HMGB1/TNFR1/NF-κB Axis in Prostate cancer: Clinical and Biological Correlation

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Research

Keywords: Prostate cancer, HMGB1, TNFR1, NF-kappa B

Posted Date: July 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-40868/v1

Abstract

Background

High mobility group box 1 protein (HMGB1) is known to be associated with progression, metastasis, and poor prognosis of several solid tumors, but the role of HMGB1 in prostate cancer (PCa) has remained unclear. Thus, we aimed to evaluate the clinical significance and the biological mechanism of HMGB1 in PCa.

Methods

To explore the clinical significance of HMGB1 in PCa, we performed IHC analyses using paraffin-embedded tissues from patients with low-, intermediate-, and high-risk PCa and from patients with BPH. Biological role and mechanism of HMGB1 in PCa were analyzed using cell viability, cell cycle, Western blot analyses, proteome profiler antibody array, and co-immunoprecipitation assay.

Results

We showed that increases in the expression of HMGB1 correlated with an increased risk of aggressive PCa, and high expression of HMGB1 was associated with poor biochemical recurrence-free survival in a Korean cohort. The inhibition of HMGB1 expression significantly reduced cell proliferation and increased cell cycle arrest in the sub-G0 phase. It also inhibited the invasive capacity of PCa cells *in vitro*. The above processes were mediated through the binding with TNFR1, leading to tumor progression by activation of the NF-κB signaling pathway.

Conclusions

We identified that HMGB1 is a critical factor in the development and progression of PCa by regulating the HMGB1/TNFR1/NF-κB signaling pathway. HMGB1/TNFR1 could serve as a novel therapeutic target for improving PCa therapy.

1. Background

Prostate cancer (PCa) is the most common male cancers and the incidence of PCa has been increasing over the last few decades [1]. Patients with locally advanced or metastatic PCa or recurrent PCa after definitive treatment receive androgen deprivation therapy. However, most of the patients eventually progress to castration-resistant PCa (CRPC) followed by metastasis [2]. The prognosis for CRPC is extremely poor and identifying more effective treatment strategies is 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 the effective therapeutic and diagnostic targets of CRPC.

High mobility group box 1 protein (HMGB1), a chromatin-associated non-histone nuclear protein, has been reported to play multiple roles, including in transcription, DNA repair, replication, and genomic stability, through interaction with DNA [4]. HMGB1 can also play as a cytokine related role in cell death and survival on inflammation through binding with multiple receptors, including receptor 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 different pro-inflammatory cytokines, such as IL-6, IL-8, and tumor necrosis factor-α (TNF-α) via the activation of NF-κB pathways [7]. Also, the release and aberrant expression of HMGB1 correlates with cancer cell survival and metastasis in different solid tumors, including gastric cancer, colon cancer, and bladder cancer, by modulating gene transcription and several signaling pathways [8–10]. Although it has been previously reported that the overexpression of HMGB1 is associated with tumor progression, metastasis formation, and poorer prognosis in PCa [11, 12], there have been few studies of the role of HMGB1 in PCa. 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) prostate tissue, and low-, intermediate-, and high-risk PCa and identified the prognostic significance of HMGB1 expression. We also explored the biological role of HMGB1 in the growth and invasion of PCa as well as its molecular mechanism. Our study indicated that HMGB1 plays an important role in PCa progression and metastasis by regulating the HMGB1/TNFR1/NF-κB signaling pathway.

2. Methods

2.1 Patient samples

After Institutional Review Board approval at the Catholic University of Korea, Seoul St. Mary's Hospital (IRB No. KC18SESI0795), formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from the Korean prostate Bank from patients with low- (n=22), intermediate- (n=41), and high-risk (n=36) PCa according to the National Comprehensive Cancer Network risk group [13] and from patients with BPH (n=28). Tumor tissue and the corresponding adjacent prostate tissue were collected separately from the PCa patients. All patients with PCa underwent radical prostatectomy, and their median follow-up duration was 38 months (range 6 to 55). We reviewed and collected the baseline demographics, clinicopathologic results, and follow-up outcomes.

2.2 Immunohistochemistry

Immunohistochemistry (IHC) was carried out to confirm the 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 two hours at room temperature and counterstained with hematoxylin. Digital images were obtained using an Olympus BX50 optical microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The areas positively stained with HMGB1 (%) were measured by ParaView 1.15.1 (Sandia Corporation, Kitware Inc., Albuquerque, NM, USA).

2.3 Cell lines and culture

The RWPE1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in complete Keratinocyte-Serum Free Media (K-SFM) (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract. DU145, PC3, LNCaP, and HEK 293 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The DU145, PC3, and LNCaP cells were routinely maintained in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin. The HEK 293 cells were routinely maintained in DMEM medium (Gibco) containing 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.5 Transfection

Small-interfering RNAs (siRNAs) of human HMGB1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc. CA, USA). DU145 and PC3 transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per 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 pCMV3 Negative Control, CV013) were purchased from Sino Biological (Sino Biological, Inc., Beijing, China). Transfection with plasmids was carried out using Lipofectamine 3000 (Invitrogen) per the manufacturer's protocol.

2.6 Cell viability

The cells (DU145, 8 x10^3/well; PC3, 1 x10^4/well) were seeded in 96-well plates and then transfected with siRNAs in a CO_2 incubator at 37 °C. At 0 h, 24 h, 48 h, 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.

2.7 Cell invasion assay

Cell invasion assays were performed using 24-well transwell chambers (8μm, Corning Inc., NY, USA) and the upper chambers were coated with 25μg/ml Matrigel (Corning). The cells were added to the pre-coated upper chambers at a density of 5×10^4 /well. Then, the lower chambers were filled with culture medium containing 20% FBS. After incubation in a CO_2 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 with 20% acetic acid. The solubilized color was measured at 570nm.

