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HMGB3 small interfere RNA suppresses mammosphere formation of MDA-MB-231 cells by down-regulating expression of HIF1α

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Abstract. – OBJECTIVE: Breast cancer has been proven as the most common malignancy influencing the health of females. This research aimed to clarify the effects of high-mobility group box 3 (HMGB3)-small interfere RNA (HMGB3-siRNA) on the proliferation of breast cancer cells.

MATERIALS AND METHODS: HMGB3-mimic and HMGB3-siRNA lentiviral vectors were structured. The above lentiviral vectors were then transfected into normal breast cells (MCF10A) and breast cancer cells (MDA-MB-231). Cell counting kit-8 (CCK-8) analysis was employed to assess proliferative viabilities of cells. The formation of the mammosphere in breast cancer cells was examined using mammosphere-forming assay. The mRNA expression of Nanog, Sox2, and OCT-4 genes was evaluated using quantitative real time-PCR (qRT-PCR). CD44 positive/CD24 negative ($CD44^+/CD24^-$) cell levels were evaluated using flow cytometry assay. The correlation between HMGB3 and hypoxia-inducible factor 1α (HIF1α) was analyzed using Linear-Regression analysis. The interaction between HMGB3 and HIF1α expression was determined using the Dual-Luciferase assay.

RESULTS: HMGB3 expression was remarkably enhanced in breast cancer cells compared to that in normal cells ($p<0.05$). HMGB3-siRNA significantly decreased the proliferative activity and remarkably suppressed the mammosphere formation compared to that in single MDA-MB-231 cells ($p<0.05$). HMGB3-siRNA remarkably reduced Nanog, SOX2, and OCT-4 and significantly enhanced $CD44^+/CD24^-$ cells compared to single MDA-MB-231 cells ($p<0.05$). HMGB3-siRNA significantly weakened the expression of HIF1α in MDA-MB-231 cells compared to single MDA-MB-231 cells ($p<0.05$). HMGB3 was positively correlated with HIF1α expression ($p<0.05$). There was an interaction between HMGB expression and HIF1α expression.

CONCLUSIONS: HMGB3 small interfering RNA suppressed the formation of mammosphere in MDA-MB-231 cells by downregulating the expression of HIF1α.

Key Words:
Breast cancer, Proliferative viability, HMGB3, HIF1α, Mammosphere formation.

Introduction

Breast cancer has been proven to be the most frequently occurred malignancy influencing health of females in the whole world[1,2]. In China, breast cancer dominants appropriately 17% of all human cancers and has become the 6th reason for death[3]. In recent years, although the mortality of breast cancer has been remarkably decreased, there are also more than 70,000 deaths every year in China[4]. The metastasis of breast cancer cells to the other organs or tissues, such as lymph nodes and distal tissues, has been proven to be a critical cause for death of patients[5]. Clinically, breast cancer patients, especially the triple-negative breast cancer (TNBC) patients, demonstrate 5-year survival rate of 62% and poor prognosis[6]. Therefore, investigating and discovering the targeting molecules that participate in breast cancer pathogenesis are of great significance to treat cancer cells.

According to the previous studies[7,8], the formation of mammosphere is proven to be a specific biomarker for metastasis and proliferation of breast cancer cells. There are many biomarkers of mammosphere that have been discovered, such as *Nanog, Sox2, OCT-4*, CD44 positive molecule ($CD44^+$) and CD24 negative molecule ($CD24^-$)[9,10]. A former investigation[11] found that the majority of breast cancer patients could form mammospheres and demonstrate evident metastasis. Ma et al[12] also discovered plenty of pheno-typical $CD44^+/CD24^-$ breast cancer cells in TNBC, which promote progression of breast cancer.

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High-mobility group box 3 (HMGB3) involves DNA repair, cellular transcription and development of cancers[13-15]. Moreover, HMGB3 commonly expresses in cancer cells and participates in progression, metastasis, recurrence, and migration of cancers[16,17]. Meanwhile, HMGB3 also involves chemo-resistance and radio-resistance of cancers[16,17]. However, there are no studies[10,16,17] focus on the effects of HMGB3 on breast cancer. Also, the pathological mechanism of HMGB3 in cancers has not been fully clarified. Furthermore, the development of breast cancer has been identified to be associated with over-expression of hypoxia-inducible factor 1α (HIF1α)[18,19]. Meanwhile, the poor prognosis of breast cancer is also related to the high expression of HIF1α[18,19].

Therefore, this study explored the potential effects of HMGB3 and HIF1α on the formation of mammosphere and breast cancer cell proliferation. Moreover, the promising mechanisms of HMGB3 modulated mammosphere formation have also been investigated.

