KDM1A represses apoptosis in osteosarcoma cells via demethylating Bcl-2/c-Myc

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Abstract

Introduction: Considering the poorly understood role of lysine demethylase 1A (KDM1A) in osteosarcoma (OS), we conducted this study to elucidate the underlying mechanism.
Material and methods: Following the appropriate transfection, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and flow cytometry assays were used to determine the viability and apoptosis of MG-63 OS cells, in which the generation of reactive oxygen species (ROS) and the binding between KDM1A and Bcl-2/ cellular Myc (c-Myc) were separately confirmed via the DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) method and chromatin immunoprecipitation-PCR. Reverse-transcription quantitative PCR and western blot were finally introduced to quantify the levels of KDM1A/Bcl-2/c-Myc and endoplasmic reticulum (ER) stress-related factors.
Results: Overexpressed KDM1A enhanced the viability (48 h) yet repressed the apoptosis and ROS generation, with downregulation of ER stress-related factors (C/EBP homologous protein [CHOP]; proline-rich extensin-like receptor kinase (PERK) and activating transcription factor 4 [ATF4]) but elevation of Bcl-2/c-Myc, while its depletion exerted contrary effects. More importantly, KDM1A could act as the demethylase of Bcl-2/c-Myc, as reflected by the results showing that the depletion of KDM1A decreased the enrichment of Bcl-2/c-Myc promoter using the antibody against KDM1A yet increased the enrichment by the antibody targeting H3K9me2. Bcl-2/c-Myc silencing, conversely, promoted ROS generation and apoptosis, elevated the levels of ER stress-related factors and abolished the effects of KDM1A on OS cells.
Conclusions: KDM1A exerts a repressive effect on the apoptosis of MG-63 OS cells by inhibiting ROS generation and ER stress via demethylation of Bcl-2 and c-Myc.

Key words: osteosarcoma, lysine demethylase 1A, apoptosis, reactive oxygen species, endoplasmic reticulum stress, demethylation, Bcl-2/c-Myc.

Introduction

As the most prevalent primary malignancy of the bone associated with a high propensity for local invasion and metastasis, osteosarcoma (OS) is a rare tumor for which complex multidisciplinary management

is needed [1, 2]. However, due to the diverse histological patterns and the shortage of diagnostic biomarkers for most cases, the diagnosis of OS can be challenging [3]. As such, the identification of the underlying mechanism concerning the evolution of OS will be of help in the better management of this rare malignancy [4].

Recent years have also witnessed increasing research highlighting the possibility to cluster OS on the basis of identifying candidate driver genes and developing targeted therapy using elaborated sequencing analyses [5]. The genes B-cell lymphoma-2 (Bcl-2) and cellular Myc (abbreviated as c-Myc and also known as MYC) have been widely reported to participate in the control of the cell cycle and/or programmed cell death, in addition to the suggestion of their impact on the biological behaviors of malignancies, OS, for instance [6, 7]. Specifically, the expression patterns of Bcl-2 and c-Myc and their correlation with the prognosis of patients have been discussed in an existing study [7]. It has been underlined that Bcl-2 exerted a regulatory effect on the mitochondrial metabolism for the generation of reactive oxygen species (ROS), the accumulation of which induces the apoptosis of OS cells via mediating endoplasmic reticulum (ER) stress, while such generation of ROS is indicated to be suppressed by MYC, another prognostic biomarker and therapeutic target of OS [8–11]. Linking the keywords "Bcl-2/c-Myc", "ROS accumulation", "ER stress" and "OS" together, we have reason to believe that Bcl-2/c-Myc may pose regulatory effects on both ROS accumulation and ER stress in OS. In this study we investigated the possible mechanisms implicated and the potential regulator(s).

Furthermore, the possible efficacy of DNA demethylation therapy in OS has been indicated, with the underlying mechanism vague [12]. Considering the pivotal role of DNA methylation status in the most prevalent molecular alterations during tumorigenesis and the effect of DNA methylation on the obstruction of the promoter region (which hampers the transcription of gene and leads to gene silencing), it remains a challenge to identify the optimal biomarkers which either regulate or are regulated by DNA methylation/demethylation during the progression of OS [13, 14]. Lysine demethylase 1A (KDM1A), also known as lysine-specific histone demethylase 1 (LSD1), symbolizes the first example of the identified nuclear protein with histone demethylase activity [15]. Specifically, the special role of KDM1A in the epigenetic regulation of gene expression has been discovered and discussed, as reflected by its capability of removing methyl groups from mono- and demethylated lysine 4 and/or lysine 9 on histone H3 (i.e., H3K4me1/2 and H3K9me1/2) and its behaviors as the repressor or activator of gene expression [15, 16]. A previous study showed that cisplatin directly down-regulated KDM1A protein expression in a dose-response manner, suggesting that KDM1A is a downstream target of cisplatin [17]. Indeed, the promoting effects of KDM1A on the occurrence of OS has been proposed, with a suggestion regarding its inhibitory effect on the expression of E-cadherin (CDH-1) by its function as the demethylase [18, 19]. Considering the dearth of research addressing the possible implication of KDM1A/Bcl-2/c-Myc and the role of KDM1A as the demethylase of Bcl-2/c-Myc in OS, we aimed to solve this mystery and commenced our research, the results of which are reported as follows.

Material and methods

Cell culture

Human OS cell line MG-63 (TCHu124, Shanghai Cell Bank, Chinese Academy of Science, Shanghai, China) was cultured in minimal essential medium (MEM, E600020, Sangon Biotech, Shanghai, China) blended with 1.5 g/l sodium bicarbonate ($NaHCO_3$, A100865, Sangon Biotech, China), 0.11 g/l sodium pyruvate ($C_3H_3NaO_3$, A100342, Sangon Biotech, China) and 10% bovine calf serum (BCS, E600001, Sangon Biotech, China), as recommended by the supplier. The Heracell VIOS 160i CO_2 incubator (51033559, Thermo Fisher, Waltham, MA, USA) was used to ensure the growth of cells, with the culture condition adjusted to 37°C with 5% CO_2.

Transfection

Prior to the transfection, the pcDNA 3.1 vector (V790-20, Invitrogen, Carlsbad, CA, USA) was used to construct the overexpression vector of KDM1A (hereafter represented as "KDM1A" in the figures) via inserting the whole sequence, with the empty vector without insertion as the negative control (hereafter stylized as "NC" in the figures). Meanwhile, the short hairpin RNA against KDM1A (abbreviated as shKDM1A, C02001) and the corresponding negative control (shNC, C03002) were synthesized and obtained from GenePharma (Shanghai, China), where the small interfering RNAs (A01001) against Bcl-2 (siBcl-2) and MYC (siMYC) as well as their negative control (siNC, A06001) were available as well. The sequences used here were provided in Table I for reference.