2.8 Flow cytometric analysis of cell cycle

Forty-eight hours after transfection, the cells were harvested and fixed with 70% cold ethanol at 4 °C for 1 h. After staining with 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).

2.9 Western blot analysis

Forty-eight hours after transfection, the cells were harvested and lysed with RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protease inhibitor. The protein concentrations were confirmed using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. After transfer, the membranes were blocked and then incubated with primary antibodies against ERK (Cell Signaling Technology), phosphor-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. After incubation, the membranes were washed and then incubated with a secondary antibody conjugated to HRP for 2 h at room temperature. The chemiluminescence method (Amersham, Arlington Heights, IL, USA) was used to develop the protein bands.

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

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

2.11 Proteome profiler antibody array

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 protocol provided. Briefly, 72 h after transfection, 500ug 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 incubated with a reconstituted detection antibody cocktail and horseradish peroxidase-conjugated streptavidin. The membranes were exposed to X-ray film and the mean intensity of each spot was quantified using Image-J software (Rasband W; National Institute of Health, Bethesda, MD, USA: http://rsbweb.nih.gov/ij/index.html).

2.12 Co-immunoprecipitation assay

For the co-immunoprecipitation (Co-IP) assay, plasmids were transfected into HEK293 cells using Lipofectamine 3000 reagent. One day after transfection, the 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 and Western blotting was conducted to detect HA (Abcam) and Flag (Abcam).

2.13 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 the statistical analyses. The data are expressed as the mean (± standard deviation [SD]) for continuous variables and the number of patients (proportions) for dichotomous variables. The differences between the groups were examined by independent t-test, one-way analysis of variance (ANOVA) 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. Value of $p<0.05$ was considered statistically significant.

3. Results

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

We evaluated the expression of HMGB1 in the BPH tissues, PCa tumor tissues, and adjacent normal tissues. Representative images of the HMGB1 expression in prostate tissues are shown in Figure 1A. Compared to the adjacent normal prostate tissues, the expression of HMGB1 was significantly higher in PCa tumor tissues. Also, the proportion of positive HMGB1 expression was significantly different among the patients with BPH, low-, intermediate-, and high-risk PCa (6.6 ± 3.7, 11.2 ± 3.5, 19.0 ± 4.9, 23.4 ± 8.2 %, respectively, $p<0.001$).

Table 1 shows the baseline demographics and clinicopathologic characteristics of the patients according to the HMGB1 expression levels. Most of the patients with BPH had low HMGB1 expression levels. However, 65.7% of the patients with PCa had high HMGB1 expression levels ($p<0.001$). Also, patients with high HMGB1 expression levels had advanced pathologic tumor stage ($p=0.093$), higher pathologic Gleason scores ($p<0.05$), and more perineural invasion ($p<0.010$) than 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 (Fig. 1B). The BCR-free survival of the low HMGB1 expression cohort was significantly longer than that of the high HMGB1 expression cohort ($p<0.05$).

3.2 Suppression of HMGB1 expression inhibits the cell proliferation of prostate cancer cells

To confirm the data obtained from patient tissue, we first evaluated the difference in HMGB1 expression between human prostate cancer cell lines (DU145, PC3, and LNCaP) and a normal prostate epithelial cell line (RWPE-1) using Western blotting. HMGB1 expression was up-regulated in all three PCa cell lines compared with the normal prostate epithelial cell line (Fig. 2A). To determine whether HMGB1 plays a role in PCa cells, we suppressed endogenous HMGB1 expression in the DU145 and PC3 cells using siRNA (Fig. 2B). The down-regulation of HMGB1 expression resulted in decreased cell proliferation and cell invasion (Fig. 2C, D). As shown in Figure 1E, the down-regulation of HMGB1 expression induced a

different cell cycle pattern. The percentage of sub-G1 phase cells was significantly increased after suppressing endogenous HMGB1 expression (DU145: 16.70 ± 1.22%, PC3: 13.93 ± 1.58%) compared to the control groups (DU145: 4.23 ± 2.45 %, DU145-NC: 3.76 ± 1.15%, PC3: 2.76 ± 1.02 %, and PC3-NC: 3.26 ± 1.10%, $p<0.05$). Additionally, the downregulation of HMGB1 expression caused a decrease in phopsho-Akt and phospho-ERK expression. In contrast, cleaved-caspase 3 expression was increased after the suppression of endogenous HMGB1 in DU145 and PC3 cells (Fig. 2F). Taken together, our results revealed that HMGB1 was associated with tumor growth, survival, and metastasis in PCa.

3.4 Suppression of HMGB1 expression inhibits the NF-κB pathway in prostate cancer cells

The role of the NF-κB signaling pathway has been well established. NF-κB signaling plays a pivotal role in cancer cell proliferation, progression, and metastasis in PCa cells [14]. An association between HMGB1 and the NF-κB signaling pathway has been widely evaluated in several solid cancers and inflammation, but rarely in PCa [15]. For this reason, we next investigated if HMGB1down-regulation could affect NF-κB signaling in prostate cancer cells using a human NF-κB proteome profiler array. As shown in Figure 3A, HMGB1 down-regulation resulted in changes in the expression level of different proteins involved with the NF-κB pathway. Important genes related to the NF-κB pathway, including p65 and IKK-β, were decreased in HMGB1-downregulated cells compared to control cells and the expression of p53 and phosph-p53 was increased. The Western blots also confirmed that the down-regulation of HMGB1 decreased the levels of p65 and IKK-β in the DU145 and PC3 cell lines (Fig. 3B). These results indicate that the down-regulation of HMGB1 in PCa cells suppressed growth by regulating the expression of proteins related to the NF-κB pathway.

