

## Original Article

# HMGB1 promotes tumor progression and invasion through HMGB1/TNFR1/NF- $\kappa$ B axis in castration-resistant prostate cancer

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**Abstract:** Prostate cancer (PCa) is the most common male cancer. Most patients treated with androgen deprivation therapy progress to castration-resistant PCa. To overcome the limitations of this treatment, there is an urgent need to identify more effective treatment targets. High mobility group box 1 protein (HMGB1) is known to be associated with progression, metastasis, and poor prognosis of several solid tumors; however, its role in PCa remains unclear. Thus, we aimed to evaluate the clinical significance and biological roles and mechanism of HMGB1 in PCa. We showed that increased expression of HMGB1 correlated with increased risk of aggressive PCa, and high expression of HMGB1 was associated with poor biochemical recurrence-free survival in a Korean cohort. Additionally, the inhibition of HMGB1 expression significantly reduced cell proliferation, invasive capacity, and NF- $\kappa$ B signaling *in vitro*. Our results indicated that HMGB1 is a critical factor in the development and progression of PCa. Moreover, we found that HMGB1 directly interacts with TNFR1, and TNFR1 overexpression in HMGB1 knockdown cells reversed the effects of HMGB1 knockdown. Importantly, our results suggest that HMGB1 binding to TNFR1 promotes tumor progression by activating the NF- $\kappa$ B signaling pathway in PCa; therefore, the HMGB1/TNFR1/NF- $\kappa$ B signaling pathway could serve as a novel therapeutic target for improving PCa therapy.

**Keywords:** Prostate cancer, HMGB1, TNFR1, NF- $\kappa$ B

## Introduction

Prostate cancer (PCa) is the most common male cancer, and its incidence has been increasing over the last few decades [1]. Patients with locally advanced, metastatic, or recurrent PCa receive androgen deprivation therapy after definitive treatment. However, most of the patients eventually progress to castration-resistant PCa (CRPC), followed by metastasis [2]. The prognosis for CRPC is extremely poor, so more effective treatment strategies are urgently needed [3]. It is imperative to elucidate the underlying mechanism of CRPC progression, such as the role of the important genes associated with CRPC, to discover effective therapeutic and diagnostic targets of CRPC.

High mobility group box 1 protein (HMGB1), a chromatin-associated, non-histone nuclear pro-

tein, has been reported to play multiple roles, including transcription, DNA repair, replication, and genomic stability, upon its interaction with DNA [4]. HMGB1 can also play a cytokine-related role in cell death and survival during inflammation by binding to multiple receptors, including those for advanced glycation end products (RAGE) and the toll-like receptor family (TLR; TLR2, TLR4, and TLR9) [5, 6]. This binding contributes to the production of multiple pro-inflammatory cytokines, including IL-6, IL-8, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) via the activation of NF- $\kappa$ B pathways [7]. In addition, aberrant expression and release of HMGB1 correlate with cancer cell survival and metastasis in different solid tumors, including gastric, colon, and bladder cancers, by modulating gene transcription and several signaling pathways [8-11]. Although it has been previously reported that the overexpression of HMGB1 is associated

with tumor progression, metastasis formation, and poor prognosis in PCa [12, 13], there have been only a few studies on the roles and potential targets of HMGB1 in PCa [14, 15]. Thus, studies on the role and mechanism of HMGB1 in PCa for more effective treatment strategies are needed.

In this study, we examined the different HMGB1 expression patterns in benign prostatic hyperplasia (BPH), low-, intermediate-, and high-risk PCa tissues, and identified the prognostic significance of HMGB1 expression. We also explored the biological role of HMGB1 in the growth and invasion of PCa cells. Notably, HMGB1 activates the NF- $\kappa$ B signaling pathway through its interaction with TNFR1.

Our study indicated that HMGB1 plays an important role in PCa progression and metastasis by regulating the HMGB1/TNFR1/NF- $\kappa$ B signaling pathway. The HMGB1/TNFR1/NF- $\kappa$ B axis could be a crucial target for the diagnosis and treatment of PCa.

### Materials & methods

#### *Patient samples*

This study was approved by the Institutional Review Board of the Catholic University of Korea, Seoul St. Mary's Hospital (IRB No. KC18SESI0795). De-identified formalin-fixed, paraffin-embedded (FFPE) tissues and their corresponding clinical information were obtained from the Korean Prostate Bank, at nationally designated biobank that is regularly inspected by the Ministry of Health and Welfare of Korea. All biomaterials were procured with informed consent. Prostate tissues from patients with low- (n = 22), intermediate- (n = 41), and high-risk (n = 36) PCa, categorized by the criteria of the National Comprehensive Cancer Network risk group [16], as well as patients with BPH (n = 28) were used for this study. Tumor tissue and the corresponding adjacent prostate tissue were collected separately from PCa patients. All patients with PCa underwent radical prostatectomy, and their median follow-up duration was 38 (range: 6-55) months. We analyzed baseline demographics, clinicopathologic results, and follow-up outcomes.

#### *Immunohistochemistry*

Immunohistochemistry (IHC) was performed to confirm HMGB1 expression in the prostate tissues. The FFPE tissue sections were blocked and incubated with anti-HMGB1 antibody (Abcam, Cambridge, England) overnight at 4°C. After washing, the samples were incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) for 2 h at 25°C and counterstained with hematoxylin. Digital images were obtained using an Olympus BX50 optical microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Areas positively stained with HMGB1 (%) were measured using ParaView 1.15.1 (Sandia Corporation, Kitware Inc., Albuquerque, NM, USA).

#### *Cell lines and culture*

RWPE1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in complete Keratinocyte-serum free medium (K-SFM) (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5 ng/mL epidermal growth factor and 50  $\mu$ g/mL bovine pituitary extract. DU145, PC3, LNCaP, and HEK293 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). PC3, and LNCaP cells were routinely maintained in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin, while DU145 and HEK293 cells were routinely maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

#### *Transfection*

Small-interfering RNAs (siRNAs) against HMGB1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc. CA, USA). An endoribonuclease-prepared siRNA (esiRNA) against human HMGB1 and a control esiRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). DU145 and PC3 transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Plasmid vectors (human HMGB1-Flag, HG10326-CF; tumor necrosis factor receptor-1/TNFR1-HA, HG10872-CY; TNFR3-HA, HG10581-CY; TNFR5-HA, HG10872-CY; and

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**Table 1.** PCR primer sequences

