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SENP1 inhibition induces apoptosis and growth arrest of multiple myeloma cells through modulation of NF-κB signaling

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ABSTRACT

SUMO/sentrin specific protease 1 (Senp1) is an important regulation protease in the protein sumoylation, which affects the cell cycle, proliferation and differentiation. The role of Senp1 mediated protein desumoylation in pathophysiological progression of multiple myeloma is unknown. In this study, we demonstrated that Senp1 is overexpressed and induced by IL-6 in multiple myeloma cells. Lentivirus-mediated Senp1 knockdown triggers apoptosis and reduces viability, proliferation and colony forming ability of MM cells. The NF-κB family members including P65 and inhibitor protein IkBα play important roles in regulation of MM cell survival and proliferation. We further demonstrated that Senp1 inhibition decreased IL-6-induced P65 and IkBα phosphorylation, leading to inactivation of NF-κB signaling in MM cells. These results delineate a key role for Senp1in IL-6 induced proliferation and survival of MM cells, suggesting it may be a potential new therapeutic target in MM.

1. Introduction

Multiple myeloma (MM) is a common blood cancer of plasma cells. Current therapeutic strategies such as immunotherapy, high-dose melphalan, thalidomide, lenalidomide and bortezomib have improved significantly the outcome of patients with MM [1]. However, MM is a still incurable hematopoietic malignancy with high mortality and relapse rates. Extensive molecular and genomic characterization of signal molecules and pathways of MM cells will provide novel drugable targets for its therapy [2].

Bone marrow microenvironments, which are composed of a cellular compartment and the liquid milieu (such as stromal cells and IL-6), confer proliferation, survival, migration and drug resistance of MM cells [3]. Specifically, the interaction between MM cells and stromal cells triggers aberrant expression of interleukin-6 (IL-6) and activation of NF-κB signaling pathway [4]. IL-6 plays a critical role in cell growth and survival through activation of MEK/MAPK, JAK/STAT3, and PI3K/Akt signaling pathways in MM cells [5]. NF-κB activation contributes to MM cell proliferation, inhibition of apoptosis, and emergence of therapy resistance. Both genetic mutations and aberrant extracellular stimuli result in constitutive activation of NF-kB pathway in MM. Several therapeutic agents with NF-κB inhibitory activity, including proteasome inhibitors, thalidomide, lenalidomide and arsenic trioxide, and novel inhibitor DTP3 are effective for the treatment of MM [6,7]. Novel selective inhibitor of NF-κB shows significant therapeutic effects in mouse myeloma xenograft models [7]. However, functional analysis of link molecules between IL-6 aberrant expression and NF-kB deregulation is less well described.

Small ubiquitin-like modifier (SUMO) is a novel ubiquitin-like protein that can reversibly modify proteins. SUMO modification has emerged as a novel regulatory mechanism during signal transduction and affected a large number of cellular processes [8,9]. Sumoylation is a dynamic process that is mediated by activating (E1), conjugating (E2), and ligating (E3) enzymes and is readily reversed

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by a family of SUMO-specific proteases (Senps) [10]. Among seven Senp members (Senp1-7) reported, Senp1 is a SUMO-specific protease with functions of processing of mature SUMO and deconjugating them from targeted proteins [11]. It mediated desumoylation of various proteins such as Sirt1,p53, HADC et al. [12,13].

Sumoylation of NF-kB complex proteins is a novel mechanism for its activation. Given the importance of Senp1 in regulation of activation in growth factor signaling, we hypothesized that Senp1 might also be involved in IL-6 signaling and NF-kB activation. In this study, we have detected the expression of Senp1 in MM cells and shown that IL-6 upregulates its expression in MM cells. We further demonstrated that Senp1 activation mediates the IL-6 induced activation of NF-kB in MM cells.

2. Materials and methods

2.1. Cell lines

Human embryonic kidney 293T cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (HyClone Laboratories, Logan, UT, USA). Human multiple myeloma cell lines, XG-7 and RPMI-8226, were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS and 3 ng/ml IL-6.

2.2. Patient samples and CD138$^+$ cell isolation

Bone marrow (BM) samples from health donor and MM patients were obtained under protocols approved by the Institutional Ethics Board at Beijing Institute of Radiation Medicine. Mononuclear cells were prepared by Ficoll-Hypaque (Sigma–Aldrich, St Louis, MO, USA) gradient centrifugation. CD138$^+$ cells were isolated as previously described [14].

