High mobility group box 1 promotes the epithelial-to-mesenchymal transition in prostate cancer PC3 cells via the RAGE/NF-κB signaling pathway

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Abstract. High mobility group box 1 (HMGB1), a critical damage-associated molecular pattern molecule, has been implicated in several inflammatory diseases and cancer types. The overexpression of HMGB1 protein occurs in prostate cancer, and is closely associated with the proliferation and aggressiveness of tumor cells. However, the underlying mechanisms of HMGB1-induced tumor metastasis in prostate cancer remain unclear. In the present study, it was demonstrated that the expression of HMGB1 was high in prostate cancer samples, particularly in the metastatic tissues. Furthermore, recombinant HMGB1 (rHMGB1) enhanced the invasive and metastatic capabilities of the prostate cancer cells. Molecular phenotype alterations of epithelial-to-mesenchymal transition (EMT) and elevated expression levels of matrix metalloproteinase (MMP)-1, -3 and -10 were observed. In addition, advanced glycosylation end-product specific receptor (RAGE) and its downstream molecule nuclear factor (NF)-κB pathway were activated during rHMGB1-induced metastasis. Silencing RAGE or NF-κB reversed the upregulation of MMP and EMT marker expression levels, thus reducing the migration and invasiveness of tumor cells. Taken together, these results suggest that highly expressed HMGB1 drives EMT and the overexpression of MMP-1, -3, -10 via the RAGE/NF-κB signaling pathways, which facilitates the metastasis of prostate cancer and may be a potential therapeutic target for metastatic prostate cancer.

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Abbreviations: HMGB1, high mobility group box 1; RAGE, advanced glycosylation end-product specific receptor; EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; BPH, benign prostatic hyperplasia; CTGF, connective tissue growth factor

Key words: high mobility group box 1, epithelial-to-mesenchymal transition, prostate cancer, advanced glycosylation end-product specific receptor, nuclear factor-κB

Introduction

Prostate cancer is the most common type of malignant tumor and the third leading cause of cancer-associated mortality in males in Western countries (1). Almost all mortalities of patients diagnosed with prostate cancer are attributed to a failure to stop metastatic progression. According to Cancer Statistics 2017, the 5-year relative survival rate for localized and regional stage prostate cancer is >99%, while that for distant-stage prostate cancer is 29% (1). Thus, distant metastasis, particularly to the lung and liver, impairs the life quality of patients and results in high mortality rates. In addition, medical care costs are relatively high for patients with distant tumor metastasis, thus leading to heavy social burdens (2,3). Therefore, it is essential to gain comprehensive knowledge of the mechanisms underlying prostate cancer metastasis in order to improve early prediction techniques and targeted therapies.

Accumulating evidence suggests that human cancer genesis and progression are associated with chronic inflammation, in which inflammatory cytokines, including tumor necrosis factor-α, interleukin-6 and transforming growth factor-β, serve important roles (4,5). These inflammatory cytokines facilitate the acquisition of a metastatic phenotype and progression to an advanced stage (6,7). Among them, intranuclear high mobility group box 1 (HMGB1) is a highly conserved nuclear DNA-binding protein that regulates gene expression and nucleosome stability. However, when actively secreted by cells or released from necrotic tumor cells, extracellular HMGB1 functions as a pro-inflammatory mediator,

modulating inflammatory responses, and consequently driving the development and progression of a range of cancer types, including, but not limited to, malignant cervical cancer, hepatocellular carcinoma and melanoma (8-10). In prostate cancer, the overexpression of HMGB1 has been observed in cancer tissues and cell lines (DU145, PC3 and LNCaP), and confirmed to be positively associated with cancer progression and a poor prognosis (11,12). It has been reported that HMGB1 induces inflammatory responses, activates key oncogenic genes, such as the androgen receptor, and promotes prostate tumor cell proliferation (13). In addition, androgen deprivation therapy has been demonstrated to induce the secretion of HMGB1 from prostatic stromal cells, subsequently promoting prostate cancer metastasis (14). However, the underlying mechanisms of HMGB1-driven metastasis of prostate cancer requires further investigation.

Epithelial-to-mesenchymal transition (EMT) is a dynamic process that regulates cell differentiation, embryo implantation, organ development and tissue regeneration (15). This process allows a polarized epithelial cell to undergo biochemical changes transitioning to the mesenchymal cell phenotype, which allows the cells to migrate under certain physical and pathological conditions, serving an essential role in the primary steps of cancer metastasis (15). Accordingly, the expression levels of epithelial junction proteins, including E-cadherin and vitamin D3 receptor (VDR) are downregulated, whereas the expression levels of mesenchymal adhesion proteins, including N-cadherin, vimentin, connective tissue growth factor (CTGF) and fibronectin 1, are highly expressed in the EMT process (15,16). Additionally, the matrix metalloproteinase (MMP) family degrades extracellular matrix components, upregulates the expression levels of tumorigenic transcription factors and triggers a cascade of molecular phenotype alterations, subsequently causing EMT (17-19).

Previous studies have demonstrated that HMGB1 induces EMT, thus promoting the invasiveness and metastatic properties of several cancer types, including hepatocellular, hypopharyngeal and colorectal carcinoma (20-22). However, it remains unknown whether extracellular HMGB1 serves an essential role in EMT-mediated metastasis in prostate cancer. The present study aims to investigate whether HMGB1 can promote the prostate cancer metastasis via inducing EMT and if so which signaling pathway is involved.

Materials and methods

Patient samples. Prostate tissue samples were obtained from 12 benign prostatic hyperplasia (BPH) patients, 12 localized prostate cancer patients and 12 metastatic prostate cancer patients diagnosed by biopsy or postoperative pathological examination at the Department of Urology of Xijing Hospital (Xi'an, Shaanxi, China) between January 2016 and December 2016. Mean calculated ages of the three groups were 71±5.6 years (range, 62-83 years), 67±6.4 years (range, 53-77 years) and 66±4.4 years (range, 59-72 years), respectively. Written informed consent and permission to collect prostate tissues were obtained from all subjects enrolled in the study. All analyses of human materials were approved by the Research Ethics Committee of the Fourth Military Medical University (Xi'an, Shaanxi, China), and conducted according to the principles of the Declaration of Helsinki. Tissue samples were fixed with a concentration of 10% formalin at room temperature overnight, paraffin-embedded and cut into 4-μm thick sections for further staining analysis. Tissue samples that were used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were stored at -80˚C until RNA isolation.

Cell culture and stimulation. The human prostate cancer PC3 cell line was obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China), and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified 5% CO_2 incubator at 37˚C. Briefly, PC3 cells at 60-80% confluency were stimulated with 0.5, 1 or 2 μg/ml recombinant (r) HMGB1 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h at 37˚C. For the RAGE inhibition experiment, RAGE neutralizing antibody (R&D Systems, Inc., Minneapolis, MN, USA) was used at 20 μg/ml for 24 h at 37˚C.

Wound healing assay. Cells were seeded into 6-well plates at 300,000 cells/well and incubated for 24 h in a humidified 5% CO_2 incubator at 37˚C to form cell monolayers. Next, a sterile 10- or 100-μl plastic pipette tip was used to create a scratch wound across the monolayer, following which the detached cells were removed by washing with cold phosphate-buffered saline [PBS; 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4 and 1.47 mM KH_2PO_4 (pH 7.4)] three times. Cells were then cultured in serum-free DMEM with/without rHMGB1 stimulation in a humidified 5% CO_2 incubator at 37˚C for 24 h, and the cells that had migrated into the wound area were imaged with a phase-contrast microscope (Nikon Corporation, Tokyo, Japan). The area of the wound at 24 h, as compared with the initial wound area, was calculated using the Fiji package of ImageJ (National Institutes of Health, Bethesda, MD, USA).