For the transfection, all processes hereafter were strictly repeated based on the manufacturer's protocols. In brief, MG-63 cells (1×10^6 cells per well in the 6-well plates) were allowed to grow until confluence of 90% was reached prior to transfection. The transfection reagent lipofectamine 2000 (11668-030, Invitrogen, USA) and the

plasmids were pre-diluted in Opti-MEM medium (22600-050, Gibco, Grand Island, NY, USA) in advance, followed by the addition of plasmids into the transfection reagent for 5-minute incubation at room temperature and the introduction of the plasmid-lipid complex to MG-63 cells for the final incubation at 37°C for 48 h. All cells were subsequently collected for further analyses.

Cell viability and apoptosis assay

In order to evaluate the viability and apoptosis of MG-63 cells following the different interventions, a commercial 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) cell proliferation assay kit (E606334) and Annexin V apoptosis detection kit (E606336) were purchased from Sangon Biotech.

For the viability assay, MG-63 cells were maintained in 96-well plates at the density of 2×10^3 cells per well and incubated for 48 h, and then the prepared MTT solution (5 mg/ml) in the final volume of 10 µl was added to the plates for an additional 4-hour incubation. The formazan solubilization solution provided with the kit was added following the careful removal of culture medium in each well, after which gentle shaking was conducted for 10 min until the formazan formed was fully dissolved. The absorbance at 570 nm was read using the iMark microplate absorbance reader (1681135, Bio-Rad, Hercules, CA, USA), and the viability of MG-63 cells was calculated as appropriate.

For the cell apoptosis assay, the binding buffer was pre-diluted and added to MG-63 cells to suspend the cells to the required density of 2×10^5 cells per milliliter (ml). The working solution of Annexin V-FITC (5 µl) was then added to the cells for incubation at room temperature for 15 min without light. After rinsing with 200 µl of binding buffer and centrifugation at 100 rpm for 5 min, the supernatant was discarded and cells were continued to be resuspended in 190 µl of binding buffer, and 10 µl of propidium iodide working solution was added. A CytoFLEX S flow cytometer (C09765, Beckman Coulter, Indianapolis, IN, USA) was used to acquire the data, which were finally processed using the affiliated CytExpert software (Beckman Coulter, USA).

Cellular ROS generation determination assay

A DCFDA cellular ROS assay kit (ab113851, Abcam, Cambridge, UK) was employed to assess the generation of ROS in MG-63 cells with different interventions [20]. For the determination, MG-63 cells were allowed to adhere overnight in the 96-well plates with the indicated density of 2.5×10^4 cells per well, following which the medium was removed and cells were dyed using 100 µl/well of the diluted DCFDA working solution at 37°C for 45 min in the dark. The working solution was then replaced with the buffer and the supplement buffer (two separate buffers provided with the kit) at the same concentration. The excitation wavelength/emission wavelength (Ex/Em) at 485/535 nm was read in a fluorescence plate reader (PHERAstar FSX, BMG Labtech, Cary, NC, USA).

Table I. Sequences for transfection

Gene	Target sequence (5′-3′)
shKDM1A	CTCTCAGAAGATGAGTATTAT
shNC	CATGTCTCAGAAGATAGTATT
siBcl-2#1	ATCTGGAAATCCTCCTAATTTTT
siBcl-2#2	TGGAAATCCTCCTAATTTTTACT
siBcl-2#3	CTGATTCATTGGGAAGTTTCAAA
siMYC#1	CTGCTTAGACGCTGGATTTTTTT
siMYC#2	TGCTTAGACGCTGGATTTTTTTC
siMYC#3	GAGGAAGAAATCGATGTTGTTTC
siNC	CTGAAGGATTCATTCAGAAGTTT

Chromatin immunoprecipitation-PCR (ChIP-PCR) analysis

A ChIP kit (ab500) ordered from Abcam was used and all processes conducted here were based on the protocols of the producer and previous research [21]. In detail, MG-63 cells in the 6-cm dish (3×10^6 cells) were transfected with the shKDM1A/shNC for 2 days as appropriate, followed by harvesting and fixation in formaldehyde (HCHO, A501912, Sangon Biotech, China) for 10 min. Then the cell pellets were sonicated, with the removal of residue via centrifugation. Following the incubation of primary antibodies against IgG (ab172730, Abcam, UK), KDM1A (ab195405, Abcam, UK) and H3K9me2 (PA5-120810, Invitrogen, USA) at 4°C overnight, cells were continued to be treated with proteinase K (A004220, Sangon Biotech, China) and the DNA was harvested. SYBR Green Fast qPCR mix (K1070, APExBio, Houston, TX, USA) was used to determine the relative enrichment in the promoter of both Bcl-2 and MYC as guided by the manufacturer. The primers used are listed in Table II.

RNA extraction and reverse-transcription quantitative PCR

The total RNA of MG-63 cells with different intervention was extracted using the conventional method with the TriZol reagent (15596-026, Invitrogen, USA) and preserved at –80°C until use. Following the assay to quantify its concentration with the spectrophotometer (ND-2000, Thermo Fisher,

Table II. Primer sequences

Gene	Primer sequence (5′-3′)	
	Forward	Reverse
Bcl-2	GATGACTGAGTACCTGAACC	AGCAGAGTCTTCAGAGACAG
MYC	GTAGTGGAAAACCAGCAG	CTCTTTTCCACAGAAACAAC
KDM1A	CACAGTTATTTAGAGCGTCA	CTTTCTCTTTAGGAACCTTG
GAPDH	ATTGACCTCAACTACATGGT	CATACTTCTCATGGTTCACA

USA), the cDNA synthesis kit (B300537, Sangon Biotech, China) was employed to synthesize the cDNA. PCR was finally run using SYBR Green Fast qPCR mix and the CFX96 PCR system (1845096, Bio-Rad, USA) at the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, as recommended by the manuals. mRNA levels were quantified using the $2^{-\Delta\Delta CT}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping control [22]. The primers used are listed in Table II.

Western blot

The protein expression levels of Bcl-2/MYC and ER stress-related factors in MG-63 cells with the indicated intervention were quantified via western blot as appropriate [23]. In detail, the total protein of MG-63 cells was extracted with the RIPA lysis buffer (C500005, Sangon Biotech, China) and its concentration was evaluated using a bicinchoninic acid (BCA) protein assay kit (C503021, Sangon Biotech, China) as appropriate.