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
HMGB1	CCTTCTTCTTCTGCTGAGTA	CATAAGGCTGCTTGCATCTGC
TNFRSF1A	GCTTCAGTCCCACTCCAGGC	CGCTGTCCTCCCACTTCTGA
TNFRSF3	GCCCATCCATACTCCCTGAC	GGTCCAAGTCCGCGCTCC
TNFRSF5	GTCCATCCAGAACCACCCACT	GTGGCAGTGTCTCTCTGTTC
β-actin	GAGACCTTCAACCCCGAGC	ATGAGGTAGTCAGTCAGGTCCC

### Western blotting

Forty-eight hours after transfection, cells were harvested and lysed with RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protease inhibitor. Protein concentrations were measured using a BCA Protein

Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Proteins were separated using SDS-PAGE and transferred onto nitrocellulose membranes. Following the transfer, membranes were blocked and incubated with primary antibodies against ERK (Cell Signaling Technology), phospho-ERK (Cell Signaling Technology), Akt (Cell Signaling Technology), phospho-Akt (Cell Signaling Technology), Ikkβ (Abcam), cleaved-caspase-3 (Cell Signaling Technology), p65 (Abcam), and β-actin (1:2500, Santa Cruz) at 4°C overnight. The membranes were washed and incubated with a secondary antibody conjugated to HRP for 2 h at room temperature. Chemiluminescence (Amersham, Arlington Heights, IL, USA) was used to visualize the protein bands.

pCMV3 Negative Control, CV013) were purchased from Sino Biological (Sino Biological, Inc., Beijing, China). Transfection with plasmids was carried out using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

### Cell viability

Cells (DU145,  $8 \times 10^3$ /well; PC3,  $1 \times 10^4$ /well) were seeded in 96-well plates, then were transfected with siRNAs in a CO<sub>2</sub> incubator at 37°C. At 0, 24, 48, and 72 h post-transfection, cell viability was measured using EZ-CYTOX (Daeil Lab Service Co. Ltd, Seoul, Korea) according to the manufacturer's instructions.

### Cell invasion assay

Cell invasion assays were performed using 24-well transwell chambers (8 μm, Corning Inc., NY, USA), and the upper chambers were coated with 25 μg/ml Matrigel (Corning). Cells were added to the pre-coated upper chambers at a density of  $5 \times 10^4$ /well. Subsequently, the lower chambers were filled with culture medium containing 20% FBS. After incubation in a CO<sub>2</sub> incubator for 48 h at 37°C, the cells on the lower surface of the upper chamber were fixed and stained with 0.1% crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA). The membranes were observed under light microscopy and dissolved in 20% acetic acid. The solubilized stain was measured at 570 nm.

### Flow cytometry

Forty-eight hours after transfection, cells were harvested and fixed with 70% cold ethanol at 4°C for 1 h. After incubating in 10 μg/mL propidium iodide and 10 mg/mL RNase at room temperature for 30 min in the dark, the percentage of cells in each cell cycle phase was determined using a FACS Canto II system (BD Biosciences, San Jose, CA, USA).

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA using the Prime Script™ RT reagent Kit (Takara Bio Inc., Shiga, Japan). The cDNA products were amplified by PCR. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized using a Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA). The DNA sequences of PCR primer pairs are shown in **Table 1**.

### Proteome profiling

The Proteome Profiler™ Human NF-κB Pathway Array (ARY029) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Proteome profiler antibody microarray analysis was performed according to the manufacturer's protocol. Briefly, 72 h after transfection, 500 μg of each sample was incubated overnight at 4°C on the dot blot membrane of the human NF-κB pathway array. The membrane was washed with 1X washing buffer and incu-

bated with a reconstituted detection antibody cocktail and HRP-conjugated streptavidin. Membranes were exposed to X-rays film, and the mean intensities of each spot were quantified using Image-J software (Rasband W; National Institutes of Health, Bethesda, MD, USA: <http://rsbweb.nih.gov/ij/index.html>).

#### *Co-immunoprecipitation (Co-IP)*

For the co-immunoprecipitation (Co-IP) assay, plasmids were transfected into HEK293 cells using Lipofectamine 3000 reagent. One day after transfection, cells were harvested and whole-cell extracts were incubated with Dynabead Protein G (Invitrogen) conjugated to an antibody against Flag overnight at 4°C. After washing the beads and eluting with 2X sample buffer, the immunoprecipitated sample was subjected to SDS-PAGE. Western blotting was conducted to detect HA (Abcam) and Flag (Abcam).

#### *Statistical analysis*

GraphPad Prism Software v5 (GraphPad Prism Software Inc., San Diego, CA, USA) and SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analyses. Data are expressed as means ( $\pm$  standard deviations) for continuous variables and number of patients (proportions) for dichotomous variables. Differences between groups were examined using the independent t-test, one-way analysis of variance test followed by Tukey's post-test, and the chi-squared test. Kaplan-Meier analysis with a log-rank test was performed to evaluate biochemical recurrence (BCR)-free survival. Values of  $P < 0.05$  were considered to be statistically significant.

## **Results**

### *HMGB1 is overexpressed in PCa tissues and associated with adverse pathologic features and poor prognosis*

We evaluated the expression of HMGB1 in BPH, PCa, and adjacent normal tissues. Representative images of HMGB1 expression in BPH, low-, intermediate-, and high-risk PCa tissue are shown in **Figure 1A**. Relative to the adjacent normal prostate tissues, the expression of HMGB1 was significantly higher in PCa tissues. The proportion of positive HMGB1 expression was significantly different among patients with

BPH, low-, intermediate-, and high-risk PCa ( $6.6 \pm 3.7\%$ ,  $11.2 \pm 3.5\%$ ,  $19.0 \pm 4.9\%$ ,  $23.4 \pm 8.2\%$ , respectively;  $P < 0.001$ ).

