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HuR targeted by miR-29b-3p suppresses the malignant biological behaviors of AML cells via the NF-κB and JAK/STAT signaling pathways

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Abstract

Background: HuR/ELAVL1 (embryonic lethal abnormal vision 1) protein exerts important prognostic effects of involving in the pathogenesis and development of acute myeloid leukemia (AML). This study aims to investigate the role of HuR targeted by miR-29b-3p in biological behaviors of AML cells and the involvement of the NF-κB and JAK/STAT signaling pathways.

Methods: The expressions of HuR and miR-29b-3p were determined using real-time quantitative PCR and Western blot analysis, and the association between them was analyzed using the Spearman's coefficient correlation. Next, the potential relationship between HuR and miR-29b-3p was predicted based on data from different biological information databases and verified by the Dual-luciferase reporter gene assay. The effect of miR-29b-3p-meidated HuR expressions on the biological behaviors of AML cells was explored after transfecting lentiviruses, mimics, and inhibitors against miR-29b-3p into AML cells. Then, the expression patterns of Bcl-2 and Bax were detected to understand the apoptosis effect of HuR on AML cells. Phosphorylation levels of NF-κB /p65, IκBα, STAT1, STAT3 and STAT5 were determined to assess the influence of HuR on AML as well as the relationship between the NF-κB and JAK/STAT signaling pathways.

Results: HuR was negatively correlated with miR-29b-3p, which was thereby identified as a downstream target of miR-29b-3p in AML. When miR-29b-3p was overexpressed in AML cells, HuR expression was lowered, accompanied by inhibited cell proliferation, migration and invasion, decreased Bcl-2 and Bax levels, as well as inhibited phosphorylation levels of p65, IκBα, STAT1, STAT3 and STAT5.

Conclusion: HuR is a direct target of miR-29b-3p. Lowered HuR protein expression by miR-29b-3p inhibits the malignant biological behaviors of AML cells via the inactivation of the NF-κB and JAK/STAT pathways.

Keywords: HuR, miR-29b-3p; malignant biological behaviors; the NF-κB and JAK/STAT signaling pathways; acute myeloid leukemia

Background

HuR (also called ELAV-like protein 1[Elavl1]) is encoded by *elavl1* gene located on chromosome 19p13.2, and it ubiquitously expressed in mammals and functionally involved in modulating mRNA stability and translational efficiency.[1,2] It stabilizes or destabilizes mRNAs which are closely related to tumor progression including the cell cycle disorder, excessive cell proliferation and invasion, and cell resistance to apoptosis.[2] HuR overexpression has been detected in almost all types of cancer tissues, including acute myeloid leukemia (AML) as well.[3] One of the mechanisms to restrain AML is the inhibition of a key protein nuclear factor κB (NF-κB) with the involvement of the JAK/STAT signaling pathway, which transcriptionally regulates stress-response genes. IκBα mRNA has a long AU-rich 3′UTR containing a number of predicted hits that target a previously identified HuR motif. A previous study reported that IκBα 3′UTR transcripts were specifically associated with HuR, and HuR overexpression increased IκB-α protein levels, which in turn downregulated NF-κB in the nucleus.[4] Moreover, HuR has been reported to destabilize STAT3 and STAT5 mRNAs ,[5] which implies the underlying involvement of the JAK/STAT signaling pathway. Thus, elucidating the regulation of HuR expression is critical for a better understanding of the molecular mechanism behind the pathogenesis of AML.

Recently, post-transcriptional regulators of gene expression have gained solicitous attentions. MicroRNAs (miRNAs) are a class of endogenous highly conserved non-coding small molecule RNAs with approximately 19-25 nucleotides. Previous studies proved that miRNAs involved in the regulation of genes related to the hematopoietic system via a complex regulatory network.[6-8] Strikingly, abnormal miRNA expressions have been observed in cases of AML as well. For instance, miR-29b over-expression has significantly suppressed the development of AML .[9] In addition, it is found that miR-29b as a tumor suppressor is down-expressed in AML cell lines and primary AML blasts, and induces cell apoptosis in tumor cells and dramatically reduces tumorigenicity in a xenograft leukemia model.[10] In the current study, unexpectedly, we predicted that HuR 3′UTR had some conservative targets of miR-29b using bioinformatics resources. Furthermore, previous studies reported that

HuR was a downstream target of miR-29b in some cancer cells.[11,12] Currently, whether HuR is regulated by miR-29b in AML and the mechanism of their interactions are still unclear.

In this study, we aimed to explore the effects of HuR targeted by miR-29b-3p on malignant biological behaviors of AML and the involvement of the NF-κB and JAK/STAT signaling pathways, in the hope of providing a novel target for the treatment of AML. We demonstrate for the first time that HuR is the direct target of miR-29b-3p, responsible for excessive cell proliferation and resistance to apoptosis by mediating activities of the NF-kB and JAK/STAT pathways in the pathogenesis of AML.

Methods

Cell lines and cultures

Human myeloid leukemia cell lines including K562, NB4, U937, HL-60 and imatinib-resistant K562/G01 and HEK 293T cells were selected from CCTCC (China Center for Type Culture Collection, Wuhan, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Myeloid leukemia cell line Kasumi-1 (kindly provided by Prof. Ligen Liu, the Fifth People's Hospital of Shanghai, Fudan University, Shanghai, China) was cultured in RPMI-1640 medium supplemented with 15% FBS. K562/G01 cells were grown in an imatinib-free culture medium (4 μM) for at least two weeks for each experiment. HEK 293T cell line was cultured in DMEM (Invitrogen) containing 1% penicillin/streptomycin and 10% FBS. All cell lines were incubated at 5% CO_2 and 37℃.

Construction of miR-29b-3p over- and down-expression vectors

The lentivirus vector containing overexpressed and scrambled miR-29b-3p was constructed by GeneChem Company (Shanghai, China), the sequences of miR-29b-3p synthetic vectors as following: UAGCACCAUUUGAAAUCAGUGUU. miR-29b-3p mimic, inhibitor and relevant negative controls were synthesized by GenePharma Company (Shanghai, China), the sequences of synthetic vectors as following:

miR-29b-3p mimic: UAGCACCAUUUGAAAUCAGUGUU, *miR-29b-3p* inhibitor: AACACUGAUUUCAAAUGGUGCUA.

Cell transfection

According to the lentivirus infection protocol, the optimal infection conditions of K562 and U937 cells respectivly included the multiplicity of infection were 20 and 50, supplemented with enhancer infection supplement (ENI.S.) and polybrene. Stably infected cell lines were selected with 1.7 μg/ml puromycin (Sigma-Aldrich). Then the cells were divided into the miR-29b-3p group (transfected with a miR-29b-3p lentivirus vector), the NC group (transfected with a scramble-miR-29b-3p lentivirus vector) and the CON group (blank control). miR-29b-3p stably transfected cells were harvested at 96h post-infection for various assays subsequently reported in this study.

Electric transfection reagents containing cells transfected with miR-29b-3p mimics, inhibitor or negative controls using the TransEasy electrical transfection kit (Cellapy Biotechnology, China) according to the manufacturer's instruction were added to electrode cups, which were placed in an X Unit (Lonza Nucleofector™4D, Switzerland) to switch on the procedure. The transfected cells were incubated at 5% CO2 and 37℃ for 48h.

Dual-luciferase reporter gene assay

HuR-3'UTR luciferase reporter plasmids with wild-type HuR-3'UTR (wt UTR) and mutated HuR-3'UTR (mut UTR) in the predicted miR-29b-3p binding site were constructed by GenePharma (Shanghai, China), the sequences of synthetic vectors as following: *HuR* wt: CTCTAGTCGCAGCTCTGTGACTGATTCCCTCCCGGGTGC TGAGTCCCCTCCCCGGCCACC, *HuR* mut: CTCTAGTCGCAGCTCTGTGACT GATTCCCTCCCGCCACGAGAGTCCCCTCCCCGGCCACC. The dual luciferase reporter plasmids with HuR wtUTR, or with mutUTR and miR-29b-3p or scramble mimics, which were co-transfected into HEK 293T cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instruction. After 48h of transfection, the firefly and renilla luciferase activities were measured using the

Dual-Luciferase reporter assay system (Promega, USA). The relative luciferase unit (RLU) activity was determined as follows, with the cell lysate of the reporter gene regarded as the blank control and the Renilla luciferase as the internal control: RLU = RLU firefly luciferase / RLU Renilla luciferase.

Real-time quantitative PCR analysis

Total RNAs were extracted using Trizol reagent according to the manufacturer's protocol. The reverse transcriptase (RT) reactions of *miR-29b-3p* and *HuR* were respectively amplified using a Bluge-Loop™ miRNA qRT Starter Kit (RiboBio, Guangzhou) and a RevertAid First Strand cDNA Synthesis Kit (Thermo ScientificTM). Quantitative PCR (qPCR) analyses for *miR-29b-3p* and *HuR* were performed on a 7500 Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA), using a Bluge-Loop ™ miRNA qPCR Starter Kit (RiboBio, Guangzhou) and UltraSYBR Mixture (Low Rox), respectively. The primer sequences of *HuR* for qPCR were shown as flowing: Forward: GGCGCAGAGATTCAGGTTCT, Reverse: TCCTGCCCCAGGTTGTAGAT. The relative quantification of *miR-29b-3p* and *HuR* expressions normalized to U6 or GAPDH was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total protein was extracted from cells and lysed using RIPA buffer (Boster, USA), and the protein concentration was determined by the BCA Kit (Boster, USA). Nuclear and cytoplasmic extracts were prepared using the nuclear and cytoplasmic protein extraction kit (Cwbio, China) following the manufacturer's protocol. Equal amounts from the cell lysates were separated by SDS-PAGE and transferred electrophoretically onto PVDF membranes. After blocking, the membranes were incubated with the following specific primary antibodies overnight: anti-HuR (ab200342, Abcam), anti-p65 (ab32536,Abcam), anti-phospho-p65 (Ser536) (#3031S,CST), anti-IκBα(#9242S, CST), anti-phospho-IκBα (Ser32/36)(5A5) (#9246S, CST), anti-STAT1 (ab109320, Abcam), anti-phospho-STAT1(Y701) (ab30645,Abcam),

anti-STAT3 (ab68153, Abcam), anti-phospho-STAT3 (Tyr705) (#8204, CST), anti-STAT5 (D3N2B) (#25656, CST), anti-phospho-STAT5 (Tyr694) (D47E4) (#4322, CST). Afterward, the membranes were incubated with the HRP-conjugated secondary antibody (SSA016, Sino biological) for 2h at room temperature. Protein bands were visualized and quantitated using the Image J 1.43 software (NIH, MD, USA), and data were normalized to GAPDH (#5174, CST) and PCNA (10205-2-AP, Proteintech).

MTS assay

Cells were seeded to 96-well plates and were detected at different times points (24h, 48h, 72h and 96h) using the MTS assay (Promega, USA). The absorbance was measured at 492/630 nm using a Microplate Reader (MK3, Thermo Fisher Scientific, USA).

Cell clone formation assay

The cells were cultured in 24-well plates in RPMI-1640 medium containing 1.6% methyl cellulose (Sigma, USA) for 7-10 days. The number of colonies (containing ≥ 40 cells) was counted and the efficiency of colony formation was assessed.

