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Original Article

Down-regulation of HMGB1 induces apoptosis and inhibits invasion and migration of MCF-7 breast cancer cells through targeting SMARCC1

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Abstract: High-mobility group box 1 (HMGB1) is a multifunctional protein with intranuclear and extracellular functions associated with most cancers including breast cancer. To investigate the effects of down-regulation of HMGB1 expression, MCF-7 breast cancer cells were infected with HMGB1-specific RNAi lentiviral vectors. The gene silencing effects on HMGB1 expression were subsequently confirmed by RT-PCR and western blotting analyses. HMGB1-specific silencing significantly decreased cell proliferation. The impact on proliferation was observed at the cell cycle level-the number of cells in the G2/M phase increased, whereas that in G0/G1 phases decreased. Down-regulation of HMGB1 expression also increased apoptosis of MCF-7 cells, Bax/Bcl-2 ratio and Caspase-3 expression. Finally, silencing of HMGB1 expression significantly resulted in the inhibition of cellular metastatic ability and MMP-9 expression. However, these effects of HMGB1 down-regulation on proliferation, apoptosis, cell cycle and metastasis of MCF-7 cells were significantly inhibited by SMARCC1 overexpression. These findings suggest that HMGB1 is critical for the cellular survival and metastasis of breast cancer and a mechanism referred possibly through targeting SMARCC1, therefore presenting a potential therapeutic target for the treatment of breast cancer.

Keywords: Breast cancer, HMGB1, SMARCC1, apoptosis, metastasis

Introduction

Breast cancer is the common malignancy and the leading cause of cancer-related death in women worldwide with approximately 1.67 million new cases in 2012 [1], which makes it the second most frequent cancer in the world after lung cancer. It is estimated that up to 56% of patients with advanced breast cancer undergo recurrence in distant organs [2, 3], including bones, lungs and brain, and is often accompanied by distant metastasis, contributing to more than 90% of death in breast cancer [4]. Although current treatments for breast cancer include radiation, chemotherapy, hormone therapy and targeted HER2 therapy is effective [5, 6], but unfortunately treatment is stopped due to drug toxicity and drug resistance by many mechanisms including interruption of the apoptotic signaling pathway [7, 8]. Thus the need for effective, safe and cost-effective alternatives is necessary.

High-mobility group box 1 (HMGB1) is a nonhistone chromosomal protein present in all mammals and belonging to the HMG protein family. HMGB1 is a multifunctional factor, implicated in a variety of biologically diverse processes including DNA repair, recombination, replication, transcription, differentiation, development and extracellular signaling [9, 10]. Variations in HMGB1 levels are shown to be associated with several disease conditions including arthritis [11], sepsis [12], Alzheimer's disease [13], ischemia-reperfusion injury [14] and cancer [15]. HMGB1 has a complex role in carcinogenesis because it has both pro- and anti-tumorigenic bioactivities. Down-regulation of HMGB1 inhibited cell proliferation, migration and invasion and induced apoptosis and cell cycle arrest in a number of cancer, including glioma [16], prostate [17], breast [18], gastric [19], bladder cancer [20]. HMGB1 suppresses tumorigenesis by interacting with p53, p73, the retinoblastoma (RB) protein, members of the Rel/NF-κB family

and nuclear hormone receptors including the estrogen receptor (ER) stimulates carcinogenesis [1, 21]. These findings indicated that HMGB1 might be a new potential therapeutic target for the treatment of human malignancies. However, the mechanism of HMGB1 involved in breast cancer apoptosis and migration is still unknown.

SMARCC1 (BAF155) protein, a part of the intranuclear SWI/SNF complex, is a major complex of ATP-dependent chromatin remodeling factors which contribute to the regulation of gene expression by altering chromatin structure [22]. SMARCC1 expression is significantly up-regulated in prostate cancer cells and correlates positively with Gleason score, tumor stage and time to recurrence [23]. Moreover, knockdown of endogenous SMARCC1 in breast cancer cells significantly decreased cell growth and migration, an effect that was rescued by re-expression of SMARCC1 [24]. A number of elements that can influence the activity of SWI/SNF complex have been described, including HMG proteins among which HMGB1 enhances binding of SWI/SNF to the nucleosome [25]. It is currently unknown whether SMARCC1 associated with the outcomes of HMGB1-induced cell apoptosis and migration in breast cancer.

In this study, we attempted to explore the effects of HMGB1 gene silence on the proliferation, cell cycle distribution, apoptosis, migration and invasion of breast cancer MCF-7 cells and clarify a role for SMARCC1 on its possible mechanisms involved in.