For subsequent analyses, the protein sample was detached on SDS-PAGE (C631100, Sangon Biotech, China) and transferred to the PVDF membrane (F619537, Sangon Biotech, China), after which the membrane pre-blocked with 5% non-fat milk was probed with the primary antibodies (Abcam, UK) against Bcl-2 (ab182858, 26 kDa, 1 : 2000), MYC (ab32072, 57 kDa, 1 : 1000), C/EBP homologous protein (CHOP, ab11419, 31 kDa, 1 : 2000), proline-rich extensin-like receptor kinase (PERK, ab79483, 125 kDa, 1 : 1000), activating transcription factor 4 (ATF4, ab184909, 50 kDa, 1 : 1000) and housekeeping control GAPDH (ab226408, 36 kDa, 1 : 1000) at 4°C overnight. Furthermore, for the incubation of horseradish peroxide-conjugated secondary antibodies (which was carried out at room temperature for 1 h), the antibodies of goat anti-rabbit IgG (D110058, Sangon Biotech, China) and goat anti-mouse IgG (D110087, Sangon Biotech, China) were used at the dilution ratio of 1 : 5000.

For the visualization process, the membrane which had been treated with the ECL luminescence reagent (C510043, Sangon Biotech, China) was exposed to the ChemiDoc Touch imaging system (Bio-Rad, USA) and the grey value of the membrane was determined in the affiliated Image Lab Touch software as needed.

Statistical analysis

All data of at least three independent assays were expressed as mean ± standard deviation (SD) and analyzed in GraphPad 8 (GraphPad, Inc., La Jolla, CA, USA). Data between two groups or among multiple groups were compared with the independent *t* test and one-way analysis of variance, with the Bonferroni *post hoc* test employed as well. Statistical significance was defined as a *p*-value lower than 0.05.

Results

KDM1A overexpression inhibited ROS generation and apoptosis yet promoted the viability of MG-63 OS cells, while its knockdown led to contrary results

Initially, we transfected the overexpression plasmid of KDM1A (presented as KDM1A in the figures) and shRNA against KDM1A (stylized as shKDM1A) into MG-63 OS cells to reveal the effects of KDM1A on the malignant behaviors of OS cells, which was proved to be successful, as reflected by the elevated or decreased KDM1A level in MG-63 cells following different transfection (Figure 1 A, $p < 0.001$). Meanwhile, based on the MTT (Figure 1 B) and flow cytometry assay (Figures 1 C, D), the knockdown of KDM1A led to suppressed viability at 48 h and increased apoptosis in MG-63 cells (Figures 1 B–D, $p < 0.001$), while its overexpression acted conversely to promote viability and inhibit apoptosis (Figures 1 B–D, $p < 0.01$). Considering the role of ROS production in the induction of apoptosis in OS cells [9], the production of ROS in MG-63 cells with either the knockdown or the overexpression of KDM1A was determined. It was found that the overexpression of KDM1A diminished ROS production (Figure 1 E, $p < 0.05$), whereas the knockdown of KDM1A produced opposite results, aggravating ROS production (Figure 1 E, $p < 0.001$). These results thus indicated that KDM1A may aggravate the malignant behaviors of OS cells.