Materials and Methods

Cell Culture

In this research human breast cancer cells (MDA-MB-231 and MCF7) and normal breast cell (MCF-10A) (Cell Bank of China Academy of Sciences, Shanghai, China) were cultured to verify HMGB3 expression. MCF-10A cells were cultured in mammary epithelial cell growth medium (MEGM, Gibco BRL. Co. Ltd., Grand Island, NY, USA). MDA-MB-231 cells were cultured in L-15 medium (Gibco BRL. Co. Ltd., Grand Island, NY, USA). MCF7 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco BRL. Co. Ltd., Grand Island, NY, USA). The above cells were cultured supplementing with 1% penicillin-streptomycin (Beyotime Biotech., Shanghai, China) and 10% fetal bovine serum (FBS; Gibco BRL. Co. Ltd., Grand Island, NY, USA) at 37°C with 5% CO_2.

Construction and Transfection of HMGB3 Mimic and HMGB3 siRNA Plasmids

The pG-LV5 lentiviral and pG-LV3 lentiviral vector (GenePhama Co. Ltd, Shanghai, China) were applied to construct LV5-HMGB3-mimic and LV3-HMGB3-siRNA, basing on the synthesized oligonucleotides (Figure 1A, 1B). All above processes of gene fragment synthesis, DNA clone, and double-chains synthesis were conducted by GenePhama Co. Ltd (Shanghai, China). LV5-HMGB3-mimic and LV3-HMGB3-siRNA plasmids, packing plasmids (PG-P2-REV, PG-p1-VSVG and PG-P3-RRE), and RNAi-mate (GenePhama Co., Ltd, Shanghai, China) were mixed together, according to previous study[20]. Finally, packaged lentiviral LV5-HMGB3-mimic and LV3-HMGB3-siRNA were transfected into MCF-10A, MDA-MB-231 and MCF7 cells, at final multiplicity of infection (MOI) of 15.

Cell Counting Kit 8 (CCK-8) Assay

Proliferation of MCF-10A, MDA-MB-231, and MCF7 cells was measured using CCK-8 Commercial Kit (Sigma-Aldrich, Cat. No. 96992, St. Louis, MO, USA), due to manufacturer's protocol. Briefly, the above cells were seeded onto 96-well plates (Corning, Corning, NY, USA) and cultured at a concentration of 1×10^5 cells/ml medium. Then, LV5-HMGB3-mimic and LV3-HMGB3-siRNA lentivirus were transfected into above cells for 24 h, 48 h, and 72 h. The final dosage of 10 μl/ml CCK-8 was added into the above cells and cultured at 37°C for 4 h. Eventually, the proliferation of cells was assessed with a micro-plate (enzyme linked immunosorbent assay, ELISA) reader (Thermo Fisher Scientific, Hudson, NH, USA) at wavelength of 450 nm.

Quantitative Real-Time RT-PCR (qRT-PCR)

The total RNAs of cells were extracted with TRIzol regents (Beyotime Biotech., Shanghai, China) and complementary DNA (cDNA) was synthesized using Reverse Transcription Kit (Western Biotech., Chongqing, China). The SYBR Green I kit (Western Biotech., Chongqing, China) was applied to qRT-PCR assay for amplifying targeting genes, including *Nanog, Sox2, OCT-4*, and *β-actin*. The whole process of qRT-PCR was conducted using a professional RT-PCR Amplification System purchased from Funglyn Biotechnology (Mode: FTC-3000P, Toronto, Canada). The primers and gene length for the above genes were illustrated in Table I. The amplified gene products were loaded and run onto 1.5% agarose gel (Beyotime Biotech., Shanghai, China). The amplified products in agarose gel were captured with an Image Scanning System (Mode: GDS8000, UVP. Biotech., Sacramento, CA, USA). The relative gene expression (mRNA level) was normalized to gene expression of β-actin according to previously established Comparative-threshold ($2^{-\Delta\Delta CT}$) approach[21].

Evaluation for Formation of Mammosphere

The formation of mammosphere for breast cancer cells was evaluated according to the former reported protocol[10,22]. In brief, the cells grow to about 50% confluence, which was detached with StemPro Accutase (Thermo Fisher Scientific, Waltham, MA, USA). Then, the cells were cultured onto 6-well plates (Corning, Corning, NY, USA) at final concentration of 10000 cells/ml medium containing hydrocortisone and heparin. The images of formed mammospheres were captured. The mammosphere formation was calculated by comparing mammosphere accounts in blank breast cancer cells, due to previous study described[9].

Evaluation for CD44+/CD24- with Flow Cytometry Assay

Accounts of $CD44^+/CD24^-$ expressed breast cancer cells were evaluated with the previously reported document[23]. Briefly, the cells were digested using Trypsin (Beyotime Biotech., Shanghai, China) to form single-cell suspension. The cell suspensions were mixed with allophycocyanin (APC) labeled rabbit anti-human CD24 monoclonal antibody (Cat No. #17-0247-42, eBioscience, Santiago, CA, USA) for 30 min at 4°C in the dark. At the same time, the cell suspensions were also mixed with phycoerythrin (PE) labeled rabbit anti-human CD44 monoclonal antibody (Cat. N. #12-0441-82, eBioscience, Santiago, CA, USA) together for 30 min at 4C° in the dark. Eventually, $CD44^+/CD24^-$ positive breast cancer cells were evaluated using a Flow Cytometer (Mode: FACSCanto II, BD Biosciences, San Jose, CA, USA) within 60 min post PE/APC staining.

