shanghai, China) was added. The amount of lentivirus added was calculated on the basis of the required multiplicity of infection (MOI). The optimal MOI for lentivirus of short hairpin (Sh)- ZNRF3 (Sh-ZNRF3) and the lentivirus of control (Sh-control) were 30 and 10, respectively, as determined by the preliminary experiments. The culture medium was changed within 24 h after infection, and 1% Puromycin (Sigma, St. Louis, MO, USA) was added to the complete medium to kill the wide-type RA-FLSs. About 5 days later, the majority of the wild-type cells had died. The efficiency of infection was determined by western blot.

Cell Viability Assay

The impact of Sh-ZNRF3 on cell viability was assessed by the Cell Count Kit-8 (Dojindo, Kumamoto, Japan). The infected RA-FLSs were incubated in 96-well plates (8×10^3 cells/well) for 24 h in the presence or absence of 10 ng/mL TNF- α . Cells were washed with PBS, and then 10 μ L CCK-8 solution and 90 μ L DMEM/well were added. After incubation at 37 °C for 3 h, the absorbance of the supernatant of the culture medium was measured at 450 nm wavelength with a spectrophotometer.

Measurement of Apoptosis

RA-FLS often impairs apoptosis which leads to hyperplastic pannus [15]. Apoptosis was assessed by the Annexin V-APC/7-AAD kit (Multisciences, Hangzhou, China). The cells were divided into two groups, those infected with Sh-ctr or Sh-ZNRF3. Briefly, cells were washed with PBS and then reconstituted in a $1 \times$ binding buffer. This was followed by treatment with Annexin V-APC and 7-AAD for 15 min at RT in the dark and then

analyzed by flow cytometric (BD, Franklin Lakes, NJ, USA).

Cell Migration

The effects of Sh-ZNR3 on the migration ability of RA-FLSs were determined using the Transwell cell migration test and healing assay. After seeding cells on 6-well plates, a cell wound was created by scraping the surface with a sterile 1 mL pipette tip. The wound-healing process was analyzed at 0 h and 24 h by phase-contrast microscope (Leitz, Germany) as reported before [16]. The cell migration was calculated based on the healed area with image J at various time points. Transwell cell migration test was performed by the Transwell invasion chambers (Corning, USA) in 24-well plates according to a previous study [16]. The number of RA-FLSs was counted in five randomly selected fields per well. The cell migration rate was determined as the average number of invading cells compared to the Sh-ctr group.

qPCR Assays

RA-FLSs samples were divided into four groups, and two of which were infected by Sh-ctr and Sh-ZNR3. The two groups infected by lentivirus and the negative control group were treated with 10 ng/mL TNF- α for 24 h. The total RNA was extracted with the RNA extraction kit (Fastagen, Shanghai, China) and transcribed to cDNA by PrimeScript reverse transcription kit (Takara, Shiga, Japan). qPCR was carried out by SYBR Green (Takara, Shiga, Japan) on a Fluorescence quantitative system (Roche, Basel, Switzerland). The following primer sequences were used: IL-1 β forward, 5'- TTCGACAC ATGGGATAACGAGG-3', and reverse, 5'- TTTTGGCT GTGAGTCCCGGAG-3'; IL-6 forward 5'- CCTTCGGT CCAGTTGCCTTCTC-3', and reverse 5'- AGAGGTGA GTGGCTGTCTGTGT-3'; IL-8 forward 5'- ACTGAGAG TGATTGAGAGTGGAC-3', and reverse 5'- AACCTCT GCACCCAGTTTTTC-3'; GAPDH forward, 5'- CATGAGAAGTATGACAACAGCCT-3', and reverse, 5'- AGTCCTTCCACGATAACCAAAGT-3'. The relative mRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method.

Measurement of Inflammatory Cytokines Release

The supernatants containing cell-infected RA-FLSs were treated in the presence or absence of 10 ng/mL TNF- α (Propotec, USA) for 24 h and then analyzed with ELISA to determine the levels of IL-1 β , IL-6, and IL-8. The serums obtained from CIA mice were examined by the Multi-Analyte Flow Assay Kit (Biolegend, USA).

Western Blot

Proteins isolated from the synovial tissues and infected RA-FLSs were resolved by the SDS-PAGE gels electrophoresis and then electrotransferred to PVDF membranes. Then the membranes were blocked with 5% fat-free milk and then treated with corresponding primary and secondary antibodies. Next, the blots were exposed to the enhanced chemiluminescence detection system (GE, USA). The following antibodies were used: HRP-conjugated secondary antibody and GAPDH antibodies (Servicebio, Wuhan, China); antibody of p65, p-p65, I κ B, p-I κ B, IKK- α (Cell Signaling (Beverly, MA, USA)); antibody of C-myc (Millipore, Boston, MA, USA); antibody of β -catenin (Cambridge, MA, USA); and antibody of ZNR3 (Biorbyt, Cambridge, England).

