Complement regulatory protein CD46 promotes bladder cancer metastasis through activation of MMP9

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Received February 19, 2024; Accepted May 8, 2024

DOI: 10.3892/ijo.2024.5659

Abstract. CD46, a transmembrane protein known for protecting cells from complement-mediated damage, is frequently dysregulated in various types of cancer. Its overexpression in bladder cancers safeguards the cancer cells against both complement and antibody-mediated cytotoxicity. The present study explored a new role of CD46 in facilitating cancer cell invasion and metastasis, examining its regulatory effect on matrix metalloproteases (MMPs) and their effect on the metastatic capability of bladder cancer cells. Specifically, CD46 alteration positively influenced MMP9 expression, but not MMP2, in several bladder cancer cell lines. Furthermore, CD46 overexpression triggered phosphorylation of p38 MAPK and protein kinase B (AKT), leading to enhanced activator protein 1 (AP-1) activity via c-Jun upregulation. The inhibition of p38 or AKT pathways attenuated the CD46-induced MMP9 and AP-1 upregulation, indicating that the promotion of MMP9 by CD46 involved activating both p38 MAPK and AKT. Functionally, the upregulation of MMP9 by CD46 translated to increased migratory and invasive capabilities of bladder cancer cells, as well as enhanced *in vivo* metastasis. Overall, the present study revealed a novel role for CD46 as a metastasis promoter through MMP9 activation in bladder cancers and highlighted the regulatory mechanism of CD46-mediated MMP9 promotion via p38 MAPK and AKT activation.

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Abbreviations: AKT, protein kinase B; AP-1, activator protein 1; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunoabsorbent assays; ERK, extracellular signal-regulated kinases; Ets, E-twenty six; FBS, fetal bovine serum; H&E, hematoxylin and eosin; JNK, c-Jun N-terminal kinases; KRT13, keratin 13; MAPK, mitogen-activated protein kinase; MGP, matrix Gla protein; MMLV, moloney murine leukemia virus; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; MMPs, matrix metalloproteinases; NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositol 3-kinase; RT-qPCR, reverse transcription-quantitative PCR; SP-1, specificity protein 1; SPAK, Ste20/SPS-1-related kinase

Key words: CD46, bladder cancer, matrix metalloproteinase 9, metastasis, 5637

Introduction

CD46, a transmembrane protein, plays pivotal roles in various biological processes, including immune activation and the modulation of adaptive immunity. Notably, it regulates complement activity, a crucial component of the immune system that targets invading pathogens and damaged cells (1). CD46 functions as a co-factor in the degradation of complement components, thereby inhibiting complement activation. This inhibition is essential to prevent excessive or inappropriate activation of the complement system, which could result in tissue damage and autoimmune disorders. CD46 is widely distributed in tissues, being expressed by almost all human cell populations, with the exception of erythrocytes (2). It is commonly expressed in four distinct isoforms, resulting from alternative mRNA splicing of a single gene transcript (3).

Beyond its role in complement regulation, CD46 has been linked to several types of cancer and other diseases (4-9). A number of tumor types exhibit CD46 overexpression. For instance, in hepatocellular carcinoma, CD46 expression is ~6 times higher than in liver cirrhosis and chronic hepatitis (10). High concentrations of CD46 have been observed in the sera of cancer patients (11). CD46 expression is an unfavorable prognostic factor in ovarian cancer, breast cancer and hepatocellular carcinoma (12-14). Additionally, CD46 contributes to the tumorigenesis and development of bladder cancer (15). Our previous research demonstrated that restoring CD46 expression in bladder cancer cells conferred protection against cetuximab-mediated inhibition of phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) phosphorylation (16). This restoration also protected cells from complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity induced by

cetuximab. Thus, CD46 shows a protective effect in cancer cells against both direct (via involvement of PBMC or complement) and indirect cytotoxic activities of cetuximab in bladder cancer cells.

Studies have underscored the importance of matrix metalloproteinases (MMPs) in tumor growth and metastasis, with a focus on MMP2 and MMP9 (17,18). Researchers have characterized promoter region of *MMP9*, identifying binding sites for several transcription factors such as activator protein 1 (AP-1), specificity protein 1 (SP-1), E-twenty six (Ets) and nuclear factor kappa B (NF-κB) (19). Further research indicates that AP-1 activation is essential for sustaining cancer cell invasion and aiding epithelial wound healing (20-22). This activation predominantly occurs through the mitogen-activated protein kinase (MAPK) cascades. The MAPK family is divided into three primary groups: ERK, c-Jun N-terminal kinase (JNK) and p38 MAPK. Certain MAPK family members, including ERK1/2 and p38 MAPK, along with PI3K/AKT, play a pivotal role in upregulating MMP9 expression (23-27). Additionally, AP-1 activation is primarily facilitated by the PI3K/AKT and JNK pathways, working in synergy (28).

Despite significant progress in understanding CD46, a number of aspects of its functions and the molecular mechanisms involved in cancer cell metastasis remain unclear. Our previous research demonstrated that CD46 is highly expressed in bladder cancers, where it helps protect cancer cells from various cytotoxic insults (16,29). Moreover, CD46 appears to influence cell migration by altering the expression of several cytoskeletal proteins (30). Given the strong correlation between MMPs, particularly MMP9, and the progression and recurrence of bladder tumors (18,31,32), the present study aimed to investigate the impact of CD46 on MMP9 in bladder cancer and to elucidate the specific mechanisms related to CD46 and MMP9 in bladder cancer cells.

Materials and methods

Cell lines and cell culture. Human bladder cancer cell lines [J82, 5637, HT-1376 (cat. no. CRL-1472), UM-UC-3], melanoma cells (M010119), prostate cancer cells (LNCaP), colon cancer cells (HCT116) and lung cancer cells (A549) were procured from the American Type Culture Collection. 253J cells were kindly provided by Dr Wun-Jae Kim (Chungbuk National University, Korea). J82, 5637, HT-1376, UM-UC-3, 253J, M010119 and LNCaP cells were cultured in RPMI 1640 medium (Welgene, Inc.) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). HCT116 and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Inc.) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cultivation was at 37˚C in an atmosphere containing 5% CO_2, with media changes every 2-3 days. To establish cell lines with CD46 overexpression, the lentiviral vector pBlasti-eGFP-CD46 kindly provided by Dr Silvio Hemmi (Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland) was co-transfected with helper in the 2nd generation transfection system into 293T cells, as detailed in a previous study (29). The 293T cells were cultured in DMEM supplemented with 10% FBS at a temperature of 37˚C and 5% CO_2. Following a 48 h incubation period at 37˚C; the supernatant of the 293T cells were collected and the lentivirus was concentrated by subjecting to centrifugation at 2,100 x g for 5 min at 4˚C and filtering the supernatant through a 0.45 μm filter. The harvested lentiviral vectors were then introduced into the target cells for transduction. Subsequently, cell lines were plated into a 6-well plate and the cells were cultured until they reached 80% confluence. To generate stable cell lines, cells were transduced with these lentiviral vectors in a milieu containing polybrene at a concentration of 8 μg/ml. Then, lentivirus was added and co-cultured with the cells at 37˚C for 24 h (Multiplicity of infection, 30). After that, the medium was replaced, and then culture continued in a 5% CO_2 and 37˚C incubator for another 48 h. Following a 72 h incubation period, cells underwent a selection process using 10 μg/ml blasticidin (MilliporeSigma), over a course of 3 days.

