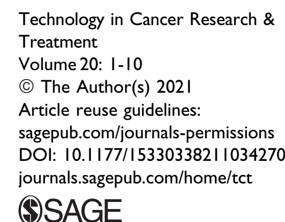
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Downregulation of LEF1 Impairs Myeloma Cell Growth Through Modulating CYLD/ NF-κB Signaling

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Abstract

Aberrant expression of lymphoid enhancer-binding factor-1 (LEF1) has been identified in various hematological malignancies including multiple myeloma (MM). However, the exact role of LEF1 in MM remains largely unknown. Here, we showed that knockdown of LEF1 could apparently impair the proliferation, induce apoptosis and promote the ROS production in MM cell lines, suggesting that LEF1 might be involved in maintaining MM cell growth and survival. Moreover, we observed that the mRNA level of the deubiquitinase cylindromatosis (CYLD), a well-recognized tumor suppressor in MM, was significantly increased following LEF1 depletion in myeloma cells. Further study showed that LEF1 could directly associate with the promoter of CYLD gene and thus repress its transcription in MM cells. Intriguingly, LEF1 depletion-mediated CYLD upregulation was sufficient to negatively modulate NF-κB signaling pathway in MM cells. Moreover, the decrease in NF-κB activity following LEF1 knockdown could be largely rescued when CYLD was silenced in MM cells. Taken together, our study provided the compelling evidence to show that LEF1 may augment the proliferation and survival of MM cells through direct repression of CYLD transcription and subsequent activation of NF-κB signaling pathway, corroborating that LEF1 may become a potential therapeutic target against MM.

Keywords

multiple myeloma (MM), LEF1, CYLD, NF-κB signaling

Abbreviations

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; CHIP, chromatin immunoprecipitation; CLL, chronic lymphocytic leukemia; CYLD, cylindromatosis; MM, multiple myeloma; mRNA, messenger RNA; ROS, reactive oxygen species; WB, Westernblot.

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Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by proliferation of clonal plasma cells secreting M protein in the bone marrow and development of osteolytic lesions.[1] Although the outcome of patients has been considerably improved with the introduction of novel reagents including proteasome inhibitor and immunomodulatory agents as well as monoclonal antibodies, most patients still relapse due to drug resistance and ultimately succumb to this disease.[2] Therefore, it is essential to dissect the pathogenesis of MM and identify novel molecular targets for improved therapies.

Lymphoid enhancer factor 1 (LEF1) is a member of the LEF/T-cell factor family of transcription factors which act as a key mediator of the canonical Wingless-type (Wnt) signaling pathway.[3] Aberrant expression of LEF1 has been reported in various types of hematological malignancies. High LEF1 expression has been identified as an independent adverse

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prognostic factor in adult B precursor acute lymphoblastic leukemia (ALL).[4] In chronic lymphocytic leukemia (CLL), LEF1 is markedly upregulated in neoplastic cells as compared to normal B cells, and high LEF1 expression was linked to inferior outcome. LEF1 knockdown in CLL cells resulted in a dramatic increased necroptotic cell death in response to TNFa tzVAD treatment.[5,6] In acute myeloid leukemia (AML), LEF1 was highly expressed in a subset of cytogenetically normal AML (CN-AML) patients, and interestingly, high LEF1 expression was found to be associated with significantly better relapse-free survival, overall survival and event-free survival in CN-AML patients.[7,8] In acute promyelocytic leukemia (APL) patients, high LEF1 expression was also associated with better prognosis.[9] In contrast, the expression pattern as well as potential role of LEF1 in MM remains largely unexplored in multiple myeloma. Based on a relatively limited number of clinical samples, we have previous reported[10] that LEF1 was highly expressed in malignant plasma cells from MM patients as compared to that in normal plasma cells from healthy volunteers; and more importantly, MM patients exhibiting high LEF1 expression had a reduced overall survival than those with low LEF1 expression. This line of evidence raised the possibility that LEF1 may be also implicated in the pathogenesis of MM.

In this study, we firstly examined the relationship between LEF1 expression and MM patient survival status with 2 publicly available microarray-based database. Then we characterized the role of LEF1 in myeloma cell growth and survival by inhibiting LEF1 expression through shRNA-mediated gene knockdown. Lastly, we examined the impact of LEF1 knockdown on the expression of CYLD gene as well as NF-kB signaling pathway. We confirmed that high LEF1 expression correlated with inferior patient survival, and observed that LEF1 may promote myeloma cell growth and survival through downregulating CYLD expression and consequently enhancing NF-kB signaling pathway, corroborating that LEF1 may serve as a promising therapeutic target in MM.

Materials and Methods

Cell Culture

Human MM cell lines including U266 and RPMI8226 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and grown in RPMI1640 medium in the presence of 10% fetal bovine serum (FBS) and antibiotics at 37 degree in a 5% CO_2 humidified environment.

Plasmid Construction

The scrambled non-mammalian control shRNA (SHC007) and 2 human LEF1 specific shRNAs (TRCN0000020162 and TRCN0000020163) cloned into pLKO.1.puro vector were purchased from Sigma-Aldrich (USA). The target sequences for SHC007, TRCN0000020162 and TRCN0000020163 were CGCTGAGTACTTCGAAATGTC, GCACGGAAAGAAAGACAGCTA and CCATCAGATGTCAACTCCAAA, respectively. A promoter construct encompassing the region −1500 bp to +85 bp relative to the transcription start site (TSS) of human CYLD gene was PCR-amplified from genomic DNA of U266 cells using 5’-GGGGTACCGCCTAAAAGGACCCCAAATCTC-3’ forward and 5’-CCCTCGAGTAGTATTTACCTCCGACGCGC-3’ reverse primers. The PCR fragment was then subcloned into pGL3-Basic vector (Promega, USA) following enzymatic digestion with KpnI and XhoI (recognition sites were underlined), yielding pGL3-CYLD-pro plasmid. The mutation was introduced into the LEF1 responsive element (CTTTGTT was changed to CCCCGCC, underlined in the primers as below) in the pGL3-CYLD-pro plasmid using the site-directed mutagenesis kit (Agilent, USA) according to the manufacturer’s instruction with the primers as follows: 5’-CTGTGACCTCTGAGTCCTACCAGCCCCGCCGGAGAATCCAGATGTAGATGAGAA-3’ (Forward primer) and 5’-TTCTCATCTACATCTG GATTCTCCGGCGGGGCTGGTAGGACTCAGAGGTCACAG-3’ (Reverse primer).