Dual-Luciferase Assay

In this study, interaction between HIF1α and HMGB3 expression was determined with Dual-Luciferase assay in the 293T cells (Cell Bank of CAS, Shanghai, China). Briefly, pcDNA3.1-HMGB3 and pGL3-HIF1α-Promoter were transfected into breast cancer cells firstly. Then, the cells were incubated with a Dual-Luciferase reporter assay system (Cat. No. E1910, Promega, Madison, MI, USA) to evaluate the Luciferase activity. The intensity of the Dual-Luciferase experiment produced fluorescence was examined using a micro-plate (ELISA) reader (Mode: MCC/340, Thermo Fisher Scientific, Waltham, MA, USA). Eventually, Luciferase activity was analyzed as the ratio of Firefly Luciferase comparing to *Renilla* Luciferase plasmids (Firefly/*Renilla* Luciferase ratio).

Statistical Analysis

Data were defined as mean±standard deviation (SD) and analyzed using professional SPSS software (version: 20.0, SPSS Inc., IBM Corp., Armonk, NY, USA). The statistical difference between the two groups was analyzed using the Student's *t*-test. Meanwhile, the statistical difference among more than 3 groups was analyzed with analysis of variance (ANOVA) analysis validated by the Tukey's post-hoc test. The Linear Regression Analysis was conducted to evaluate the correlations between HIF1α and HMGB3 expression. The $p<0.05$ was defined as statistical significance.

Table I. Sequences for the RT-PCR assay.

Genes		Sequences	Length (bp)
HMGB3	Forward	ACAGTGAAAAGCAGCCTTACATC	124
	Reverse	CGGGCAACTTTAGCAGGAC	
Nanog	Forward	ATGGATCTGCTTATTCAGGACAG	115
	Reverse	GTTTCTTGACCGGGACCTTG	
Sox2	Forward	AGTGGAAACTTTTGTCGGAGAC	150
	Reverse	GCAGCGTGTACTTATCCTTCTTC	
OCT-4	Forward	AAGGGCAAGCGATCAAGC	166
	Reverse	AAGGGACCGAGGAGTACAGTG	
β-actin	Forward	TGACGTGGACATCCGCAAAG	205
	Reverse	CTGGAAGGTGGACAGCGAGG	

A

Sense: 5'-GATCC-(GN18)-(TTCAAGAGA)-(N18C)-TTTTTG-3'

Anti-sense: 3'-G(CN18) -(AAG TTCTCT)-(N18G)-AAAAAACTTAA-5'

Targeting sequence of siRNA: TGAGAAGGATGTTGCTGACTATA

B

Forward primer: 5'-AGGGTTCCAAGCTTAAGCGGCCGCGCCACCAT GGC AAAGGT GACCCCAAGAAAC-3'

Reverse primer: 5'- GATCCATCCCTAGGTAGATGCATTTA TTCATC CTCCTCCTCCTCCTCCTCCTCT-3'

C

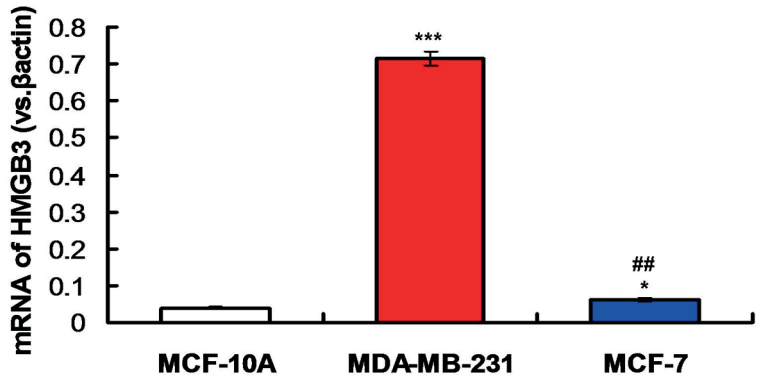

Figure 1. Evaluation for HMGB3 mRNA expression in normal and breast cancer cells. **A,** Oligonucleotides for siRNA. **B,** Primers for amplifying HMGB3-mimic fragments. **C,** Statistical analysis for the HMGB3 mRNA expression in cells. *p<0.05, ***p<0.001 *vs*. MCF-10A cells. $^{\#}p$<0.05 *vs*. MDA-MB-231 cells.

Results

HMGB3 Expression Was Remarkably Enhanced in Breast Cancer Cells

We firstly examined HMGB3 expression in MCF10A cells, basal-like breast cell (MDA-MB-231), and luminal breast cell (MCF7), using qRT-PCR method. Our findings demonstrated that HMBG3 expression in MDA-MB-231 cells (p<0.001) and MCF7 cells (p<0.05), was remarkably higher compared to that in the normal breast cell (MCF10A) (Figure 1C). Furthermore, expression of HMBG3 in MDA-MB-231 cells was also remarkably higher compared to that of MCF7 cells (Figure 1C, p<0.01).

