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Nuclear factor I-C aggravates acute myelogenous leukemia by inhibiting SRY-box transcription factor 1 to activate autophagy

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Despite advances in chemoradiotherapy and hematopoietic stem cell transplantation, the treatment of acute myeloid leukemia (AML) remains challenging due to significant side effects and poor prognosis. This study aimed to investigate the role of nuclear factor I-C (NFIC) in AML progression by evaluating whether NFIC exacerbates AML through the inhibition of SRY-box transcription factor 1 (SOX1) and activation of autophagy, thereby providing potential insights for clinical treatment. NFIC and SOX1 expression levels in AML and normal samples were analyzed using bioinformatics, ELISA, RT-qPCR, and western blotting, and the interaction between NFIC and SOX1 was assessed through RNA pull-down and RNA-binding protein immunoprecipitation assays. Moreover, CCK-8 assay, FITC/PI apoptosis detection, immunofluorescence staining, RT-qPCR, and western blotting were conducted to assess cell viability, apoptosis, and the expression of NFIC, SOX1, Bax, Bcl-2, LC3-I, LC3-II, p62, and Beclin-1 following gene transfection. NFIC expression was significantly upregulated in AML samples while SOX1 expression was downregulated compared to normal controls. High NFIC levels were associated with poor prognosis in AML patients, and it was found to regulate SOX1 expression in KG-1 and NB4 cells negatively. Silencing NFIC or overexpressing SOX1 resulted in reduced cell viability and autophagy, and increased apoptosis in KG-1 and NB4 cells. Importantly, NFIC knockdown did not affect apoptosis in bone marrow mononuclear cells. The adverse effects of NFIC overexpression were reversed by SOX1 overexpression, highlighting the interplay between these factors in AML. This study demonstrates that NFIC promotes AML progression by activating autophagy and suppressing apoptosis in KG-1 and NB4 cells by inhibiting SOX1, providing a potential basis for therapeutic strategies targeting NFIC and SOX1 in AML.

Key words: acute myeloid leukemia; autophagy; Nuclear factor I-C; SRY-box transcription factor 1

Acute myeloid leukemia (AML) is a hematologic malignancy originating in the bone marrow, characterized by the uncontrolled proliferation of hematopoietic stem cells, and its incidence and associated mortality have increased in recent years [1]. AML occurs in individuals across all age groups, but its prognosis is particularly poor in elderly patients, with an overall 5-year survival rate of less than 21% [2]. The complex mechanisms are involved in the abnormal proliferation, survival, and differentiation of AML cells, such as cell apoptosis, autophagy, cell metabolism, DNA methylation modification, signal transduction, and chromatin remodeling [3]. Current clinical approaches for AML treatment primarily include chemotherapy, such as cytarabine combined with erythromycin, molecular targeted therapy, and allogeneic hematopoietic stem cell transplantation [4]. Despite advancements in molecular targeted therapies in recent years, the prognosis for AML patients remains unsatisfactory [5]. Furthermore, the high costs, severe toxic side effects of chemotherapy, and challenges in matching donors for hematopoietic stem cell transplantation restrict the widespread clinical application of these treatments [6]. Therefore, there is an urgent need to investigate the mechanisms underlying AML pathogenesis to identify new therapeutic strategies and improve clinical outcomes.

Autophagy is a unique and highly regulated cellular process in eukaryotic cells. Under conditions such as nutrient deprivation, growth factor deficiency, hypoxia, or endoplasmic reticulum stress, cells initiate autophagy by forming autophagosomes, which encapsulate misfolded proteins and damaged organelles. These autophagosomes then fuse with lysosomes to form autolysosomes, facilitating the degradation and recycling of cellular components [7, 8]. Under normal physi-

ological conditions, autophagy is maintained at a low basal level, responding to various stimuli to enhance cell survival in adverse environments [9]. However, excessive autophagy can cause organelle damage and even lead to autophagic cell death [10]. In AML, autophagy exhibits a dual role by both promoting cell survival and inducing cell death [11]. It was reported that neratinib, an anti-cancer agent, induced autophagy to inhibit proliferation and enhance apoptosis of AML cells [12]. Conversely, autophagy-related E1 ligase 7 can upregulate autophagy to prevent apoptosis in AML cells, leading to chemoresistance against cytarabine [13]. These findings highlight the essential role of autophagy regulation in AML progression and chemoresistance, emphasizing its potential as a target for therapeutic intervention.

Nuclear factor I (NFI) family transcription factors consist of four members: NFIA, NFIB, NFIC, and NFIX, all of which contain a highly conserved N-terminal DNA-binding domain [14]. Among these, NFIC, the first identified member of the NFI family, is located on human chromosome 19p13.3 and exerts its physiological effects by specifically regulating downstream gene expression [15]. While current research predominantly focuses on the role of NFIC in tooth development, emerging evidence suggests that NFIC also has significant regulatory functions in various cancers [16]. For instance, the downregulation of NFIC has been shown to promote epithelial-mesenchymal transition (EMT), proliferation, and migration in esophageal squamous cell carcinoma cells [17]. Similarly, the knockdown of NFIC enhances the proliferation of lung squamous cell carcinoma cells by modulating the expression of lncRNA CASC2 [18]. Additionally, NFIC has been reported to inhibit EMT, invasion, and migration in breast cancer [19]. In the context of AML, NFIC overexpression has been implicated in promoting disease progression, and its role in regulating autophagy has also been documented [20, 21]. SRY-box transcription factor 1 (SOX1), a member of the SOX gene family, contains a highly conserved DNA-binding domain and plays significant roles in embryonic and postnatal development [22]. Notably, SOX1 has been identified as an inhibitory factor in various cancers, including breast cancer, esophageal squamous cell carcinoma, cervical carcinoma, and colorectal carcinoma [23–26]. Despite these findings, the role of SOX1 in AML and its potential interaction with NFIC have not yet been elucidated.

