Long Noncoding RNA NORAD/MiR-26a-5p/NAMPT (Visfatin) Axis Regulates Proliferation and Apoptosis of Human Osteosarcoma Cells

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Research

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Abstract

Background: Visfatin is a novel adipokine, also known as a nicotinamide phosphorybosyltransferase (NAMPT) that is reported to promote the progression of osteosarcoma. The research sought to determine the regulatory network underlying NAMPT on osteosarcoma (OS).

Method: The OS tissues and paired normal controls were collected from 45 OS patients. The binding relationship between microRNA-26a-5p (miR-26a-5p) and two genes (NAMPT and NORAD) were predicted by TargetScan V7.2 and confirmed by dual-luciferase reporter assay. To reveal the function of long noncoding RNA NORAD/miR-26a-5p/NAMPT axis on cell viability, cell cycle and apoptosis of U2OS cells, CCK-8 and flow cytometry assays, examination of apoptosis-associated molecules (Bcl-2, Bax, cleaved (C)-caspase-3) were performed. The mRNA and protein levels were separately examined by RT-qPCR and Western blot.

Results: NAMPT and NORAD expressions were increased in OS tissue samples, while miR-26a-5p expression was decreased. Functionally, NORAD functions as a ceRNA of miR-136-5p to competitively target NAMPT. Furthermore, miR-26a-5p overexpression inhibited viability, cell cycle and apoptosis resistance of U2OS cells by down-regulating NAMPT along with change the expressions of apoptosis-related molecules. NORAD overexpression promoted viability, cell cycle and apoptosis resistance of U2OS cells by down-regulating MiR-26a-5p along with changes of the expressions of apoptosis-related molecules.

Conclusion: LncRNA NORAD, serving as a ceRNA of miR-26a-5p, promoted proliferation and apoptosis resistance of U2OS cells by upregulation of NAMPT.

Introduction

Osteosarcoma (OS) is the most frequent malignant tumor of bone and affects adolescents and children worldwide (1). The five-year survival rate is about 50–60% for OS patients, and nearly 40% of these patients succumb to lung metastases (2). At present, the standard strategy for OS patients is a combination of treatments consisting of surgical resection, systemic chemotherapy and restoration of limb function (3). However, anti-metastasis therapy is not yet satisfactory; systemic chemotherapy may lead to severe side effects, and has different degrees of injury on organs of the human body; surgery resection with a large impact on limb function often make it difficult on the patients. Therefore, targeted therapy of OS has become an urgent problem for researchers to solve. The therapy at gene level is always the hot-spot of OS research and is crucial for research to find the basis for OS development and effective gene therapy.

Visfatin is an adipokine also known as a nicotinamide phosphorybosyltransferase (NAMPT) that is able to modulate different processes, including lipid and glucose metabolism, oxidative stress, inflammation, and insulin resistance (4). NAMPT serves important effects on various metabolic and stress responses, and displays activities on proliferation, apoptosis, inflammation and angiogenesis (5). Accumulated

evidence indicated that NAMPT serves significant effects in different types of cancer and inhibition of NAMPT may be used as a therapeutic method in cancer (6–8). Moreover, Wang et al. (9) indicated that NAMPT promotes the migration of OS cells *in vitro* by activating NF-κB/IL-6 signals. Previous studies by our research team have shown that NAMPT serves significant effects on epithelial-mesenchymal transition, migration and invasion of OS cells (10). Additionally, we also found that NAMPT triggers nuclear translocation of NF-κB in OS cells (10). NF-κB has involvement with the control of cell growth, apoptosis and cell cycle progression (11). Cell proliferation is cell cycle-dependent (12). In eukaryotic cells, progression of cell cycle must occur in an effective and timely manner so that cell proliferation can be sustained (13). Therefore, NAMPT may also play a significant regulatory role in proliferation and apoptosis of OS cells.

Nevertheless, the functions of NAMPT on proliferation and apoptosis of OS cells remain unclear so far. In the current research, we aimed to explore the effect and associated regulatory network of NAMPT on cell proliferative and apoptotic abilities of OS cells.

Materials And Method

OS specimens

The OS tissue specimens and paired normal controls were collected from 45 patients who were underwent wide-excision surgery in Yantaishan Hospital from January 2015 to January 2016. The postoperative pathology specimens were all confirmed for osteosarcoma.

Cells culture and grouping

U2OS cells (OS cell line, ATCC, USA) were incubated in DMEM (12100, Solarbio, China) with supplement of 10% FBS (11011-8611, Solarbio) in a 37 ℃ incubator with 5% CO_2.

First, to reveal the function of microRNA-26a-5p (miR-26a-5p) on phenotypes of U2OS cells, U2OS cells were divided into Blank, MC and M groups. Cells in blank group were only cultured with DMEM. Cells in MC group or M group were separately subjected to mimic control or miR-26a-5p mimic transfection and then cultured in DMEM for the specified times.

To determine whether miR-26a-5p could bind to NAMPT to regulate phenotypes of U2OS cells, U2OS cells were divided into Blank, MC, M, NC, NAMPT, M+NAMPT groups. Blank, MC, M groups were carried out according to described above. NC group: cells were subjected to pcDNA (negative control) transfection and then cultured in DMEM for the specified times; NAMPT group: cells were transfected with pcDNA-NAMPT and then cultured in DMEM for the specified times; M+NAMPT group: cells were co-transfected with miR-26a-5p mimic and pcDNA-NAMPT and then cultured in DMEM for the specified times.

To determine whether lncRNA NORAD (NORAD) could target miR-26a-5p to regulate phenotypes of U2OS cells, U2OS cells were divided into Blank, MC, M, NORAD-NC, NORAD, NORAD+M group. Blank, MC, M

groups were performed as described above. NORAD-NC group: cells were transfected with pcDNA empty vector and then cultured in DMEM for the specified times; NORAD group: cells were transfected with pcDNA-NORAD and then cultured in DMEM for the specified times; NORAD+M group: cells were subjected to miR-26a-5p mimic and pcDNA-NORAD transfection and then cultured in DMEM for the specified times.

Transfection

The miR-26a-5p mimic (miR10000082-1-5), mimic control (miR1N0000001-1-5) were purchased from RiboBio Co., Ltd. (Guangzhou, China). The overexpression plasmid containing the NAMPT sequence (pcDNA-NAMPT), or the NORAD sequence (pcDNA-NORAD), and pcDNA (empty vector) were acquired from GenePharma Co., Ltd. (Shanghai, China). U2OS cells were transfected for 48 h with 50 μM miR-26a-5p mimic and mimic control, 5 mg/L pc-DNA plasmids using lipofectamine 2000 (11668, Invitrogen). After that, the transfected cells were collected and used for other research.