Lentiviruses Packaging and Infection

For generation of lentiviruses, 293 T cells were co-transfected with pLKO.1-puro expressing LEF1-specific shRNA (shLEF1) or the scrambled non-mammalian shRNA (shCon) together with packaging plasmids including psPax2 and pMD2.G (Addgene, USA) using Lipofectamining 2000 reagent (ThermoFisher Scientific, USA). The fresh viral supernatants harvested after 48 hour of transfection were used to transduce myeloma cells in the presence of 6 μg/mL polybrene (Sigma-Aldrich, USA). After lentiviral infection, the cells were maintained in the culture medium containing 1μg/mL puromycin (Sigma-Aldrich, USA) for 2 days before they were used for functional assays.

Cell Viability Assay

Cell counting kit (Beyotime, China) was used to measure cell viability as we previously described.[11] Myeloma cells with or without LEF-1 knockdown were seeded into 96-well plates (8000 cells/well), and maintained in RPMI 1640 medium. Ten microliters of CCK-8 solution was added in each well. The plates were incubated for 3 hours and then the absorbance was measured at 450 nm with 650 nm as a reference wavelength on an Synergy HTX absorbance microplate reader (Biotek Instruments, USA).

Brdu Cell Proliferation Assay

Cell proliferation rate was measured by bromodeoxyuridine (BrdU) incorporation during DNA synthesis in cultured myeloma cells using the recommendation included in the commercial assay (11647229001, Roche Diagnostics, Switzerland) as previously described by others.[12] Data were shown as the relative absorbance at 370 nm, with 492 nm as reference

wavelength using an Synergy HTX absorbance microplate reader (Biotek Instruments, USA).

Apoptosis Detection by Flow Cytometry

Myeloma cells with or without LEF1 knockdown were stained with APC-Annexin-V (Biolegend, USA) and 7AAD (Biolegend, USA) in Annexin V Binding Buffer for 15 minutes at room temperature, and then the stained cells were analyzed by an BD LSRII flow cytometer. Annexin V-positive but 7-AAD-negative cells were considered to be early apoptotic; and Annexin V/7-AAD double positive cells late apoptotic.

Dual-Luciferase Reporter Assay

Myeloma cells with or without LEF1 knockdown were transiently transfected with 5 μg of human CYLD promoter construct or NF-κB reporter p-NF-κB-luc (Stratagene, USA) together with 0.1 μg of PRL-CMV plasmid (Promega, USA) by electroporation using Neon transfection system (ThermoFisher Scientific, USA). When indicated, cells were co-transfected with the siRNA specific for human LEF1 (sc-35804, Santa Cruz, USA) or the control siRNA (sc-37007, Santa Cruz, USA) at the final concentration of 20 nM. Luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instruction, and firefly luciferase values were normalized to Renilla luciferase values and expressed as relative luciferase units (RLU).

Chromatin Immunoprecipitation (Chip) Assay

Chip assay was carried out with a SimpleChIP Enzymatic Chromatin IP Kit (Cell signaling Technologies, USA) according to the manufacturer's instruction as previously described by others.[13] Briefly, crosslinked DNA-protein complexes from U266 cells were immunoprecipitated using an equal amount of the anti-LEF1 antibody (#76010, Cell Signaling Technology, USA) or normal rabbit IgG (#2729, Cell Signaling Technology, USA). After reversal of cross-linking, the immunoprecipitated chromatin was amplified by Q-PCR using the SYBR green system (ThermoFisher Scientific, USA) with the following primers: TCCTTCCTCCTTATCGTATG (forward); GGCAACACCGTTCTCATCTA (reverse). Relative occupancy values were determined by calculating the ratio of the amount of immunoprecipitated DNA to that of the input sample. The values were then normalized to the values of IgG control, which was defined as 1.

Western Blotting Analysis

Protein extraction and Western Blotting Analysis were performed as previously described.[11] Whole cell extracts were prepared using the M-PER reagent (Life Technologies, USA). Proteins were separated by SDS-PAGE (Bio-Rad Laboratories, USA), transferred to polyvinylidene fluoride membranes (Millipore, USA), blocked for 1 hour in buffer containing 5% nonfat dry milk (Lab Scientific, USA), and incubated with the primary antibodies purchased from Cell Signaling Technology (USA) including anti-LEF1 antibody (#76010, 1:1000 dilution), anti-CYLD antibody (#8462, 1:1000 dilution), anti-p65 antibody (#8242, 1:1000 dilution), anti-pp65 antibody (#3031, 1:500 dilution), anti-PARP antibody (#9532, 1:1000 dilution), anti-cleaved caspase 3 antibody (#9661, 1:1000 dilution)and anti-β-actin antibody (#4970, 1:2000 dilution) overnight. The HRP-conjugated anti-rabbit antibody(#7074, Cell Signaling Technology, 1:2000 dilution)was applied the next day and the signal was visualized on a Molecular Imager ChemidocXRS (Bio-Rad Laboratories, USA) using the Pierce Supersignal West Pico chemiluminescent substrate (Life Technologies, USA). Images were obtained using Quantity One software (Bio-Rad Laboratories, USA).