In this study, we aimed to determine whether NFIC aggravates AML by activating autophagy through targeting SOX1 to provide novel insights into the molecular mechanisms underlying AML progression and identify potential targets for its clinical treatment.

Patients and methods

Bioinformatics analysis. RNA sequencing data were obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) database (https://gepia.cancer-pku.cn/detail.php) to compare NFIC expression levels between normal tissues (n=70) and AML tissues (n=173). Kaplan-Meier survival analysis was performed to assess the prognostic impact of NFIC expression levels, and Spearman correlation analysis was employed to examine the relationship between NFIC and SOX1 expression levels.

Patients and clinical specimens. A total of 30 patients (including 16 PML-RARA positive patients, 10 AML1-ETO positive patients, and 4 patients with other karyotypes) were diagnosed with AML in our hospital from May 2021 to May 2023, and 10 normal control ones were selected. The inclusion criteria were: 1) diagnosis of AML based on the NCCN Clinical Practice Guidelines in Oncology (Version 3.2019) [27], and 2) availability of complete clinical data. The exclusion criteria were: 1) diagnosis of other types of leukemia, 2) presence of other malignant tumors, and 3) pregnancy or lactation. Blood samples were collected before treatment using a 21-gauge needle and BD Vacutainer® tubes and were centrifuged at 3,000 × g for 10 min to isolate sera. Written informed consent was obtained from all participants, and ethical approval for this study was obtained from the Ethics Committee of The First Affiliated Hospital, Hengyang Medical School, University of South China.

ELISA. The levels of NFIC in serum and human bone marrow mononuclear normal cells (BMMNCs), as well as SOX1 in serum, were determined using human ELISA kits for NFIC (#MBS7201183) and SOX1 (#MBS006666, MyBioSource, San Diego, USA), following the manufacturer's instructions. Briefly, 100 µl serum samples and BMNC lysates (prepared after ice bath cracking and centrifugation) were first added to wells pre-coated with corresponding antibodies. Then, NFIC-HRP conjugate (or HRP-conjugated antibody) was supplemented to each well, and the plates were incubated at 37 °C for 1 h. After washing the plates five times, substrate solutions A and B were added sequentially to each well and incubated for 15 min at 37 °C. The reaction was terminated with a stop solution, and the optical density (OD) values at 450 nm were measured using a microplate reader (Molecular Devices Spectra MAX Plus 384, Molecular Devices, San Jose, USA) to determine NFIC and SOX1 levels.

Cell culture and cell transfection. Human BMMNCs and human AML cell lines, including OCI-AML3, KG-1, Kasumi-1, NB4, ME-1, and MOLM-14 cells, purchased from the Chinese Academy of Sciences, were cultured in RPMI 1640 medium (#11875093, Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (#A5670701, Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Transfection was performed using Lipofectamine 2000 (#11668500, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. The following constructs were synthesized and obtained from Aibosi Life Technology: sh-NC (5'-TTCTCCGAACGTGTCACGT-3'), sh-NFIC-1 (5'-GATGGACAAGTCACCATTCAA-3'), sh-NFIC-2 (5'-CCCGGTGAAGAAGACAGAGAT-3'),

oe-NC (pcDNA3.1), oe-NFIC (pcDNA3.1-NFIC, NM_001245002.2, GenBank™), and oe-SOX1 (pcDNA3.1-SOX1, NM_005986.3, GenBank™). These constructs were transfected into the AML cells and incubated for 48 h for subsequent experiments.

Cell experiment protocol. To compare the gene and protein expression levels of NFIC between BMMNCs and AML cell lines, all cells were divided into seven groups: the BMMNC group, OCI-AML3 group, KG-1 group, Kasumi-1 group, NB4 group, ME-1 group, and MOLM-14 group. To assess the effects of NFIC downregulation on BMMNCs and AML cells, specifically KG-1 and NB4 cells, the cells were randomly assigned to three groups: the sh-NC group (negative control), the sh-NFIC-1 group, and the sh-NFIC-2 group. To determine whether NFIC promotes AML progression by targeting SOX1, KG-1, and NB4 cells were further divided into four groups: the oe-NC group (negative control), the oe-NFIC group (NFIC overexpression), the oe-SOX1 group (SOX1 overexpression), and the oe-NFIC+oe-SOX1 group (co-overexpression of NFIC and SOX1). All transfections were performed using the corresponding constructs.

Cell viability assay. Cell viability was evaluated using the CCK-8 assay. Following transfection, KG-1, and NB4 cells were incubated with the CCK-8 reagent (#CA1210, Solarbio, Beijing, China) at 37 °C, and the optical density (OD) values were measured using a microplate reader at 450 nm to determine cell viability.

Cell apoptosis assay. The apoptosis rates of BMMNCs, KG-1, and NB4 cells were determined using the FITC/PI apoptosis detection kit (#556547, BD Biosciences, California, USA). Briefly, after transfection, the cells were collected and resuspended in 100 μl of 1× binding buffer. Subsequently, 5 μl of Annexin V-FITC and 5 μl of PI dye were added to the suspension, and the cells were incubated in the dark for 15 min at room temperature. Following incubation, 400 μl of 1× binding buffer was added to each sample. Apoptosis rates were then measured using a flow cytometer (CytoFLEX, Beckman Coulter, Brea, USA).

Immunofluorescence (IF) staining. After transfection, the KG-1 and NB4 cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 5% bovine serum albumin (BSA) to reduce nonspecific binding and incubated overnight at 4 °C with the primary antibody against LC3 (1: 500, #AF5402, Affinity, Ohio, USA). The following day, the cells were incubated with fluorescently labeled secondary antibodies for 30 min at room temperature in the dark. Then, their nuclei were counterstained with DAPI solution for 15 min, and lastly, the stained cells were visualized and imaged using a fluorescence microscope (Laite LF50, Laite, Guangzhou, China).