Dual-luciferase reporter assay

The targeting relationships between miR-26a-5p and two genes (NAMPT and NORAD) were predicted by TargetScan V7.2 (http://www.targetscan.org/vert_72/). The 3-UTR of NAMPT and the mutated sequence were designed and synthesized by GenePharma Co., Ltd. The fragments were inserted into the pmirGLO control vector (E1330, Promega, USA) to construct the NAMPT-wild-type (WT) vector and NAMPT-mutant (MUT) vector, respectively. Similar procedures were performed to construct reporter plasmids NORAD-WT and NORAD-MUT. For the dual-luciferase reporter assay, either a WT or MUT reporter plasmid and either miR-26a-5p mimic or mimic control were co-transfected into U2OS cells for 48 h using lipofectamine 2000 (11668, Invitrogen). Dual-luciferase reporter assay kit (E1910, Promega) was applied to determine luciferase activity of transfected cells.

CCK-8

Transfected U2OS cells were harvested and cultured in 96-well plates (5×10^3 cells/well). Cell viability was indicated at 24 h, 48 h and 72 h with a CCK-8 Kit (CA1210, Solarbio). The viability of U2OS cells was evaluated by monitoring the absorbance at 490 nm using an enzyme-labeling instrument (Bio-Rad, USA).

Flow cytometry assay

Cell cycle of U2OS cells was examined with propidium iodide (PI) kit (CA1510, Solarbio). Transfected U2OS cells (5×10^4/dish) were cultured in 6 cm dishes. After 72 h culture, cells were fixated in 70% ethanol overnight at 4°C, washed by PBS and suspended in 100 μL R RNase A solution. Next, after incubation at 37 °C for 30 min, cells were treated with 400 μL PI for 30 min at 4 °C. Cell populations in in each of the cell cycle phases were quantified using FACS Caliber cytometer (BD Biosciences) and the data were analyzed by the Modfit LT software (Verity Software House, USA).

For apoptosis experiment, transfected U2OS cells were harvested and dyed by both Annexin V-FITC and PI as manufacturers' protocol (CA1020, Solarbio). Briefly, harvested U2OS cells were centrifuged at 300×

g for 10 min. After discarding the supernatant, the cells were resuspended in 1 ml 1 × binding buffer and the cell concentration was adjusted to 1×10^6 cells/ml. 100 μL of cells (1×10^5) was added to each labelled tube, incubated with 5 μL Annexin V-FITC for 10 min and then incubated with 5 μL PI for 5 min. Apoptosis of U2OS cells was examined by FACS Caliber cytometer (BD Biosciences) within 1 h.

Reverse transcription quantitativepolymerase chain reaction (RT-qPCR)

Total RNA was isolated from OS tissues and paired normal tissues and U2OS cells with TRIzol reagent (15596-026, Invitrogen). RT-qPCR was carried out to synthesize cDNA using total RNA with cDNA Synthesis Kit (RR036B, Takara). Then, qPCR was carried out with TB Green® Premix Ex Taq™ II (RR820Q, Takara) in an Thermal Cycler Dice Real Time System.

For miRNA analysis, miRNA was extracted using RNAiso for Small RNA (9753A, Takara). CDNA was synthesized using t Mir-X miRNA First-Strand Synthesis Kit (638315, Clontech). And qPCR was performed using Mir-X miRNA qRT-PCR TB Green® Kit (638316, Clontech) in athermal Cycler Dice Real Time System. The nucleotide sequence of the forward primer for miR-26a-5p, is as follows (TTCAAGTAATCCAGGATAGGCT). A universal reverse primer was provided in the kit. GAPDH or U6 was served as internal control for mRNA and miRNA quantification, respectively. The genes levels of were determined by the $2^{-\Delta\Delta Ct}$ method (14). The nucleotide sequences of primer used in the qRT-PCRs are listed in **Table 1**.

Western blot

Treated U2OS cells were lysed in a RIPA buffer (R0010, Solarbio) containing 1 mM PMSF. The supernatant Protein concentration in each group was qualified by BCA Protein Assay Kit (C0020, Solarbio). After being isolated on 12% SDS-PAGE, soluble protein was transferred to PVDF membranes (YA1701, Solarbio). Protein markers (PR1910 (11-180kDa) and PR1920 (11-245KD)) were acquired from Solarbio Science & Technology Co., Ltd. (Beijing, China). After separation and transfer, the blots were blocked with 5% nonfat dry milk and reacted with primary antibodies (listed in **Table 2**) overnight at 4°C. Then membranes were reacted with secondary antibody (ab205718, 1:2000, Abcam) for 2 h. Blots were visualized by ECL Western Blotting Substrate (32209, Thermo Fisher Scientific) and digitized by NIH Image (National Institutes of Health, Bethesda, MD).

Statistical analysis

All cell-based experiments were performed independently and repeated at least three times. The data were shown as mean ± standard deviation (S.D.) and analyzed by GraphPad Prism 6.0 (GraphPad). Paired *t*-test was used for OS tissues and paired normal tissues. All *P* values were analyzed using Student's *t* test or one-way ANOVA followed by post hoc analysis using Bonferroni's test. A *p*-value of less than 0.05 was taken as statistical significance.

Results

MiR-26a-5p overexpression inhibited viability, cell cycle and apoptosis resistance of U2OS cells

Firstly, the findings showed that miR-26a-5p level was lower in OS tissues than that in paired normal tissues (Fig. 1A, $p < 0.001$). Subsequently, we examined the effect of miR-26a-5p mimic on viability, cell cycle and apoptosis resistance of U2OS cells. MiR-26a-5p mimic obviously inhibited viability of U2OS cells at 72 h (Fig. 1B, $p < 0.001$) and decreased cells percentage at G2/M and S phases (Fig. 1C and 1E, $p < 0.001$), but elevated cells percentage at G0/G1 phase (Fig. 1C and 1E, $p < 0.001$); cell apoptosis was significantly induced by miR-26a-5p mimic (Fig. 1D and 1F, $p < 0.001$). Finally, the findings indicated that miR-26a-5p mimic significantly decreased Bcl-2 at mRNA and protein levels (Fig. 2A-2C, $p < 0.001$), but increased Bax at mRNA and protein levels (Fig. 2A-2C, $p < 0.001$) and C-caspase-3 at protein level (Fig. 2B and 2C, $p < 0.001$).

MiR-26a-5p directly targeted NAMPT

TargetScan V7.2 predicted that miR-26a-5p had binding sites to NAMPT (Fig. 3A). Moreover, dual-luciferase report assay showed that luciferase activity was remarkably decreased in M+NAMPT-WT group as compared to MC+NAMPT-WT group (Fig. 3B, $p < 0.001$).

MiR-26a-5p overexpression inhibited viability, cell cycle and apoptosis resistance of U2OS cells by down-regulating NAMPT

We first found that NAMPT was highly expressed in OS tissues compared to paired normal tissues (Fig. 4A, $p < 0.001$). The findings from Western blot showed that miR-26a-5p mimic obviously decreased AMPT protein expression (Fig. 4B and 4C, $p < 0.001$), while NAMPT transfection increased AMPT protein expression (Fig. 4B and 4C, $p < 0.001$), which was partially abrogated by miR-26a-5p mimic (Fig. 4B and 4C, $p < 0.001$). Moreover, NAMPT overexpression remarkably promoted viability (Fig. 4F, $p < 0.001$) at 72 h, cell cycle (Fig. 4D and 4G, $p < 0.001$) and apoptosis resistance (Fig. 4E and 4H, $p < 0.001$); by contrast, these effects were partially abrogated by miR-26a-5p mimic (Fig. 4D and 4H, $p < 0.01$). The findings from Western blot showed that NAMPT overexpression remarkably increased the mRNA and protein levels of Bcl-2 (Fig. 5A-5C, $p < 0.001$), but decreased the mRNA and protein levels of Bax (Fig. 5A-5C, $p < 0.001$), and the protein level of cleaved (C)-caspase-3 (Fig. 5B and 5C, $p < 0.001$); by contrast, these effects were partially abrogated by miR-26a-5p mimic (Fig. 5A-5C, $p < 0.001$).