Materials and methods

Cell culture

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS, Hyclone, USA), 100 mg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified 5% CO_2 atmosphere.

Production of lentiviral vector

shRNA targeting HMGB1 mRNA (shRNA, 5'-TGGTGATGTTGCGAAGAAA-3'), a non-specific scramble shRNA sequence (NC, 5'-CCTAAGGTTAAGTCGCCCTCG-3') and SMARCC1 coding sequence obtained from Shanghai Generay Biotech Co., Ltd. (Shanghai, China) were cloned into lentiviral vector (pCMV-G&NR-U6, Shanghai Genechem Co., Ltd., Shanghai, China), respectively. The constructs were then transfected into HEK293T cells (ATCC) with lentiviral packaging vectors by using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. After 48 h transfection, MCF-7 cells were infected specific or negative control lentiviral vectors at one multiplicity of infection. The cells were analyzed 48 h after infection.

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. The first-strand cDNA was synthesized using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (RT-qPCR) was performed using SYBR green master mix (Roche Diagnostics GmbH, Mannheim, Germany)on ABI7300 (Applied Biosystem, Shanghai, China) thermal cycler. The relative level of HMGB1 gene was calculated by comparing threshold cycle (Ct) values of samples to that of the reference. All data were normalized to standard GAPDH. Primers for HMGB1 and SMARCC1 were as follows: HMGB1 forward 5'-TGATGTTGCGAAGAAACTG-3', and reverse 5'-GCTTTCCTTTAGCTCGATATG-3'; SMARCC1 forward 5'-TCATGCGGATGCTCCTACCA-3', and reverse 5'-AAACCTCCGCCATCCCTGTT-3'. The GAPDH primers used were forward 5'-CACCCACTCCTCCACCTTTG-3', and reverse 5'-CCACCACCCTGTTGCTGTAG-3'.

Western blot analysis

MCF-7 cells were harvest and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rcf for 10 min at 4°C and the supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies specific for HMGB1, SMARCC1, Bax, Bcl-2, Caspase-3,

