RESEARCH ARTICLE

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hsa_circRNA_092488 Exacerbates the Progression of Deep Vein Thrombosis Through the NLRP3/NF-κB Signaling Pathway

hsa_circRNA_092488, NLRP3/NF-κB Sinyal Yolu Aracılığı ile Derin Ven Trombozunun İlerlemesini Şiddetlendirir

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Abstract

Objective: Deep vein thrombosis (DVT) is a vascular disorder with an incidence rate of about 0.1%. Endothelial progenitor cells (EPCs) are precursor cells of endothelial cells and contribute to vascular repair and regeneration. Circular RNA (circRNA) has become a new focus of research as circRNAs are involved in various biological processes including the progression of DVT. This study explored the upregulation of hsa_circRNA_092488 in DVT patients.

Materials and Methods: The expression of hsa_circRNA_092488 was evaluated in venous blood samples obtained from DVT patients (n=42) and healthy controls (n=42). Gain- and loss-of-function studies of hsa_circRNA_092488 were carried out. The expression levels of related RNAs and proteins were examined by quantitative real-time reverse-transcription polymerase chain reaction, western blotting and immunofluorescence assays. The proliferation, migration, cell cycle progression, and apoptosis of transfected cells were measured by CCK-8 assay, transwell assay, and flow cytometry. The association of hsa_circRNA_092488 and NOD-like receptor protein 3 (NLRP3) in EPCs was revealed using RNA pull-down analysis. Furthermore, the stability of NLRP3 mRNA was examined in transfected EPCs.

Results: Upregulation of hsa_circRNA_092488 was detected in blood samples from DVT patients and it had the ability to suppress the proliferation and migration of EPCs, induce cell cycle arrest from the S to the G0/G1 phase, and trigger cellular apoptosis. Furthermore, NLRP3 was identified as the potential downstream target molecule of hsa_circRNA_092488 and it could exert its regulatory functions by activating the NLRP3/nuclear factor (NF)-κB signaling pathway. Overexpression of hsa_circRNA_092488 in cells notably elevated the protein expression of caspase-1, interleukin-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα, while knockdown of hsa_circRNA_092488 significantly reduced the levels of those proteins in EPCs.

Conclusion: hsa_circRNA_092488/NLRP3/NF-κB signaling could be a novel therapeutic candidate for the treatment of DVT.

Keywords: hsa_circRNA_092488, NLRP3, NF-κB, Deep vein thrombosis

Öz

Amaç: Derin ven trombozu (DVT), yaklaşık %0,1'lik bir insidans oranına sahip bir vasküler hastalıktır. Endoteliyal progenitor hücreler (EPC), endoteliyal hücrelerin öncü hücreleridir ve vasküler onarım ve rejenerasyona katkıda bulunurlar. Dairesel RNA (circRNA), DVT'nin ilerlemesi de dahil olmak üzere çeşitli biyolojik süreçlerde yer alması nedeniyle yeni bir araştırma odağı haline gelmiştir. Bu çalışmada, DVT hastalarında hsa_circRNA_092488'in yukarı düzenlenmesi araştırıldı.

Gereç ve Yöntemler: hsa_circRNA_092488 ekspresyonu DVT hastalarından (n=42) ve sağlıklı kontrollerden (n=42) alınan venöz kan örneklerinde değerlendirildi. hsa_circRNA_092488'in fonksiyon kazanımı ve kaybı çalışmaları yapıldı. İlgili RNA ve proteinlerin ekspresyon düzeyleri kantitatif gerçek zamanlı ters transkripsiyon polimeraz zincir reaksiyonu, western blotting ve immünofloresan analizleri ile incelendi. Transfekte hücrelerin proliferasyonu, göçü, hücre döngüsü ilerlemesi ve apoptozu CCK-8 analizi, transwell analizi ve akış sitometrisi ile ölçüldü. hsa_circRNA_092488 ve NOD benzeri reseptör protein 3'ün (NLRP3) EPC'lerdeki ilişkisi RNA çekim analizi kullanılarak ortaya kondu. Ayrıca, NLRP3 mRNA'nın stabilitesi transfekte EPC'lerde incelendi.

Bulgular: DVT hastalarının kan örneklerinde hsa_circRNA_092488'in yukarı yönlü düzenlenmesi tespit edildi ve bu proteinin EPC'lerin çoğalmasını ve göçünü baskılama, hücre döngüsünün S'den G0/G1 fazına kadar durmasını sağlama ve hücresel apoptozu tetikleme yeteneği olduğu görüldü. Ayrıca, NLRP3, hsa_circRNA_092488'in potansiyel alt akış hedef molekülü olduğu ve düzenleyici işlevlerini NLRP3/nükleer faktör (NF)-κB sinyal yolunu aktive ederek uygulayabileceği tanımlandı. Hücrelerde hsa_circRNA_092488'in aşırı ekspresyonu, kaspaz-1, interlökin-1β, P-NF-κB-p65/NF-κB-p65 ve P-IκBα/IκBα'nın protein ekspresyonunu belirgin şekilde yükseltirken, hsa_circRNA_092488'in baskılanması, bu proteinlerin EPC'lerdeki seviyelerini önemli ölçüde azalttı.

Sonuç: hsa_circRNA_092488/NLRP3/NF-κB sinyal iletimi DVT tedavisinde yeni bir tedavi adayı olabilir.

Anahtar Sözcükler: hsa_circRNA_092488, NLRP3, NF-κB, Derin ven trombozu

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Introduction

Deep vein thrombosis (DVT) is a multifactorial disorder that may be triggered by numerous risk factors including pregnancy, immobility, and thrombophilia [1]. The risk factors for DVT particularly include elements that favor the formation of thrombi, including venous stasis, vascular injury, and hypercoagulability [2]. However, the detailed mechanisms underlying the onset and progression of DVT are not fully understood. It has been reported that alterations in venous blood flow, endothelial activation, adhesion of platelets and leukocytes, and activation of coagulation play essential roles in the pathogenesis of DVT [1,2]. The diagnosis is based on a high degree of suspicion considering the risk factor history, D-dimer measurements, and ultrasound scans showing the presence of deep vein clots [1]. Other relevant tests include pulmonary artery computed tomography scans and ventilation-perfusion scans when pulmonary embolism is suspected [1,2].