2.3. Q-PCR assay

Total RNAs from XG-7, RPMI-8226 cells were extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Real-time PCR was carried out in ABI 7500 fast system by using SYBR-Green I as a double-strand DNA-specific binding dye. PCRs were performed in triplicate for each. The mRNAs of Senp1 and house-keeping gene actin RNA were quantified in separate tubes. The value represents the relative level of target gene expression. The primers for PCR Senp1forward:ATCAGGCAGTGAAACGTTGGAC, reverse: GCAGGCTTC ATTGTTTATCCCA. β-actin forward: CATCCTCACCCTGAAGTACCC, reverse: AGCCTGGATAGCAACGTACATG.

2.4. Lentivirus vector production and transduction of MM cells

The Senp1 shRNA 1 and shRNA2 (shRNA1: CCGGAACTACATCTTCGTGTACCTCCTCGAGGAGGTACACGAAGATGTAGTT TTTTT, shRNA2: CCGGGCGCCAGATTGAAGAACAGAACTCGAGTTCTGTT CTTCAATCTGGCGCTTTTT) were synthesized and cloned into a pLKO.1 lentivirus vector. An IRES-GFP construct was inserted this vector from Kpn1 site under puromycin resistant gene. Lentivirus particles were produced by co-transfecting 293T cells with lentivirus vectors and psPAX2, pMD2.G packaging plasmids. Supernatants containing virus particles were collected, filtered and concentrated using PEG. Titration of infectious virus particles was performed on HT1080 cells.

2.5. Colony-forming assay

RPMI-8226 and XG-7 cells were transduced with pLKO.1-Senp1 shRNAs and control vectors. These cells were dispensed into methycellulose culture medium and incubated in 24 well plates for 7 days in a humidified incubator. Colonies with at least 50 cells were counted using an inverted microscope.

2.6. Cell proliferation and apoptosis assays

MM cells transduced with pLKO.1-Senp1-shRNAs and control vector were cultured in completed culture medium at 37 °C for 48 h. The apoptotic cells were detected using APC-conjugated Annexin-V (BD Biosciences, San Jose,CA) and propidiumiodide (PI). Cells were washed with cold PBS and resuspended in Annexin-V binding buffer at a concentration of 1×10^6 cells/ml. 100 μl cells (1×10^5 cells) were added with 5 μl of Annexin V-APC with PI and incubated for 15 min. The apoptotic cells were detected by flowcytometry.

2.7. Western Blot

Protein extracts were prepared and resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in PBS for 2 h and labeled with primary antibodies anti-GAPDH, Anti-SUMO-1, anti-SUMO-2, anti-SENP1, anti-IKBα, anti-P-65 (Cell signaling, MA, USA) followed by horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (ZhongShan Golden Bridge Biotechnology, Beijing, China). Antibody detection was performed using enhanced chemiluminescence (Pirece Biotechnology, IL,USA).

2.8. Measurement of NF-κB nuclear translocation and DNA binding activity

The MM cell lines XG-7 and RPMI-8226 were transduced with pLKO.1-Senp1shRNAs and Ctrl vector. The nuclear protein of these transduced cells was extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Sangon Biotech, Shanghai, China). The probes of NF-κB (NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, 3′-TCAACTCCCCTGAAA GGGTCCG-5′) and OCT-1 binding site (5′-TGT CGA ATGCAA ATCACTAGA A-3′,3′-ACAGCTTACGTTTAGTGATCTT-5′) was label with biotin using EMSA probe biotin labeling kit (Beyotime,Nantong,JS,China) and mix with 20 μl nuclear extract for 20 min. The mixtures were then separated by 4% nondenaturing polyacrylamide gel and transferred to nylon membrane. The probe bound or either was detected using Chemiluminescent EMSA Kit.

2.9. Statistics

Data obtained from multiple experiments were reported as the mean ± SEM. Significance levels were determined by Student's *t* test and ANOVA analysis.

3. Results

3.1. Human MM cells express high level of Senp1 and IL-6 induces Senp1 expression via JAK/STAT signaling

To identify the Senp1 expression in MM cells, we analyzed their mRNA and protein levels in normal bone marrow mononuclear cells, MM cell lines and CD138$^+$ primary cells from MM patients. As shown in Fig. 1A, the Senp1 mRNA level in XG-7, RPMI-8226 and primary CD138 + MM cells were higher than that of normal mononuclear cells. The protein level of Senp1 overexpression was further confirmed by Western Blot (Fig. 1B).