HMGB3 siRNA Decreased Proliferative Activity of MDA-MB-231 Cells

Our results illustrated that at 72 h post HMGB3-mimic treatment, the cell viability of MCF10A cells was significantly increased compared to that in the MCF10A group (Figure 2A, p<0.05). However, at both 48 h and 72 h post HMGB3-siRNA treatment, cell viability of MDA-MB-231 cells was decreased compared with MDA-MB-231 cells (Figure 2A, p<0.05).

HMGB3 siRNA Suppressed Formation of Mammosphere

According to the previous literature[16], mammosphere formation could reflect migration of cancer cells. Therefore, the formation of mammosphere was evaluated in this study. The findings here exhibited that HMGB3-mimic treatment remarkably enhanced mammosphere amounts compared to that in MCF10A cells, at 3rd day, 5th day, and 7th day (Figure 2B, p<0.05). However, HMGB3-siRNA remarkably reduced mammosphere amounts comparing with that in the MDA-MB-231 cells (Figure 2C, p<0.05).

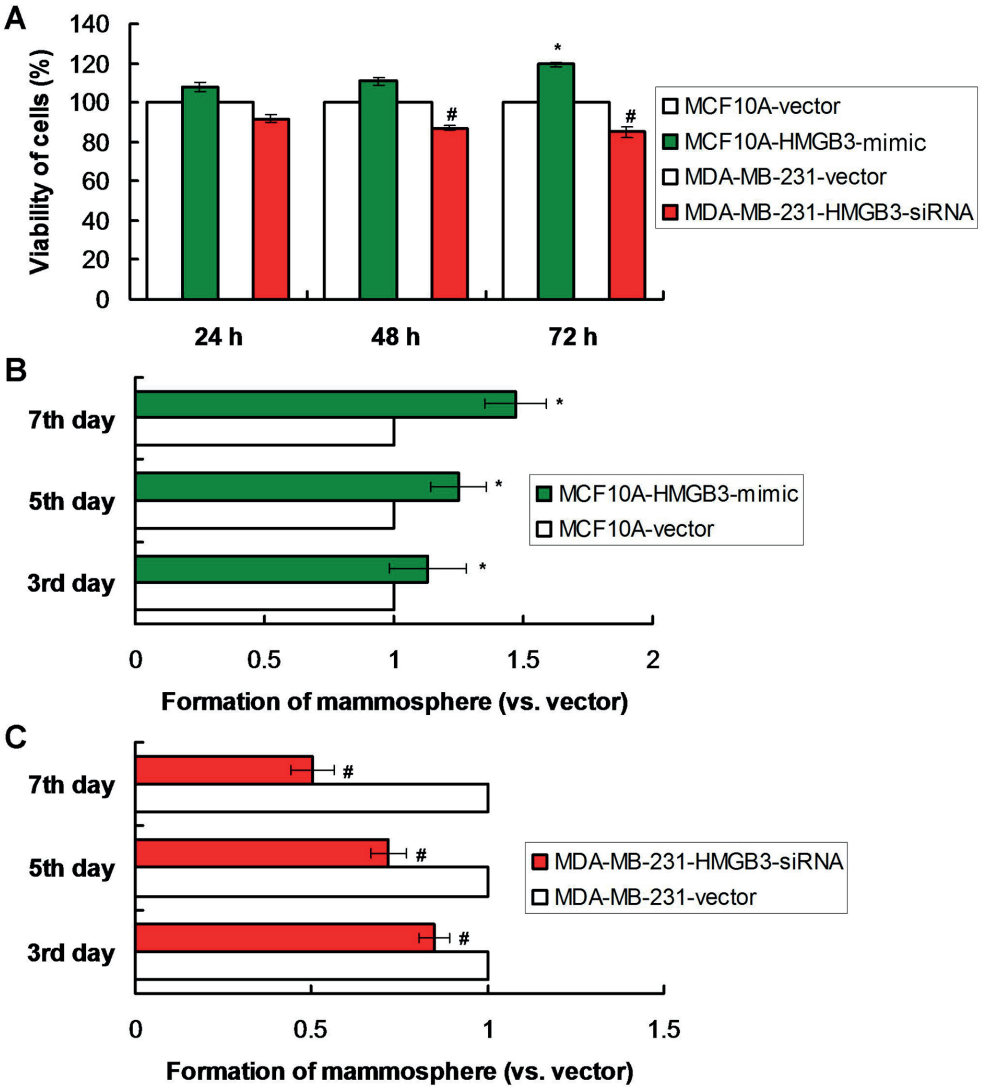

Figure 2. Determination of HMGB3-mimic and HMGB-siRNA effects on cell proliferative viability and mammosphere formation. **A,** Statistical analysis for cell proliferative viability in HMGB-mimic or HMGB3-siRNA administrated cells. **B,** Statistical analysis of mammosphere formation in HMGB3-mimc administrated MCF10A cells. **C,** Statistical analysis of the HMGB3-siRNA administrated MDA-MB-231 cells. *p<0.05 vs. MCF-10A cells. #p<0.05 *vs.* MDA-MB-231 cells.