Endothelial progenitor cells (EPCs) are precursor cells of endothelial cells and contribute to vascular repair and regeneration [3]. Under physiological or pathological stimulation, they can be mobilized from the bone marrow into the peripheral blood to participate in the repair of damaged blood vessels [4]. Studies have shown that EPCs play important roles in cardiovascular and cerebrovascular diseases, peripheral vascular diseases, tumor angiogenesis, and wound healing [5].

Circular RNAs (circRNAs) are associated with vascular dysfunction in several vascular disorders and could be used as disease biomarkers [6]. They constitute a group of essential post-transcriptional regulators and they are formed by the head-to-tail splicing of exons, reflecting the unrecognized regulatory potential of coding sequences [6]. For instance, circ_0020123 and hsa_circ_0001020 may affect EPC migration, invasion, and tube formation during the development of DVT [7,8]. The knockdown of circ_0020123 enhanced the proliferation and angiogenesis of human umbilical vein endothelial cells in vitro and it was considered as a putative biomarker for the onset of DVT in pregnant women [7]. In addition, the upregulation of hsa_circ_0001020 was observed in both DVT patients and a mouse model, and it suppressed the migration and invasion of EPCs as well as tube formation by regulating the miR-29c-3p/MDM2 signaling pathway [8]. The knockdown of hsa_circ_0001020 in mice with DVT inhibited thrombosis and enhanced the homing ability of EPCs into thrombi [8], while the upregulation of hsa_circRNA_092488 was observed in DVT and its involvement in inflammation was also reported [9,10].

In this study, we explore the upregulation of hsa_circRNA_092488 in patients with DVT and its ability to inhibit the proliferation and migration of EPCs, cause a shift in the cell cycle from the S to the G0/G1 stage, and induce apoptosis. In addition, the ability of hsa_circRNA_092488 to exert its regulatory functions via the NOD-like receptor protein 3 (NLRP3)/nuclear factor (NF)-κB pathway was evaluated.

Materials and Methods

Characterization of CircRNAs

For reverse transcription, oligo-dT and random primers were mixed with isolated RNA and then the levels of circular and linear RNAs were evaluated. The incubation of total RNA (~2 μg) and RNase R (3 U/μg; Epicentre Technologies, Madison, WI, USA) was carried out at 37 °C for 30 min. After treatment with RNase R, RNA expression was measured using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).

Patient Specimens and Cell Cultures

The expression of hsa_circRNA_092488 was evaluated in venous blood samples obtained from patients with DVT (n=42) and healthy individuals (n=42) who had presented to the First Affiliated Hospital of Jinzhou Medical University between June 2018 and May 2020. Venous blood samples were collected from the median cubital vein after 6 h of fasting immediately after diagnosis. The median value of hsa_circRNA_092488 expression was used as the cutoff. Samples with values below the median were classified as "low" and those above the median were classified as "high." Demographic factors including sex, age, history of smoking, and obesity were assessed and no significant differences in these factors were observed between the DVT patients with low-level and high-level hsa_circRNA_092488. The clinical features of the analyzed patients are summarized in Table 1. This study included patients who were diagnosed with DVT using color Doppler ultrasound and lower extremity angiography. Individuals with a history of diabetes mellitus, hypertension, other chronic diseases, and recent immobilization or surgery were excluded, as were those using anticoagulants or platelet inhibitors, undergoing hormone therapy, or having concurrent tumors. All procedures were performed in line with the Declaration of Helsinki and the protocols were reviewed by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University (decision no: 202047). Informed consent was obtained from all analyzed patients and controls.

To isolate mononuclear cells (MNCs), circulating blood (~100 mL) was obtained from the patients with DVT and the healthy controls using Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and samples were kept in the dark before being processed. The isolation of MNCs was performed at 5003 x g for 30 min using a density-gradient centrifuge (Biocoll, Biochrom, Berlin, Germany). Samples were then rinsed with phosphate-buffered saline (PBS) three times. Isolated cells were inoculated on cell culture dishes precoated with commercial fibronectin (Sigma, Darmstadt, Germany) and cultured using EGM growth medium for endothelial cells (GE Healthcare Life

Table 1. Clinical features of analyzed patients.

Parameter	Number of patients	Low hsa_circRNA_092488 expression (n=21)	High hsa_circRNA_092488 expression (n=21)	p
Age, years				
≤40	22	12	10	0.54
>40	20	9	11	
Sex				
Female	21	11	10	0.76
Male	21	10	11	
Smoking				
Yes	19	8	11	0.35
No	23	13	10	
BMI (kg/m^2)		24.62±6.17	25.24±6.35	0.75

BMI: Body mass index.

Sciences, Chicago, IL, USA), which contains human epidermal growth factor (10 ng/mL), bovine brain extract (12 mg/mL), hydrocortisone (1 mg/mL), human insulin-like growth factor-1 (50 ng/mL), and penicillin/streptomycin (all from Gibco, Grand Island, NY, USA). In addition, heparin (10 U/mL) was added to prevent platelet coagulation, and cells were incubated at 37 °C with 5% CO_2. EPC colonies were visible after about 2 weeks.

Transfection

The annealing of small hairpin RNA segments was carried out and then the plasmids were cloned into the pU6-Luc-Puro lentivirus vector (Genepharm Co. Ltd., Pallini, Greece). To produce a cell model with the overexpression of hsa_circRNA_092488, WT (oe- hsa_circRNA_092488) and MUT (oe-NC) fragments were amplified by PCR and then the segments were inserted into the PLCDH-cir expression vector (Invitrogen, Waltham, MA, USA). To verify the transfection efficiencies, down- or upregulation of hsa_circRNA_092488 was confirmed using qRT-PCR. Briefly, cells were seeded onto a 24-well plate and 1 μg of plasmids were used per well for each transfection.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

The extraction of total RNA was performed using TRIzol reagent (Sobao Biotechnology, Shanghai, China). Subsequently, reverse transcription was carried out using the PrimeScript RT Reagent Kit (Invitrogen, Shanghai, China), and the SYBR Green PCR reagent was used for qRT-PCR on the 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA). Paired primers were produced by Xinbei Biotechnology (Shanghai, China) as follows: hsa_circRNA_092488, forward 5′-GCAGGTTGTTCATCGGGCATTC-3′, reverse 5′-GGCTTACAGC-ACGGAAGTGTTTC-3′; Ki-67, 5′-AGCGGCTCTCTTTAACACAGT-3′, 5′-TGACCCCCAAAGGATACACG-3′; NLRP3, 5′-GGACTGAAGCA-CCTGTTGTGCA-3′, 5′-TCCTGAGTCTCCCAAGGCATTC-3′; GAPDH, 5′-GTCTCCTCTGACTTCAACAGCG-3′, 5′-ACCACCCTGTTGCTGT-AGCCAA-3′; U6, 5′-CTCGCTTCGGCAGCACATA-3′, 5′-AACGATTCA-CGAATTTGCGT-3′. Endogenous U6 or GAPDH was used as the control for the normalization of the expression of miRNA or mRNA. For mRNA expression analysis, untreated RNA samples were used. For circRNA expression analysis, RNA samples treated with RNase R were used to ensure the removal of linear RNA.