3.5 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 downregulation of HMGB1 suppressed the expression of TNFR1, TNFR3, and TNFR5 (Fig. 4A). The RT-PCR and Western blot results were consistent (Fig. 4A) with those of the proteome profiler array. A recent study reported that TNF-α/TNFR-mediated signaling played an important role in cell survival and therapy resistance [16] and that HMGB1 stimulated TNF-α/TNFR-mediated signaling [17]. However, no study has been conducted to examine the interaction between TNFR and HMGB1. To identify which TNFR was responsible for HMGB1 binding, we performed a Co-IP assay on HEK 293 cells after co-transfection with FLAG-HMGB1 and HA-TNFR1, 3, or 5. As shown in Figure 4B, HMGB1 was coimmunoprecipitated with TNFR1 but not with TNFR3 or TNFR5. Combined, these findings indicate that HMGB1 binds to TNFR1.

3.6 HMGB1 binding with TNFR1 increases cell survival in PCa

To validate the role of the HMGB1/TNFR1 axis in PCa, we examined whether TNFR1 overexpression would reverse the effects of HMGB1 knockdown in PCa cells. We co-transfected with si-HMGB1 and v-TNFR1 and then confirmed cell viability. The cell viability analysis results showed that TNFR1 overexpression in the HMGB1-knockdown cells reversed the effects of HMGB1-knockdown (Fig 5).

Consistent with the cell viability assay, immunoblotting showed reversal of p65 expression in TNFR1 overexpression of HMGB1-knockdown cells. These data demonstrated that HMGB1 binding with TNFR1 activated the NF-κB signaling pathway, promoting the cell survival and invasion of PCa (Fig. 6).

Discussion

HMGB1 has been shown to be associated with inflammation and cancer [7-10]. HMGB1 in PCa has been reported to be highly expressed and associated with tumor progression, metastasis, and poorer prognosis [11, 12]. However, the specific role and molecular mechanisms of HMGB1 in PCa development and progression remain poorly understood. This study revealed the clinical correlation and molecular role of HMGB1 in PCa. More interestingly, we provided novel evidence regarding the association between HMGB1/TNFR/NF-κB signaling pathway and PCa for the first time.

We performed IHC to identify HMGB1 expression patterns in patient tissue and explore the clinical significance of HMGB1 in PCa. Several researchers have reported that high HMGB1 expression was associated with pathological features, such as Gleason score, pathological stage, PSA level, and metastasis [18-20]. 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 the distinct tissue-specific expression pattern in BPH, PCa, and adjacent normal tissue according to low-, intermediate and high-risk groups. Our results demonstrated that HMGB1 was highly expressed in PCa compared to BPH and that HMGB1 expression was increased according to increased 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 HMGB1in PCa, we performed diverse assays using silenced HMGB1 expression in PCa cells. Our results showed that the down-regulation of HMGB1 in PCa cells led to decrased cell proliferation and invasion. These results indicate that HMGB1 might promote PCa development and progression.

The activation of NF-κB signaling pathway induces cytokine release and differential transcription modification, which contribute to the immune response, cancer initiation, progression, and metastasis [21-23]. The phosphorylated IKK complex leads to the phosphorylation induced degradation of IκB. The degradation of IκB induces the translocation of p65 to the nucleus. This process triggers NF-κB signaling activation (23). Previous studies have demonstrated that NF-κB pathways are important for PCa cell growth, invasion, and the development of treatment resistance [14, 24]. Moreover, numerous researchers have demonstrated that the HMGB1/NF-κB signaling pathway plays an important role in inflammation and several solid cancers [25-27]. Huang et al. showed that suppressing the gene expression of HMGB1 inhibited proliferation, migration, and invasion in bladder urothelial carcinoma via the NF-κB signaling pathway [28]. Zhang et al. showed that HMGB1 was associated with tumor metastasis in gastric adenocarcinoma through the NF-κB pathway. High HMGB1 expression was correlated with survival and tumor metastasis in non-small cell lung cancer via activation of the NF-κB pathway [29]. However, little information is available regarding the HMGB1/NF-κB signaling pathway in PCa. Our results showed that HMGB1 blocking in PCa decreased the expression of p65 and IKK-β. Also, HMGB1 blocking in PCa

decreased the expression of Ikκ-α, cIAP1, IκB-ε, and IκB-α, whereas the expression of phospho-p53 and p53 was increased. These results showed that HMGB1 is associated with PCa proliferation and invasion by regulating NF-κB signaling. Our research revealed the underlying mechanisms of the HMGB1/NF-κB signaling pathway in PCa and provides a basis for future research.

Interestingly, we found that the knockdown of HMGB1 decreased TNFR expression in PCa cells. TNFR, including TNFR1, TNFR2, TNFR3, TNFR5, and CD95 (TNFR6), is a cytokine receptor and its receptor-ligand interaction regulates a variety of pathways, such as immune processes, tissue homeostasis, survival, proliferation, and death [30]. TNFR is expressed on most cells in the body and is activated by a soluble ligand [31]. Cytokine binding to TNFR1 stimulated classical NF-κB signaling and enhanced inflammation, cancer cell proliferation, and tumor metastasis [32]. TNFR1 knockout in mice reduced the incidence of PCa and inhibited the expression of AR, mTOR, and p65 [33]. HMGB1 was previously reported to promote diverse pathological and physiological functions through its receptor, which include RAGE, TLRs, TIM3, and CXCR4 [34]. Specifically, HMGB1-RAGE and HMGB1-TLR signaling contribute to tumor cell proliferation and metastasis through inducing NF-κB activation in cancer [35, 36]. Both HMGB1 and TNFR have been shown to be closely related with the NF-κB pathway (33-35). However, whether HMGB1 interaction with TNFR and it affects NF-κB signaling has not yet been clarified. We questioned whether HMGB1 would bind with TNFR, followed by stimulation of the NF-κB signaling pathway. To further verify this question, we conducted a Co-IP assay that showed that HMGB1 directly bound only with TNFR1. Also, TNFRSF1A overexpression in HMGB1 knockdown cells reversed the effects of HMGB1 knockdown and p65 expression. Notably, this is the first study to elucidate the HMGB1 binding with TNFR1 could activate NF-κB signaling in PCa.