HMGB3 siRNA Reduced Nanog, SOX2, and OCT-4 mRNA Levels in MDA-MB-231 Cells

Cancer mammosphere formation associated biomarkers[24] in induced pluripotent stem cells (iPSCs), including *Sox2, Oct-4,* and *Nanog*, were examined. The results illustrated that mRNA levels o*f Nanog* (Figure 3A), *SOX2* (Figure 3B), and *OCT-4* (Figure 3C) were increased in the HMGB3-mimic treated MCF10A group compared to that in the MCF10A-vector group (p<0.05). Moreover, mRNA levels o*f Nanog* (Figure 3A), *SOX2* (Figure 3B), and *OCT-4* (Figure 3C) in HMGB3-siRNA treated MDA-MB-231 cells were remarkably reduced compared to the single MDA-MB-231 cells group (p<0.05).

HMGB3-siRNA Downregulated CD44-Positive and CD24-Negative (CD44$^+$/CD24$^-$) Breast Cancer Cells

As the tumor mammosphere are also associated with CD44$^+$/CD24$^-$ cancer cell levels, they have been evaluated using flow cytometry approach. Notably, HMGB3-mimic remarkably increased

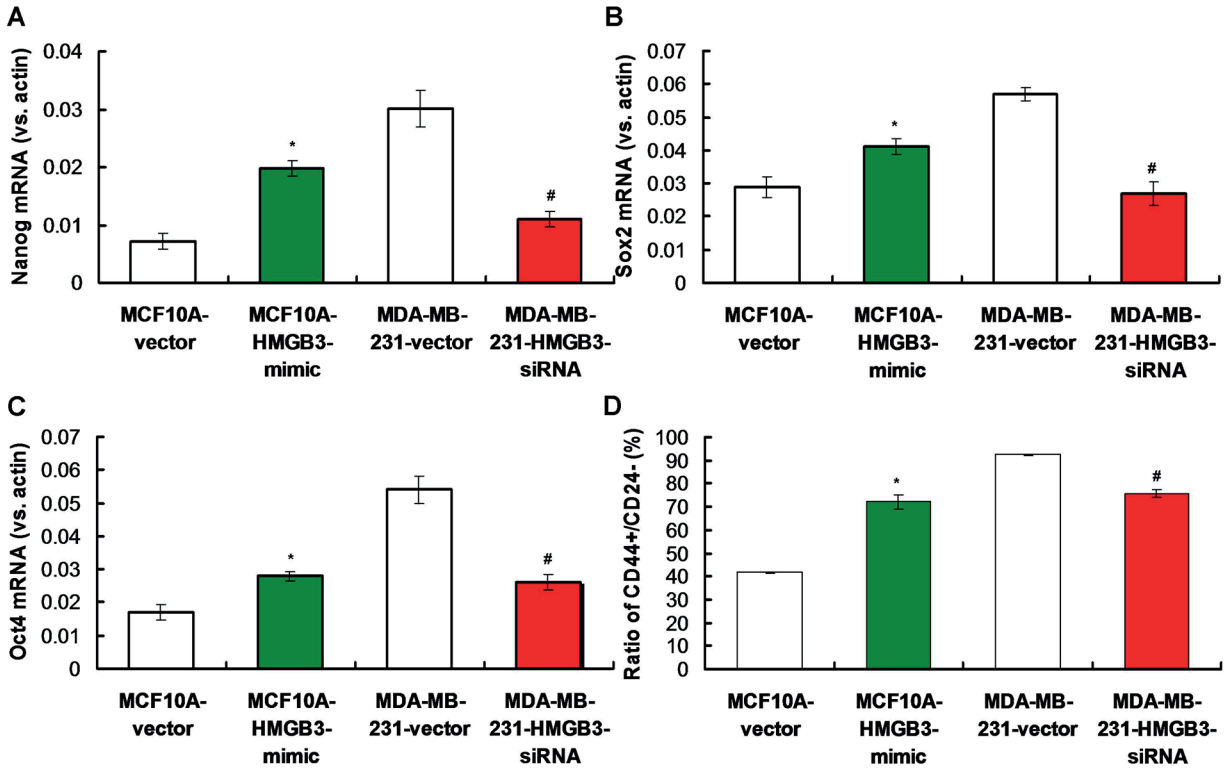

Figure 3. Effects of HMGB3-mimic or HMGB3-siRNA administration on expression of iPSCs biomarkers using qRT-PCR assay. **A,** Statistical analysis of Nanog mRNA expression in both HMGB3-mimic or HMGB3-siRNA treated cells. **B,** Statistical analysis of the Sox2 mRNA expression in both HMGB3-mimic or HMGB3-siRNA treated cells. **C,** Statistical analysis of the Oct-4 mRNA expression in both HMGB3-mimic or HMGB3-siRNA treated cells. **D,** Statistical analysis of the CD44+/CD24- cells. *$p<0.05$ *vs.* MCF-10A cells. #$p<0.05$ *vs.* MDA-MB-231 cells.