Western blotting. For the cancer cells, protein extraction was performed using Pro-Prep (cat. no. 17081; Intron Biotechnology, Inc.). The quantification of proteins was conducted using the BCA Protein Assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). Proteins (15 μg) were subjected to separation on a 7.5% SDS-polyacrylamide gel, using the Bio-Rad electroporation system (Bio-Rad Laboratories, Inc.), followed by transfer to PVDF membranes (cat. no. IPVH00010; MilliporeSigma). After the membranes were blocked with 3% BSA (BioShop) in tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h and then incubated at 4˚C overnight with primary antibodies. The primary antibodies used included: CD46 (1:2,000; cat. no. ab108307; Abcam); MMP9 (1:1,000; cat. no. 7-11C; Santa Cruz Biotechnology, Inc.), phosphorylated (p-)AKT (1:1,000; cat. no. 05-669; MilliporeSigma), p38α (1:1,000; cat. no. sc-535; Santa Cruz Biotechnology, Inc.), JNK1/3 (1:1,000; cat. no. sc-474; Santa Cruz Biotechnology, Inc.), p-JNK (1:1,000; cat. no. sc-6254 Santa Cruz Biotechnology, Inc.), ERK1 (1:1,000; cat. no. sc-271269 Santa Cruz Biotechnology, Inc.), c-Fos (1:1,000; cat. no. sc-166940; Santa Cruz Biotechnology, Inc.), p-c-Fos (1:1,000; cat. no. sc-81485; Santa Cruz Biotechnology, Inc.), c-Jun (1:1,000; cat. no. sc-1694; Santa Cruz Biotechnology, Inc.), p-c-Jun (1:1,000; cat. no. sc-822; Santa Cruz Biotechnology, Inc.); p-p38 (1:2,000; cat. no. 05-1059; MilliporeSigma), AKT (1:2,000; cat. no. 9272; Cell Signaling Technology, Inc.); MMP2 (1:2,000; cat. no. NB200-114; Novus Biologicals; Bio-Techne); p-ERK (1:2,000; cat. no. 05-797R; MilliporeSigma); and β-actin (1:2,000; cat. no. 26628-22-8; MilliporeSigma). After washing three times with TBST, the membranes were incubated at room temperature for 1 h with the secondary antibody (Anti-mouse IgG HRP-linked antibody; 1:5,000; cat. no. 7076S; Anti-rabbit IgG HRP-linked antibody; 1:10,000; cat. no. 7074; Cell Signaling Technology, Inc.). Following secondary antibody incubation, the membranes were washed four times again and then completely covered with a 1:1 mixture of HRP substrate peroxide solution/HRP substrate Luminol reagent (cat. no. WSKLS0500; MilliporeSigma). Band visualization was conducted using the Fusion Solo system (Vilber Lourmat Deutschland GmbH) and analyzed with ImageJ software (version 1.53m; National Institutes of Health, USA).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manual instructions. cDNA was generated with M-MLV reverse transcriptase (cat. no. M170B; Promega Corporation). mRNA expression was determined by qPCR on a CFX96 real-time PCR System (Bio-Rad Laboratories, Inc.) using TOPreal SYBR Green qPCR premix kit (cat. no. RT500M; Enzynomics), using GAPDH as the internal loading control. The primer sequences for PCR were as follows: human CD46 (Forward: 5'-CCA CGACCATTTGAAGCTAT-3', Reverse: 5'-TCCAGGTGC TGGATCACAC-3'), human MMP-9 (Forward: 5'-CATCGT CATCCAGTTTGG-3', Reverse: 5'-GATGGATTGGCCTTG GAA-3') and human GAPDH (Forward: 5'-GAAGGTGAA GGTCGGAGTC-3', Reverse: 5'-GAAGATGGTGATGGG ATTTC-3'). PCR cycling conditions for all samples included: 10 min at 95˚C for enzyme activation, followed by 40 cycles consisting of melting (95˚C; 15 sec) and annealing/extension (72˚C; 30 sec) phases. The data were analyzed as previously described (33). Briefly, using the comparative CT method, the Ct values obtained were converted to picogram quantities based on each gene's standard curve from target cDNA. The quantity of CD46 and MMP9 were normalized to GAPDH and adjusted by subtracting values from no reverse transcriptase controls, averaging the result for each triplicate (34).

Transient of CD46 short interfering (si)RNA. Two variants of CD46 siRNA (CCAAAACCCUACUAUGAGA, GGAUAC UUCUAUAUACCUCUU) and negative control siRNA (UGC AGGUUUAUAGUCCACAUU) were procured from Bioneer Corporation. 5637 cells underwent transfection with these siRNAs using Lipofectamine® RNAiMAX transfection reagent (cat. no. 13778-075; Invitrogen; Thermo Fisher Scientific, Inc.), adhering to the manufacturer's protocol. Cells were cultured in six-well plates until they reached 60% confluence. To prepare the transfection mix, 7.5 μl Lipofectamine® RNAiMAX was diluted in 150 μl Opti-MEM Medium (Gibco; Thermo Fisher Scientific, Inc.) and mixed. DNA siRNA (25 pmol) was diluted in 150 μl Opti-MEM Medium and mixed. The diluted DNA was then combined with the previously diluted Lipofectamine® RNAiMAX, at a 1:1 ratio, to form a DNA-lipid complex. This complex was allowed to incubate for 15 min at room temperature to a stable DNA-lipid complex. Then, the complex was administered to the cultured cells. Following transfection, the cells were incubated at 37˚C. At 6 h post-transfection, the cells were chanced and incubated at 37˚C with media containing 5% FBS. At 48 h post-transfection, the cells were lysed for western blot analysis as described earlier.

Wound migration assay. 5637, J82 and 253J cells were cultured in six-well plates until they reached 90% confluence in 2 ml of growth medium. The cells were then washed and incubated in RPMI containing 0.5% FBS for 16 h. A sterile 200 μl pipette tip was used to scrape the cell monolayer. Subsequently, the cells were gently washed thrice with PBS and FBS-free medium was added for starvation culture at 37˚C. Images were captured at 0, 12 and 24 h post-scraping using an inverted microscope (Olympus IX51; Olympus Corporation). The open wound area percentages were measured and calculated with ImageJ software (version 1.53m; National Institutes of Health).

Transwell assays. The migration devices (cat. no. 3422; Corning, Inc.) and invasion devices (cat. no. 354483; Corning, Inc.) were subjected to room temperature equilibration for 10 min, then the two devices were incubated for 2 h in a 37˚C incubator, and 300-μl serum-free RPMI was added in the upper chamber and 600 μl in the lower chamber. Cells at 80-90% confluence were washed and incubated in RPMI with 0.1% FBS for 16 h prior to their application to the chambers. Subsequently, 5637 and J82 cells ($5x10^4$) in 200 μl of serum-free medium were seeded into the upper chamber and the lower chambers were filled with 600 μl of RPMI media containing 10% fetal bovine serum. After a 24-h incubation, cells on the bottom membrane of the chamber were gently wiped with a cotton swab and migrating cells were stained with Diff-Quik stain solution (cat. no. 38721; Sysmex Corporation) at room temperature. After drying, images were captured under a light microscope (Olympus IX51; Olympus Corporation) and cells were counted in four fields per chamber.

Cell proliferation assay. Cell proliferation was assessed using the WST-1 assay (EZ-Cytox Cell Viability Assay kit; ITSBio, Inc.), following the manufacturer's instructions. Briefly, cells were seeded in 96-well plates with 100 μl of RPMI media containing 10% FBS and cultured at 37˚C. Then, at the indicated time points (0, 24, 48 and 72 h), 10 μl of the kit solution was added to the cells, followed by a 30-min incubation at 37˚C. Cell viability was measured at 450 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc.).

Gelatin zymography. Cells at 80-90% confluence were washed and incubated in RPMI without FBS for 24 h in 60 mm plates. The samples were then electrophoresed on a 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin. Post-electrophoresis, the gel was washed twice for 2 h at room temperature using a washing buffer [50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 2.5% Triton X-100] and then briefly rinsed with H_2O. Subsequently, the gel was incubated with 1X of Zymogram developing buffer 10X, pH 7.45 (cat. no. 42620000-2; Bioworld Technology, Inc.) at 37˚C for 24 h. After incubation, the gels were stained with Coomassie Brilliant Blue R-250 Staining Solution (cat. no. 1610436; Bio-Rad Laboratories, Inc.) and destained with Coomassie Brilliant Blue R-250 Destaining Solution (cat. no. 1610438; Bio-Rad Laboratories, Inc.).