RNA Extraction and qRT-PCR

RNA isolation and qRT-PCR were performed as previously described.[11] Total cellular RNA was extracted using Nucleic acid PrepStation (Tiangen Biotech, China) according to the manufacturer's instruction. cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA) following manufacturer's instructions. The mixture was then incubated at 42°C for 1 h. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in a total volume of 20 μL, containing 50 ng cDNA, 1 μM of each gene-specific primer, 1 μM SYBR Green (ThermoFisher Scientific, USA), and 10 μL 2×Taq PCR Master-mix. Amplification was performed in an Exicycler TM 96 qPCR system (Bioneer Corporation, Korea). The primers used for amplification of individual genes were as follows: LEF1-Forward: CCAGACAAGCACAAACCTCTC, Reverse: CGGGCACTTTATTTGATGTTCTC; CYLD-Forward: CTCTTTACCATTCAGTCTCACC, Reverse: CTCATCTTCCAGTTCCAGTCC; CCND1-Forward: CCTC TAAGATGAAGGAGACCA, Reverse: AAATGAACTTCA-CATCTGTGGC; Bcl-2-Forard: GAGAGTGCTGAAGATT GATGG, Reverse: ACTTGATTCTGGTGTTTCCC; Bcl-xL-Forward: AATTCCTGTGTCGCCTTCTG, Reverse: GAAAG AGATTCAAATCCGCCT; β-actin-Forward: AAGATCAAG ATCATTGCTCCTCC, Reverse: GTCATAGTCCGCCTAGA AGCA.

The amplification conditions included a denaturation step at 95°C for 5 minutes followed by 40 cycles of 15 seconds at 95°C, 45 seconds at 60°C, and 45 seconds at 72°C. Samples were tested in triplicates and the mean cycle numbers were included in the analysis. The relative fold change was normalized against β-actin.

Detection of Intracellular ROS Accumulation

Intracellular ROS production was measured using a ROS assay kit (Beyotime, China) according to the manufacturer's instruction as described previously.[14] Cells were incubated with 10 μM DCFH-DA solution at 37°C for 30 min in darkness, followed by 3 washes with PBS. The non-fluorescent probe

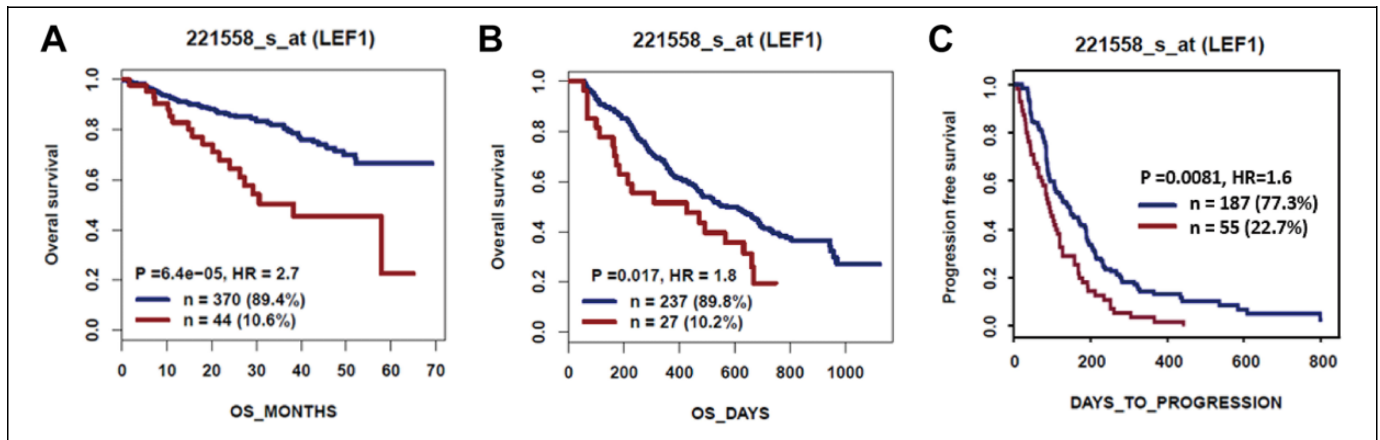

Figure 1. Association between LEF1 mRNA expression with survival status of MM patients. A, The overall survival curve exhibited the overall survival of MM patients with low or high expression levels of LEF1 mRNA using GenomicScape software (www.genomicscape.com). B, The progression-free survival (Left) and overall survival (Right) status of MM patients with low or high expression levels of LEF1 mRNA using GenomicScape software (www.genomicscape.com). Red line: high expression group, blue line: low expression group. *P* values were denoted on the plot.

DCFH-DA can permeate cell membranes and is cleaved by esterase to yield non-fluorescent DCFH, which can be oxidized in the presence of ROS to generate the highly fluorescent 2,7-dichlorofluorescein (DCF).[14] The stained cells were analyzed with a BD LSRII flow cytometer. Results were processed using CellQuest program (BD Biosciences, USA).

Statistical Analysis

SPSS software version 16.0 for Windows (SPSS Inc., USA) was used for all analyses. All numerical data were expressed as mean $\pm$ SD from at least 3 independent replicates. Survival was compared using Log-rank test. *t*-test and one-way ANOVA were used to compare 2 or 3 independent groups, respectively. A *P* value of 0.05 or less was considered statistically significant.

Results

High LEF1 Expression Predicted Poor Prognosis in Patients With Myeloma

To explore the relation between LEF1 expression and disease outcome, we analyzed a publically available microarray dataset containing 414 newly diagnosed myeloma patients (GSE2658). As shown in Figure 1A, there was a significant association between high LEF1 expression and inferior overall survival. In addition, analysis of a cohort of relapsed myeloma patients (GSE9782, n = 256) showed that both progression-free survival and overall survival were reduced in the cases exhibiting high LEF1 expression (Figure 1B). All these findings indicated that upregulation of LEF1 in both newly diagnosed and relapsed myeloma patients was associated with disease progression and inferior outcome.

LEF1 Downregulation Inhibited Myeloma Cell Proliferation In Vitro

To evaluate the functional role of LEF1, 2 human myeloma cell lines U266 and RPMI8226 were infected with lentiviruses encoding 2 different short hairpin RNAs (shRNAs) against LEF-1 or scramble shRNA as a control. After confirming that LEF1 mRNA and protein levels could be efficiently downregulated in both cell lines following transduction of LEF-1 specific shRNA (Figure 2A), we examined the cell viability by CCK8 assay, which demonstrated that depletion of LEF-1 resulted in a remarkable decrease in the viability of U266 and RPMI8226 cells (Figure 2B). To determine whether knockdown of LEF1 may affect cell proliferation, we performed Brdu incorporation ELISA assay. As shown in Figure 2C, silencing of LEF-1 led to decreased Brdu incorporation in U266 and RPMI8226 cells. These results suggested that LEF1 downregulation exhibited a negative effect on the proliferation of myeloma cells.

