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Original Article

LncRNA AL645608.3 mediates malignant progression of acute myeloid leukemia

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Abstract: Objective: To investigate the role of lncRNA AL645608.3 in the malignant progression of acute myeloid leukemia (AML) cells and explore relevant molecular mechanisms. Methods: The expression level of AL645608.3 was measured in AML cell lines (THP-1, HL-60, KG-1, and AML-193) via real-time quantitative polymerase chain reaction (RT-qPCR). Small hairpin RNA (shRNA) and open reading frame of AL645608.3 were cloned into lentiviral vectors and were infected into THP-1 and AML-193 cells. The expression of casitas B-lineage lymphoma (CBL), interferon regulatory factor 6 (IRF6), and interferon beta 1 (IFNB1) was detected through RT-qPCR, and western blot. Co-immunoprecipitation (Co-IP) on IRF6 was conducted. Matrix metalloprotease-9 (MMP-9) activity was evaluated via gelatin zymography assay. Results: LncRNA AL645608.3 was expressed in the four AML cell lines (THP-1, HL-60, KG-1, and AML-193). Silencing AL645608.3 mitigated the expression of IRF6 and IFNB1 but elevated the expression of CBL in THP-1 cells. Oppositely, AL645608.3 overexpression up-regulated the expression of IRF6 and IFNB1 but decreased the expression of CBL in AML-193 cells. Co-IP results proved that AL645608.3 could directly mediate IRF6 activity in THP-1 and AML-193 cells. MMP-9 activity was decreased by AL645608.3 knockdown and was improved by AL645608.3 overexpression in AML-193 cells. Conclusion: AL645608.3 is expressed in different AML cell lines, and mediates the expression of CBL, IRF6, IFNB1, and MMP-9. These findings might deepen our comprehension of the molecular mechanisms underlying AML.

Keywords: AL645608.3, acute myeloid leukemia, lncRNA, CBL, IRF6, IFNB1, MMP-9

Introduction

Acute myeloid leukemia (AML) is a genetically heterogenous malignancy arising from uncontrolled proliferation of clonal hematopoietic cells [1, 2]. AML primarily comprises acute erythroid leukemia and acute megakaryoblastic leukemia [3]. The five-year overall survival is around 30% and varies notably between different age groups, which reaches 50% in younger patients but less than 10% in patients aged under 60 [4]. This is partly due to the standardization of intensive cytarabine- and anthracycline-based chemotherapy (known as "7 + 3") as the treatment for AML, but the tolerance of older and sicker patients is poor [5]. In addition to age and comorbidities, the prognostic outcomes of AML patients depend much upon the biology of malignancy. The field of AML treatment is gradually shifting from unified methods based solely upon intensive chemotherapy towards precision therapy. Currently, the treatment of AML can be personalized in accordance with patient features and cytogenetic/molecular characteristics. The treatment landscape for AML has improved with the approval of novel molecular targeted treatment, such as FMS-like tyrosine kinase 3 (FLT3) inhibitors [6, 7], isocitrate dehydrogenase (IDH) 1 and 2 inhibitors [8, 9], a Hedgehog pathway inhibitor [10], and B-cell lymphoma 2 (BCL-2) inhibitor venetoclax [11, 12]. In-depth research into the molecular mechanisms of AML will contribute to notable advances in the comprehension of this malignancy.

Long noncoding RNAs (LncRNAs) have greater than 200 nucleotides in length that transcribed

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Table 1. Primer information for RT-qPCR

Primer name	Primer information	Primer sequence (5'-3')		Tm value	CG%	Product length
H-GAPDH	NM_001256799.2	Sense	CATCATCCCTGCCTCTACTGG	59.4	57.1	259
		Antisense	GTGGGTGTCGCTGTTGAAGTC	60.1	57.1	
H-CBL	NM_005188.4	Sense	CAAGAAGATGGTGGAGAAGTGC	58.9	50	247
		Antisense	CTTGAAGAGGCTTATGGTTTGC	58.8	45.5	
H-IRF6	NM_001206696.2	Sense	ATGCCATTTATGCCATCAGG	58.3	45	273
		Antisense	GAGCCACTACTGGAATGACCTG	58.8	54.5	
H-IFNB1	NM_002176.4	Sense	GACAGGATGAACTTTGACATCCC	60	47.8	156
		Antisense	CTCAACAATAGTCTCATTCCAGCC	60	45.8	
Lnc-AL645608.3	ENST00000423619.2	Sense	TCACCAGACTTTCAGAATTAAGCG	60.8	41.7	144
		Antisense	AGACCCAGCTTGGCTGTTCTC	60.8	57.1	

by RNA polymerase II with diverse characteristics: without open reading frame, inability to encode proteins, cap structure at the 5' end, and polyadenylation at the 3' end [13, 14]. Accumulated evidence has suggested that lncRNA transcripts are linked with and mediate malignant phenotypes in AML [15]. For instance, NPM1 mutation-mediated lncRNA HOTAIRM1 facilitates leukemia cell autophagy and proliferation through targeting EGR1 and ULK3 [16]. Activated HOXBLINC leads to leukemogenesis in NPM1-mutant AML [17]. Suppression of GAS6-AS1-mediated YBX1/MYC signaling hinders cell propagation and disease progression of AML [18]. Cytoplasmic NEAT1 mitigates self-renewal and leukemogenesis of AML stem cells via inactivating the Wnt pathway [19]. Nevertheless, the implication of lncRNA AL645608.3 in AML remains indistinct. The present study analyzed the expression of AL645608.3 in AML cells as well as its influence on casitas B-lineage lymphoma (CBL), interferon regulatory factor 6 (IRF6), interferon beta 1 (IFNB1), and matrix metalloprotease-9 (MMP-9).

Materials and methods

Cell culture

AML cell lines (THP-1, HL-60, KG-1, and AML-193) purchased from the American Type Culture Collection (Manassas, USA) were cultivated in RPMI 1640 medium (#SH30809.01; Hyclone, Utah, USA) with fetal bovine serum (FBS; #141215; Hangzhou Tianhang Biotechnology Co., LTD., Zhejiang, China). All cells were grown in a 5% CO_2 atmosphere at 37°C.