Animal Studies

Twenty-two SPF male 6 weeks old DBA/1 mice were purchased from SLRC Laboratory Animals (Shanghai, China). All animal procedures and handling were approved by the Animal Care and Use Committee of Changhai hospital. Six mice were assigned to the normal group, two mice were infected with lentivirus, and 14 were used to establish the collagen-induced arthritis (CIA) as previously described [17]. The Cri Maestro *in-vivo* Imaging System (Caliper Life Sciences) was employed to determine the efficiency of the lentiviral transfection into the knee joint of mice. Clinical arthritis was observed about a week after the second immunization. Twelve typical arthritic mice were selected from the mice with CIA according to the severity score of arthritis [17] and categorized into 2 groups on the 28th day. Sh-ZNR3 and Sh-ctr were administered separately into the knee joints of mice of Sh-ZNR3 and Sh-ctr groups once a week for 3 weeks. Finally, the mice were euthanized on the 49th.

Histopathology of Mice

Knee joints were fixed in 4% paraformaldehyde for 48 h followed by decalcification in 14% EDTA glycerol at 37 °C for 30 days in couveuse. H&E staining was performed to examine the changes and extent of synovitis according to the reports [18, 19].

Data Analysis

Data are shown as mean \pm SD and were analyzed with SPSS 19.0. Differences between two and three groups were assessed by t-test and one-way analysis of variance