Transwell assay. Cell migration and invasion assays were performed using Transwell chambers (pore size, 8 μm; Costar; Corning Incorporated, Corning, NY, USA), whereby the base was coated with 1 mg/ml BD Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) for the invasion assay. Cells were diluted at a cell density of $2x10^5$ cells/ml. A total of 200 μl DMEM containing rHMGB1 was added to the upper chamber and 600 μl DMEM was added to the lower chambers. After 24 h of culture, the cells that invaded into the lower chamber were fixed with 4% formalin for 15 min at room temperature. The cells on the inner layer were then gently removed with a cotton swab and the adherent cells on the lower surface of the insert was stained with 0.1% crystal violet for 30 min at room temperature. The filters were washed with water several times and images were captured (original magnification, x100). Invaded cells on the lower surface were counted under a light microscope (Nikon Corporation).

Silencing PC3 cells. The small interfering RNAs (siRNAs) for RAGE, NF-κB p65 and normal control siRNA (si-NC) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). PC3 cells were transfected with siRNA (5 nM) and Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific,

Inc.) according to the manufacturer's protocol. Subsequent to 48 h of culture at 37˚C, control cells were left untreated, and rHMGB1 at 1 μg/ml was added to the remaining wells for stimulation.

Immunohistochemical and immunofluorescence staining. For immunohistochemistry and immunofluorescence staining of prostate samples, 4-μm sections of paraffin-embedded tissues were blocked at room temperature with 5% goat serum in PBS for 30 min. Sections were then incubated with rabbit anti-HMGB1 (1:100; catalog no. ab18256; Abcam, Cambridge, UK) or anti-RAGE (1:50; catalog no. sc-365154; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight, followed by a horseradish peroxidase-labeled goat anti-rabbit antibody (1:200; catalog no. CW0156; CWBio, Beijing, China) or a Cy3-conjugated goat anti-rabbit antibody (1:200; catalog no. ab6939; Abcam) for 1 h at room temperature. DAB developing system (Gene Tech Biotechnology Co., Ltd., Shanghai, China) was used for detecting the biotinylated antibodies at room temperature for 30 sec. Immunohistochemistry images were acquired with an Olympus VS120 slide scanner (Olympus Corporation, Tokyo, Japan). Two pathologists who were blinded to the clinical characteristics of the patients scored HMGB1 expression independently. As previously reported (20), the intensity of HMGB1 staining was expressed as four grades: 0, None; 1, weak; 2, moderate; and 3, strong. The percentage of HMGB1-positive cells was expressed as five grades: 0, <5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. The score of the HMGB1 expression was evaluated by multiplying the intensity by the percentage. In addition, confocal images were acquired using the Olympus Fluoview 1000 microscope (Olympus Corporation) with a PlanApo N lens (x40 with and without x2.5 digital zoom).

For cell immunofluorescence staining, PC3 cells were seeded and incubated at 37˚C, in 5% CO_2, followed by stimulation with rHMGB1 (1 μg/ml) for 24 h. Cells were then washed with ice-cold PBS and fixed immediately with 4% paraformaldehyde for 15 min at room temperature, then permeabilized with 0.1% Triton X-100 for 15 min. Cells were incubated with anti-E-cadherin (catalog no. 14472) or vimentin (catalog no. 5741) (both 1:100; Cell Signaling Technologies, Inc., Danvers, MA, USA), or fibronectin 1 (1:100; catalog no. ab2413; Abcam) overnight at 4˚C. Cy3-conjugated goat anti-rabbit antibody (1:200; catalog no. ab6939; Abcam) was used as the secondary antibody. Nuclear DNA was detected by incubating cells with Hoechst 33342 (1:1,000; Sigma-Aldrich; Merck KGaA) for 20 min at room temperature.

RT-qPCR. The total RNA from the tissues and cells was extracted using TRIzol (Takara Bio, Inc., Otsu, Japan), and cDNA was synthesized using reverse transcriptase from a Perfect Real-Time PrimeScript™ RT Reagent kit (Takara Bio, Inc.). The relative content of mRNA was amplified via qPCR with SYBR-Green (SYBR® Premix Ex Taq™ II; Takara Bio, Inc.) on a Chromo4 continuous fluorescence detector with a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Thermal cycling conditions were as follows: Initial denaturation for 30 sec at 95˚C, followed by 40 cycles of denaturation for 5 sec at 95˚C and extension for 30 sec at 60˚C; the absorbance value was read at the extension stage. All values were normalized to the expression of β-actin. The primers used are listed in Table I. Relative quantification was performed according to the ΔΔCq method, and results were expressed in the linear form using the formula $2^{-\Delta\Delta Cq}$ (23). Results were considered significant when at least a 2-fold difference in expression levels was detected.

Table I. Primer sets used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primers
MMP-1	5'-GAGTGCCTGATGTGGCTCAG-3'
	5'-TTCTCAATGGCATGGTCCAC-3'
MMP-2	5'-CCAAGTCTGGAGCGATGTGA-3'
	5'-GGAGTCCGTCCTTACCGTCA-3'
MMP-3	5'-CAGCCAACTGTGATCCTGCT-3'
	5'-ACGCCTGAAGGAAGAGATGG-3'
MMP-7	5'-GACTCACCGTGCTGTGTGCT-3'
	5'-TGAGATAGTCCTGAGCCTGTTCC-3'
MMP-8	5'-TCACCTCTCATCTTCACCAGGA-3'
	5'-AGGCATGAGCAAGGATTCCA-3'
MMP-9	5'-TCATCTTCCAAGGCCAATCC-3'
	5'-GCAGAAGCCGAAGAGCTTGT-3'
MMP-10	5'-TGTGGAGTTCCTGACGTTGG-3'
	5'-GCCTGGAGAATGTGAGTGGA-3'
CTGF	5'-GGCTTACCGACTGGAAGACA-3'
	5'-AGGAGGCGTTGTCATTGGTA-3'
Vimentin	5'-CAGGACTCGGTGGACTTCTC-3'
	5'-GTCGATGTAGTTGGCGAAGC-3'
N-cadherin	5'-TCCTTGCTTCTGACAATGGA-3'
	5'-TTCGCAAGTCTCTGCCTCTT-3'
VDR	5'-ACTTGCATGAGGAGGAGCAT-3'
	5'-ACGTCTGCAGTGTGTTGGAC-3'
E-cadherin	5'-TTGAGCACGTGAAGAACAGC-3'
	5'-GGCGTTGTCATTCACATCAG-3'
β-actin	5'-GGCTACAGCTTCACCACCAC-3'
	5'-TGCGCTCAGGAGGAGC-3'
Fibronectin 1	5'-CAAACTCCGTCACCCTCAGT-3'
	5'-GGTGCCAGTGGTTTCTTGTT-3'

MMP, matrix metalloproteinase; CTGF, connective tissue growth factor; VDR, vitamin D3 receptor.