Cell counting kit-8 (CCK-8)

The cells were inoculated into a 96-well plate at 1×10^4 cells per well. 10 μL CCK-8 solution (#C0038; Beyotime, Shanghai, China) was added to each well and incubated for 2 h. The optical absorption at 450 nm was measured by ELISA detector (Diatek, Illinois, USA) to calculate the relative proliferation capacity of the cells.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cells, and the first strand cDNA was synthesized using EntiLink™ 1st strand cDNA synthesis kit (#EQ003; ELK Biotechnology, Wuhan, China). RT-qPCR was carried out via QuantStudio 6 Flex System PCR instrument and EnTurbo™ SYBR Green PCR SuperMix (#EQ001; ELK Biotechnology). The information of primers is listed in **Table 1**. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Lentiviral vector construction

Three small hairpin RNAs (shRNAs) (sh-AL645608.3#1, 5'-AGCGATTCCTGGCCAAGAATTCAAGAGATTCTTGGCCAGGAATCGCTTTTTT-3'; AL645608.3#2, 5'-TCACCAGACTTTCAGAATTATTCAAGAGATAATTCTGAAAGTCTGGTGATTTTT-3'; AL645608.3#3, 5'-GACTTTCAGAATTAAGCGATTTTCAAGAGAAATCGCTTAATTCTGAAAGTCTTTTT-3') that specifically targeted AL645608.3 were designed by ASPEN (Wuhan, China), and was cloned into pLVX-shRNA2 lentiviral vector, with 5'-AGCCCTCCAAGAGGTTGAATTCAAGAGATTCAACCTCTTGGAGGGCTTTTTT-3' sequence as

a negative control of shRNA. The open reading frame of AL645608.3 (ASPEN) was cloned into pLVX-IRES-ZcGreen1 lentiviral vector. The lentiviral vectors were packaged into HEK-293T cells. After incubation for 48 h, lentiviruses were gathered, filtered and concentrated. Next, the cells were infected with viruses by using polybrene and selected via puromycin.

Western blot

Protein was extracted from the cells by RIPA lysate (ASPEN). The protein concentration was determined utilizing BCA protein concentration assay kit (#AS1086; ASPEN). The protein was electrophoresed by use of SDS-PAGE and transferred onto PVDF membranes (#IPVH00010; Millipore, Massachusetts, USA). The transferred membranes were blocked for 1 h. After discarding the sealing liquid, the diluted primary antibodies against IRF6 (#sc-37704; 1:500; Santa, Texas, USA), CBL (#25818-1-AP; 1:1000; Proteintech, Wuhan, China), IFNB1 (#ab2758-80; 1:500; Abcam, Massachusetts, USA), and GAPDH (#ab181602; 1:10000; Abcam) were added and incubated overnight at 4°C. The diluted primary antibody was recovered and washed three times with TBST for 5 min each time. The diluted HRP-goat anti-rabbit or anti-mouse secondary antibody (#AS1107 or #AS1106; 1:10000; ASPEN) was added and incubated at room temperature for 30 min. TBST was utilized to wash four times in a shaker at room temperature for 5 min each time. The enhanced chemiluminescence (ECL) mixture (A:B=1:1) (#AS1027; ASPEN) was dripped onto the protein side of the membrane and exposed in a dark room. The film was scanned, and the AlphaEaseFC software processing system (Alpha Innotech, California, USA) was utilized to quantify the optical density value of the target band.

Co-immunoprecipitation (Co-IP)

The cells were lysed by IP lysate (ASPEN), and extracted protein samples were gathered, of which 1/10 was used as input, with the remaining as IP. After identifying concentration, 20 μL magnetic beads (#1614813; BIO-RAD, California, USA) were added into an appropriate volume of IP lysate into the centrifuge tube, and washed on a vertical shaker for 5 min. After that, the centrifuge tube was placed on the magnetic rack for 5-10 s to absorb the supernatant. Next, 1 μg IgG and 20 μL magnetic beads were added into the supernatant of the cell lysate and incubated at 4°C for 30 min. The supernatant of the cell lysate with the same amount as above was taken into the centrifuge tube and added with 1 μg IRF6 antibody, and then incubated at 4°C for 1 h. 20 μL magnetic beads were added and incubated at 4°C overnight. Western blot was then conducted as described above.

Gelatin zymography assay

The cell supernatant was centrifuged at 2000 rpm for 10 min at 4°C. The protein concentration was determined via the BCA method. The sample was mixed with 5 μL 4× loading buffer, and 20 μL sample was loaded to each well. Electrophoresis was performed on SDS-PAGE at 4°C, 100 V, until bromophenol blue reached the base of the gel. After that, the gel was placed in the eluent 4 times, 15 min each time, followed by being rinsed in the bleach solution by shaking twice, 20 min each time. The sample was then incubated at 37°C for 48 h. In the dye solution, the sample was dyed for 3 h. After decolorization, images were photographed.

Statistical analysis

Data from ≥3 independent experiments were exhibited as the mean ± standard deviation. GraphPad Prism (version 9.0.1) was used for statistical analysis. Difference among ≥3 groups was assessed utilizing one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered statistically significant.

Results

LncRNA AL645608.3 is expressed in distinct AML cells

The viability of four AML cells (THP-1, HL-60, KG-1 and AML-193) was determined via CCK-8 assay. As illustrated in **Figure 1A**, HL-60 cells presented the best cell viability (48 h), followed by THP-1, KG-1, and AML-193. The expression of AL645608.3 in the four AML cell lines was determined using RT-qPCR, and HL-60 cells presented the highest expression, while AML-193 cells presented the lowest expression (**Figure 1B**).

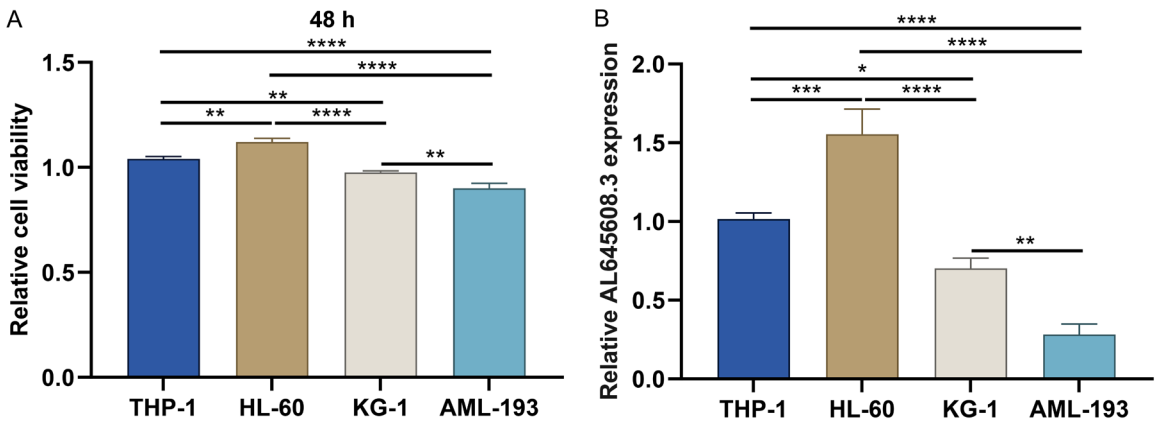

Figure 1. The expression of lncRNA AL645608.3 in various AML cells. A. CCK-8 for detecting the cell viability of THP-1, HL-60, KG-1, and AML-193 cell lines. B. RT-qPCR for measuring the expression of AL645608.3 in the above cell lines. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Construction of lentiviral vectors for silencing AL645608.3 in THP-1 cells