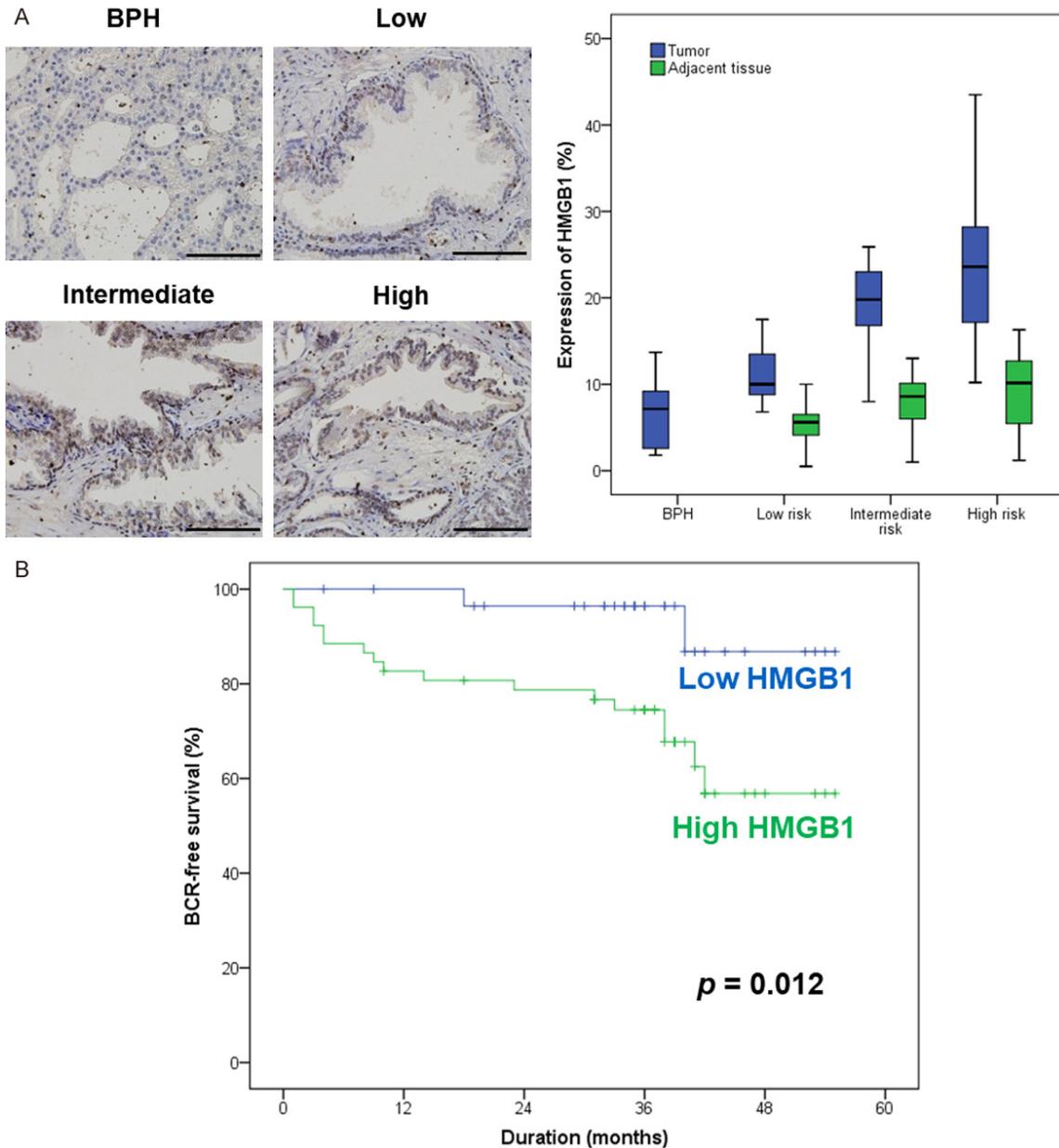
**Table 2** shows the baseline demographics and clinicopathological characteristics of the study patients according to HMGB1 expression levels. Most of the patients with BPH had low HMGB1 expression levels. In contrast 65.7% of the patients with PCa had high HMGB1 expression levels ( $P < 0.001$ ). Patients with high HMGB1 expression levels had advanced pathologic tumor stages ( $P = 0.093$ ), higher pathologic Gleason scores ( $P < 0.05$ ), and more perineural invasion ( $P < 0.010$ ) relative to those with low HMGB1 expression levels.

To explore the prognostic significance of HMGB1 in PCa, Kaplan-Meier analysis with a log-rank test was performed (**Figure 1B**). The BCR-free survival of the low-HMGB1-expression cohort was significantly longer than that of the high-HMGB1-expression cohort ( $P < 0.05$ ).

### *Suppression of HMGB1 expression inhibits PCa cell proliferation*

To confirm the data obtained from patient tissues, we first evaluated the difference in HMGB1 expression between human PCa cell lines (DU145, PC3, and LNCaP) and a normal prostate epithelial cell line (RWPE-1) using western blotting. HMGB1 expression was upregulated in all three PCa cell lines compared with the normal prostate epithelial cell line (**Figure 2A**). To characterize the function of HMGB1 in PCa cells, we suppressed endogenous HMGB1 expression in the DU145 and PC3 cells using siRNA (**Figure 2B**). This down-regulation of HMGB1 expression resulted in decreased cell proliferation and cell invasion (**Figure 2C** and **2D**). To rule out the off-target effects, we measured cell proliferation after treatment another siRNA (HMGB1 esiRNA) and performed a rescue experiment with HMGB1 add-back (**Supplementary Figure 1**). We confirmed that decreased cell proliferation was due to the suppression of the HMGB1 expression, not due to the off-target effect of siRNA. As shown in **Figure 2E**, the down-regulation of HMGB1 expression changed the cell-cycle pattern in PCa cells. The percentage of sub-G1 phase cells significantly increased after suppressing endogenous HMGB1 expression (DU145,  $16.70 \pm 1.22\%$ ; PC3,  $13.93 \pm 1.58\%$ ) compared to those in the control groups (DU145,  $4.23 \pm$

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**Figure 1.** HMGB1 in benign prostatic hyperplasia (BPH) and prostate cancer (PCa) tissues. A. Expression patterns of HMGB1 in BPH and PCa patient tissues were confirmed by IHC and representative images (left) for each group are shown (scale bar size, 100 μm; magnification 400 ×). HMGB1 shown as brown, and nuclei were counterstained. HMGB1 expression quantified as HMGB1-positive areas, as well as paired normal control prostate tissues (right,  $P < 0.001$ ). B. Biochemical recurrence (BCR)-free survival according to HMGB1 expression level quantified via Kaplan-Meier analysis in patients with PCa in a Korean cohort.

2.45%; DU145-NC,  $3.76 \pm 1.15\%$ ; PC3,  $2.76 \pm 1.02\%$ , and PC3-NC,  $3.26 \pm 1.10\%$ ;  $P < 0.05$ ). In addition, down-regulation of HMGB1 expression caused a decrease in phospho-Akt and phospho-ERK expression. In contrast, cleaved-caspase 3 expression increased after the suppression of endogenous HMGB1 in DU145 and PC3 cells (Figure 2F). Taken together, our

results suggest that HMGB1 is associated with tumor growth, survival, and metastasis in PCa.