Flow cytometry

Cell apoptosis was examined using Annexin V-PE and 7-AAD staining assays (BD Bioscience, USA) following the manufacturer's protocols, and cell cycle was analyzed using a Cell Cycle Analysis Kit (Keygen Biotech, China). Cell cycle and apoptosis were detected by flow cytometry analysis (Accuri C6, BD Bioscience, USA).

Transwell assay

In-vitro invasion and migration were analyzed using 8 μm pore transwell chambers (Coring, MA, USA). The cells were reseeded onto the matrigel-coated upper chambers (Corning, USA) containing serum-free RPMI-1640, and 10% FBS was added to the lower chambers. Cells were incubated for 24h for invasion assay. For

invasion assay, cells attaching to the lower surface of the membrane were fixed by methanol and stained with Wright-Giemsa, and the number of cells was counted under a light microscope (IX71, Olympus, Japan). For migration assay, cells migrating to the lower chambers were collected and detected using MTS.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde (DingGuo, Beijing, China) for 15min at room temperature, and permeabilized with 0.1%Triton X-100 (Sigma, USA) for 15 min. After blocking with 5% goat serum (Gibco, USA) for 2h at room temperature, cells were incubated with anti-p65 antibody (at a dilution of 1:200, Affinity Biosciences, USA) overnight at 4℃. Then cells were incubated with a 1:500 dilution of Alexa Fluor 555 fluorescein-labelled goat anti-rabbit IgG antibody (Dingjie, China) in darkness for 2h. After the cells for washing three times and stained with DAPI (Servicebio, Wuhan, Chian) for 5 min, images were captured by a camera on an inverted fluorescence microscope (Olympus, Japan).

Statistical analysis

All experiments were repeated at least three times. Data were expressed as mean± standard deviation (SD) using SPSS 24.0 statistical software. For variables with a normal distribution, comparisons between two groups and homogeneity of variance was verified using an independent samples t-test. Differences between multiple groups were compared using one-way ANOVA. For variables with a non-normal distribution, a nonparametric test was employed. Spearman's coefficient was used for determining the correlation between two variables. A P value of <0.05 was considered statistically significant.

Results

HuR is overexpressed in AML cell lines and negatively correlated with miR-29b-3p

The expressions of HuR and miR-29b-3p in AML cells were determined by RT-qPCR

and Western blot analyses and their relationship was analyzed by comparing mRNA expressions in AML cell lines versus the normal controls. The results showed that *HuR* was overexpressed in 6 myeloid leukemia cell lines including K562, NB4, U937, Kasumi-1, HL-60 and imatinib resistant K562/G01 cells (Fig. 1a), accompanied by miR-29b-3p downexpressions (Fig. 1b). The correlation analysis showed that *HuR* mRNA expression was negatively correlated with miR-29b-3p expression in these cell lines (r = -0.829, P<0.05), so was HuR protein expression with miR-29b-3p expression (Fig. 1c-d). Owing to the lower degree in HuR overexpressions in K562 and U937 cells, they were used in the following experiments.

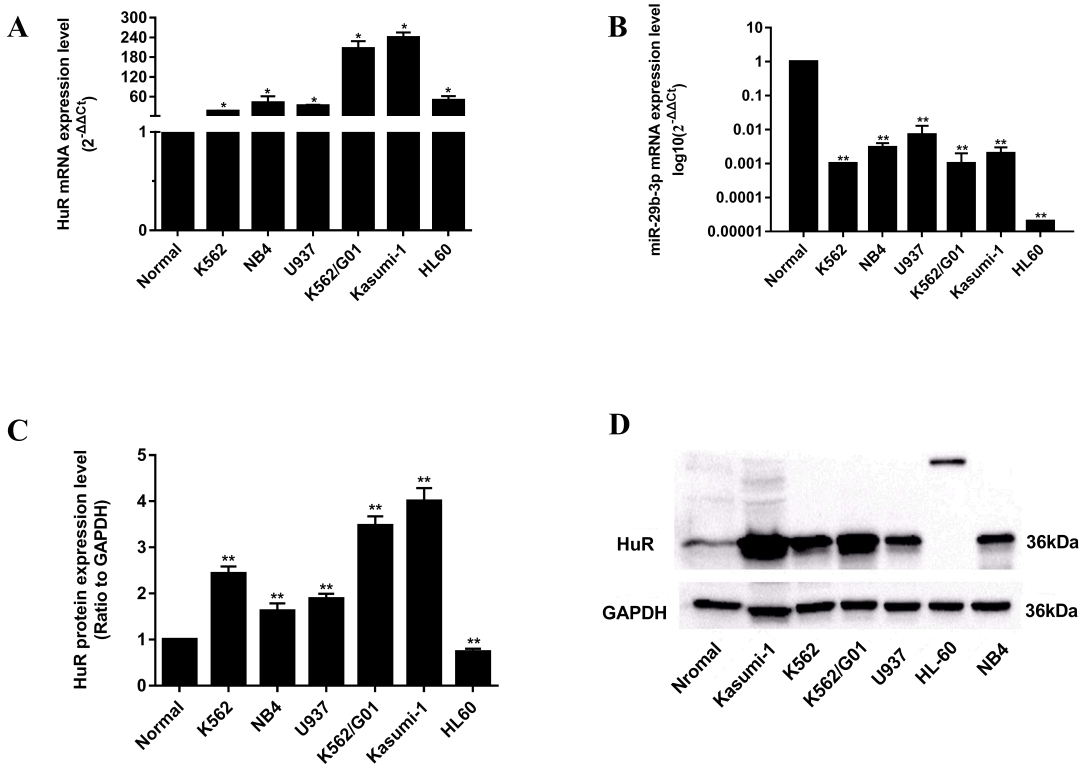

Fig. 1 Expression of HuR and miR-29b-3p in AML cell lines detected by RT-qPCR and Western blot. (a, b) Expression of HuR and miR-29b-3p in mRNA level in AML cell lines, respectively. (c, d) Protein expression of HuR in AML cell lines. (**P*<0.05, ***P*<0.01, vs. healthy normal control). The full-length blots/gels are presented in Supplementary Figure 1. Note: Normal, healthy normal control.

HuR is the directly target of miR-29b-3p

Putative binding sites in the HuR 3'UTR interacting with miR-29b-3p were predicted

using microRNA.org, miRanda and RNAhybrid2.2 (data not shown). As shown in Fig. 2a, the luciferase activity of HuR wtUTR significantly decreased in miR-29b-3p-transfected 293T cells (P<0.01). However, luciferase reporter activity was not significantly affected by HuR mutUTR (P>0.05). After miR-29b-3p overexpression (Fig. 2b) or inhibition (Fig. 2c), we validate the correlation between the endogenous expressions of miR-29b-3p and HuR.The results showed that HuR mRNA and protein levels were significantly lowered in the miR-29b-3p group and were elevated in the inhibitor group (Fig. 2d-h). This indicated that HuR was a downstream target of miR-29b-3p in AML cells.

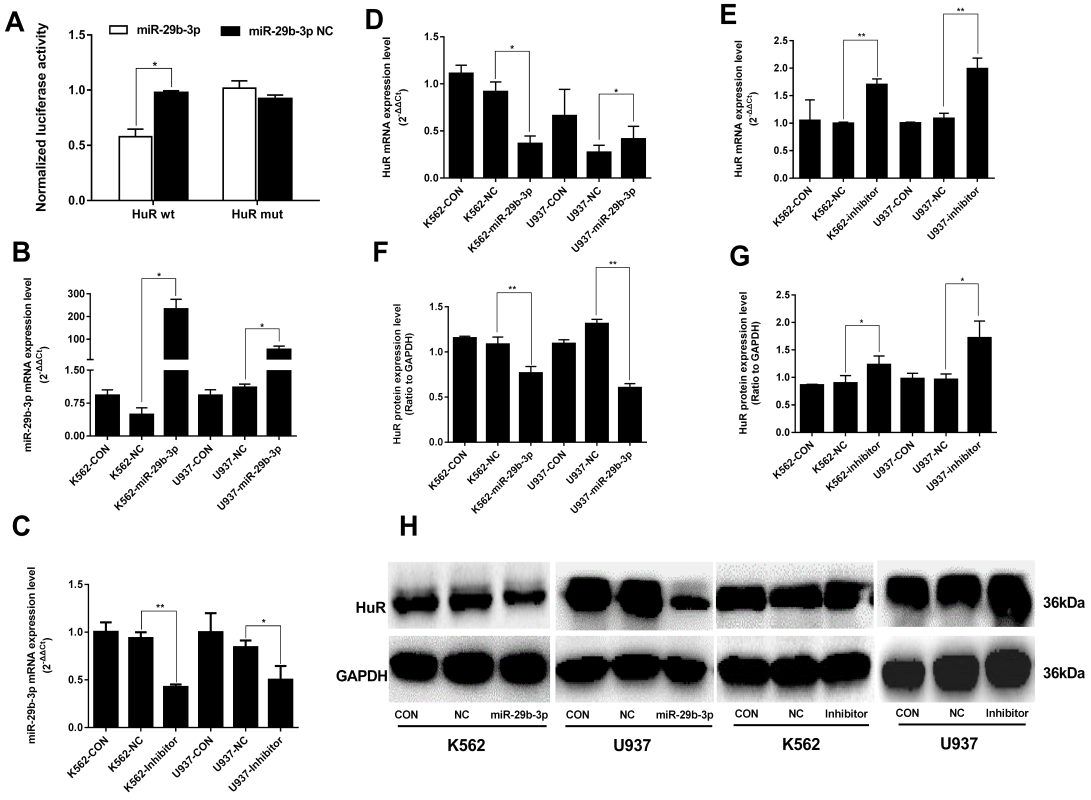

Fig. 2 HuR was verifyied the downstream target of miR-29b-3p. (a) The dual luciferase activites assay of miR-29b-3p and HuR. (b, c)The mRNA expression levels of miR-29b-3p after up-regulating or inhibiting miR-29b-3p. (d, e) The mRNA expression of HuR after up-regulating or inhibiting miR-29b-3p. (f-h) Western blot of HuR expression were detected. (*P<0.05, **P<0.01, vs. NC group). The full-length blots/gels are presented in Supplementary Figure 2.

HuR down-regulation induced by miR-29b-3p overexpression inhibits proliferation, colony formation, invasion and migration , and induces apoptosis in AML cells

A restoration of miR-29b-3p expression (HuR expressions were correspondingly down-regulated) resulted in a time-dependent inhibition of cell proliferation in K562 and U937 cells (Fig. 3a). The capability of colony formation of K562 and U937 cells significantly decreased (Fig. 3b). FCM analysis showed that the percentage of cells at the G0/G1 phase significantly increased in both the K562-miR-29b-3p group and U937-miR-29b-3p group (Fig. 3c). Additionally, the percentage of cells at the S phase significantly decreased in the K562-miR-29b-3p and U937-miR-29b-3p groups (P<0.01). These results suggested that HuR down-regulation induced by miR-29b-3p overexpression in AML cells resulted in cell cycle arrest at the G0/G1 phase. As shown in Fig. 3d, the total apoptosis ratio was 14.300±0.000 in the K562-miR-29b-3p group and 8.896±0.289 in the U937-miR-29b-3p group, compared with the NC group (P<0.01), and the percentages of early apoptotic cells were 14.033±0.578% and 5.967±0.208%, respectively. Moreover, when HuR was inhibited by miR-29b-3p overexpression, BCL-2 protein levels was lowered and Bax protein levels were elevated in K562 and U937 cells (Fig. 3e). This indicated that cell apoptosis in AML cells was triggered by HuR downexpression. The invading cells in both the K562 and U937-miR-29b-3p groups were fewer than those in the paired NC groups (P<0.01) (Fig. 3f). Furthermore, the OD value of migrating cells decreased in the K562-miR-29b-3 and U937-miR-29b-3p groups compared with the paired NC groups (P<0.01) (Fig. 3g).