Luciferase assay. Transcriptional activity was assessed using a luciferase assay system. The pGL4.17[luc 2/Neo] vector, containing the MMP9 promoter region (-924/+13), known as the MMP9-luc promoter, was used. The MMP9-luc vector was kindly provided by Dr Young-Han Lee (Konkuk University, Korea). The AP-1-luc vector was obtained from Stratagene (Agilent Sumitomo Dainippon Pharma Co., Ltd.). CD46-overexpressing and control cells were seeded in a 24-well plate at a density of $1x10^5$ cells per well and cultured for 24 h. The cells were transfected with 0.1 μg of the reporter vector and 2 ng of *Renilla* using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. At 6 h post-transfection, the cells were washed and cultured with media containing 5% FBS at 37˚C for 18 h. The addition of SB202190 or LY294002 was

at 24 h post-transfection. 24 h following addition of inhibitor, luciferase and *Renilla* activities were measured. Light signals were detected using the Dual-Luciferase Reporter Assay System (Promega Corporation) and a Glomax Navigator instrument (Promega Corporation), in accordance with the manufacturer's instructions.

MMP9 ELISA. Cell media or mouse sera were collected under sterile conditions, immediately frozen and stored at -20˚C until use. MMP9 concentration was determined using the human MMP9 Quantikine ELISA Kit (cat. no. DMP900; R&D Systems), following the manufacturer's instructions.

Xenograft model and lung metastasis model. A total of 28 female BALB/c nude mice (6-weeks-old; 17-22 g; Orient Bio, Inc.) were housed under specific pathogen-free conditions in the animal facility at the Hwasun Biomedical Convergence Center, with a 12-h light/dark cycle (light from 7:00 am to 7:00 pm) and controlled temperature maintained at 24±2 ˚C for optimal growth, while maintaining a relative humidity range of 50±10%. The mice were allowed *ad libitum* access to water and food pellets (PicoLab 5053). All procedures performed in studies were approved by the Animal Use and Care Committees at Chonnam National University Medical School (approval no. H2022-70). Mice were used to establish lung metastasis models through tail vein inoculation with $1.5x10^6$ UM-UC-3 and 5637 cells. For each cell line, mice were randomly assigned to the experimental group (overexpressing CD46) and the control group (7 mice per group). Overall survival and physiological signs of mice in the groups were monitored daily (for a period of 6 weeks). If the animals reached any of the following humane endpoints, they were sacrificed: Animal weight loss >20%; severely decreased mobility/activity, moribund behavior or wasting syndrome. Notably, none of the mice suffered from those signs of illness or mortality during the experimental process. At the end of the experiment, the mice were deeply anesthetized with 5% isoflurane (Ifran; Hana Pharm, Co., Ltd.) to minimize any potential pain or distress during the procedure, followed by cervical dislocation to sacrifice the mice. Their blood was collected via the left ventricle using a 23-25 gauge needle. The lung metastasis tumors' fluorescence was detected using the FOBI Fluorescence *In Vivo* Imaging System (Cellgentek, Co., Ltd.). Additionally, lung and liver tissues were fixed with 10% buffered formalin at 4˚C for 48 h. Then tissues were washed three times with PBS 1X and dehydrated with increasing alcohol concentration from 30 to 100% within 4 h and xylene within 1 h, embedded in paraffin and sliced into 5-μm serial sections and subsequently analyzed through H&E staining at room temperature for 1 min 40 and 15 sec and immunohistochemistry.

Immunohistochemical staining. Tumor paraffin (5 μm) sections from the mice were deparaffinized and rehydrated in graded alcohols and distilled water. Then they initially treated with 3% H_2O_2 in 60% methanol for 10 min and then incubated with Immuno-Block reagent (BioSB, Santa Barbara, CA) for 30 min at room temperature. These sections were further incubated overnight at 4˚C with primary antibodies against MMP9 (1:200; cat. no. HPA001238; Atlas Antibodies AB). For the secondary antibody, anti-rabbit immunoglobulin IgG (1:500; cat. no. 30014; Vector Laboratories, Inc.) was used and incubated for 1 h at room temperature. Immunoreactivity was then visualized using an enhanced DAB kit (Abcam). The results were observed using a light microscope.

Statistical analysis. GraphPad Prism software (Dotmatics) was employed for all statistical analyses. Comparison of two data sets was done with two-tailed unpaired Student's t-test in case of normal distributed data, or with U-Mann Whitney test in case of non-parametric data, considering P-values <0.05 as statistically significant. Multiple groups were analyzed by one-way ANOVA followed by Tukey's post hoc test or two-way ANOVA followed by Bonferroni's post hoc test. The results are presented as mean ± standard deviation.

Results

Forced expression of CD46 alters expression of MMP9 in bladder cancer cells. The observations indicated that ~50% of bladder cancers exhibit an overexpression of CD46 compared with normal urothelia (29). CD46 typically acts as a protector against complement-mediated cytotoxicity in cells. Previous studies reported that CD46 also shields cancer cells from the indirect cytotoxic effects of cetuximab (an EGFR inhibitor), by modulating AKT and ERK phosphorylation in bladder cancer cells (16). The direct role of intracellular CD46 in cancer cells, however, remains poorly understood. Recently, we found that overexpression and suppression of CD46 in bladder cancer cells regulated several genes associated with migration and invasion, as identified by DNA microarray analysis (30). These genes include matrix Gla protein (MGP) and keratin 13 (KRT13) (30). Therefore, the present study explored whether CD46 affected the expression and activity of MMPs, which are pivotal in cancer cell invasion and metastasis across various types of cancer. Focusing on MMP2 and MMP9, known to play significant roles in bladder cancer metastasis (35), the present study examined if forced CD46 expression could alter their expression levels. Previously described stable transfectants overexpressing CD46 in bladder cancer cells (5637, J82, UM-UC-3, 253J and HT1376) and melanoma cells (M010119) were used (29,36). Additionally, LNCaP prostate cancer cells, HCT116 colon cancer cells and A549 lung cancer cells were transfected with pBlasti-CD46 vectors, using pBlasti as control (vehicle group). Western blotting analysis revealed that CD46 overexpression stimulated MMP9 expression in 5637, J82 and UM-UC-3 cells, but not evidently in 253J cells (Fig. 1A). In HT1376 cells, where CD46 previously showed a stimulatory effect on migration (30), MMP9 expression remained unchanged. By contrast, most non-bladder cancer cells (LNCaP, HCT116 and A549) did not exhibit enhanced MMP9 upregulation with CD46, except for M010119 melanoma cells, which showed a marked increase in MMP9 expression. In all tested cells, CD46 stimulation of MMP2 expression was not observed, with the exception of M010119. Given the consistent upregulation of MMP9 mediated by CD46, the present study focused on three bladder cancer cell lines that exhibited increased MMP9 expression (5637, J82 and UM-UC-3). To determine the effect of CD46 overexpression on cancer cell proliferation, a cell proliferation assay was conducted over three days. The results indicated that CD46 overexpression did not significantly affect cell proliferation in these three