Conclusions

These results indicate that HMGB1 binding with TNFR1 could activate NF-κB signaling. This processing regulates PCa key events during tumor progression and metastasis. Notably, this is the first study to elucidate the direct binding of HMGB1 with TNFR1. Our study may provide a basis for investigating whether HMGB1-mediated TNFR1 may prove to be an effective strategy to treat PCa. Further molecular studies of the relationship between HMGB1 and TNFR1 should be conducted to better understand its contribution to tumor progression.

Abbreviations

BPH
benign prostatic hyperplasia
cIAP
cellular inhibitor of apoptosis protein
CRPC
castration-resistant prostate cancer
FFPE

formalin-fixed and paraffin-embedded

HMGB1

high mobility group box 1 protein

K-SFM

keratinocyte-serum free media

PCa

prostate cancer

si-HMGB1

HMGB1 siRNA

TMA

tissue microarray

TNFR

TNF receptor

v-TNFR1

TNFR1 plasmid vector

Declarations

Ethics approval and consent to participate

All the procedures carried out in the research involving human samples were approved by the Institutional Review Board of the Catholic University of Korea, Seoul St. Mary's Hospital (IRB No. KC18SESI0795). All subjects provided written informed consent.

Consent for publication

Not applicable

Availability of data and materials

Not applicale

Competing interests

The authors have declared that no competing interest exists.

Funding

This study was supported by Research Fund of Seoul St. Mary’s Hospital, The Catholic University of Korea (ZC19EISI0692).

Authors' contributions

Yong Hyun Park designed this study; Ae Ryang Jung and Ga Eum Kim performed experiments; U-Syn Ha, Sung-Hoo Hong, Ji Youl Lee, and Sae Woong Kim assisted with data collection; Ae Ryang Jung and Mee Young Kim analyzed and interpreted the data; Young Hyun Park and Ae Ryang Jung wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

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Table

Please see the supplementary files section to view the table.

Figures

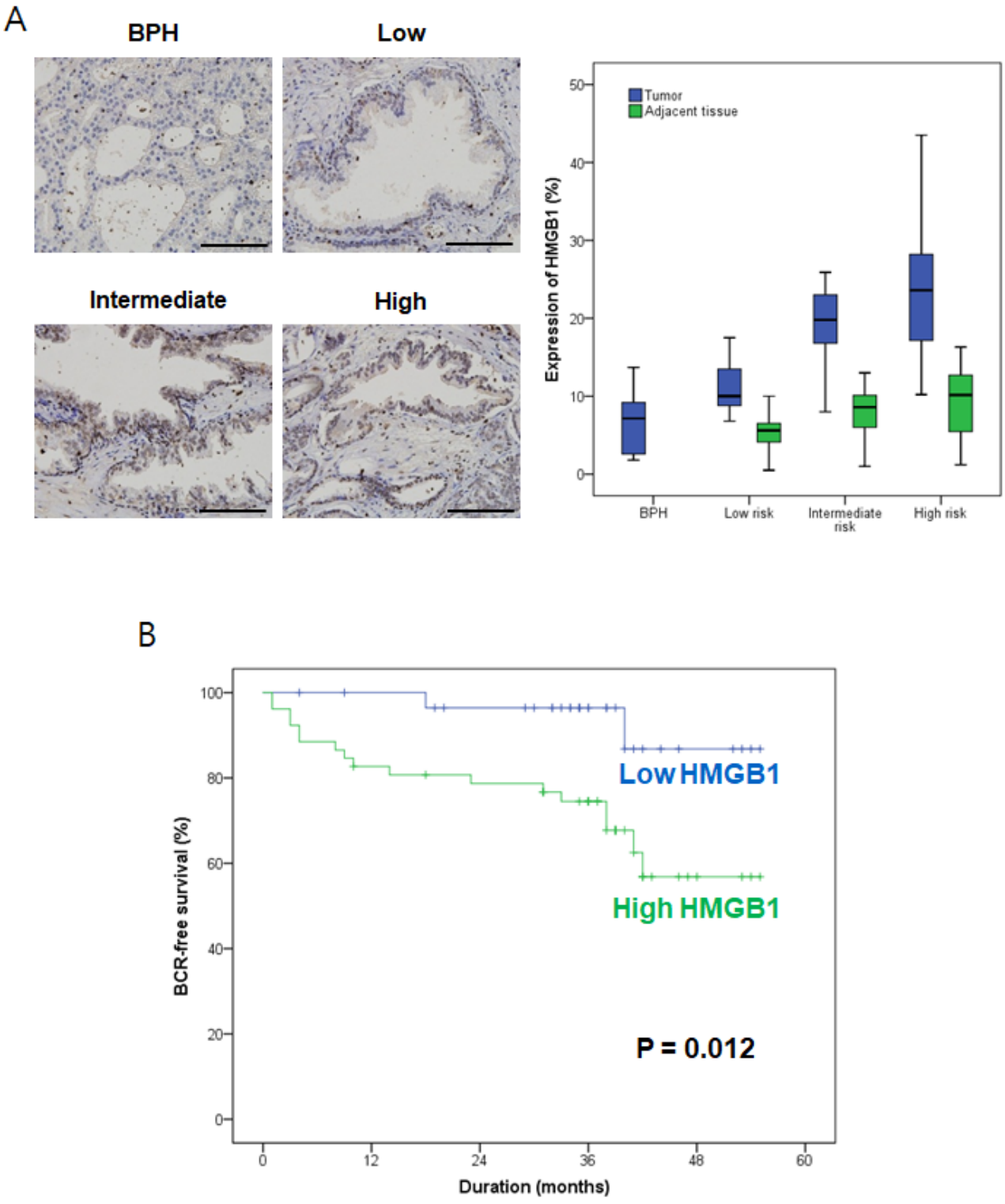


Figure 1

Immunohistochemical analyses of HMGB1 in benign prostatic hyperplasia (BPH) and prostate cancer (PCa) patients. (A) The expression of HMGB1 in BPH and PCa patient tissues was confirmed by IHC and representative images for each group are shown (scale bar size 100μm, magnification x400). HMGB1 was stained brown and the nuclei were counterstained. Graph of HMGB1 quantification expressed as the HMGB1-positive areas in BPH and PCa patient tissues and paired normal prostate tissues ($p<0.001$). (B)

BCR-free survival according to HMGB1 expression level was quantified by Kaplan-Meier analysis in patients with PCa in a Korean cohort.