### *Suppression of HMGB1 expression inhibits the NF-κB pathway in PCa cells*

NF-κB has been shown to play a pivotal role in cancer cell proliferation, progression, and

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**Table 2.** Baseline demographics and pathologic outcomes according to the expression of HMGB1

	Overall	Low HMGB1	High HMGB1	P-value
Diagnosis (%)				< 0.001
BPH	28 (22.0)	26 (92.9)	2 (7.1)	
PCa	99 (78.0)	34 (34.3)	65 (65.7)	
Age, yrs (SD)	68.7 (7.0)	69.4 (7.5)	67.6 (6.0)	0.148
BMI, kg/m <sup>2</sup> (SD)	23.6 (2.9)	23.4 (2.5)	23.8 (3.1)	0.428
PSA, ng/mL (SD)	13.0 (19.6)	11.3 (20.7)	15.6 (16.7)	0.197
Prostate vol (mL)	43.4 (23.3)	49.5 (26.7)	35.9 (15.9)	0.002
pT stage (%)				0.093
2a	5 (5.1)	4 (11.8)	1 (1.5)	
2b	2 (2.0)	0 (0)	2 (3.1)	
2c	53 (53.5)	19 (55.9)	34 (52.3)	
3a	12 (12.1)	1 (2.9)	11 (16.9)	
3b	24 (24.2)	9 (26.5)	15 (23.1)	
4	3 (3.0)	1 (2.9)	2 (3.1)	
pN stage (%)				0.127
x	53 (53.5)	22 (64.7)	31 (47.7)	
0	27 (27.3)	9 (26.5)	18 (27.7)	
1	19 (19.2)	3 (8.8)	16 (24.6)	
pGleason score (%)				0.010
≤ 6	16 (16.2)	11 (32.4)	5 (7.7)	
7 (3 + 4)	38 (38.4)	10 (29.4)	28 (43.1)	
7 (4 + 3)	30 (30.3)	7 (10.6)	23 (35.4)	
≥ 8	15 (15.2)	6 (17.6)	9 (13.8)	
Tumor volume (%)				0.296
≤ 1 cc	14 (14.1)	5 (14.7)	9 (13.8)	
1-5 cc	37 (37.4)	16 (47.1)	21 (32.3)	
> 5 cc	48 (48.5)	13 (38.2)	35 (53.8)	
Lymphovascular inv. (%)	19 (19.2)	4 (11.8)	15 (23.1)	0.175
Perineural inv. (%)	71 (71.7)	18 (52.9)	53 (81.5)	0.003

metastasis in PCa cells [17]. The association between HMGB1 and NF-κB signaling has been widely evaluated in several solid cancers and inflammation, but rarely in PCa [18]. For this reason, we investigated whether HMGB1 down-regulation could affect NF-κB signaling in PCa cells using a human NF-κB proteome profiler array. As shown in **Figure 3A**, HMGB1 down-regulation changed expression levels of different proteins involved in the NF-κB pathway. Important genes related to the NF-κB pathway, including p65 and IKK-β, were decreased in HMGB1 down-regulated cells compared to those in control cells, while expression levels of p53 and phospho-p53 increased. Western blotting also confirmed that the down-regula-

tion of HMGB1 decreased the levels of p65 and IKK-β in the DU145 and PC3 cell lines (**Figure 3B**). These results indicate that the down-regulation of HMGB1 in PCa cells suppresses growth by regulating the expression of proteins related to the NF-κB pathway.

### *HMGB1 is a novel ligand of TNFR1*

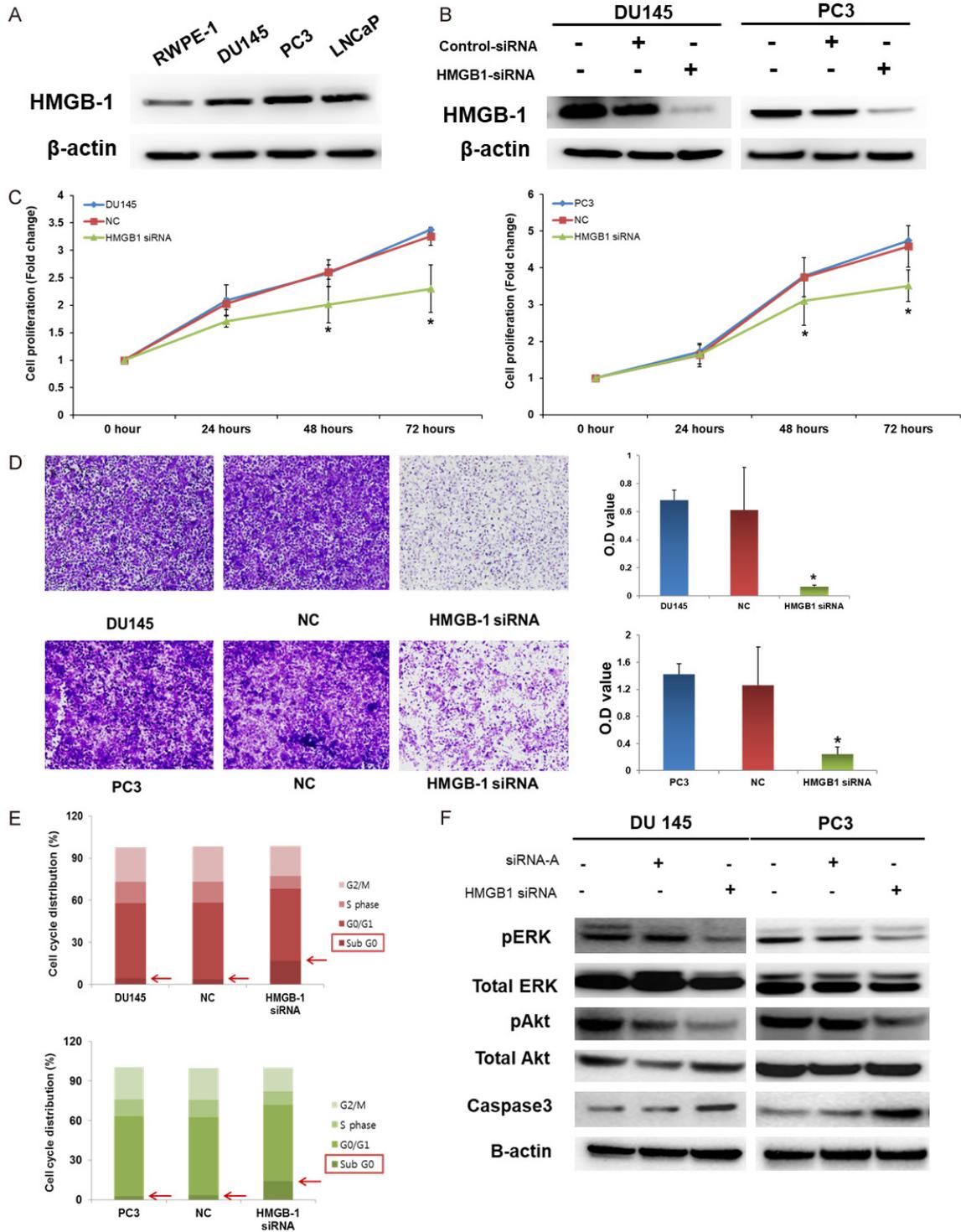
In the human NF-κB proteome profiler array, we evaluated the changes in the NF-κB pathway mediated by HMGB1 in PCa. Interestingly, the down-regulation of HMGB1 suppressed the expression of TNFR1, TNFR3, and TNFR5 (**Figure 3A**). As shown in **Figure 4A**, RT-PCR and western blotting results were consistent with those of the proteome profiler array. Furthermore, recent studies have shown that TNF-α/TNFR-mediated signaling plays an important role in cell survival and therapy resistance [19], and that HMGB1 stimulates TNF-α/TNFR-mediated signaling [20]. However, to our knowledge, no study has been conducted to examine