NORAD directly targeted miR-26a-5p

TargetScan V7.2 showed that miR-26a-5p had binding sites to NAMPT (Fig. 6A). Moreover, dual-luciferase report assay showed that luciferase activity was remarkably decreased in M+NORAD-WT group as compared to MC+ NORAD-WT group (Fig. 6B, $p < 0.001$).

NORAD overexpression stimulated viability, cell cycle and apoptosis resistance of U2OS cellsby down-regulating miR-26a-5p

NORAD expression was obviously higher in OS tissues than that in paired normal tissues (Fig. 7A, $p < 0.001$). NORAD transfection remarkably increased NORAD expression in U2OS cells (Fig. 7B, $p < 0.001$), but miR-26a-5p mimic had no effect on NORAD expression (Fig. 7B); miR-26a-5p mimic remarkably elevated miR-26a-5p expression in U2OS cells (Fig. 7C, $p < 0.001$), whereas NORAD overexpression remarkably decreased miR-26a-5p expression (Fig. 7C, $p < 0.001$) and partially abrogated the effect of miR-26a-5p mimic on miR-26a-5p expression (Fig. 7C, $p < 0.001$). NORAD overexpression inhibited miR-26a-5p expression in U2OS cells. NORAD overexpression promoted viability at 72 h (Fig. 7D, $p < 0.01$), cell cycle (Fig. 7E and 7G, $p < 0.001$) and apoptosis resistance (Fig. 7F and 7H, $p < 0.001$) of U2OS cells; by contrast, these effects were partially abrogated by miR-26a-5p overexpression (Fig. 7D-7H, $p < 0.001$). Moreover, the results from Western blot showed that NORAD overexpression remarkably elevated the mRNA and protein levels of Bcl-2 (Fig. 8A-8C, $p < 0.001$), but decreased the mRNA and protein levels of Bax (Fig. 8A-8C, $p < 0.01$), and the protein level of C-caspase-3 (Fig. 8B and 8C, $p < 0.001$); however, these effects were partially abrogated by miR-26a-5p mimic (Fig. 8A-8C, $p < 0.01$).

Discussion

Abnormal expression of NORAD is found in various cancers and NORAD has involvement with several processes related to carcinogenesis, including proliferation, invasion, metastasis, and apoptosis (15). Furthermore, previous report demonstrated that NORAD modulates proliferation and migration in human OS by endogenously competing with miR-199a-3p (16). miRNAs, a group of short non-coding RNAs with roughly 18 to 24 nucleotides (nt), are abundant in eukaryotic cells (17). MiR-26a-5p is a member of the miRNA family, and negatively regulates proliferation and positively regulate apoptosis in several cancers (18, 19). miR-26a-5p has also involvement with proliferation and migration of OS cells (20). Through bioinformatics prediction, we discovered that NAMPT and NORAD were direct binding genes of miR-26a-5p. A competing endogenous RNA (ceRNA) has been regarded as a new mechanistic type of lncRNA in recent years (21). LncRNA can acts as a miRNA sponge via ceRNA activity to regulate target gene expression by inhibiting miRNA activity. We, therefore, assumed that NORA could regulate NAMPT by sponging miR-26a-5p, thereby promoting proliferation and apoptosis resistance of OS cells.

By using TargetScan V7.2 and dual-luciferase reporter assay in our work, we predicted and confirmed that miR-26a-5p possess a binding site of NORAD and NAMPT, respectively. NAMPT is elevated in OS compared to benign bone (22). NORAD is observed to be conspicuously overexpressed in both OS cells and OS tumors (16). MiR-26a-5p expression was higher in OS cell lines, particularly U2OS cells, than that in non-tumor cells (23). The present data suggested that miR-26a-5p level was decreased in OS tissues, while both NAMPT and NORAD levels were increased. Moreover, we founded that miR-26a-5p bound to NAMPT to inhibit its expression. NORAD inhibits miR-26a-5p expression by direct targeting. Moreover, NORAD shared identical binding sites of miR-26a-5p with NAMPT, which indicated that NORAD might function as a ceRNAs to modulate NAMPT expression by sponging miR-26a-5p. Similarly, lncRNA TTN-AS1 facilitates MBTD1 expression by binding miR-134-5p, thereby regulating cell growth and apoptosis in OS (24). Zhang et al. (25) suggested that lncRNA DLX6-AS1/miR-129-5p/DLK1 axis deteriorate stemness

of OS. In considering the role of lncRNA/miRNA/mRNA axis in phenotypes of OS, we therefore further explore roles of NORAD/miR-26a-5p/NAMPT axis in proliferation and apoptosis of OS cells.

Control of cell proliferation generally occurs during the G1 phase of the division cycle in eukaryotic cells (26). The present results suggested that miR-26a-5p overexpression in U2OS cells inhibited viability at 72 h, and increased cell percentage at G0/G2 phase, and thus accurately reflected miR-26a-5p overexpression inhibited the cell proliferation through inhibiting viability and cell cycle. Various factors, especially Bcl-2 and Bax, can regulate apoptosis, and apoptosis is activated by apoptotic cascade via caspase-3 whose cleaved protein has been reported as an executioner of apoptotic pathway and is needed for stimulation of apoptosis (27). In our research, miR-26a-5p overexpression in U2OS cells elevated apoptosis rate following by decreasing Bcl-2 expression and elevating Bax and C-caspase-3 expressions. These findings reflected miR-26a-5p overexpression promoted the cell apoptosis through regulating Bcl-2, Bax and C-caspase-3 expressions. Two basic physiological processes, cell apoptosis and proliferation, basically maintain the dynamic equilibrium of the cell numbers in the body (28). Apoptosis always maintains the dynamic equilibrium between proliferating cells and programmed cell death under normal circumstances (29). Therefore, inhibited proliferation and promoted apoptosis caused by miR-26a-5p overexpression in OS cells accurately decreased the quantity of OS cells.

Furthermore, we confirmed that the inhibitory functions of miR-26a-5p overexpression on proliferation and apoptosis resistance of U2OS cells, which was partially reversed by NAMPT overexpression and NORAD overexpression. Collectively, the present study suggested that NORAD regulated NAMPT by sponging miR-26a-5p, thereby promoting proliferation and apoptosis resistance of OS cells. Our data extended previous observations that NAMPT promotes migration and invasion by NF-κB/Snail/EMT signaling (10). Our new findings provide the first evidence to suggest that NORA targeted miR-26a-5p to regulate NAMPT,thereby regulating proliferation and apoptosis of OS cells. These will provide new ideas for developing new antitumor drugs or one single specific therapeutic method for OS. However, further research needed be performed on appropriate animal model in the future.