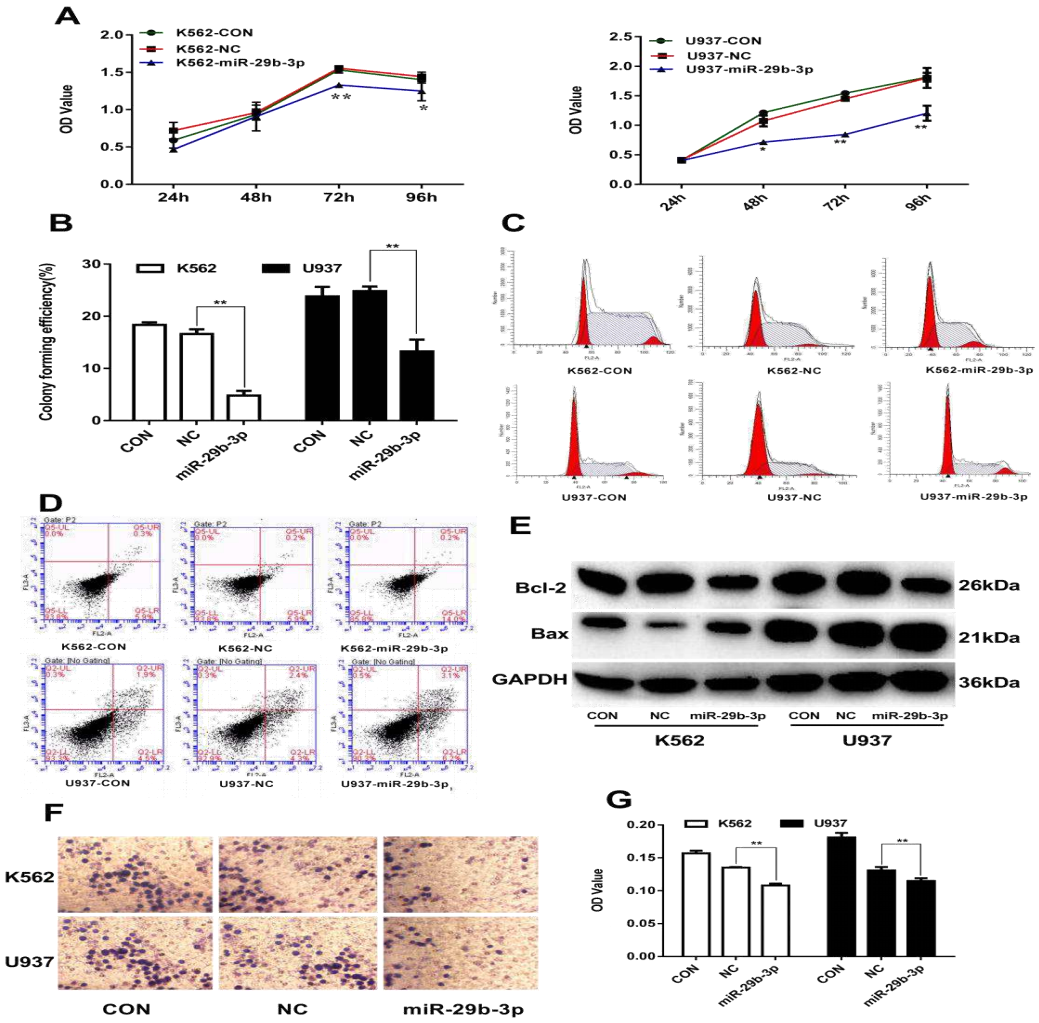

Fig. 3 Biologic effects of HuR after overexpression miR-29b-3p evaluated in K562 and U937 cells. (a) Grow curve of proliferation based on the OD value after miR-29b-3p restoration at different time points, resceptivly. (b) Colonies containing ≥40 cells were counted on day 7~10 using a microscope (×200). (c) Cells were labeled by PI and analyzed using FCM. (d) Apoptotic cells were measured by FCM. Dot plots show 7-AAD (y-axis) vs. Annexin-V (x-axis). (e) The protein expression of BCL-2 and Bax were detected by Western blot. (f) Wright-Giemsa stained invading cells were observed under microscope (×200). (g) The OD values (proportional to cell numbers) of migrating cells were measured by MTS assay. (*P<0.05, **P<0.01, vs. NC group).

HuR up-regulation induced by inhibition of miR-29b-3p promots cell proliferation, colony formation, migration and invasion in AML cells

When miR-29b-3p expression was suppressed by the inhibitor (HuR expressions were correspondingly up-regulated), cell proliferation and colony formation in both K562 and U937 cells were correspondingly promoted (Fig. 4a, b). As shown in Fig.4c-e, miR-29b-3p inhibition didn't affect the cell cycle, but promote resistance to apoptosis in both K562 and U937 cells. Moreover, the invaded and migrated cells markedly increased in response to miR-29b-3p inhibition. The above data suggested that miR-29b-3p inhibition targeting HuR was able to promote cell proliferation, colony formation, as well as the invasion and migration abilities in AML cells (Fig. 4f, g).

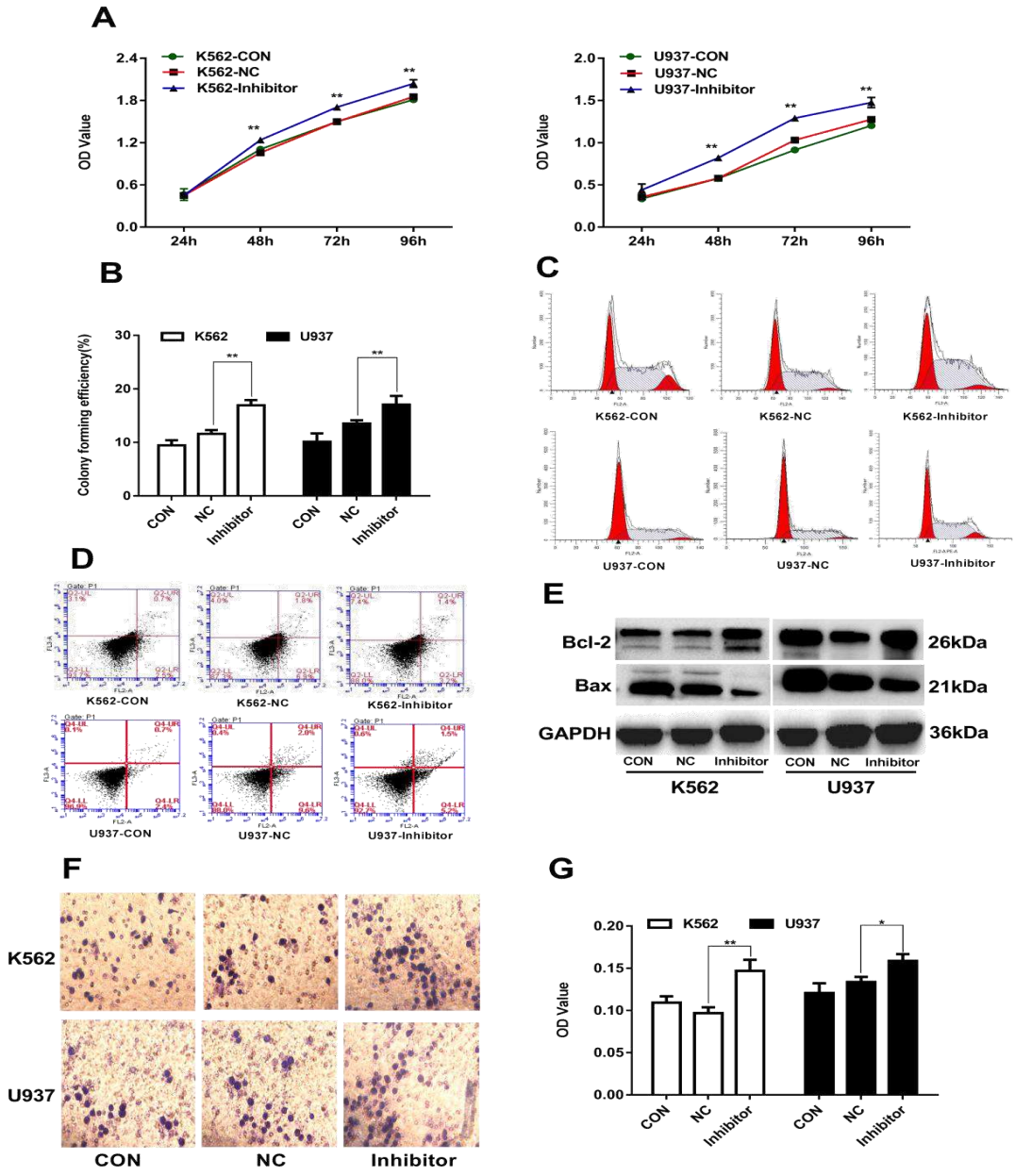

Fig. 4 Biologic effects of HuR after inhibition miR-29b-3p evaluated in K562 and

U937 cells. (a) Grow curve of proliferation based on the OD value after miR-29b-3p suppression at different time points, resceptivly. (b) Colonies containing ≥40 cells were counted on day 7~10 using a microscope (×200). (c) Cells were labeled by PI and analyzed using FCM. (d) Apoptotic cells were measured by FCM. Dot plots show 7-AAD (y-axis) vs. Annexin-V (x-axis). (e) The protein expression of BCL-2 and Bax were detected by Western blot. The full-length blots/gels are presented in Supplementary Figure 3. (f) Wright-Giemsa stained invading cells were observed under microscope (×200). (g) The OD values (proportional to cell numbers) of migrating cells were measured by MTS assay. (*P<0.05, **P<0.01, vs. NC group).

HuR targeted by miR-29b-3p inhibits the expression of p65 in nuclear and NF-κB signaling pathway in AML cells

According to the result of cellular immunofluorescence experiment as shown in Fig. 5, the relative fluorescence rate of p65 in K562 (0.230±0.007) and U937 (0.241±0.013) cells were significantly reduced in miR-29b-3p group compared with NC groups (P<0.01), while the rate of inhibited miR-29b-3p in K562 (1.986±0.113) and U937 (1.766±0.045) was increased (P<0.01). The result of Western blot confirmed the significant decrease of total p65 (P<0.05) in nuclear protein (P<0.01) for K562 and U937 cells, as shown in Fig. 6a-d. In order to further investigate the activity of p65, its phosphorylation protein expression was examined by Western blot (Fig. 6h,i). Consistently, HuR was inhibited by miR-29b-3p, thus suppressing the phosphorylation of p65 significantly (P<0.01) . In this case, the phosphorylation of IκBα was detected to further confirm the activation of NF-κB signaling pathway. The results indicated that the phosphorylation of IκBα was reduced (P<0.05) in miR-29b-3p groups(Fig. 6j). Total IκBα remained unchanged after the transfection of miR-29b-3p ($P > 0.05$) (data not shown). The opposite results were obtained when miR-29b-3p was inhibited(Fig. 6a,e-g,k,l). It was revealed that the HuR modulated by miR-29b-3p may contribute to inhibiting p65 expression in nucleus, thus deactivating the NF-κB signaling pathway in AML cells.