Protein extraction and western blot analysis. Western blot assays were performed using whole cell lysates from cultured PC3 cells. Cells were washed twice with ice-cold PBS and lysed in 2X sodium dodecyl sulfate (SDS) sample buffer (2% mercaptoethanol, 20% glycerol, 4% SDS in 100 mM Tris-HCl buffer, pH 6.8) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and a phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.), and incubated for 15 min on ice. The supernatants were collected

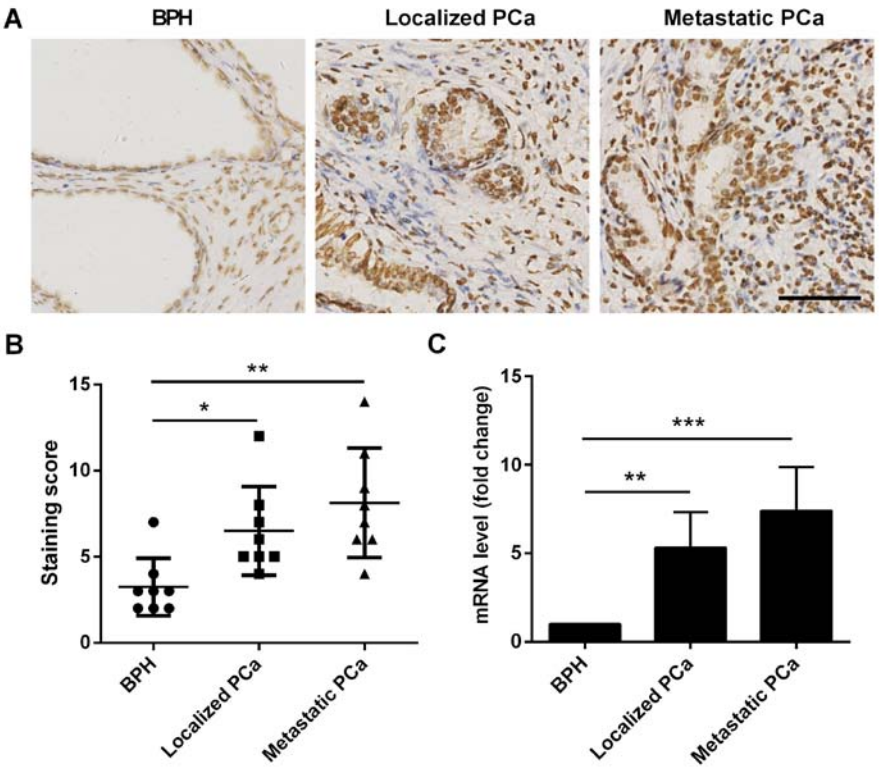

Figure 1. HMGB1 is highly expressed in prostate cancer tissues. (A) Immunohistochemical detection of HMGB1 expression in BPH tissues, localized prostate cancer tissues and metastatic prostate cancer tissues. The brown color represents positive HMGB1 expression. Scale bar, 100 μm. (B) The mean staining score of HMGB1 in tissues as indicated in (A). (C) The expression of HMGB1 at the mRNA level in tissues of BPH, localized prostate cancer and metastatic prostate cancer. One-way analysis of variance. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. PCa, prostate cancer; HMGB1, high mobility group box 1; BPH, benign prostatic hyperplasia.

by centrifugation at 12,000 x g for 15 min at 4˚C, and a BCA protein assay kit was used to measure the protein content. Protein samples (20 μg) were loaded and separated with 10% SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes. Subsequently, membranes were blocked with Tris-buffered saline-Tween-20 buffer containing 5% skimmed milk for 1 h at room temperature. Membranes were incubated at 4˚C overnight using the following antibodies: RAGE (catalog no. sc-365154; Santa Cruz Biotechnology, Inc.), NF-κB p65 (catalog no. 8242), phospho-NF-κB p65 (catalog no. 3033), E-cadherin (catalog no. 3195), N-cadherin (catalog no. 13116), vimentin (catalog no. 5741) (all from Cell Signaling Technology, Inc.) and fibronectin 1 (catalog no. ab2413; Abcam), with β-actin (catalog no. CW0264; CWBio) as an internal control. The membranes were then developed with an enhanced chemiluminescence detection kit (Advansta, Inc., Menlo Park, CA, USA).

Cell counting kit-8 (CCK-8) assay. Cells were trypsinized and seeded at 3,000 cells per well in 96-well plates. Cell proliferation was then measured using the CCK-8 kit (catalog no. C0038; Beyotime Institute of Biotechnology, Shanghai, China) every 24 h according to the manufacturer's protocols. Absorbance was detected at a wavelength of 450 nm. Three wells were measured for cell viability in each treatment group.

Statistical analysis. Unless stated otherwise, all experiments were performed three times. All statistical analyses were performed using GraphPad Prism version 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using the Student's unpaired two-tailed t-test when two groups were compared. Statistical significance was also determined using analysis of variance where indicated in the figure legends. The number of sampled units (n) is also indicated in the figure legends. P<0.05 was considered to indicate a statistically significant difference.

Results

HMGB1 is highly expressed in prostate cancer tissues. To examine the expression of HMGB1 in the tissues of BPH, localized prostate cancer and metastatic prostate cancer, immunohistochemical staining and RT-qPCR were performed. As the immunochemistry results showed, the protein level of HMGB1 was markedly high in localized prostate cancer tissues and metastatic prostate cancer tissues, and less detectable in BPH tissues, with H-Scores of 228.8±12.88, 269.4±7.223 and 105.6±12.94, respectively (Fig. 1A and B). These results were confirmed by RT-qPCR, which demonstrated significantly elevated HMGB1 mRNA in localized and metastatic prostate cancer tissues compared with that in BPH tissues (Fig. 1C). Immunohistochemical staining and RT-qPCR results indicated a moderate and significant elevation, respectively, in the HMGB1 levels in metastatic prostate cancer tissues compared with that in primary prostate cancer tissues (Fig. 1B and C). Consistent with previous studies, these findings indicated that HMGB1 was overexpressed in prostate cancer tissues, and particularly predominant in metastatic prostate cancer tissues.