the interaction between TNFR and HMGB1. Therefore, to identify the TNFR responsible for HMGB1 binding, we performed Co-IP on HEK293 cells extracts following co-transfection with FLAG-HMGB1 and HA-TNFR1, 3, or 5. As shown in **Figure 4B**, HMGB1 co-immunoprecipitated with TNFR1 but not with TNFR3 or TNFR5. Taken together, these findings indicate that TNFR1 interacts physically with HMGB1.

### *HMGB1 binding to TNFR1 increases cell survival in PCa*

To validate the role of the HMGB1/TNFR1 axis in HMGB1-mediated NF-κB signaling, we examined whether TNFR1 overexpression would

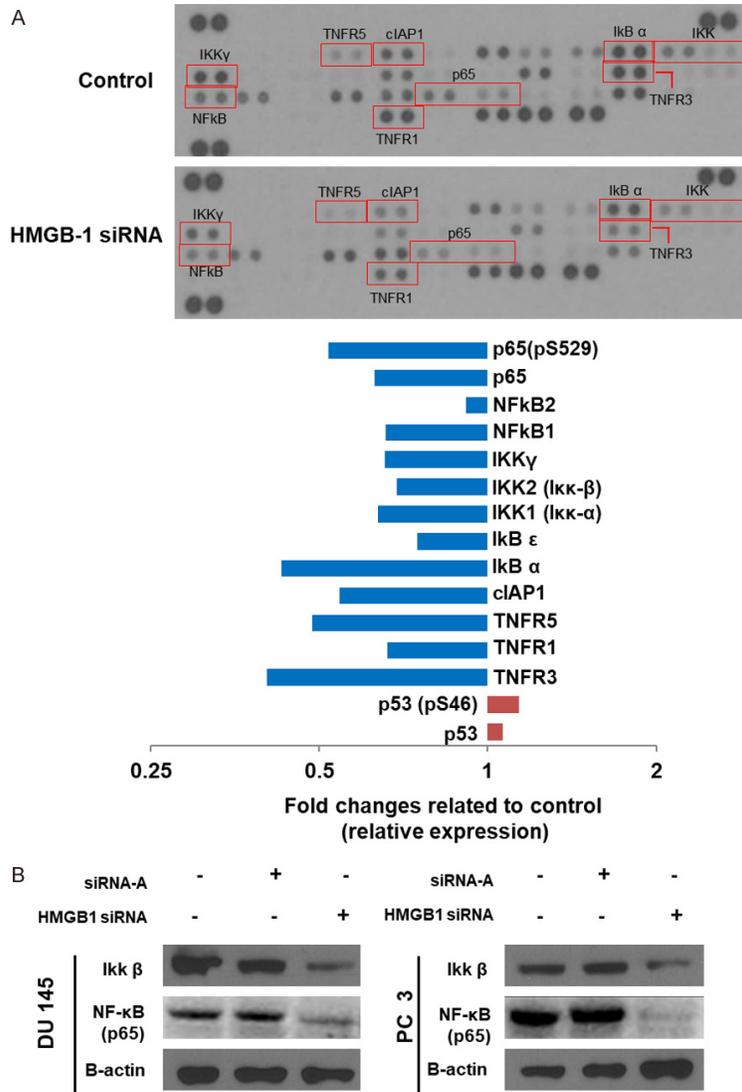
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**Figure 2.** Suppression of HMGB1 expression mediates the response of prostate cancer cells. **A.** HMGB1 expression levels in a normal prostate cell line (RWPE-1) and prostate cancer cell lines (LNCaP, DU145 and PC3). **B.** HMGB1 knockdown in DU145 and PC3 cells transfected with HMGB1 or negative control (NC) siRNAs. HMGB1 expression was determined by western blotting and normalized to that of  $\beta$ -actin. **C.** Cell proliferation in DU145 and PC3 cells after transfection with HMGB1 or NC siRNA. Cell proliferation is shown as fold-change ( $*P < 0.01$  compared with NC). No significant difference between non-transfected cells and NC. **D.** Cell invasion assays using the DU145 and PC3 cells following transfection with HMGB1 or NC siRNA. Representative images are shown for each group (X200). Graph of invasive ability was produced by calculating the optical density of migrated cells using 20% acetic acid ( $*P < 0.01$  compared with NC). **E.** Proportion of sub G0, G0/G1, S, and G2/M phase cells in the PC3 and DU145

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cells after transfection with HMGB1 or NC siRNA. Cell cycle distribution analysis was performed via FACS analysis of propidium iodide-stained cells. The histograms and data show the percentage of cells in different cell cycle stages. F. ERK, p-ERK, AKT, p-AKT, caspase-3, and β-actin expression in DU145 and PC3 cells after transfection with HMGB1- or NC siRNAs.