Conclusion

In conclusion, the data presented in this research elucidated that miR-26a-5p level was decreased in OS tissues, while NAMPT and NORAD levels were increased. Moreover, NORAD competitively interacted with miR-26a-5p to NAMPT, thereby promoting proliferation and apoptosis resistance of OS cells. Our results might offer a new theoretical basis for OS therapy.

Method

The OS tissues and paired normal controls were collected from 45 OS patients. The binding relationship between microRNA-26a-5p (miR-26a-5p) and two genes (NAMPT and NORAD) were predicted by TargetScan V7.2 and confirmed by dual-luciferase reporter assay. To reveal the function of long noncoding RNA NORAD/miR-26a-5p/NAMPT axis on cell viability, cell cycle and apoptosis of U2OS cells,

CCK-8 and flow cytometry assays, examination of apoptosis-associated molecules (Bcl-2, Bax, cleaved (C)-caspase-3) were performed. The mRNA and protein levels were separately examined by RT-qPCR and Western blot.

Abbreviations

NAMPT : nicotinamide phosphorybosyltransferase

OS : osteosarcoma

miR-26a-5p : microRNA-26a-5p

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

Substantial contributions to conception and design:

Data acquisition, data analysis and interpretation:

Drafting the article or critically revising it for important intellectual content:

Final approval of the version to be published: All authors

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved:

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Availiability of Data and Materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

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Tables

Table 1
Primer sequences used for reverse transcription quantitative polymerase chain reaction (RT-qPCR) for human.

Genes		Primer sequences(5′-3′)
NAMPT	forward	CGGCAGAAGCCGAGTTCAA
	reverse	GCTTGTGTTGGGTGGATATTGTT
NORAD	forward	TGCCTGTACTTGTCCACTG
	reverse	CCTTGAATCATTCCCTCCTC
Bcl-2	forward	GGTGGGGTCATGTGTGTGG
	reverse	CGGTTCAGGTACTCAGTCATCC
Bax	forward	CCCGAGAGGTCTTTTTCCGAG
	reverse	CCAGCCCATGATGGTTCTGAT
GAPDH	forward	CCATCTTCCAGGAGCGAGATC
	reverse	GCCTTCTCCATGGTGGTGAA
U6	forward	CTCGCTTCGGCAGCACA
	reverse	TGGTGTCGT GGAGTCG

Table 2
List of primary antibodies used for western blots.

Protein	Antibody	Catalog Number	Company	Antibody Dilution
Bcl-2	Rabbit Anti-Bcl-2 antibody	ab32124	Abcam	1:1000
Bax	Rabbit Anti-Bax antibody	ab32503	Abcam	1:1000
C-caspase-3	Rabbit Anti-Cleaved Caspase-3 antibody	ab2302	Abcam	1:500
NAMPT	Rabbit Anti-Visfatin antibody	ab45890	Abcam	1:250
GAPDH	Rabbit Anti-GAPDH antibody	ab181602	Abcam	1:10000

Figures

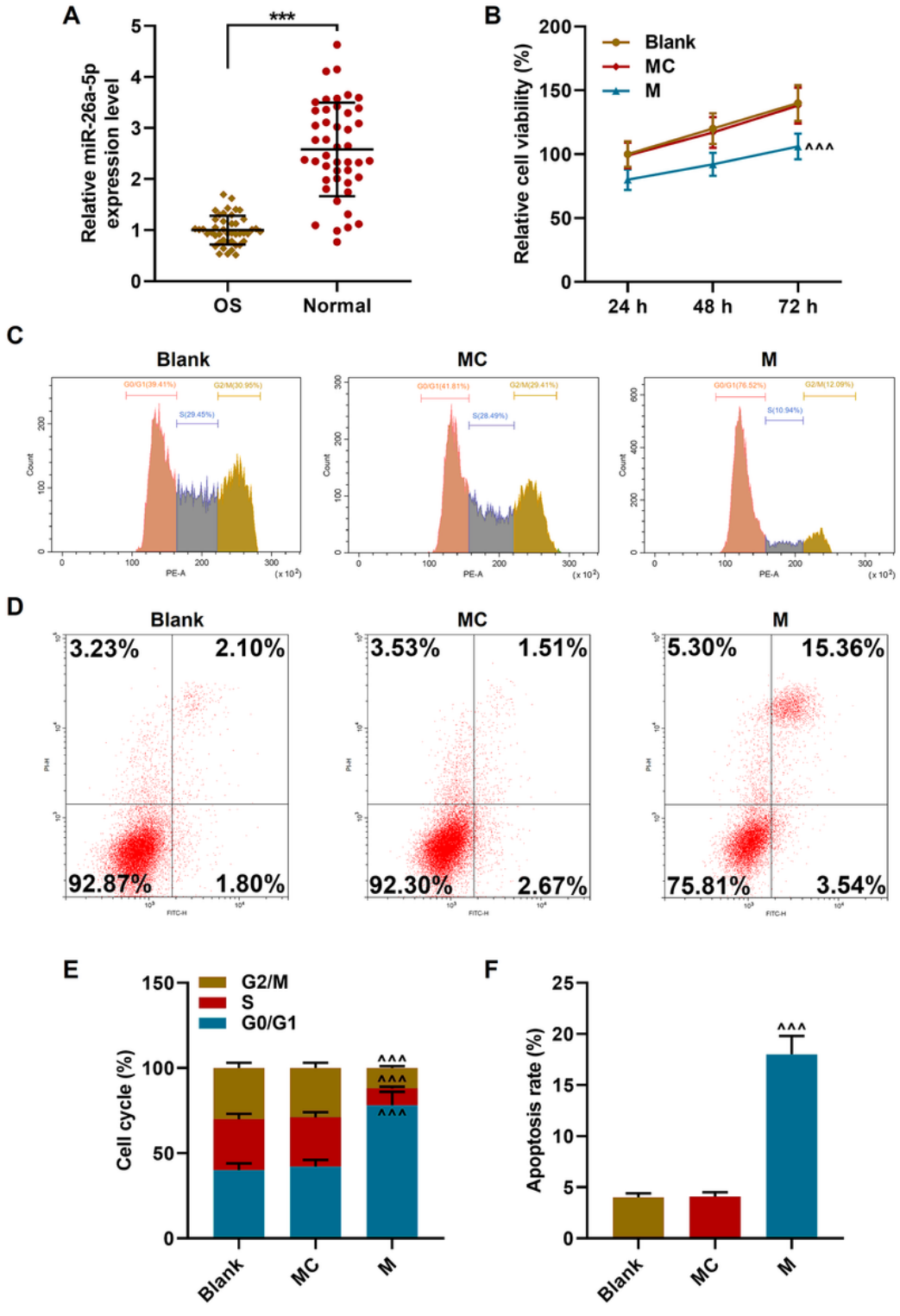

Figure 1

MiR-26a-5p overexpression inhibited viability, cell cycle and apoptosis resistance of U2OS cells. (A) MiR-26a-5p expression in osteosarcoma (OS) tissue samples and paired normal controls was examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B-F) U2OS cells were treated with

mimic control (MC) or miR-26a-5p mimic (M). Cell viability (B) in U2OS cells was determined by CCK-8 assay. Cell cycle (C and E) and apoptosis (D and F) in U2OS cells were determined by flow cytometry assay. ***p < 0.001 vs. OS; ^^^p < 0.001 vs. MC. U6 expression was used as the endogenous control. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