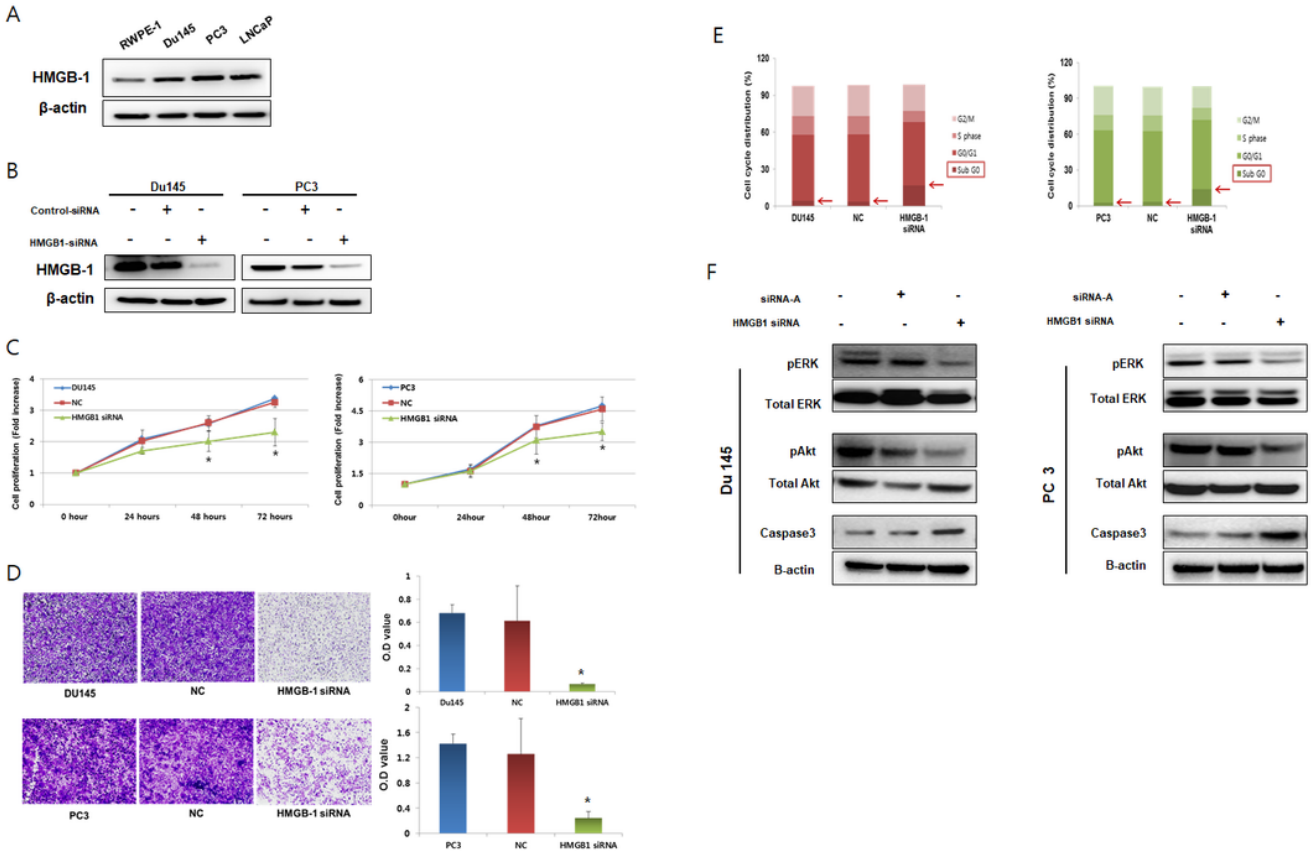
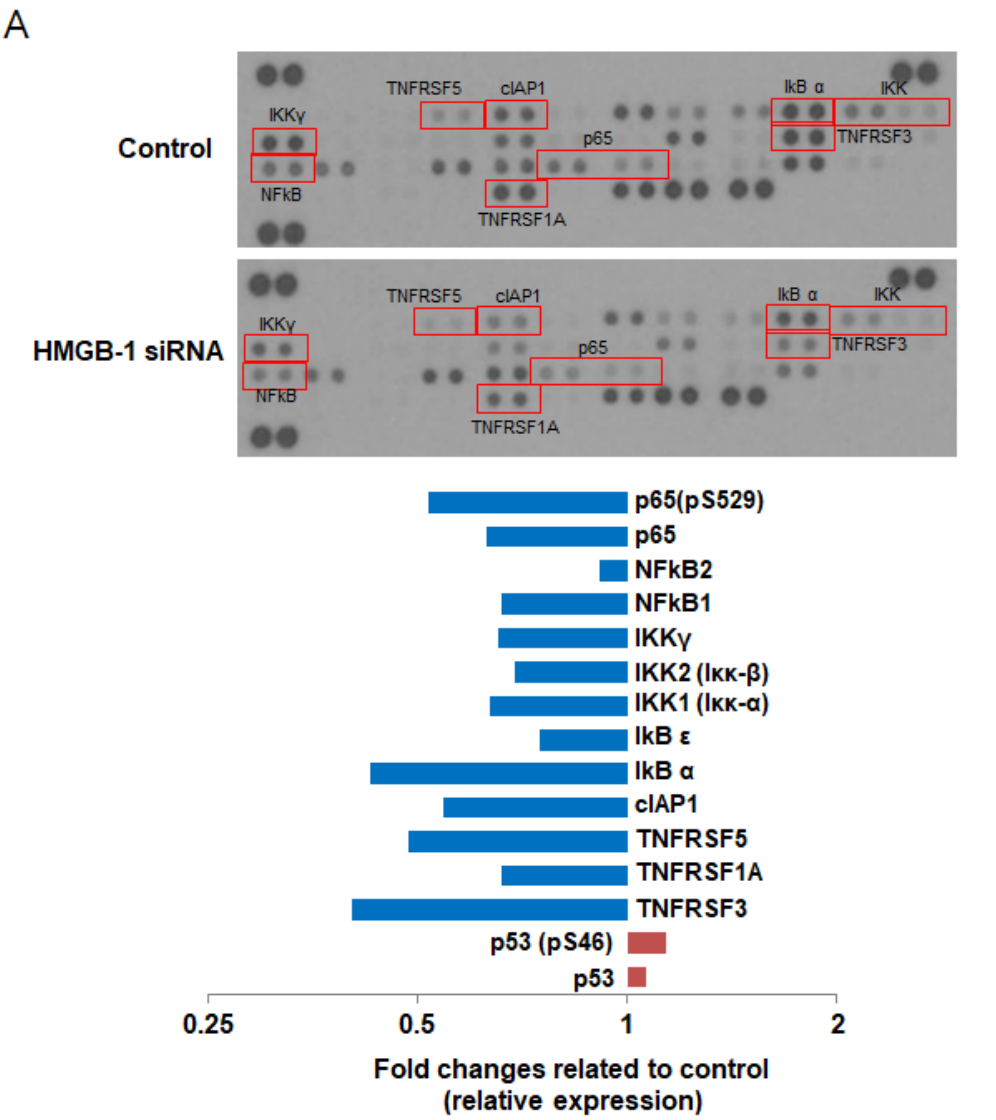