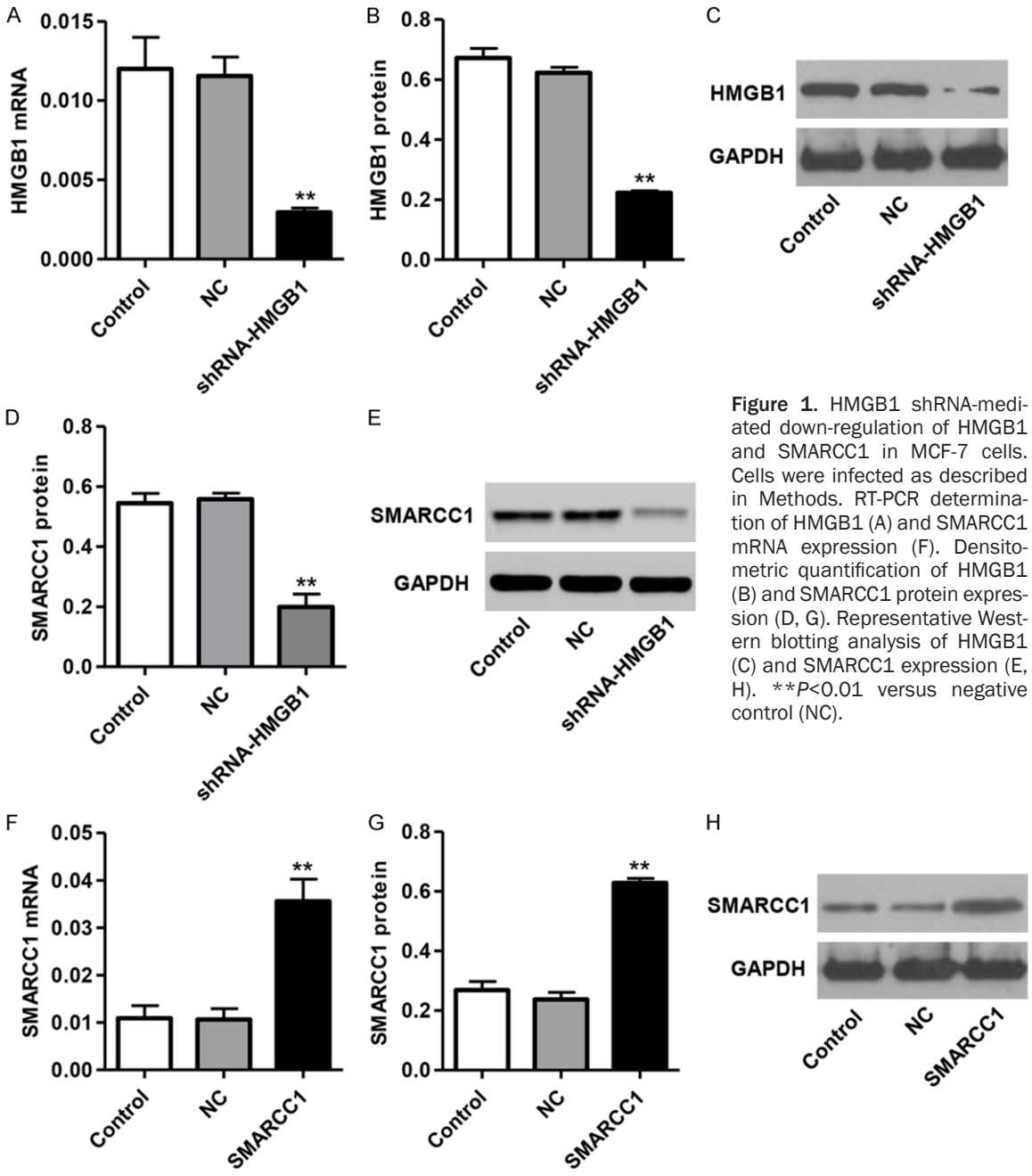

Figure 1. HMGB1 shRNA-mediated down-regulation of HMGB1 and SMARCC1 in MCF-7 cells. Cells were infected as described in Methods. RT-PCR determination of HMGB1 (A) and SMARCC1 mRNA expression (F). Densitometric quantification of HMGB1 (B) and SMARCC1 protein expression (D, G). Representative Western blotting analysis of HMGB1 (C) and SMARCC1 expression (E, H). $^{**}P<0.01$ versus negative control (NC).

MMP-9, E-cadherin and GAPDH overnight at 4°C. Then the blots were incubation with HRP-conjugated anti-rabbit IgG for 1 h, respectively. Immunoreactive bands were detected using an ECL detection kit, and an LAS-4000 mini system (Fujifilm Corporation, Tokyo, Kumamoto, Japan) was used for visualization.

Cell proliferation by CCK-8 assay

Cell viability was determined using a CCK-8 assay (SAB, Nanjing, China). Briefly, cells (3×10^3/well) were seeded in 96-well plates and cultured for 0, 12, 24 and 48 h at 37°C, respectively. After that, 100 μl serum free DMEM containing 10% CCK-8 reagents (v/v) was added in each well, and cells were cultured for 1 h in 5% CO_2 at 37°C. Finally, optical density values (OD) were read at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was done in triplicate.

Cell cycle distribution analysis

MCF-7 cells (3×10^4/well) were digested and washed, followed by 70% pre-cooled ethanol

for 12 h and RNA was removed by using 1 mg/ml RNaseA. Then cells were stained with propidium iodide (PI) for 10 min and the DNA content was measured using flow cytometry so as to determine the proportion of cells in each stage of cell cycle.

Cell apoptosis assay

MCF-7 cells (3×10^4/well) were formed single cell by 0.25% trypsin and washed by 10% PBS, followed by the centrifugation at 1000 RCF for 5 min. Supernatant was discarded and then the cells were incubated with Annexin-V fluorescein isothiocyanate (FITC) apoptosis detection Kit (eBioscience, Inc., San Diego, CA, USA) for 10 min at room temperature without light. Cell apoptotic rate was measured and the data was obtained using flow cytometry (BD Biosciences, San Diego, CA, USA).

Migration and invasion assay

Migration and invasion assays were performed in uncoated or Matrigel (BD Biosciences)-coated 24-well Transwell chamber (8-μm pore size, Corning Life Sciences, Corning, NY, USA). Briefly, MCF-7 cells (3×10^4/well) in 200 μl serum-free medium were seeded into the upper chamber, while 600 μl medium with 10% FBS were added into the lower chamber. After 24 h of incubation at 37°C and 5% CO_2, cells remaining in the upper chamber were completely removed using a cotton swab. Cells attached to the bottom of the membranes were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. The degree of migration/invasion was expressed as the average number of cells counted in five randomly selected filed.

Statistical analysis

All data are presented as means ± SD. Statistical significance was performed by using the GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and determined by Student's t test. $P<0.05$ was considered significant.