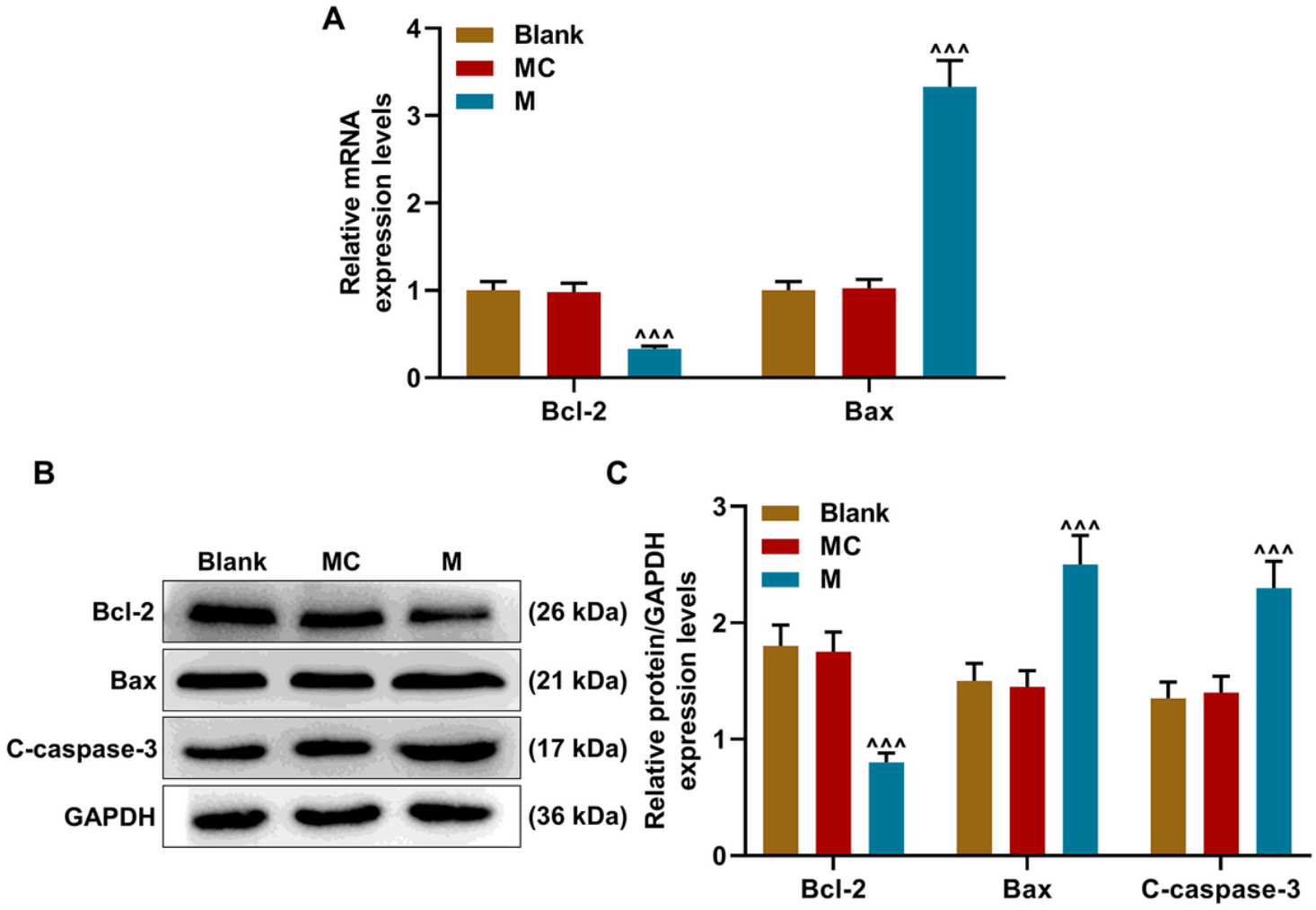

Figure 2

MiR-26a-5p overexpression regulated apoptosis-related molecules in U2OS cells. (A-C) U2OS cells were treated with mimic control (MC) or miR-26a-5p mimic (M). (A) The mRNA levels of Bcl-2 and Bax in U2OS cells were examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B and C) The protein levels of Bcl-2, Bax, cleaved (C)-caspase-3 in U2OS cells were examined by Western blot. ^^^p < 0.001 vs. MC. GAPDH was used as the endogenous control. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

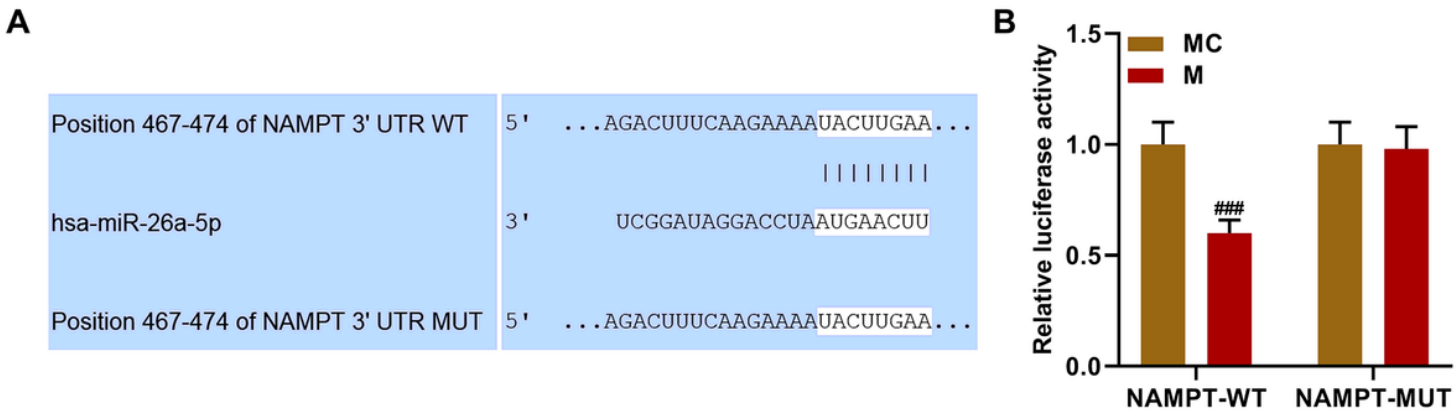

Figure 3

MiR-26a-5p directly targeted NAMPT. (A) The binding relationship between miR-26a-5p and NAMPT was predicted by TargetScan V7.2. (B) Either a NAMPT-WT or NAMPT-MUT reporter plasmid and either miR-26a-5p mimic (M) or mimic control (MC) were co-transfected into U2OS cells. ###p < 0.001 vs. MC. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

