Collagen type VI-α1 and 2 repress the proliferation, migration and invasion of bladder cancer cells

XUAN-MEI PIAO[1], BYUNGDOO HWANG[2], PILDU JEONG[1], YOUNG JOON BYUN[1], HO WON KANG[1,3], SUNG PHIL SEO[1,3], WON TAE KIM[1,3], JONG-YOUNG LEE[4], YUN-SOK HA[5,6], YOUNG-SUK LEE[7], ISAAC Y. KIM[8], YUNG HYUN CHOI[9], EUN-JONG CHA[10], SUNG-KWON MOON[2], SEOK JOONG YUN[1,3] and WUN-JAE KIM[1,11]

[1]Department of Urology, College of Medicine, Chungbuk National University, Cheongju, Chungcheongbuk 28644; [2]Department of Food Science and Technology, Chung-Ang University, Anseong, Gyeonggi 456-756; [3]Department of Urology, Chungbuk National University Hospital, Cheongju, Chungcheongbuk 28644; [4]Oneomics Institute, Seoul 04158; [5]Department of Urology, School of Medicine, Kyungpook National University; [6]Department of Urology, Kyungpook National University Hospital, Daegu 41566; [7]Department of Urology, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon, Gyeongsangnam 51353, Republic of Korea; [8]Section of Urologic Oncology and Dean and Betty Gallo Prostate Cancer Center, The Cancer Institute of New Jersey and Robert Wood Johnson Medical School, New Brunswick, NJ 08903-2681, USA; [9]Department of Biochemistry, College of Oriental Medicine, Dong-Eui University, Busan 614-052; [10]Department of Biomedical Engineering, Chungbuk National University College of Medicine; [11]Institute of Urotech, Cheongju, Chungcheongbuk 28644, Republic of Korea

Received October 28, 2020; Accepted April 16, 2021

DOI: 10.3892/ijo.2021.5217

Abstract. The bladder cancer (BCa) microenvironment comprises heterogeneous tumor cell populations, the surrounding stroma and the extracellular matrix (ECM). Collagen, the scaffold of the tumor microenvironment, regulates ECM remodeling to promote tumor infiltration, angiogenesis, invasion and migration. The present study examined how collagen type VI-α (COL6A) 1 and 2 function during BCa pathogenesis and progression, with the aim of facilitating the development of precision therapeutics, risk stratification and molecular diagnosis. *COL6A1* and *COL6A2* mRNA expression in non-muscle invasive BCa (NMIBC) and MIBC tissue samples was measured using reverse transcription-quantitative PCR. In addition, the tumor-suppressive effects of *COL6A1* and *COL6A2* in human BCa EJ cells (MGH-U1) were assessed. Compared with normal controls, *COL6A1* and *COL6A2* mRNA expression was downregulated in both NMIBC and MIBC tissue samples ($P<0.05$, respectively). *COL6A1* and *COL6A2* effectively inhibited the proliferation of human BCa EJ cells (MGH-U1) and induced cell cycle arrest at the G_1 phase. Additionally, *COL6A1* and *COL6A2* served roles in MAPK and AKT signaling by increasing p38 MAPK phosphorylation and decreasing AKT phosphorylation. Finally, *COL6A1* and *COL6A2* inhibited wound healing and invasion by suppressing the activity of matrix metalloproteinase (MMP)-2 and MMP-9. In conclusion, COL6A1 and COL6A2 may act as classical collagens by forming a physical barrier to inhibit BCa tumor growth and invasion.