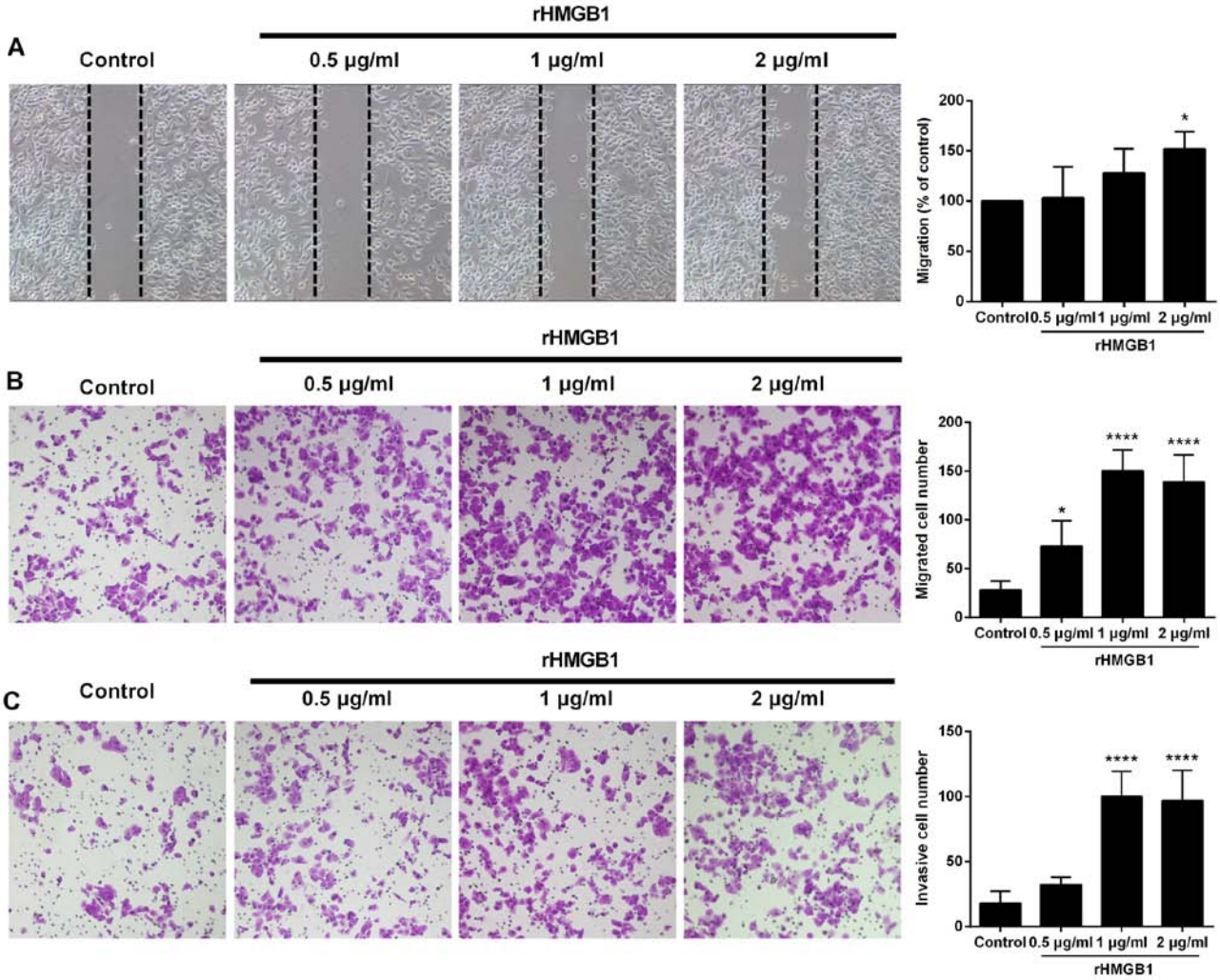

Figure 2. HMGB1 promotes the migration and invasion of PC3 cells. (A) A wound-healing assay was employed to detect the migration of PC3 cells, which were treated with 0.5, 1 or 2 μg/ml rHMGB1, respectively. Cells were imaged at 24 h. The area of the wound at 24 h, as compared with the initial wound area, was calculated using ImageJ software. (B) PC3 cells were cultured with 0.5, 1 or 2 μg/ml rHMGB1 in the upper chambers of a Transwell plate. After 24 h, the number of migrated cells was calculated. (C) PC3 cells were cultured with 0.5, 1 or 2 μg/ml rHMGB1 in the upper chambers of the Transwell plate. After 24 h, the number of cells that migrated through the Matrigel was calculated. One-way analysis of variance (adjusted for Dunnett's test). Data are expressed as the mean ± standard error of the mean (n=3). *P<0.05 and ****P<0.0001 vs. control. rHMGB1, recombinant high mobility group box 1.

HMGB1 promotes the invasion and migration of PC3 cells. The study next investigated whether HMGB1 was involved in the invasion and metastasis of PC3 cells using a wound-healing assay, as well as Transwell migration and invasion assays. Following stimulation by recombinant HMGB1 (rHMGB1) at 0.5, 1 and 2 μg/ml, PC3 cells exhibited gradually increased migratory capacity in the wound-healing and Transwell assays (Fig. 2A and B). In the Transwell experiments aimed at detecting PC3 cell invasion, the stimulating effects of 1 μg/ml rHMGB1 on PC3 cells were most evident compared with that in the other groups, inducing significant PC3 cell invasion through the Matrigel matrix (Fig. 2C). The quantitative analyses of the migration and invasion of the cells are also presented in Fig. 2. Taken together, these data revealed that HMGB1 enhanced the metastatic and invasive capabilities of PC3 cells.

HMGB1 induces EMT in PC3 cells. Next, the underlying mechanism of HMGB1-induced migration and invasion in PC3 cells was investigated. EMT is recognized as an essential process that is involved in cancer metastasis by providing tumor cells with enhanced mobility (15). Therefore, the expression levels of EMT markers were evaluated by RT-qPCR, western blot analysis and cell immunofluorescence. As the CCK-8 assay results showed, rHMGB1 at 2 μg/ml enhanced the proliferation of PC3 cells in a time-dependent manner compared with that in groups of lower concentrations (data not shown). PC3 cells treated with rHMGB1 at different concentrations showed significant changes in epithelial markers in the 1-μg/ml group (data not shown). Therefore, rHMGB1 at 1 μg/ml was chosen as the optimal stimulus to investigate the mechanism underlying HMGB1-induced migration and invasion.

In the present study, it was demonstrated that treatment of PC3 cells with 1 μg/ml rHMGB1 for 24 h led to a significant decrease in the mRNA expression of epithelial markers, including E-cadherin and VDR, and an evident increase in the expression levels of mesenchymal markers, including N-cadherin, vimentin, CTGF and fibronectin 1 (Fig. 3A). Furthermore, western blot analysis demonstrated the downregulation of E-cadherin and upregulation of N-cadherin,