Results

Down-regulation of HMGB1 decreased SMARCC1 expression in MCF-7 cells

To investigate the effects of HMGB1 expression on breast cancer cell biological function, we infected MCF-7 cells with shRNA targeting HMGB1 plasmid vector. Infection with the HMGB1 shRNA lentiviral vector efficiently and specifically reduced the HMGB1 expression by 75.4% and 66.9% at mRNA and protein levels in MCF-7 cells compared with that of negative control (NC), respectively (**Figure 1A-C**). Interestingly, down-regulation of HMGB1 showed significant decrease in the protein expression of SMARCC1 in MCF-7 cells (**Figure 1D** and **1E**), suggesting an important link between HMGB1 and SMARCC1 was presence. Then, SMARCC1 was therefore overexpressed in HMGB1 silenced MCF-7 cells. As shown in **Figure 1F-H**, infection with the SMARCC1 expressing vector efficiently and specifically increased the SMARCC1 expression by 2.27-fold and 1.34-fold at mRNA and protein levels in MCF-7 cells compared with that of NC, respectively.

Down-regulation of HMGB1 decreased cell viability and induced cell cycle arrest of MCF-7 cells

Following knock down of HMGB1 gene with shRNA, we also measured the cell viability in infected MCF-7 cells. These studies showed that knock down of HMGB1 resulted in decreased proliferation of MCF-7 cells by 14.4%, 22.8% and 35.6% at 12, 24 and 48 h, respectively, compared with cells that were infected with a NC plasmid (**Figure 2A**). However, SMARCC1 up-regulation markedly increased the cell viability of HMGB1-silencing MCF-7 cells by 10.0%, 15.6% and 25.4% at 12, 24 and 48 h, respectively (**Figure 2A**). These data suggest that SMARCC1 overexpression inhibited HMGB1 silence induced decrease in MCF-7 cell viability.

Moreover, we also investigate the effect of HMGB1 shRNA lentiviral vector on cell cycle distribution of MCF-7 cells. As shown in **Figure 2B** and **2C**, HMGB1 shRNA lentiviral vector infection in MCF-7 cells showed decreased cell number of G0-G1 phase, but cell number of G2-M phase was increased significantly. HMGB1 shRNA lentiviral vector infection didn't influence the cell number of S phase in MCF-7 cells. Importantly, SMARCC1 up-regulation markedly inhibited cell cycle arrest through increasing cell number of G0-G1 phase and decreasing that of G2-M phase (**Figure 2B** and **2C**). These data suggest that SMARCC1 overexpression inhibited HMGB1 silence induced MCF-7 cell cycle arrest.

