

Effects of high-mobility group box 1 knockdown on proliferation, migration and invasion of the HONE-1 human nasopharyngeal carcinoma cell line

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Abstract. The present study was designed to investigate the effects of high-mobility group box 1 (HMGB1) knockdown on the proliferation, migration and invasion of the HONE-1 human nasopharyngeal carcinoma cell line and explore the possible underlying mechanisms. HMGB1-knockdown HONE-1 cells were generated by lentiviral transfection, and HMGB1 expression was demonstrated to be obviously decreased in these cells. A Cell Counting kit-8 assay was used to determine cell proliferation, while flow cytometric analysis was employed to determine the apoptotic rate. In addition, *in vitro* wound healing, cell adhesion and invasion assays were performed to evaluate the metastatic potential of the cells. Western blot analysis was used to determine the protein expression of apoptosis signaling proteins caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein, receptor for advanced glycation end products (RAGE) as well as phosphorylated and total extracellular signal-regulated kinase 1/2 in HONE-1 cells. The results of the present study demonstrated that HMGB1 knockdown suppressed the proliferation, migration and invasion of HONE-1 cells, the mechanisms of which may be associated with the induction of mitochondria-mediated apoptosis and inhibition of HMGB1/RAGE pathways.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common type of nasopharyngeal malignant tumor in men and women in southern China, with an incidence rate of 15-50/100,000 (1-4). Etiological investigations indicated that the genesis of NPC

is highly complex, and that the Epstein-Barr virus as well as environmental and genetic factors are correlated with the development of NPC (5-7). Furthermore, patients present with only few obvious symptoms in the early stage of NPC, and ~70% NPC patients present with a locally advanced stage at the time of final diagnosis (1,8,9). In addition, it is known that NPC bears a substantial risk of invasion and metastasis, which remain the leading cause of NPC-associated mortality. Although concurrent chemotherapy and radiotherapy for the treatment of NPC has improved the overall survival rate, local relapse post-treatment and distant metastasis are common, to which NPC patients eventually succumb (10-12). Thus, there is an urgent requirement for effective and reliable therapeutic methods to cure NPC.

High-mobility group box 1 (HMGB1) belongs to the HMGB protein family, is associated with various biological processes and has important roles in inflammation, immunity, cell proliferation, cell migration, cell death, wound healing and progression of malignant tumors (13,14). Previous studies have revealed that HMGB1 is commonly overexpressed in malignant tumor cells and that knockdown of HMGB1 markedly inhibited cancer cell proliferation, invasion and metastasis (14-16). In addition, a preliminary screening study by our group found that HMGB1 is overexpressed in the HONE-1 NPC cell line, rendering it an ideal cell line for studies on the roles of HMGB1 in NPC. The aim of the present study was to determine the effects of HMGB1 knockdown on the proliferation, migration and invasion of the HONE-1 cell line in order to explore its suitability as a therapeutic target for treating NPC.

Materials and methods

Reagents and cell lines. The HONE-1 and 293T cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-BRL (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and fetal bovine serum (FBS) was purchased from Sijiqing Biotech. Co. (Hangzhou, China). TRIzol reagent was purchased from Invitrogen Life Technologies, Inc. and a Cell Counting kit-8 (CCK-8) was purchased from Dojindo Biochem (Shanghai, China). An