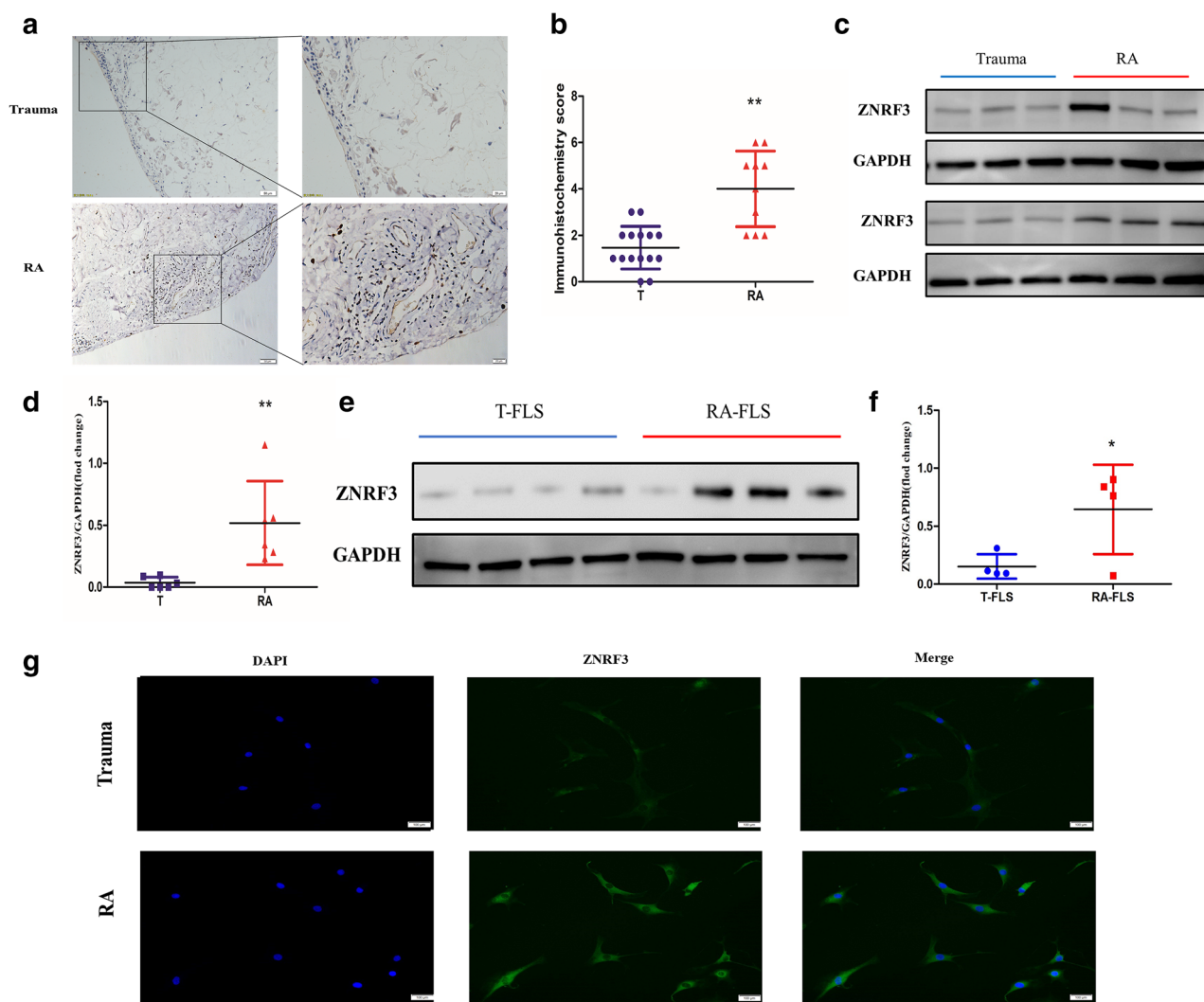


Fig. 1. The expression of ZNRF3 in synovial and FLSs samples from RA patient. **a** Typical images of immunohistochemistry which was used to detect the expression of ZNRF3 in synovial extracts from trauma ($n = 15$) and RA ($n = 10$) patients. **b** The scores of immunohistochemistry in trauma group was lower in RA group. **c** Western blot results for GAPDH, ZNRF3 of synovial tissues from trauma ($n = 6$) and RA ($n = 6$) patients. **d** Analysis of protein levels shown above. **e** Western blot results for GAPDH, ZNRF3 of FLSs from trauma ($n = 4$) and RA ($n = 4$) patients. **f** the protein expression levels shown above. **g** Immunofluorescence was used to observe the expression of ZNRF3 in FLSs of RA and trauma patients. ($n = 3$).

(ANOVA), respectively. P -values < 0.05 were considered statistically significant * $P < 0.05$, ** $P < 0.01$.

ZNRF3 Expression Is Increased in Synovial Tissues and FLSs of RA Patients

The immunohistochemical (Fig. 1a,b) and western blotting (Fig. 1c,d) assays were employed to measure the ZNRF3 expression in the synovial specimen from RA patients to Trauma patients. Further, western blotting (Fig. 1e,f) and immunofluorescence (Fig. 1g) were

performed to quantify the expression of ZNRF3 in FLSs of the two groups. The results indicated that ZNRF3 was localized in the plasma membrane, and it was elevated in the synovial tissues and FLSs of RA group compared to the trauma group.

Infection of RA-FLSs with Sh-ZNRF3

The infection rate of RA-FLSs with Sh-ZNRF3 was approximately 60% when infected at the MOI of 30 for 48 h (Fig. 2a). Five days after infection, the effect of

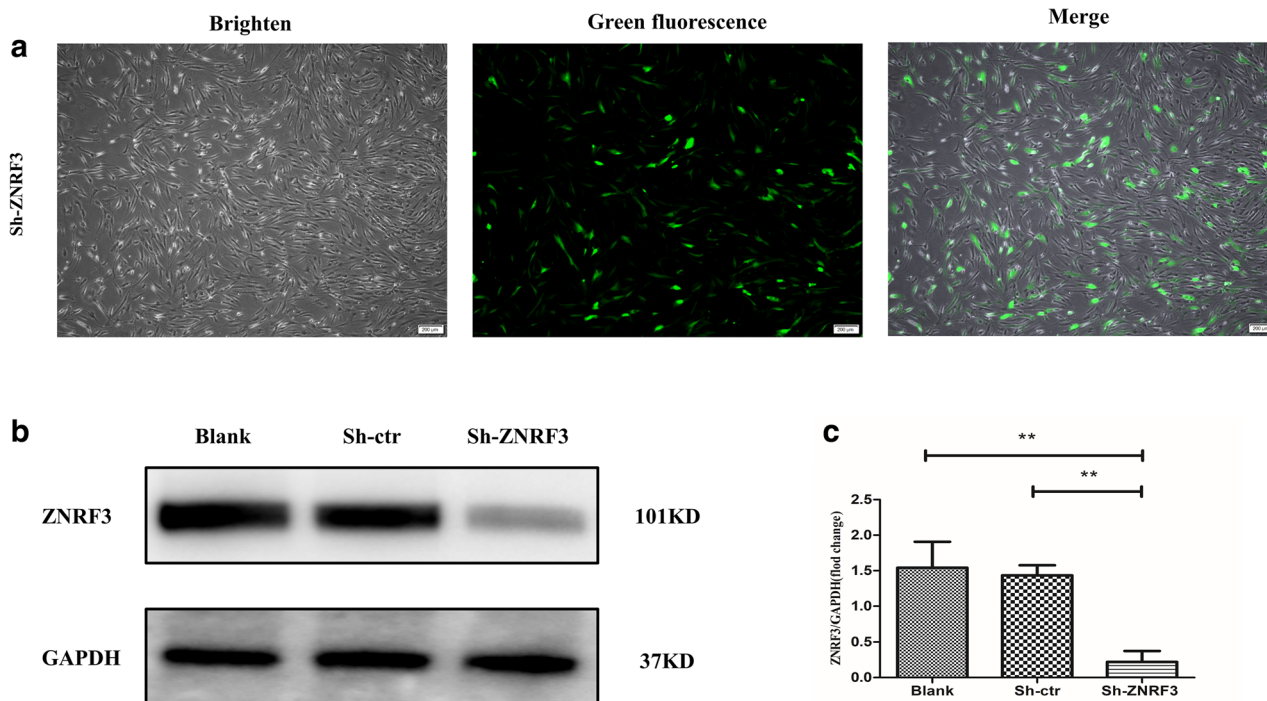


Fig. 2. The results of infection with Sh-ZNRF3. **a** the infection rate of GFP in the RA-FLSs was observed by fluorescence microscope when the MOI was 30. **b** The effects of Sh-ZNRF3 infection on protein expression. **c** Relative ratios of ZNRF3 to GAPDH. (*n* = 3).