Western Blotting

Concentrations of extracted protein were determined using a BCA Kit (Dingguo Biotechnology, Guangzhou, China). The separation of proteins (~40 μg) was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, samples were transferred to a PVDF membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membranes were then blocked with 5% skimmed milk at room temperature for 1 h and then incubated using primary antibodies against NLRP3 (1:1000; Cat No. ab263899; Abcam, Cambridge, UK), caspase-1 (1:500; Cat No. ab207802; Abcam), IL-1β (1:500; Cat No. ab283818; Abcam), p-NF-κB-p65 (1:500; Cat No. 3033; Cell Signaling Technology, Danvers, MA, USA), NF-κB-p65 (1:500; Cat No. 3034; Cell Signaling Technology), P-IκBα (1:1000; Cat No. 2859; Cell Signaling Technology), IκBα (1:1000; Cat No. 4818; Cell Signaling Technology), and GAPDH (1:2000; Cat No. 4967; Cell Signaling Technology) in a cold room overnight. The next day, incubation was performed using anti-mouse IgG (1:2000; Cat No. ab6728; Abcam) or anti-rabbit IgG (1:2000; Cat No. ab6721; Abcam) for 1 h.

CCK-8 Assay

Cell proliferation under various transfection conditions was measured by CCK-8 assay. Transfected cells were inoculated using 96-well plates and then 10 μL of the CCK-8 mixture (Dojindo, Kumamoto, Japan) was added to the cells. Cell proliferation was measured at different time points (days 1, 2, 3, and 4). Further

incubation of the cells was then carried out for an additional 2 h and the absorbance at 450 nm was recorded by microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Transwell Experiment

A transwell kit (ChenGong Biotechnology, Shanghai, China) was used to evaluate cell migration. A cell suspension (~5x10^5 cells) was added to the upper chamber (pore size: 8 µm; Becton Dickinson), which was precoated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 500 µL of culture medium supplemented with 10% fetal bovine serum was loaded in the lower chamber. After 48 h, non-migratory cells were discarded using a cotton bud. Fixation of the cells that remained in the lower chamber was carried out using ice-cold methanol for 15 min. Cells were then stained using 0.5% crystal violet solution.

Cell Cycle Analysis and Measurement of Apoptosis

Cells were seeded at a density of 5x10^5 cells/well on 6-well plates. Subsequently, they were spun down at low speed (1000 rpm) at 4 °C for 5 min. The obtained pellets were then washed three times and suspended using PBS, and then fixation of the cells was performed using 70% ice-cold ethanol and samples were stored at 4 °C for 48 h. Lysis of the cells was then performed before flow cytometry and the cells were centrifugated and suspended using PI staining solution with 50 µL/mL PI and 250 µL/mL RNase A (Sigma-Aldrich). The distribution of cell cycle phases was evaluated using a flow cytometry device (Becton Dickinson) and the data were analyzed using FlowJo 7.6 software (FlowJo LLC, Becton Dickinson). For cell apoptosis, the cell suspension was incubated at 4 °C in the dark for 30 min and staining was performed using 5 µL of annexin V-FITC (JingMei Biotech, Beijing, China).

RNA Pulldown Assay

The probes of NLRP3 and the negative control, which were labeled with biotin, were generated by GenePharma (Shanghai, China). The labeling of cell lysates was carried out with Dynabeads M-280 Streptavidin (Thermo Fisher Scientific). Beads conjugated with immobilized NLRP3 were treated using ethylenediaminetetraacetic acid (10 mM).

Assessment of mRNA Stability

To prevent the additional synthesis of RNA, treatment with actinomycin D (5 µg/mL; MedChemExpress, Monmouth Junction, NJ, USA) was applied to the cells. Subsequently, treatment with actinomycin D was carried out at different time points. The extraction of RNA was then performed and samples were subsequently used for qRT-PCR. Remaining levels of NLRP3 RNA at different time points were normalized to the level at the start of the treatment.

Immunofluorescence Analysis

The fixation of cells was conducted with prechilled acetone (Sigma-Aldrich) for 20 min. Subsequently, fixed cells were washed using PBS and further incubated in blocking buffer for 30 min. Incubation with primary anti-Ki67 antibody (1:500; Cat. No. ab15580; Abcam) was then carried out at 4 °C overnight. The following day, samples were washed three times and incubation of the cells was carried out for 60 min with a secondary antibody conjugated with Alexa-Fluor 568 (1:1000, Molecular Probes, Eugene, OR, USA). Subsequently, nuclei were stained with DAPI solution and the stained cells were washed three times and mounted on slides using 10% Mowiol D488 reagent (Calbiochem, Nottingham, UK).

Statistical Analysis

Data obtained in the experiments of this study are shown as mean ± standard error of the mean. Data were analyzed using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). Statistical significance was determined with Student's t-test or one-way analysis of variance (ANOVA). The post-hoc Tukey test was conducted after ANOVA. The Pearson correlation test was used to examine correlations of relative gene expression. The chi-square test was used to assess significant differences between the low- and high-hsa_circRNA_092488 expression groups. The measurement data conformed to normal distribution.

Results

CircRNA Characterization in Endothelial Progenitor Cells

For the characterization of circRNAs in EPCs, the levels of linear RNAs were measured following the addition of oligo-dT primer, whereas circRNAs were not observed (Figure 1A). Furthermore, linear RNAs were sensitive to digestion induced by RNase R but not circRNAs (Figure 1B).