Figure 2

Suppression of HMGB1 expression mediated the response of prostate cancer cells. (A) Western blots were performed to confirm the difference in HMGB1 expression in a normal prostate cell line (RWPE-1) and prostate cancer cell lines (DU145, PC3, and LNCaP). (B) The effect of HMGB1 knockdown in DU145 and PC3 cells. DU145 and PC3 cells were transfected with HMGB1- or control (negative control, NC) siRNA. HMGB1 expression was determined by Western blotting and normalized to β-actin. (C) Cell proliferation in the DU145 and PC3 cells after transfection with HMGB1 evaluated by the WST assay. Cell proliferation is shown as fold-change (*P< 0.01 compared with NC). No significant difference was found between non-tranfected cells and NC. (D) Cell invasion assays in the DU145 and PC3 cells after 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 the migrated cells using 10% acetic acid (*P< 0.01 compared with NC). (E) Proportion of sub G0, Go/G1, S, and G2/M phase cells in the PC3 and DU145 cells after transfection with HMGB1 or NC siRNA. The cell cycle distribution analysis was performed by 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 in the DU145 and PC3 cells after transfection with HMGB1- or NC siRNA.



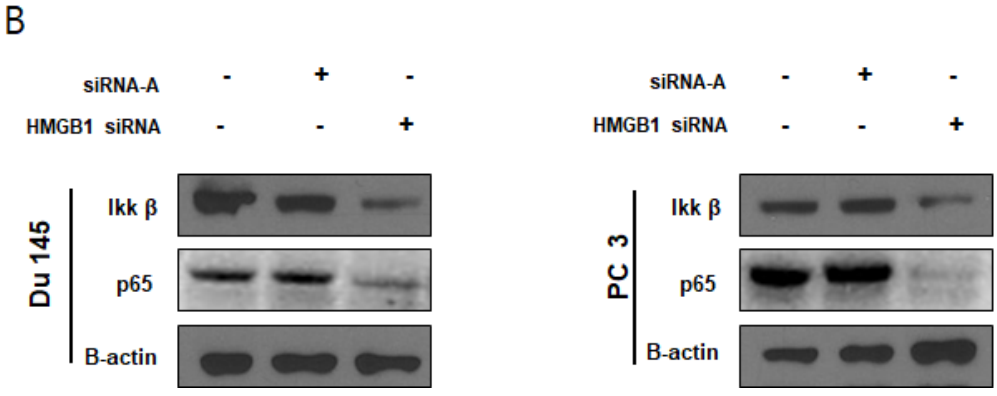


Figure 3

Suppression of HMGB1 expression changed several NF-κB factors. (A) Several NF-κB factors were evaluated using a proteome profiler array kit (R&D Biosystems) after 48 h of transfection with HMGB1 siRNA. Quantitative analysis of the spots was performed by densitometic analysis and fold-changes compared to the internal controls (mean) were considered significant. (B) IKK-β, p65, and β-actin in DU145 and PC3 cells after transfection with HMGB1 or NC siRNA.