lentivirus transduction was examined by the western blot. Results showed that the expression of ZNRF3 was significantly decreased in RA-FLSs compared with the NC group and Sh-ctr group (Fig. 2b,c).

Sh-ZNRF3 Increases Apoptosis and Decreases the Proliferation of RA-FLS

The effect of Sh-ZNRF3 on RA-FLS viability was determined by the CCK-8 assay. The results showed that, compared to the Sh-ctr group, the cell viability of Sh-ZNRF3 group was decreased. This inhibitory effect exists after treatment with 10 ng/ml TNF- α (Fig. 3a). The apoptosis was examined by flow cytometry. It was found that the proportion of apoptotic cells in Sh-ZNRF3 group exceeded that of the V-ctr group (Fig. 3b,c).

TNF- α -Stimulated Sh-ZNRF3 Inhibited the Release of Inflammatory Cytokines from RA-FLSs

The release of inflammatory cytokines from RA-FLSs promotes the development of RA [20]. ELISA and qPCR assays were performed to assess the impact of Sh-

ZNRF3 on the proteins and genes associated with inflammation, respectively. Data analysis revealed that the level of IL-8, IL-1 β , and IL-6 in the TNF- α -stimulated Sh-ZNRF3 cells was lower than those in the TNF- α -stimulated Sh-ctr group (Fig. 3d).

Sh-ZNRF3 Inhibits the Cell Migration of RA-FLSs

The migration of RA-FLSs cells promotes tissue destruction [21]. Moreover, RA-FLS migration and invasion are important characteristics that distinguish it from FLS. The effects of ZNRF3 on cell migration were determined using the wound healing assay (Fig. 4a,b) and the Transwell cell migration assays (Fig. 4c,d), respectively, at two-dimensional and three-dimensional levels. The results showed that cell migration was decreased in Sh-ZNRF3 compared to Sh-ctr group.

Sh-ZNRF3 Alleviates Inflammation and Prevents Knee Joint Destruction in CIA Mice

In this study, a CIA mouse model was developed to explore the therapeutic effect of Sh-ZNRF3 *in vivo*. As

shown in Fig. 5a, the lentivirus (3.19×10^8 IU/mL) successfully infected mice knee joint as evidenced by the high expression of ZNR3 in the synovium (Fig. 5b). Figure 5c shows the typical histopathological lesions in the knee joints. Results revealed that the number of infiltrating inflammatory cells, the level of synovial hyperplasia, pannus formation, and the narrowing of the joint space were smaller in the CIA mice injected with Sh-ZNR3 compared to CIA mice injected with Sh-ctr. Finally, the Multi-Analyte Flow Assay revealed that the serum levels of TNF- α , IL-6, and IL-1 β were significantly decreased in Sh-ZNR3 group compared to the Sh-ctr group. (Fig. 5d).

Sh-ZNR3 Plays a Role in RA-FLSs by Regulating Wnt/ β -Catenin and NF- κ B Pathways

Having found that ZNR3 knockdown eliminates inflammation both *in vivo* and *in vitro*, the impact of Sh-ZNR3 on NF- κ B and Wnt signaling in RA-FLS was examined. It was found that in the Wnt pathway, Sh-ZNR3 upregulated the expression of p-GSK3 β but downregulated the expression of β -catenin and C-myc (Fig. 6a,b). In the NF- κ B pathway, Sh-ZNR3 downregulated the phosphorylated I κ B α , phosphorylated p65, and IKK α protein expression (Fig. 6c,d).