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Annexin V/fluorescein isothiocyanate (FITC) kit and Matrigel were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Crystal violet, Giemsa, trypsinase, acrylamide, SDS and phosphate-buffered saline (PBS) were purchased from JRDun Biotech (Shanghai, China). Transwell well culture chambers were purchased from Corning (Corning, NY, USA). A bicinchoinic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and nitrocellulose filter membranes were purchased from Millipore (Billerica, MA, USA). Antibodies against caspase-3 (cat. no. Ab32351; rabbit monoclonal; 1:1,000), HMGB1 (cat. no. Ab79823; rabbit monoclonal; 1:800) and advanced glycation end products (RAGE; cat. no. Ab3611; rabbit polyclonal; 1:1,000) were purchased from Abcam (Cambridge, MA, USA); monoclonal antibodies against extracellular signal-regulated kinase (ERK)1/2 (cat. no. 9102; rabbit polyclonal; 1:1,000), phosphorylated (p)-ERK1/2 (cat. no. 4376; rabbit IgG; 1:1,000) and GAPDH (cat. no. 5174; rabbit monoclonal; 1:2,000) were purchased from CST Biotech (Shanghai, China); monoclonal antibodies against B-cell lymphoma 2 (Bcl-2; cat. no. Sc-492; rabbit polyclonal; 1:400) and Bcl-2-associated X protein (Bax; cat. no. Sc-493; rabbit IgG; 1:500) were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Goat-anti-rabbit/rat horseradish peroxidase-conjugated secondary antibodies were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture. HONE-1 cells were cultured in DMEM containing 15% (v/v) heat-inactivated FBS, 100 mg/ml streptomycin (Corning Incorporated, Corning, NY, USA) and 100 U/ml penicillin (Corning Incorporated) at 37°C in a humidified atmosphere containing 5% CO₂.

Plasmid construction and transient transfection. HMGB1-specific small interfering (si)RNA overexpression vector was constructed for RNA interference as previously described (17). The resulting recombinant lentiviruses were transfected into HONE-1 cells to inhibit the expression of HMGB1. The siRNA target sequence was as follows: 5'-TGGTGATGTTGCGAAGAAA-3'. HMGB1 expression in untreated HONE-1 cells, cells treated with control vector (Mock group), and HONE-1 cells with HMGB1 knockdown (HMGB1-KD) was detected using real-time fluorogenic reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Real-time fluorogenic RT-qPCR analysis of HMGB1. HONE-1 cells were harvested and total RNA was extracted using TRIzol according to the manufacturer's instructions. Total RNA was used for cDNA synthesis of HMGB1 and GAPDH by reverse transcription using an ABI-7300 real-time PCR apparatus (Applied Biosystems, Thermo Fisher Scientific). All mRNA primers were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by JRDun Biotech. Primers used in for PCR are listed in Table I. qPCR was performed according to the manufacturer's instructions of the quantitative real-time RT-PCR reaction kit (SYBR Green; Thermo Fisher Scientific). Thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 45 sec. Gene expression levels were quantified using the $\Delta\Delta C_t$ method.

Table I. Primers used for quantitative polymerase chain reaction analysis.

Gene	Primer sequence	Size (bp)
HMGB1	F: 5'-TGATGTTGCGAAGAACTG-3'	134
	R: 5'-GCTTTCCTTTAGCTCGATATG-3'	
GAPDH	F: 5'-CACCCACTCCTCCACCTTTG-3'	110
	R: 5'-CCACCACCCTGTTGCTGTAG-3'	

F, forward primer; R, reverse primer.

Western blot analysis. HONE-1 cells were harvested and total protein was extracted using radioimmunoprecipitation acid lysis buffer (JRDUN Biotechnology Co., Ltd., Shanghai, China). The protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (20 μ g) were separated by 10 or 15% SDS/PAGE, electrotransferred onto a nitrocellulose filter membrane and probed with various primary antibodies overnight at 4°C, followed by detection with horseradish-peroxidase-conjugated secondary antibodies and electrochemiluminescence development (Millipore) with X-ray film (Kodak, Rochester, NY, USA) exposure. To normalize for protein loading, antibodies directed against GAPDH were used, and the proteins expression levels were expressed as a relative value to that of GAPDH.

CCK-8 assay. Cells (5x10³/100 μ l) were seeded in 96-well plates and cultured for 0, 12, 24 or 48 h at 37°C. Subsequently, 100 μ l serum-free DMEM containing 10% CCK-8 reagent (v/v) was added to each well, and cells were cultured for 1 h at 37°C. Finally, optical density values (OD) were determined at 450 nm using a 96-well plate reader (Multiskan MK3; Thermo Fisher Scientific) (18).