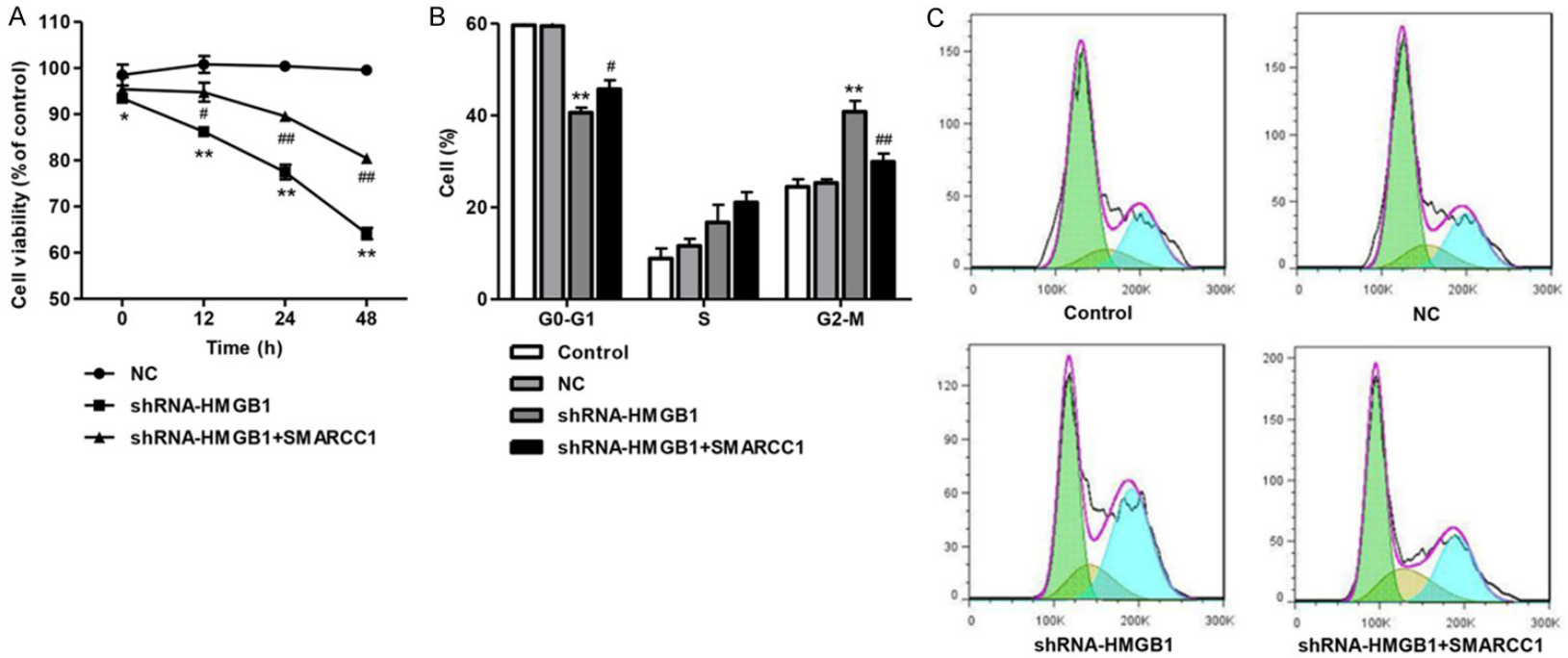

Figure 2. HMGB1 gene silencing reduces MCF-7 cell viability and arrests cell cycle. A. The viability of infected MCF-7 cells was measured by CCK-8 assay on 0, 12, 24 and 48 h post-infected. B, C. Cell cycle was measured by flow cytometry. **P*<0.05, ***P*<0.01 versus negative control (NC). #*P*<0.05, ##*P*<0.01 versus shRNA-HMGB1.

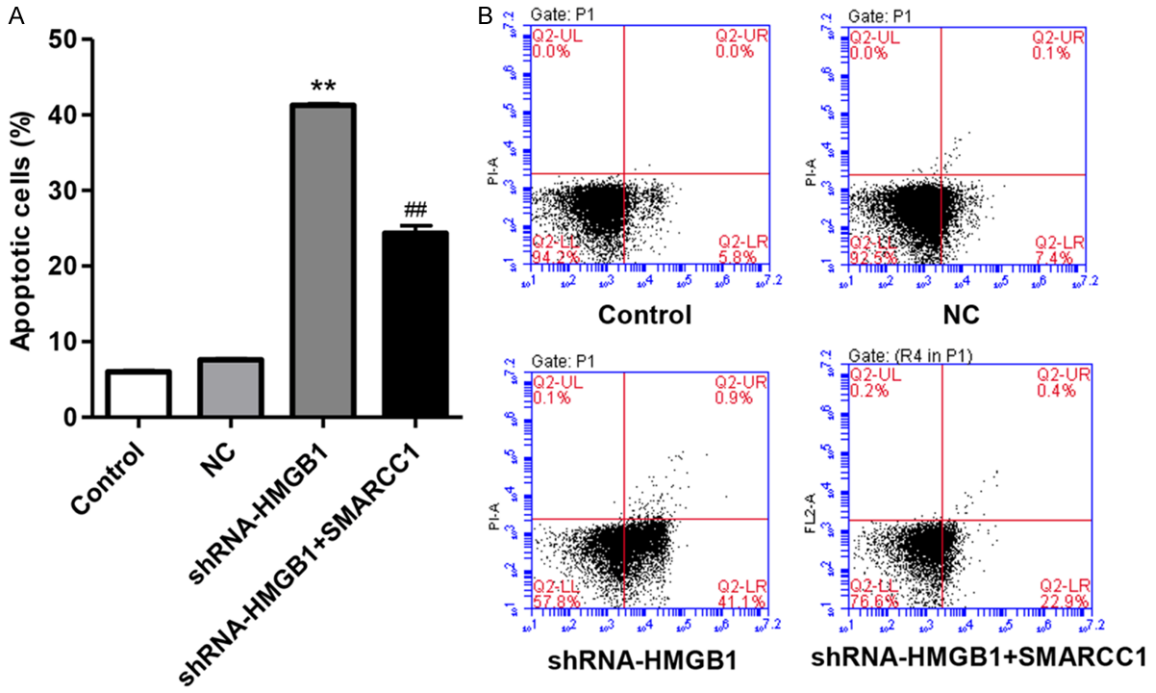

Figure 3. HMGB1 gene silencing induces MCF-7 cell apoptosis. A, B. Cell cycle was measured by flow cytometry. $^{**}P<0.01$ versus negative control (NC). $^{**}P<0.01$ versus negative control (NC). $^{\#\#}P<0.01$ versus shRNA-HMGB1.