Upregulation of hsa_circRNA_092488 Revealed in Samples from Patients with Deep Vein Thrombosis

Levels of hsa_circRNA_092488 were remarkably elevated in patients with DVT compared to the healthy controls (Figure 1C). To explore the roles of hsa_circRNA_092488 upregulation in the progression of DVT, function experiments were carried out. Cell models with hsa_circRNA_092488 overexpression and knockdown were respectively generated (Figures 1D and 1E).

Involvement of hsa_circRNA_092488 in Biological Behavioral Changes of Endothelial Progenitor Cells

The results of the CCK-8 assay revealed that overexpression of hsa_circRNA_092488 significantly inhibited the proliferation of EPCs (Figure 2A), while knockdown of hsa_circRNA_092488 remarkably promoted the proliferative activity of the cells (Figure 2B). In addition, levels of Ki-67 were decreased in EPCs with

hsa_circRNA_092488 overexpression (Figures 2C and 2D) and were enhanced in cells treated with sh-hsa_circRNA_092488 (Figures 2E and 2F).

Furthermore, the migration of EPCs was suppressed by the overexpression of hsa_circRNA_092488 (Figures 3A and 3B), while cell migration was elevated after transfection with sh-hsa_circRNA_092488 (Figures 3C and 3D). The results of cell cycle phase distribution suggested the shift of EPCs from the S to the G0/G1 phase following treatment with oe-hsa_circRNA_092488 (Figures 3E and 3F). In contrast, the proportion of cells at the G0/G1 phase was reduced and the proportion at the S phase was elevated after the knockdown of hsa_circRNA_092488 (Figures 3G and 3H).

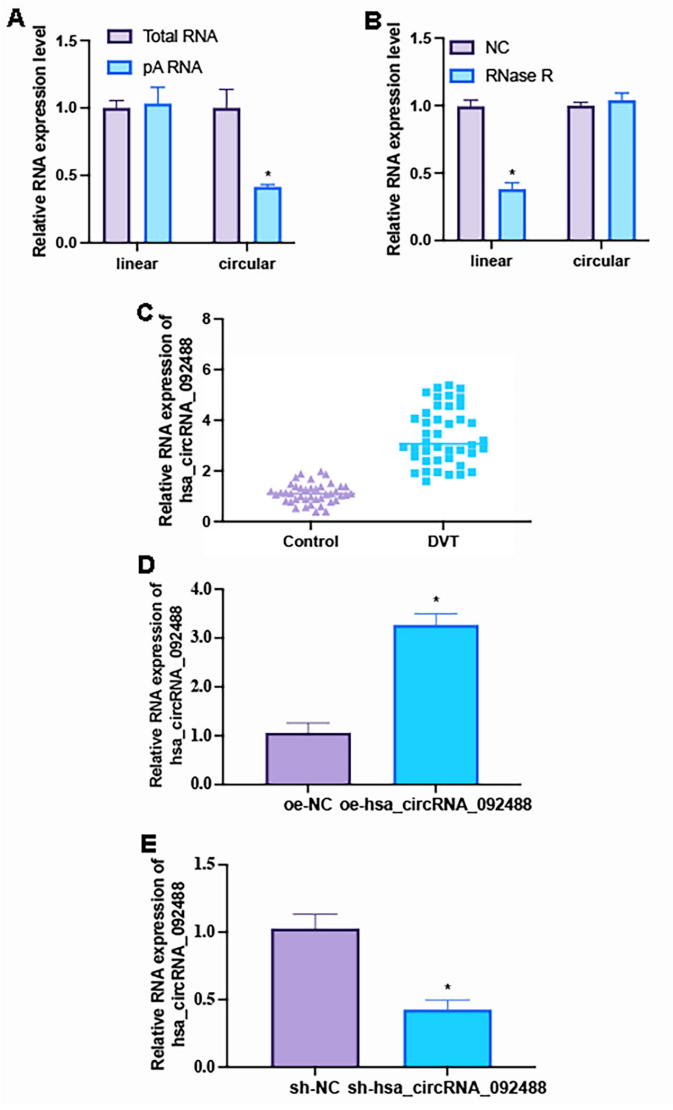

Figure 1. Characterization of circular RNAs (circRNAs) in endothelial progenitor cells and upregulated hsa_circRNA_092488 in deep vein thrombosis. A) Linear RNAs were observed after adding oligo-dT primer while there was no expression of circRNAs. B) The results revealed the resistance of circRNAs to digestion induced by RNase R but not linear RNAs. C) hsa_circRNA_092488 was upregulated in cases of deep vein thrombosis compared to the healthy control group. D, E) Cells with hsa_circRNA_092488 overexpression and knockdown were produced using lentiviral vectors.

*: p<0.05; NC: negative control; DVT: deep vein thrombosis.

Flow cytometry revealed that hsa_circRNA_092488 overexpression triggered cell apoptosis in EPCs (Figures 4A and 4B). In contrast, the apoptosis rate was decreased after transfection of sh-hsa_circRNA_092488 (Figures 4C and 4D).

NLRP3 as the Novel Target of hsa_circRNA_092488

The association of hsa_circRNA_092488 and NLRP3 in EPCs was revealed using RNA pull-down analysis (Figure 5A). The stability of NLRP3 mRNA in EPCs was found to be enhanced after transfection with oe-hsa_circRNA_092488 (Figure 5B). In contrast, the mRNA stability was decreased in EPCs with hsa_circRNA_092488 knockdown (Figure 5C). In addition, NLRP3 expression was enhanced in samples obtained from patients with DVT (Figure 5D), and the levels of hsa_circRNA_092488 and NLRP3 were positively correlated in those samples (Figure 5E; $r=0.602$, $p<0.05$). Western blotting demonstrated that NLRP3 protein levels were increased in EPCs treated with oe-hsa_circRNA_092488 and reduced in cells transfected with sh-hsa_circRNA_092488 (Figures 5F and 5G).

Functioning of hsa_circRNA_092488 Through the NLRP3/NF-κB Pathway

The protein expression of NLRP3/NF-κB-associated molecules was examined (Figure 6). The results of western blotting showed that overexpression of hsa_circRNA_092488 in cells notably elevated the protein expression levels of caspase-1, IL-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα, while the knockdown of hsa_circRNA_092488 significantly reduced the levels of these proteins in EPCs.