IL-6 is aberrantly expressed by MM cells and in tumor microenvironments. It stimulates the growth and survival of MM cells via activation of multiple signaling cascades. We further tested

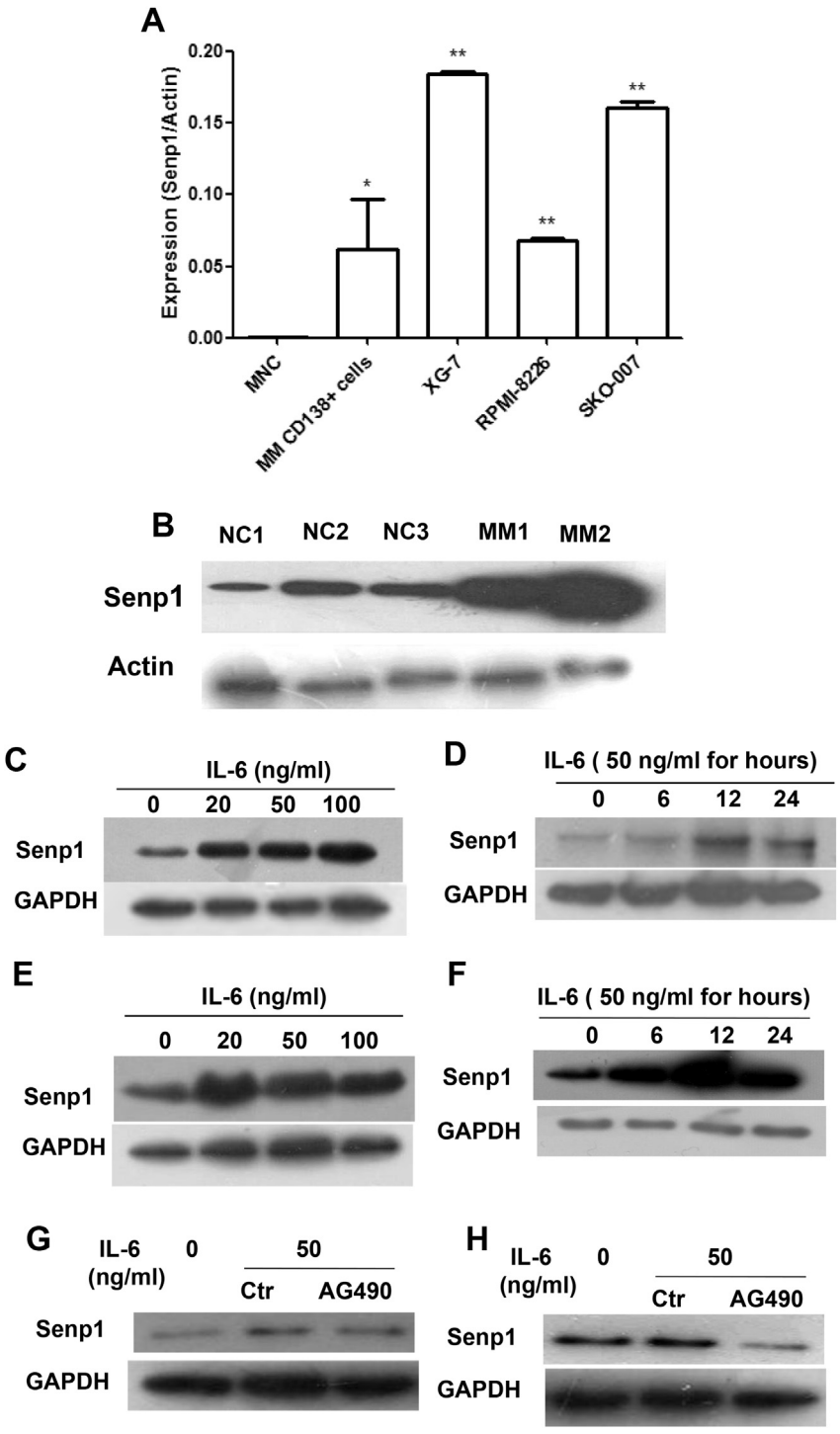

Fig. 1. Increased SENP1 levels in MM cells and IL-6 induces SENP1 expression in MM cell lines. (A)The Senp1 expression in MM cell line XG-7, RPMI-8226, SKO-007, primary MM CD138 + cells (n = 6) and normal bone marrow nuclear cells were evaluated by Q-RT-PCR.*$p < 0.05$, **$p < 0.01$. (B)The Senp1 protein level of primary MM CD138 + cells and normal bone marrow nuclear cells (NC) were determined by Western Blot. (C) XG-7 cells were treated with various concentrations of IL-6 for 12 h and Senp1 expression was determined by Western Blot. (D)XG-7 cells were treated with 50 ng/ml IL-6 for different periods indicated. Senp1 expression was determined by Western Blot. (E) RPMI-8226 cells were treated with various concentrations of IL-6 for 12 h and Senp1 expression was determined by Western Blot. (F) RPMI-8226 cells were treated with 50 ng/ml IL-6 for different periods indicated. Senp1 expression was determined by Western Blot. XG-7 (G) and RPMI-8226 cells (H) were pretreated with AG590 at a concentration of 100 μM for 1 h, then the cells were treated with 50 ng/ml IL-6 for 24 h, the Senp1 protein level were determined by Western Blot.

whether IL-6 could induce Senp1 expression in MM cells. The IL-6 dependent MM cell lines XG-7 and RPMI-8226 were starved overnight in serum-free medium, then treated with IL-6 at various concentrations and times. Senp1 expression was assayed by Western Blot. As shown in Fig. 1C–F, IL-6 induces Senp1 expression in a concentration and time dependent manner in both XG-7 and RPMI-8226 cells. The Senp1 upregulation reaches maximal level at 12 h post IL-6 treatment. AG490 is a specific and potent inhibitor that modulates JAK-Stat activation in various types of cells. We further pretreated XG-7 cells with AG490, and then assayed the IL-

6-induced Senp1 protein level. AG490 could block the upregulation of Senp1 induced by IL-6 (Fig. 1G and H). It is indicated that JAK-Stat is a central mediator in IL-6 induced-upregulation of Senp1.

3.2. Senp 1 knockdown inhibits the growth of MM cells