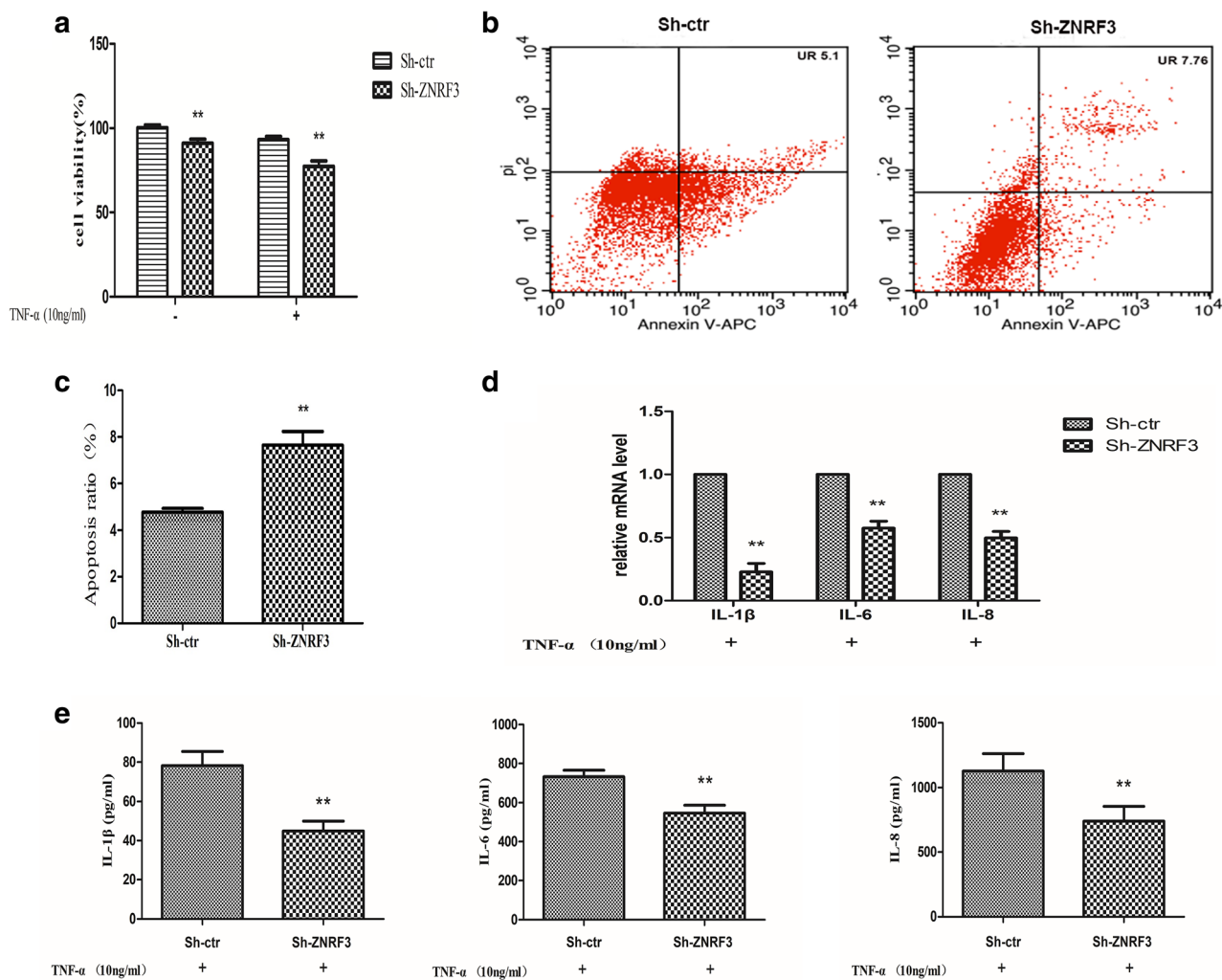


Fig. 3. Impact of Sh-ZNR3 on apoptosis, viability, and inflammation of RA-FLS. **a** The results of CCK-8 assay revealed that the cell viability in Sh-ZNR3 group was lower compared with Sh-ctr group. **b** The typical image of apoptosis in RA-FLSs. **c** Results of apoptosis rate showed that Sh-ZNR3 induced apoptosis in RA-FLSs. The relative mRNA **d** and protein levels **e** of IL-1 β , IL-6 and IL-8. ($n = 3$).