RNA pull-down assay. To perform the RNA pull-down assay, the TranscriptAid T7 High Yield Transcription Kit (#K0441, Thermo Fisher Scientific) was first used to synthesize NFIC-sense and NFIC-antisense RNAs. After that, the biotin was labeled onto the surface of target RNAs to generate biotin-labeled RNA probe complexes with the help of the Pierce™ RNA 3' end desulfurization biotinylation kit (#20163, Thermo Fisher Scientific). The biotin-labeled RNAs were bound to streptavidin-agarose beads (#88816, Thermo Fisher Scientific) and subsequently incubated with protein extracts from KG-1 and NB4 cells. After washing, the RNA-protein complexes were boiled in sodium dodecyl sulfate (SDS) buffer to release bound proteins, and the extracted proteins were analyzed using western blotting.

RNA binding protein immunoprecipitation (RIP) assay. The RIP assay was conducted using the Magna RIP reagent kit (#17-704, Millipore, Beverly, USA), following the manufacturer's instructions. Briefly, KG-1 and NB4 cells were lysed using RIPA buffer containing RNase inhibitors. The cell lysates were incubated overnight at 4 °C with magnetic beads conjugated to anti-SOX1 (#DF8196, Affinity, Ohio, USA) or anti-IgG (#ab133470, Abcam, Cambridge, UK) antibodies. The immunoprecipitated complexes were released from the beads using proteinase K for 30 min. Total RNA was then extracted from the complexes using the TRIzol reagent. The enrichment of NFIC RNA was quantified using RT-qPCR.

Real-time quantitative PCR (RT-qPCR). Total RNA was extracted from BMMNCs, OCI-AML3, KG-1, Kasumi-1, NB4, ME-1, and MOLM-14 cells using the TRIzol reagent (#R0016, Beyotime, Shanghai, China). The extracted RNA was reverse transcribed into cDNA using a reverse transcription kit (#4366596, Invitrogen, California, USA). RT-qPCR was performed using 2× Taq PCR Master Mix (#FY16606, Feiyu Bio, Nantong, China) with specific primers (Table 1) on a QuantStudio 3 RT-qPCR instrument (Thermo Fisher Scientific). The relative expression levels of NFIC and SOX1 were quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blot. Proteins from BMMNCs, OCI-AML3, KG-1, Kasumi-1, NB4, ME-1, and MOLM-14 cells were extracted using RIPA lysis buffer and denatured by boiling. The denatured proteins were separated using SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with skim milk and incubated overnight at 4 °C with primary antibodies, including anti-NFIC (1: 1000, #ab228909), anti-SOX1 (1: 1000, #DF8196), anti-Bax (1: 2000, #AF0120), anti-Bcl-2 (1: 2000, #AF6139), anti-p62 (1:

Table 1. Primer sequences used for RT-qPCR.

Genes	Forward (5'-3')	Reverse (5'-3')
NFIC	CGACTTCCAGGAGAGCTTTG	GTTCAGGTCGTATGCCAGGT
SOX1	GAGATTCATCTCAGGATTGAGATTCTA	GGCCTACTGTAATCTTTTCTCCACT
GAPDH	TCCAGAGTGCAAGGCTTCAG	ACAGCACGCAGTAGCA

1000, #AF5384), anti-Beclin-1 (1: 1000, #AF5128), anti-LC3 (1: 1000, #AF5402), and anti-GAPDH (1: 2000, #AF7021). Except for anti-NFIC, which was purchased from Abcam, all other primary antibodies were bought from Affinity. After incubation with appropriate HRP-conjugated secondary antibodies, the membranes were treated with ECL chemiluminescent substrate (#G2161, Servicebio, Wuhan, China). The protein bands were visualized, and their grayscale intensity was analyzed using ImageJ software (version 1.8.0.112).

Statistical analysis. All experiments were performed independently in triplicate, and the results are presented as mean ± standard deviation. Statistical analysis was conducted using GraphPad Prism software (version 8.0.2). Differences between groups were evaluated using one-way analysis of variance (ANOVA), and statistical significance was determined at $p<0.05$.

Results

NFIC is highly expressed in AML patients and AML cell lines. To investigate the expression level of NFIC in AML, RNA sequencing data were obtained from the GEPIA database. A comparison between normal tissues (n=70) and AML tissues (n=173) revealed that NFIC expression was significantly higher in AML tissues than in normal tissues (Figure 1A). To validate these findings, the NFIC levels in clinical specimens were further examined using ELISA, and the results confirmed that NFIC levels in the serum of AML patients were significantly elevated compared to those in healthy controls (Figure 1B). Additionally, NFIC levels were analyzed in AML patients with different fusion genes. Among the 30 AML patients, 16 had PML-RARα fusion genes, 10 had AML1-ETO fusion genes, and 4 had other fusion genes. Regardless of the fusion gene type, NFIC levels in all patient groups were significantly higher than those in healthy controls (Supplementary Figure S1A). To assess the prognostic value of NFIC, Kaplan-Meier survival analysis was performed. As shown in Figure 1C and Supplementary Figure S1B, patients with high NFIC expression exhibited significantly lower progression-free survival and overall survival rates compared to those with low NFIC expression over a 60-month follow-up period. These findings suggest that high NFIC expression is associated with a poor prognosis in AML patients.