To explore the function of Senp1 in regulating growth of MM cells, we constructed pLKO.1-Senp1-shRNA (1 and 2) lentiviral vector to block its expression in MM cells. Transduction of XG7 cells with pLKO.1−Senp1 ShRNA-1 and shRNA-2 results in decreased mRNA and protein level of Senp1 in MM cells (Fig. 2A and B). These transduced MM cells were also assayed for growth and colony formation ability. Senp1 shRNA1 and shRNA2 expressing cells generated lower cell numbers in liquid culture compared to control vector expressing cells (Fig. 2C and D). Consistent with these results, knockdown of Senp1 significantly inhibited MM cell colony formation in semi-culture systems (Fig. 2E). Expression of Senp1 shRNA also resulted in significantly reduced cell division and the percentage of MM cells in S phase of the cell cycle (Fig. 2F and G).

3.3. Senp1silence induces apoptosis of MM cells

We further investigated the role of Senp1 knockdown on the apoptosis of MM cells. Senp1 shRNA transduction induces apoptosis of both XG-7 and RPMI-8226 cells (Fig. 3A and B). The average apoptosis rate was 34.4% for XG-7-Senp1-shRNA cells compared to 3.3% for XG-7-Ctr cells. Similar results were obtained in RPMI-8226 cells with Senp1 knockdown. The characteristic morphological features of apoptosis such as cell shrinkage, membrane blebbing and chromatin condensation were observed in these cells transduced with Senp1 shRNA (Fig. 3C). Our data indicated that senp-1 knockdown increases apoptosis of MM cells.

3.4. Senp1 shRNA increases protein sumoylation and blocked IL-6 -induced NF-κB activation

To clarify the roles of Senp1 in protein SUMO-modification in MM cells, we detected the total sumoylated protein MM cells. As shown in Fig. 4A, the Senp1 shRNA transduction significantly increases total SUMO-2 proteins in XG-7 cells or RPMI-8226 cells transduced with Senp1 shRNA. Protein sumoylation affects the cell cycle, proliferation and differentiation by regulating cellular signals including NF-kB pathway. There are several family members in NF-kB signal pathways including P65. We further checked the influences of Senp-1 knockdown on P65 phosphorylation. Senp1 shRNA transduction significantly reduced the P65 and P-IKBα phosphorylation in both XG-7 and RPMI-8226 cells (Fig. 4B).