Correspondence to: Professor Wun-Jae Kim, Institute of Urotech, Chungdae-ro 1, Seowon, Cheongju, Chungcheongbuk 28644, Republic of Korea
E-mail: wjkim@chungbuk.ac.kr

Abbreviations: AP-1, activator protein 1; BCa, bladder cancer; CDK, cyclin-dependent kinase; CKI, cyclin kinase inhibitor; COL6A1/2, collagen type VI-α1/2; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IL, interleukin; JNK, c-Jun N-terminal kinase; MIBC, muscle invasive BCa; MMP, matrix metalloproteinase; NMIBC, non-MIBC; SP-1, specificity protein 1; TAM, tumor-associated macrophage; TME, tumor microenvironment; TUR, transurethral resection

Key words: COL6A, BCa, tumor suppressor, pathogenesis, progression

Introduction

At present, >400,000 patients worldwide are newly diagnosed with bladder cancer (BCa) each year (1). Among them, ~75% have non-muscle invasive BCa (NMIBC) and 25% have muscle invasive bladder cancer (MIBC), at the time of presentation (2). NMIBC is not immediately life-threatening, but its propensity for recurrence leads to costly clinical surveillance throughout life. The 5-year progression rate of NMIBC is 10-30%; however, once progression occurs, the cancer-specific survival rate falls to ~35% (3,4). Thus, it is important to better understand the mechanisms underlying BCa progression and to identify practical biomarkers for early diagnosis, treatment and prognosis (5). In our previous study, next generation sequencing and microRNA (miRNA/miR) microarray assays were used to identify several differentially expressed miRNAs and their target genes in BCa (6). Among them, the expression levels of

genes encoding collagen type VI-α1 and 2 (COL6A1 and 2) were downregulated, while the expression levels of *miR-6124* and *miR-4651* were upregulated in BCa compared with in normal controls (6). Additionally, the expression of cell-free *miR-6124* in BCa urine was higher than that in non-cancer patients with hematuria, indicating that urinary *miR-6124* may be a promising non-invasive diagnostic biomarker for BCa (7).

The interaction between tumor cells and the microenvironment can affect disease initiation and progression (8). The extracellular matrix (ECM) is an essential part of the tumor microenvironment (TME), which interacts with cancer cells at each step of the metastatic process (9). Collagen is the major component of the ECM, and dysregulation of collagen is associated with increased malignancy (10,11). COL6 is a major component of the bladder ECM (12). In lung, pancreatic, breast and ovarian cancer, *COL6A1* and *COL6A2* act as oncogenes (13). In particular, *COL6A1* expression is associated with a poor prognosis in some types of cancer, including kidney, prostate, lung and cervical cancer (14-17), and COL6A2 protein expression is suppressed in UPII-SV40Tag mice (a model of invasive BCa) (18). A weighted gene co-expression network analysis and protein-protein interaction network analysis demonstrated that increased expression levels of six potential biomarkers (COL3A1, fibronectin 1, COL5A1, fibrillin 1, COL6A1 and thrombospondin 2) were significantly associated with a worse overall survival in patients with BCa (19). In addition, a bioinformatic analysis using three GSE datasets identified six collagen members (COL1A1, COL1A2, COL5A2, COL6A1, COL6A2 and COL6A3) that were upregulated in BCa, which all resided in the ECM-receptor interaction signaling pathway (20). However, although COL6A1 and COL6A2 serve diverse roles in different types of cancer, the exact functions of COL6A1 and COL6A2 in the pathogenesis and progression of BCa remain unclear.

The present study examined the expression levels of *COL6A1* and *COL6A2* in BCa using reverse transcription-quantitative (RT-q) PCR. Additionally, the molecular signaling cascades by which COL6A1 and COL6A2 may suppress proliferation, migration and invasion of human BCa EJ cells (MGH-U1) were investigated. To do this, the current study analyzed different signaling pathways, cell cycle modulation and transcription factors that regulate matrix metalloproteinase (MMP)-2 and MMP-9.