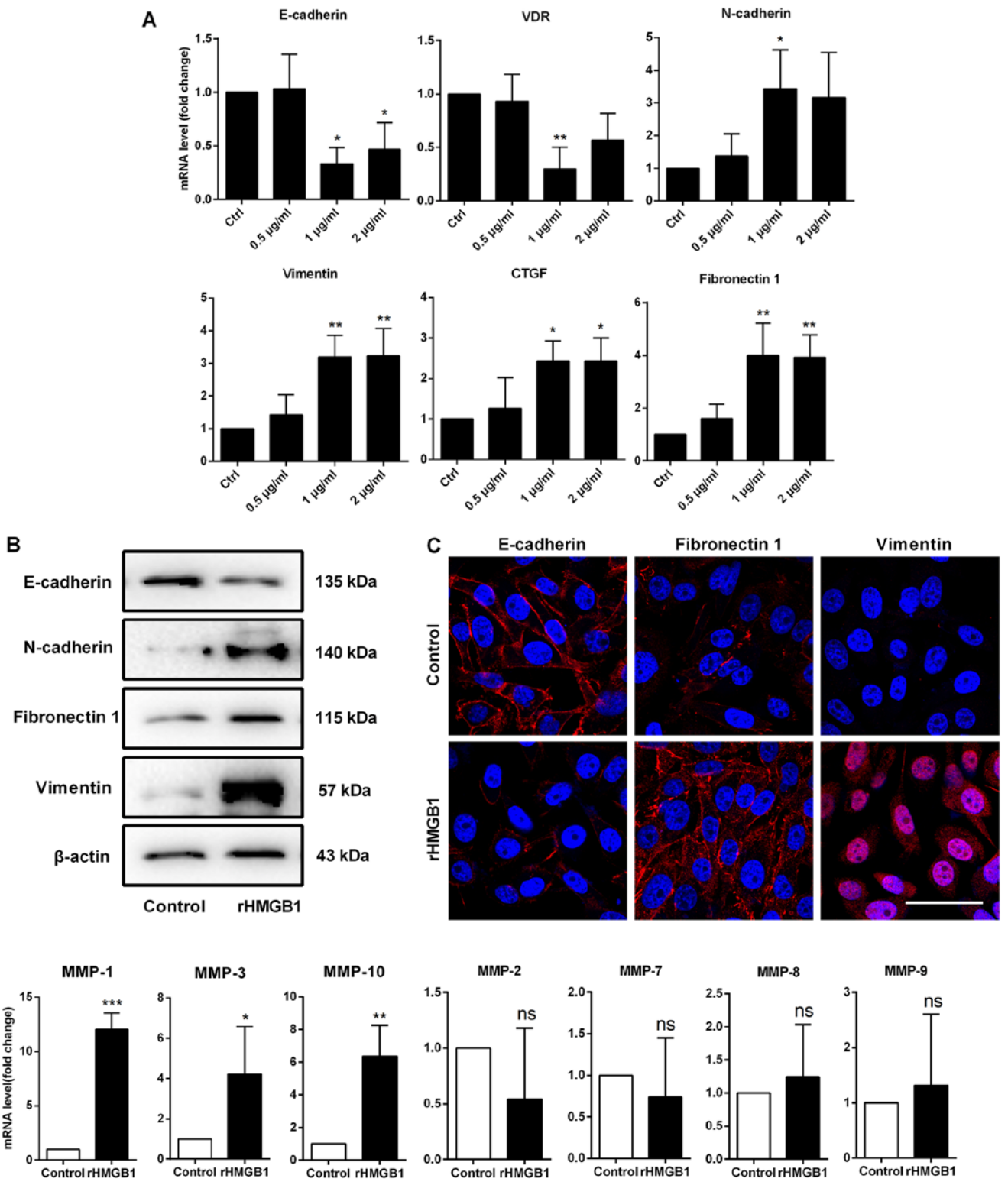

Figure 3. HMGB1 induces EMT in PC3 cells. (A) The mRNA levels of the EMT markers in PC3 cells stimulated by rHMGB1 at 0.5, 1 or 2 μg/ml for 24 h. One-way analysis of variance. Data are expressed as the mean ± SD (n=3). (B) Protein levels of EMT markers in PC3 cells with/without rHMGB1 stimulation. (C) Immunofluorescence staining of vimentin, fibronectin 1 and E-cadherin in PC3 cells treated with or without rHMGB1. Red fluorescence indicates positive expression; blue fluorescence represents Hoechst 33342-labeled nuclei. Scale bar, 10 μm. (D) The mRNA expression of MMPs in PC3 cells co-cultured with rHMGB1 or control medium. Two-tailed Student's t-test. Data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001. rHMGB1, recombinant high mobility group box 1; EMT, epithelial-to-mesenchymal transition; SD, standard deviation; MMP, matrix metalloproteinase; ns, not significant; ctrl, control; VDR, vitamin D3 receptor; CTGF, connective tissue growth factor.

fibronectin 1 and vimentin in PC3 cells stimulated with 1 μg/ml rHMGB1 for 24 h (Fig. 3B). In addition, the cell immunofluorescence results showed that the expression levels of the mesenchymal markers fibronectin 1 and vimentin were higher in rHMGB1-treated PC3 cells compared with those in untreated normal cells, while E-cadherin ectodomain

immunofluorescence was reduced (Fig. 3C). As the MMP family has long been recognized as an important mediator of tumor metastasis (24), the mRNA expression levels of several primary MMPs were detected in PC3 cells to elucidate the MMPs that were involved in HMGB1-induced metastasis. As presented in Fig. 3D, the mRNA expression levels of MMP-1, -3 and -10, but not other MMPs, in the PC3 cells were significantly upregulated by rHMGB1 stimulation (Fig. 3D). Collectively, the aforementioned findings revealed that HMGB1 induced the EMT process and overexpression of MMPs in PC3 cells, thus serving a pathogenic role in the metastasis of prostate cancer.

HMGB1 activates RAGE to induce EMT in PC3 cells. To define the mechanisms by which HMGB1 induced EMT to promote cancer metastasis, the receptors for HMGB1 were then detected in PC3 cells. As reported previously, extracellular HMGB1 binds to several cell surface receptors, including RAGE and toll-like receptor 2 and 4 (TLR2 and 4) (25). Therefore, RT-qPCR and western blotting were performed to examine the expression levels of possible receptors in rHMGB1-treated PC3 cells. It was demonstrated that the receptor RAGE, but not TLR2 or TLR4, was significantly upregulated (Fig. 4A and B). The expression and localization of RAGE in BPH tissues and metastatic prostate cancer tissues were then evaluated by tissue immunofluorescence, which revealed strong positive staining of RAGE in prostate cancer and stromal cells in the metastatic prostate cancer tissues, and moderate staining in the BPH tissues (Fig. 4C). To further determine whether RAGE participated in the stimulatory effects of HMGB1 on PC3 cells, siRNA targeting RAGE was applied to reduce its expression level (data not shown). The results of the wound healing and Transwell assays demonstrated that the previously elevated invasive and metastatic capabilities of rHMGB1-treated PC3 cells were markedly attenuated by RAGE siRNA (Fig. 4D and E). The rHMGB1-induced expression changes in EMT markers, including E-cadherin, VDR, N-cadherin, vimentin, CTGF and fibronectin 1, were reversed by the siRNA targeting RAGE (Fig. 4F). In addition, following the application of a neutralizing antibody against RAGE in PC3 cells, similar outcomes were observed to that of silencing the receptor using siRNA (Fig. 5A and B). As determined by CCK-8 assay, the neutralizing antibody of RAGE did not influence the viability of PC3 cells at 24, 48, 72 and 96 h (data not shown). Furthermore, the increased expression levels of MMP-1, -3 and -10 induced by rHMGB1 were significantly lowered in the cells that were transfected with the siRNA targeting RAGE or pre-treated with RAGE antibody (Figs. 4F and 5C). Collectively, these observations revealed that the HMGB1/RAGE axis contributed to the EMT process and MMP secretion, thus leading to the migration and invasion of PC3 cells.

RAGE/NF-κB pathway is responsible for HMGB1-induced EMT. As previously reported, the activation of different signaling pathways, including mitogen-activated protein kinase, phosphoinositide-3 kinase/protein kinase B, Smad and NF-κB, contributes to the development of EMT in various cancer types (15). To elucidate which pathway was involved in HMGB1-induced EMT in prostate cancer, the activation of the aforementioned signaling pathways was determined by western blot analysis. As presented in Fig. 6A, rHMGB1 significantly triggered NF-κB p65 phosphorylation in PC3 cells, peaking at 20 min (Fig. 6A). Other signaling pathways were not significantly activated in PC3 cells by rHMGB1 (data not shown). In addition, silencing RAGE with siRNA or blocking RAGE with neutralizing antibody reduced the phosphorylation of NF-κB in rHMGB1-stimulated PC3 cells (Fig. 6B), and indicated that rHMGB1 activated RAGE and its downstream NF-κB pathway to induce EMT in prostate cancer.

Next, NF-κB was knocked down using siRNA to identify its pathogenic role (data not shown), and the results of the wound-healing and Transwell assays demonstrated that the numbers of migrated and invasive cells were markedly decreased in rHMGB1-stimulated PC3 cells due to NF-κB silencing (Fig. 6C and D). Furthermore, siRNA targeting NF-κB reversed the mRNA expression levels of these EMT markers and MMPs in PC3 cells triggered by rHMGB1, as demonstrated by the RT-qPCR results (Fig. 6E). Therefore, we hypothesize that HMGB1 activates the RAGE/NF-κB signaling pathway to induce EMT and upregulate MMP expression, thus promoting the progression of prostate cancer.