To further evaluate NFIC expression, its gene and protein levels were compared between AML cell lines and normal BMMNCs. RT-qPCR and western blot analyses demon-

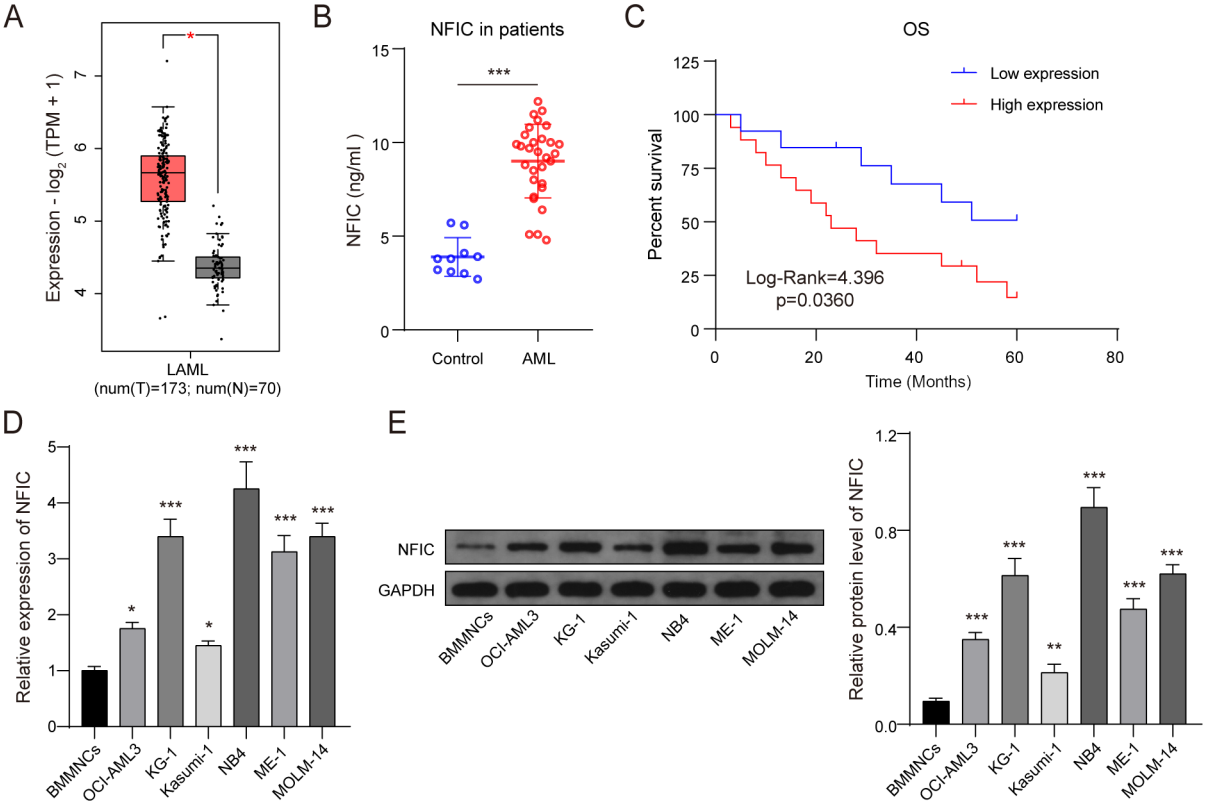

Figure 1. NFIC is highly expressed in AML patients and AML cell lines. A) Comparison of NFIC expression levels between AML tissues (n=173) and normal tissues (n=70). B) Comparison of NFIC levels in serum between AML patients (n=30) and healthy controls (n=10). C) Kaplan-Meier survival analysis showing the effect of NFIC expression on AML prognosis. D) Comparison of NFIC gene expression levels between BMMNCs and AML cell lines (n=3). E) Comparison of NFIC protein expression levels between BMMNCs and AML cell lines (n=3). */**/*p<0.001/0.01/0.05 vs. the first group**

strated that NFIC gene and protein expression were significantly upregulated in AML cell lines, including OCI-AML3, KG-1, Kasumi-1, NB4, ME-1, and MOLM-14, compared to BMMNCs (Figures 1D, 1E). Notably, among the AML cell lines tested, NB4 and KG-1 cells exhibited the highest NFIC expression levels. Based on these observations, NB4 and KG-1 cells were selected for subsequent experiments. Collectively, these results demonstrate that NFIC is highly expressed in AML patients and cell lines, and its elevated expression is closely associated with poor prognosis in AML.

Effects of knocking down NFIC on KG-1 and NB4 cells. To verify the successful transfection of sh-NC, sh-NFIC-1, and sh-NFIC-2 into KG-1 and NB4 cells, the gene and protein expression levels of NFIC were analyzed. As shown in Figures 2A and 2B, the gene and protein expression levels of NFIC in the sh-NFIC-1 and sh-NFIC-2 groups were significantly reduced compared to the sh-NC group, confirming that sh-NC, sh-NFIC-1, and sh-NFIC-2 were successfully transfected into KG-1 and NB4 cells. Given that NFIC expression was elevated in AML, the effects of NFIC knockdown on cell viability, apoptosis, and autophagy were further investigated in KG-1 and NB4 cells. CCK-8 assay results revealed that the cell viability of KG-1 and NB4 cells in the sh-NFIC-1 and sh-NFIC-2 groups was significantly lower than in the sh-NC group over 72 hours (Figure 2C). Flow cytometry analysis showed that the apoptosis rates of KG-1 and NB4 cells were significantly higher in the sh-NFIC-1 and sh-NFIC-2 groups compared to the sh-NC group (Figure 2D). Immunofluorescence staining for LC3 demonstrated that the fluorescence intensity of LC3-positive areas was notably weaker in the sh-NFIC-1 and sh-NFIC-2 groups than in the sh-NC group, indicating reduced autophagy levels (Figure 2F). Furthermore, western blot analysis revealed significant changes in the expression of apoptosis and autophagy-related proteins. Specifically, the protein expression levels of Bax and p62 were significantly upregulated, while the expression levels of Bcl-2, LC3-II/LC3-I, and Beclin-1 were significantly downregulated in the sh-NFIC-1 and sh-NFIC-2 groups compared to the sh-NC group (Figures 2E, 2G). Moreover, NFIC knockdown did not affect the apoptosis rate of BMMNCs (Supplementary Figures S2A, S2B). These findings demonstrate that NFIC knockdown inhibits cell viability and autophagy while promoting apoptosis in KG-1 and NB4 cells, suggesting that NFIC plays an important role in the survival and autophagic activity of AML cells.