Down-regulation of HMGB1 induced cell apoptosis of MCF-7 cells

We then analyzed whether reduction in cell viability was due to cell death and if so whether they are undergoing apoptosis. Results showed that following infection of MCF-7 cells with HMGB1 shRNA constructs resulted in increase in apoptosis of these cells by 5.88-fold compared with NC (**Figure 3A** and **3B**). While, SMARCC1 up-regulation markedly decreased the cell apoptosis of HMGB1-silencing MCF-7 cells by 40.9% (**Figure 3A** and **3B**). These data suggest that SMARCC1 overexpression inhibited HMGB1 silence induced MCF-7 cell apoptosis.

Down-regulation of HMGB1 inhibited cell migration and invasion of MCF-7 cells

To evaluate the effect of HMGB1 inhibition on cell invasion, migration and invasion assays were performed. The results indicated that infection with HMGB1 shRNA lentiviral vector significantly reduced the number of MCF-7 cells that invaded through the membrane. HMGB1 silencing caused a 72.6% and 77.9% decrease in the migratory and invasive cell number when compared with the NC (**Figure 4A-D**). However, SMARCC1 up-regulation markedly increased the cell migration and invasion by 1.71-fold and 1.95-fold in HMGB1-silencing MCF-7 cells, respectively (**Figure 4A-D**). These data suggest that SMARCC1 overexpression inhibited HMGB1 silence induced decrease in MCF-7 cell migration and invasion.

Effects of HMGB1 down-regulation on apoptotic and migratory mediators in MCF-7 cells

As Bcl-2 family and Caspase-3 are important mediators of tumor cell apoptosis, we investigated the impact of gene silencing on Bcl-2, Bax and Caspase-3 expression. Western blot analysis revealed that the expression of Bax and Caspase-3 protein was increased, but Bcl-2 protein was decreased in stable HMGB1-silencing MCF-7 cells compared with NC (**Figure 5A** and **5B**). In addition, the expression of important mediator of tumor cell invasiveness and metastasis, MMP-9 protein, was also decreased in stable HMGB1-silencing MCF-7 cells compared with NC, but E-cadherin protein was increased (**Figure 5A** and **5B**). However, SMARCC1 up-regulation markedly inhibited HMGB1 silence induced protein expression in MCF-7 cells.