**Figure 3.** Suppression of HMGB1 expression alters the NF-κB signaling pathway. A. Several NF-κB factors evaluated using a proteome profiler array kit after 48 h of transfection with HMGB1 or NC siRNA, quantitative analysis of the spots was performed via densitometry and fold-changes compared to internal controls (mean) were considered significant. B. IKK-β, NF-κB (p65), and β-actin in DU145 and PC3 cells after transfection with HMGB1 or NC siRNA.

reverse the effects of HMGB1 knockdown in PCa cells. We co-transfected the cells with a HMGB1 siRNA and a TNFR1 vector, and confirmed p65 expression. Following the induction of TNFR1 overexpression in HMGB1 knockdown cells, immunoblotting showed a reversal

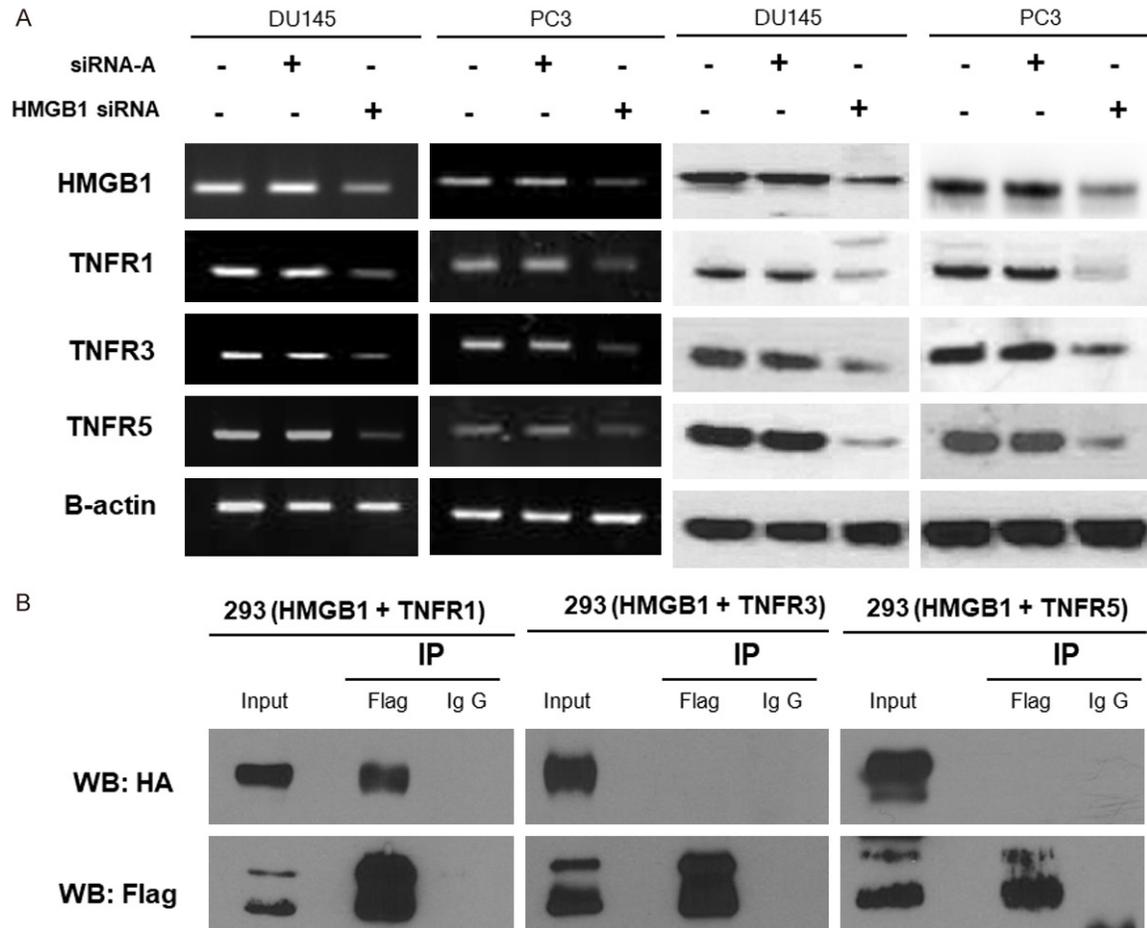
of p65 expression compared to that in HMGB1 knockdown cells, in which TNFR1 was not overexpressed (**Figure 5A**). To determine whether HMGB1-mediated TNFR1 influences cell viability, we assessed cell viability after co-transfection with HMGB1 siRNA and TNFR1 vector. Consistent with the above results, cell proliferation in HMGB1 knockdown cells was significantly decreased compared with that in the other groups (\* $P < 0.01$ ). In contrast, following the co-transfection of HMGB1 siRNA and TNFR1 vector, TNFR1 overexpression in the HMGB1 knockdown cells reversed the effects of the HMGB1 knockdown (**Figure 5B**). These data suggest that HMGB1 mediates NF-κB signaling in PCa cells via physical interaction with TNFR1, which promotes the survival and invasion of PCa cells (**Figure 6**).

### Discussion

HMGB1 has been shown to be associated with inflammation and cancer [7-10]. In PCa, HMGB1 has been reported to be highly expressed and associated with tumor progression, metastasis, and poor prognosis [12, 13]. However, the specific role and molecular mechanisms of HMGB1 in PCa development and progression remain poorly understood [14,

15]. This study revealed the clinical correlation and molecular role of HMGB1 in PCa. Interestingly, to our knowledge, our study is the first to provide novel evidence regarding the association between the HMGB1/TNFR/NF-κB signaling pathway and PCa.

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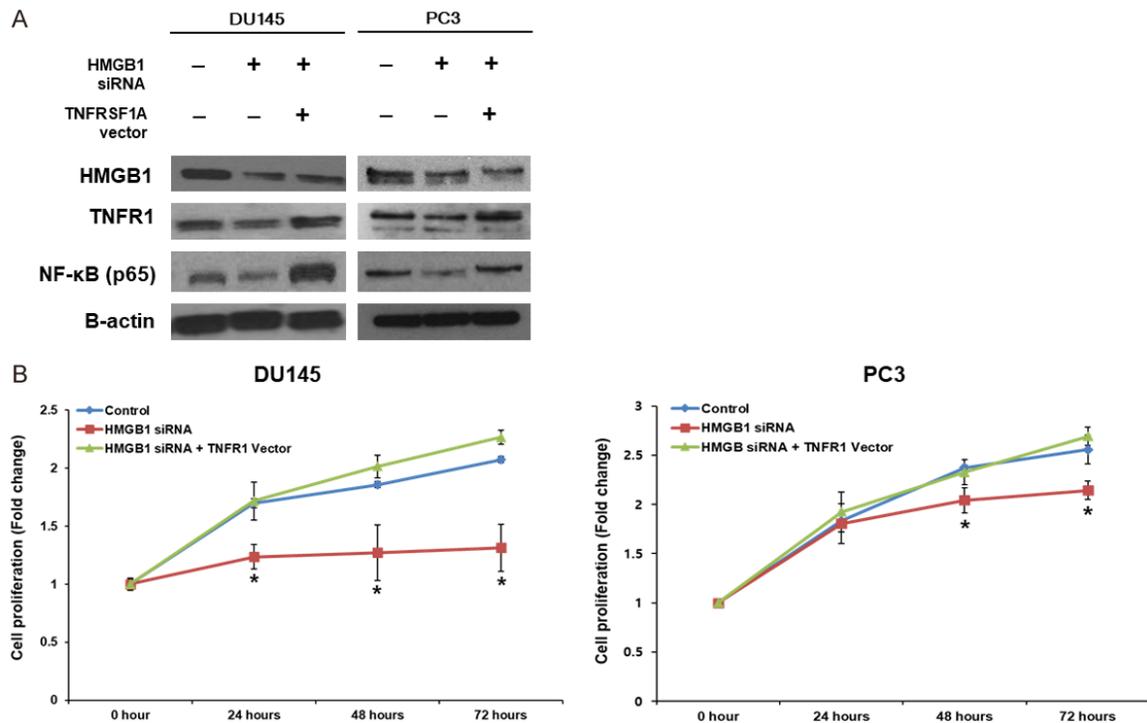
**Figure 4.** Identification of the interaction between HMGB1 and TNFRSF1A. **A.** Following HMGB1 knockdown in DU145 and PC3 cells using a siRNA for 48 h, HMGB1, TNFR1, TNFR3, TNFR5, and  $\beta$ -actin were analyzed by RT-PCR and western blotting. **B.** A total of 239 cells were co-transfected with HMGB1 plasmid vector and the indicated TNFR plasmid vectors. Forty-eight hours after transfection, cell lysates were precipitated with anti-Flag in conjunction with Dynabead Protein G and detected via western blotting using anti-HA (top lane) and anti-Flag (bottom lane).