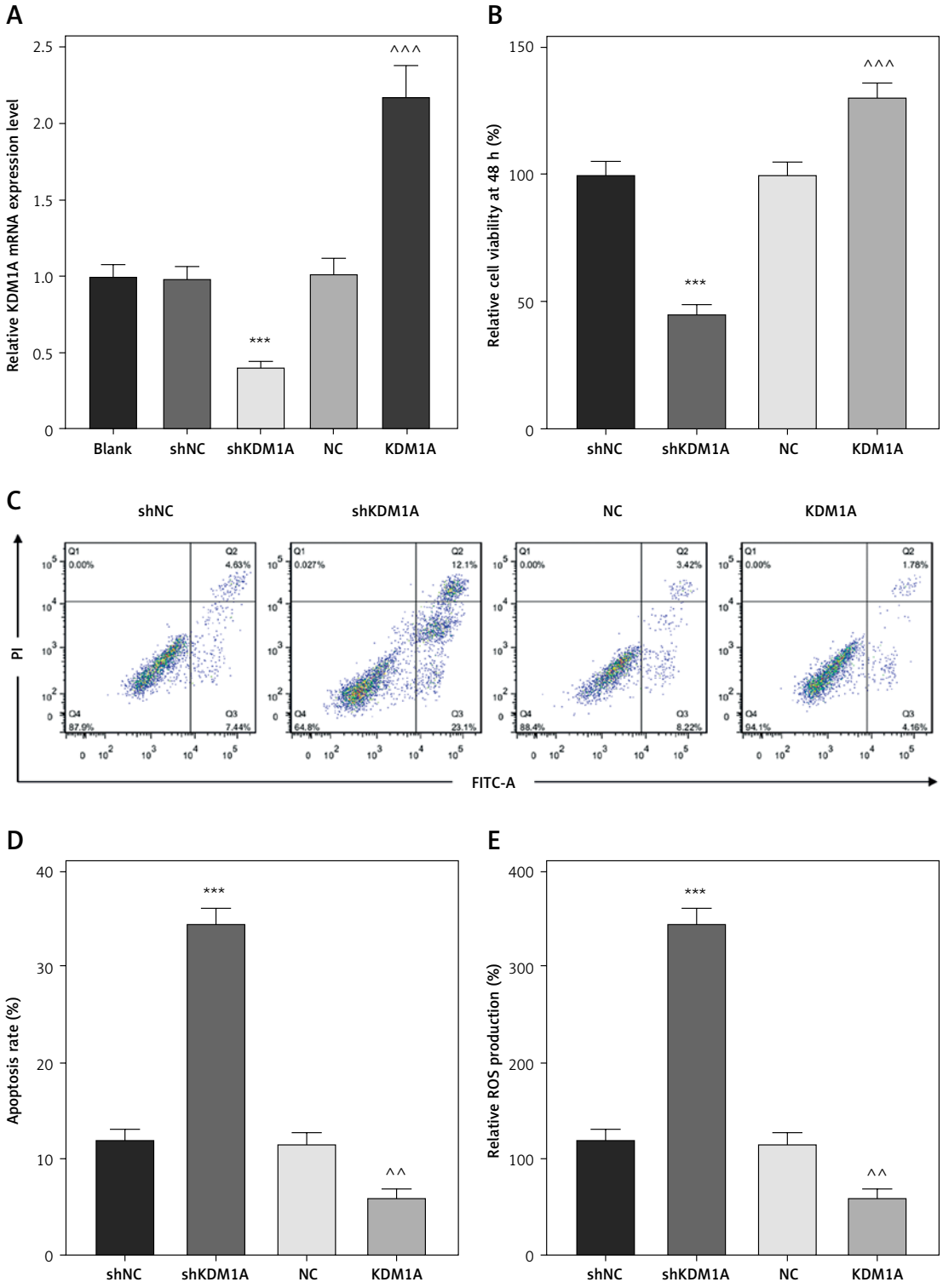

Figure 1. Lysine demethylase 1A (KDM1A) overexpression inhibited reactive oxygen species (ROS) generation and apoptosis but promoted viability of MG-63 OS cells, while its knockdown led to contrary results. First, the overexpression plasmid of KDM1A (presented as KDM1A in the figures) and short hairpin RNA (shRNA) against KDM1A (stylized as shKDM1A) were transfected into MG-63 osteosarcoma (OS) cells to reveal the effects of KDM1A on the malignant behaviors of OS cells. **A** – The transfection was proved to be successful, as reflected by the results of reverse-transcription quantitative PCR, with GAPDH as the housekeeping control. **B**–**D** – Meanwhile, based on the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (**B**) and flow cytometry assay (**C**, **D**), the effects of KDM1A knockdown or overexpression on viability at 48 h and apoptosis in MG-63 cells were evaluated. **E** – The production of ROS in MG-63 cells with either knockdown or overexpression of KDM1A was finally calculated. All data of three independent tests were expressed as mean ± standard deviation. $^{***}p < 0.001$, vs. shNC; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, vs. negative control (NC)

KDM1A overexpression inhibited ER stress but promoted expression of Bcl-2/MYC in MG-63 OS cells, while its silencing had the opposite effects

The significant role of ER stress in the apoptosis of OS cells has also been emphasized [9]. Herein, we measured some related factors, including CHOP, PERK, and ATF4. These were quantified in MG-63 cells after the intervention as appropriate. It was found that the silencing of KDM1A increased the expression of all these factors (Figures 2 A, B, $p < 0.001$), whilst KDM1A overexpression produced contrary results (Figures 2 A, B, $p < 0.001$). Further-

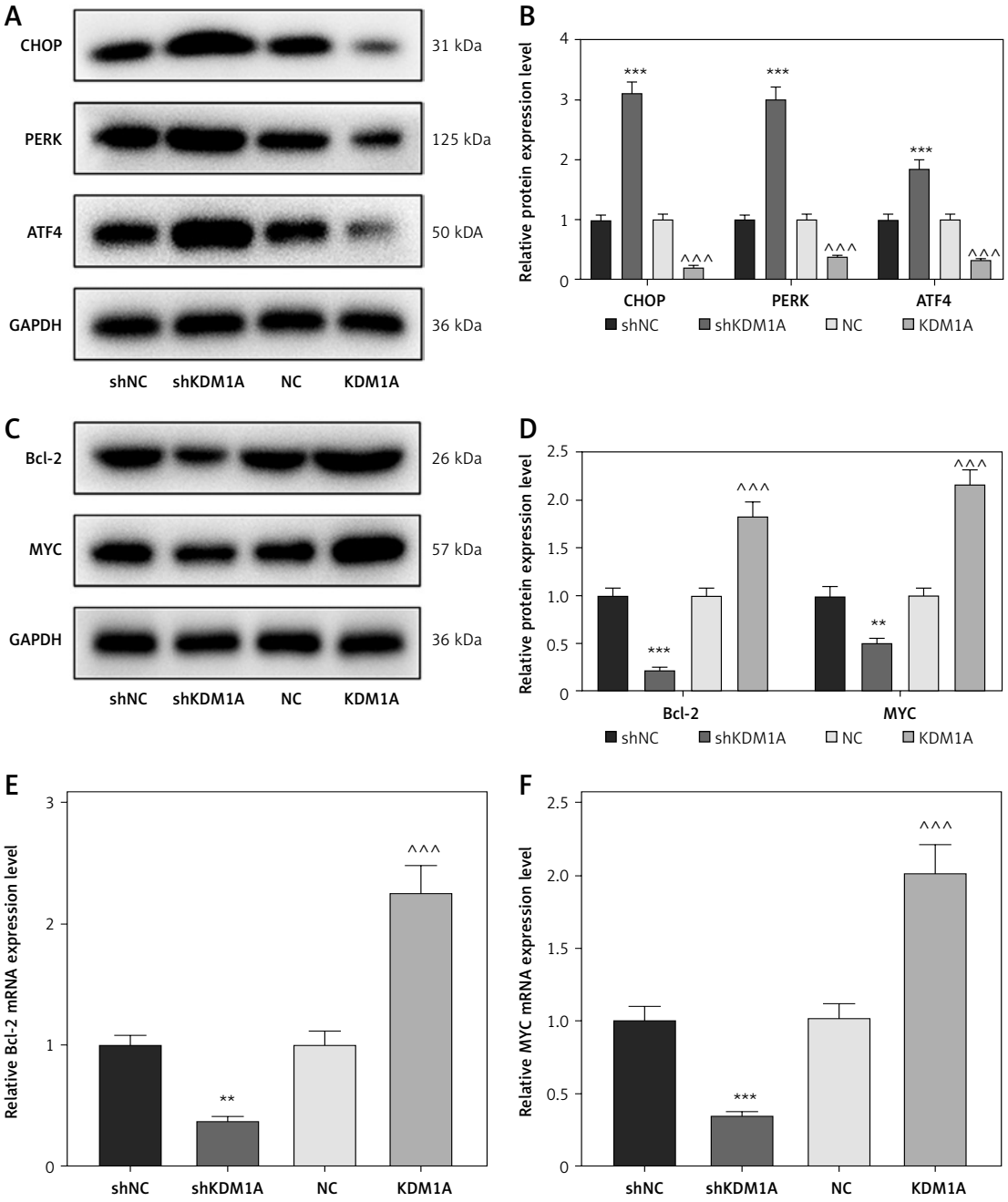

Figure 2. Effects of KDM1A knockdown/overexpression on endoplasmic reticulum (ER) stress and expression of B-cell lymphoma-2 (Bcl-2)/cellular-Myc (MYC) in MG-63 OS cells. Likewise, the significant roles of ER stress and Bcl-2/MYC in OS cells have been emphasized. **A**, **B** – Herein, some ER stress-related factors were measured, including C/EBP homologous protein (CHOP), proline-rich extensin-like receptor kinase (PERK), and activating transcription factor 4 (ATF4), based on the western blot assay, with GAPDH as the housekeeping control. **C**–**F** – Meanwhile, the protein and mRNA levels of Bcl-2/MYC were quantified via western blot (**C**, **D**) and reverse-transcription quantitative PCR (**E**, **F**), with GAPDH as the housekeeping control. All data of three independent tests were expressed as mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. shNC; $^{\wedge\wedge\wedge}p < 0.001$, vs. NC

more, with the proposal on the prognostic value of Bcl-2/MYC in OS patients [7], we assumed that Bcl-2/MYC might participate in the mechanism by which KDM1A exerted its effects on OS cells. Accordingly, the levels of Bcl-2/MYC were quantified. It was observed that silencing of KDM1A inhibited the expression of Bcl-2/MYC (Figures 2 C–F, $p < 0.01$). Such effects were opposite to those following the overexpression of KDM1A (which promoted the levels of Bcl-2/MYC) (Figures 2 C–F, $p < 0.001$), proving the regulatory effects of KDM1A on ER stress and Bcl-2/MYC in OS.