Specific shRNAs targeting AL645608.3 were designed and cloned into pLVX-shRNA2 lentiviral vectors (**Figure 2A**). To determine the optimal lentivirus titer, THP-1 cells were infected by lentiviruses with different MOI (0, 1, 10, 40 and 100). As depicted in **Figure 2B**, **2C**, when MOI=100, the infection effect was the best. RT-qPCR data demonstrated that AL645608.3 expression was the lowest in THP-1 cells with sh-AL645608.3#3 (**Figure 2D**). Thus, sh-AL645608.3#3 was utilized for subsequent experiments.

Construction of lentiviral vectors for overexpressing AL645608.3 in AML-193 cells

To overexpress AL645608.3, the open reading frame of AL645608.3 was cloned into pLVX-IRES-ZcGreen1 lentiviral vectors (**Figure 3A**). For determining the optimal lentivirus titer, AML-193 cells were infected by lentiviruses with different MOI (0, 1, 10, 40 and 100). When MOI=100, the infection effect was the best (**Figure 3B**, **3C**). RT-qPCR confirmed that AL645608.3 was successfully overexpressed in AML-193 cells (**Figure 3D**).

The expression of IRF6 and CBL in different AML cells

The IRF6 gene encodes a member of the interferon regulatory transcription factor (IRF) family [20]. IRF6 mRNA was observed in the four AML cell lines, among which HL-60 cells presented the highest mRNA level, and AML-193 cells presented the lowest mRNA level (**Figure 4A**). CBL that encodes an E3 ubiquitin ligase and signaling adaptor modulates receptor and non-receptor tyrosine kinases [21]. The mRNA expression of CBL was detected in four AML cells, and among them, HL-60 cells exhibited the lowest mRNA level, and AML-193 cells showed the highest mRNA level (**Figure 4B**). The similar expression patterns of IRF6 and CBL in above AML cells were also investigated at the protein level (**Figure 4C-E**).

Effects of AL645608.3 on the expression of IRF6, IFNB1 and CBL in AML cells

As indicated by q-PCR results, in THP-1 cells, AL645608.3 knockdown notably decreased the mRNA levels of IRF6 and IFNB1 but elevated the mRNA level of CBL (**Figure 5A-C**). In AML-193 cells, it was observed that after AL645608.3 overexpression, the mRNA level of IRF6 and IFNB1 increased prominently while CBL decreased prominently (**Figure 5D-F**).

Western blot experiment also suggested that AL645608.3 knockdown significantly inhibited the protein expression of IRF6 and IFNB1 but promoted the protein expression of CBL in THP-1 cells, while overexpressing of AL645608.3 significantly elevated the protein expression of IRF6 and IFNB1 but decreased the mRNA expression of CBL in AML-193 cells (**Figure 6**).