LEF1 Depletion Induced Apoptosis of Myeloma Cells In Vitro

Next we sought to determine whether LEF1 depletion could trigger apoptosis of myeloma cells. We observed that knockdown of LEF1 could induce apparent apoptosis in U266 and RPMI8226 cells, as determined by flow cytometric analysis following Annexin V/7AAD staining (Figure 3A). In accordance with this, immunobloting analysis revealed increased cleavage of caspase 3 and PARP in U266 and RPMI8226 cells following depletion of LEF1 (Figure 3B), suggesting that increased apoptosis may also contribute to decreased cell viability induced by LEF1 depletion. Since ROS generation is an important factor facilitating mitochondrial apoptosis, we evaluated the total intracellular ROS generation in myeloma cells following LEF1 depletion. As shown in Figure 3C, ROS levels

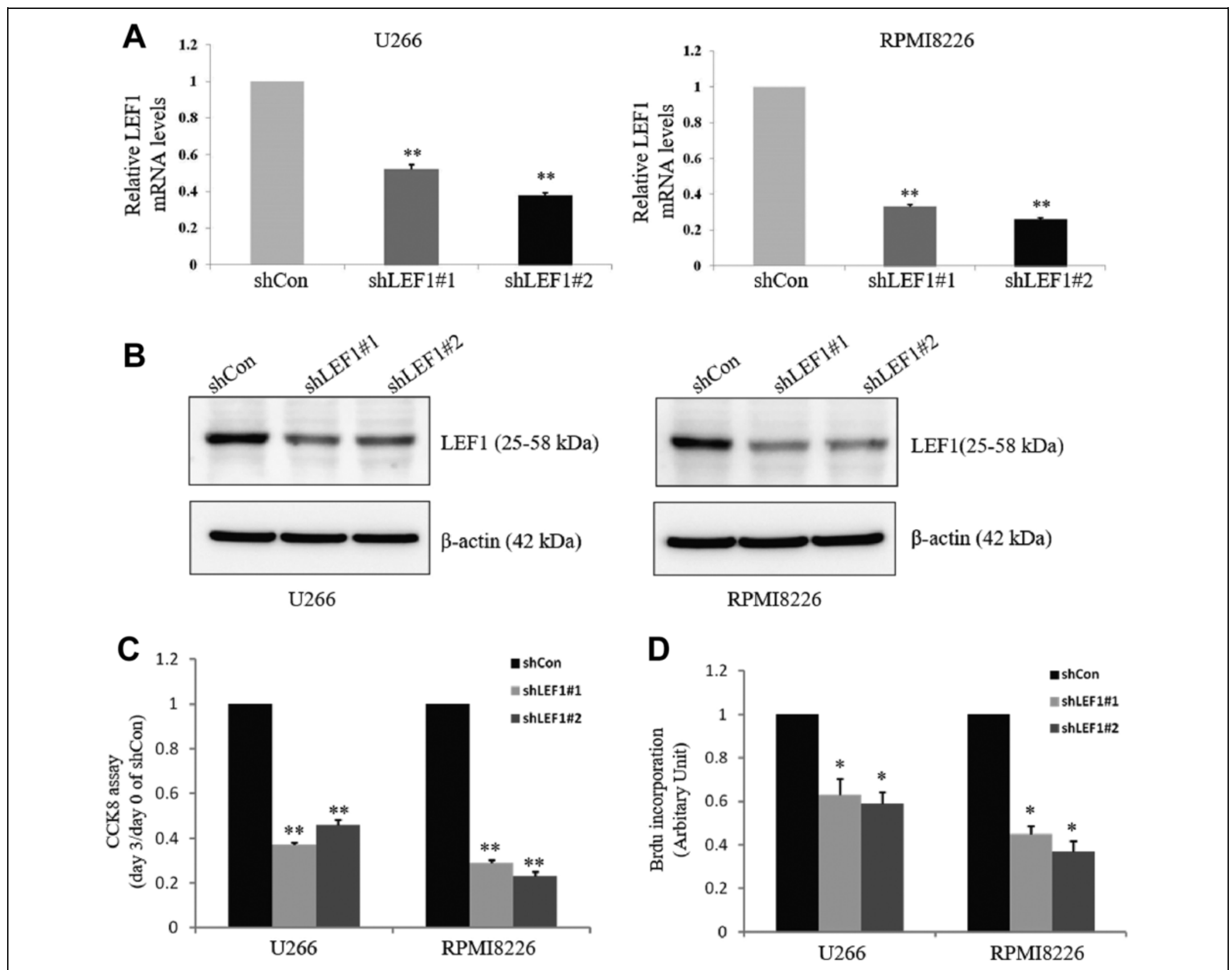

Figure 2. Knockdown of LEF1 suppressed growth of U266 and RPMI8226 myeloma cells. A, The mRNA expression level of LEF1 in U266 and RPMI8226 cells infected with LEF1 shRNA (shLEF1) or scramble shRNA as the control (shCon) for 48 h was assessed by quantitative real-time RT-PCR (qRT-PCR). B, The protein expression level of LEF1 in U266 and RPMI8226 cells infected with shLEF1 or shCon for 72 h was detected by Westernblot analysis. C, The cell viability of U266 and RPMI8226 cells following transduction with shLEF1 or shCon was determined by CCK-8 assay. D, Brdu incorporation was determined in U266 and RPMI8226 cells transduced with shLEF1 or shCon. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus shcon group.

were significantly elevated in myeloma cells subject to LEF1 depletion, suggesting that LEF1 may prevent apoptosis of myeloma cells by inhibiting intracellular ROS generation. These results supported that LEF1 was essential for both proliferation and survival of myeloma cells.