KDM1A could demethylate Bcl-2/MYC in MG-63 OS cells

The role of KDM1A as the demethylase of CDH-1 in OS led us to speculate whether KDM1A could also demethylase Bcl-2/MYC in OS cells [19]. Herein, ChIP-PCR analysis revealed that the depletion of KDM1A led to the decreased enrichment of Bcl-2/MYC promoter using the antibody against KDM1A yet caused increased enrichment by the antibody against H3K9me2, an important epigenetic modifier of several processes (Figures 3 A–D, $p < 0.01$) [24]. It was thus suggested that KDM1A exerted negative regulatory effects on Bcl-2/MYC in an H3K9-dependent manner.

Silencing of Bcl-2/MYC abolished the effects of KDM1A on ROS production, ER stress and apoptosis of OS cells

With the hope to investigate the interplay between KDM1A and Bcl-2/MYC, the siRNA against Bcl-2/MYC was transfected into OS cells, where it was found that all these siRNAs decreased the levels of Bcl-2/MYC in OS cells (Figures 3 E, F, $p < 0.01$),

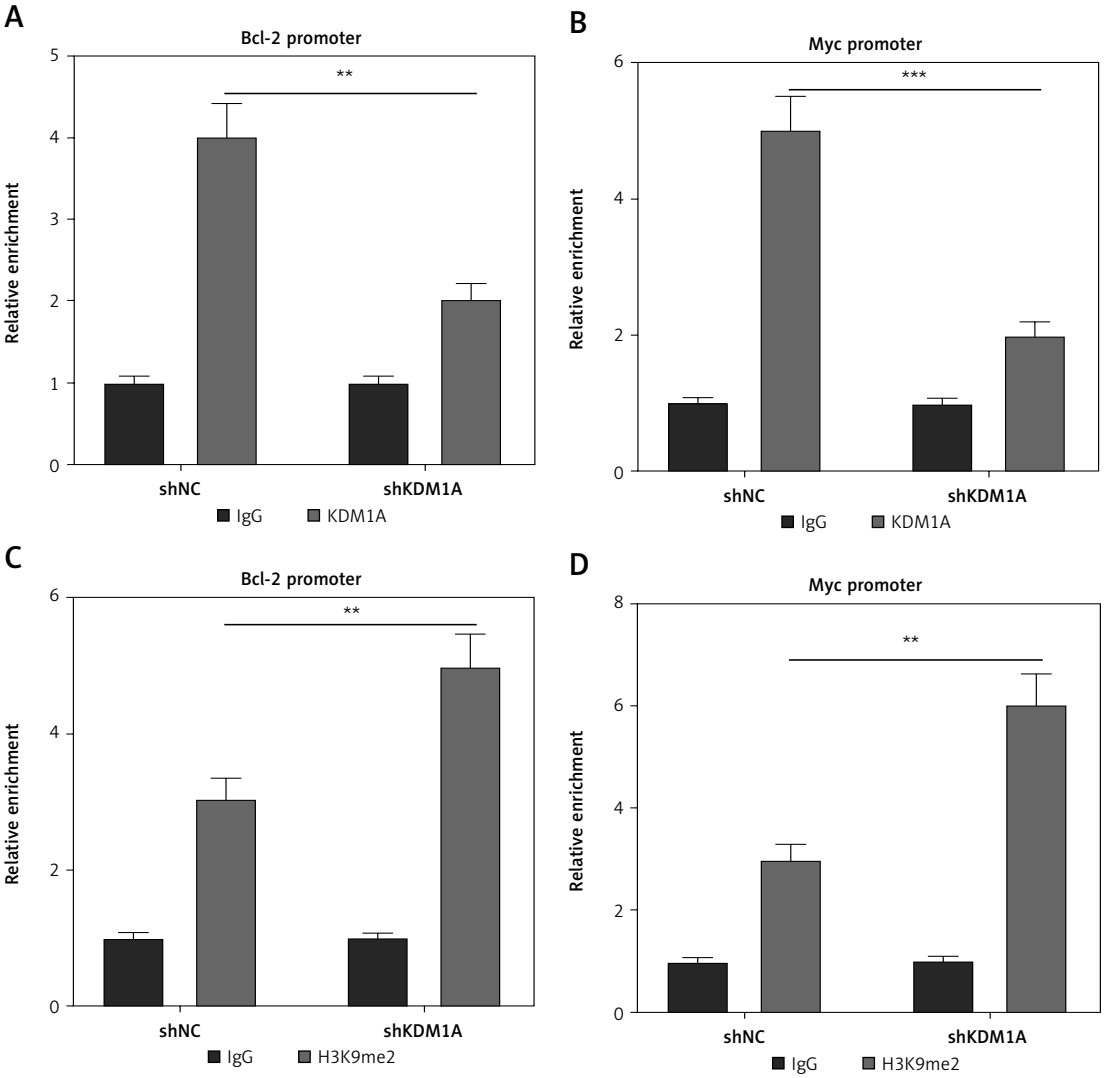

Figure 3. Analysis of KDM1A/Bcl-2/MYC axis in OS cells. **A–D** – Chromatin immunoprecipitation-PCR (ChIP-PCR) analysis was performed to determine the enrichment of Bcl-2/MYC promoter using the antibody against KDM1A or H3K9me2. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. shNC; $^{++}p < 0.01$, $^{+++}p < 0.001$, vs. siNC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. NC + shNC; $^{\Delta\Delta\Delta}p < 0.001$, vs. siNC + KDM1A; $^{\&\&\&}p < 0.001$, vs. siBcl-2 + NC; $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siMYC + NC