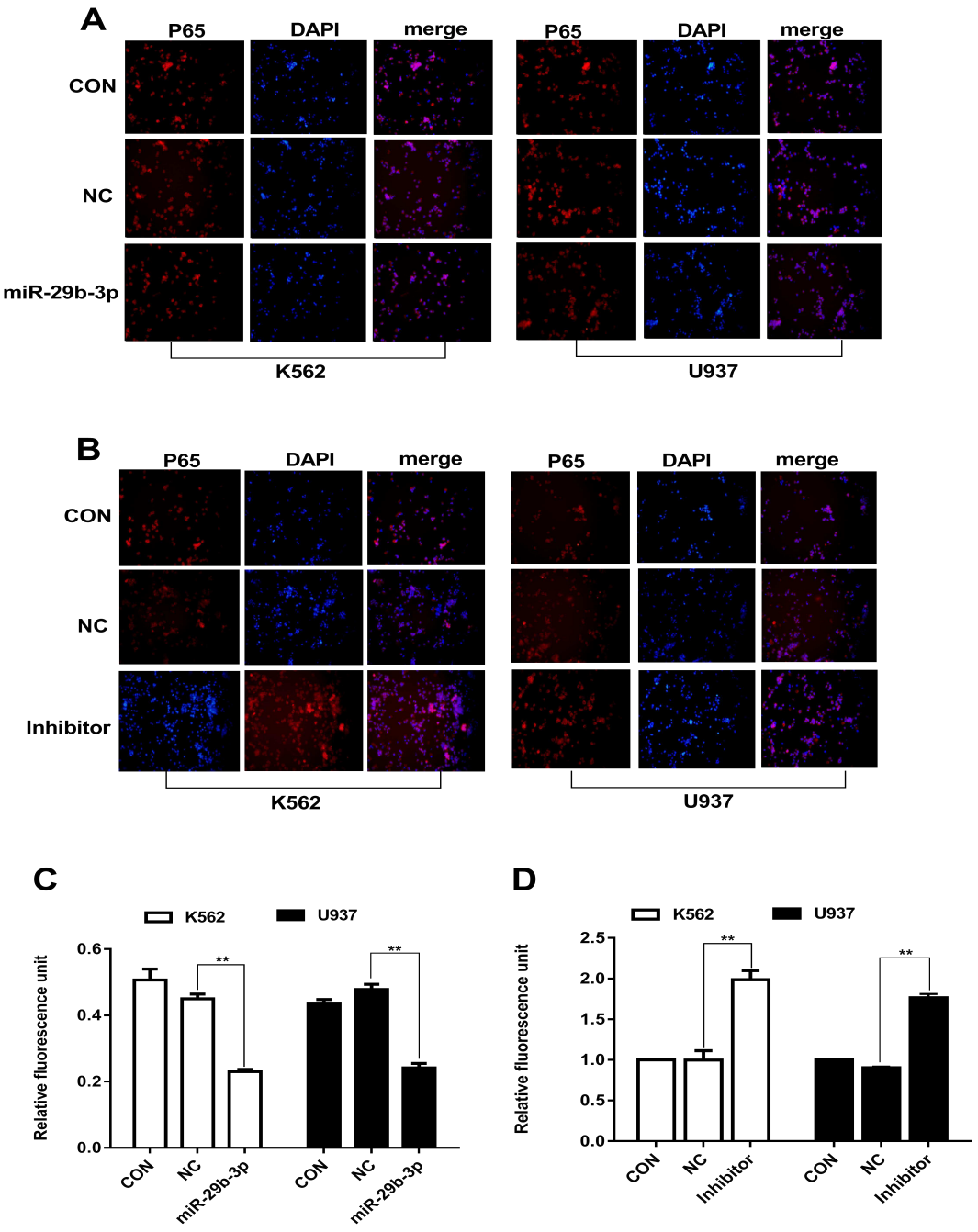

Fig. 5 Effects of HuR targeting by miR-29b-3p on the expression of p65 were evaluated via cellular immunofluorescence experiment in K562 and U937 cells. (a, b) Fluorescent expressions of p65 were observed using fluorescence microscope (×200), images were longitudinally aligned reprented as total pattern, nuclear and merge pattern, respectivly. (c, d) Fluorescent expressions of merge pattern deviding by total pattern represented as the relative fluorescence rate of p65. (**P*<0.05, ***P*<0.01, vs. NC group).

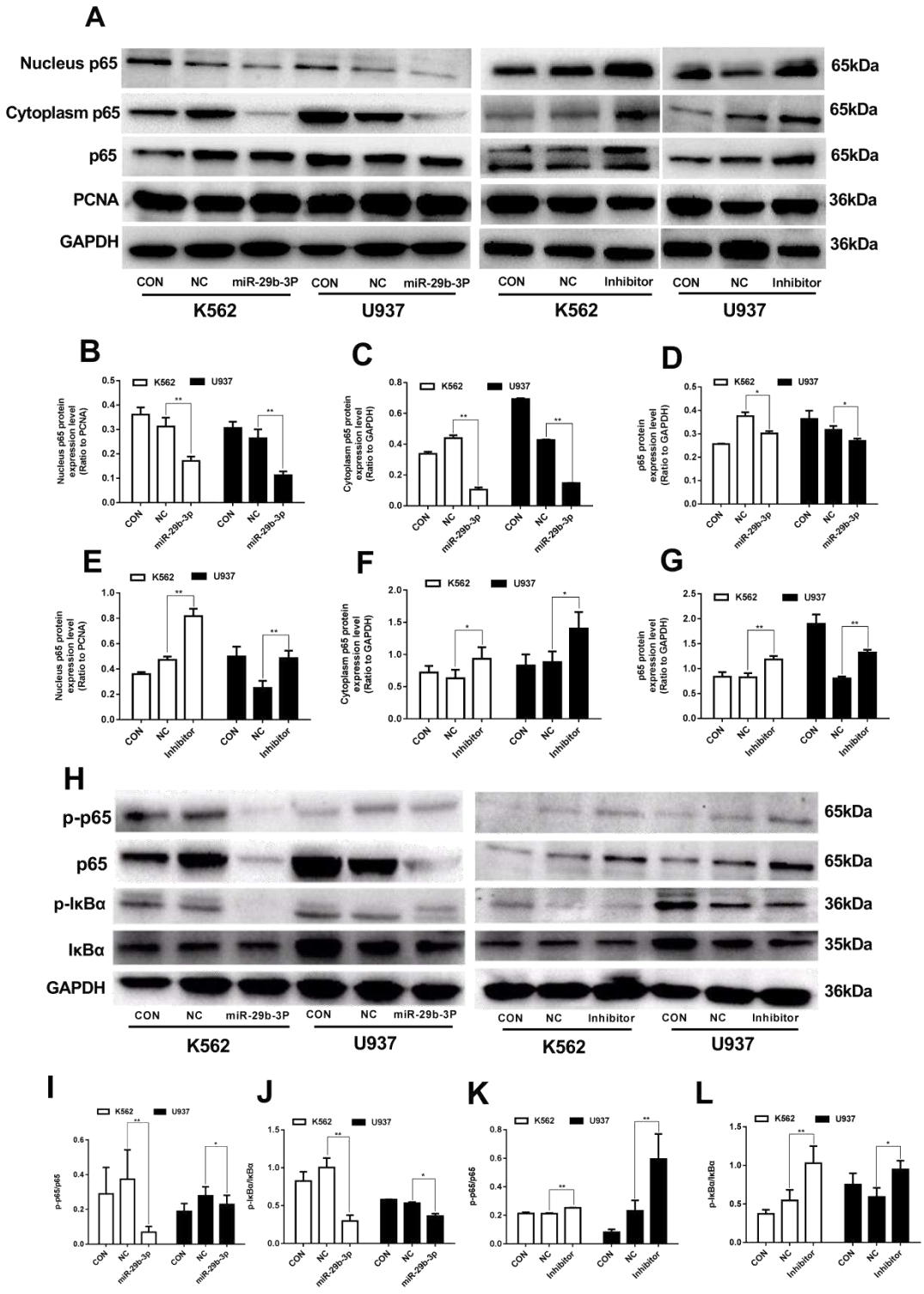

Fig. 6 Effects of miR-29b-3p on the protein expression of p65 in in different cellular components and on the phosphorylation of p65 and IκBα expressed in K562 and U937 cells. (a) p65 at nuclear, cytosol and total cellular proteins were detected by western blot. The full-length blots/gels are presented in Supplementary Figure 4.

(b–g) Quantification of nucleus p65 was normalized to PCNA, quantification of cytoplasm and total p65 were normalized to GAPDH. (*P<0.05, **P<0.01, vs. NC group). (h) p65, IκBα and their phosphorylation proteins expression were detected by western blot. (i-j) Quantification of phosphorylated p65 and phosphorylated IκBα were normalized to p65 and IκBα after miR-29b-3p overexpression, respectivly. (k-l) Quantifications of phosphorylated p65 and phosphorylated IκBα were normalized to p65 and IκBα after miR-29b-3p inhibition, respectivly. (*P<0.05, **P<0.01, vs. NC group).

HuR targeted by miR-29b-3p reduces the activity of STAT signaling pathway in AML cells

As shown in Fig. 7, the reduction expression of HuR via up-regulated miR-29b-3p led to a sharp decline in the phosphorylation of STAT1 (P<0.05), STAT3 (P<0.01) and STAT5 (P<0.05) for K562 and U937 cells. However, total STAT1, STAT3 and STAT5 remained unchanged after the transfection of miR-29b-3p (P＞0.05) (data not shown). The opposite results were obtained when miR-29b-3p was inhibited. According to the results, the HuR regulated by miR-29b-3p suppressed the phosphorylation of STAT1, STAT3 and STAT5, which is speculated to reduce the constitutive activation of STATs signaling in AML cells.