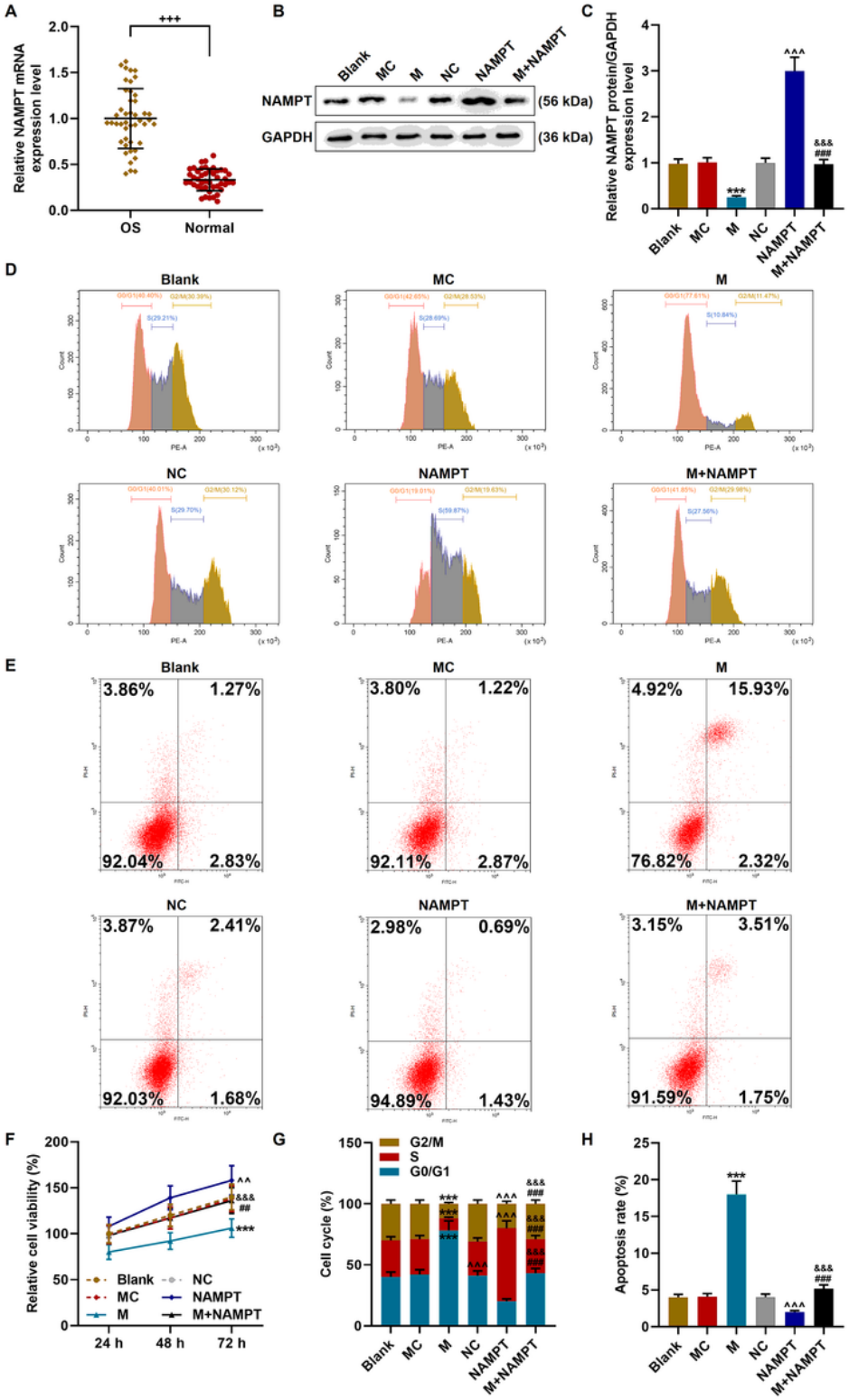

Figure 4

MiR-26a-5p overexpression inhibited viability, cell cycle and apoptosis resistance of U2OS cells by down-regulating NAMPT. (A) NAMPT expression in osteosarcoma (OS) tissue samples and paired normal controls was examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B-H) U2OS cells were treated with mimic control (MC) or miR-26a-5p mimic (M), pcDNA (NC), pcDNA-NAMPT (NAMPT), and M+NAMPT. NAMPT expression (B and C) and cell viability (F) at 24h, 48 h and 72 h in

U2OS cells were determined by Western blot and CCK-8 assays. respectively. Cell cycle (D and G) and apoptosis (E and H) in U2OS cells were determined by flow cytometry assay. +++p < 0.001 vs. OS; ***p < 0.001 vs. MC; ^^^p < 0.001 vs. NC; &&&p < 0.001 vs. M; ##p < 0.01 or ###p < 0.001 vs. NAMPT. GAPDH was used as the endogenous control. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