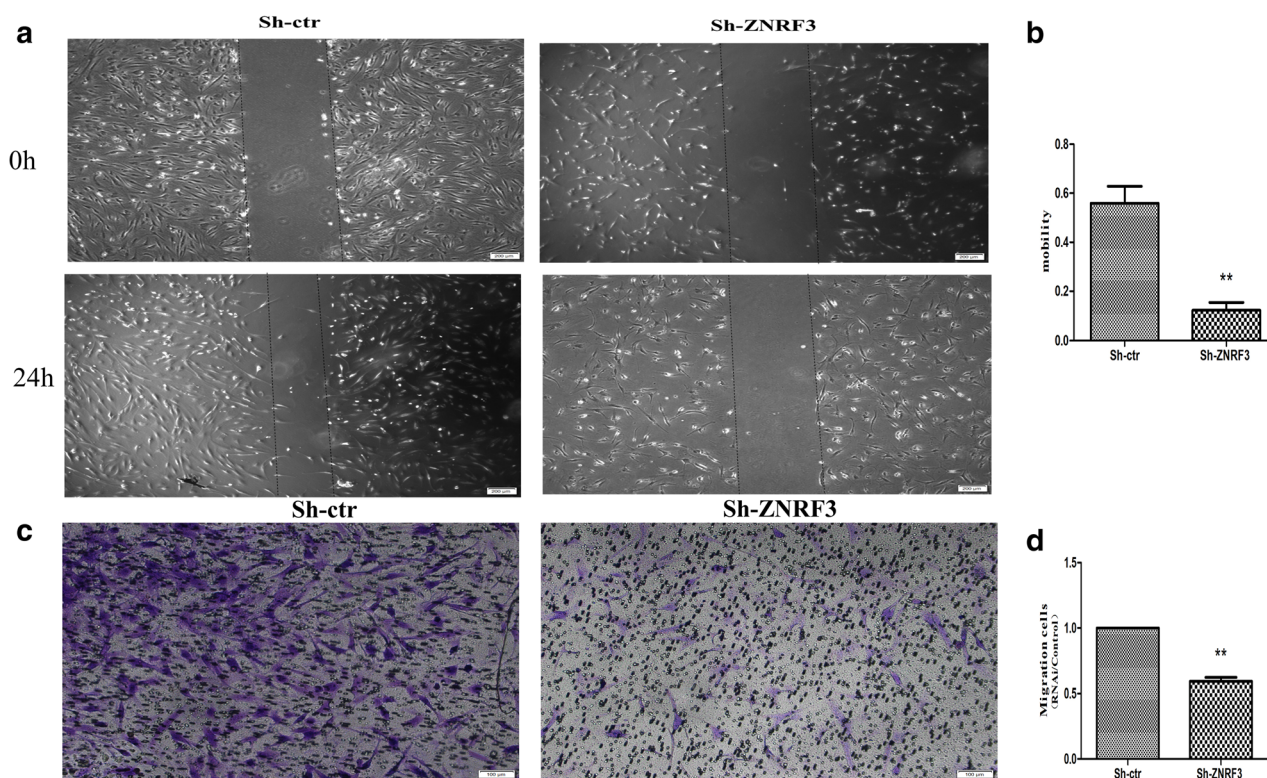


Fig. 4. The effect of Sh-ZNR3 on migration of RA-FLS. **a** The results of wound healing assay. **b** The mobility of RA-FLSs infected by Sh-ZNR3 was decreased. **c** The results of Transwell cell migration test in RA-FLSs. **d** The number of migration cells in Sh-ZNR3 group was lower than Sh-ctr group. ($n = 3$).

DISCUSSION

ZNR3 is a E3 ubiquitin ligase that contains an intracellular RING domain, a transmembrane domain, an extracellular domain, and a signal peptide [22]. ZNR3 and its homolog RNF43 have a sequence similarity of up to 39% [8]. Previous studies have demonstrated that ZNR3 inhibits Wnt/ β -catenin signaling cascade [7, 8]. When the Wnt pathway is activated, it enhances the expression of β -catenin and promotes the transcription of TCF/LEF-1. ZNR3 is released in the cytoplasm where it binds FZD on the plasma membrane, thereby inhibiting Wnt signaling [7, 9].

Wnt signaling pathway is a conserved pathway that modulates the mature tissue homeostasis and embryonic development in all large animals. It is also involved in cell differentiation, migration, adhesion, and proliferation. A number of studies have shown that β -catenin is highly expressed in the

synovial tissues and FLSs of RA patients, implying that Wnt pathways may be abnormally activated in the synovitis of RA [23, 24]. Therefore, we selected ZNR3 as a target gene of this pathway in this study.

Results showed that ZNR3 was highly expressed in the synovial tissues and FLS of RA patient. ZNR3 inhibited the Wnt/ β -catenin pathway, whereas Wnt pathway was overactivated in RA synovitis. Hence, we speculated that the increase in ZNR3 expression occurred *via* a negative feedback mechanism. Unexpectedly, we observed that the knocked down of ZNR3 (Sh-ZNR3) decreased cells proliferation, increased apoptosis, inhibited migration, and alleviated inflammation in RA-FLS, indicating its therapeutic effect on CIA mice. Mechanistically, Sh-ZNR3 increased the expression of p-GSK3 β but decreased the expression of c-Myc and β -catenin. Further tests showed that Sh-ZNR3 inhibited the NF- κ B signaling cascade in RA-FLS.