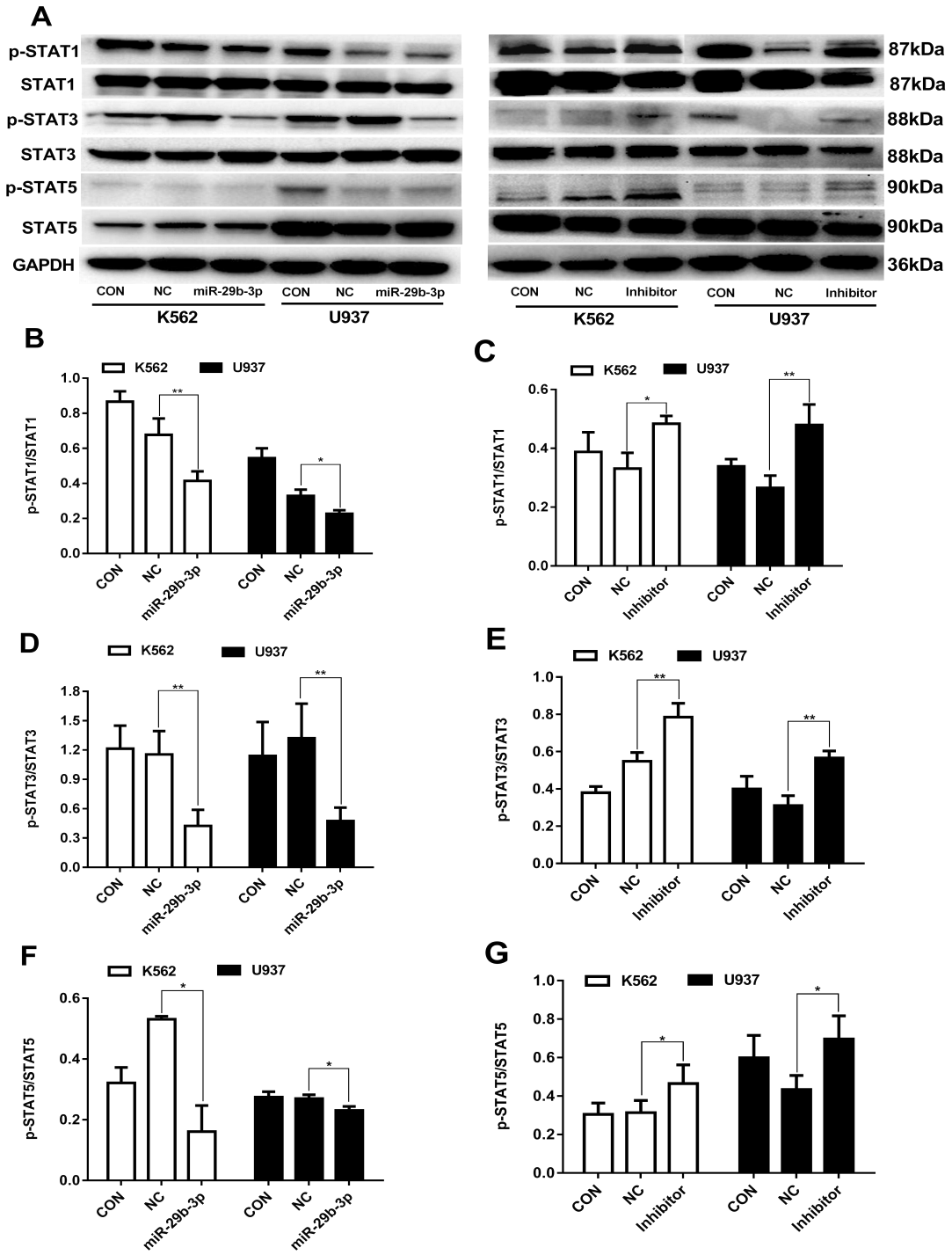

Fig. 7 Effects of miR-29b-3p on the protein phosphorylation of STA1, STAT3 and STAT5 expressed in K562 and U937 cells. (a) STA1, STAT3, STAT5 and their phosphorylation proteins expression were detected by western blot. The full-length blots/gels are presented in Supplementary Figure 4. (b–g) Quantifications of phosphorylated STA1, STAT3, STAT5 were normalized to STA1, STAT3, STAT5 after miR-29b-3p overexpression and inhibition, respectivly. (**P*<0.05, ***P*<0.01, vs. NC group).

Discussion

Abnormal HuR expressions have gained much attention as its diverse roles closely related to tumor progression including activation of proto-oncogene, disturbed cell cycle, excessive proliferation and invasion of tumor cells, and tumor resistance to apoptosis. Although HuR is crucial in the post-transcriptional regulation of many genes, the regulation of its own function and expression remains obscure. In the current study, our data showed that HuR was overexpressed in AML cell lines. This suggests that HuR can serve as a potential diagnostic biomarker or therapeutic target of AML. Moreover, we demonstrated HuR was inhibited by miR-29b-3p via the NF-κB and JAK/STAT signaling pathways, so that the malignant progression of AML was thwarted.

Non-coding RNAs particularly miRNAs post-transcriptionally modify the expression pattern of cancer-specific proteins.[13] Many studies to date have demonstrated that certain miRNAs differentially expressed in cancer cells are linked to the apoptotic death, disturbed cell cycle or the invasiveness and migration of these cells and to the progression of cancers through mechanisms associated with RNA-binding proteins (RBPs).[13,14] Further research is warranted to better understand the role of miRNAs in AML so as to better identify potential therapeutic targets. Recently, the diagnostic and therapeutic implications of miR-29b has become a research hotspot in multiple malignancies especially hematological malignancies. The miR-29b isoform consists of two subtypes miR-29b-1 and 2 at loci from chromosome 7q32 and 1q32, respectively. Though mature miR-29bs are encoded by two different stem precursor sequences, the translated mature miR-29bs are identical.[10] In our current study, miR-29b-3p was recognized as one of the critical factors. miR-29b is gaining prominence because of its emerging roles in a variety of physiological and pathological progresses including cell growth and differentiation, aging, transfer, immune regulation and cell cycle regulation and apoptosis.[15-19] Moreover, miR-29b acting as a tumor suppressor in AML has been confirmed.[9,10] As such, there is a great interest in the specific mechanism of miR-29b-3p in the development of AML. There is an overlap in function between HuR and miR-29b-3p, hinting a potential close

association between them. Furthermore, some studies have mentioned that miRNA may control the expression patterns of RBP in cancers,[15] and previous evidences have confirmed a miR-29b-mediated post-transcriptional regulation of HuR expression in cancer cells.[11,12] Likewise, in this study, we verified that HuR 3′UTR was specifically associated with miR-29b-3p using the dual luciferase reporter assay. The results showed that miR-29b-3p overexpression suppressed both HuR mRNA and protein expressions, when miR-29b-3p was downexpressed, HuR expressions significantly increased. These findings indicate that HuR is a downstream target of miR-29b-3p in AML.

To assess the role of how miR-29b-3p-mediated HuR expression involves in the pathogenesis of AML, further experiments on malignant biological behaviors were implemented in our study. It has been reported that the diverse functions of HuR in cancer development and progression are strongly associated with regulations of the stability or translation of target mRNAs that encode multiple cancer-related proteins including c-myc, GM-CSF, EGF, p21, p27, p53, cyclin A/B1/D1/E, and BCL-2.[2,5] Previous clinical studies have demonstrated that HuR is associated with lymph node metastasis in malignant tumors such as non-small cell lung carcinoma,[20] colon carcinoma,[21] upper urinary tract urothelial carcinoma.[22] In addition, it is found that matrix metalloproteinase-9 (MMP-9) and MTA1 are regulated by HuR,[2] and previous observation support the possibility that HuR downexpression in HL-60 cells may deteriorate the stability of MMP-9, resulting in diminished synthesis and secretion of MMP-9.[23] This supports our findings that miR-29b-3p-mediated HuR inhibition in turn suppressed cell migration and invasion in AML cells. Moreover, Bcl-2 protein is usually highly expressed in cancer cells through a process that induces stabilization of *bcl-2* mRNA. Considerable evidences indicate that there exists interactions between trans-acting factors and an ARE element in the *bcl-2* mRNA 3′-UTR to make stabilization change happens. Stabilization of *bcl-2* mRNA by HuR has been verified in HL60 both *in vitro* and *in vivo*.[24] This suggests that targeting HuR may be an effective way to down-regulate Bcl-2 expression in malignant cells. Consistent with previous reports, HuR downexpression induced by miR-29b-3p overexpression in the

current study lowered BCL-2 protein levels and increased Bax protein expression in K562 and U937 cells. Similarly, miR-29b levels were down-regulated in AML cell lines, accompanied by suppressed cell proliferation, colony formation and invasion, which functioned as a tumor suppressor in AML.[9,10] Evidences support that miR-29b-induced HuR inhibition drives apoptosis in cancer cells.[11,12] In line with these findings, our data revealed that HuR expression was inhibited by miR-29b-3p, inducing reduced cell viability, increased apoptosis, cell cycle block, suppressed migration and invasion in AML cells.

One of the most central findings of the current study was that miR-29b-3p-induced HuR inhibition suppressed oncogenic activation of the NF-κB and JAK/STAT signaling pathways. Classical NF-κB activation, whose main effector is RelA (p65)/p50, is usually a rapid and transient response to a wide range of stimuli. Previous studies have clearly proven that NF-κB signaling pathway often shows an abnormally high activation in AML cell lines.[25] Just as continuous activation of NF-κB may activate anti-apoptotic proteins Bcl-2 and Bcl-xL, suppressing NF-κB activity may activate its pre-apoptotic signals, thus increasing chemotherapy sensitivity of AML.[26] NF-κB transcriptionally regulates relevant important genes controlling cell differentiation, proliferation, cell cycle, apoptosis and invasion to affect tumor progression. Interestingly, the involvement of the NF-κB and JAK/STAT pathways in HuR inhibition has recently been observed. The conventional mechanism of triggering the NF-κB signaling pathway is associated with NF-κB and its inhibitor IκBα. IκBα phosphorylation induces the polyubiquitination and degradation of target molecules. The IκBα mRNA 3′UTR contains numerous predicted hits that target a previously identified HuR motif, and HuR is able to destabilize STAT3 and STAT5 mRNAs.[5] Previous evidence has revealed that HuR can bind specifically to the IκBα mRNA 3'UTR to modulate the translation of IκBα mRNA leading to a change in NF-κB protein expressions,[4] which in turn down-regulates NF-κB in the nucleus. In our study, HuR inhibition induced by miR-29b-3p overexpression lowered nuclear p65 levels in K562 and U937 cells. Normally, HuR is located in the nuclei, however, stimuli (e.g., hypoxia, glucose deprivation, chemotherapy) in the tumor

microenvironment will cause HuR relocation from the nucleus to the cytoplasm and promote HuR to bind to U- or AU-rich sequences in the 3'UTR of target mRNAs which confer cellular responses including cell growth, apoptosis and cell cycle .[27] It is therefore conceivable that HuR inhibition prevents AML development and progression via certain signaling pathways. Given that HuR has never been reported to be mutated and that NF-κB is up-regulated in AML, it is conceivable that the cytoplasmic abundance of HuR can be closely associated with the oncogenic activation of the NF-κB signaling. In addition, Mertens C et al. report that unphosphorylated STAT1 and STAT3 in the JAK/STAT signaling can be activated by the binding of NF-κB transcription factors.[28]

The JAK/STAT pathway is also related to the formation of the blood system and immune responses, which can identify specific cytokines and transmit anti-apoptotic, proliferation, differentiation and immune regulatory signals, and is critical to the formation of the hemopoietic system.[29] Indeed, the NF-κB and JAK-STAT pathways possibly have exhibited constitutive signaling activities and hypersensitivity to cytokine stimulation. Sustained activation of NF-κB can initiate STAT3 phosphorylation on tyrosine residues dependent on the NF-κB-induced production of IL-6 which is a target gene of both NF-κB and STAT3.[30,31] The crosstalk between the JAK/STAT and NF-κB signal pathways may expose interlinked feedbacks for controlling cell fate decisions in cancer cellular populations. Consistent with this notion, the correlation between STAT5 and p65/RELA phosphorylation has also been observed in myelofibrosis and secondary AML.[32] Furthermore, miRNAs have been reported to prevent cancer progression through inactivating the JAK/STAT signaling pathway.[33-35] Unexpectedly, a previous study demonstrated that miR-29b reduced NF-κB activity which was functionally related to STAT3.[36] In our study, HuR inhibition induced by miR-29b-3p overexpression resultantly decreased nuclear p65 activation and expression to make consistent suppression of the phosphorylation of the associated molecules in the NF-κB and JAK/STAT signal pathways. This indicates possible connections between miR-29b-3p-mediated HuR inhibition and the crosstalk between the NF-κB and JAK/STAT pathways. The Further study series will be

warranted to better understand the underlying mechanisms based on HuR inhibition and the involvement of the NF-κB and JAK/STAT signaling pathways in AML.