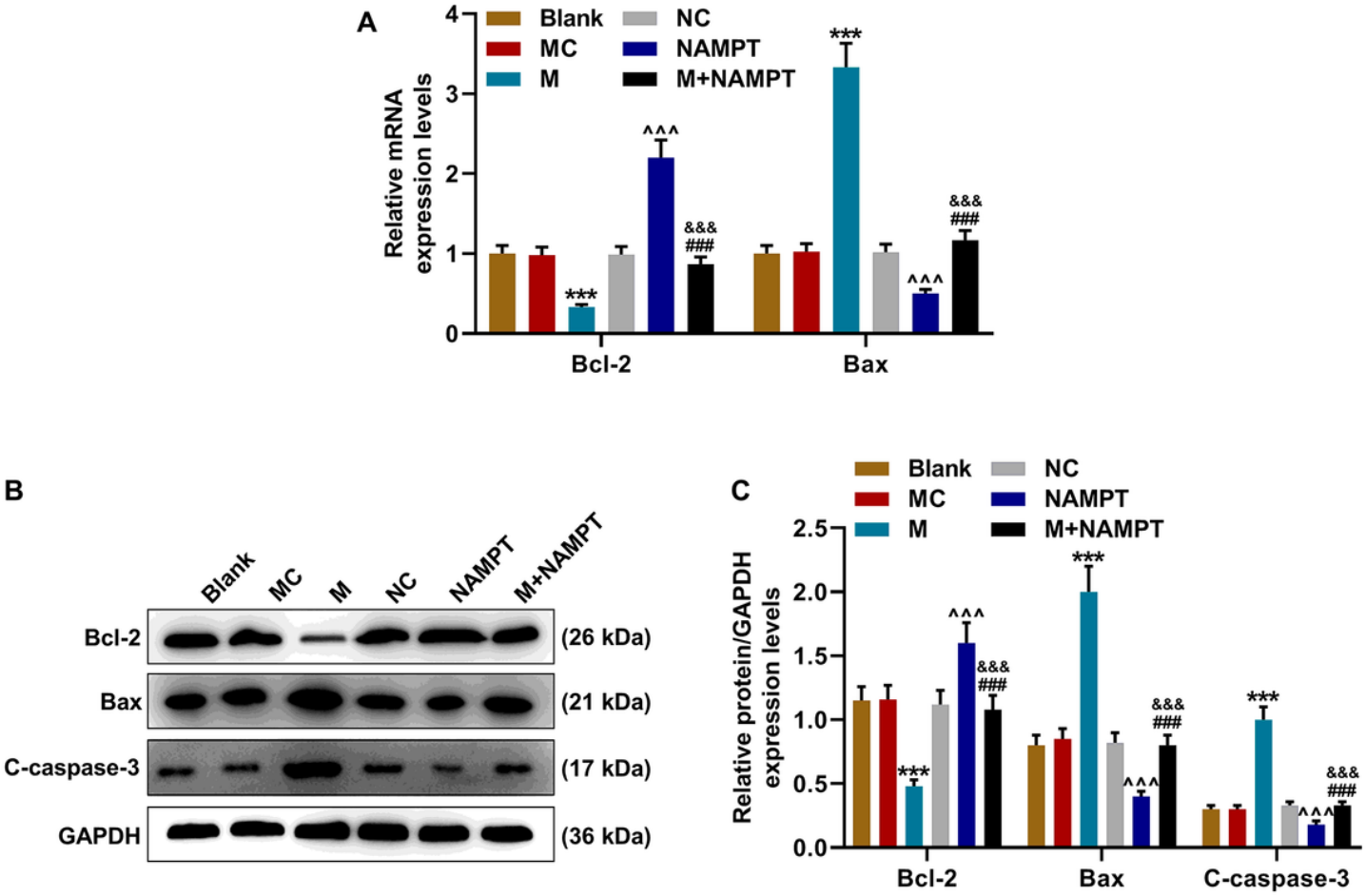

Figure 5

MiR-26a-5p overexpression regulated apoptosis-related molecules in U2OS cells by down-regulating NAMPT. (A-C) U2OS cells were treated with mimic control (MC) or miR-26a-5p mimic (M), pcDNA (NC), pcDNA-NAMPT (NAMPT), and M+NAMPT. (A) The mRNA levels of Bcl-2 and Bax were examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B and C) The protein levels of Bcl-2, Bax, cleaved (C)-caspase-3 in U2OS cells were examined by Western blot. ***p < 0.001 vs. MC; ^^^p < 0.001 vs. NC; &&&p < 0.001 vs. M; ###p < 0.001 vs. NAMPT. GAPDH was used as the endogenous control. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

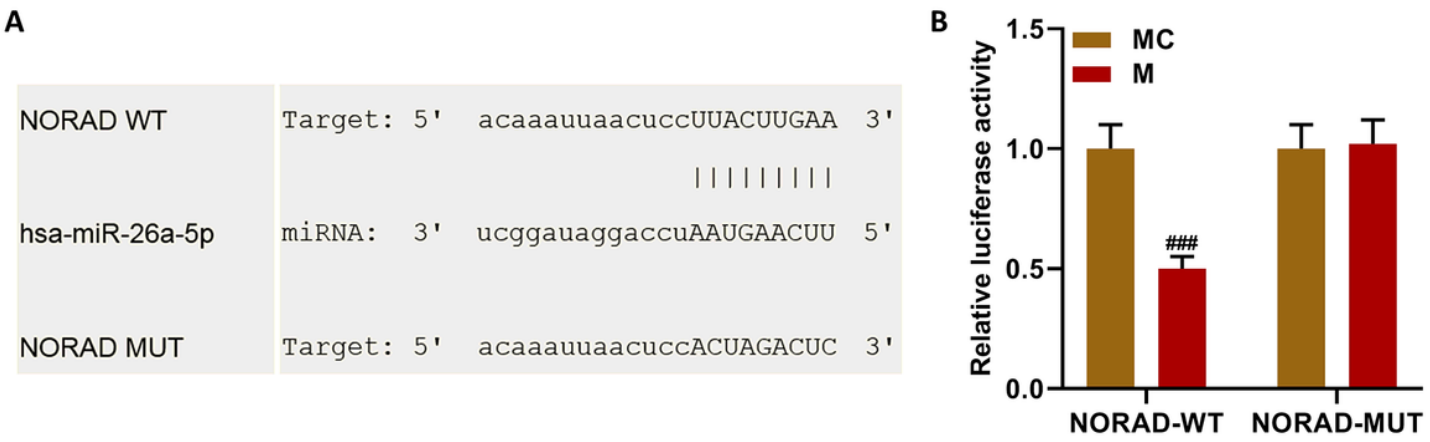

Figure 6

NORAD directly targeted miR-26a-5p. (A) The binding relationship between miR-26a-5p and NORAD was predicted by TargetScan V7.2. (B) Either a NORAD-WT or NORAD-MUT reporter plasmid and either miR-26a-5p mimic (M) or mimic control (MC) were co-transfected into U2OS cells. ###p < 0.001 vs. MC. MC, mimic control; M, miR-26a-5p mimic. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