Discussion

In previous studies, circRNAs were shown to bind to miRNA to regulate the expression of target genes; thus, they have become a new focus of research as they are involved in various biological processes [6]. For example, circ_0020123 and hsa_circ_0001020 modulated EPC migration and tube formation and regulated the progression of DVT [7,8]. Upregulated hsa_circRNA_092488 was detected in DVT and its involvement in inflammation was reported [9,10]. In the present study, the biological functions of hsa_circRNA_092488 in DVT were explored and the underlying mechanisms were evaluated.

In our experiments, upregulated hsa_circRNA_092488 was confirmed in DVT patients and it inhibited EPC proliferation and migration, triggered the shift of the cell cycle from the S phase to the G0/G1 phase, and induced cell apoptosis. Similarly, upregulation of hsa_circ_0001020 accelerated the

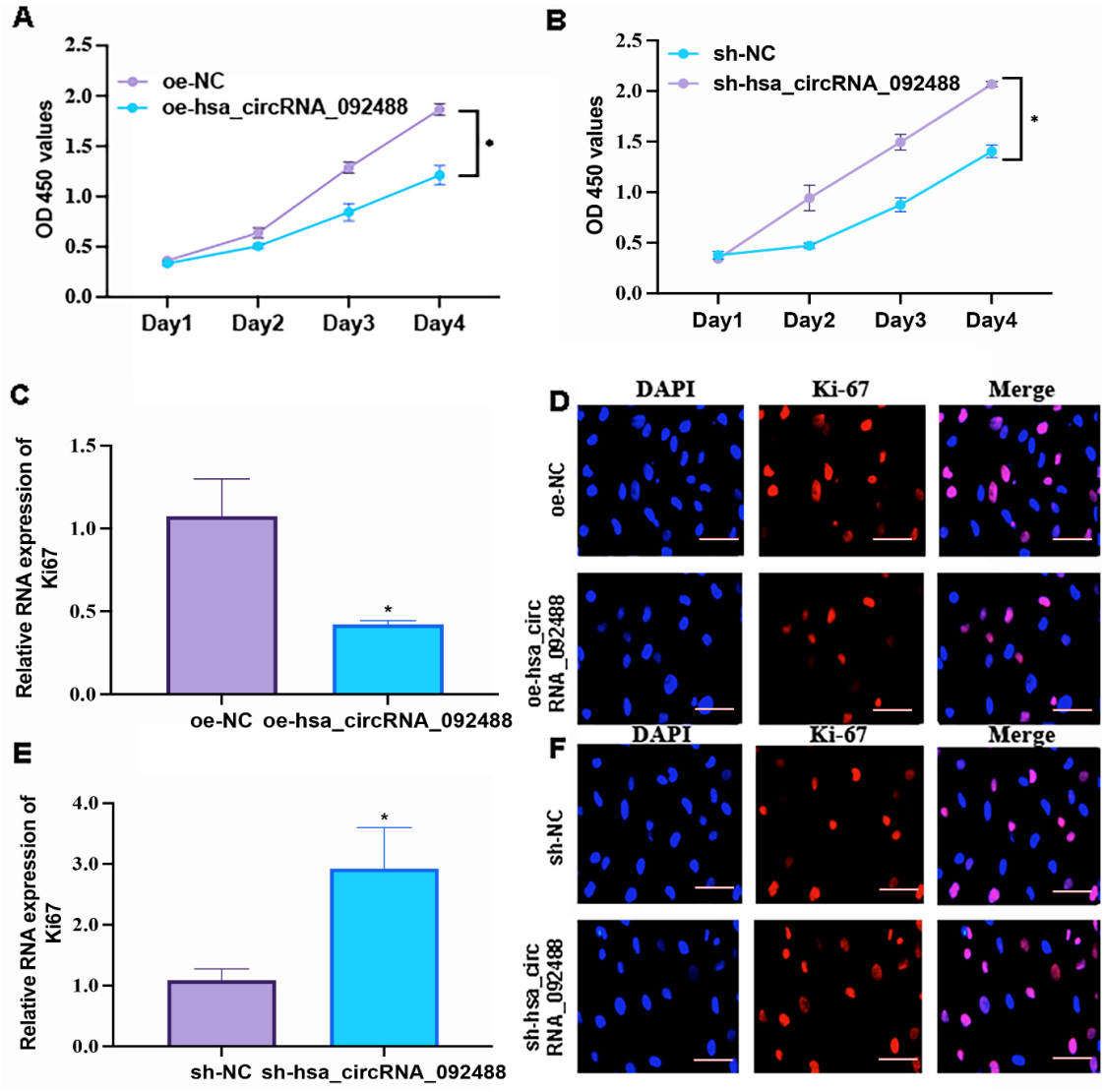

Figure 2. hsa_circRNA_092488 participated in the regulation of biological behaviors of endothelial progenitor cells (EPCs). A) Overexpression of hsa_circRNA_092488 notably downregulated the proliferative activity of EPCs. B) Knockdown of hsa_circRNA_092488 enhanced cell proliferation. C-F) Subcellular staining with Ki-67 was also evaluated in EPCs following treatment with oe-hsa_circRNA_092488 or sh-hsa_circRNA_092488.

*: p<0.05; magnification for (D): 40×; scale bar: 50 μm.

development of DVT by sponging miR-29c-3p to promote the expression of MDM2 [8]. In addition, the long non-coding RNA CRNDE promoted DVT by sequestering miR-181a-5p away from thrombogenic Pcyox1l [11]. In that study, both CRNDE and Pcyox1l levels were enhanced in the blood of mice with DVT and miR-181a-5p was the novel target of CRNDE. Furthermore, the knockdown of CRNDE and restoration of miR-181a-5p both inhibited inflammatory injury, therefore suppressing the formation of thrombi in the mice [11].