Materials and methods

Patients and tissue samples. Table I lists the baseline characteristics of the study subjects. A total of 237 bladder tissue samples were obtained from the National Biobank of Korea at Chungbuk National University Hospital (Cheongju, South Korea). Among these, 140 samples were from patients with primary BCa (age range, 24-89 years) and were verified histologically as transitional cell carcinoma. The remaining 97 samples (used as the control set) comprised normal bladder mucosa samples (n=37), which were taken from patients with continuous hematuria who were suspicious of BCa (the final diagnoses were benign diseases, mostly stress urinary incontinence) or normal tissues from the area surrounding BCa (n=60); among them, 36 were obtained from the aforementioned patients with BCa and 24 were from patients with BCa not included in the present study (3 cm from tumor; age range, 19-89 years). To reduce the chances of confounding factors affecting the analyses, patients diagnosed with concomitant carcinoma *in situ* or carcinoma *in situ* lesions alone were excluded. Voided urine cytology was tested before surgical treatment to assist BCa diagnosis and/or prognosis. Fresh-frozen specimens were obtained during surgical resection of transitional cell carcinoma at Chungbuk National University Hospital (Cheongju, South Korea) between April 2000 and October 2010. All tumors were macro-dissected, typically within 15 min of surgical resection. Each specimen was confirmed by pathological analysis of a part of fresh-frozen specimens obtained from radical cystectomy and transurethral resection of the bladder tumor. Tumors were staged (2002 TNM Classification) and graded (2004 World Health Organization Classification), according to standard criteria (21). In cases of NMIBC (n=97), transurethral resection (TUR) of the tumor was performed. If incomplete, or when a high-grade or T1 tumor was detected, a second TUR was performed 2-4 weeks after the initial resection. Patients with intermediate- or high-risk NMIBC received one cycle of intravesical Bacillus Calmette-Guérin immunotherapy. In cases of MIBC (n=43), radical cystectomy and complete pelvic lymph node dissection were performed. Patients with pT3 or pT4, or node-positive disease (based on analysis of radical cystectomy specimens), received at least four cycles of cisplatin-based chemotherapy. Neither clinically metastatic disease, nor non-cystectomy cases were excluded from the study. Each patient was followed and managed according to standard recommendations (22,23). Recurrence was defined as recurrence of primary NMIBC of the same pathological stage. Progression of NMIBC or MIBC was defined as progression of TNM stage after disease relapse. The mean follow-up period for patients with NMIBC was 72.95 months (range, 3.2-172.2 months). The mean follow-up period for patients with MIBC was 36.18 months (range, 3.0-141.4 months). Collection and analysis of all samples were approved by the Institutional Review Board of Chungbuk National University (approval no. GR2010-12-010), and written informed consent was obtained from each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki.

BCa cell line. Human BCa EJ cells (MGH-U1) were kindly provided by Dr Wun-Jae Kim (Department of Urology, Chungbuk National University, Cheongju, South Korea) and were authenticated using short tandem repeat analysis. After authentication, this cell line matched 100% with T24 cells. Cells were grown at 37˚C in a 5% CO_2 humidified incubator in DMEM (cat. no. 10-017-CV; Corning, Inc) supplemented with 10% fetal bovine serum (FBS; cat. no. 35-015-CV; Corning, Inc), 100 U/ml penicillin and 100 μg/ml streptomycin.

RNA extraction and RT-qPCR. Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and stored at -80˚C. Subsequently, cDNA was synthesized from 1 μg total RNA using the First Strand cDNA Synthesis kit (Clontech Laboratories, Inc.) following the manufacturer's protocol. Relative gene expression was analyzed using qPCR and the $2^{-\Delta\Delta Cq}$ method (24). qPCR was performed using a Rotor Gene 6000 instrument (Qiagen

Table I. Clinicopathological features of tissues from 140 patients with primary bladder cancer (97 with NMIBC and 43 with MIBC) and 97 controls (surrounding normal tissues and normal bladder mucosae).