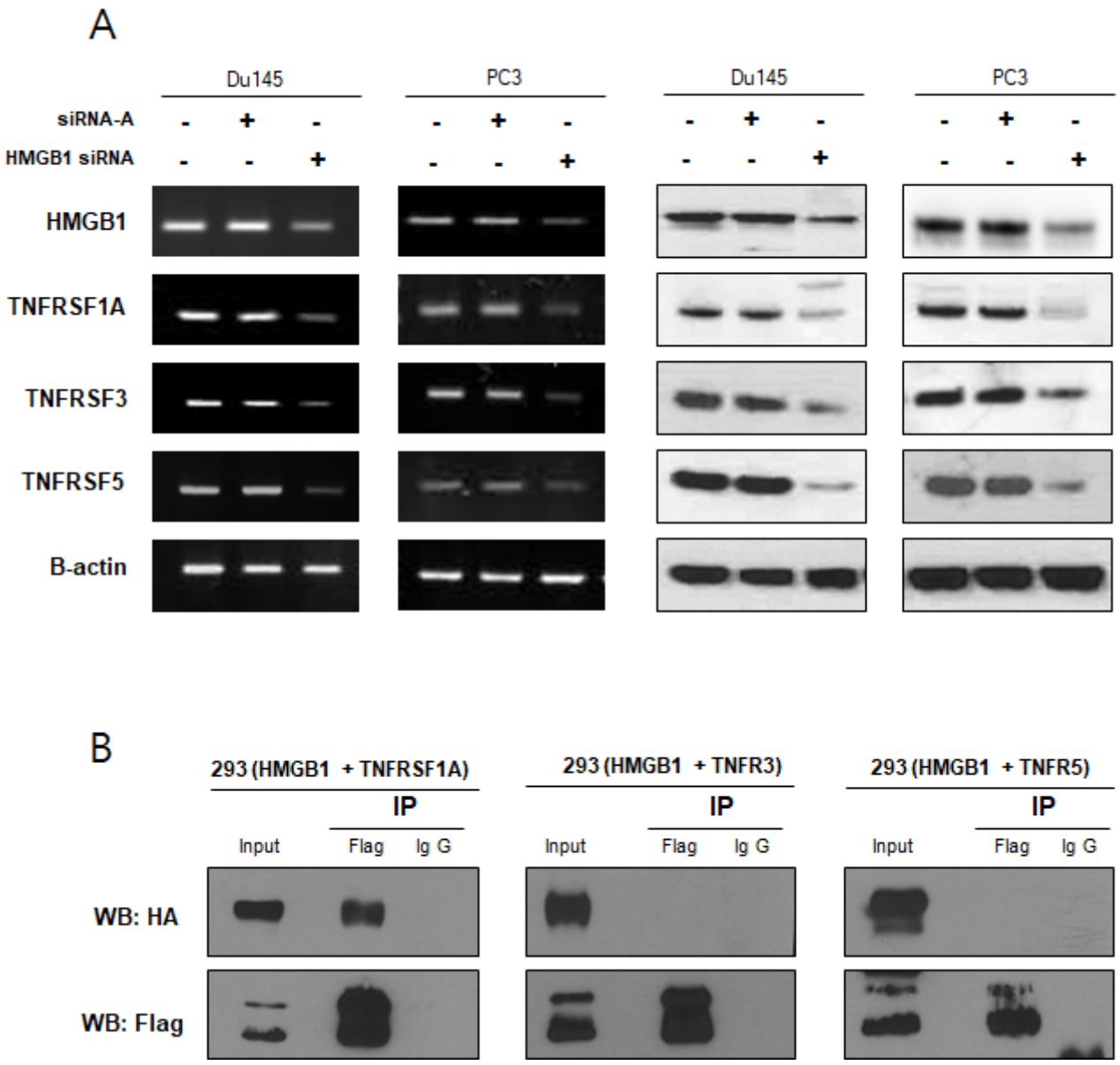


Figure 4

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

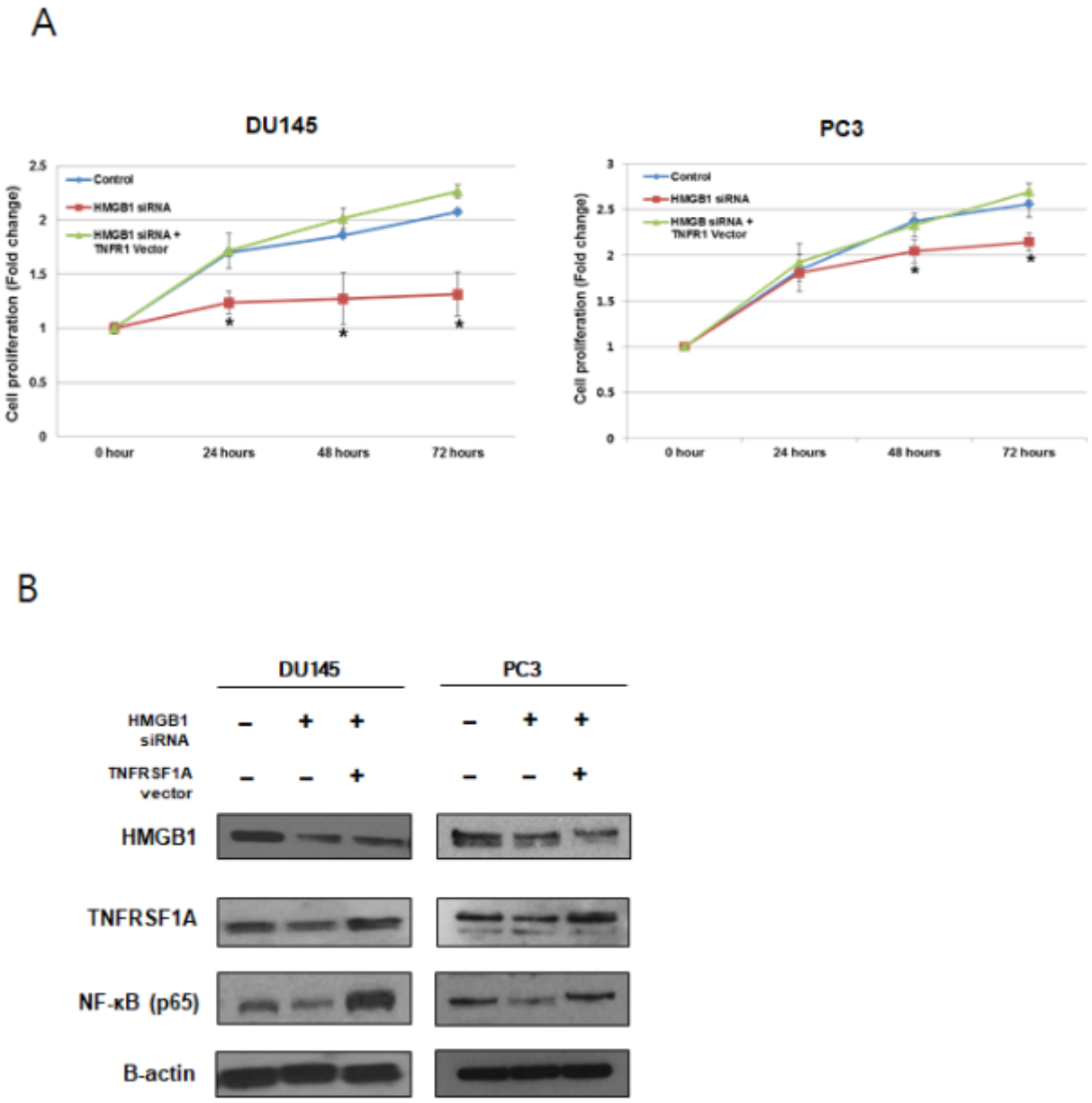


Figure 5

TNFR1 overexpression in the HMGB1 knockdown cells reversed the effects of HMGB1-knockdown. (A) Cell proliferation in the DU145 and PC3 cells after co-transfection with HMGB1 siRNA and TNFR1 plasmid DNA evaluated by the WST assay. Control cells were co-transfected with control siRNA and control plasmid vector. Cell proliferation is shown as fold-change. Cell proliferation in HMGB1 siRNA group significantly decreased compared with the control and HMGB1 siRNA + TNFR1 vector group (*P< 