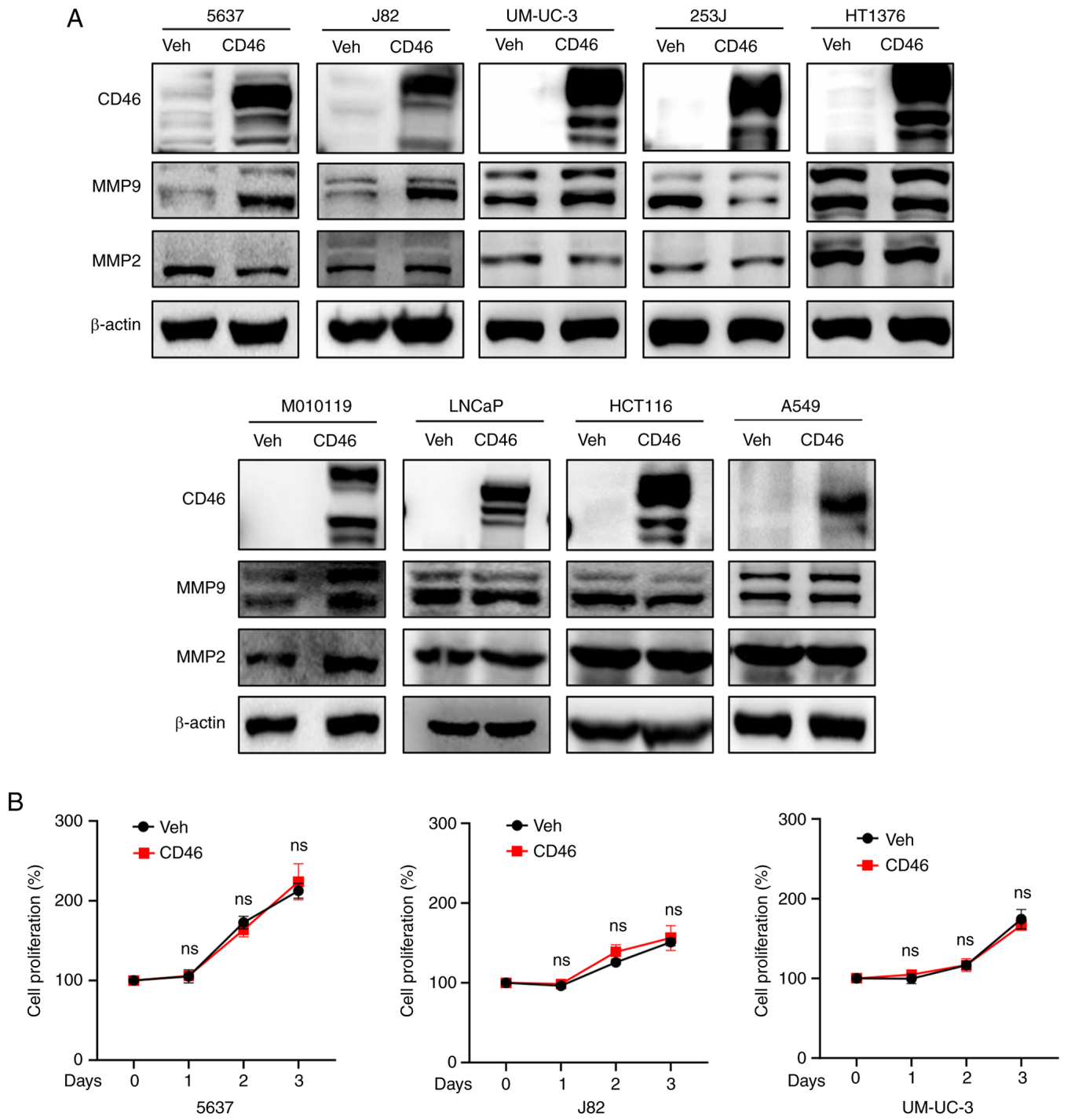

Figure 1. Expression of MMP9 by CD46 in bladder cancer cells. (A) Observation of MMP2 and MMP9 expression in five bladder cancer cell lines with CD46 overexpression, analyzed by western blotting. (B) Assessment of CD46 overexpression's impact on cell growth using an *in vitro* proliferation assay in cells overexpressing MMP9 due to CD46. Data are presented as mean ± SD. P-values were obtained using the U Mann Whitney test (^{ns}P>0.05). MMP9, matrix metalloproteinase 9; veh, vehicle; MMP2, matrix metalloproteinase 2.

bladder cancer cells (Fig. 1B; P>0.05 by Mann-Whitney U test), thus underscoring the CD46-induced MMP9 overexpression. These findings suggested that CD46 selectively enhances the expression of MMP9, a key factor in cancer metastasis, in bladder cancer cells.

CD46 promotes phosphorylation of p38 MAPK, PI3K/AKT and AP-1. To elucidate the mechanism behind CD46's role in elevating MMP9 expression, the present study examined several key signaling pathways that govern MMP9 regulation. There is mounting evidence indicating the involvement of both MAPK and PI3K/AKT cascades in MMP9 regulation (27,37,38). The primary constituents of the MAPK family include ERK, p38 and JNK, while PI3K/AKT is crucial for cell survival and growth (39). Investigating the potential link between CD46 and MMP9, the present study assessed alterations in the MAPK and PI3K/AKT pathways in bladder cancer cells exhibiting CD46-induced MMP9 expression.

As depicted in Fig. 2A, CD46 stimulation not only enhanced MMP9 expression but also increased phosphorylation levels of p38 MAPK and AKT proteins in all three cell lines examined. The right panel of Fig. 2A presents a quantitation graph corresponding to the left panel. No significant changes were observed in JNK and ERK phosphorylation across the cell lines, with the exception of p-ERK in UM-UC-3 cells (Student's t-test; P<0.0001). To minimize potential nonspecific effects of overexpression via viral vectors, we introduced CD46 siRNA (siCD46) into 5637 cells through transient transfection. CD46 suppression effectively reduced MMP9 expression and decreased phosphorylation of p38 and AKT (Fig. 2B; Student's t-test; P<0.0001). Jun and Fos family proteins form a dimeric complex of activator protein-1 (AP-1), influenced by both MAPK and PI3K/AKT pathways (37,38,40,41). Fig. 2A illustrated that CD46 overexpression also led to increased phosphorylation of c-Jun, but not c-Fos, in all three cell lines tested (Student's t-test; P<0.0001). These results indicated that CD46 enhanced the activation of p38 MAPK and PI3K/AKT in bladder cancer cells, which appears to be associated with increased MMP9 expression, suggesting a potential role of CD46-mediated activation in bladder cancer metastasis through the p38 MAPK and PI3K/AKT pathways.

CD46 activates p38 MAPK and PI3K/AKT through phosphorylation of c-Jun to promote expression of MMP9. To examine the influence of CD46 on MMP9 transcription, the present study conducted RT-qPCR in 5637 cells. The results showed that CD46 overexpression significantly increased MMP9 mRNA levels (Fig. 3A; P<0.01). Additionally, reporter gene transcription assays with MMP9 or AP-1 promoters revealed CD46-induced upregulation of both MMP-9 and AP-1 promoter activities in all three cell types examined (Fig. 3B; P<0.01). Subsequently, the present study explored if CD46-mediated MMP9 overexpression involved p38 MAPK and PI3K/AKT pathway activation. UM-UC-3 cells were treated with specific inhibitors: p38 inhibitor SB202190 (20 μM) and AKT inhibitor LY294002 (10 μM) for 24 h. The results, depicted in Fig. 3C, demonstrated that both inhibitors significantly reduced CD46-induced MMP9 promoter activity (P<0.0001) and AP-1 activity (P<0.01). Moreover, these inhibitors also decreased CD46-stimulated MMP-9 protein overexpression in 5637 and J82 cells, as confirmed by western blot analysis (Fig. 3D; P<0.0001). Collectively, these findings suggested that CD46 enhanced MAPK and AKT signaling, leading to AP-1-mediated transcription and, consequently, increased MMP9 expression in bladder cancer cells.