Flow cytometric apoptosis assay. HONE-1 cells (5x10⁴ per group treated as above) were harvested, washed with PBS and stained using the Annexin V/FITC kit according to the manufacturer's instructions. Apoptosis was detected using a FACScalibur flow cytometer (BD Biosciences). The percentage of cells in early apoptosis was the population of Annexin V-positive and PI-negative cells, while the percentage of cells in late apoptosis was resembled by Annexin V-positive and PI-positive cells (19).

Wound-healing assay. A wound-healing assay was performed according to a previously reported method with minor modifications (20,21). HONE-1 cells were treated with control lentivirus (Mock group) or HMGB1 siRNA lentivirus (HMGB1-KD), and seeded onto 35-mm² petri dishes, and a sterile pipette (200 μ l) was used to scratch the cell monolayer to generate a line-shaped wound. Cells were washed once with cold PBS, and incubated for 48 h. Migration of the cells was evaluated at 0, 24, 48 and 72 h.

Cell adhesion assay. A cell adhesion assay was performed in 12-well plates according to a previous method with minor modification (22,23). The wells were pre-coated with

Table II. Effect of HMGB1 knockdown for different time periods on the proliferation of HONE-1 cells.

Group	0 h	12 h	24 h	48 h
HONE-1	0.224±0.0042	0.304±0.0047	0.447±0.0035	0.703±0.006
MOCK	0.220±0.0025	0.307±0.0038	0.448±0.0032	0.700±0.004
HMGB1-KD	0.209±0.0026	0.262±0.0038 ^{a,b}	0.346±0.0046 ^{a,b}	0.451±0.006 ^{a,b}

Values are expressed as the mean ± standard deviation (n=3). ^aP<0.01, compared to HONE-1 group; ^bP<0.01, compared to MOCK group. Groups: HONE-1, untreated cells; MOCK, cells treated with control vector; HMGB1-KD, HMGB1-knockdown HONE-1 cells. HMGB1, high-mobility group box 1.

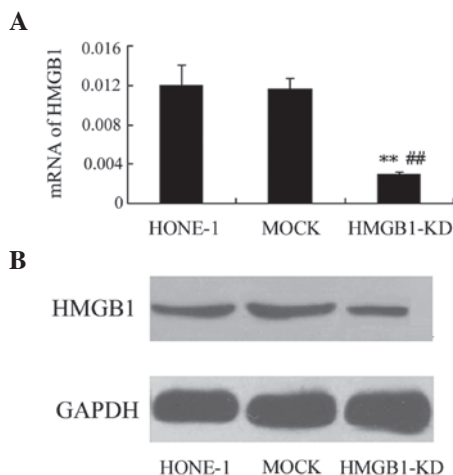


Figure 1. Expression of HMGB1 in HONE-1 cells after transient transfection with plasmid for the expression of HMGB1-targeting small interfering RNA. (A) mRNA expression of HMGB1 as determined by reverse-transcription quantitative polymerase chain reaction. Values are expressed as the mean ± standard deviation. ^{**}P<0.01, compared to HONE-1 group; ^{##}P<0.01, compared to MOCK group. (B) Protein expression of HMGB1 determined by western blot analysis. Groups: HONE-1, untreated cells; MOCK, cells treated with control vector; HMGB1-KD, HMGB1-knockdown HONE-1 cells. HMGB1, high-mobility group box 1.

fibronectin (BD Biosciences, Franklin Lakes, NJ, USA) overnight at room temperature. HONE-1 cells were harvested and re-suspended in DMEM containing 10% FBS. Subsequently 1×10^5 cells were seeded into each well and incubated at 37°C for 1 h. The wells were washed twice with warm PBS to remove the unattached cells, and the attached cells were then stained with Giemsa for 10 min. The cells were observed by using an optical Olympus IX50 microscope (Olympus, Tokyo, Japan).