NFIC targets and inhibits the expression of SOX1. Analysis of clinical specimens demonstrated that the level of SOX1 in the serum of AML patients was significantly lower than that in the serum of healthy controls (Figure 3A). Furthermore, Spearman correlation analysis indicated a negative correlation between NFIC and SOX1 expression (Figure 3B). To determine whether NFIC directly targets and regulates SOX1 in AML, RNA pull-down and RNA-binding protein immunoprecipitation (RIP) assays were performed. As shown in Figures 3C and 3D, the SOX1 protein in KG-1 and NB4 cells was pulled down by the NFIC probe, and the NFIC mRNA was enriched in the complex immunoprecipitated by the SOX1 antibody. In addition, the gene and protein expression levels of SOX1 in KG-1 and NB4 cells were significantly higher in the sh-NFIC-1 and sh-NFIC-2 groups compared to the sh-NC group (Figures 3E, 3F). These results suggest that NFIC targets and inhibits the expression of SOX1 in AML cells.

Effects of overexpressing NFIC and SOX1 on KG-1 and NB4 cells. To confirm the successful transfection of oe-NC, oe-NFIC, and oe-SOX1 into KG-1 and NB4 cells, the gene and protein expression levels of SOX1 were first measured. As shown in Figures 4A and 4B, compared to the oe-NC group, SOX1 gene and protein expression levels were significantly downregulated in the oe-NFIC group and dramatically upregulated in the oe-SOX1 group. Furthermore, in the oe-NFIC + oe-SOX1 group, SOX1 expression levels were significantly lower than those in the oe-SOX1 group. These findings confirm the successful transfection of oe-NC, oe-NFIC, and oe-SOX1 into KG-1 and NB4 cells.

The effects of NFIC and SOX1 overexpression on cell viability, apoptosis, and autophagy in KG-1 and NB4 cells were further investigated. Compared to the oe-NC group, the cell viability of KG-1 and NB4 cells was significantly increased in the oe-NFIC group but substantially decreased in the oe-SOX1 group (Figure 4C). Conversely, the apoptosis rate was markedly reduced in the oe-NFIC group but dramatically elevated in the oe-SOX1 group (Figure 4D). Immunofluorescence staining showed that LC3 fluorescence intensity, reflecting autophagy activity, was significantly enhanced in the oe-NFIC group but prominently reduced in the oe-SOX1 group compared to the oe-NC group (Figure 4F). Western blot analysis further corroborated these findings, demonstrating that compared to the oe-NC group, the protein expression levels of Bax and p62 were significantly reduced in the oe-NFIC group but notably increased in the oe-SOX1 group. In contrast, the expression levels of Bcl-2, LC3-II/LC3-I, and Beclin-1 were significantly elevated in the oe-NFIC group but substantially decreased in the oe-SOX1 group (Figures 4E, 4G). Interestingly, the co-transfection of oe-NFIC and oe-SOX1 effectively reversed the effects of SOX1 overexpression on cell viability, apoptosis, and autophagy in KG-1 and NB4 cells (Figures 4C–4G). Taken together, these results demonstrate that overexpressing NFIC promotes cell viability and autophagy while inhibiting apoptosis in KG-1 and NB4 cells, primarily by suppressing SOX1.

Discussion

AML is a highly heterogeneous hematological malignancy characterized by clonal proliferation disorders of hematopoietic stem cells and is the most common type of acute leukemia in adults [28]. Its incidence has been reported to increase with age, with patients over 60 years old accounting for more than 50% of all AML cases, and the median age of