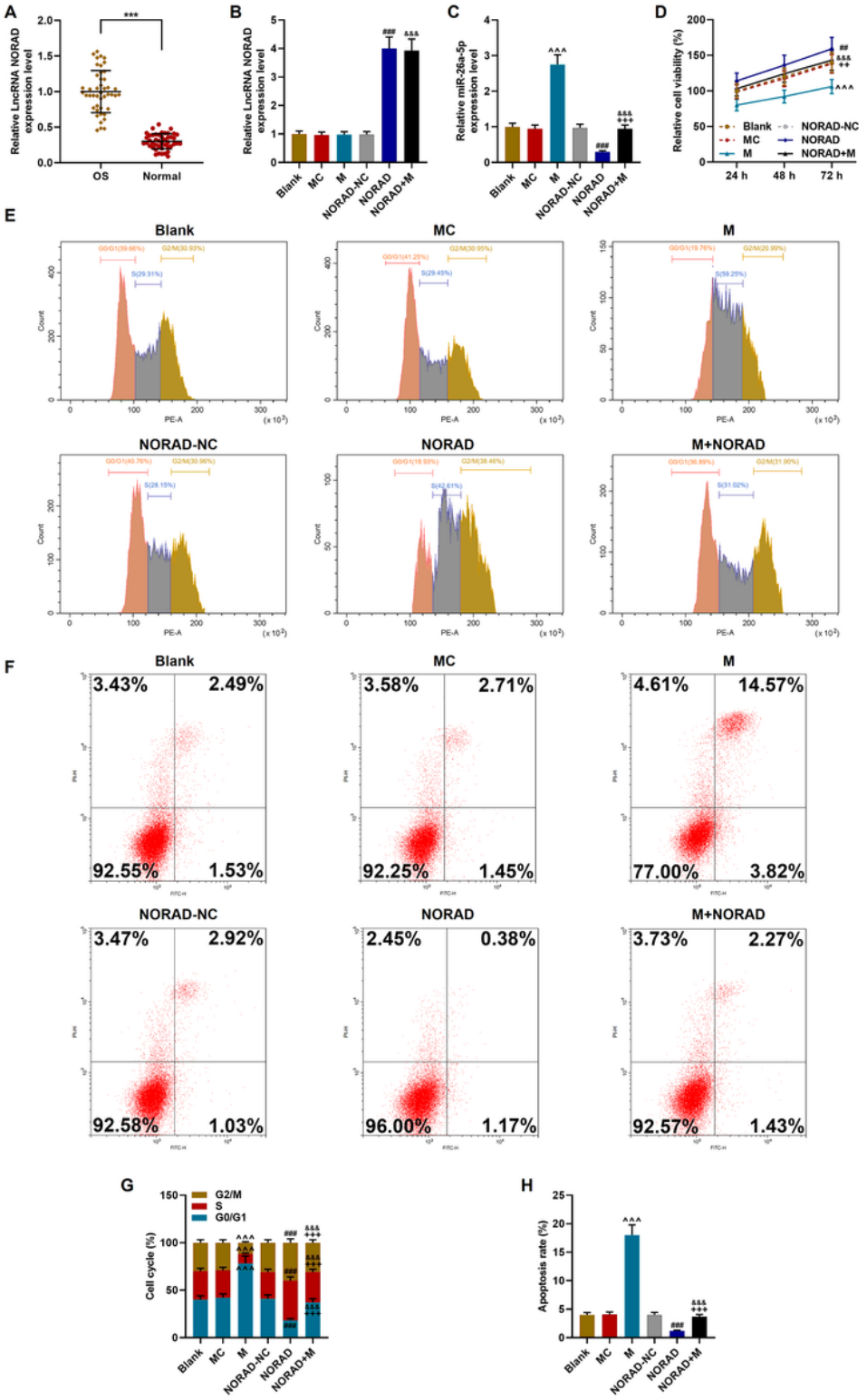

Figure 7

NORAD overexpression promoted viability, cell cycle and apoptosis resistance of U2OS cells by down-regulating miR-26a-5p. (A) NORAD expression in osteosarcoma (OS) tissue samples and paired normal controls was examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B-H) U2OS cells were treated with mimic control (MC) or miR-26a-5p mimic (M), pcDNA-NORAD (NORAD)+ pcDNA (NORAD-NC), NORAD, and M+NORAD. NORAD (B) and miR-26a-5p (C) expressions in U2OS cells

were examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Cell viability (D) in U2OS cells at 24h, 48 h and 72 h was determined by CCK-8 assay. Cell cycle (E and G) and apoptosis (F and H) in U2OS cells were determined by flow cytometry assay. ***p < 0.001 vs. OS; ^^^p < 0.001 vs. MC; ##p < 0.01 or ###p < 0.001 vs. NORAD-NC; &&&p < 0.001 vs. M; ++p < 0.01 or +++p < 0.001 vs. NORAD. GAPDH or U6 was used as the endogenous control. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).

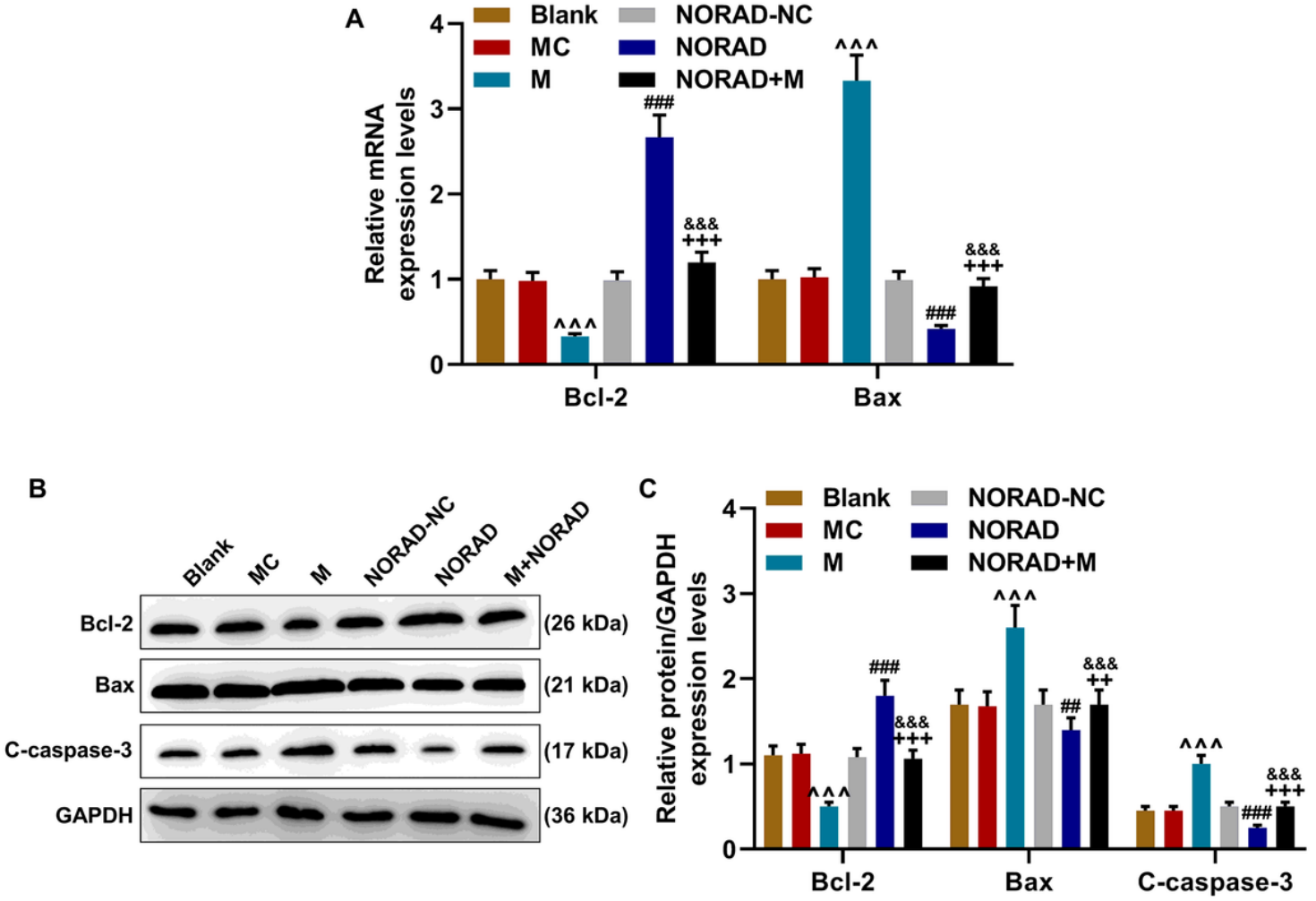

Figure 8

NORAD overexpression regulated apoptosis-related molecules in U2OS cells by down-regulating miR-26a-5p. (A-C) U2OS cells were treated with mimic control (MC) or miR-26a-5p mimic (M), pcDNA-NORAD (NORAD)+pcDNA (NORAD-NC), NORAD, and M+ NORAD. (A) The mRNA levels of Bcl-2 and Bax were examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). (B and C) The protein levels of Bcl-2, Bax, cleaved (C)-caspase-3 in U2OS cells were examined by Western blot. ^^^p < 0.001 vs. MC; ##p < 0.01 or ###p < 0.001 vs. NORAD-NC; &&&p < 0.001 vs. M; +++p < 0.001 vs. NORAD. GAPDH was used as the endogenous control. All experiments were performed independently at least three times in duplicate. The data were expressed as mean ± standard deviation (S.D.).