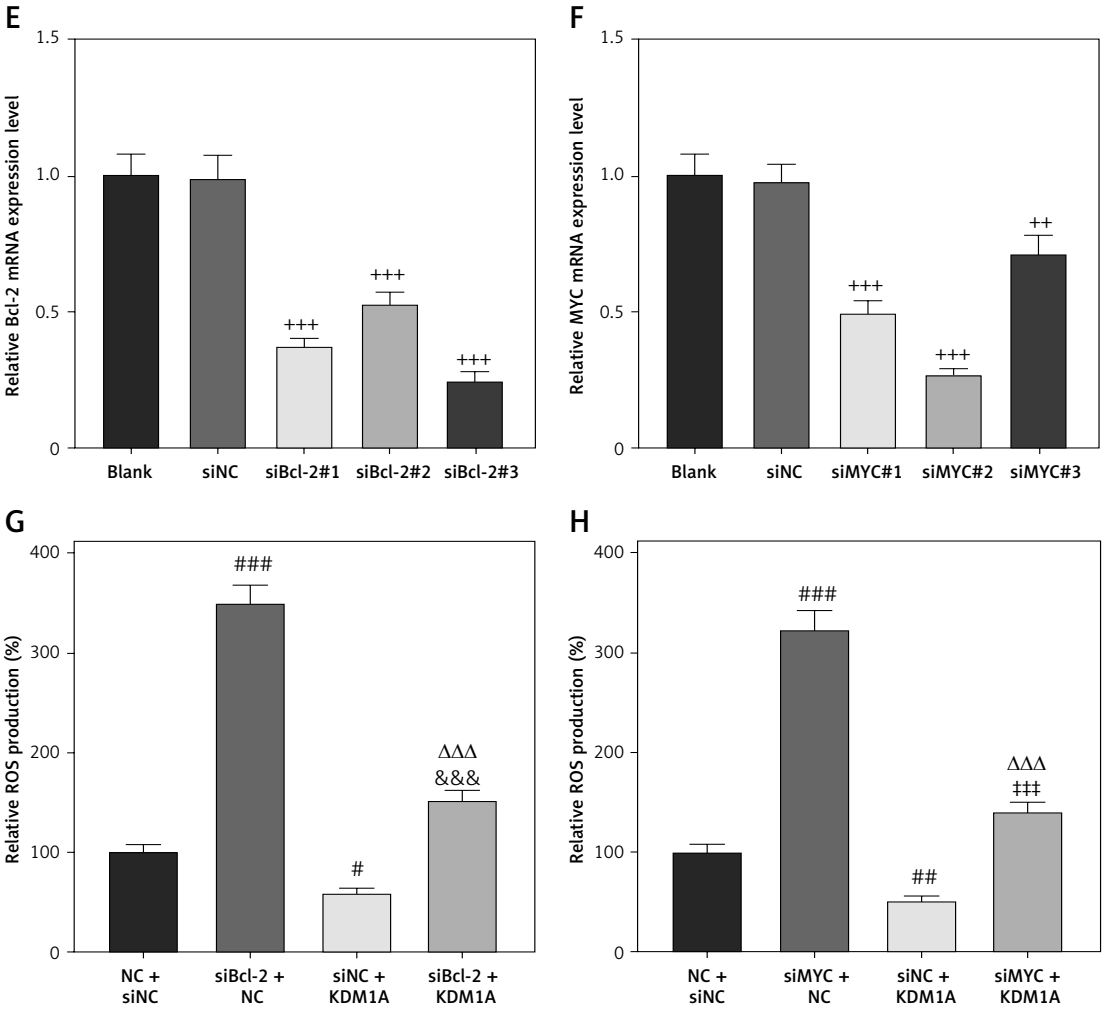

Figure 3. Cont. **E**, **F** – Evaluation of transfection efficiency of Bcl-2/MYC siRNA via reverse-transcription quantitative PCR, with GAPDH as the housekeeping control. **G**, **H** – Interplay between KDM1A and Bcl-2/MYC in ROS production in MG-63 OS cells. All data of three independent tests were expressed as mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. shNC; $^{++}p < 0.01$, $^{+++}p < 0.001$, vs. siNC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. NC + shNC; $^{\Delta\Delta\Delta}p < 0.001$, vs. siNC + KDM1A; $^{\&\&\&}p < 0.001$, vs. siBcl-2 + NC; $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siMYC + NC

and those with the most significant knockdown effects (siBcl-2#3 and siMYC#2) were employed for subsequent assays.

Regarding ROS production, Bcl-2/MYC silencing led to a sharp increase of ROS production in MG-63 OS cells (Figures 3 G, H, $p < 0.001$), whereas the overexpression of KDM1A acted oppositely (Figures 3 G, H, $p < 0.05$). Furthermore, we found that such effects of overexpressed KDM1A on ROS production in OS cells were diminished via Bcl-2/MYC silencing (Figures 3 G, H, $p < 0.001$).

As regards the protein expression of ER stress-related factors, the silencing of Bcl-2/MYC aggravated the levels of CHOP, PERK, and ATF4 (Figures 4 A–D, $p < 0.01$), the results of which were contrary to those following the overexpression of KDM1A (Figures 4 A–D, $p < 0.01$). Likewise, the silencing of Bcl-2/MYC also neutralized the effects of KDM1A overexpression on the ER stress-related factors in OS cells (Figures 4 A–D, $p < 0.01$).

Finally, based on the flow cytometry data, KDM1A overexpression led to a suppressive effect on the apoptosis of MG-63 OS cells (Figures 5 A–D, $p < 0.001$), while Bcl-2/MYC silencing had the opposite effect and abolished the suppressive effects of overexpressed KDM1A on the apoptosis of MG-63 OS cells (Figures 5 A–D, $p < 0.05$). All considered, we concluded that KDM1A exerted its effects on ROS production and ER stress and OS cells so as to modulate apoptosis via demethylating Bcl-2/MYC.

Discussion

Previous research has underlined the role of KDM1A as a histone demethylase during carcinogenesis, showing the emerging option of targeting KDM1A as a therapeutic option for cancers [25]. Indeed, KDM1A has been suggested to be associated with different aspects of tumors, epigenetic regulation of epithelial-to-mesenchymal