Discussion

HMGB1 has been reported to be highly expressed in several cancer types, including melanoma, hepatoma and breast cancer, contributing to the aggressiveness of tumor cells (26). However, the underlying mechanisms of HMGB1-triggered metastasis have been poorly investigated. In prostate cancer, the highly-expressed HMGB1 in the tumor samples has been revealed to be positively correlated with clinicopathological features, including Gleason score, lymph node metastasis and distant metastasis (11,14,27). Furthermore, the dying DU145 tumor cells or PC3 cells that are caused by chemotherapeutic agents can release HMGB1 (28). In the present study, we hypothesized that extracellular HMGB1 induces EMT in PC3 prostate cancer cells to promote metastasis through activating the RAGE/NF-κB signaling pathway, which may be targeted for future therapies.

The intracellular and extracellular forms of HMGB1 have been universally studied in the formation and progression of tumors (29). In inactive cells, HMGB1 is a non-histone DNA-binding protein and highly conserved among mammalian species. In the state of sterile inflammation and cancer, HMGB1 may be actively secreted from damaged cells or passively released from autophagic or necrotic cells into the extracellular environment, binding to various receptors, including RAGE and TLR4, thereby increasing cell proliferation, enhancing cell invasion and migration, activating neovascularization and inducing the inflammatory microenvironment (30-32). Over the past two decades, the high expression of HMGB1 in tissues and its aforementioned effects have been confirmed in several cancer types, including hepatocellular carcinoma, breast cancer and bladder cancer (25). For instance, HMGB1 is reportedly abundantly secreted from ultraviolet radiation-damaged epidermal keratinocytes, subsequently causing angiotropism and metastasis in malignant melanoma (10).

In cases of prostate cancer, extracellular HMGB1 has been reported to be overexpressed in metastatic tumors and

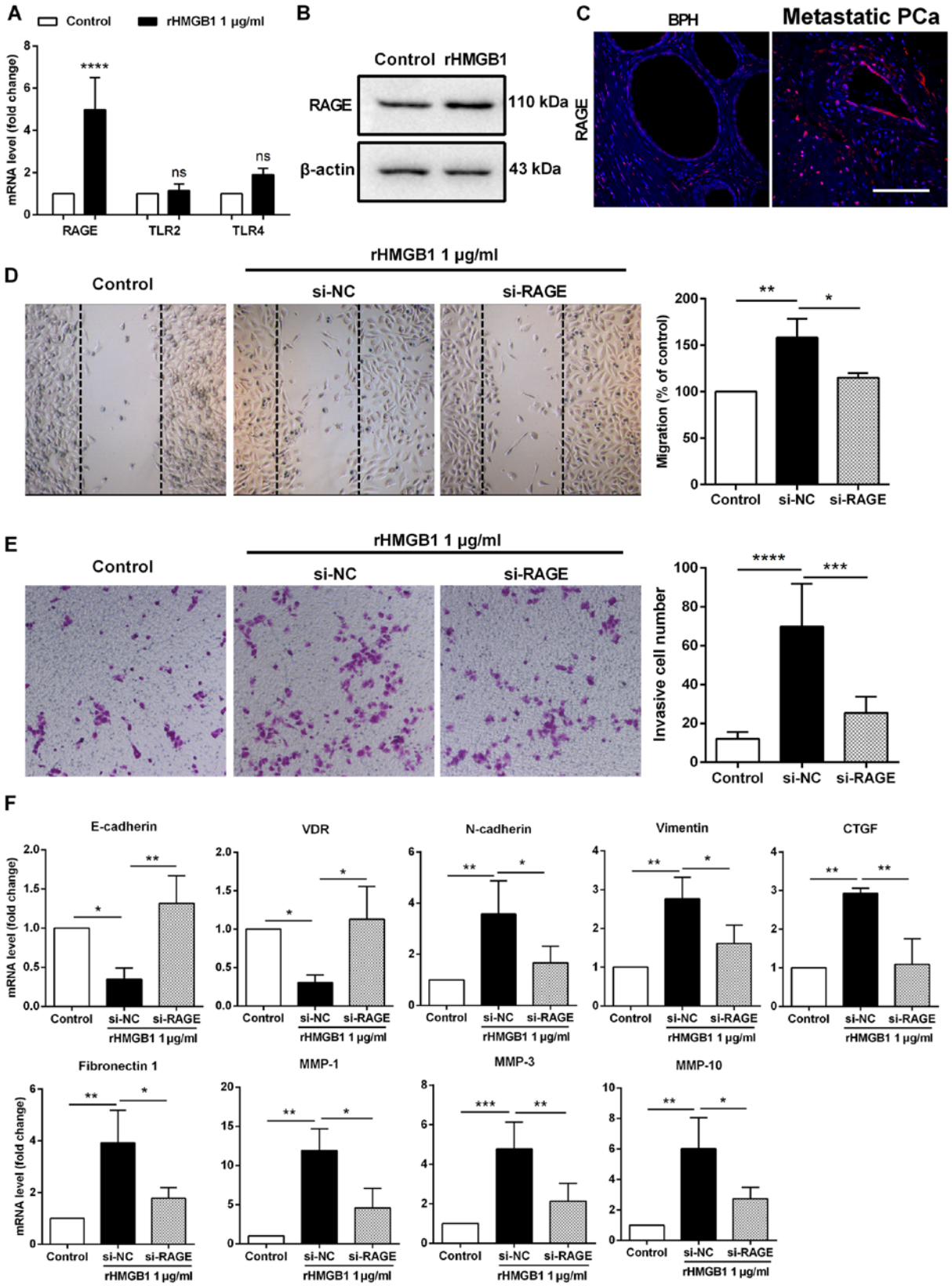

Figure 4. HMGB1 activates RAGE to induce EMT in PC3 cells. (A and B) PC3 cells were treated with or without 1 μg/ml rHMGB1 for 24 h, and the mRNA and protein expression of certain receptors was detected by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis, respectively. (C) The expression and localization of RAGE in the tissues of BPH and metastatic PCa. Scale bar, 100 μm. (D) PC3 cells were transfected with RAGE siRNA or control siRNA, and then scratches were made in the cell monolayer. Cells were stimulated with 1 μg/ml rHMGB1 and observed at 24 h (magnification, x10). (E) A Transwell assay was employed to detect the invasiveness of PC3 cells transfected with RAGE siRNA or control siRNA, followed by stimulation with 1 μg/ml rHMGB1 (magnification, x20). (F) The mRNA levels of EMT markers and MMPs in RAGE siRNA or control siRNA transfected PC3 cells in response to rHMGB1 stimulation. Two-way analysis of variance. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control. rHMGB1, recombinant high mobility group box 1; RAGE, advanced glycosylation end-product specific receptor; EMT, epithelial-to-mesenchymal transition; BPH, benign prostatic hyperplasia; PCa, prostate cancer; siRNA, small interfering RNA; MMP, matrix metalloproteinase; ns, not significant; VDR, vitamin D3 receptor; CTGF, connective tissue growth factor; NC, negative control.