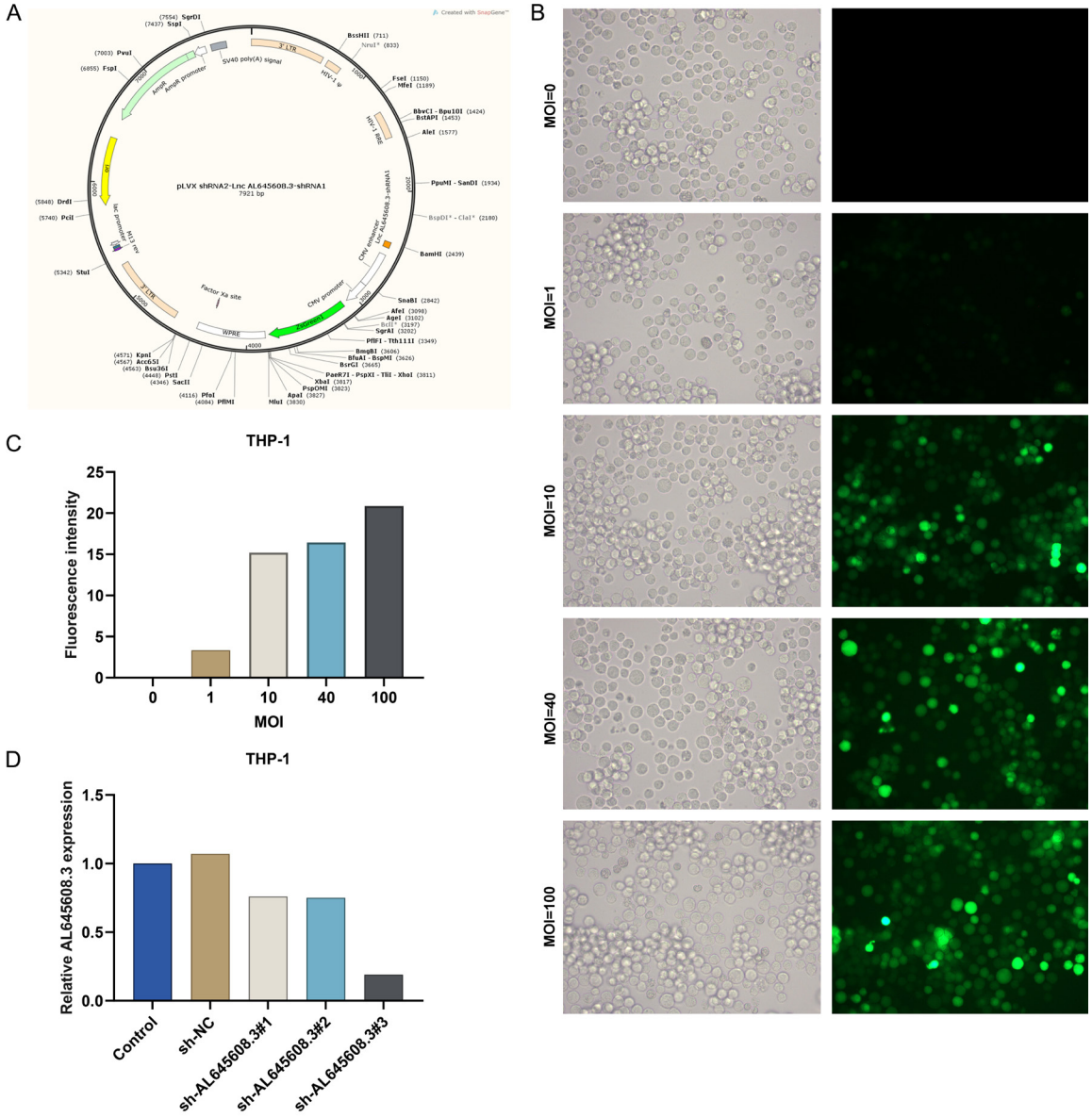

Figure 2. Construction of lentiviral vectors for silencing AL645608.3 in THP-1 cells. A. Design of shRNAs of AL645608.3. B, C. Cellular morphology and fluorescence intensity of THP-1 cells under light microscope or immunofluorescence microscope. Magnification, 200×. D. RT-qPCR for evaluation of AL645608.3 expression in THP-1 cells with infection of pLVX-shRNA2 lentiviral vectors. ns, P>0.05; ***P<0.001; ****P<0.0001.

AL645608.3 directly enhanced IRF6 expression in AML cells

The Co-IP experiment demonstrated that IRF6 expression was notably decreased in sh-AL645608.3 lentivirus-infected THP-1 cells, with elevated ubiquitin expression. Oppositely, IRF6 expression was notably elevated in AL645608.3-overexpressed AML-193 cells, with reduced ubiquitin expression (**Figure 7A-E**). This unveils that AL645608.3 potentially modulates IRF6 expression in AML cells through ubiquitination.

AL645608.3 up-regulated MMP-9 activity in AML cells

MMP-9 is one of the most widely studied matrix metalloproteinases (MMPs) [22]. Gelatin zymography assay was conducted for measuring the activity of MMP-9 in the four AML cell lines. As illustrated in **Figure 8A**, **8B**, MMP-9

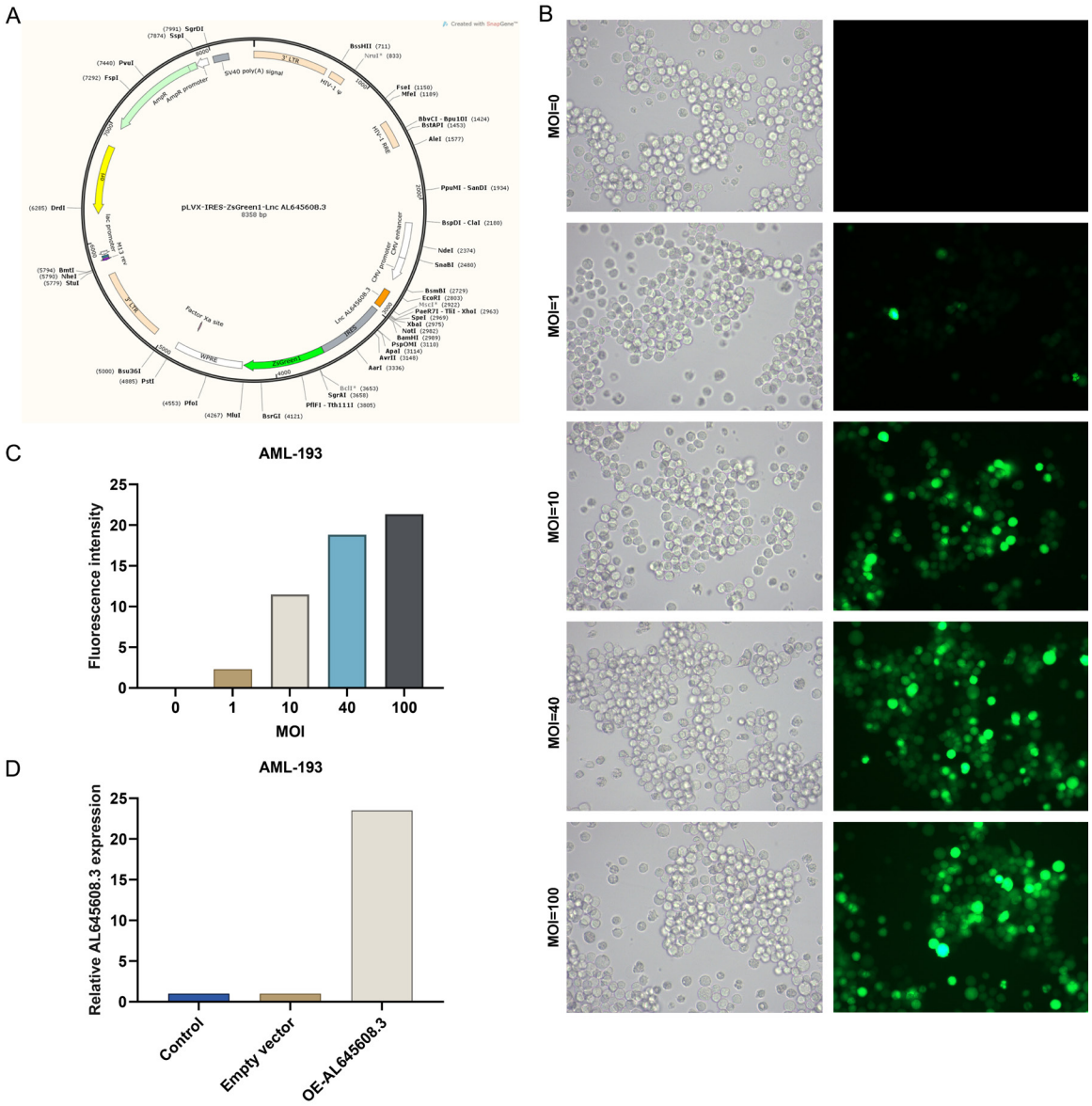

Figure 3. Construction of lentiviral vectors for overexpressing AL645608.3 in AML-193 cells. A. Design of the open reading frame of AL645608.3. B, C. Cellular morphology and fluorescence intensity of AML-193 cells under light microscope or immunofluorescence microscope. Magnification, 200×. D. RT-qPCR for evaluation of AL645608.3 expression in AML-193 cells with infection of pLVX-IRES-ZcGreen1 lentiviral vectors. ns, P>0.05; ****P<0.0001.

exhibited the strongest activity in HL-60 cells, with the weakest activity in AML-193 cells. In THP-1 cells, MMP-9 activity was suppressed by sh-AL645608.3 (**Figure 8C-E**). On the contrary, AL645608.3 overexpression improved the activity of MMP-9 in AML-193 cells. Thus, AL645608.3 was capable of potentially modulating MMP-9 activity in AML cells.