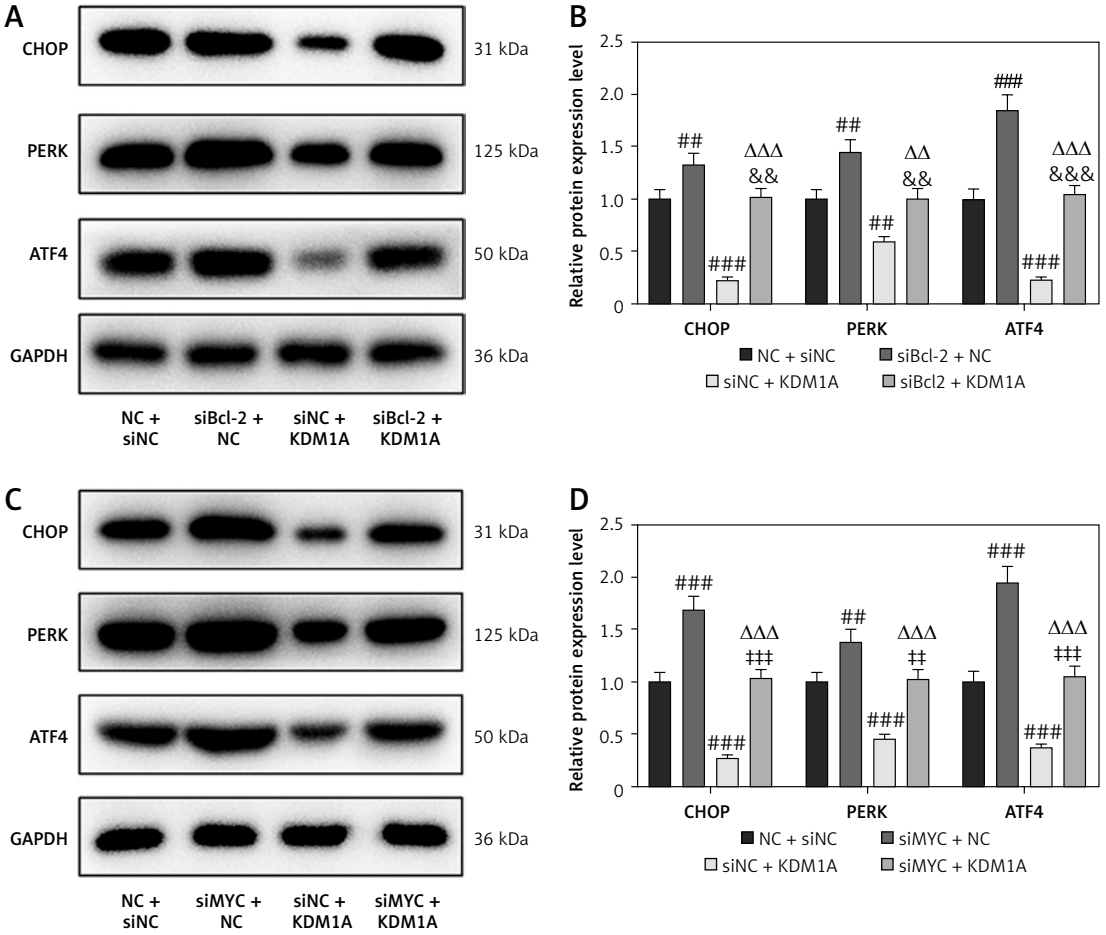

Figure 4. Evaluation of KDM1A/Bcl-2/MYC axis in ER stress in OS cells. **A–D** – As regards protein expression of ER stress-related factors CHOP (**B**), PERK (**C**), and ATF4 (**D**) in MG-63 OS cells, their levels following the silencing of Bcl-2/MYC and the overexpression of KDM1A were determined via western blot. GAPDH was the housekeeping control used. All data of three independent tests were expressed as mean ± standard deviation. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. NC + siNC; $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$, vs. siNC + KDM1A; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, vs. siBcl-2 + NC; $^{\ddagger\ddagger}p < 0.01$, $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siMYC + NC

transition, stemness and drug resistance, to name a few [26–28]. Furthermore, the promoting effect of KDM1A on the occurrence of OS has been well reported, and more importantly, it has been stressed that KDMI1A, being a demethylase, exerted an inhibitory effect on the expression of CDH-1 [18, 19]. Considering the above, we believe there must exist other candidate(s) that could both be demethylated by KDM1A and participate in the mechanism underlying the effects of KDM1A on OS. Upon seeking the candidate(s), Bcl-2 and c-Myc caught our attention in that they have been widely reported to participate in different malignancies, including OS [6, 7]. Further experiments have led us to conclude that KDM1A could indeed demethylate Bcl-2/c-Myc so as to exert its effects on OS, which, to some extent, additionally supported the participation of KDM1A in OS.

Previous investigation has underlined the fact that excessive apoptosis may result from the ROS and ER stress, and both ROS and mitochondria play critical roles in the induction of apoptosis under pathologic and physiologic conditions [29, 30]. In other words, at high levels, ROS may induce oxidative stress, resulting in oxidative damage in the mitochondria and the onset of certain human pathologies such as tumors, despite the fact that a low level of ROS can be beneficial [31–33]. More importantly, what distinguishes malignant cells from normal cells is their capability of producing an increasing number of ROS and their elevated dependence on the antioxidant defense system [33]. Also, one should be aware that, apart from mitochondria, the ER, being regulated by ROS as well, is a crucial regulator of the cell death signaling pathway, with mitochondria being another [34]. Under prolonged and severe ER stress, the unfolded protein response (UPR), a sequence of reactions noted to restore homeostasis in cells, may become cytotoxic instead of cytoprotective, triggering some unique signaling transduction mechanisms [35]. CHOP is also known as growth arrest-