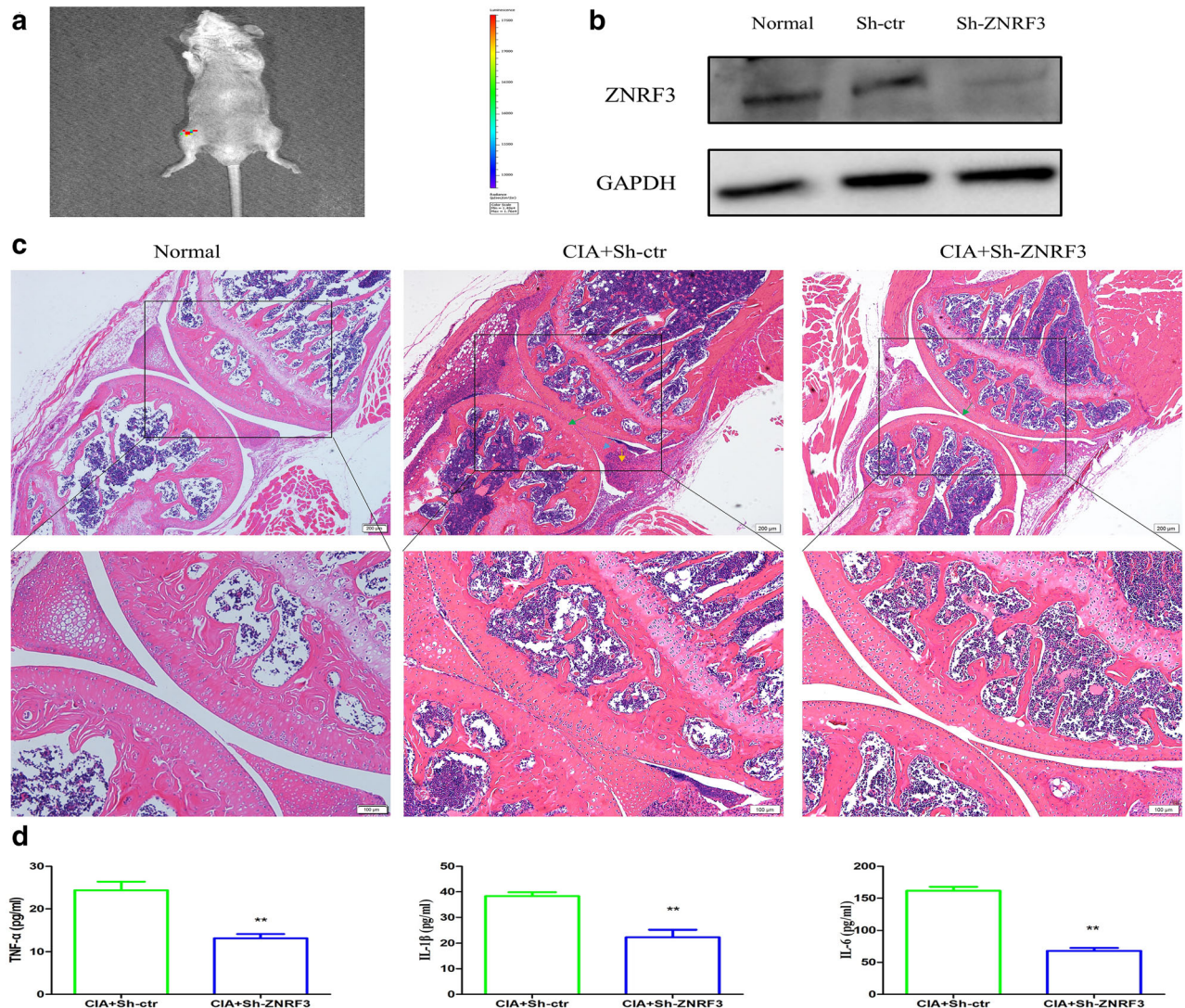


Fig. 5. The effect of Sh-ZNRF3 on CIA mice. **a** The expression of Sh-ZNRF3 in the knee joint *in vivo* imaging (right knee injected with Sh-ZNRF3, left knee injected with PBS). **b** The expression of ZNRF3 in the knee joint detected by WB. **c** The typical H&E images of knee joint (yellow arrow stands for inflammatory cell infiltration, green arrow stands for joint space, and blue arrow for synovial hyperplasia and pannus). **d** The protein levels of IL-1 β , IL-6, and IL-8 in the serum were tested by Multi-Analyte Flow Assay Kit. ($n = 3$).

NF- κ B plays a role in inflammation, proliferation, and apoptosis of RA and is stimulated by TNF- α [25, 26]. Suppressing the activation of NF- κ B alleviates inflammation both in FLSs and animal models of RA [13, 16, 27]. Several studies have demonstrated that there is a crosstalk between the Wnt/ β -catenin and the NF- κ B pathway [28]. NF- κ B signal pathway activates Wnt/ β -catenin pathway by fine-tuning the expression

of genes related to this pathway and the activity of IKK- α [29–31]. IKK- α increases the turnover of β -catenin [29] and up-regulates the expression of beta-catenin/TCF [30]. Here, we speculated that in RA-FLSs, Sh-ZNRF3 promoted the ubiquitination mediated β -catenin breakdown by suppressing IKK α and down-regulating the expression of β -catenin and its target genes. In addition, Sh-ZNRF3 inhibited the