Discussion

Evidence has suggested that aberrant expression of non-coding RNAs are biological tumorigenesis-relevant mechanisms and promising targets for treating cancers [23-27]. This study investigated the expression of lncRNA AL645608.3 in four AML cells: THP-1, HL-60, KG-1 and AML-193. In addition, AL645608.3 participated in modulating the expression of CBL, IRF6, IFNB1, and MMP-9 in AML cells.

IRF6 is aberrantly activated in AML cells [28]. Several lncRNAs (ENSMUST_147219 [29], and Lin28A [30]) are capable of post-transcriptionally modulating IRF6 expression. In our study, IRF6 expression was measured in four AML cell

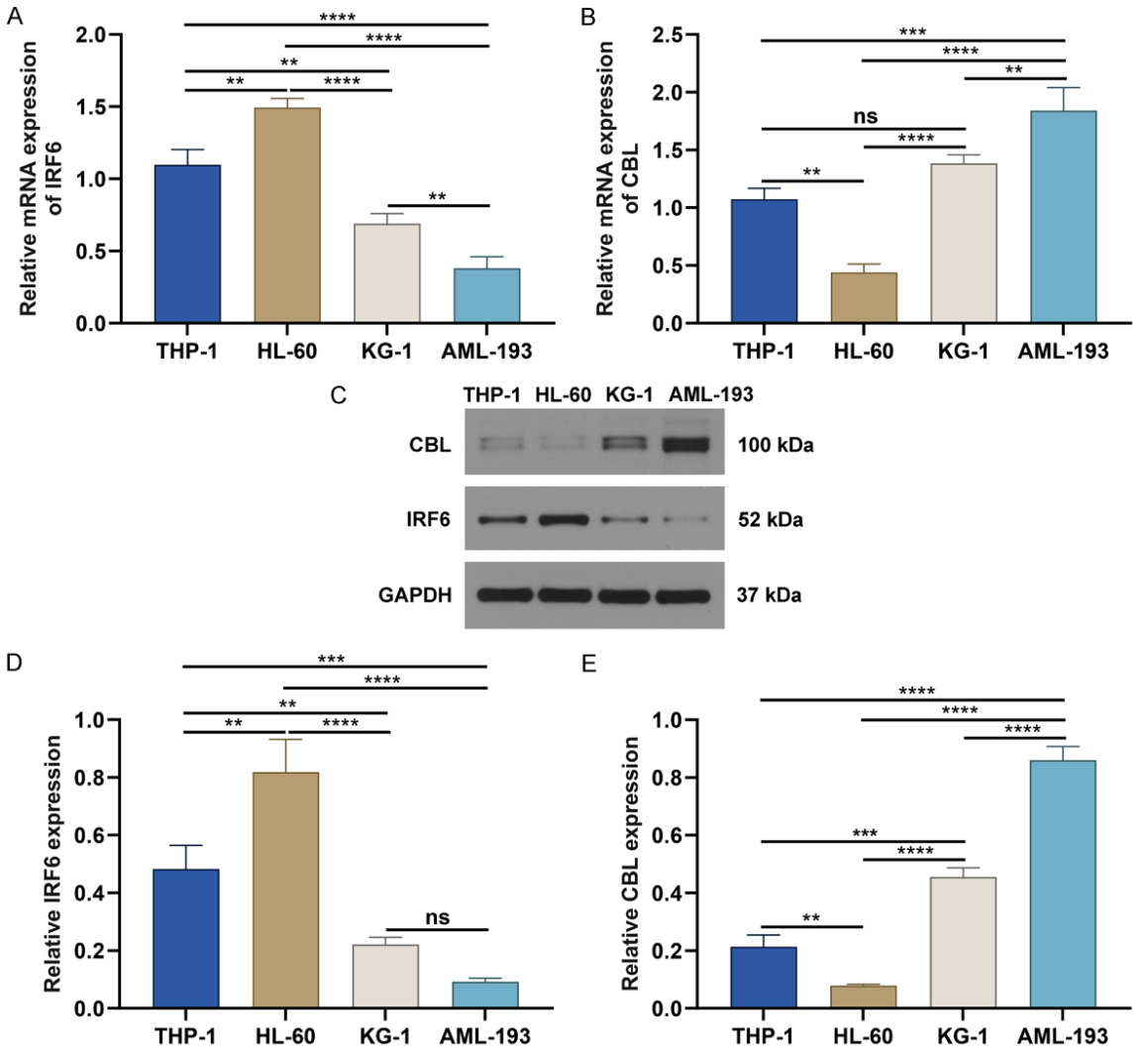

Figure 4. Expression of IRF6 and CBL in diverse AML cells. A. RT-qPCR for detection of the mRNA level of IRF6 in THP-1, HL-60, KG-1, and AML-193 cell lines. B. RT-qPCR for measuring the mRNA level of CBL in the above AML cells. C-E. Western blot of the protein levels of IRF6 and CBL in the above AML cells. ns, P>0.05; **P<0.01; ***P<0.001; ****P<0.0001.

lines. It was demonstrated that IRF6 presented the highest expression in HL-60 cells and the lowest expression in AML-93 cells. Silencing AL645608.3 prominently mitigated the expression of IRF6 in THP-1 cells, while AL645608.3 overexpression promoted the expression of IRF6 in AML-193 cells. The Co-IP results also proved the role of AL645608.3 in up-regulating IRF6 expression in AML cells.