NLRP3 has been explored as the putative target of hsa_circRNA_092488. NLRP3 is a protein encoded by the *NLRP3* gene located on the long arm of chromosome 1 [12]. NLRP3 is abundantly detected in macrophages and is a component of inflammasomes. Research has indicated that various inflammatory diseases are associated with NLRP3 inflammasomes [12]. NLRP3 inflammasomes could be activated by a variety of stimuli, such as mitochondrial dysfunction and elevated levels of reactive oxygen species [13]. Dysregulated activation of NLRP3 inflammasomes has been implicated in numerous disorders including diabetes, atherosclerosis, and Alzheimer disease. Small-molecule inhibitors targeting NLRP3 inflammasomes have shown certain therapeutic potential but their clinical feasibility still requires further exploration. Once activated, NLRP3, along with the adaptor protein ASC and the effector protein caspase-1, initiates the assembly

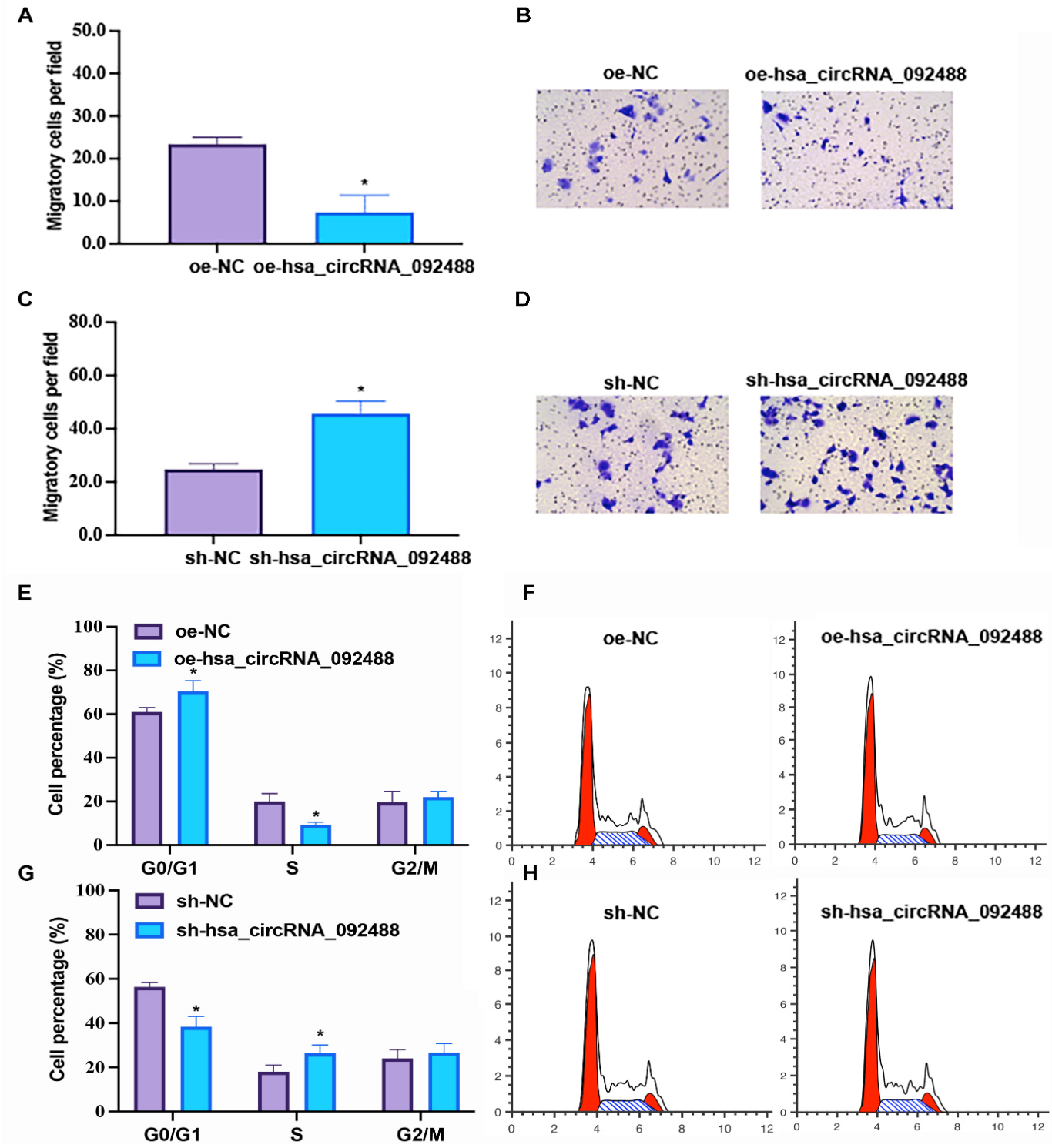

Figure 3. The migration and the cell cycle phase distribution of endothelial progenitor cells (EPCs) were affected by hsa_circRNA_092488. A-D) The migrative ability of EPCs was evaluated following treatment with oe-hsa_circRNA_092488 or sh-hsa_circRNA_092488. E-H) Cell cycle phases of EPCs were also evaluated after transfection with oe-hsa_circRNA_092488 or sh-hsa_circRNA_092488.

*: $p<0.05$.

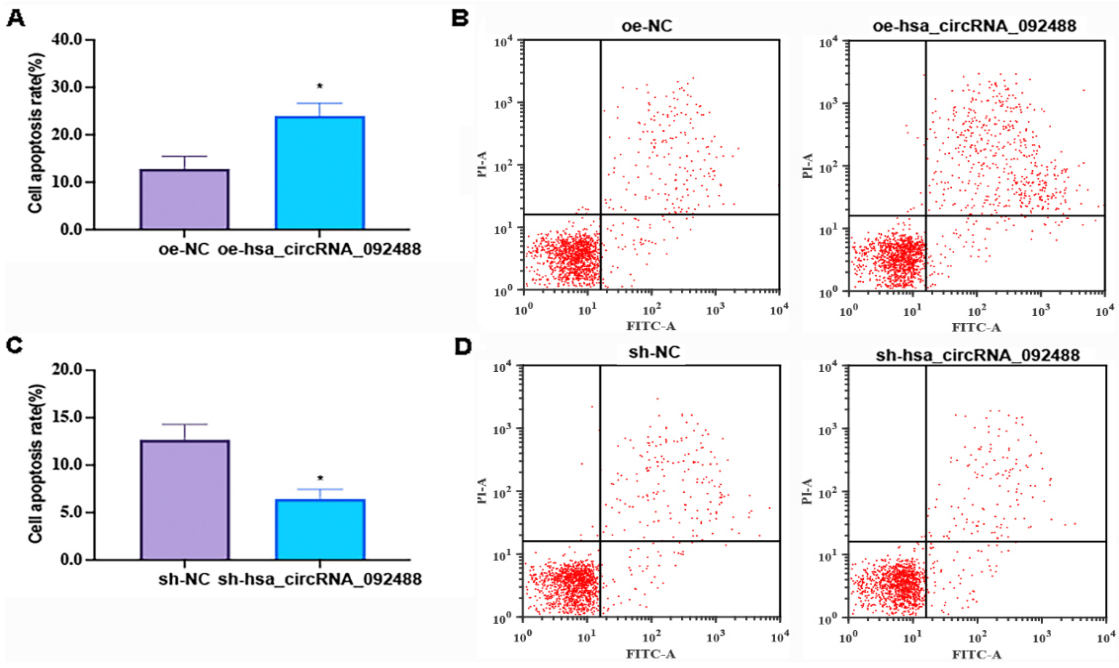

Figure 4. Cell apoptosis was triggered by upregulated hsa_circRNA_092488. A, B) The rate of cell apoptosis was elevated in endothelial progenitor cells transfected with oe-hsa_circRNA_092488. C, D) Apoptosis was inhibited by sh-hsa_circRNA_092488.