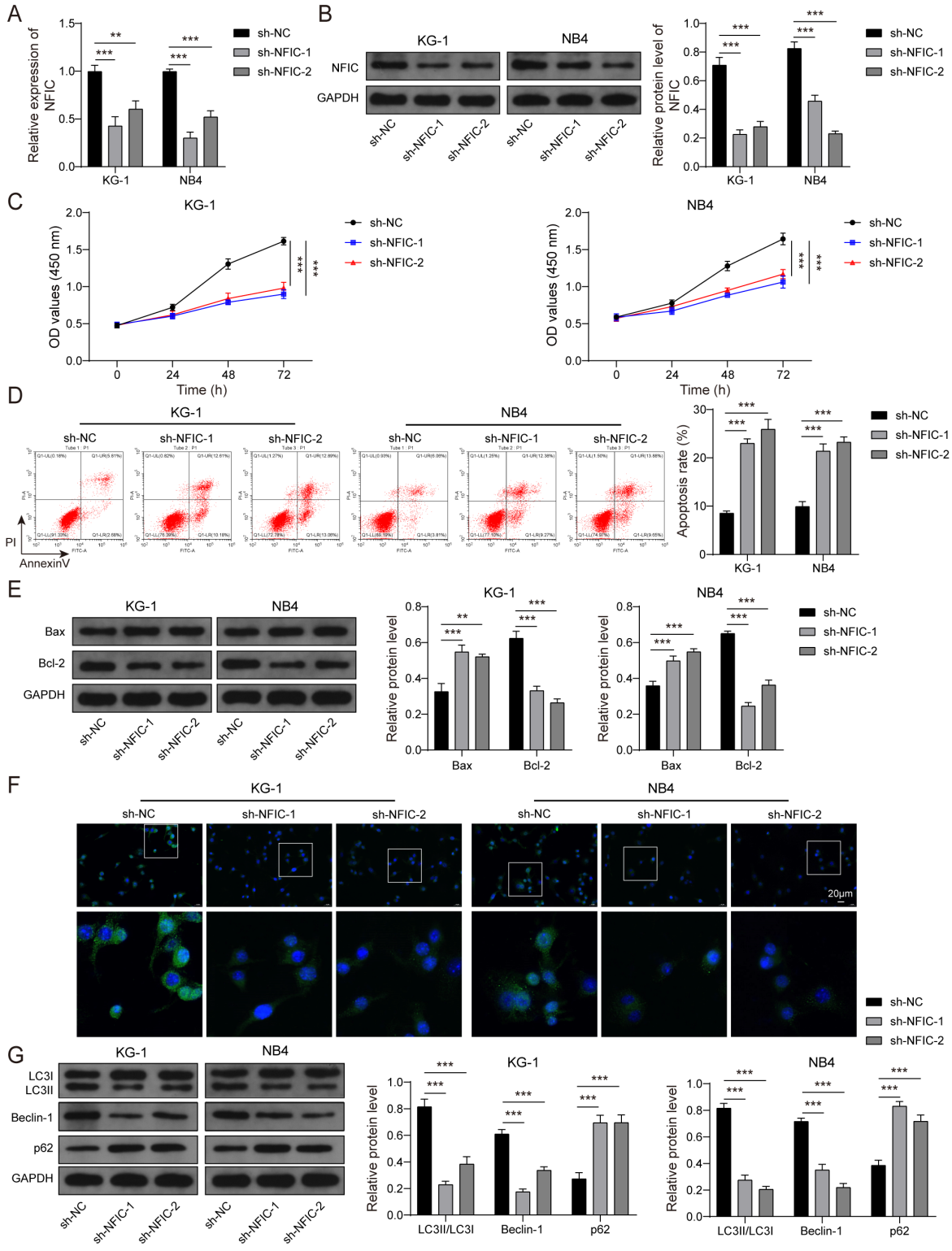

Figure 2. Effects of knocking down NFIC on KG-1 and NB4 cells. A) NFIC gene expression levels in KG-1 and NB4 cells after transfection with sh-NC, sh-NFIC-1, and sh-NFIC-2 (n=3). B) NFIC protein expression levels in KG-1 and NB4 cells after transfection with sh-NC, sh-NFIC-1, and sh-NFIC-2 (n=3). C) Cell viability of KG-1 and NB4 cells after NFIC knockdown (n=3). D) Apoptosis rates of KG-1 and NB4 cells after NFIC knockdown (n=3). E) Protein expression levels of Bax and Bcl-2 in KG-1 and NB4 cells after NFIC knockdown (n=3). F) Immunofluorescence analysis of LC3 in KG-1 and NB4 cells after NFIC knockdown. G) Protein expression levels of LC3-I, LC3-II, p62, and Beclin-1 in KG-1 and NB4 cells after NFIC knockdown (n=3). */**p<0.001/0.01 vs. the sh-NC group**

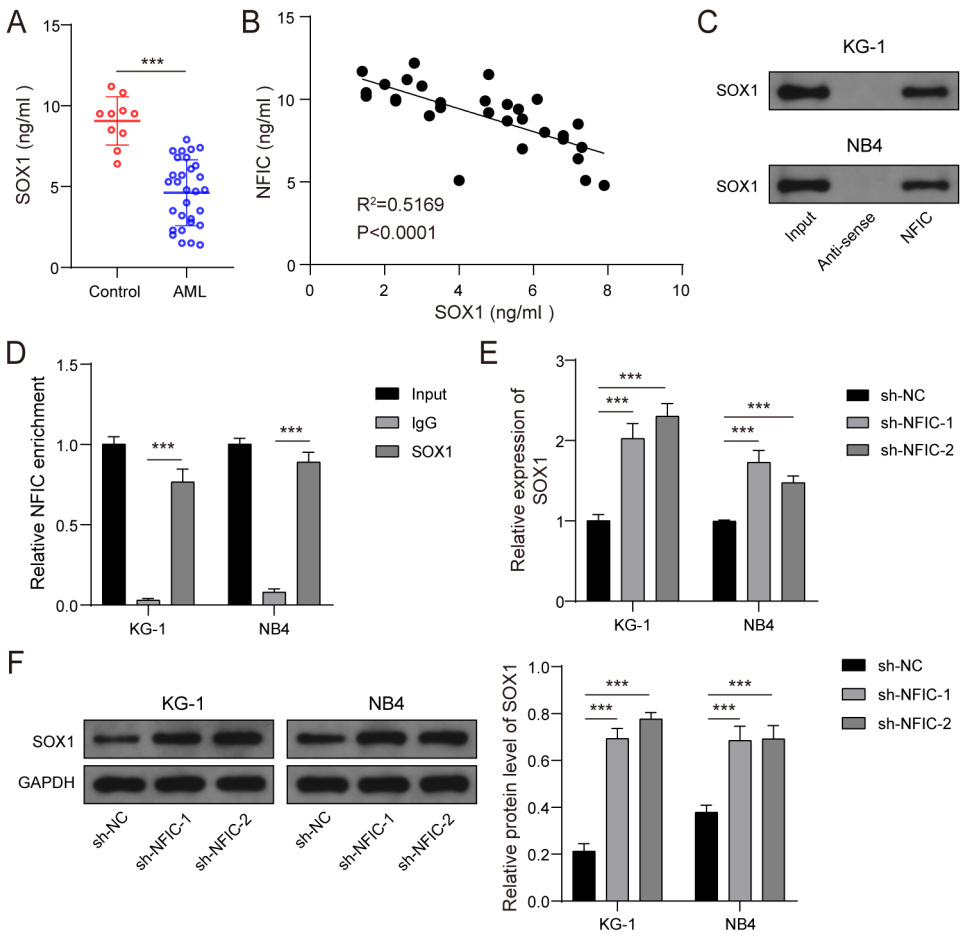

Figure 3. NFIC targets and inhibits the expression of SOX1. A) Comparison of SOX1 levels in serum between AML patients (n=30) and healthy controls (n=10). B) Spearman correlation analysis showing the relationship between NFIC and SOX1 expression levels. C) RNA pull-down assay results showing the interaction between NFIC and SOX1 in KG-1 and NB4 cells. D) RIP assay results confirming the interaction between NFIC and SOX1 (n=3). E) SOX1 gene expression levels in KG-1 and NB4 cells after NFIC knockdown (n=3). F) SOX1 protein expression levels in KG-1 and NB4 cells after NFIC knockdown (n=3). ***p<0.001 vs. the first group