	Bladder cancer			
Variable	NMIBC	MIBC	Control	P-value
Mean age ± SD, years	63.45±13.79	67.60±9.84	61.98±14.32	0.083[a]
Sex, n (%)				0.975[b]
Male	80 (82.5)	36 (83.7)	81 (83.5)	
Female	17 (17.5)	7 (16.3)	16 (16.5)	
Operation, n (%)				<0.0001[b]
TUR-BT	97 (100.0)	17 (39.5)		
Radical cystectomy	0 (0.0)	26 (60.5)		
Tumor size, n (%)				0.003[c]
≤1 cm	16 (16.5)	2 (4.7)		
2-3 cm	37 (38.1)	11 (25.6)		
>3 cm	37 (38.1)	28 (65.1)		
NA	7 (7.3)	2 (4.7)		
Multiplicity, n (%)				0.108[c]
Single	52 (53.6)	30 (69.8)		
2-7	28 (28.9)	7 (16.3)		
>7	11 (11.3)	4 (9.3)		
NA	6 (6.2)	2 (4.6)		
Grade (2004 WHO grading system), n (%)				<0.0001[b]
Low	72 (74.2)	8 (18.6)		
High	25 (25.8)	35 (81.4)		
Stage, n (%)				<0.001[c]
TaN0M0	26 (26.8)			
T1N0M0	71 (73.2)			
T2N0M0		13 (30.2)		
T3N0M0		6 (14.0)		
T≥4 or N≥1 or M1		24 (55.8)		
Chemotherapy, n (%)				<0.0001[b]
No	97 (100.0)	23 (53.5)		
Yes	0 (0.0)	20 (46.5)		
BCG therapy, n (%)				<0.0001[b]
No	56 (57.7)	38 (88.4)		
Yes	40 (41.2)	5 (11.6)		
Recurrence, n (%)				
No	59 (60.8)	-		
Yes	38 (39.2)	-		
Progression, n (%)				0.125[b]
No	79 (81.4)	30 (69.8)		
Yes	18 (18.6)	13 (30.2)		
Survival, n (%)				0.009[c]
Alive	64 (66.0)	21 (48.8)		
Non-cancer-specific death	18 (18.6)	3 (7.0)		
Cancer-specific death	15 (15.5)	19 (44.2)		
Mean follow-up time (range), months	72.95 (3.20-172.20)	36.18 (3.00-141.40)		

[a]P-value obtained using the Kruskal-Wallis H test. [b]P-values obtained using the χ^2 test. [c]P-values obtained using the Mann-Whitney U test. BCG, Bacillus Calmette-Guerin; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; TUR-BT, transurethral resection of bladder tumor; WHO, World Health Organization; NA, not available.

GmbH) to amplify mRNA from tissue. Microtubes (Qiagen GmbH) containing SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.) were used for the qPCR reactions. The primers used for amplifying candidate genes were as follows: *COL6A1* sense, 5'-CGTGGACCTGTTCTTTGTG-3' and antisense, 5'-CGTCACTGTAGTGCAGCG-3'; *COL6A2* sense, 5'-CAGGAGGTCATCTCGCCG-3' and antisense, 5'-GTT CTGCAGCTGGCTGATG-3'; *miR-6124* sense, 5'-GGAAAA GGAAGGGGGAGGA-3'; and *miR-4651* sense, 5'-CGG GGUGGGUGAGGUCGGGC-3'. A universal primer with a poly-A tail was used as the miRNA antisense primer. Control *GAPDH* (used for target mRNA normalization) primers were as follows: Sense, 5'-CATGTTCGTCATGGGTGTGA-3' and antisense, 5'-ATGGCATGGACTGTGGTCAT-3'. Since there are no consensus housekeeping miRNAs to date, and the utility of most commonly used miRNA internal controls, such as U6, may lead to varied results (7,25,26), miRNA expression was normalized to the total RNA concentration measured using Quant-iT™ RiboGreen™ RNA Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.).