$CD44^+/CD24^-$ cell levels compared with that in the single MCF10A cells (Figure 3D, $p<0.05$). However, HMGB3-siRNA treatment remarkably downregulated $CD44^+/CD24^-$ cell levels compared with that in the single MDA-MB-231 cells (Figure 3D, $p<0.05$).

HMGB3-siRNA Weakened Expression of HIF1α in Breast Cancer MDA-MB-231

Due to the relationship between HIF1α and cell proliferation in cancer cells, HIF1α expression was determined using qRT-PCR. The data demonstrated that HMGB3-mimic treatment remarkably enhanced the HMGB3 mRNA levels compared to that of the single MCF10A cells (Figure 4A, $p<0.05$). Meanwhile, HMGB3-siRNA administration remarkably decreased HMGB3 mRNA levels in single MDA-MB-231 cells (Figure 4A, $p<0.05$). Moreover, HMGB3-mimic treatment significantly increased the HMGB3 mRNA levels and HMGB3-siRNA administration remarkably weakened HMGB3 mRNA levels (Figure 4B, $p<0.05$).

HMGB3 Was Positively Correlated with HIF1α Expression

According to Linear Regression Analysis, we found that HMGB3 expression was positively correlated with HIF1α expression in MCF10A cells (Figure 5A, $p<0.05$). Furthermore, Linear Regression Analysis findings also showed that HMGB3 was also correlated with HIF1α expression in MDA-MB-231 cells (Figure 5B, $p<0.05$).

There Was an Interaction Between HMGB and HIF1α Expression

We firstly synthesized pGL3-HIF1α reporter and pcDNA3.1-HMGB3, both of which were then transfected into MDA-MB-231 cells (Figure 6A). Then, the pGL3-HIF1α reporter co-transfected together with pcDNA3.1-HMGB3 into MCF10A cells (Figure 6B) and MDA-MB-231 cells (Figure

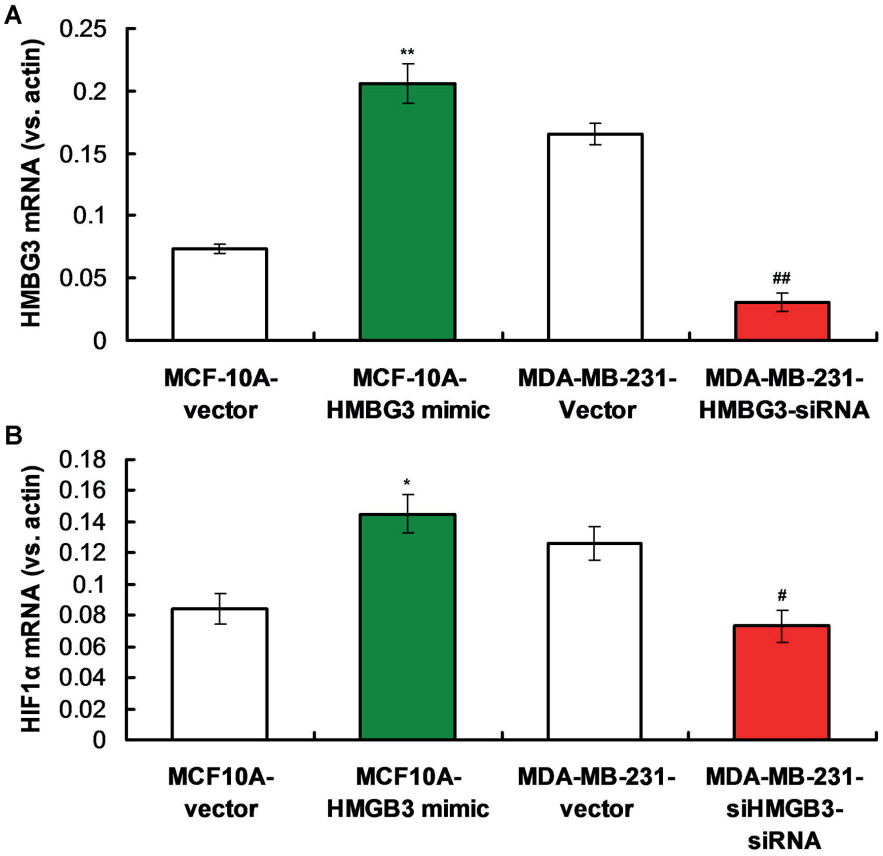

Figure 4. HMGB3-siRNA suppressed expression of HIF1α in MDA-MB-231 cells. **A,** HMGB3 mRNA expression in both MCF10A and MDA-MB-231 cells. **B,** Statistical analysis of the HIF1α mRNA expression in HMGB3-mimic or HMGB3-siRNA administrated cells. *p<0.05, **p<0.01 *vs.* MCF-10A cells. $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* MDA-MB-231 cells.