CD46-stimulated MMP9 promotes migratory and invasive potential of bladder cancer cells in vitro. To explore the functional impact of CD46-stimulated MMP9 expression, the bioactivity of MMP9 secreted by 5637 and J82 cells was analyzed using gelatin zymography. This revealed that CD46 markedly elevated the gelatinase activity of MMP9 in cells overexpressing CD46 (Fig. 4A). It was also observed that CD46 is actively secreted into the cell culture media in CD46-overexpressed cancer cells (bottom panel of Fig. 4A). MMP9 is known to actively cleave membrane CD46 to increase soluble CD46 (42,43). Additionally, MMP9 levels secreted into the media were quantified by ELISA, finding significantly elevated MMP9 levels in the media of both cell types (Fig. 4B; P<0.01 for both 5637 and J82). The influence of CD46 on cell migration and wound healing was further investigated using wound-healing assays. Here, the width of the healed gap was measured after a specified incubation period. As depicted in Fig. 4C, CD46 overexpression significantly accelerated wound repair in 5637 and J82 cells at each time point (P<0.001). In 253J cells in which CD46 did not upregulate MMP9 expression, CD46 did not affect the wound repair capability of cells (Fig. S1). Furthermore, to confirm the role of CD46 in promoting bladder cancer cell migration and invasion, Transwell migration and invasion assays were conducted. Post-incubation, filters were stained and examined under a microscope at randomly chosen areas to count cells. CD46 overexpression substantially increased both migration and invasion in 5637 and J82 cells (Fig. 4D; P<0.01). Correspondingly, suppression of CD46 by siRNA caused 5637 cells to slow wound repair and decreased migration and invasion (Fig. S2). These findings suggested that the upregulation of MMP9 expression induced by CD46 was linked with enhanced bioactivity and secretion of MMP9 in bladder cancer cells.

CD46 promotes bladder cancer metastasis in vivo. To explore the effect of CD46 overexpression in a live experimental metastasis model, 5637 cells were injected into the tail vein of nude mice, with seven mice in each group. Over a period of six weeks following the injection, the weight of the mice was monitored. The results indicated that CD46 overexpression did not significantly alter the weight of the mice groups (Fig. S3A; P>0.05; Mann-Whitney U test). At the end of the six-week period, the mice were sacrificed and examined for metastatic tumor nodules in the lung and liver. Gross and fluorescent examinations of the lung surfaces were conducted (Fig. S3B and C). The lungs of the mice injected with 5637 cells displayed multiple positive fluorescence foci, as shown in a representative image (Fig. 5A). The lung tissues were fixed and embedded in paraffin to identify metastatic tumor nodules within the lung parenchyma. Serial lung tissue sections, taken every 1-2 mm up to 5 sections, were stained with H&E and the tumor nodules counted. The number of lung metastases was significantly higher in the CD46-5637 cells group, with 6 out of 7 mice showing more than one micrometastatic nodule, in contrast to almost none in the control group (Fig. 5B; P=0.04). The average tumor area in lung metastatic nodules of the CD46-5637-injected mice was 11.99±0.19 mm^2, compared with >1 mm^2 in control cell-injected mice (data not shown). The entire microscopic examination of the lung tissues is shown in Fig. S3D. Fig. 5C (a-f) shows a representative lung tissue with a tumor nodule. Immunohistochemical staining revealed that the metastasized tumor cells did not significantly overexpress MMP9 (Fig. 5C, g-h). No tumor nodules were detected in the liver of any mice. Additionally, serum analysis during sample collection showed that mice injected with CD46-overexpressing cells had higher blood levels of MMP9 compared with those injected with control cells, as determined by ELISA (Fig. 5D). These results demonstrated a significant increase in lung metastasis in mice due to CD46 overexpression. While overexpression of MMP9 in these metastatic tumor nodules was

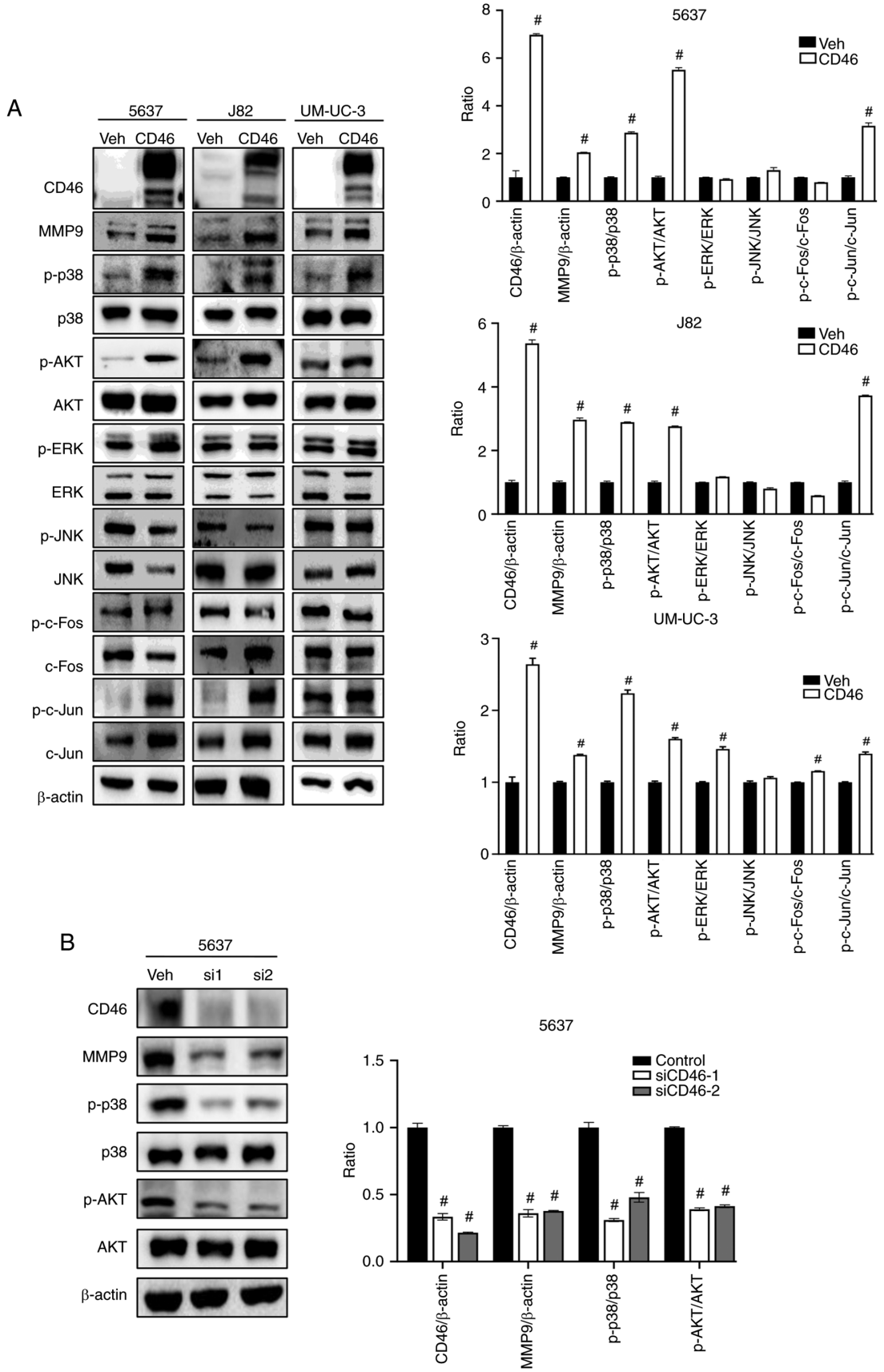

Figure 2. Effects of CD46 on MAPK and PI3K/AKT pathways. (A) Western blot analysis of bladder cancer cells overexpressing CD46 for indicated proteins. The right panel quantitatively displays the proteins from the left panel. Phosphorylated protein levels were normalized to total protein levels. (B) Treatment of 5637 cells with two types of CD46 siRNA and subsequent western blot analysis for p38 and AKT. Quantitative results are shown in the right panel. Phosphorylated protein levels were normalized to total protein levels. Each bar represents mean ± SD. Statistical significance between groups was assessed using a two-tailed unpaired Student's t-test for A and one-way ANOVA with Tukey multiple comparisons test for B. #P<0.0001 vs. control cells. veh, vehicle; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; si, short interfering; NF-κB, nuclear factor kappa B; p-, phosphorylated.