To further check the roles of Senp1 in IL-6-induced activation of NF-kB in MM cells. We treated the Senp1 shRNA transduced cells with IL-6 and then detect the P65 phosphorylation. Senp1 knockdown significantly reduces IL-6 -induced P65 phosphorylation (Fig. 4C). Meanwhile, the DNA binding activity of NF-kB detected by EMSA was blocked in Senp1 shRNA transduced cells (Fig. 4D). These results demonstrated that Senp1 regulated IL-6-induced activation of NF-kB in MM cells through Sumo-modulation of NF-κB members.

4. Discussion

Sumoylation is a highly transient post-translational protein modification that affects the cell cycle, proliferation, apoptosis and differentiation [15]. Senps which removed SUMO intact from its substrates are the central players in the regulation of protein sumolytiom balances [16]. In this study, we identified that both MM cell lines and primary CD138^{+} tumor cells express high levels of

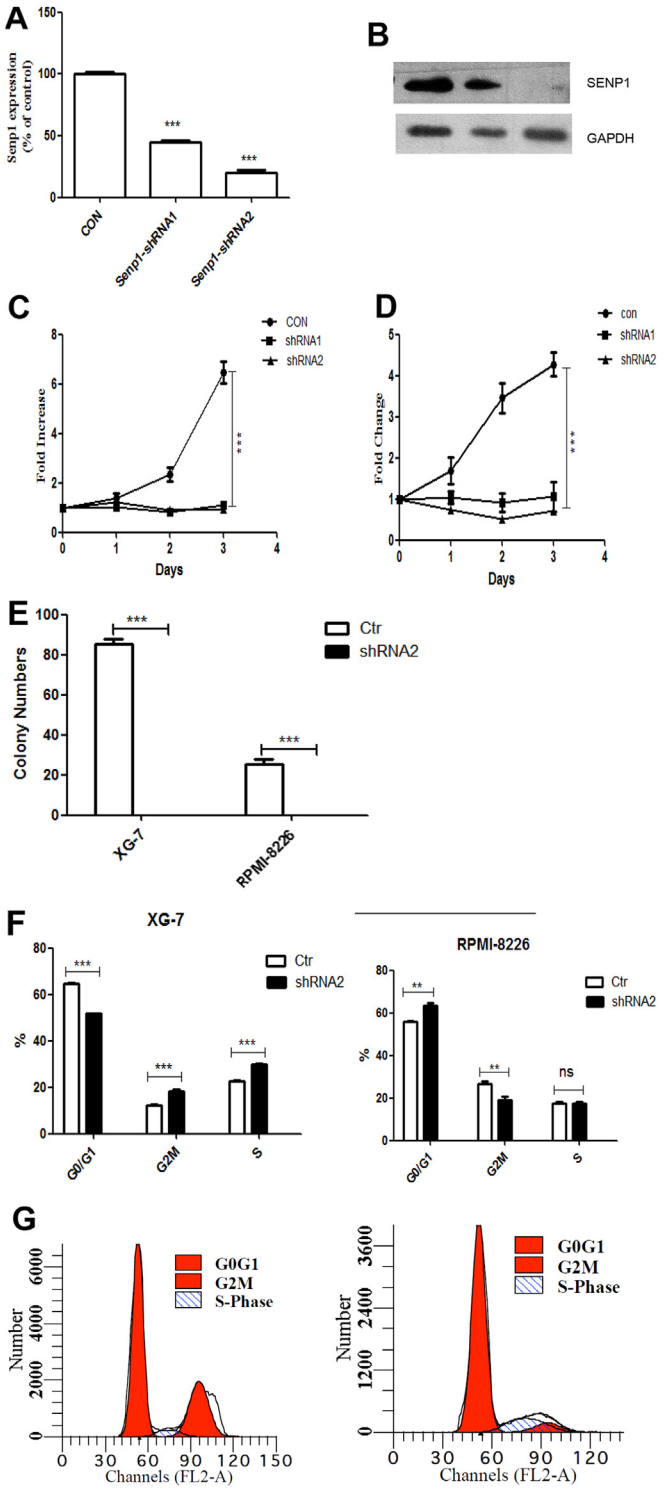

Fig. 2. Senp1 silence inhibits the growth and induces cell cycle arrest in MM cells. XG-7 cells were transduced with pLKO.1-Senp1-shRNA1, shRNA2 and control vector respectively. The mRNA (A) and protein level (B) of Senp1 expression were determined. The growth curve of XG-7 cells (C) and RPMI-8226 cells (D) transduced with Senp1 shRNA1 shRNA2 and control vectors. (E) The colony formation ability of XG-7 cells and RPMI-8226 cells transduced with Senp1 shRNA1 shRNA2 and control vectors. (F) The cell cycle distribution of XG-7 cells and RPMI-8226 cells transduced with Senp1 shRNA2 and control vectors. (G) The representative plots of cell cycle distribution of XG-7 cells transduced with Senp1 shRNA2 and control vectors.