Materials. Antibodies specific for $p21^{WAF1}$ (cat. no. sc-756), $p27^{KIP1}$ (cat. no. sc-528), p53 (cat. no. sc126), cyclin-dependent kinase (CDK) 2 (cat. no. sc-6248), CDK4 (cat. no. sc-23896), Cyclin D (cat. no. sc-8396), Cyclin E (s cat. no. c-377100) and β-actin (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. Antibodies specific for extracellular signal-regulated kinase (ERK; cat. no. 9102), p38 MAPK (cat. no. 9212), Jun N-terminal kinase (JNK; cat. no. 9258), AKT (cat. no. 9272), phospho-ERK (cat. no. 9101), phospho-p38 MAPK (cat. no. 9211), phospho-JNK (cat. no. 9251) and phospho-AKT (cat. no. 9271) were purchased from Cell Signaling Technology, Inc. All primary antibodies were diluted in TBS at 1:1,000. Goat anti-rabbit IgG-HRP (cat. no. sc-2004), goat anti-mouse IgG-HRP (cat. no. sc-2005) and donkey anti-goat IgG-HRP (cat. no. sc-2020) were purchased from Santa Cruz Biotechnology, Inc. All secondary antibodies were diluted at 1:3,000. The nuclear extract kit and the electrophoretic mobility shift assay (EMSA) kit (cat. nos. E3050 and E3300, respectively) were obtained from Promega Corporation. The cDNA for COL6A1-pcNSD2, pcNSD2, COL6A2-pOTB7 and pOTB7 was obtained from the Korea Human Gene Bank.

Transfection. Cells were transfected with cDNA using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells ($1x10^5$ cells/well) were seeded into 6-well plates and incubated for 24 h at 37˚C. A total of 2 μg of each cDNA of *COL6A1* and *COL6A2*, as well as empty vectors (EVs; *pcNSD2* and *pOTB7*) were mixed with 1X OPTI-MEM (Thermo Fisher Scientific, Inc.) containing 15 μl Lipofectamine 2000 transfection reagent and incubated for 5 min at room temperature. The mixture was added to cells, which were incubated for 48 h at 37˚C before subsequent experiments. The EV was used to evaluate if the elements on the EV alone (without inserted gene cassette) would affect the transfection results.

MTT assay. Cell viability was measured using the assay. Briefly, cells ($5x10^3$ cells/well) were plated in 96-well plates and incubated for 24 h at 37˚C. The medium was removed and fresh medium containing 100 μl of 5 mg/ml MTT (cat. no. M2128; Sigma-Aldrich; Merck KGaA) was added. After 2 h, the medium was removed and 200 μl DMSO was added. Absorbance at 570 nm was measured using a microplate reader.

Cell cycle analysis. Cells were harvested and fixed for 3 h at 4˚C in 70% ethanol. After washing the cells with ice-cold PBS, harvested cells were incubated using the Muse™ Cell Cycle kit for 30 min at room temperature to assess the relative number of cells at each phase of the cell cycle according to the manufacturer's protocol. Samples were analyzed using the Muse® Cell Cycle Analyzer (Merck KGaA). Data was generated with the Muse Cell Cycle Software 1.5.0.0 (Merck KGaA).

Immunoblot analysis. Whole cell extracts from transfected cells were prepared using lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 0.1 mM PMSF, 0.1 mM 10% glycerol, 0.1% Tween-20, 10 μg/ml leupeptin and 2 μg/ml aprotinin). Protein concentration was determined using the BCA protein assay reagent kit (Thermo Fisher Scientific, Inc.). Proteins (30-40 μg/lane) were separated by 4-20% SDS-PAGE under denaturing conditions and then transferred onto nitrocellulose membranes (Hybond; Cytiva). Non-specific binding to the membrane was blocked by incubation in 5% skim milk for 2 h at room temperature, after which the membranes were incubated overnight with primary antibodies at 4˚C. HRP-conjugated secondary antibodies were applied to the membranes for 2 h at room temperature. Immunocomplexes were analyzed using Western Lightning Plus-ECL (PerkinElmer, Inc.). The values of the blots were measured using the ImageJ software v1.50i (National Institutes of Health).