Mutations in CBL are frequent in AML [31]. The oncogenic mutations of CBL activate growth and survival signaling in AML dependent upon enhanced metabolism [32]. In addition, CBL exon 8/9 mutants are capable of activating FLT3 signaling and clustering in core binding factor/11q deletion AML [33]. Among 41 adult patients with AML, CBL was overexpressed in 20% of the patients and also down-regulated in 20% of them [34]. Here, we found that HL-60 cells had the lowest CBL expression, while AML-193 cells had the highest CBL expression. CBL can be post-transcriptionally regulated by several lncRNA transcripts, such as DUXAP9-206 [35] and DANCR [36]. CBL expression was heterogeneous in diverse AML cells, with the lowest expression in HL-60 cells and the highest expression in AML-193 cells. AL645608.3 knockdown significantly elevated the expression of CBL in THP-1 cells, while its overexpres-

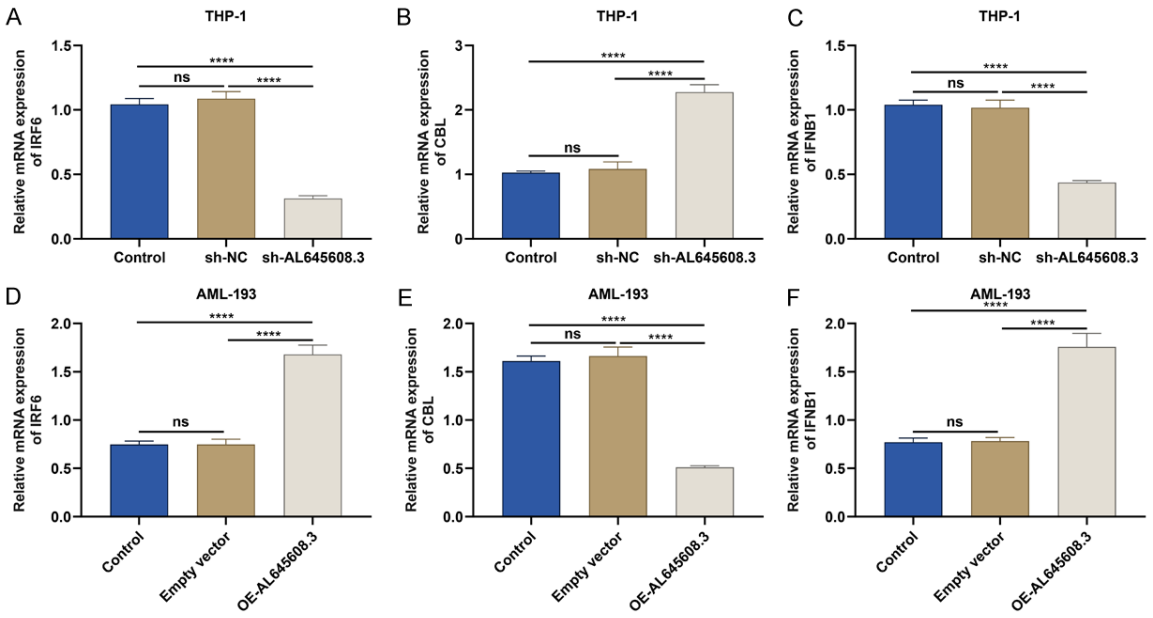

Figure 5. Effects of AL645608.3 on the mRNA expression of IRF6, CBL and IFNB1 in AML cells. A-C. The mRNA expression of IRF6, CBL and IFNB1 in THP-1 cells infected with sh-AL645608.3 lentiviruses. D-F. The mRNA expression of IRF6, CBL and IFNB1 in AML-193 cells infected with AL645608.3 overexpression lentiviruses. ns, P>0.05; ****P<0.0001.

sion significantly attenuated the expression of CBL in AML-93 cells, indicative of the inhibitory role of AL645608.3 in CBL expression in AML cells.

The IFNB1 gene encodes a cytokine belonging to the interferon family of signaling proteins that is released as a part of the innate immune response to pathogens [37]. The encoded protein belongs to type I interferon that is of importance for defending against viral infections [38]. Moreover, type I interferon is involved in cell differentiation and anti-tumor defense. In response to pathogens, type I interferon binds to homologous receptor complexes and induces transcription of genes encoding inflammatory cytokines and chemokines [39]. Overactivated type I interferon secretion associates with autoimmune diseases [40]. lncRNA transcripts are capable of modulating type I interferon signaling, such as TSPOAP1-AS1 [41], RFPL1S-202 [42] and ZFAS1 [43]. In THP-1 cells, silencing AL645608.3 prominently inhibited the expression of IFNB1. Meanwhile, overexpressing AL645608.3 prominently enhanced the expression of IFNB1 in AML-193 cells. Altogether, AL645608.3 can potentially modulate IFNB1 in AML cells.

MMP family proteins participate in the breakdown of extracellular matrix in normal physiological processes (e.g., embryonic development, reproduction and tissue remodeling) and in disease processes (e.g., cancer) [44]. Most MMPs can be secreted as inactive proproteins that are activated when cleaved by extracellular proteases [45]. For cancer, the major roles of MMPs in angiogenesis, tumor growth and metastases include degradation of extracellular matrix and release and/or activation of growth factors via degradative activity, eventually leading to cancer progression [46]. The enzyme encoded by MMP-9 is capable of degrading type IV and V collagens [47]. The expression of MMP-9 can be post-transcriptionally modulated by lncRNAs, e.g., TP73-AS1 [48], BC200 [49], and FOXF1-AS1 [50]. MMP-9 exhibited the highest activity in HL-60 cells and the lowest activity in AML-193 cells. In THP-1 cells, knockdown of AL645608.3 notably decreased the activity of MMP-9. Meanwhile, overexpression of AL645608.3 notably improved the activity of MMP-9 in AML-193 cells. Thus, we inferred that AL645608.3 can mediate the activity of MMP-9 in AML cells. However, the limitations of this study should be pointed out. Firstly, the biological role of AL645608.3 in AML needs to be investigated