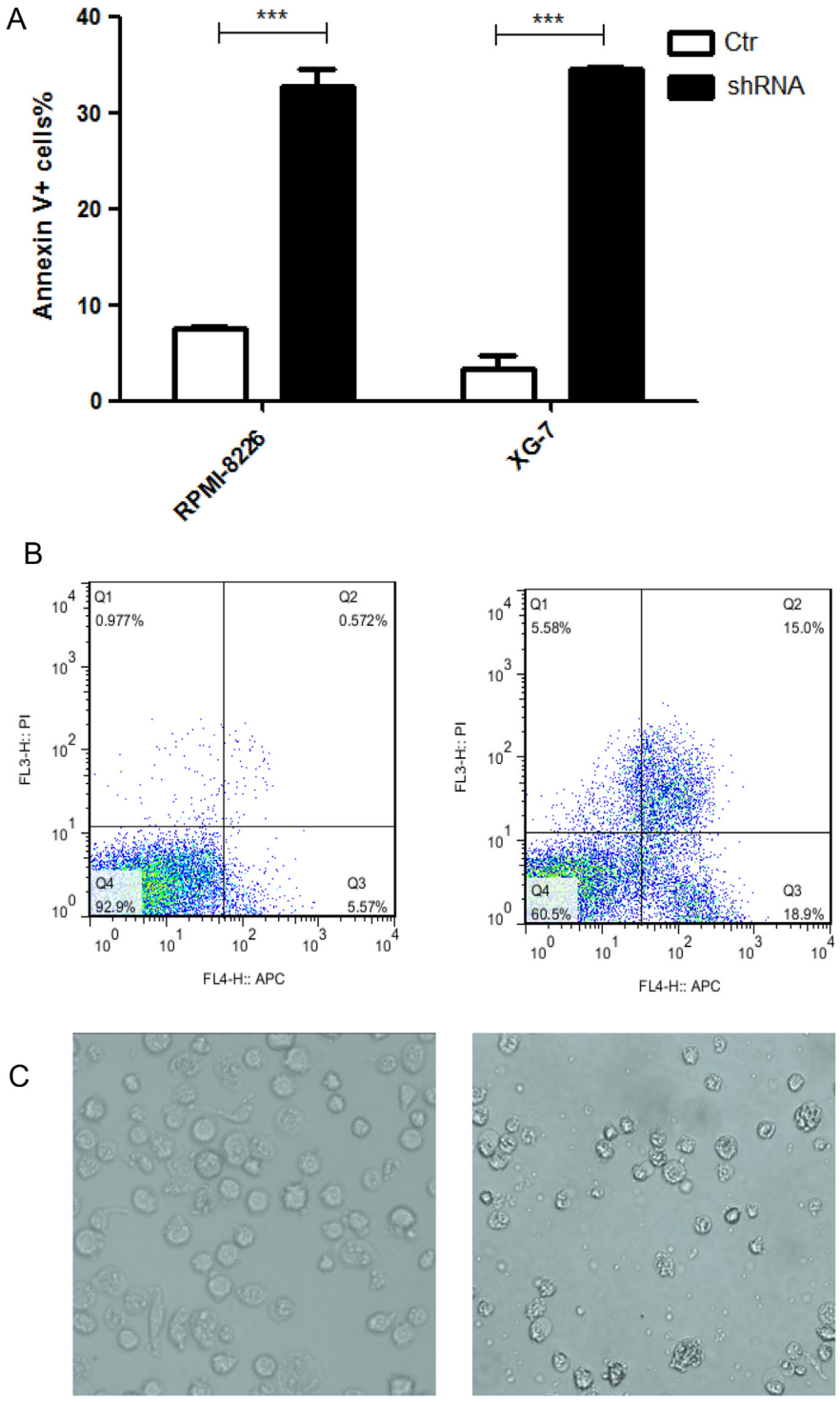

Fig. 3. Senp1 shRNA induces apoptosis of MM cells. (A)The XG-7 cells and RPMI-8226 cells transduced with Senp1 shRNA2 and control vector were assayed for apoptosis by Annexin V-APC labeling. (B) Representative flow cytometry plots are shown. (C)The morphological changes of XG7 cells transduced with Senp1 shRNA and Control vector.

Senp1. It is known that aberrant expression of IL-6 by MM cells and in tumor microenvironments are the major events in the pathophysiological progression of MM. We further confirmed that Senp1 upregulation is associated with IL-6 signaling. IL-6 triggers the Jak-STAT, MAPK and PI3K/AKT kinase pathways in MM cells [17]. By employing JAK2 specific inhibitor AG490, we clarify the contributions of the JAK/Stat signaling pathways in IL-6-induced Senp1 upregulation.

Senp1 is essential for the development of early T and B cells through regulation of STAT5 activation [18]. It is also over expressed by many types of tumors, including prostate cancer, lung carcinoma and hematopoietic malignancies [19–21]. Senp1 is shown as a marker of radioresistance of lung carcinoma radiotherapy [22]. Given the key role of Senp1 in carcinogenesis, we sought to investigate the roles and detail mechanisms that regulating the proliferation and survival of MM cells. We developed lentiviral