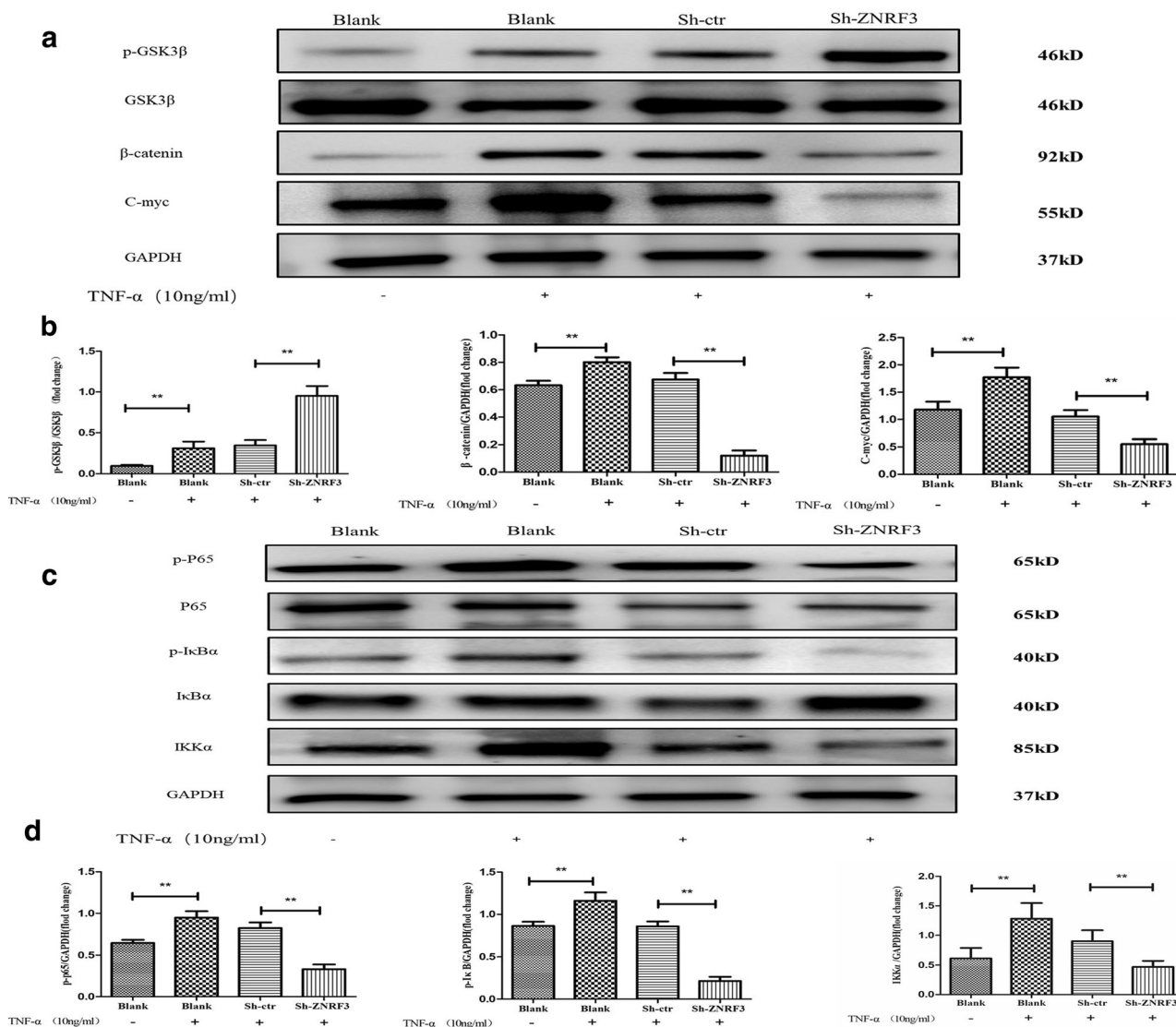


Fig. 6. Effects of Sh-ZNRF3 on Wnt and NF-κB pathways activation in RA-FLS. All the cells were treated in the presence or absence of 10 ng/mL TNF-α for 24 h before Western blotting. **a** The protein level of β-catenin, GAPDH, and C-myc. **b** Quantitative data of the protein levels of GAPDH, β-catenin, and C-myc. **c** The protein expression of phospho-p65, phospho-IκBα, IκBα, GAPDH, p65, and IKKα. **d** the protein levels of phospho-p65, phospho-IκBα, and IKKα (n = 3).

degradation of phosphorylated IκB and p65 by inhibiting the activity of IKK-α, leading to the suppression of NF-κB signaling. This may explain the protective role of Sh-ZNRF3 observed *in vivo* and *in vitro*.

Taken together, this study shows that ZNRF3 protects against RA by regulating the crosstalk between Wnt/ β-catenin and NF-κB signaling pathways. Therefore, Sh-ZNRF3 might be a potential therapeutic target for RA

patients. Other unidentified mechanisms that orchestrate the regulatory role of ZNRF3 on RA remain to be elucidated in future studies.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing and Conflicting Interests. The authors declare that there is no conflict of interest associated with this work.

Disclosures.

None
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Changhai hospital+ No. CHEC2017159).

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