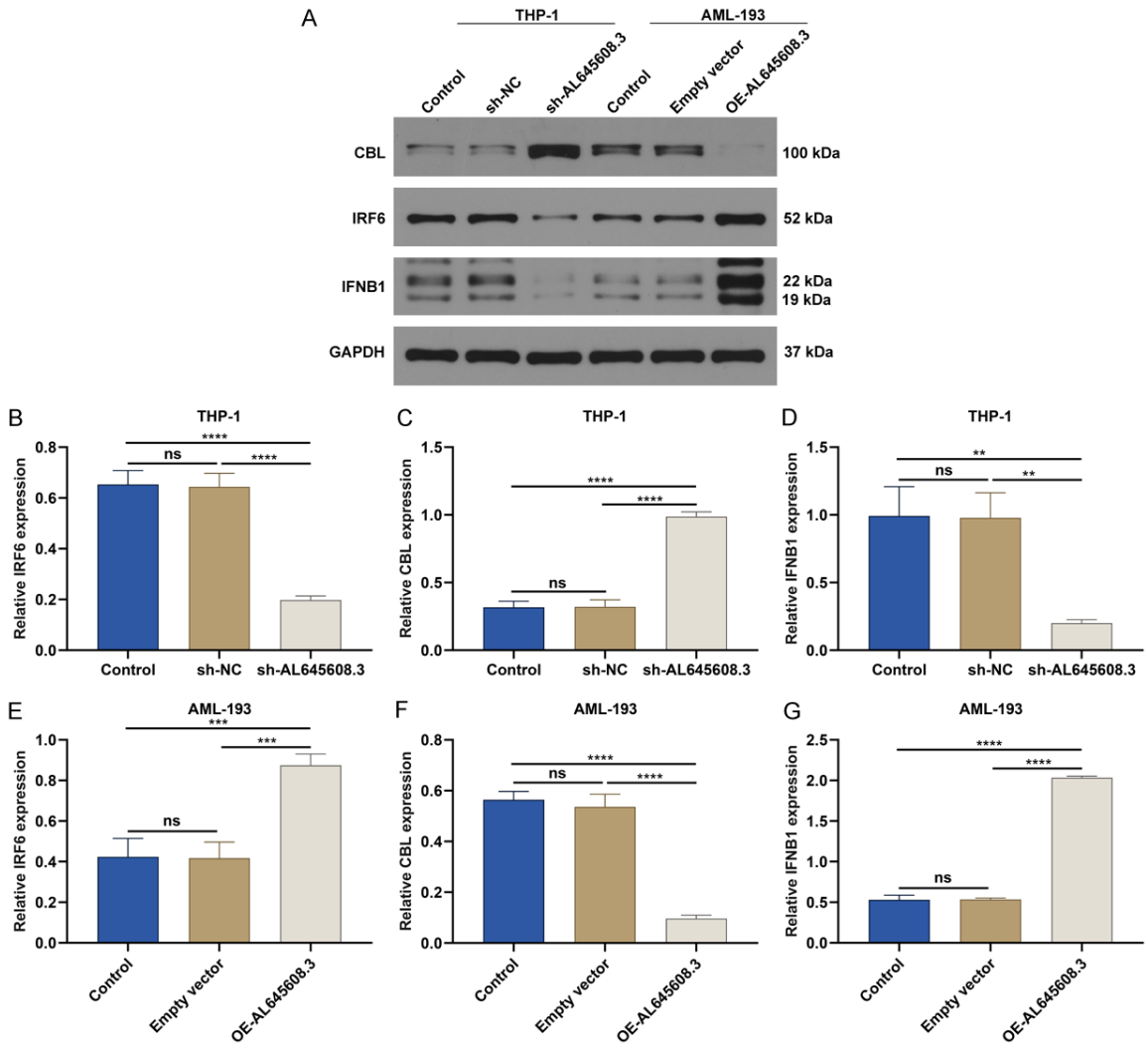

Figure 6. Effects of AL645608.3 on the protein expression of IRF6, CBL and IFNB1 in AML cells. A. Representative western blot images for IRF6, CBL and IFNB1 in THP-1 cells with sh-AL645608.3 lentiviruses infection and in AML-193 cells with AL645608.3 overexpression lentiviruses infection. B-D. Western blot for the detection of protein levels of IRF6, CBL and IFNB1 in THP-1 cells infected with sh-AL645608.3 lentiviruses. E-G. Western blot for the detection of protein levels of IRF6, CBL and IFNB1 in AML-193 cells infected with AL645608.3 overexpression lentiviruses. ns, P>0.05; **P<0.01; ***P<0.001; ****P<0.0001.

in more experiments. Secondly, clinical relevance as well as potential as a therapeutic target should be further validated. Thirdly, more experiments are required to be implemented for evaluating relevant molecular mechanisms underlying AL645608.3 in AML. In our future studies, we will continue to study the role and molecular mechanisms of AL645608.3 in AML.

Conclusion

Collectively, this study investigated the expression of lncRNA AL645608.3 in diverse AML cell lines. Its aberrant expression can influence the expression of CBL, IRF6, IFNB1, and MMP-9 in AML cells. Altogether, our findings might deepen the comprehension of the molecular mechanisms of AML.

Acknowledgements

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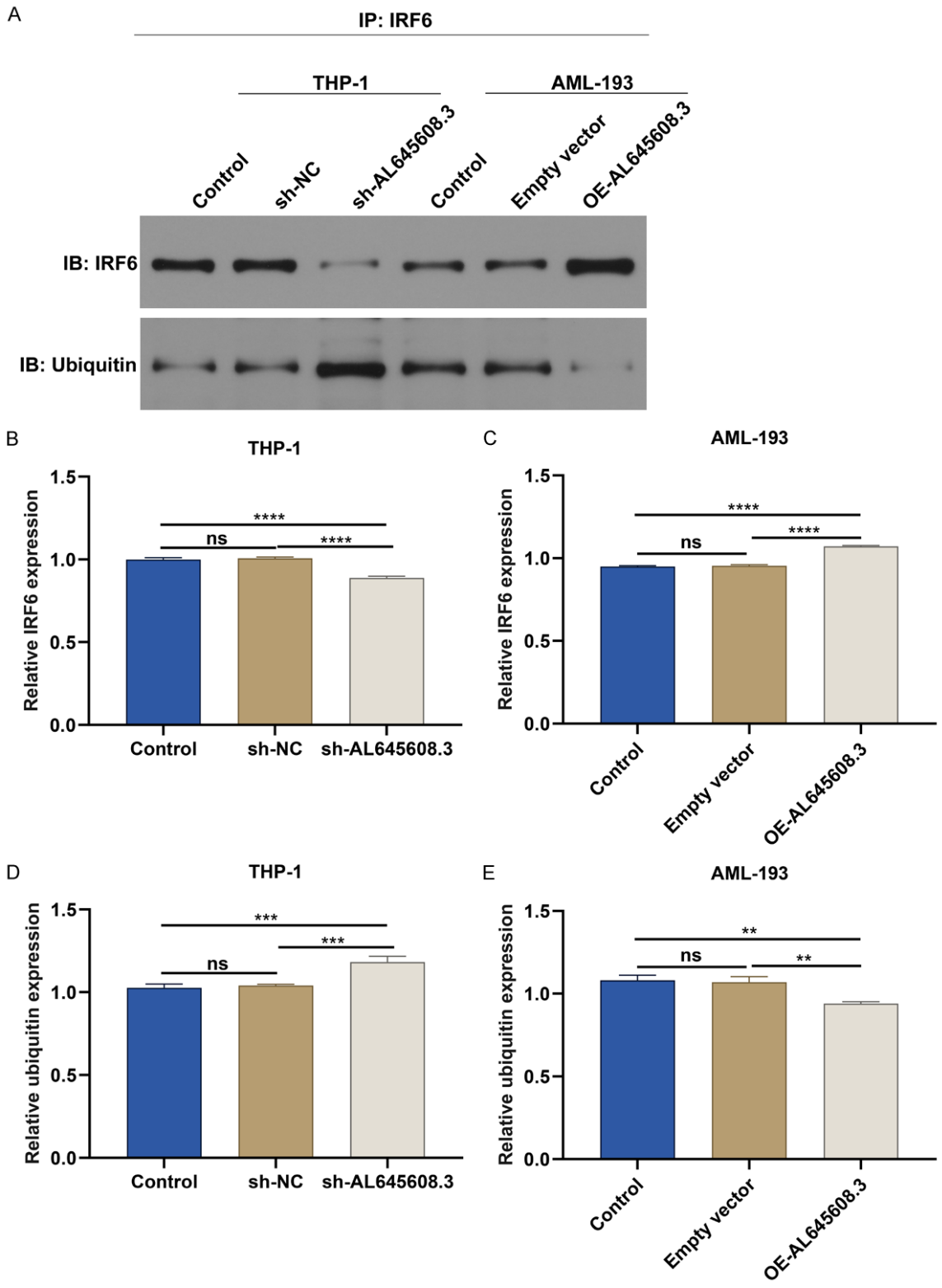