Wound healing migration assay. Transfected cells ($3x10^5$) were seeded into 6-well plates in 2 ml DMEM containing 10% FBS and then grown to 90% confluency. To inhibit cell proliferation, cells were pre-incubated for 2 h with 5 μg/ml mitomycin C (Sigma-Aldrich; Merck KGaA). An assigned area of the cell surface was scratched with a 2-mm pipette tip. After three washes with 1X PBS, the plates were incubated with culture medium containing 10% FBS (required for cells) for 24 h at 37˚C. The number of cells migrating into the wound surface was evaluated as the width of the remaining wounded area relative to the initial wounded area. Migration of cells into the scratched area was observed and compared with that of control plates. Morphological changes were photographed under an inverted light microscope at x40 magnification.

Invasion assay. The cell invasion assay was performed using SPL Insert Hanging 24 wells inserts (SPL Life Sciences). First, the insert membrane was coated with 0.1% gelatin solution (cat. no. G1393; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Next, cells ($3.5x10^4$) were resuspended in serum-free DMEM and added to the upper chamber. DMEM containing 10% FBS was added to the lower chamber and incubated for 24 h at 37˚C. Cells that had invaded the membrane were fixed with 4% formaldehyde for 30 min at room temperature and stained for 1 h at room temperature

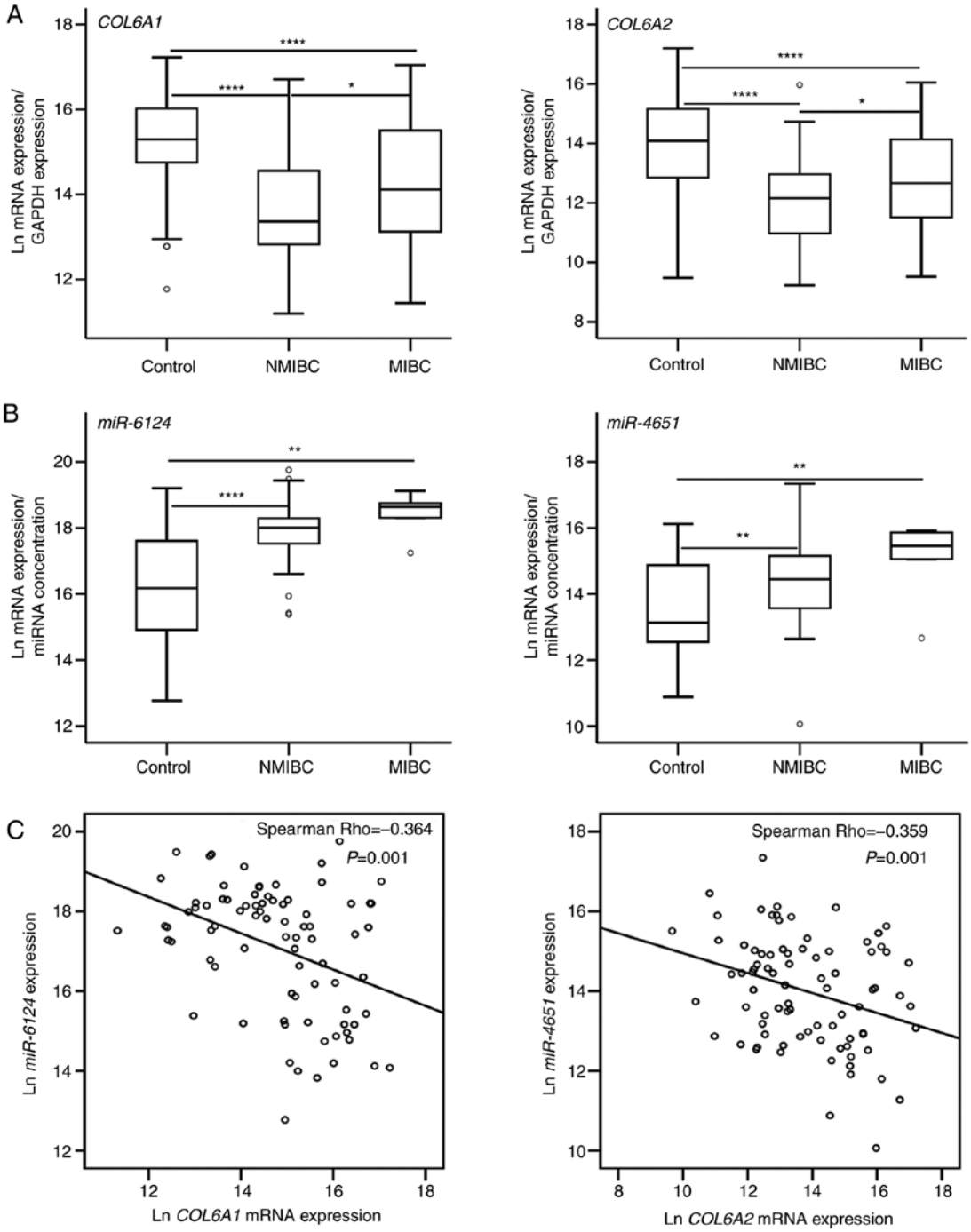