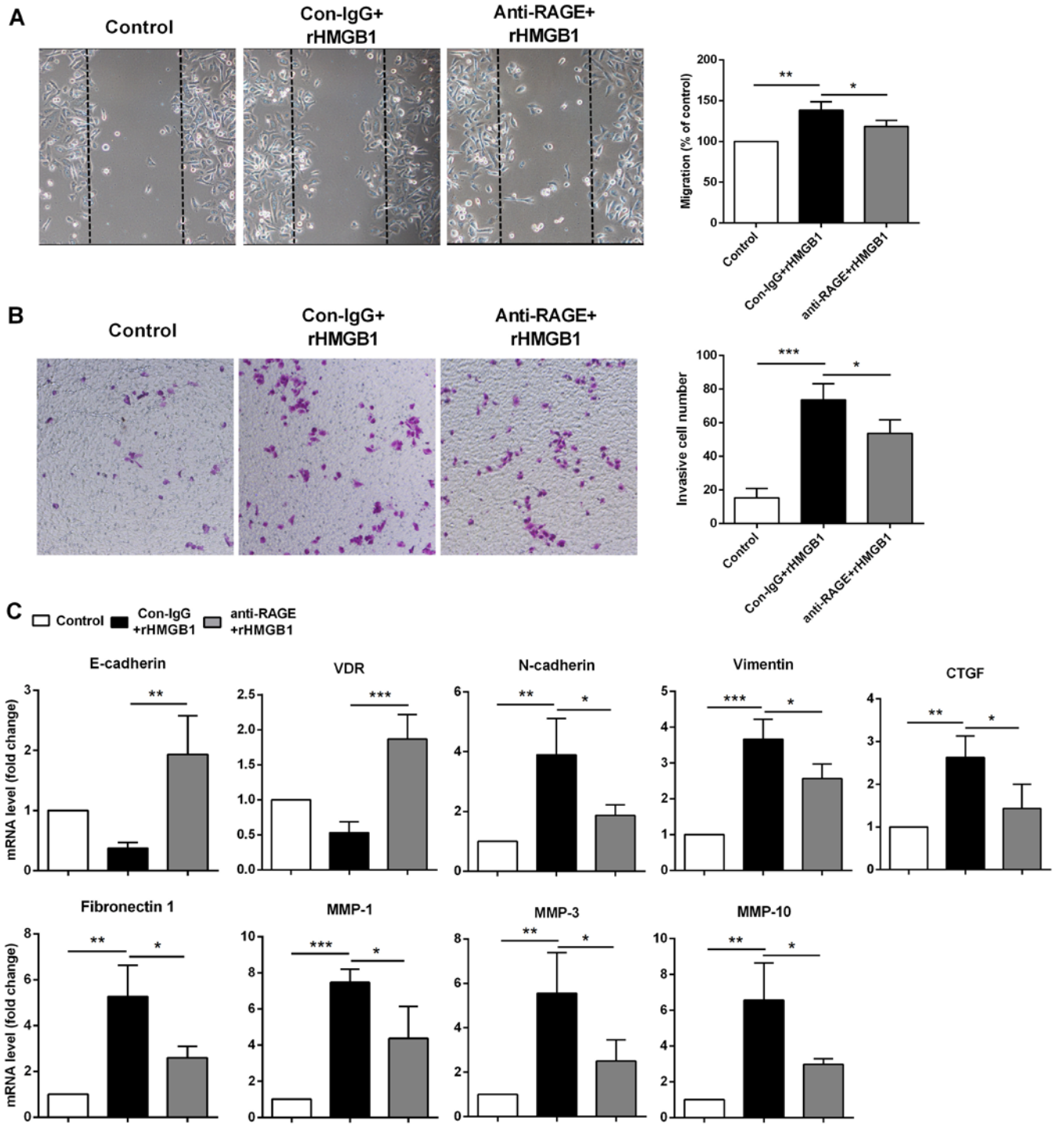

Figure 5. Neutralizing RAGE attenuates rHMGB1-induced migration and invasion in PC3 cells. (A and B) PC3 cells were pre-treated with a RAGE antibody and then stimulated with 1 μg/ml rHMGB1. The migration and invasion of the PC3 cells was evaluated (magnification, x20). (C) Expression of EMT markers and MMPs in RAGE-neutralizing PC3 cells in response to rHMGB1. Two-way analysis of variance. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001. rHMGB1, recombinant high mobility group box 1; RAGE, advanced glycosylation end-product specific receptor; EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; VDR, vitamin D3 receptor; CTGF, connective tissue growth factor.

positively associated with tumor aggressiveness in rats and humans. Previous studies demonstrated that the mRNA level of HMGB1 is higher in Matlylu tumors, a highly aggressive rat prostate tumor, compared with that in the Dunning R-3327-H subline, a benign rat prostatic tumor (33,34). Leman *et al* (35) further confirmed this result in a transgenic mouse model, claiming that the expression of HMGB1 is only detectable in late-stage prostate cancer in mice. In human prostate cancer, several studies suggested that HMGB1 and RAGE are more overexpressed in metastatic cancer tissues compared with non-metastatic cases (14,36). Furthermore, high levels of HMGB1 have been reported to be associated with certain clinico-pathological features, including pathological stage, Gleason score, preoperative prostate-specific antigen concentration, biochemical recurrence, lymph node metastasis and distant metastasis (11,27,37).