In vitro invasion assay. The invasive abilities of HONE-1 cells were evaluated according to a previously described method with minor modifications (24). Cell invasion assays were performed using Transwell culture chambers, which were pre-coated with 80 ml Matrigel. The coated filters were thoroughly washed with PBS and dried immediately prior to use. 0.75 ml DMEM containing 10% FBS was placed in the lower chamber, while 0.5 ml cell suspension (1×10^5 /ml) in DMEM containing 1% FBS was placed in the upper chamber followed by incubation for 48 h at 37°C in an atmosphere containing 5% CO₂. The number of the cells which had transgressed

through the Matrigel-coated filter was evaluated by counting cells stained with 0.5% crystal violet solution under an optical microscope (Olympus, Tokyo Japan).

Statistical analysis. Values are expressed as the mean ± standard deviation, and statistical analyses were performed via a one-way analysis of variance followed by post-hoc Dunnett's test. SPSS software version 18.0 (International Business Machines, Armonk, NY, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference between values.

Results

Knockdown of HMGB1 in HONE-1 cells. As shown in Fig. 1A, the expression of HMGB1 was significantly down-regulated in HONE-1 cells transfected with HMGB1 siRNA expression plasmid, compared to that in the untreated and mock-transfected groups (P<0.01). Furthermore, western blot analysis demonstrated that the protein expression of HMGB1 was obviously decreased after transfection, which indicated the successful construction of HONE-1 cells with HMGB1 knockdown (Fig. 1B).

HMGB1 knockdown reduces the proliferation of HONE-1 cells. HONE-1 cell proliferation was detected using the CCK-8 assay after transfection. As shown in Table II, no significant difference in proliferation was observed between the untreated and mock-transfected HONE-1 cells (P>0.05). However, the proliferation of HONE-1 cells with HMGB1 knockdown was significantly inhibited at 12, 24 and 48 h (P<0.01), compared to that of the untreated and mock-transfected HONE-1 cells. Therefore, the results of the present study indicated that HMGB1 knockdown significantly inhibited the proliferation of HONE-1 cells.

HMGB1 knockdown induces apoptosis of HONE-1 cells. To assess whether the anti-proliferative effects of HMGB1 knockdown on HONE-1 cells were due to induction of apoptosis, flow cytometric analysis following staining with PI and Annexin V-FITC was performed. As shown in Fig. 2, the apoptotic rate in the HMGB1-knockdown group was 42.4%, which was significantly higher than that in untreated (6.2%) and in the mock-transfected HONE-1 cells (7.8%). This result revealed that HMGB1 knockdown significantly induced apoptosis in HONE-1 cells.

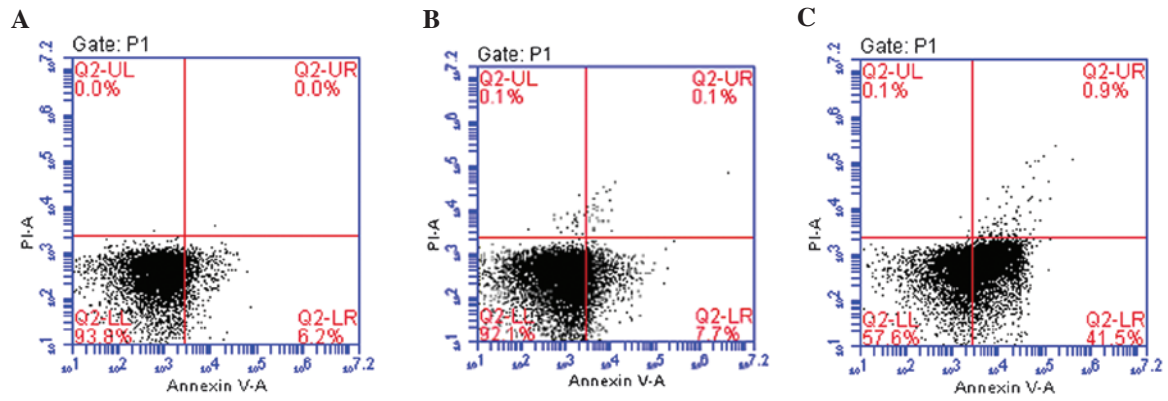


Figure 2. Apoptosis of HONE-1 cells determined by flow cytometric analysis. (A) Untreated HONE-1 cells, (B) cells transfected with control vector and (C) HONE-1 cells with high-mobility group box 1 knockdown. PI, prodidium iodide; Q, quadrant; UL, upper left; LR, lower right.