Conclusions

Our study illustrates the potential mechanism of miR-29b-3p-mediated HuR inhibition and its correlation with signal pathways in AML. The results demonstrate that HuR inhibition targeted by the tumor suppressor miR-29b-3p hinders the tumorigenesis and development of AML via the NF-κB and JAK/STAT signaling pathways. However, limited by the small sample size and experimental conditions in this study, the underlying correlations between this HuR inhibition and the crosstalk between the two signalings need to be verified in further intensive studies. HuR inhibition has the potential to diminish tumor resistance to cytotoxic agents, small-molecule antagonists, and molecular targeted agents, which can be a new mechanism of chemotherapy sensitivity in cancers. Although miRNA-based therapeutic methods are still in their infancy, miR-29b-3p-mediated HuR inhibition will inspire more promising therapeutic strategies for AML.

Abbreviations

HuR/ELAVL1: Embryonic lethal abnormal vision 1; AML: Acute myeloid leukemia; Bcl-2: B-cell lymphoma-2; Bax: BCL2-Associated X; PCNA: Proliferating Cell Nuclear Antigen; STAT: Signal transducers and activators of transcription

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Authors’ contributions

Study design: YT and DL. Data collection: YT and WW. Data analysis: YT, WW and YX.Manuscript composition: YT, WW and YX. Manuscript revision: ZC, JX and DL. Figure Drafting: QC, SL and ZZ. All authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that all data used or analysed during the current study are available on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors have declared that no competing interest exists.

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Figures

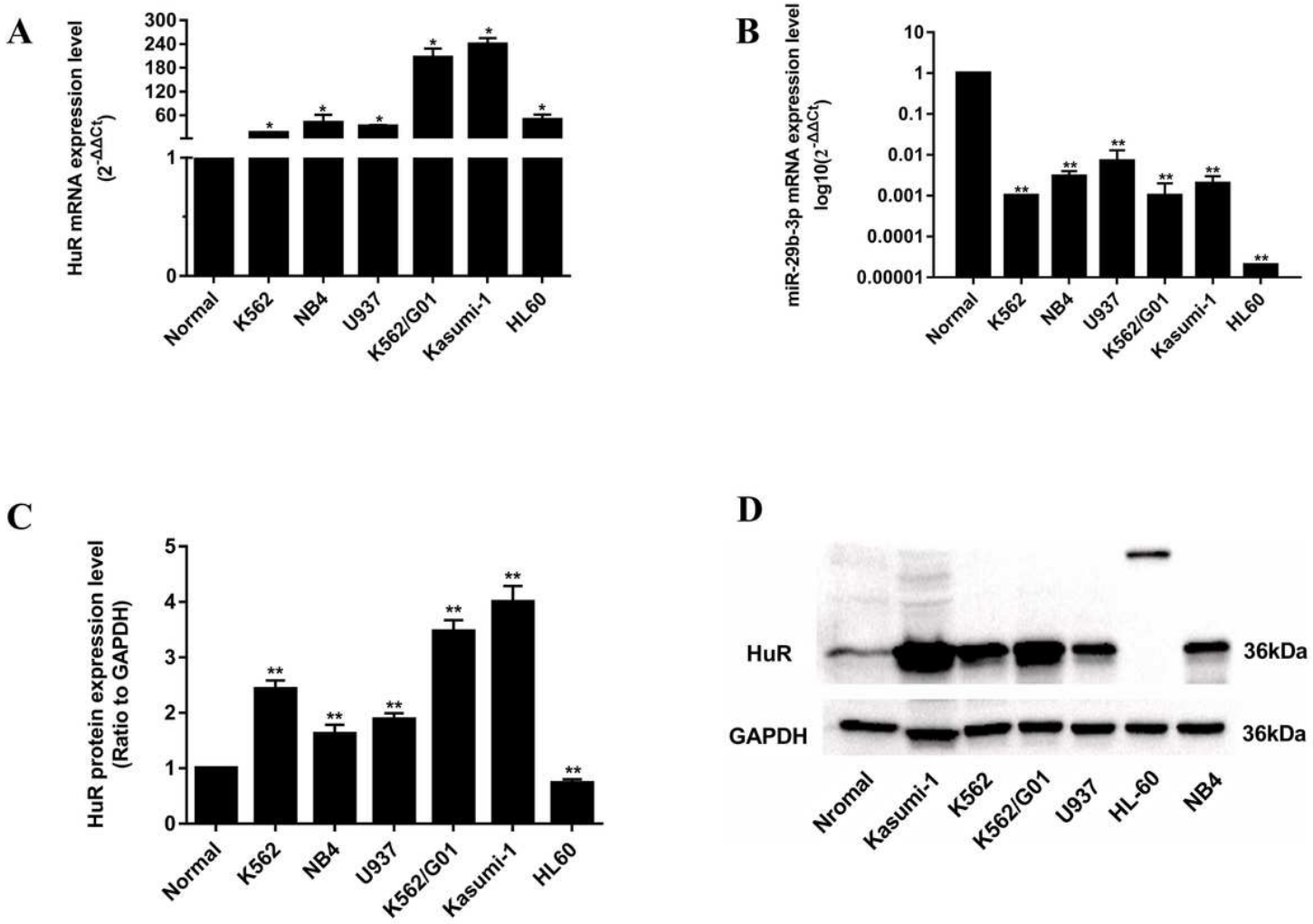

Figure 1

Expression of HuR and miR-29b-3p in AML cell lines detected by RT-qPCR and Western blot. (a, b) Expression of HuR and miR-29b-3p in mRNA level in AML cell lines, respectively. (c, d) Protein expression of HuR in AML cell lines. (*P<0.05, **P<0.01, vs. healthy normal control). The full-length blots/gels are presented in Supplementary Figure 1. Note: Normal, healthy normal control.

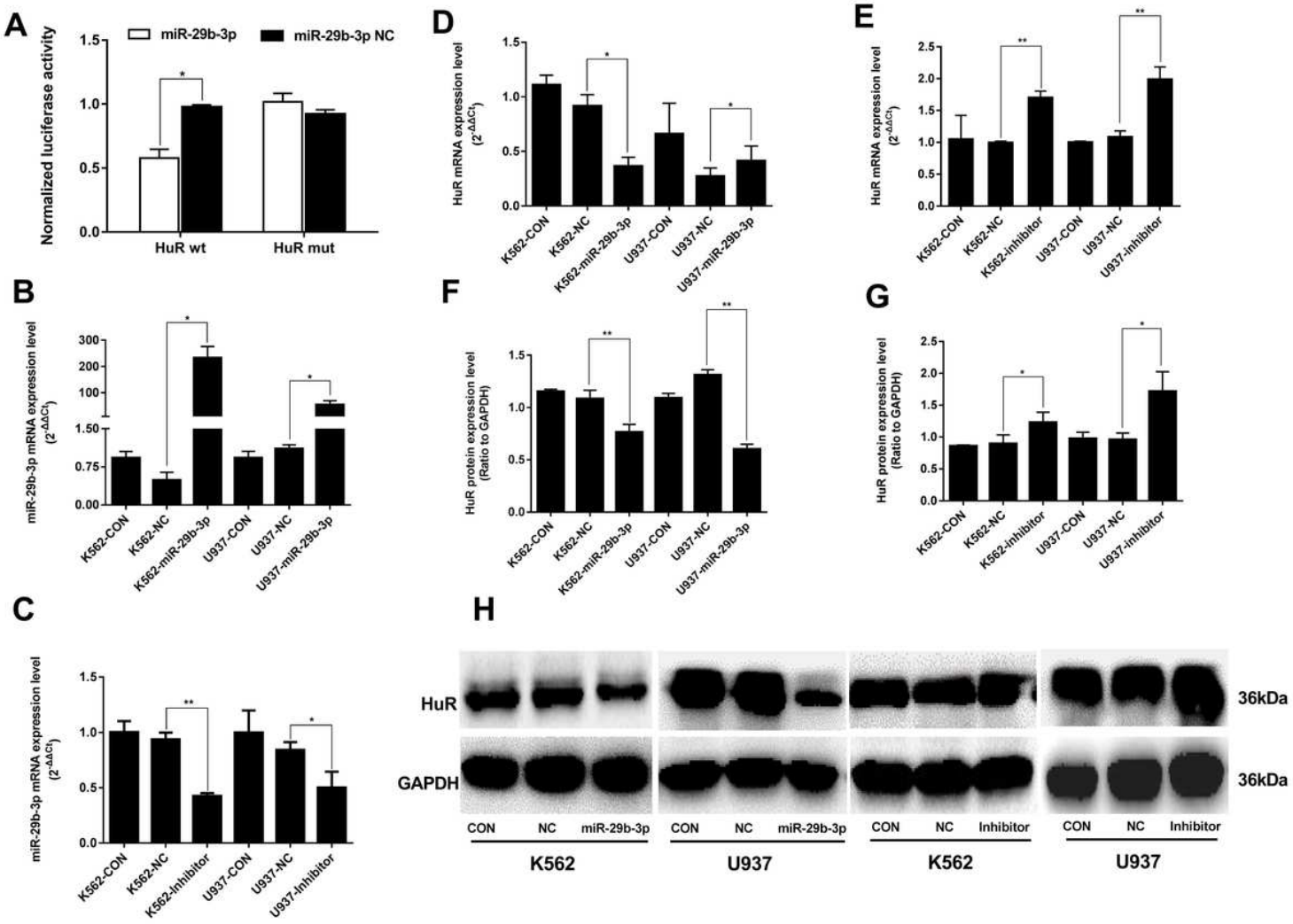

Figure 2

HuR was verifyied the downstream target of miR-29b-3p. (a) The dual luciferase activites assay of miR-29b-3p and HuR. (b, c)The mRNA expression levels of miR-29b-3p after up-regulating or inhibiting miR-29b-3p. (d, e) The mRNA expression of HuR after up-regulating or inhibiting miR-29b-3p. (f-h) Western blot of HuR expression were detected. (*P<0.05, **P<0.01, vs. NC group). The full-length blots/gels are presented in Supplementary Figure 2.