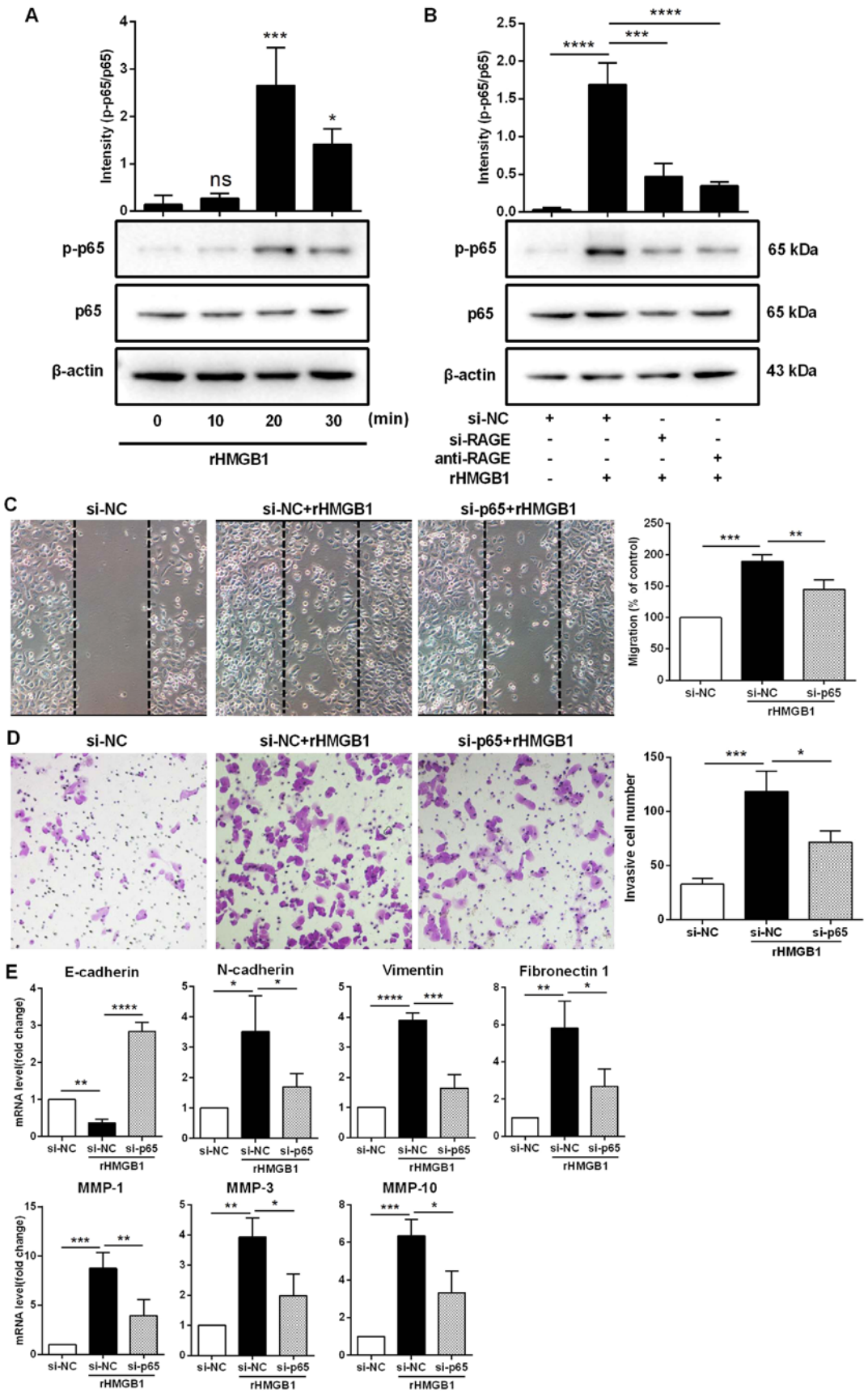

Figure 6. RAGE/NF-κB signaling pathway is responsible for HMGB1-mediated EMT. (A) Activation of phospho-NF-κB p65 in PC3 cells stimulated by rHMGB1 (1 μg/ml). (B) Inhibitory effect of RAGE-targeting siRNA and a RAGE antibody on the level of phosphorylated NF-κB p65 in rHMGB1-induced PC3 cells. (C) Migration and (D) invasion of NF-κB p65-silenced PC3 cells in response to rHMGB1 stimulation. (E) The mRNA expression of EMT markers and MMPs in NF-κB p65-silenced PC3 cells with or without rHMGB1 stimulation. Two-way analysis of variance. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001. rHMGB1, recombinant high mobility group box 1; RAGE, advanced glycosylation end-product specific receptor; EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; siRNA, small interfering RNA; ns, not significant; NC, negative control.

Certain mechanisms have been suggested to be responsible for the pathogenic effects of HMGB1 on prostate cancer metastasis. Firstly, HMGB1 has been demonstrated to activate sex steroid hormone receptors, including androgen receptor, which serves an essential role in the development of prostate cancer (38,39). In addition, androgen deprivation results in the secretion of HMGB1 from prostatic stromal cells and subsequently enhances the metastatic ability of prostate cancer cells (14). Secondly, HMGB1 directly interacts with Ets transcription factor, resulting in the enhancement of Ets target gene transcriptional activity (38,40). The Ets transcription factors have been confirmed to contribute to the angiogenesis and metastasis of prostate cancer (41). Thirdly, chronic inflammation has been reported as one of the key risk factors in prostate carcinogenesis in studies focusing on the histopathological, epidemiological and genetic epidemiological aspects (6). As a proinflammatory cytokine, extracellular HMGB1 recruits and activates T cells, which express cytokine lymphotoxin α1β2 (LT) on the cell surface, and macrophages to the tumor tissue, thus initiating an adaptive immune response, and ultimately enhancing the malignant progression via the LTβ-receptor pathway (42). Lastly, studies have suggested that HMGB1 serves an essential role in MMP generation, which modulates the tumor microenvironment and promotes cancer metastasis (6,24). For instance, one study revealed that HMGB1 induces MMP-9 expression and increases the cellular metastatic ability in non-small cell lung cancer cell lines (43). Furthermore, the present study results indicated that treatment of HMGB1 upregulated the secretion of MMP-1, -3 and -10 in prostate cancer PC3 cells, promoting tumor cell migration and invasion.

In the past decades, growing evidence has indicated that EMT is an association between inflammation and cancer metastasis (44). It is also recognized as a driver of cancer metastasis primarily by the provision of migratory and invasive properties to cells (44,45). Several studies reported that the infiltration of inflammatory mediators, including cytokines, chemokines and MMPs, in the tumor microenvironment facilitate the occurrence of EMT, aggravating cancer metastasis. For instance, as an essential transcription factor in inflammation and cancer, the activation of NF-κB caused by inflammatory mediators modulates the expression of transcription factors, including Twist1 and cell adhesion molecules, such as selectins and integrins, thereby facilitating EMT and further promoting metastasis (46). In addition, MMPs have been reported to remodel the extracellular matrix and trigger a cascade of molecular alterations, thus inducing EMT and promoting distant metastasis (17-19). For example, MMP-3 has been reported to cause a reduction in E-cadherin, catenin and cytokeratin, and upregulation of vimentin and endogenous MMPs. Another study also revealed that MMP-3 induces EMT by increasing the expression of Rho-related protein Rac1b and reactive oxygen species (47). Other MMPs, including MMP-1, -2, -9 and -14, have similar effects (24). The present study suggested that extracellular HMGB1 stimulated MMP-1, -3 and -10, and EMT marker expression levels, thus contributing to the migration and invasion of PC3 cells. However, the exact functions of MMP-1 and MMP-10 in cancer invasion require further investigation.

Based on previous studies and the results of the present study, we hypothesize that the tumorigenic effects of HMGB1 render this protein a therapeutic target in prostate cancer. The current data confirmed that the inhibition of the RAGE/NF-κB signaling pathway effectively suppressed HMGB1-induced EMT, and consequently reduced the migration and invasion of prostate cancer cells, which requires further verification in a transgenic adenocarcinoma mouse prostate (TRAMP) mouse model or other appropriate mouse models. A number of studies have also reported favorable results for inhibiting HMGB1 in prostate cancer. For example, in prostate cancer DU-145 cells, treatment with 18-α glycyrrhetinic acid, a natural HMGB1 inhibitor, inhibited proliferation by inducing apoptosis, downregulating the expression levels of NF-κB (p65), vascular endothelial growth factor and MMP-9, and upregulating the expression of non-steroidal anti-inflammatory gene-1 (48). Furthermore, using antisense and RNA interference technology, silencing HMGB1 gene expression exhibited effective results with regard to inhibiting the proliferation and metastasis of prostate cancer cells *in vitro* and *in vivo* in nude mice (12). Moreover, administration of HMGB1 antibody also yields therapeutic effects. In the TRAMP mouse model of prostate cancer, HMGB1 released from dying cells was shown to initiate an adaptive immune response that promoted prostate tumor aggressiveness, which could be suppressed by neutralizing HMGB1 (49). Taken together, these results show that HMGB1 can be a promising candidate for the treatment of prostate cancer.

In summary, the present study demonstrated that HMGB1 was highly expressed in metastatic prostate cancer samples and able to enhance the aggressiveness of PC3 cells. In addition to previously reported and proven mechanisms, we hypothesize that HMGB1 promotes the EMT process and upregulates the expression levels of MMP-1, -3 and -10 by activating the RAGE/NF-κB signaling pathway in PC3 cells, thereby facilitating cancer metastasis. Therefore, further studies on the roles of HMGB1 in the carcinogenesis and metastasis of prostate cancer may aid in developing novel therapeutic strategies for patients with prostate cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ, SShao and DH designed and performed the experiments, analyzed the data and wrote the manuscript. YX, DJ, JW and FY collected and provided patient samples, and analyzed the data of wound-healing assay and Transwell assay. YG, SShi and YL revised the manuscript and performed the CCK-8

assay. WQ in and WW conceived and supervised this study, provided critical suggestions and discussions throughout the study, and revised the manuscript with comments from co-authors.

Ethics approval and consent to participate

All analyses of human materials were approved by the Research Ethics Committee of the Xijing Hospital (Xi'an, Shaanxi, China), and conducted according to the principles of the Declaration of Helsinki. All patients provided written informed consent.

Consent for publication

All patients provided written informed consent for the purpose of the anonymized evaluation and publication of their data.

Competing interests

The authors declare that they have no competing interests.

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