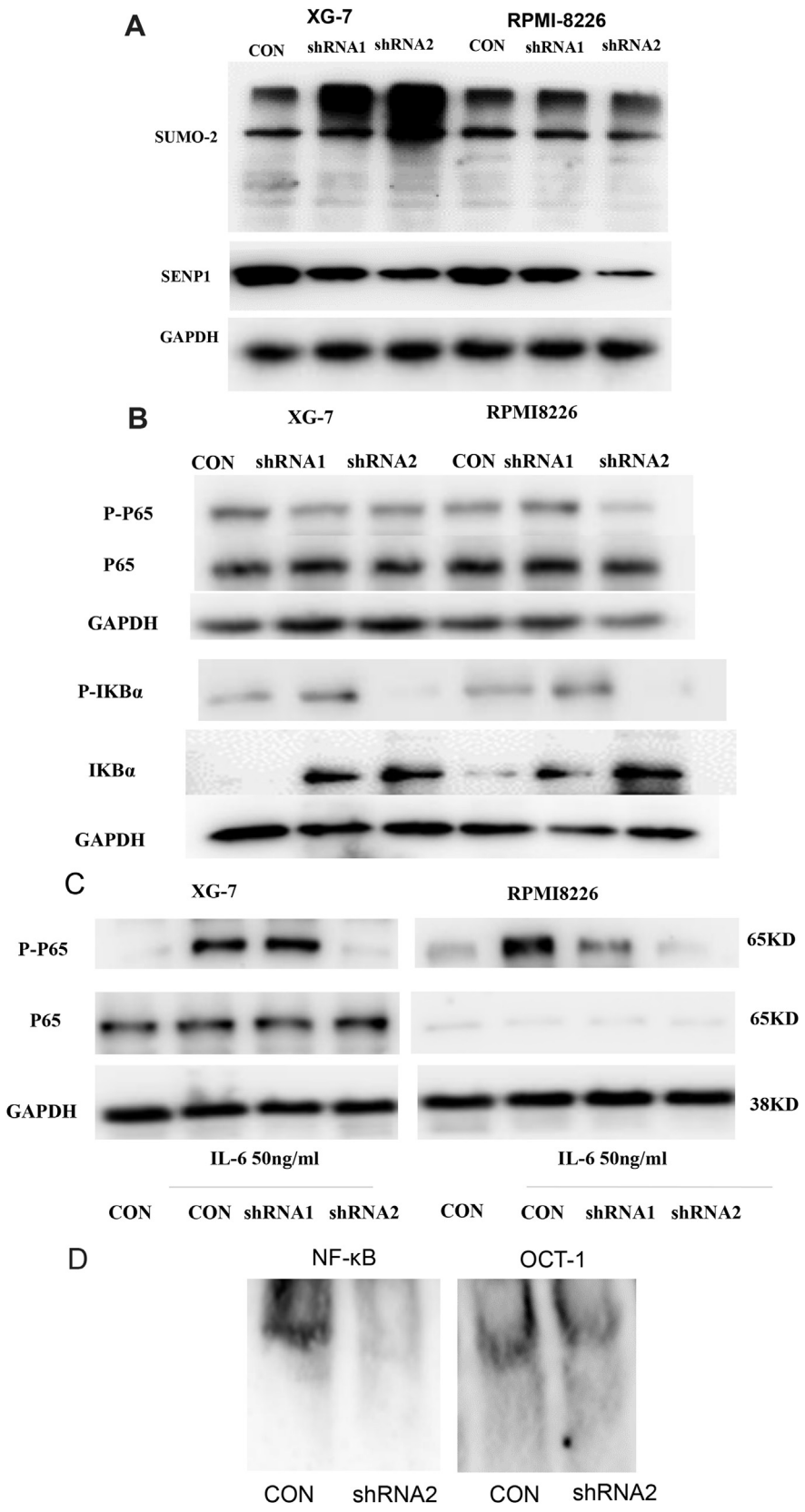

Fig. 4. SENP1 shRNA blocks the IL-6 induced NF-κB activation in MM cells. (A)XG-7 cells and RPMI-8226 cells were transduced with Senp1 shRNA2 and control vector and cultured for 48 h. The total protein-SUMO2 was determined by Western Blot. (B) The IKBα and p65 phosphorylation were determined by western Blot. (C) These transduced cells were treated with 50 ng/ml IL-6, then p65 phosphorylation were determined by Western Blot. (D) The DNA binding activity (NF-kB and OCT-1) of nuclear protein from RPMI-8226 cells transduced Senp1 shRNA2 or control vector were determined by EMSA.

vectors carrying Senp1 shRNA and detected their silence efficiency in MM cells. Both Senp1 shRNA1 and shRNA2 could significantly decrease the proliferation and increase apoptosis of MM cells, including reducing the colony forming ability, inducing cell cycle arrest and apoptosis. Importantly, both senp1 shRNA1 and shRNA2 transduction results in the loss of growth ability of MM cells. This indicates that senp1 enzyme inhibition can block the proliferative and antiapoptotic stimulation provided to multiple myeloma cells by bone marrow microenvironment.

Mechanistically, Senp1 protease inhibition with shRNA transduction was associated with increase of SUMO-modified proteins, including the NF-κB pathway members. The NF-κB pathway plays a critical role in the regulation of proliferation and survival of multiple myeloma cells. Constitutive NF-κB signaling promotes survival in MM. Several therapeutic agents such as bortezomib, a proteasome inhibitor with remarkable preclinical and clinical antitumor activity in MM patients, significantly down-regulated expression