*: $p<0.05$.

of inflammasomes, leading to the activation of caspase-1 and subsequent cleavage of interleukin (IL)-1β and IL-18. Aberrant activation of NLRP3 inflammasomes is associated with the pathogenesis of various inflammatory diseases [13]. Upon activation, NLRP3 inflammasomes could promote the activation of NF-κB and the production of cytokines in inflammatory diseases [14,15]. In a previous study, the knockdown of NLRP3 inhibited the activation of NF-κB and secretion of cytokines in both microbially induced and sterile inflammation. NLRP3 not only triggered the activation of caspase-1 post-translationally but also promoted the expression of cytokines in the innate immune system [15]. In line with those findings, our results suggest that hsa_circRNA_092488 can exert its regulatory functions by activating the NLRP3/NF-κB signaling pathway, as overexpression of hsa_circRNA_092488 enhanced the protein levels of caspase-1, IL-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα while its knockdown lowered them.

A previous study similarly revealed that NF-κB signaling could modulate proinflammatory and coagulation responses in DVT [16]. That study demonstrated that miRNAs or drugs designed to interfere with NF-κB signaling could offer promising therapeutic approaches to improve thrombosis, but the appropriate dose and possible side effects need to be considered and require further investigation [16]. In another study, the essential regulatory role of NLRP3/HIF-1α signaling within a complex network of coagulation and inflammation during the progression of thrombosis was revealed [17]. Another study indicated that NLRP3 inflammasomes were able to promote the production of IL-1β and it was elevated in the activation of platelets and the formation of thrombi [18]. Therefore, targeted therapies against NLRP3 or IL-1β could also be beneficial for the treatment of inflammation-related thrombosis.

Study Limitations

There are some limitations of the present study. To confirm the findings, the expression of apoptosis-related molecules should be examined in transfected cells in further work. Furthermore, in vivo experiments should be carried out in future work to confirm these findings.

Conclusion

The upregulation of hsa_circRNA_092488 in cases of DVT was able to suppress the proliferation and migration of EPCs, induce cell cycle arrest from the S to the G0/G1 phase, and trigger apoptosis. Moreover, hsa_circRNA_092488 could function by activating the NLRP3/NF-κB signaling pathway, as overexpressed hsa_circRNA_092488 elevated the protein levels of caspase-1, IL-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα while its knockdown lowered them. Therefore, the hsa_circRNA_092488/NLRP3/NF-κB pathway could be associated with the progression of DVT, and this novel signaling pathway may be considered as a putative therapeutic candidate for the treatment of DVT, possibly involving the targeting of circRNAs, the design of drugs