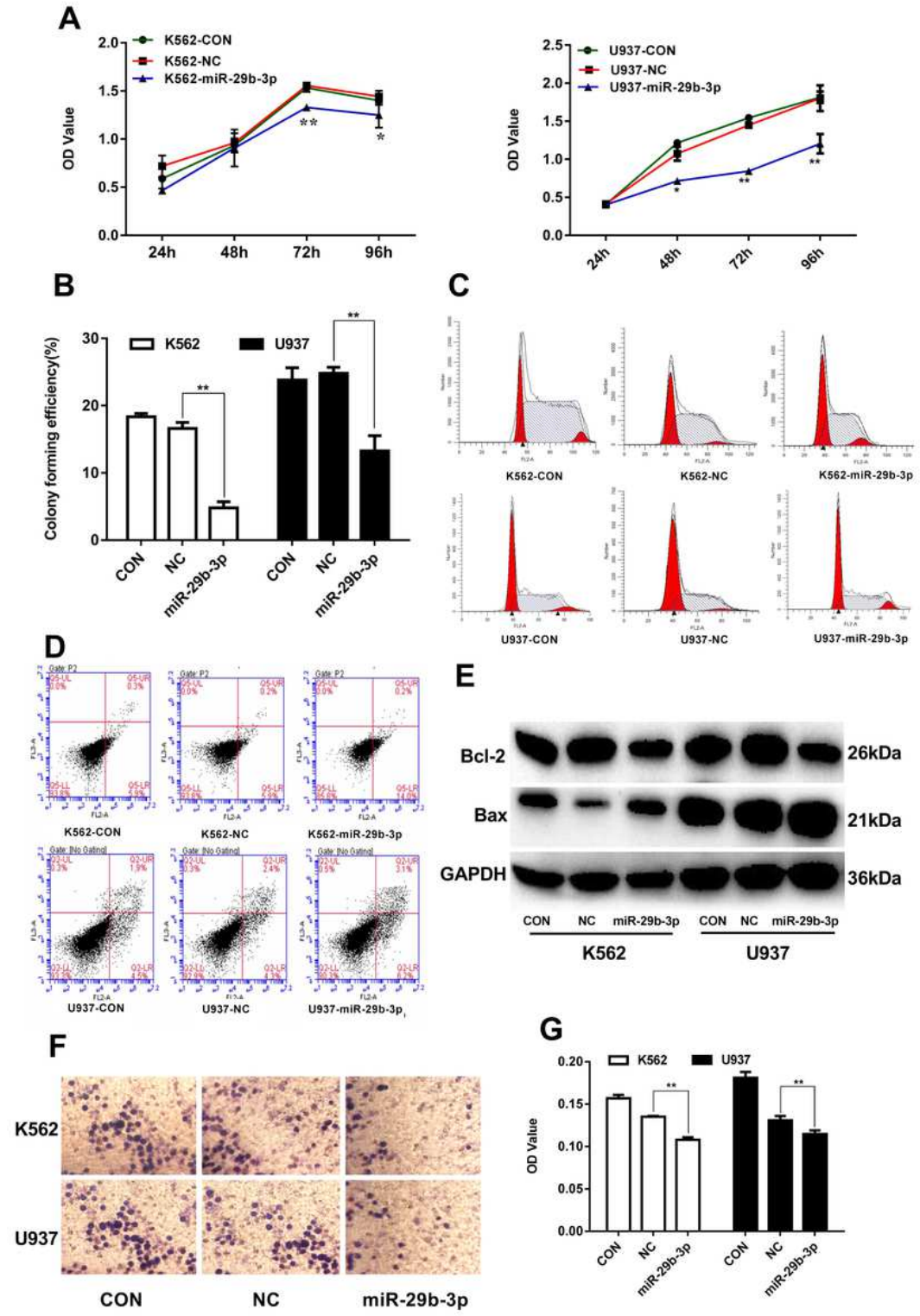

Figure 3

Biologic effects of HuR after overexpression miR-29b-3p evaluated in K562 and U937 cells. (a) Grow curve of proliferation based on the OD value after miR-29b-3p restoration at different time points, resceptivly. (b) Colonies containing ≥40 cells were counted on day 7~10 using a microscope (×200). (c) Cells were labeled by PI and analyzed using FCM. (d) Apoptotic cells were measured by FCM. Dot plots show 7-AAD (y-axis) vs. Annexin-V (x-axis). (e) The protein expression of BCL-2 and Bax were detected by

Western blot. (f) Wright-Giemsa stained invading cells were observed under microscope (×200). (g) The OD values (proportional to cell numbers) of migrating cells were measured by MTS assay. (*P<0.05, **P<0.01, vs. NC group).

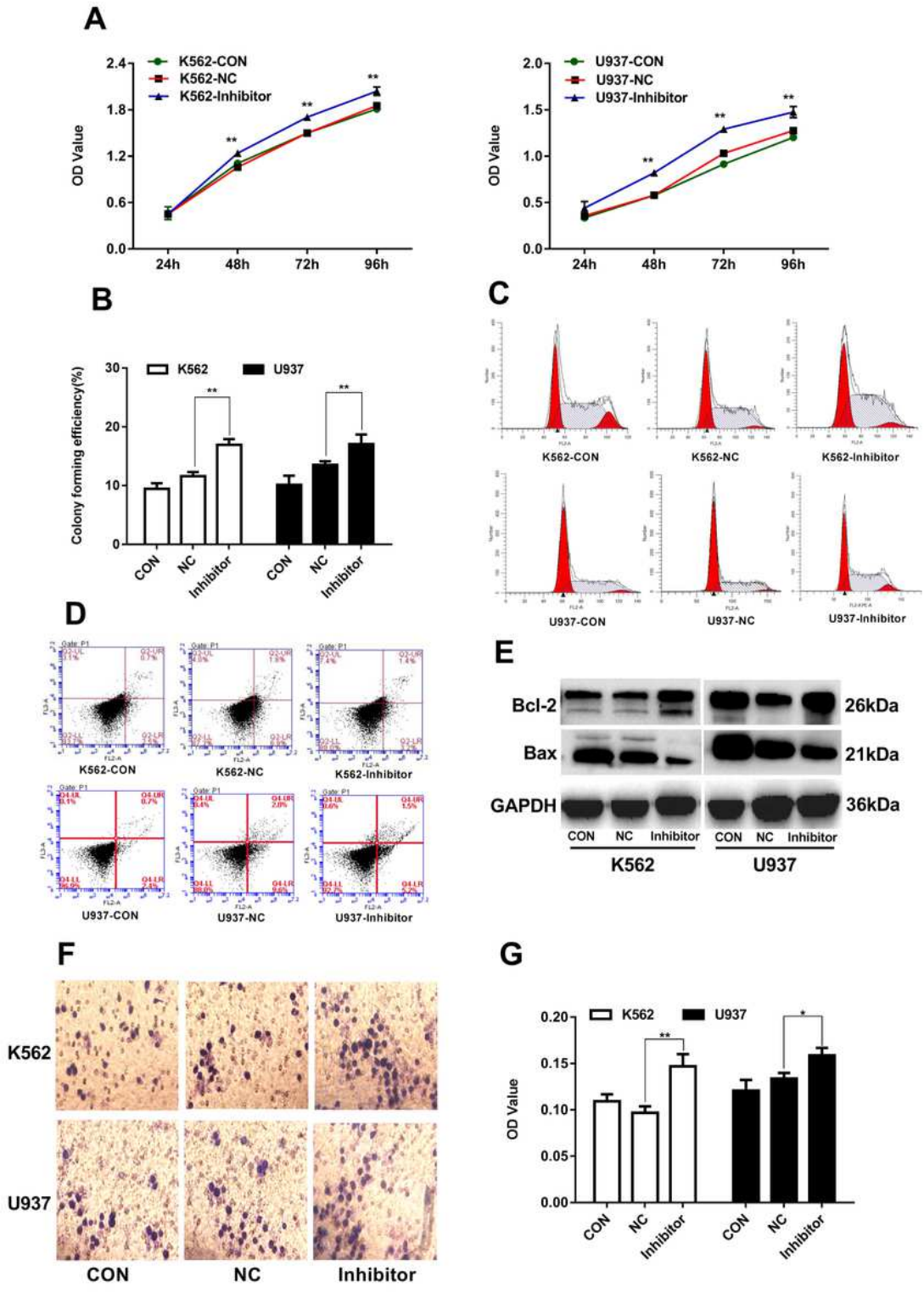

Figure 4

Biologic effects of HuR after inhibition miR-29b-3p evaluated in K562 and U937 cells. (a) Grow curve of proliferation based on the OD value after miR-29b-3p suppression at different time points, resceptivly. (b)

Colonies containing ≥40 cells were counted on day 7~10 using a microscope (×200). (c) Cells were labeled by PI and analyzed using FCM. (d) Apoptotic cells were measured by FCM. Dot plots show 7-AAD (y-axis) vs. Annexin-V (x-axis). (e) The protein expression of BCL-2 and Bax were detected by Western blot. The full-length blots/gels are presented in Supplementary Figure 3. (f) Wright-Giemsa stained invading cells were observed under microscope (×200). (g) The OD values (proportional to cell numbers) of migrating cells were measured by MTS assay. (*P<0.05, **P<0.01, vs. NC group).