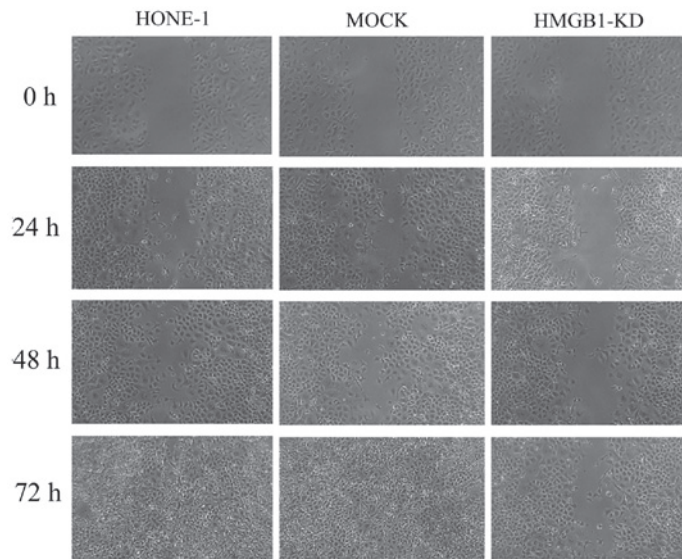


Figure 3. Wound healing assay. Migration of HONE-1 cells was evaluated at 0, 24, 48 and 72 h. Images are representative of at least three independent experiments. Magnification, x200. Groups: HONE-1, untreated cells; MOCK, cells treated with control vector; HMGB1-KD, HMGB1-knockdown HONE-1 cells. HMGB1, high-mobility group box 1.

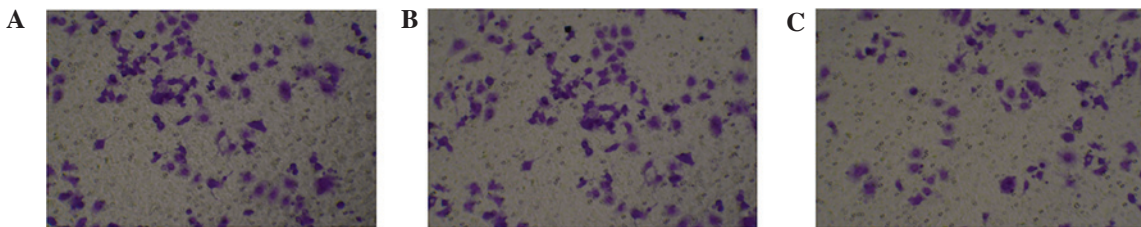


Figure 4. *In vitro* cell invasion assay. (A) Untreated HONE-1 cells, (B) cells transfected with control vector and (C) HONE-1 cells with high-mobility group box 1 knockdown. Cells were stained with crystal violet. Images are representative of at least three independent experiments. Magnification, x200.

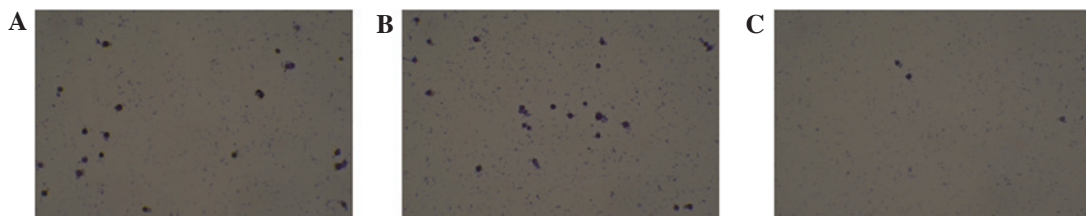


Figure 5. Cell adhesion assay. (A) Untreated HONE-1 cells, (B) cells transfected with control vector and (C) HONE-1 cells with high-mobility group box 1 knockdown. Cells were stained with Giemsa. Images are representative of at least three independent experiments. Magnification, x200.