We performed IHC to identify HMGB1 expression patterns in patient tissues and to explore the clinical significance of HMGB1 in PCa. Several researchers have reported that high HMGB1 expression is associated with pathological features, such as increased Gleason score, pathological stage, PSA level, and metastasis [21-23]. However, most of the previous studies did not analyze the expression pattern according to the stage and nuclear grade of PCa. This study is the first to show a distinct tissue-specific expression pattern in BPH, PCa, and adjacent normal tissues according to low-, intermediate-, and high-risk groups. We found that HMGB1 was highly expressed in PCa tissues compared to BPH tissues, and that HMGB1 expression increased according to increases in PCa aggressiveness. Moreover,

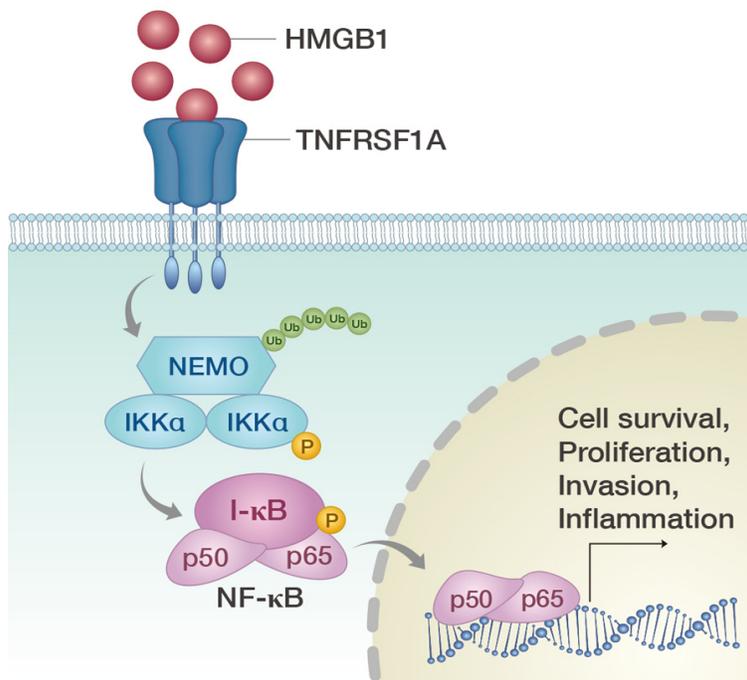
high expression of HMGB1 was found to be associated with poor BCR-free survival in the current Korean cohort. To elucidate the biological role of the increased expression of HMGB1 in PCa, we performed diverse assays using silenced HMGB1 expression in PCa cells. In accordance with previous studies [13], our results showed that the down-regulation of HMGB1 in PCa cells led to decreased cell proliferation and invasion, indicating that HMGB1 may promote PCa development and progression.

Regarding the molecular mechanism of HMGB1, we focused on the NF- $\kappa$ B pathway. Previous studies have demonstrated that NF- $\kappa$ B pathways are important for PCa cell growth, invasion, and the development of treat-

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**Figure 5.** TNFR1 overexpression in the HMGB1 knockdown cells reverses the effects of HMGB1 knockdown. A. Effect of TNFR1 overexpression in the HMGB1 knockdown cells. DU145 and PC3 cells were co-transfected with HMGB1 siRNA and/or TNFR1 plasmid vector. HMGB1, TNFR1, p65, and  $\beta$ -actin expression levels were analyzed via western blotting. B. Cell proliferation of DU145 and PC3 cells following co-transfection with HMGB1 siRNA and TNFR1 plasmid vector was evaluated by a WST assay. Control cells were co-transfected with NC siRNA and control plasmid vector. Cell proliferation is shown as fold-change (\* $P < 0.01$  compared HMGB1 siRNA + TNFR1 vector group). No significant difference in cell proliferation was found between HMGB1 siRNA + TNFR1 vector group and control group.



**Figure 6.** Schematic model of a hypothesized mechanism by which HMGB1 binding to TNFR1 stimulates cell proliferation and invasion through classical NF- $\kappa$ B signaling in prostate cancer.

ment resistance [11, 17, 24]. Moreover, numerous researchers have demonstrated that the HMGB1/NF- $\kappa$ B signaling pathway plays an important role in inflammation and several solid cancers [25-27]. Therefore, we performed a human NF- $\kappa$ B proteome profiler array. HMGB1 blocking in PCa decreased the expression of I $\kappa$  $\alpha$ , cIAP1, I $\kappa$ B- $\epsilon$ , I $\kappa$ B- $\alpha$ , p65, and IKK- $\beta$ , whereas the expression of phospho-p53 and p53 was increased (**Figure 3A**). Activation of the NF- $\kappa$ B

signaling pathway induces cytokine release and differential transcription modification, which contribute to an activated immune response, cancer initiation, progression, and metastasis [28-30]. Phosphorylated IKK complex leads to the phosphorylation-induced degradation of I $\kappa$ B. The degradation of I $\kappa$ B then induces the translocation of p65 to the nucleus. This process triggers NF- $\kappa$ B signaling activation [30]. Our results also showed that blocking HMGB1 expression in PCa decreased the expression of p65 and IKK- $\beta$  in PC3 and DU145 cells, and that HMGB1 promotes the proliferation and invasion of PCa cells by regulating the NF- $\kappa$ B signaling pathway.