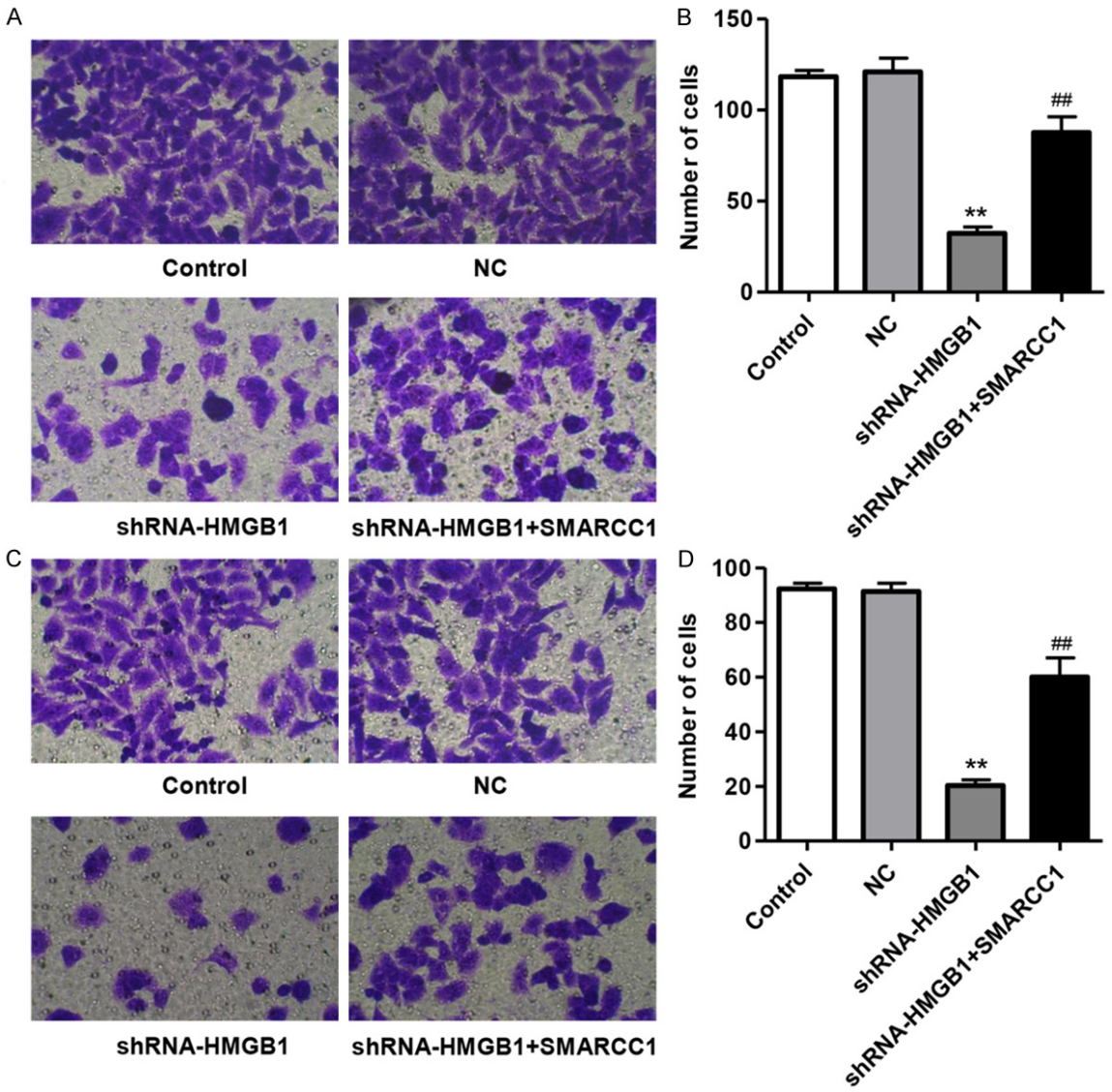

Figure 4. HMGB1 gene silencing reduces MCF-7 cell migration and invasion. A. Representative photographs of cell migration assay. B. Average migratory cell number of MCF-7 cells infected with HMGB1 shRNA lentiviral vector was significantly decreased, as compared to the cells infected with NC. C. Representative photographs of cell invasion assay. D. Average invasive cell number of MCF-7 cells infected with HMGB1 shRNA lentiviral vector was significantly decreased, as compared to the cells infected with NC. $^{**}P<0.01$ versus negative control (NC). $^{\#\#}P<0.01$ versus shRNA-HMGB1.

Discussion

The expression of HMGB1 has been described in many types of cancers [18, 26], with several studies indicating that HMGB1 is overexpressed in breast cancer. Despite these findings, relatively little is known about the function of HMGB1 in breast cancer progression. Here, we report that genetic knockdown of HMGB1 in MCF-7 breast cancer cells significantly inhibited cell proliferation, migration, invasion ability as well as SMARCC1 expression. Moreover, HMGB1 silencing caused elevated apoptosis and cell cycle arrest. However, SMARCC1 overexpression inhibited HMGB1 silencing induced apoptosis and motility in MCF-7 cells. These results suggest that HMGB1 has an essential role in the development and malignant transformation of breast cancer, which may through targeting SMARCC1 expression.

Previous study hasshowed that down-regulation of HMGB1 expression resulted in the inhibition of cell growth in LNCaP prostate cancer