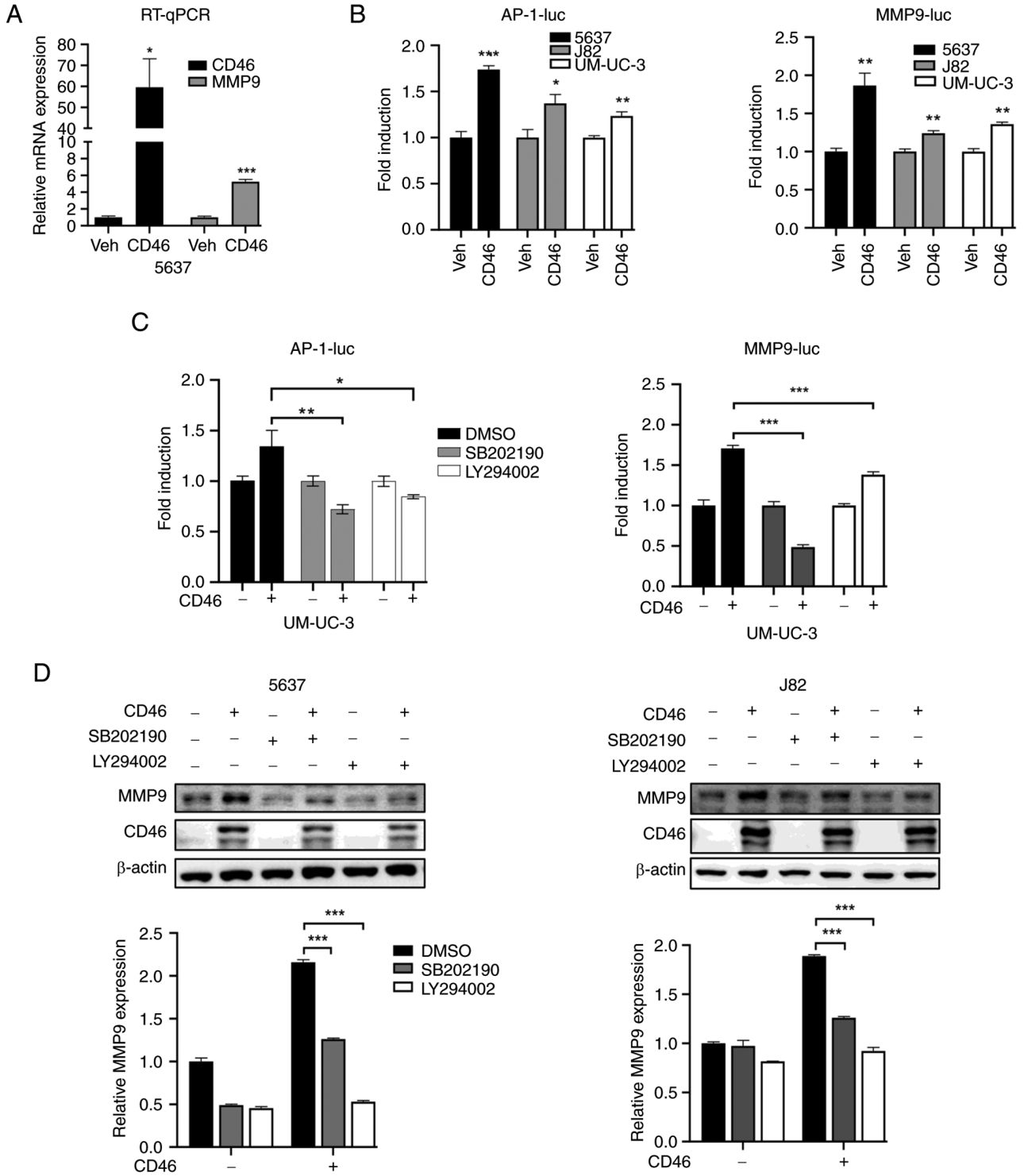

Figure 3. Effects of specific inhibitors on p38 and AKT in CD46-mediated MMP9 promotion. (A) RT-qPCR of total RNA from 5637 cells to assess CD46 and MMP9 expression levels. (B) Transfection of bladder cancer cells with either AP-1-luc or MMP9-luc plasmids, followed by reporter gene transcription analysis. (C) UM-UC-3 cells underwent a reporter gene transcription assay to evaluate the effect of p38 (SB202190) and AKT (LY294002) inhibitors on CD46-mediated transcriptional activities of AP-1 (left panel) and MMP9 (right panel). (D) Treatment of both 5637 and J82 cells with indicated inhibitors for 24 h, followed by western blot analysis. Densitometric analysis of MMP9 expression is displayed as bar graphs at the bottom of each blot. Each bar represents mean ± SD. Statistical differences between groups were determined using a two-tailed unpaired Student's t-test for A and B and two-way ANOVA with Bonferroni multiple comparisons test for C and D. *P<0.01, **P<0.001, ***P<0.0001 vs. control cells. veh, vehicle; AKT, protein kinase B; MMP9, matrix metalloproteinase 9; RT-qPCR, reverse transcription-quantitative PCR; AP-1, activator protein 1.

not definitively positive, the higher MMP9 levels in the blood of mice injected with CD46-overexpressing cells suggested a potential association between CD46, MMP9 expression and tumor metastasis.

Discussion

CD46 is a protein with diverse roles in various biological processes, notably in inhibiting complement activation.