LEF1 Repressed CYLD Transcription Through Binding CYLD Promoter in Myeloma Cells

It has been documented that LEF1 could negatively regulate CYLD expression in colorectal cancer cells.[15] Given that CYLD has been reported to function as a tumor suppressor in myeloma cells,[16] we sought to examine whether LEF1 could influence the expression of CYLD in myeloma cells. Consistent with this report, we found attenuation of LEF1 resulted in the increased expression of CYLD protein in U266 and RPMI8226 cells, as determined by western blot analysis (Figure 4A); and real-time reverse transcription-PCR analysis showed that CYLD mRNA expression was parallelly augmented in LEF1-deficient cells, suggesting that the enhanced CYLD protein expression was mainly due to increased transcription of CYLD gene following LEF1 depletion.

Inspection of proximal promoter region of human CYLD gene revealed a potential LEF1-binding site (CTTTGTT) at −1374 bp relative to the transcription start site (TSS) (Figure 4C). To explore whether LEF-1 may regulate expression of CYLD mRNA through affecting its promoter activity, the

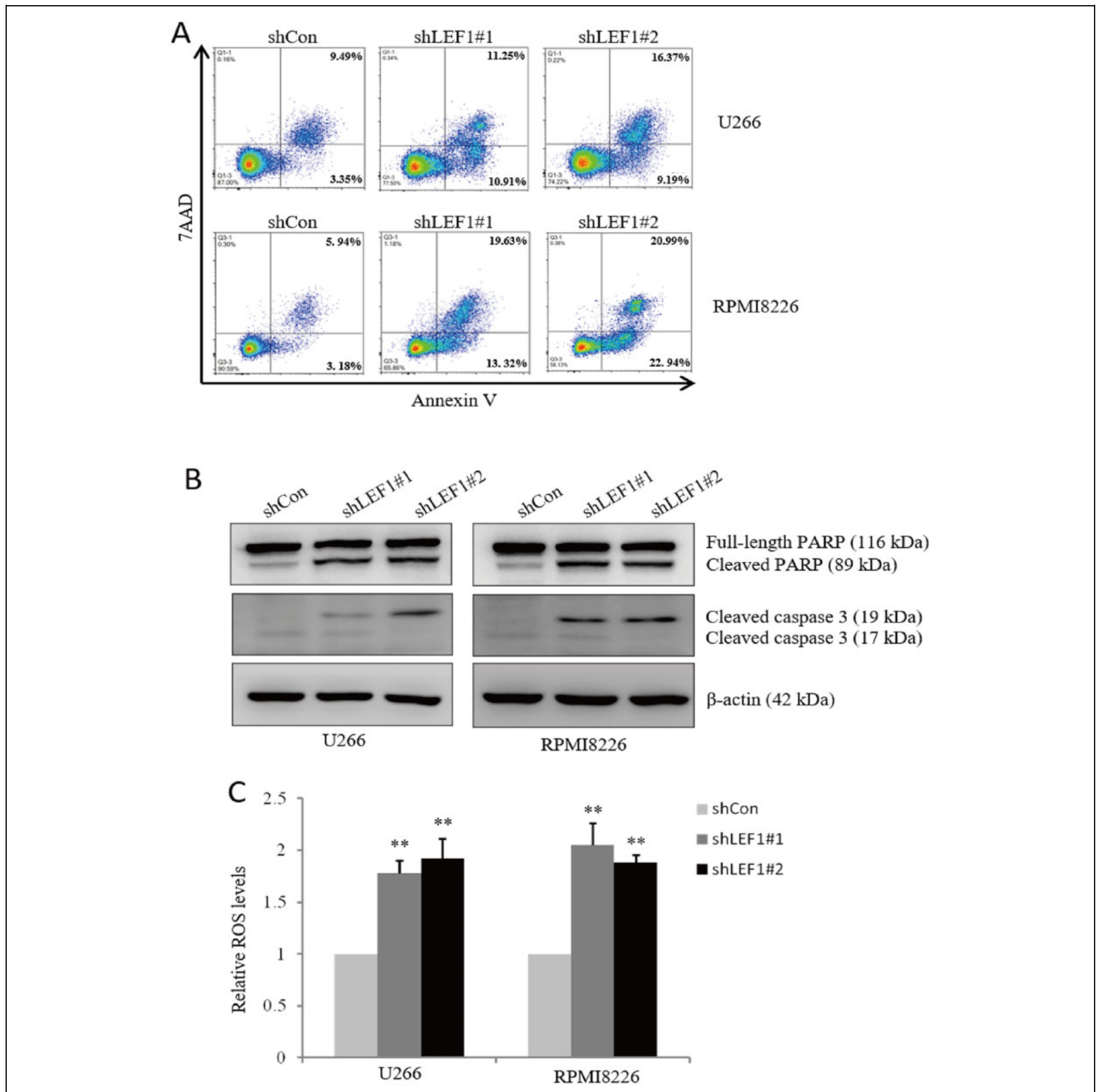

Figure 3. Depletion of LEF1 induced apoptosis of U266 and RPMI8226 myeloma cells. A-B, The cellular apoptosis was detected in U266 and RPMI8226 cells transduced with shLEF1 or shCon for 5 days by flow cytometry analysis following Annexin V/7AAD staining. C, The protein levels of PARP and cleaved caspase 3 was detected in U266 and RPMI8226 cells transduced with shLEF1 or shCon for 5 days by Westernblot analysis. D, Intracellular ROS production was determined by CM-H2DCFDA staining in U266 and RPMI8226 cells following transduction with shLEF1 or shCon for 72 h. $*P < 0.05$ and $**P < 0.01$ versus shCon group.

proximal promoter region of CYLD gene (from −1500 bp to +85 bp) was amplified and subcloned into pGL3-basic luciferase reporter vector, yielding pGL3-CYLD reporter plasmid. The dual luciferase reporter assay result showed that knockdown of LEF1 apparently augmented the CYLD promoter activity in U266 cells. Then we introduced the mutation (CTTTGTT was changed to CCCCGCC), which destroyed the potential LEF1-binding site on pGL3-CYLD plasmid, and found that introduction of this mutation largely abolished the repressive effect of LEF1 on the CYLD promoter (Figure 4D), indicating that this responsive motif was indispensible for the regulatory function of LEF1 on CYLD promoter.