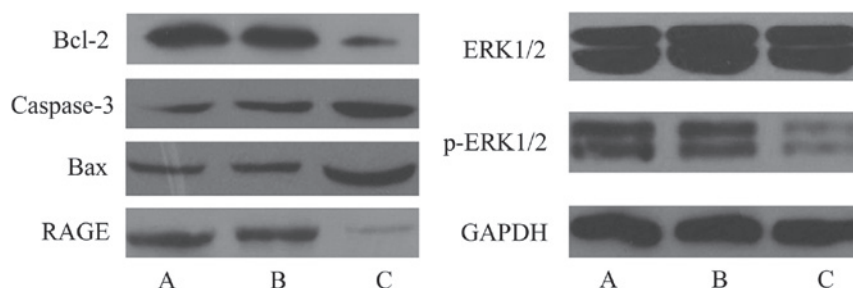


Figure 6. Western blot analysis of apoptosis-associated proteins in HONE-1 cells. Groups: A, untreated cells; B, cells treated with control vector; C, high-mobility group box 1-knockdown HONE-1 cells. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; p-ERK, phosphorylated extracellular signal-regulated kinase; RAGE, receptor for advanced glycation end products.

HMGB1 knockdown reduces the migratory, invasive and adhesive capacities of HONE-1 cells. As demonstrated by the wound healing assay, HMGB1 knockdown effectively inhibited the migration of HONE-1 cells at 24, 48 and 72 h (Fig. 3). In the untreated and mock-transfected HONE-1 cells, the wounds were obviously smaller than those in the HMGB1 knockdown group at all of the observed time-points and, by contrast to the HMGB1 knockdown group, the wounds had disappeared in the untreated and mock-transfected groups after 72 h. Furthermore, as indicated by the Transwell assay, HMGB1 knockdown effectively inhibited the invasion of HONE-1 cells (Fig. 4). As shown in Fig. 5, there was no obvious difference in cell adhesion between the untreated and mock-transfected HONE-1 cells. However, in the HMGB1 knockdown group, cell adhesion was significantly inhibited compared to that in the untreated and mock-transfected HONE-1 cells. The combined results of the wound healing, Transwell invasion and cell adhesion assay indicated that HMGB1 knockdown significantly suppressed the metastasis-forming capacity of HONE-1 cells.

HMGB1 knockdown enhances apoptosis signaling in HONE-1 cells. The results of the present study indicated that HMGB1 knockdown inhibited the proliferation, migration and invasion of HONE-1 cells, while enhancing their apoptotic rate. To investigate the possible underlying mechanisms, the expression of apoptosis-associated signaling and effector proteins was determined by western blot analysis. As shown in Fig. 6, there was no obviously difference in the expression of Bcl-2, Bax, cleaved caspase-3, RAGE, p-ERK1/2 and ERK1/2 between the untreated and mock-transfected HONE-1 cells. Western blotting was repeated three times and similar results were obtained. However, in the HMGB1-knockdown group, the expression of Bcl-2, RAGE and p-ERK1/2 was down-regulated, whereas the expression of caspase-3 and Bax was obviously upregulated compared to that in the untreated and mock-transfected HONE-1 cells. These results indicated that the mechanisms of the inhibitory effects of HMGB1 knockdown on HONE-1 cells may be associated with the induction of apoptosis via the HMGB1/RAGE pathway.