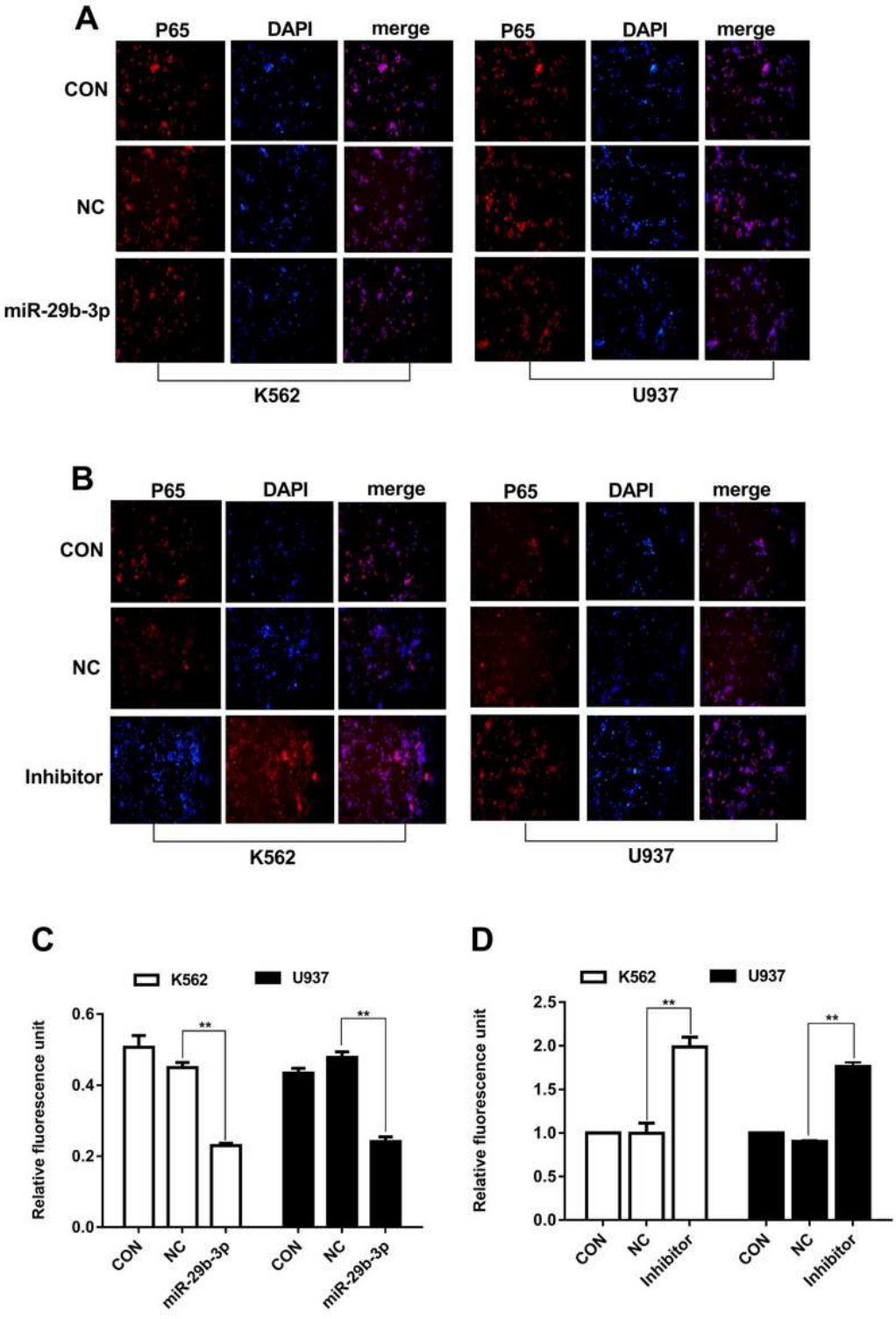

Figure 5

Effects of HuR targeting by miR-29b-3p on the expression of p65 were evaluated via cellular immunofluorescence experiment in K562 and U937 cells. (a, b) Fluorescent expressions of p65 were observed using fluorescence microscope (×200), images were longitudinally aligned reprented as total pattern, nuclear and merge pattern, respectivly. (c, d) Fluorescent expressions of merge pattern deviding by total pattern represented as the relative fluorescence rate of p65. (*P<0.05, **P<0.01, vs. NC group).

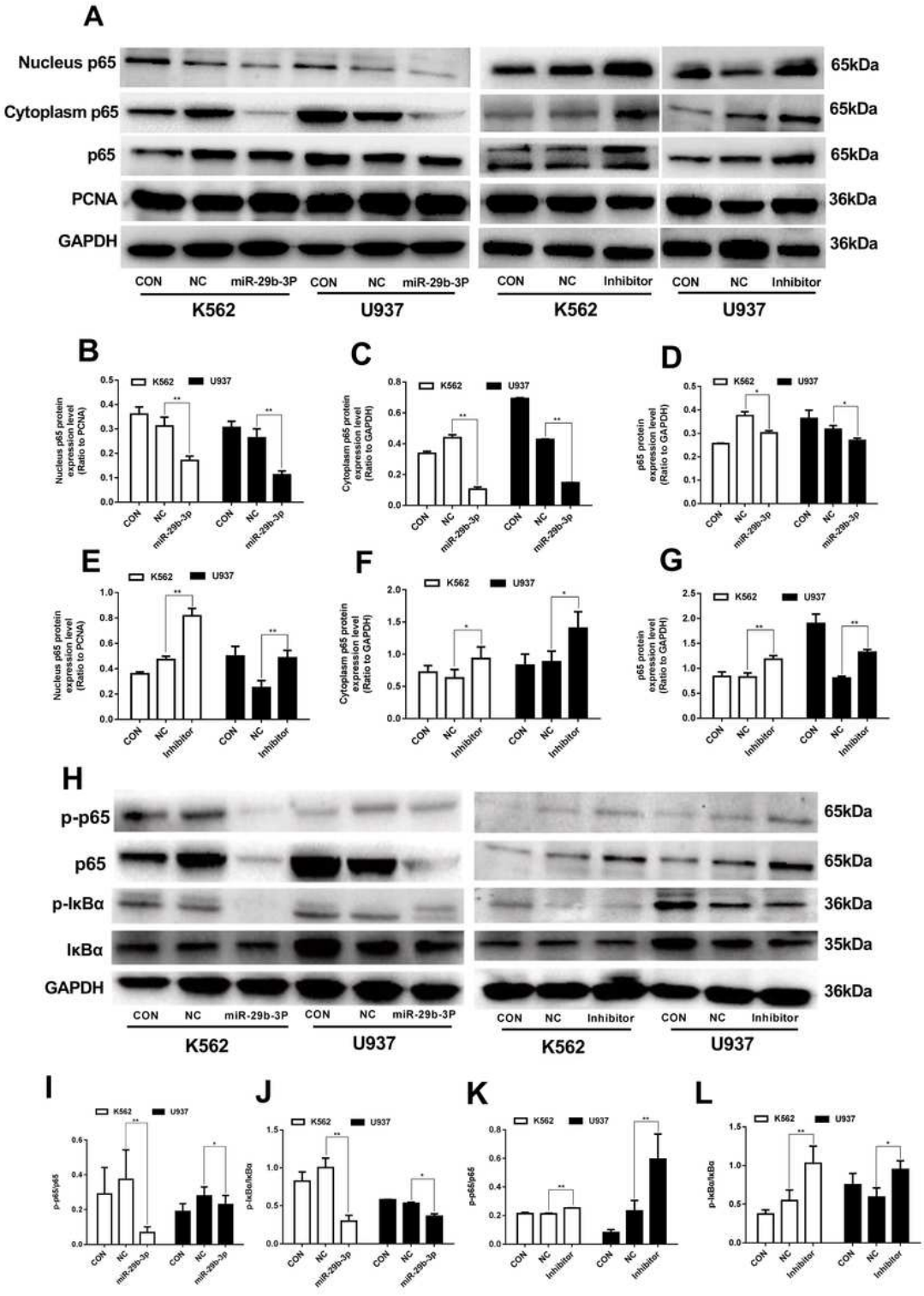

Figure 6

Effects of miR-29b-3p on the protein expression of p65 in in different cellular components and on the phosphorylation of p65 and IκBα expressed in K562 and U937 cells. (a) p65 at nuclear, cytosol and total cellular proteins were detected by western blot. The full-length blots/gels are presented in Supplementary Figure 4. (b–g) Quantification of nucleus p65 was normalized to PCNA, quantification of cytoplasm and total p65 were normalized to GAPDH. (*P<0.05, **P<0.01, vs. NC group). (h) p65, IκBα and their phosphorylation proteins expression were detected by western blot. (i-j) Quantification of phosphorylated p65 and phosphorylated IκBα were normalized to p65 and IκBα after miR-29b-3p overexpression, respectivly. (k-l) Quantifications of phosphorylated p65 and phosphorylated IκBα were normalized to p65 and IκBα after miR-29b-3p inhibition, respectivly. (*P<0.05, **P<0.01, vs. NC group).

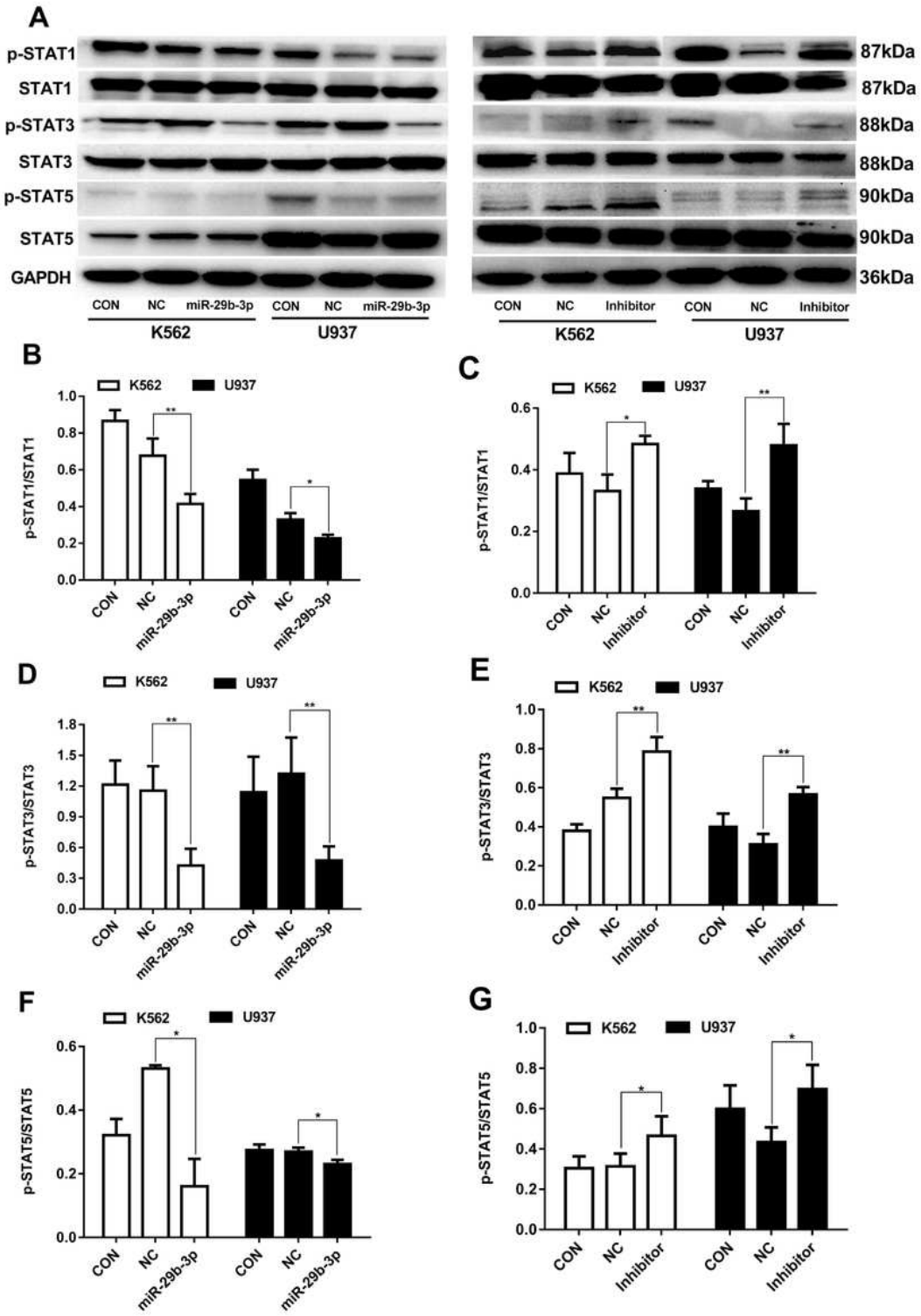

Figure 7

Effects of miR-29b-3p on the protein phosphorylation of STA1, STAT3 and STAT5 expressed in K562 and U937 cells. (a) STA1, STAT3, STAT5 and their phosphorylation proteins expression were detected by western blot. The full-length blots/gels are presented in Supplementary Figure 4. (b–g) Quantifications of phosphorylated STA1, STAT3, STAT5 were normalized to STA1, STAT3, STAT5 after miR-29b-3p overexpression and inhibition, respectivly. (*P<0.05, **P<0.01, vs. NC group).

Supplementary Files

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