onset being approximately 68 years [29]. In elderly individuals, the reduced number and impaired function of bone marrow stem cells and disruptions in the proliferation and differentiation of hematopoietic stem cells contribute to AML development [30]. Furthermore, the immune function of elderly AML patients is often compromised, weakening their anti-tumor immune response. This promotes immune escape, proliferation, and metastasis of AML cells [31]. The excessive proliferation of AML cells further exacerbates hematopoietic dysfunction. On the one hand, it disrupts the production of essential blood cells, including red blood cells, white blood cells, and platelets, leading to anemia and a bleeding tendency. On the other hand, it impairs the immune system, making patients more susceptible to infections caused by various pathogens [32]. Although chemotherapy and hematopoietic stem cell transplantation remain the primary treatment strategies for AML, these therapies are associated with significant side effects and a high recurrence rate [33]. Therefore, investigating the molecular mechanisms underlying AML progression is essential to identify novel targets for more effective and safer clinical treatments. In this present study, the relative mechanisms of AML were investigated to provide a novel insight into its clinical treatment.

Over the past decade, there has been increasing research on the role of NFIC in cancer; however, its effects vary among different cancer types. For instance, NFIC expression was reported to be markedly upregulated in gastric cancer (GC), where its overexpression further promoted GC progression [34, 35]. In contrast, previous studies have demonstrated that NFIC activation inhibits the proliferation, migration, and invasion of other cancers, such as bladder cancer and breast cancer [36, 37]. More importantly, a previous study identified NFIC as a potential target that is also significantly overexpressed in AML [21]. Consistent with these findings, our study confirmed through bioinformatics analysis, clinical specimens, and *in vitro* cellular experiments that NFIC is promi-