Discussion

To the best of our knowledge, the present study was the first to indicate that HMGB1 knockdown inhibits the proliferation, migration and invasion of HONE-1 human nasopharyngeal

carcinoma cells. In addition, the present study also suggested that the underlying mechanisms of these inhibitory effects may be mediated via induction of apoptosis and the HMGB1/RAGE pathway. Previous studies have demonstrated that HMGB1 is able to promote tumor growth, migration and invasion, and that overexpression of HMGB1 is commonly observed in various types of malignant tumor tissue (13,15). Therefore, downregulation of HMGB1 may be an obvious and feasible approach for the treatment of cancer, including NPC. Lentiviral vectors, which are among the most commonly used vectors in gene therapy, are able to effectively transfect cells for the establishment of stably transfected cell lines (25). In the present study, HMGB1 knockdown was successfully performed in HONE-1 cells by using the lentiviral transfection method.

In the present study, in order to determine the proliferation of HONE-1 cells, a CCK-8 assay was performed. The CCK-8 assay is currently considered to be an advantageous alternative to the MTT assay. The results of the present study indicated that HMGB1 knockdown significantly inhibited the proliferation of HONE-1 cells (18). Furthermore, flow cytometric analysis demonstrated that the induction of apoptosis may be the underlying reason for the anti-proliferative effects of HMGB1 knockdown on HONE-1 cells. A previous study reported that overexpression of HMGB1 suppressed apoptosis of cancer cells via inhibiting the activation of caspase-3 and -9 proteins (26). It is well known that caspase proteins, cysteine-aspartic acid proteases, are the crucial executioners of apoptosis. Caspase-3, one of the key cell death proteases, is one of the most commonly activated cysteine protease in the early stage of apoptosis, and is also considered as a marker for cells undergoing apoptosis (27). Furthermore, Bcl-2 family proteins also serve important roles in regulating intrinsic apoptosis mediated by mitochondria and governing outer mitochondrial membrane permeability and release of cytochrome C (28,29). Bcl-2 is a well-known anti-apoptotic protein, whereas Bax is a pro-apoptotic protein, with the ratio of Bcl-2 to Bax having a crucial role in apoptosis induction (30). It has been reported that HMGB1 was able to inhibit of Bax and suppress apoptosis induced by Bax in mammalian cells (31). The present study demonstrated that HMGB1 knockdown was able to downregulate the expression of Bcl-2, while upregulating the expression of caspase-3 and Bax. These results lead to the conclusion that HMGB1 knockdown promotes the induction of mitochondria-mediated apoptosis.

In the early stages of tumor metastasis formation, tumor cell invasion and reduction of the extracellular matrix (ECM) are

crucial steps, while tumor cell adhesion is another key stage in metastasis formation (32). The present study demonstrated that HMGB1 knockdown markedly inhibited the invasion of HONE-1 cells *in vitro*; in addition, it was demonstrated that HMGB1 knockdown obviously suppressed the adhesion of HONE-1 cells to fibronectin. HMGB1 and RAGE can promote cell proliferation as well as migration of malignant tumors (15). In addition, the proteolytic degradation of the ECM is a critical step during tumor invasion, and the activation of HMGB1/RAGE was shown to increase fibrinolysin (24,33). Previous studies further indicated that HMGB1 can bind to RAGE in the cell membrane and then induce the activation of endocellular RAGE via through activation of ERK; in addition, the HMGB1/RAGE pathway stimulates bioenergetics in a p-ERK1/2-dependent process to sustain the enhanced tumor cell growth and the resulting increased metabolic requirement of the tumor (15,34,35). The results of the present study showed that HMGB1 knockdown downregulate the expression of RAGE and p-ERK1/2, which indicated that HMGB1 knockdown inhibited the activation of HMGB1/RAGE pathways.

In conclusion, the present study demonstrated that HMGB1 knockdown suppressed the proliferation, migration and invasion of the HONE-1 human nasopharyngeal carcinoma cell line, and the possible underlying mechanisms may involve the induction of mitochondria-mediated apoptosis and inhibition of HMGB1/RAGE pathways.

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