0.01). In HMGB1 siRNA + TNFR1 Vector group, no significant difference was found in cell proliferation compared with the control group. (B) The effect of TNFR1 overexpression in the in the HMGB1-knockdown cells. DU145 and PC3 cells were transfected with HMGB1 siRNA and/or TNFR1 plasmid vector. HMGB1, TNFR1, p65 and β-actin were analyzed by Western blotting.

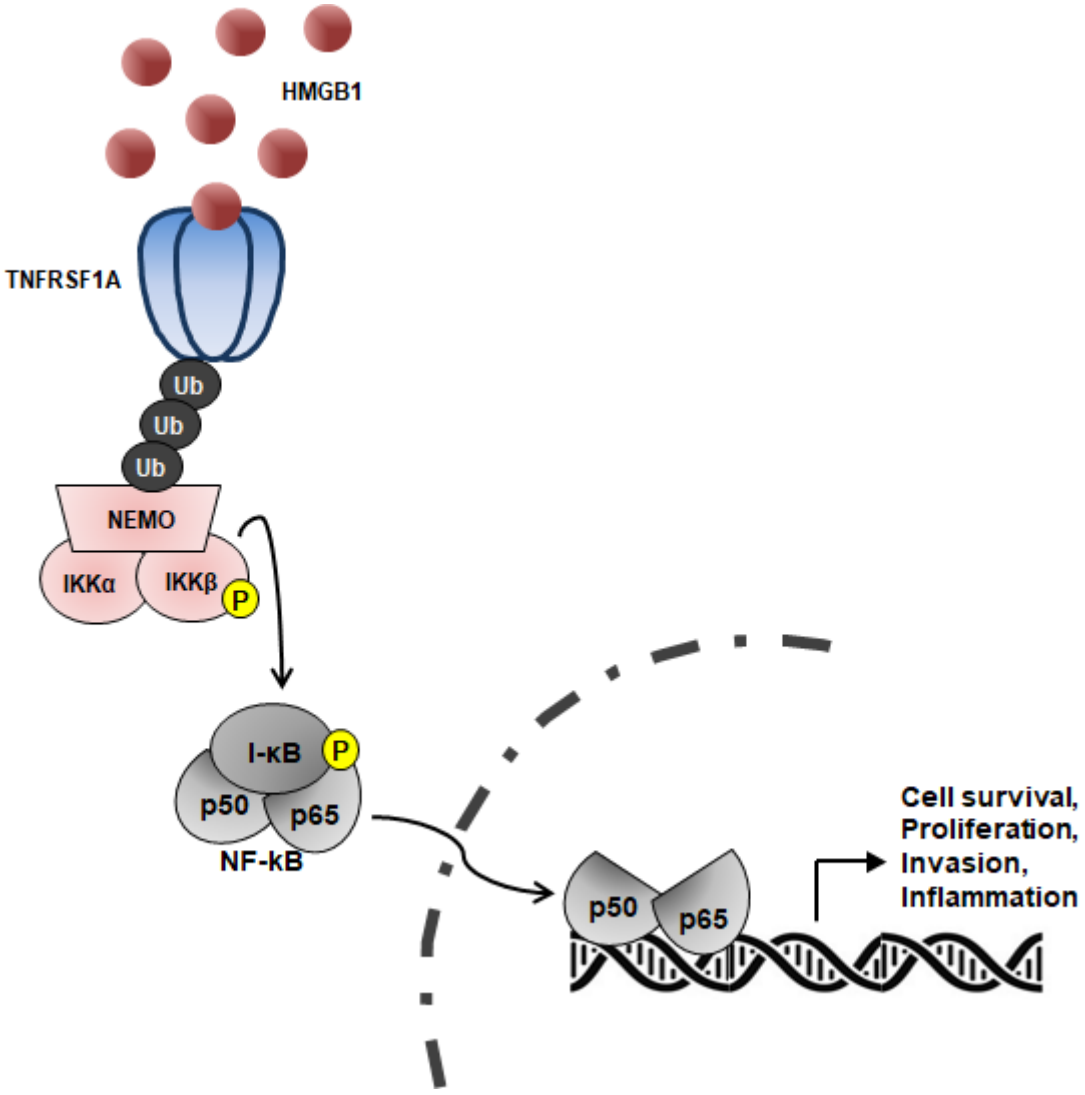


Figure 6

Schematic model of the hypothesized mechanism by which HMGB1 binding with TNFR1 stimulates cell proliferation and invasion through classical NF-κB signaling in PCa.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- suppletable1.docx