Figure 7. AL645608.3 directly enhanced IRF6 expression in AML cells. A. Co-IP for IRF6 in THP-1 cells with infection of sh-AL645608.3 lentiviruses as well as AML-193 cells with infection of AL645608.3 overexpression lentiviruses. B-E. Quantification of IRF6 and ubiquitin expression in above THP-1 and AML-193 cells. ns, P>0.05; **P<0.01; ***P<0.001; ****P<0.0001.

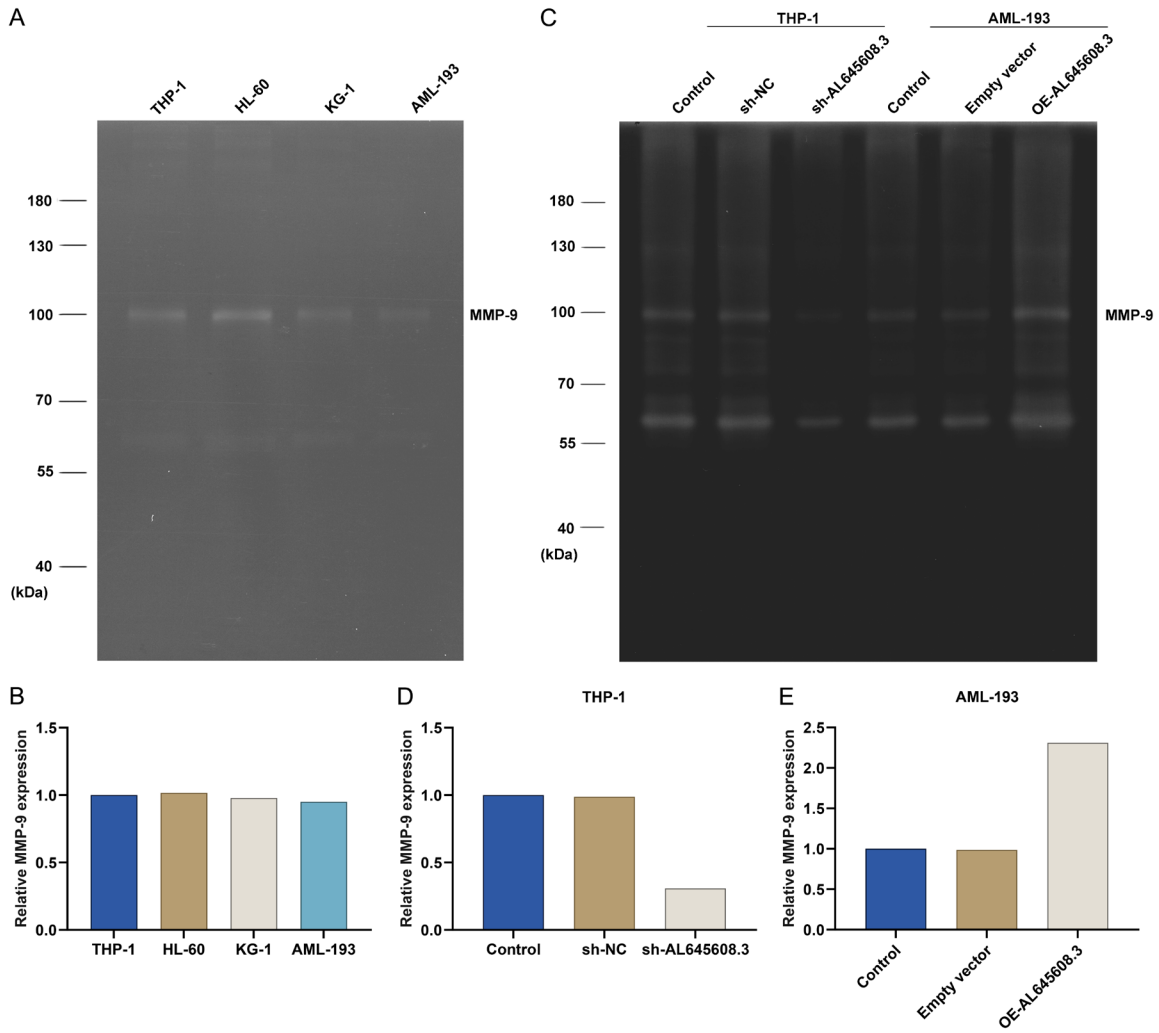

Figure 8. MMP-9 was activated in AML cells with AL645608.3 overexpression. A, B. Gelatin zymography assay for MMP-9 in THP-1, HL-60, KG-1, and AML-193 cell lines. C-E. Gelatin zymography assay for MMP-9 in THP-1 cells with infection of sh-AL645608.3 lentiviruses and AML-193 cells with infection of AL645608.3 overexpression lentiviruses. ns, P>0.05; ****P<0.0001.

Disclosure of conflict of interest

None.

Abbreviations

AML, acute myeloid leukemia; lncRNAs, long noncoding RNAs; CBL, casitas B-lineage lymphoma; IRF6, interferon regulatory factor 6; IFNB1, interferon beta 1; MMP-9, matrix metalloprotease-9; CCK-8, cell counting kit-8; RT-qPCR, real-time quantitative polymerase chain reaction; shRNA, small hairpin RNA; Co-IP, Co-immunoprecipitation; MMPs, matrix metalloproteinases.

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