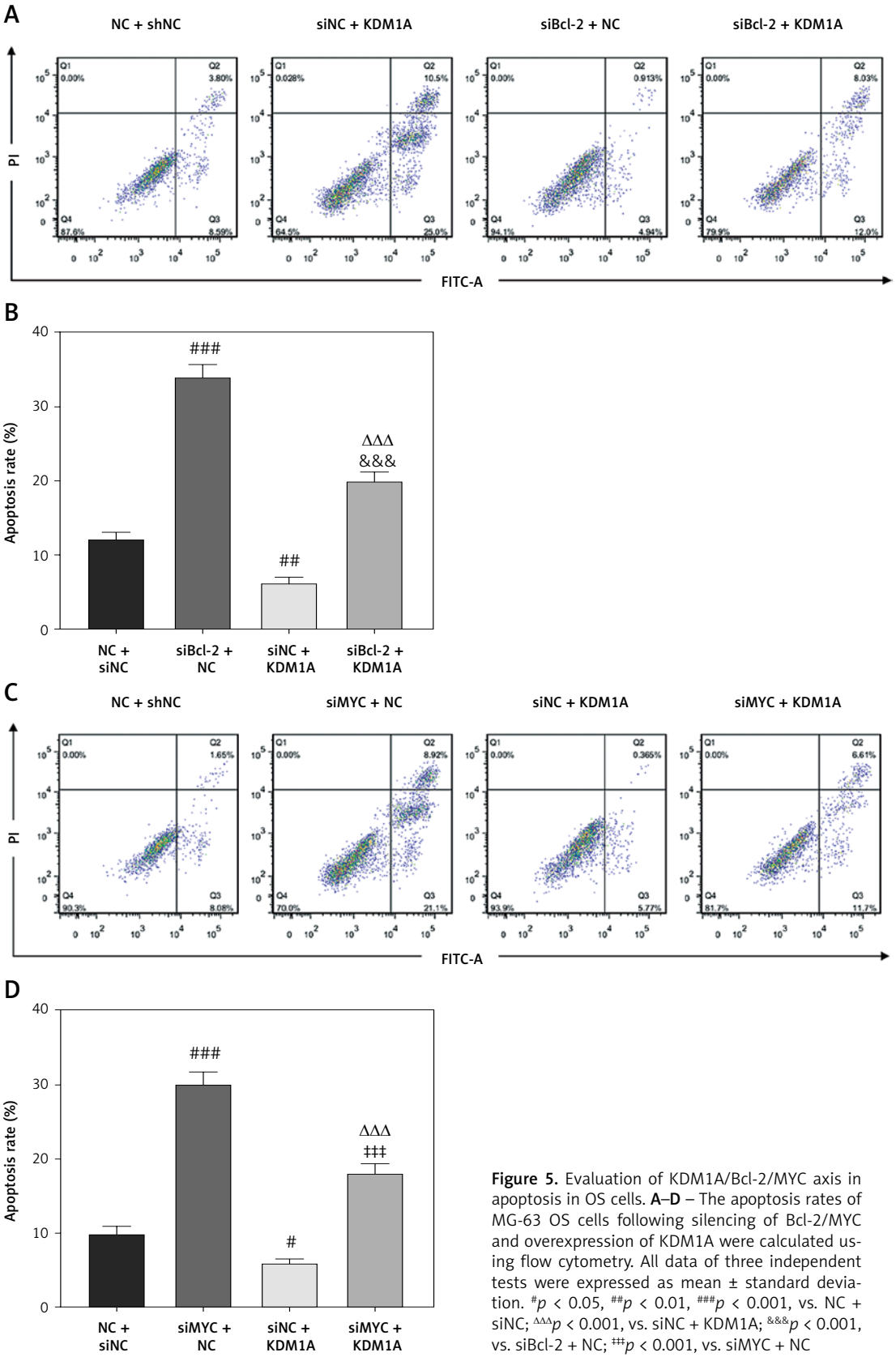

Figure 5. Evaluation of KDM1A/Bcl-2/MYC axis in apoptosis in OS cells. **A**–**D** – The apoptosis rates of MG-63 OS cells following silencing of Bcl-2/MYC and overexpression of KDM1A were calculated using flow cytometry. All data of three independent tests were expressed as mean ± standard deviation. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. NC + siNC; $^{\Delta\Delta\Delta}p < 0.001$, vs. siNC + KDM1A; $^{\&\&\&}p < 0.001$, vs. siBcl-2 + NC; $^{\ddagger\ddagger\ddagger}p < 0.001$, vs. siMYC + NC

and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3 [DDIT3]), which contains an element for the positive regulation of the mitochondrial UPR [36]. Following the failure to restore ER protein homeostasis, the prolonged activation of the UPR may initiate apoptotic cell death by up-regulating CHOP [37]. Being a master regulator with a pivotal role in the adaptation to stresses, ATF4 is both a necessity and sufficiency for the ER stress-induced upregulated expression of regulated in development and DNA damage response 1 (REDD1), a gene transcriptionally upregulated during ER stress [38, 39]. PERK, serving as the ER stress sensor and mediator of the UPR, is known to mediate cell death as well [40, 41]. When considering the association of KDM1A with both ROS and ER stress, it should be noted that RN-1, a known inhibitor of KDM1A, diminishes the content of ROS in a murine model of sickle cell disease (SCD) and that the therapeutic targeting of KDM1A in Ewing sarcoma engages the ER stress response [42, 43]. Research has furthermore highlighted the role of excessive ROS generation and ER stress in the induction of apoptosis of OS cells [44, 45]. Based on these perspectives, we additionally confirmed that overexpression of KDM1A in OS cells may result in diminished apoptosis within via inhibiting excessive ROS generation and aggravating ER stress, along with the decreased levels of ER stress-related factors CHOP, ATF4 and PERK. However, we should use a KDM1A inhibitor such as RN-1 to verify the effect of KDM1A in OS cells in the future.

DNA demethylation is defined as the enzymatic process which results in the removal of the methyl group from 5-methylcytosine (5meC) via destroying the carbon-carbon bond [46, 47]. Increasing research has highlighted the role of site-specific DNA demethylation as a possible target for the epigenetic therapy of tumors such as OS [12, 48]. More importantly, some key regulators in OS have been revealed based on the integrated genome-wide methylation and expression analysis [49]. As we mentioned earlier, KDMI1A, being a demethylase, exerted an inhibitory effect on the expression of CDH-1 [18, 19]. Considering this, we expected that there must exist other candidate(s) that could be demethylated by KDM1A and participate in the underlying mechanism by which KDM1A exerted its effects on OS. Upon seeking candidate(s), Bcl-2 and c-Myc caught our attention as they have been widely reported to participate in different malignancies, including OS [6, 7]. What really surprised us is the fact that KDM1A knockdown, in response to the stimulation of estrogen, caused the abrogated expression of Bcl-2 and c-Myc genes, based on the relevant data in the Reactome database (https://reactome.org/content/detail/R-HSA-9011984) [50–53]. Bcl-2 and c-Myc are among the most extensively investigated proteins in all biological research. Bcl-2 is a member of the Bcl-2 family, classified within the "Bcl-2-like" pro-survival proteins. On the other hand, c-Myc plays a predominant role in apoptosis and exhibits co-operative reactions with Bcl-2 [54]. Considering these observations, we strongly believe that KDM1A may demethylate Bcl-2/c-Myc in MG-63 OS cells and inversely, Bcl-2/c-Myc may be implicated in the mechanism underlying the effects of KDM1A in OS. Here, this belief was reconfirmed in OS cells, where KDM1A demethylated Bcl-2/c-Myc in an H3K9-dependent manner and the silencing of Bcl-2/c-Myc not only produced contrary results to those following the overexpression of KDM1A but also reversed the effects of overexpressed KDM1A in both ROS generation and ER stress of OS cells. Based on these results, the conclusion can be drawn that KDM1A overexpression, opposite to its knockdown, may restrain ROS generation and ER stress so as to repress the apoptosis of OS cells, the mechanism of which may be associated with the demethylation of Bcl-2/c-Myc.

In conclusion, collectively, considering these investigations together [7, 18], we propose a novel discovery: the participation of KDM1A as a demethylase in OS has been re-evaluated, suggesting that KDM1A-mediated demethylation of Bcl-2/c-Myc may regulate the ROS generation and ER stress in MG-63 OS cells. However, it is worth noting that despite the emphasis on the effects of KDM1A/Bcl-2/c-Myc on the apoptosis of OS cells from the perspectives of ROS generation and ER stress in our current research, some other mechanisms – DNA damage, for instance – may be implicated in the apoptosis of OS cells as well, as evidenced by the discoveries highlighting the involvement of DNA damage in the apoptosis and the recruitment of KDM1A to the sites of DNA damage [55, 56]. Also, we only used one OS cell line, MG-63, in our current research, and it remained unclear whether the same results would be obtained from other OS cells *in vitro*. We intend to explore these issues in our future research.

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Ethical approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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