and NF-κB activation in MM cell cells. However, the detail mechanisms for NF-κB activation and its roles in IL-6 signaling are less explored. The NF–κB family of transcript factors is composed of several members including NFkB-1, NFkB-2,Rel A (P65) and RelB. The activity of NF-kB members are regulated by posttranslational modifications comprising phosphorylationand ubiquitination. Our data shows that phosphorylation form of P65 and inhibitor protein IkBαis suppressed by Senp-1 inhibition in MM cells. Furthermore, the transcript binding activity of was blocked in MM cells transduced with Senp1 shRNA. The latest research demonstrates that sumoylation is extensively involved in the regulation of signal molecues. Although the roles of Senp1 mediated desumoylation in regulation of NF-kappaB remain unclear. Our data had identified the inhibition of Senp1 results in the increase of total SUMO2-binding proteins in MM cells. This observation is compatible with the known role of Senp1 in linking the IL-6 signings and NF-kB activation in MM cells.

Therapeutically targeting the SUMOylation, Ubiquitination and Proteasome pathways have become a novel anticancer strategy for treatment of MM [23]. This study elucidated the link that Senp1-meidated symolyation between the IL-6 signaling cascade and NF-kB activation, and further elucidated the roles of SENP1in regulation the proliferation and apoptosis of MM cells,raise the possibility of targeting Senp1 to improve therapeutic outcomes of patients with MM.

Conflict of interest

None.

Acknowledgments

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Transparency document

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