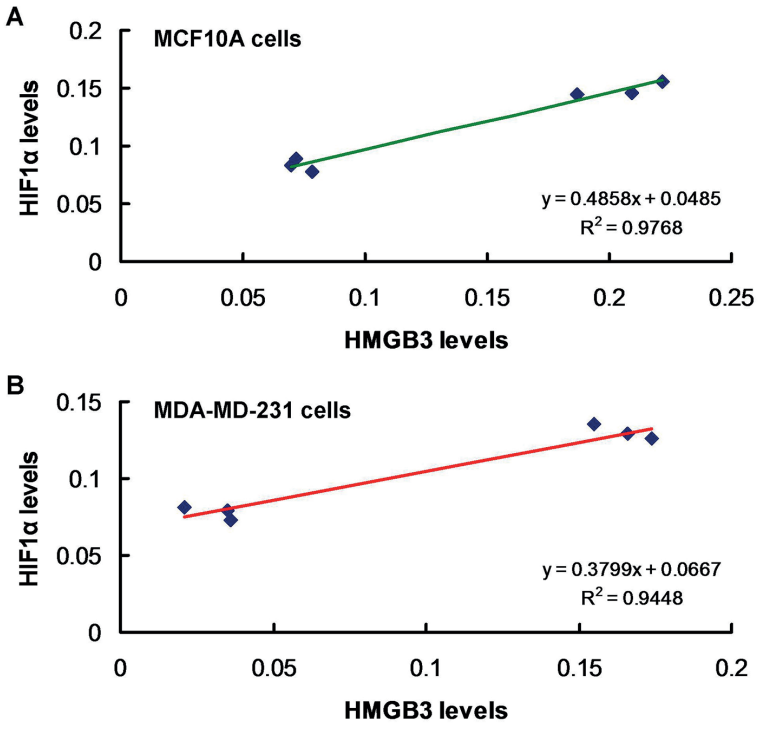

Figure 5. Correlation between HMGB3 expression and HIF1α expression in both MCF10A cells **(A)** and MDA-MB-231 cells **(B)**, using Linear Regression analysis.

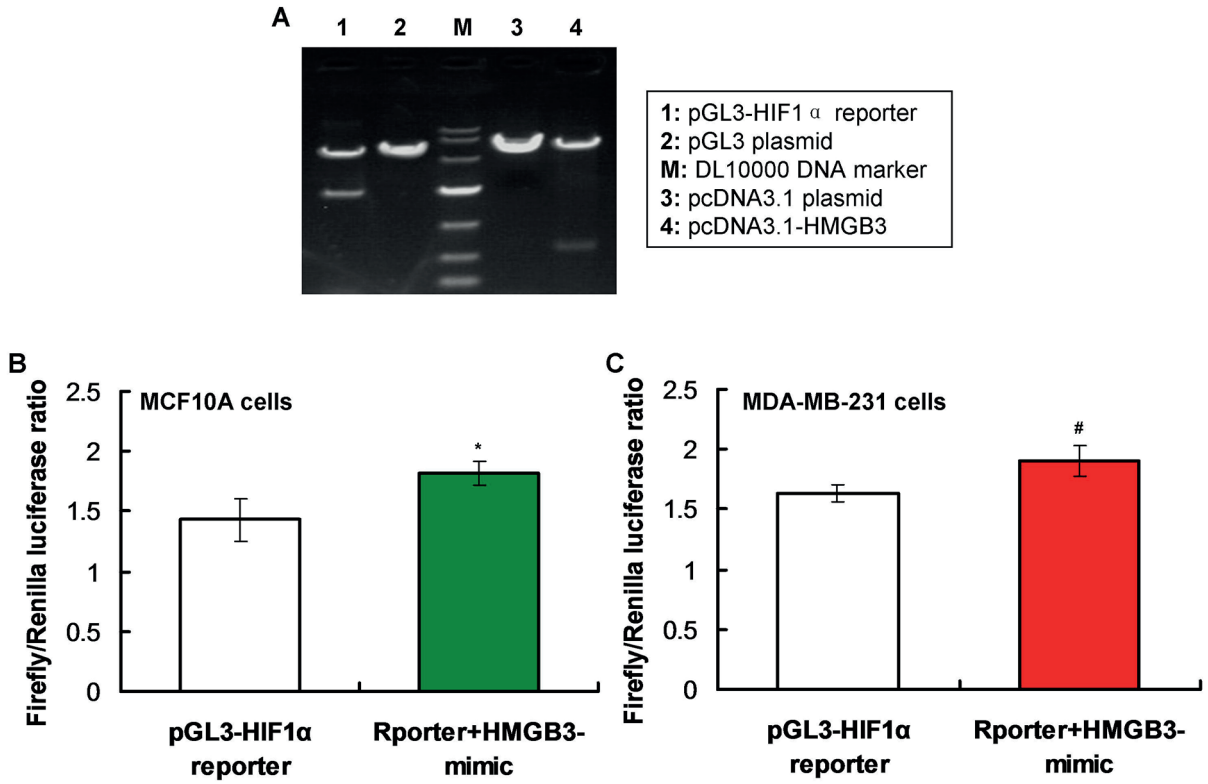

Figure 6. HMGB3 interacted with HIF1α expression in MDA-MB-231 cells. **A,** Identification for synthesis of pGL3-HIF1α reporter and pcDNA3.1-HMGB3. **B,** Statistical analysis of Firefly/Renilla Luciferase ratio in MCF10A cells. **C,** Statistical analysis of Firefly/Renilla Luciferase ratio in MDA-MB-231 cells.