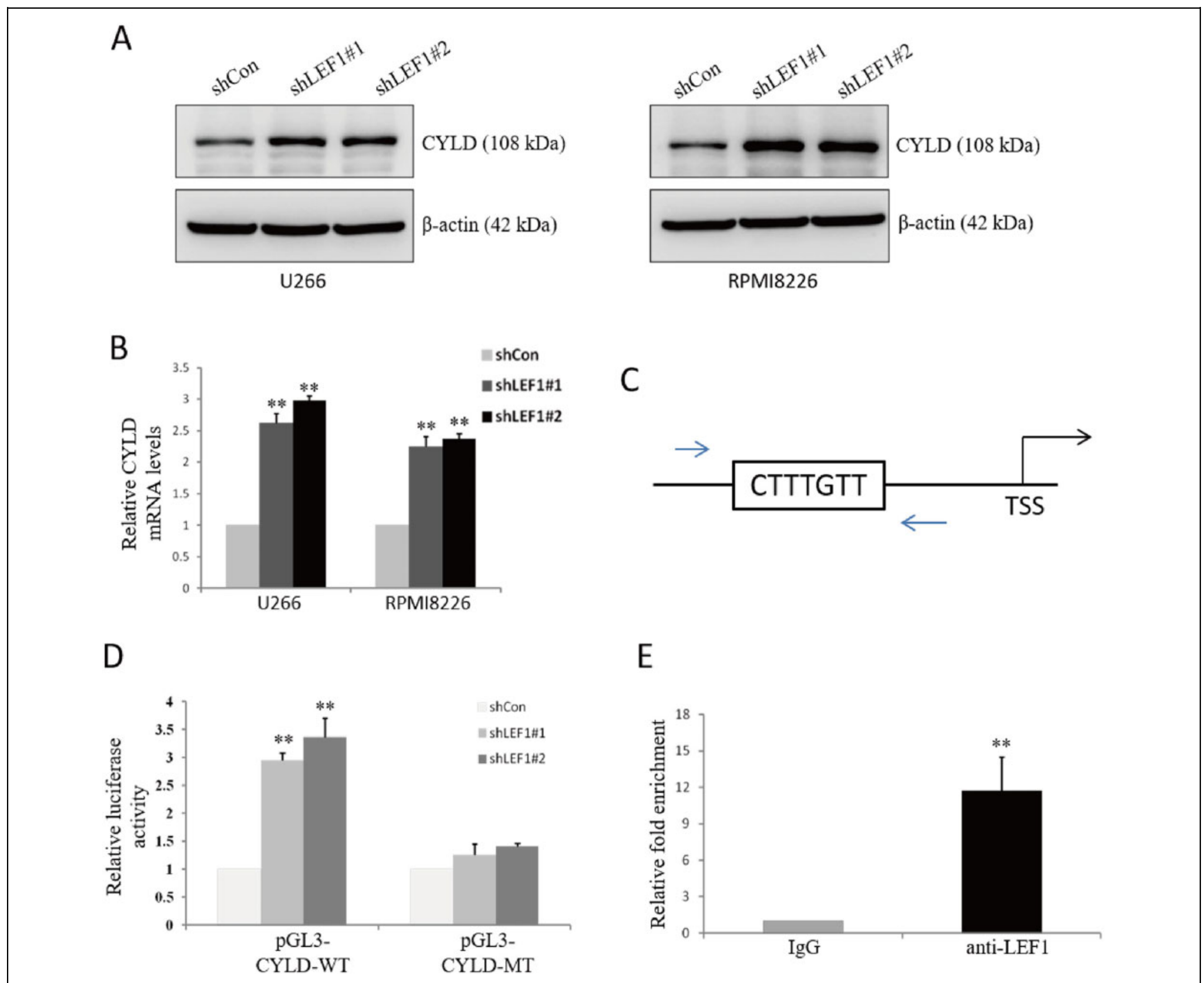

Figure 4. LEF1 inhibited transcription of CYLD gene through promoter binding. A, The protein levels of CYLD in U266 and RPMI8226 cells following transduction with shLEF1 or shCon for 72 h was measured by Westernblot analysis. B, The CYLD mRNA expression level in U266 and RPMI8226 cells transduced with shLEF1 or shCon for 72 h was determined using qRT-PCR. C, Schematic representation of potential LEF1-binding sites in CYLD promoter and the location of the primers used for ChIP assay. D, U266 cells transduced with shLEF1 or shCon were co-transfected with either wild-type CYLD luciferase reporter construct (pGL3-CYLD-wt) or the plasmid containing the mutation in the putative LEF1 binding site (pGL3-CYLD-mt) together with the pRL-CMV plasmid for normalization of transfection efficacy for 24 h, and relative luciferase activity was determined with dual-luciferase reporter assay kit. E, ChIP assay was performed to show binding of LEF1 on CYLD promoter in U266 cells. Anti-LEF1 antibody or control IgG was used to precipitate sonicated chromatin from U266 cells. The purified DNA was analyzed by real-time PCR with primers flanking potential LEF1 binding site. Data showed fold enrichment relative to control IgG. $^*P < 0.05$ and $^{**}P < 0.01$ versus shCon group.

To further explore the recruitment of LEF1 on CYLD promoter in U266 cells, we carried out a Chip assay by immunoprecipitating DNA/protein complexes with a anti-LEF1 monoclonal antibody and then amplified the purified DNA with specific primers flanking the potential LEF1 binding site. As expectedly, LEF1 was found to be recruited on the CYLD promoter region surrounding the potential LEF1-binding site (Figure 4E). Collectively, LEF1 may negatively modulate transcription of CYLD gene through directly binding CYLD promoter in myeloma cells.