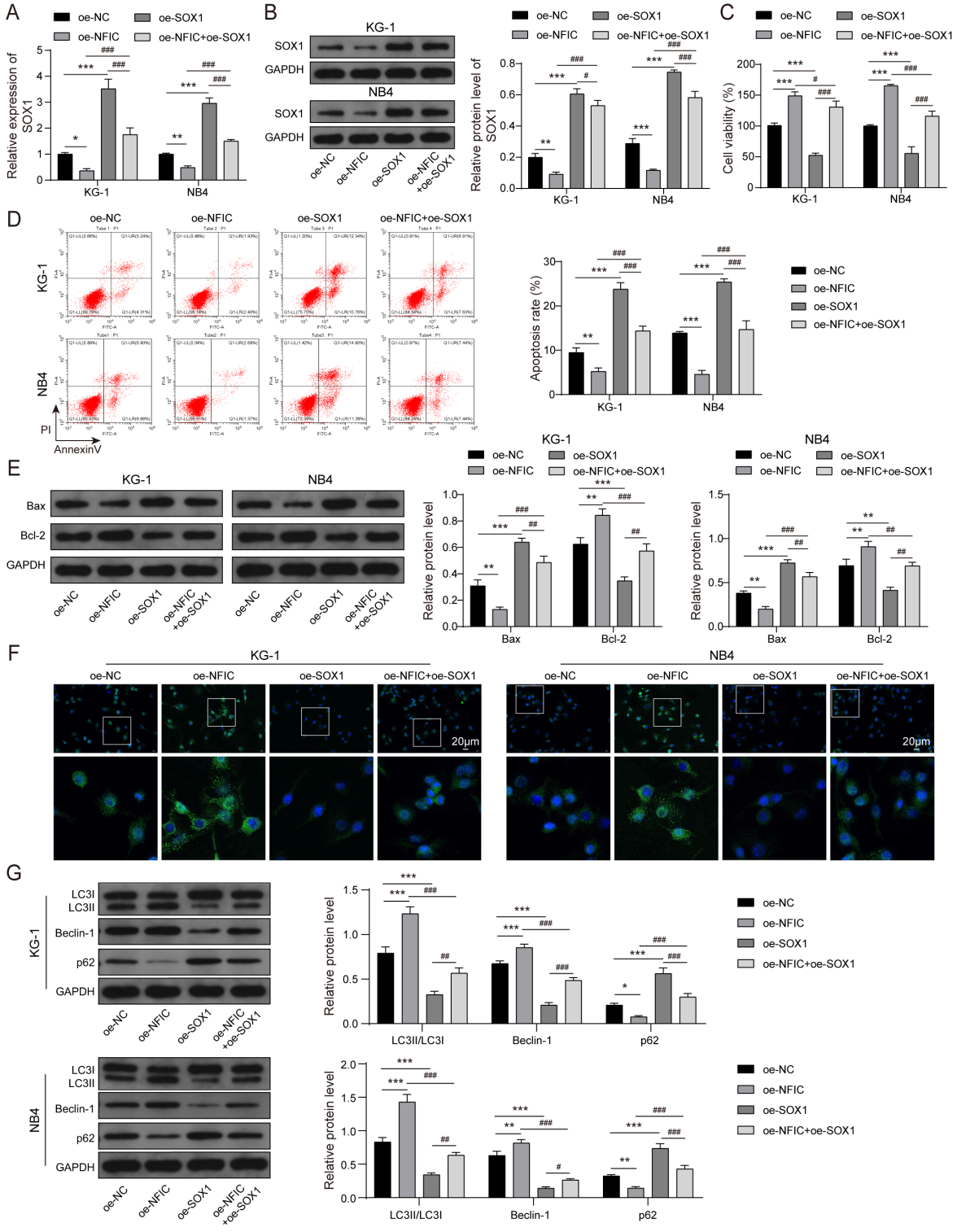

Figure 4. Effects of overexpressing NFIC and SOX1 on KG-1 and NB4 cells. A) SOX1 gene expression levels in KG-1 and NB4 cells after overexpression of NFIC and SOX1 (n=3). B) SOX1 protein expression levels in KG-1 and NB4 cells after overexpression of NFIC and SOX1 (n=3). C) Cell viability of KG-1 and NB4 cells after overexpression of NFIC and SOX1 (n=3). D) Apoptosis rates of KG-1 and NB4 cells after overexpression of NFIC and SOX1 (n=3). E) Protein expression levels of Bax and Bcl-2 in KG-1 and NB4 cells after overexpression of NFIC and SOX1 (n=3). F) Immunofluorescence analysis of LC3 in KG-1 and NB4 cells after overexpression of NFIC and SOX1. G) Protein expression levels of LC3-I, LC3-II, p62, and Beclin-1 in KG-1 and NB4 cells after overexpression of NFIC and SOX1 (n=3). */**/*p<0.001/0.01/0.05 vs. the oe-NC group; ###/##/#p<0.001/0.01/0.05 vs. the oe-NFIC+oe-SOX1 group**

nently overexpressed in AML. Moreover, our results demonstrated that NFIC overexpression is associated with a lower survival rate in AML patients. In recent years, studies have highlighted the protective role of autophagy and apoptosis in AML progression [38]. Therefore, in this study, we investigated the effects and underlying mechanisms of NFIC on AML by regulating autophagy and apoptosis. To evaluate apoptosis and autophagy in KG-1 and NB4 cells, we analyzed the expression of key proteins: Bax and Bcl-2 (apoptosis markers), LC3-I and LC3-II (autophagy markers, reflecting LC3-I lipidation), p62 (an autophagy substrate recognition protein), and Beclin-1 (a core autophagy regulator). Our results showed that NFIC knockdown reduced cell viability and autophagy while increasing apoptosis in KG-1 and NB4 cells. Importantly, NFIC knockdown did not affect apoptosis in BMMNCs, suggesting that NFIC knockdown may alleviate AML progression without harming normal bone marrow mononuclear cells. One previous study reported that abnormally elevated NFIC in neural tube defect (NTD) mice suppressed autophagy and promoted apoptosis by activating miR-200 [20]. We speculated the differences between the effects of NFIC on autophagy and apoptosis observed in our study and those in the previous NTD study could be due to the distinct biological systems and cell types involved, as NTDs primarily involve injuries to neural stem cells in the central nervous system, whereas AML is characterized by the generation of abnormal leukemia cells in the peripheral circulatory system.

SOX1 is expressed in various malignant tumors and is closely associated with tumor occurrence, progression, and prognosis. Low SOX1 expression has been reported in breast cancer, where its upregulation significantly inhibited tumor cell migration and invasion [24]. In esophageal squamous cell carcinoma, high SOX1 expression was identified as a potential therapeutic target, and its regulation was shown to improve prognosis [39]. Additionally, SOX1 overexpression inhibited proliferation, invasion, and metastasis, while promoting apoptosis in hepatocellular carcinoma by regulating the Wnt/β-catenin pathway [40]. However, the role of SOX1 in AML and its relationship with NFIC remains unclear. In this study, we further investigated the relationship between NFIC and SOX1, as well as the effects of SOX1 on AML cells. Analysis of clinical specimens revealed that SOX1 expression was significantly downregulated in AML, consistent with previous findings. Interestingly, results from our bioinformatics analysis, molecular interaction experiments (RNA pull-down and RIP assays), and *in vitro* cellular experiments demonstrated that NFIC targets and negatively regulates SOX1 in KG-1 and NB4 cells. To determine whether NFIC regulates autophagy and apoptosis through SOX1 inhibition, we investigated the effects of overexpressing NFIC, SOX1, and NFIC combined with SOX1 in KG-1 and NB4 cells. Our findings showed that NFIC overexpression increased cell viability and autophagy while reducing apoptosis in KG-1 and NB4 cells. Importantly, these effects were effectively reversed by SOX1 overexpression, which aligns with a previous study showing that SOX1 downregulation promotes autophagy and reduces apoptosis, enhancing drug resistance in non-small cell lung cancer [41]. The results of this study suggest that NFIC targets and negatively regulates SOX1, while SOX1 overexpression suppresses AML progression in KG-1 and NB4 cells by inhibiting autophagy.

Despite the interesting findings reported, some limitations need to be addressed. First, although our findings were derived from clinical specimens and *in vitro* cellular experiments, validation through *in vivo* animal experiments was not performed. Second, autophagy was evaluated by measuring the expression of LC3-I, LC3-II, p62, and Beclin-1, which provides limited evidence. Additional approaches are required to comprehensively assess autophagy, such as evaluating the expression of other autophagy-related proteins, such as ATG and ULK family proteins, and observing the formation of autophagosomes using transmission electron microscopy.

In conclusion, this study demonstrated that NFIC promotes autophagy, enhances cell viability, and inhibits apoptosis in KG-1 and NB4 cells by targeting and suppressing SOX1, supporting the potential role of NFIC in AML progression and as a potential novel target for the clinical treatment of AML.

Supplementary information is are available in the online version of the paper.

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