6C). Based on results of Dual-Luciferase assay, the Luciferase activities were remarkably higher compared to the single pGL3-HIF1α reporter transfecting group ($p<0.05$).

Discussion

Although breast cancer has become the leading reason for female's cancer-related death, the pathological mechanisms have not been fully clarified till now[25,26]. According to the former studies[27-29], HMGB3 involves in pathological processes in many cancers, especially in carcinogenesis and tumor progression, such as colon cancer, gastric cancer, and ovarian cancer. Although a few studies have investigated the roles of HMGB3 in breast cancer, the effect of HMGB3 on mammosphere formation has never been clarified. Here, the effects of HMGB3 expression and silence on mammosphere formation and proliferation were evaluated in breast cancer cells.

Our findings showed that compared with the normal breast cell (MCF10A cells), HMGB3 was significantly over-expressed in both of basal-like cell (MDA-MB-231) and luminal cell (MCF7). However, MDA-MB-231 cells were more inclined to express high levels of HMGB3. Therefore, MDA-MB-231 cells were employed to evaluate to effects of HMGB3 on cell proliferation. According to CCK-8 results, HMGB3-mimic remarkably increased cell proliferative viabilities of MCF10A cells. Meanwhile, HMGB3-siRNA remarkably decreased the proliferative viabilities of MDA-MB-231 cells. Moreover, HMGB3-mimic treatment in MCF10A cells also significantly increased the mammosphere amounts in MDA-MB-231 cells. Also, HMGB3-siRNA administration remarkably reduced the mammosphere amounts in MDA-MB-231 cells. The above findings suggest that HMGB3 silencing could significantly inhibit breast cancer cell proliferation and suppress the formation of mammosphere. Therefore, the inhibition of cell proliferation and

suppression of mammosphere are beneficial to inhibition of tumor growth[30,31].

The previous study[32] reported that specific biomarkers of iPSCs, such as *Nanog, SOX2,* and *OCT-4*, involve in processes of tumorigenesis[32], especially in breast cancer[33]. Our findings demonstrated that HMGB3-mimic treatment enhanced *Nanog, SOX2*, and *OCT-4* mRNA levels in MCF10A cells. While, HMGB3-siRNA administration reduced *Nanog, SOX2*, and *OCT-4* mRNA levels in MDA-MB-231 cells. These results suggest that iPSCs might participate in HMGB3-siRNA mediated inhibition of breast cancer cells and suppression of mammosphere. The previous studies[9,34] reported that higher levels of $CD44^+CD24^-$ cells were correlated with the formation of mammosphere and metastases of tumor cells. Our results also provided a similar finding that HMGB3-siRNA remarkably decreased $CD44^+CD24^-$ levels in MDA-MB-231 cells. These results hint that HMGB3-siRNA reduces mammosphere accounts of breast cancer cells by regulating $CD44^+CD24^-$ levels.

Moreover, in response to condition of hypoxia, tumor cells usually express HIF-1α to reverse hypoxia-damage[35]. Meanwhile, HIF-1α is also proven to be associated with iPSCs biomarkers, such as *Nanog*, *Sox2*, and *OCT-4* in cancer cells[36]. Therefore, we speculated that HMGB3 might be correlated to HIF-1α expression in the pathological processes. We have proved that HMGB3-siRNA significantly downregulated mRNA expression of HIF-1α in MDA-MB-231 cells. Meanwhile, the Linear Regression Analysis data also demonstrated that HMGB3 was positively correlated with HIF-1α expression in both MCF10A cells and MDA-MB-231 cells. Dual-Luciferase findings also illustrated that HMGB3 could remarkably interact with HIF-1α in MDA-MB-231 cells. These results suggest that the effects of HMGB3-siRNA on breast cancer cells (cancer cell proliferation and mammosphere formation) might be mediated by activating siHIF-1α molecule.

Conclusions

HMGB3-siRNA inhibited breast cancer cell proliferation, suppressed formation of mammosphere, and reduced mRNA levels of iPSCs biomarkers. HMGB3 correlated and interacted with HIF1α in breast cancer cells. In summary, HMGB3 small interfere RNA suppressed formation of mammosphere in MDA-MB-231 cells by downregulating the expression of HIF1α. The present study is the first one that evaluates the effects of HMGB3 silencing on mammosphere formation in breast cancer cells. The conclusion of this study would provide the potential targeting strategy to inhibit tumor cell growth and would be benefit to the therapy of clinical breast cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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