LEF1 Knockdown Blunted NF-kB Signaling Pathway in Myeloma Cells

NF-κB signaling pathway is frequently overactivated in multiple myeloma and plays an essential role in myeloma cell growth and survival,[17,18] and meanwhile, CYLD has been reported to repress NF-κB signaling pathway in MM cells.[16] Therefore, we were motivated to examine whether the observed CYLD upregulation following LEF1 depletion was sufficient to interfere with NF-κB signaling. Silencing of LEF1

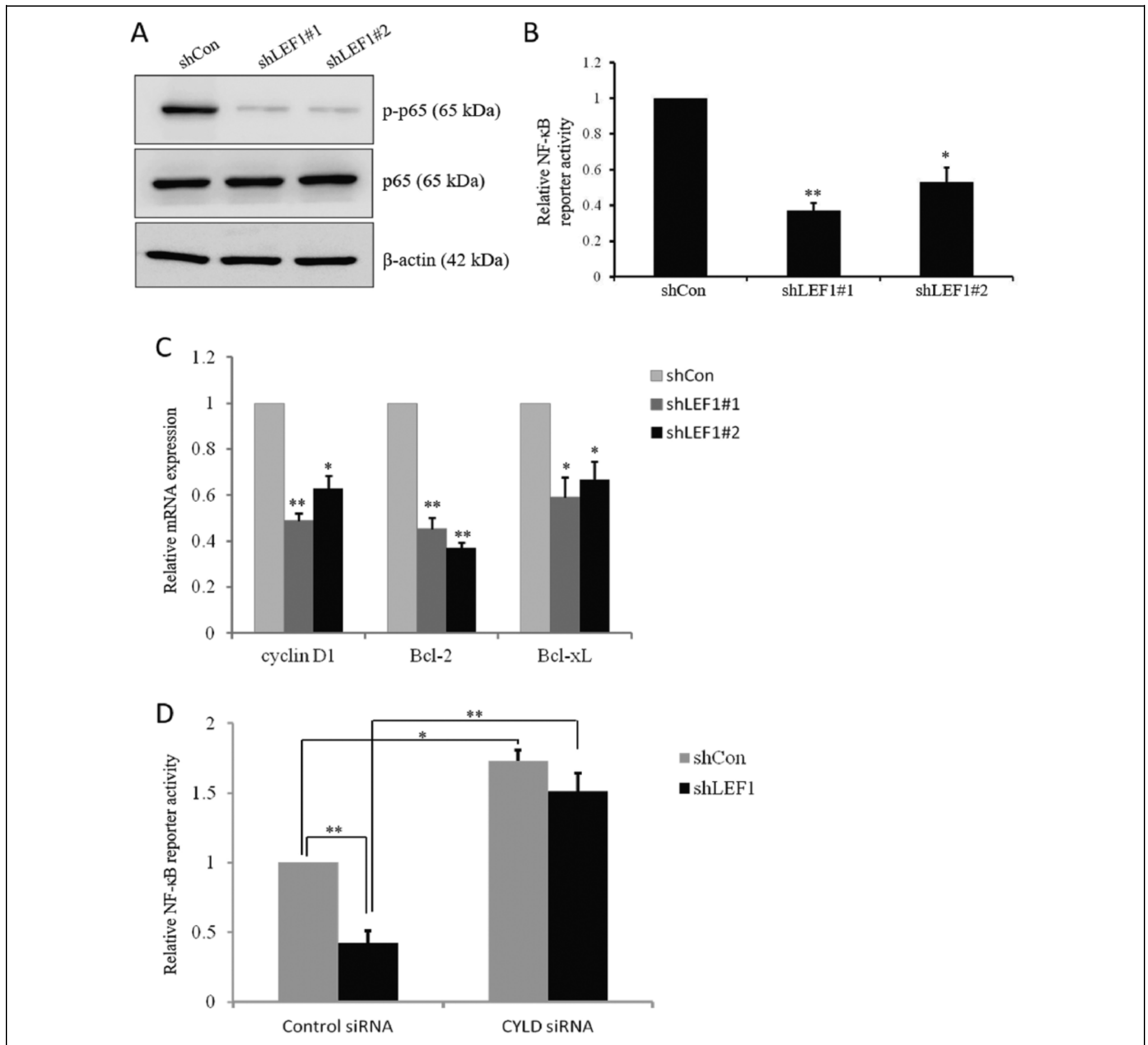

Figure 5. Depletion of LEF1 repressed NF-κB signaling pathway through modulation of CYLD in U266 cells. A, The protein levels of p65 and p-p65 in U266 cells following LEF1 knockdown for 72 h was measured by Westernblot analysis. B, U266 cells transduced with shLEF1 or shCon were co-transfected with NF-κB reporter construct (p-NF-κB-luc) together with the pRL-CMV plasmid for normalization of transfection efficacy for 24 h, and relative luciferase activity was determined using dual-luciferase reporter assay kit. C, The mRNA expression levels of Cyclin D1, Bcl-2 and Bcl-xL in U266 cells transduced with shLEF1 or shCon for 72 h were determined using qRT-PCR. $*P < 0.05$ and $**P < 0.01$ versus shCon group. D, U266 cells transduced with shLEF1 or shCon were co-transfected with NF-κB reporter construct (p-NF-κB-luc) in the presence of CYLD siRNA or the control siRNA together with the pRL-CMV plasmid for normalization of transfection efficacy for 24 h, and relative luciferase activity was determined using dual-luciferase reporter assay kit. $*P < 0.05$ and $**P < 0.01$.

significantly downregulated the protein level of phosphorylated NF-κB p65 (pS536) rather than that of the total NF-κB p65 in U266 cells as determined by western blot analysis (Figure 5A), indicative of inactivation of NF-κB p65 signaling by LEF1 knockdown. In accordance with this finding, we found LEF1-deficient U266 cells displayed significantly lower basal NF-κB luciferase reporter activity (Figure 5B). Moreover, the mRNA expression levels of 3 putative NF-κB target genes relevant to cell growth and survival, including cyclin D1, Bcl-2 and Bcl-xL,[19] were also obviously downregulated in LEF1-deficient U266 cells (Figure 5C). To further confirm the involvement of CYLD in LEF1-regulating NF-κB activity, we

employed siRNA technique to knockdown CYLD gene in U266 cells with LEF1-depletion and then check the NF-κB luciferase reporter activity. CYLD mRNA expression level was successfully reduced in U266 cells transfected with CYLD-specific siRNA as confirmed by Q-RT-PCR (data not shown). As shown in Figure 5D, downregulation of NF-κB luciferase reporter activity elicited by LEF1 depletion could be largely rescued when CYLD specific siRNA was introduced into the cells. Meanwhile, we observed that transfection with CYLD-specific siRNA was sufficient to augment NF-κB luciferase reporter activity in U266 cells, which was consistent with the previous report.[16] Hence, our data strongly corroborated that LEF1 may boost NF-κB activity through downregulation of CYLD in myeloma cells.