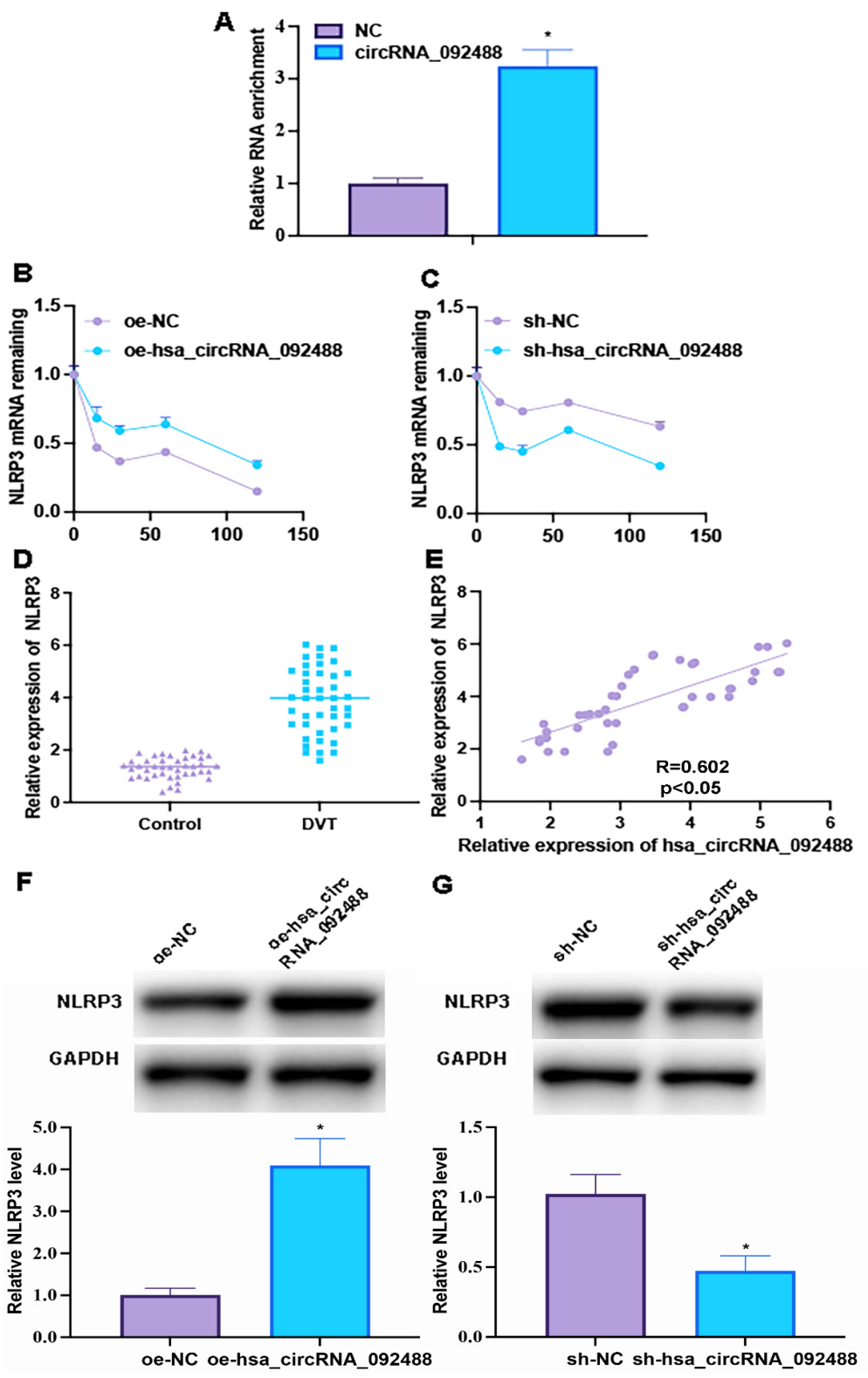

Figure 5. NOD-like receptor protein 3 (NLRP3) was the putative downstream target molecule of hsa_circRNA_092488. A) The interaction of hsa_circRNA_092488 and NLRP3 was revealed by RNA pull-down analysis. B) The stability of NLRP3 mRNA was elevated in endothelial progenitor cells (EPCs) transfected with oe-hsa_circRNA_092488. C) NLRP3 mRNA stability was reduced in EPCs by sh-hsa_circRNA_092488. D) NLRP3 expression was enhanced in cases of deep vein thrombosis (DVT). E) Levels of hsa_circRNA_092488 and NLRP3 were positively correlated in DVT. F, G) NLRP3 protein levels were increased in EPCs treated with oe-hsa_circRNA_092488 and reduced in cells transfected with sh-hsa_circRNA_092488.

*: $p<0.05$.

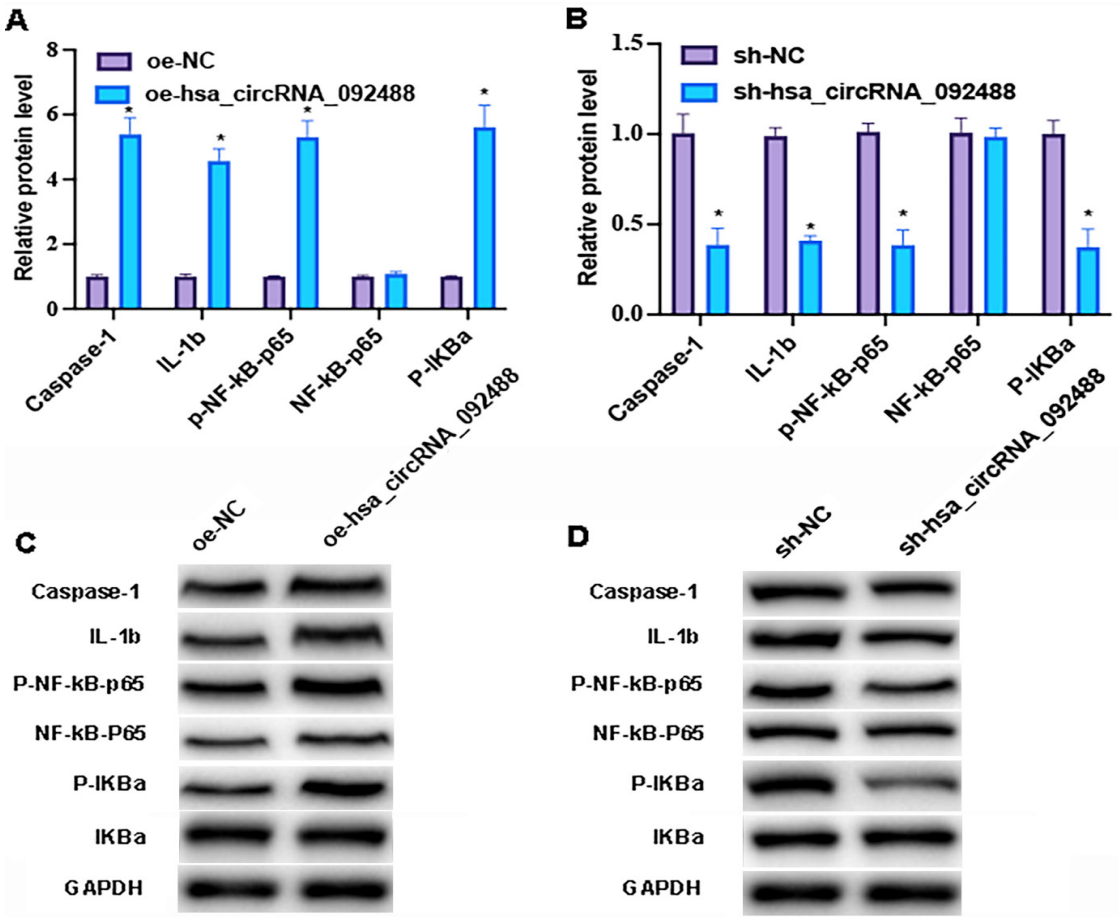

Figure 6. A-D) The levels of NOD-like receptor protein 3/nuclear factor (NF)-κB-associated proteins were affected by hsa_circRNA_092488. Overexpression of hsa_circRNA_092488 in endothelial progenitor cells remarkably increased the protein levels of caspase-1, interleukin-1β, P-NF-κB-p65/NF-κB-p65, and P-IκBα/IκBα while its knockdown lowered these protein levels.

*: $p<0.05$.

to interfere with NF-κB signaling, or targeted therapies against NLRP3 or IL-1β. However, the optimal doses and potential side effects must also be considered. These points require further investigation.

Ethics

Ethics Committee Approval: All procedures were performed in line with the Declaration of Helsinki and the protocols were reviewed by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University (decision no: 202047).

Informed Consent: Informed consent was obtained from all patients and controls.

Footnotes

Authorship Contributions

Surgical and Medical Practices: J.W., B.D.; Concept: J.W., B.D.; Design: J.W., B.D.; Data Collection or Processing: J.W., B.D.; Analysis or Interpretation: J.W., B.D.; Literature Search: J.W., B.D.; Writing: J.W., B.D.

Conflict of Interest: No conflict of interest was declared by the authors.

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