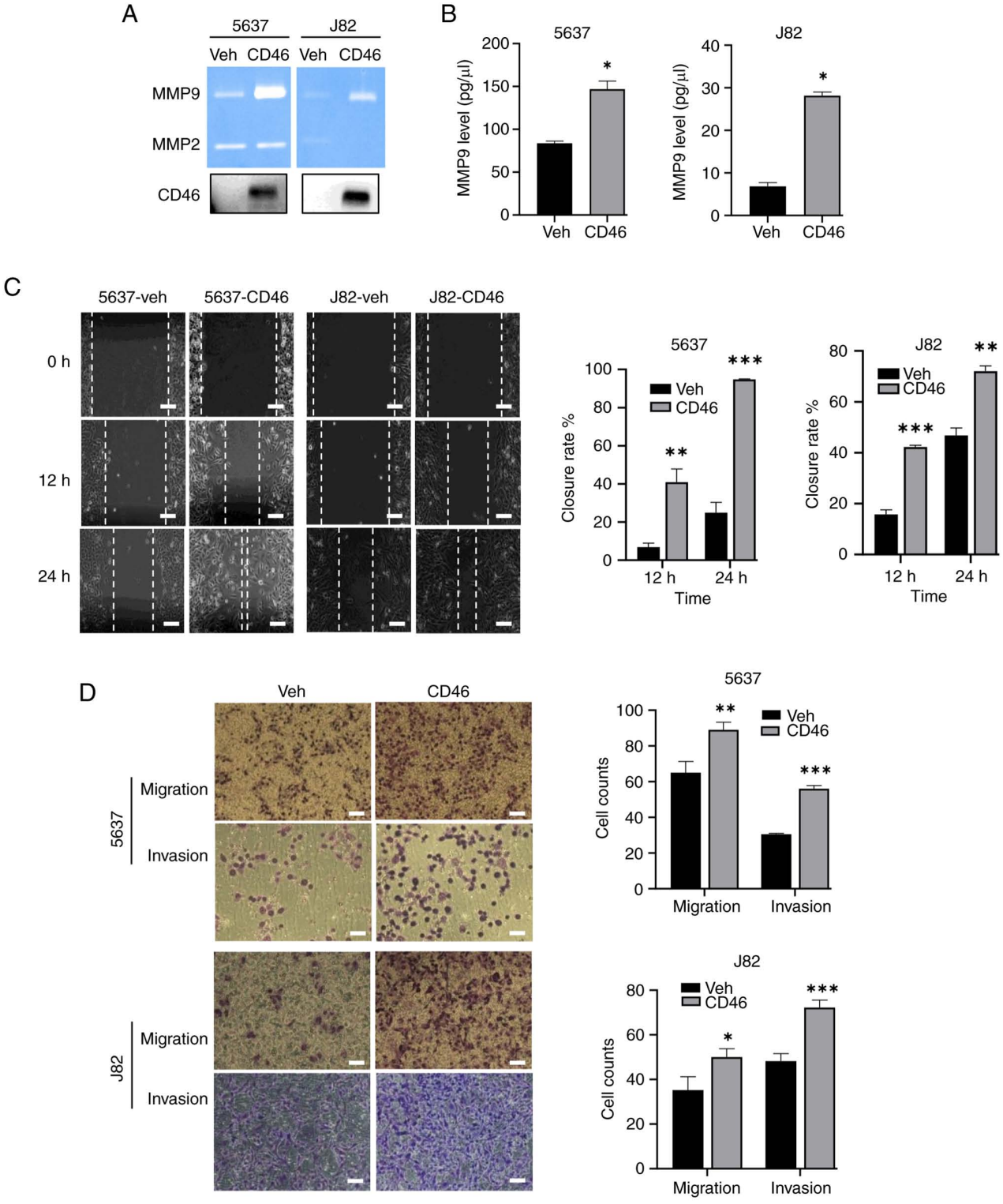

Figure 4. Effects of CD46 on the regulation of migratory and invasive potential in bladder cancer cells. (A) Media from specified cells were collected for analysis. Non-reducing gelatin SDS-PAGE was used to demonstrate gelatinase activity (top panel) and western blot analysis was conducted to show secreted CD46 (lower panel). (B) Media from cells were analyzed by ELISA assay to show secreted MMP9. (C) Bright-field images showing scratched areas (marked by white lines) in confluent monolayers of cancer cells overexpressing CD46 at different time points (left panel). Relative wound closure was assessed by measuring the width of the wounds compared with control cells (right panel). (D) Transwell assays to measure cell migration and invasion in 5637 and J82 cells transfected with CD46 (left panel). Quantification of cell migration and invasion was conducted (right panel). Each bar represents mean ± SD. All scale bars, 200 μm. Differences between groups were assessed using a two-tailed unpaired Student's t-test. *P<0.01, **P<0.001, ***P<0.0001 vs. control cells. veh, vehicle; MMP9, matrix metalloproteinase 9; MMP2, matrix metalloproteinase 2.

This function is essential for preventing excessive or inappropriate activation of the complement system, which could lead to tissue damage and autoimmune disorders. Apart from complement regulation, CD46 has been implicated in several diseases, including different types of cancer. Our previous research established that restoring CD46 expression in bladder

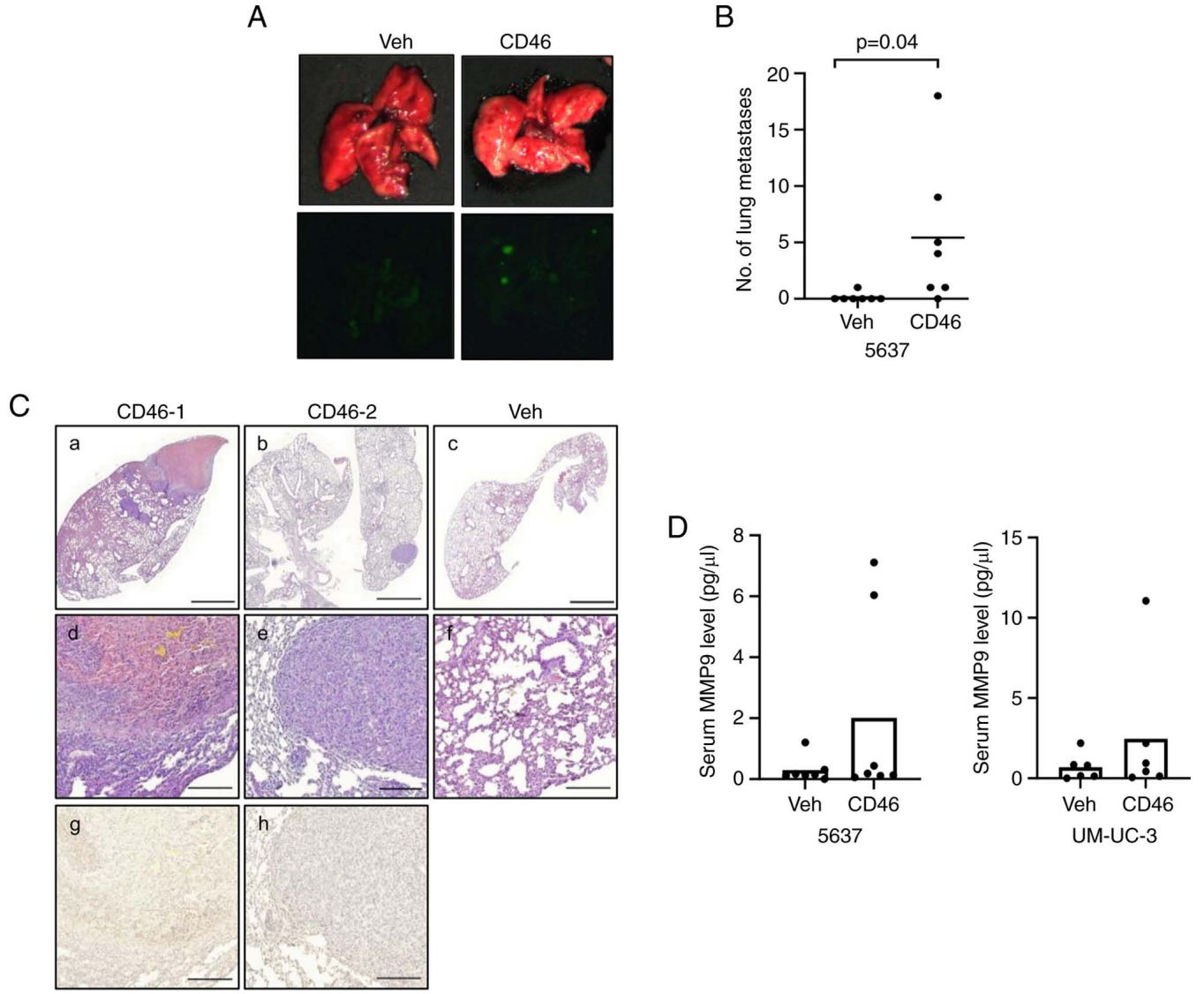

Figure 5. Effects of CD46 on experimental lung metastasis in a murine bladder cancer model. Nude mice were injected intravenously with 5637 cells. After 6 weeks, they were sacrificed for analysis of metastatic lung tumor nodules. (A) Optical images of lung metastasis in athymic nude mice injected with 5637-veh and 5637-CD46 cells (n=7 each group). (B) Quantification of lung tumor nodules in each mouse group. Each dot represents an individual mouse. (C) Histological examination of lungs from CD46-5637 and veh-5637 injected mice (a-c). High magnification images of corresponding lungs (d-f) and (g and h) immunohistochemical images for MMP9 expression in a and b. Scale bar, 2 mm (a-c), 200 μm (d-h). (D) ELISA analysis of sera from mice before termination for serum MMP9 levels. Data represented as mean ± SD. Each dot represents an individual mouse. Statistical differences determined by paired two-tailed Student's t-test. veh, vehicle; MMP9, matrix metalloproteinase 9.