Discussion

The outcome of MM has been significantly improved due to the introduction of novel agents. However, the therapeutic options are still limited for patients with relapsed or refractory MM, which have poor survival outcomes. Therefore, better delineation of the molecular mechanisms underlying development and progression of MM with the aim to develop novel therapeutic strategies is urgently desired.

Accumulating evidence has showed that high LEF1 expression is correlated with poor prognosis and overall survival of leukemia and therefore LEF1 may serve as a potential marker for predicting disease progression.[4-6] However, whether LEF1 may predict poor prognosis and overall survival in MM remains obscure. Although we have previously[10] reported that MM patients with high LEF1 expression had a poorer overall survival than those with low LEF1 expression, the study had some limitation in that it was merely based on a relatively small sample size ($n = 40$). To address this limitation, in present study we utilized 2 publically available database with relatively large sample size, including 414 newly diagnosed and 256 relapsed MM patients, respectively, to investigate the correlation between LEF1 expression level and prognosis of MM patients. We found that in both databases, high LEF1 expression was well correlated with poor overall survival and/or disease-free survival in MM patients, which was in good accordance with our previous study, strongly indicating that LEF1 expression level may be utilized as a molecular marker for clinical diagnosis of MM patients.

The functional role of LEF1 has been extensively studied in leukemia. In AML, ectopic expression of wild-type LEF1 or of a constitutively-active LEF1 mutant in murine bone marrow led to the onset of AML in transplanted mice.[7] In ALL, point mutations located in exons 2 (K86E) and 3 (P106 L) of LEF1 resulted in enhanced promoter activity and high expression of c-myc and cyclin D1, leading to accelerated leukemia cell proliferation.[20] Moreover, LEF1 has been recently found to drive the nuclear localization of β-catenin in AML cells.[21] In CLL, inhibition of nuclear translocation of LEF1 or disruption of the interaction between LEF1 and β-catenin caused apoptosis in leukemia cells, implying that LEF1 may be an attractive therapeutic target for CLL therapy.[22] However, the functional roles of LEF1 in MM remains unexplored. Herein, we explored the influence of shRNA-mediated LEF1 knockdown on myeloma cell growth *in vitro*. We found that knockdown of LEF1 by shRNA could impair the viability of myeloma, which may be attributed to impeded cell proliferation as well as increased cell apoptosis. This suggested that LEF1 was indispensible for maintaining the growth and survival of myeloma cells *in vitro*, and therefore may act as a tumor-promoting factor. Regarding the downstream target of LEF1 in myeloma cells, we focused on CYLD gene. CYLD was originally identified as a gene mutated in familial cylindromatosis, a genetic condition that predisposes patients for the development of cylindroma.[23] As a member of deubiquitinase family, CYLD can modulate various physiological processes, spanning from immune response and inflammation to cell-cycle progression as well as osteoclastogenesis.[24] In agreement with the previous finding in colon cancer cells,[15] we found LEF1 gene silencing dramatically enhanced endogenous CYLD mRNA and protein expression in MM cells, suggesting that LEF1 may also negatively modulate CYLD expression in MM cells. Our Chip assay and luciferase reporter assay further supported that the suppressive effect of LEF1 on CYLD transcription in MM cells was mediated through direct binding LEF1 responsive element within CYLD promoter. On the other hand, although LEF1 is a downstream effector of the Wnt/β-catenin signaling pathway, the inhibitory effect of LEF1 on CYLD transcription seemed to be independent of β-catenin in MM cells, which was based on our observation that knockdown of β-catenin did not significantly affect the expression level of CYLD in MM cells (data not shown). It was tempting to speculate that LEF1 may cooperate with some unknown partners to execute repressive effect on CYLD transcription, although the detailed machinery still warrants further study.

Since CYLD may function as a tumor suppressor through negative control of NF-κB signaling in MM cells, we were prompted to examine whether LEF1 depletion-mediated CYLD upregulation was sufficient to block NF-κB signaling pathway in MM cells. Our results from Westernblot and luciferase reporter assay together with Q-PCR showed that NF-κB signaling pathway was indeed activated in MM cells with LEF1 depletion. More importantly, we observed that the decrease in NF-κB luciferase reporter activity triggered by LEF1 depletion could be largely rescued when CYLD specific siRNA was introduced into MM cells, providing the compelling evidence to corroborate that LEF1 may function to activate NF-κB signaling pathway through downregulation of CYLD in MM cells. Noteworthily, the stimulatory effect of LEF1 on NF-κB signaling in MM cells might be cellular context-dependent, since it has not been reported in any other type of cells. Given that activation of NF-κB signaling plays a pivotal role in the expansion and survival of myeloma cells, we concluded that the growth-promoting effect of LEF1 on myeloma cells might be at least partly through activation of NF-κB signaling pathway.

Conclusion

In summary, the present study showed that LEF1 expression level was inversely associated with the overall survival as well as disease-free survival of MM patients. LEF1 may favor the proliferation and survival of MM cells, at least partly through direct repression of CYLD transcription and subsequent activation of NF-κB signaling pathway. Therefore, our study highlights LEF1 as a novel and attractive therapeutic target against MM.

Authors' Note

Jinge Xu and Guihua Zhang conceived and designed the experiments; Guihua Zhang, Kaige Liu and Jinyan Wu conducted the experiments; Faan Miao performed statistic analysis; Guihua Zhang and Faan Miao wrote the paper. All authors read and approved the final manuscript. Our experiments did not involve patient and animal experiments.

Declaration of Conflicting Interests

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