HMGB1 was previously reported to promote diverse pathological and physiological functions through its receptors, including RAGE, TLRs, TIM3, and CXCR4 [31]. Specifically, HMGB1-RAGE and HMGB1-TLR signaling contribute to tumor cell proliferation and metastasis by inducing NF- $\kappa$ B activation in cancer [32, 33]. This implies that HMGB1 may be involved in NF- $\kappa$ B activation by interacting with multiple receptors in cancer. Interestingly, we found that the knockdown of HMGB1 decreased TNFR expression in PCa cells (**Figures 3A and 4A**). TNFR, including TNFR1, TNFR2, TNFR3, TNFR5, and CD95 (TNFR6), are cytokine receptors, and their receptor-ligand interactions regulate a variety of pathways, such as immune processes, tissue homeostasis, survival, proliferation, and death [34]. We investigated whether HMGB1 binds to TNFR and stimulates the NF- $\kappa$ B signaling pathway. Until recently, whether HMGB1 interacts with TNFR and affects NF- $\kappa$ B signaling has not yet been clarified. Thus, we conducted a Co-IP assay for TNFR1, 3 and 5, and confirmed that HMGB1 directly binds to TNFR1 only. This result suggests that TNFR1 acts as a receptor for HMGB1. In addition, TNFR1 overexpression in HMGB1 knockdown cells reversed the effects of HMGB1 knockdown and down-regulated p65 expression. Taken together, these data suggest that HMGB1 binding to TNFR1 could activate NF- $\kappa$ B signaling in PCa.

Overall, we identified that HMGB1 is a critical factor in the progression and metastasis of PCa *in vitro* and in patients with tumors. Notably, to our knowledge, this is the first study showing that HMGB1 binding to TNFR1 could

activate NF- $\kappa$ B signaling in PCa. Our study provides a foundation for further investigation on the potential of HMGB1/TNFR1/NF- $\kappa$ B signaling as an effective strategy for the treatment of PCa.

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### Disclosure of conflict of interest

None.

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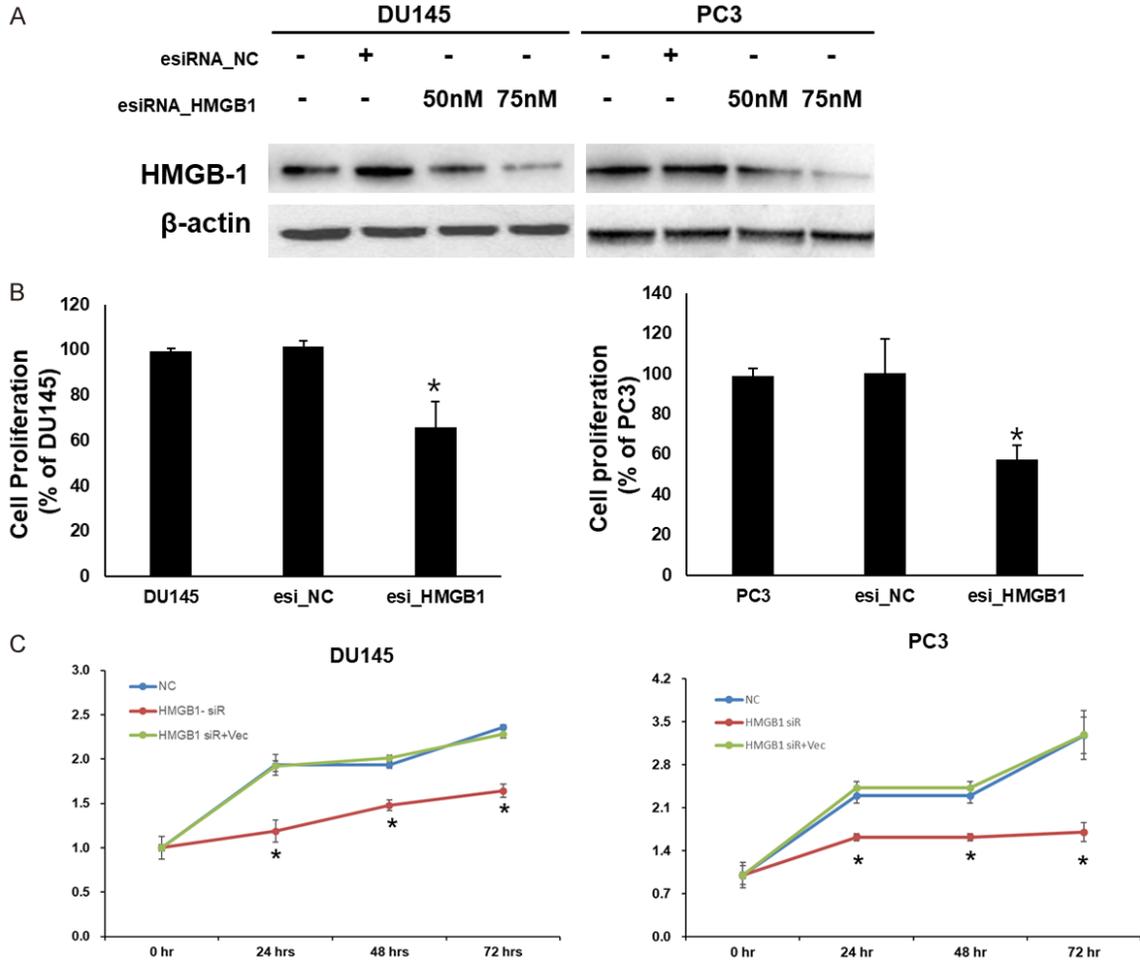
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**Supplementary Figure 1.** Control experiments to rule out off-target effects of siRNA. A. DU145 and PC3 cells transfected with HMGB1- or NC esiRNA have decreased HMGB1 expression. B. Cell proliferation after transfection with HMGB1 esiRNA (75 nM) evaluated by cell counts. Knockdown of HMGB1 expression using HMGB1 esiRNA decreased proliferation, consistent with previous results. C. Co-transfection of HMGB1 siRNA and HMGB1 vectors, with control vector and control siRNA as negative controls, respectively. Cell proliferation after co-transfection with HMGB1 siRNA and HMGB1 vector was evaluated by the WST assay, showing that the effect of the HMGB1 siRNA was significantly reversed by the HMGB1 vector. Cell proliferation is shown as fold-change (\* $P < 0.01$  compared NC control).