cancer cells offers protection against the inhibitory effects of cetuximab on AKT and ERK phosphorylation. Furthermore, this restoration shields cells from complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity induced by cetuximab. Consequently, CD46 plays a protective role in cancer, counteracting direct cytotoxicity (through peripheral blood mononuclear cells or the complement system) and indirect cytotoxicity exerted by cetuximab in bladder cancer cells. We have also shown that CD46 target gene expression profiling in HT1376 bladder cancer cells using DNA microarray identified MGP and keratin 13 (KRT13) as CD46 responsive proteins; both are associated with cell migration and invasion. Overexpression of CD46 was found to enhance the migratory potential of bladder cancer cells (30). The present study aimed to investigate the effect of CD46 on MMP9 expression and bladder cancer progression. The findings indicated that CD46 facilitates bladder cancer cell migration and invasion via MMP9 expression. In a mouse model of cancer metastasis, achieved through tail vein injection, CD46 overexpression led to a significant increase in bladder cancer lung metastatic lesions. Notably, while metastatic tumors did not markedly overexpress MMP9, elevated levels of MMP9 were detected in the serum of mice injected with CD46 overexpressing 5637 bladder cancer cells. Although a number of studies are focused on the role of CD46 as a protector for complement-mediated cytotoxicity and a viral receptor for cancer therapeutics, there are several studies reporting CD46 expression as a cancer progression and recurrence indicator. In breast cancers, expression of CD46 was positively associated with tumor grade and tumor recurrence and also indicative of decreased progression-free time and overall survival time (9,12). CD46 was also highly correlated with shorter revival-free time in

ovarian cancers and increased liver metastasis in colorectal cancers (13,42). Altogether, this suggests that upregulated MMP9 can be rapidly secreted into the extracellular matrix and bloodstream in a tumor microenvironment. Most MMPs, including MMP9, are secreted as inactive proproteins and are activated upon cleavage by extracellular proteinases (43).

The present study also demonstrated that CD46-mediated MMP9 activation occurs through p38 MAPK and PI3K/AKT, which further stimulates AP-1, particularly the activation of c-Jun protein. Currently, the exact mechanism by which CD46 activates the p38 MAPK or PI3K/AKT pathways remains to be elucidated. Nonetheless, substantial evidence links the transmembrane protein CD46 to cytoskeletal proteins and the MAPK pathway. CD46 has been long associated with the maintenance of intestinal epithelial barrier integrity (44). It directly interacts with Ste20/SPS-1-related kinase (SPAK) and the cytoplasmic part of E-cadherin (45). SPAK is also involved in the MAPK pathway. E-cadherin, a transmembrane protein, plays an essential role in cell-to-cell adhesion. Activation of CD46 on intestinal epithelial cells leads to a rapid decrease in tight junction integrity (44). Furthermore, CD46 activation results in cell proliferation and migration, aligning with the effects observed following reduced contact inhibition (46). MMPs play a role in various physiological processes, including embryonic development, reproduction, tissue remodeling and carcinogenesis. MMP9, a key MMP member, primarily degrades collagen type IV in the basement membrane. MMP9 expression, observed in various cancers, is thought to aid tumor cell metastasis (47). Activation of EGFR stimulates the phosphorylation of AKT, MAPK and JNK (37). The *MMP9* promoter region contains multiple transcription factor binding sites, including those for NF-κB, SP-1, Ets, AP-1 and retinoblastoma (19). Previous research has shown that MMP9 is upregulated in bladder cancer cells through the activation of p38 MAP kinase pathways (48) and the AKT pathway (49).

The present study focused on activating AP-1 to understand the CD46-mediated activation of p38 MAPK and/or AKT and its role in regulating MMP9. AP-1 induction, mostly driven by the JNK and p38MAPK pathways in response to various stimuli, is well-documented (50). Upon activation, JNKs move to the nucleus, phosphorylating c-Jun, which enhances its transcriptional activation capabilities through homodimerization or heterodimerization with c-Fos (51). The findings of the present study indicated that the upregulation of MMP9, stimulated by CD46, is primarily facilitated through the activation of c-Jun phosphorylation, rather than c-Fos. It is plausible that other transcription factors, such as NF-κB or SP-1, are also influenced by CD46 in regulating MMP9. The presence of AP-1 sites ~70 bp upstream from the transcriptional start site of the *MMP9* gene is considered to play a crucial role in the transcriptional activation of MMP promoters (52). Moreover, the transactivation of the MMP9 promoter necessitates the specific interaction of AP-1 with other cis-acting elements and certain transcription factors binding to these sequences (53). Further research is required to delineate the intricate transcriptional regulation of the MMP9 promoter and enhancer regions.

The major limitation of the present study is that it did not survey MMP9 and CD46 expressions in bladder cancer patients, which is a potent tool for studying CD46 progression and development. In the future, it is planned to assess the correlation of MMP9 and CD46 expressions with stages of bladder cancer patients.

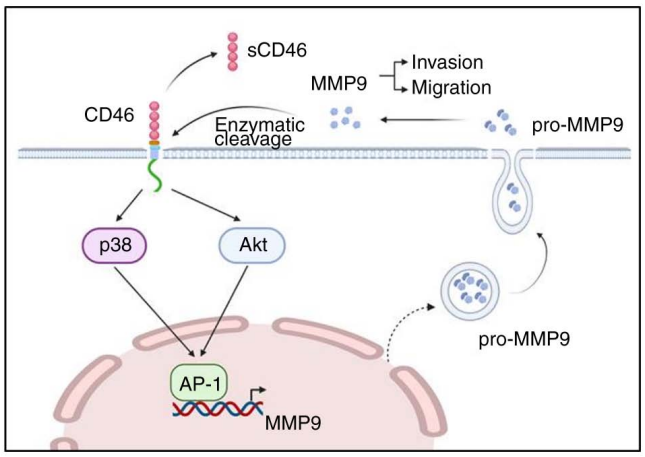

Figure 6. Model of CD46-induced MMP9 upregulation in bladder cancer cells. This figure proposes a model for CD46-mediated promotion of bladder cancer metastasis. Transmembrane receptor CD46 activates hyperphosphorylation of p38 MAPK and PI3K/AKT, leading to c-Jun activation in the AP-1 complex at the MMP9 promoter. Increased MMP9 secretion into the extracellular matrix enhances migration and invasion of bladder cancer cells. Secreted MMP9 may also cleave the extracellular domain of CD46, potentially regulating overstimulation of CD46-mediated MMP9 expression. The model was created using BioRender.com. MMP9, matrix metalloproteinase 9; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; AP-1, activator protein 1.

In conclusion, the results suggested that CD46 enhanced bladder cancer cell migration, invasion and metastasis. The present study also underscored the potential role of MMP9 in the metastatic process via the MAPK and AKT pathways activation (Fig. 6). CD46 could be integral in regulating these biological processes and may serve as a viable target for therapeutic intervention in bladder cancer metastasis.

Acknowledgements

Not applicable.

Funding

The present study was supported by a National Research Foundation of Korea grant funded by the Korean government (Ministry of Science and ICT; grant no. 2022R1A2C1003206).

Availability of data and materials

The data generated in the present study are included in the figures of this article.

Authors' contributions

TNT performed the experiments, data analysis and preparation of the original draft. HDT, VTN and SYK contributed to the investigation, methodology, review and editing of the final manuscript. CM assisted in the animal studies. ECH aided the clinical background of study and final version of the manuscript. CJ contributed to the conceptualization, supervision,

funding acquisition, review, revision and approval of the manuscript. TNT and CJ confirm the authenticity of all the raw data. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Animal Use and Care Committees at Chonnam National University Medical School (approval no. H2022-70).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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