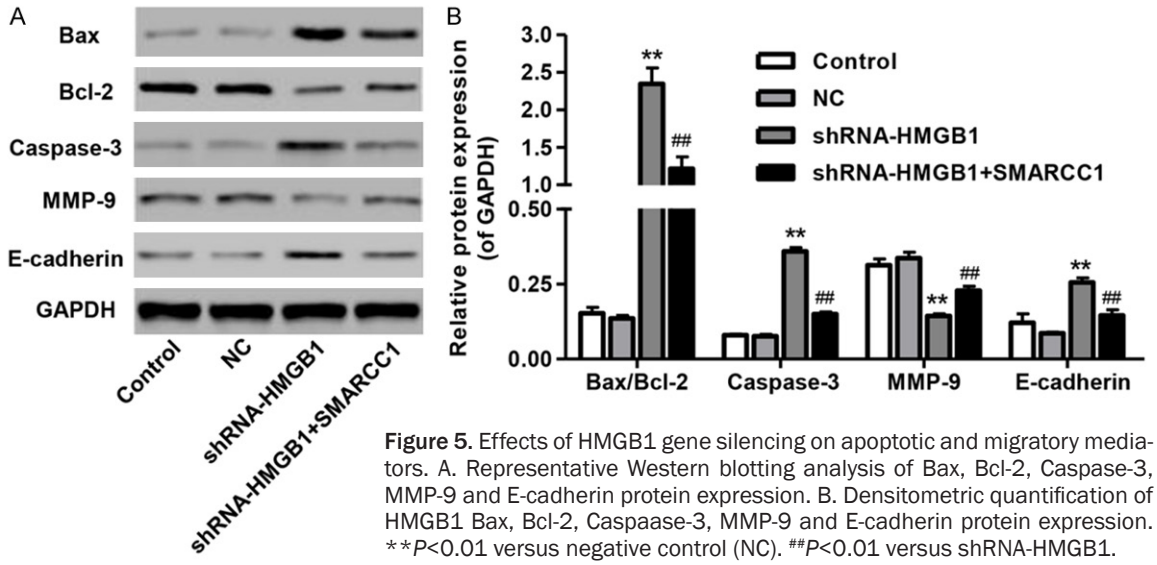

Figure 5. Effects of HMGB1 gene silencing on apoptotic and migratory mediators. A. Representative Western blotting analysis of Bax, Bcl-2, Caspase-3, MMP-9 and E-cadherin protein expression. B. Densitometric quantification of HMGB1 Bax, Bcl-2, Caspaase-3, MMP-9 and E-cadherin protein expression. $^{**}P<0.01$ versus negative control (NC). $^{\#\#}P<0.01$ versus shRNA-HMGB1.

cells and apoptosis via caspase-3-dependent pathways [17]. Attenuating the expression of RAGE and its ligand HMGB1 in human breast adenocarcinoma cells also promoted apoptosis [27]. In line with the findings that down-regulation of HMGB1 significant decrease in cell viability and induction of apoptosis through increasing Caspase-3 expression and Bax/Bcl-2 ratio in MCF-7 cells. Moreover, Ni et al. [18] reported that HMGB1 silence by siRNA didn't inhibit MCF-7 cell proliferation, but promoted apoptosis. However, the most likely explanationis that the shRNA lentiviral vector is more effective than the use of siRNA target HMGB1. HMGB1 silencing significantly decreased cell proliferation and induced increase in the number of cells in the G0/G1 phase, whereas that in S and G2/M phases decreased, which were accompanied by decreases in cyclin D1 expression [19]. However, in the present study the number of cells in the G0/G1 phase was markedly decreased and the number of cells in the G2/M phase was increased in HMGB1 silencing MCF-7 cells. The increased expression of HMGB1 conferred a suppression of growth of MCF-7 cells, G1 arrest and apoptosis in RB-containing MCF-7 cells, and elevated the radiosensitivity of breast cancer cells [21]. These suggest that the role of HMGB1 in breast cancer cells in growth and apoptosis is quite different that dependent on the different cancer types, cancer cells and cancer conditions.

Our studies reveal that the migratory and invasive ability of MCF-7 cells is significantly decreased *in vitro* by HMGB1 silencing, which is consistent with the previous study [28, 29]. Furthermore, we identify that HMGB1 silencing reduces cellular expression of MMP-9 and increased E-cadherin. Liu et al. [30] reported that HMGB1 induced MMP-9 expression via specific inhibition of the PI3K/Akt signaling pathways and that HMGB1 was closely correlated to the clinical stage of advanced non-small cell lung cancer. Our data support these observations, suggesting that HMGB1 expression plays an essential role in the metastatic ability of breastcancer cells.

A small number of studies have analyzed the effect of HMG proteins on ATP-dependent chromatin remodeling [25, 31]. Additionally, it has been recently shown that the activity of the yeast SWI/SNF complex is stimulated by rat HMGB1 [32]. In the present study, we found that SMARCC1, a part of the intranuclear SWI/SNF complex, is associated with HMGB1 silencing induced cell viability, apoptosis, migration and invasion of MCF-7 cells. SMARCC1 overexpression markedly increased cell viability, migration and invasion ability and inhibited cell apoptosis and cell cycle arrest. Conflicting evidence suggests that SMARCC1 may serve as tumor suppressor or oncogene in a tumor-type-specific manner. SMARCC1 was initially implicated as a tumor suppressor in lung and other adenocarcinomas [33]. However, mounting evidence supports that, for at least some tumor types, SMARCC1 might enhance tumor development in a context-dependent manner [24].

In conclusion, our study suggests that HMGB1 plays an essential role in the proliferative and metastatic activity of breast cancer cells. Silencing the HMGB1 gene with lentiviral vector-mediated HMGB1 shRNA can significantly inhibit proliferation, migration and invasion and, in addition, induced apoptosis and cell cycle arrest in MCF-7 cells. These effects were, in partly, through targeting SMARCC1. These data suggest that HMGB1 represents a potential target for the therapeutic intervention of breast cancer.

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Disclosure of conflict of interest

None.

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