Figure 1. *COL6A1* and *COL6A2* expression and their interaction with miRNAs in BCa tissues. (A) *COL6A1* and *COL6A2* mRNA expression in NMIBC, MIBC and controls. (B) *miR-6124* and *miR-4651* expression, which interact with *COL6A1* and *COL6A2*, respectively, in NMIBC, MIBC and controls. (C) Spearman's correlation presented negative correlation between *miR-6124* and *COL6A1* expression and between *miR-4651* and *COL6A2* expression. Box plots represent the minimum score, first (lower) quartile, median, third (upper) quartile and maximum score. Controls represent normal tissue surrounding BCa. P-values were calculated using the Kruskal-Wallis (*COL6A1* and *miR-6124* expression) and one-way ANOVA (*COL6A2* and *miR-4651* expression) with the Bonferroni correction. ****P<0.0001, **P<0.01 and *P<0.05. BCa, bladder cancer; COL6A1/2, collagen type VI-α1/2; NMIBC, non-muscle invasive BCa; MIBC, muscle invasive BCa; miR, microRNA; Ln, natural logarithm.

with 0.1% crystal violet solution (2.5 mM crystal violet, 1% MeOH and 4% paraformaldehyde). Non-invading cells on the upper surface were removed using a cotton swab. Invasive cells were photographed using an inverted light microscope at x40 magnification.

Gelatin zymography. Culture medium from transfected cells was subjected to electrophoresis on 8% polyacrylamide gel containing 1 mg/ml gelatin. The gel was rinsed twice for 30 min at room temperature with 2.5% Triton X-100 and then incubated in reaction buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5 and 10 mM $CaCl_2$) at 37˚C overnight. The gel was stained with 0.2% Coomassie Blue, de-stained with 10% acetic acid and 10% methanol in distilled water, and photographed using a charge-coupled device camera (cat. no. KCS3-63S; Korea Lab Tech) on a light box.

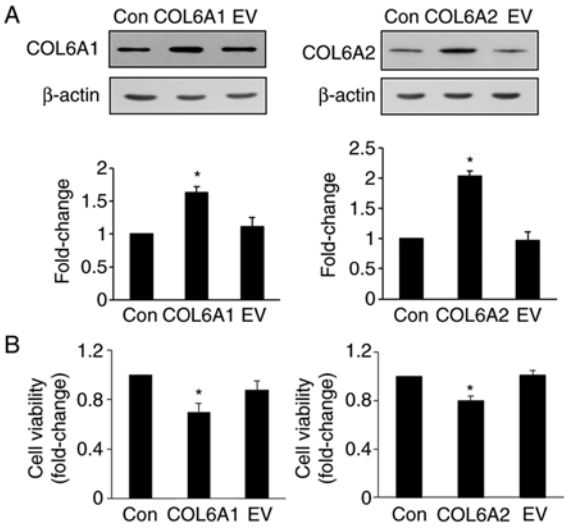

Figure 2. COL6A1 and COL6A2 suppress the proliferation of human bladder cancer EJ cells (MGH-U1). (A) Vector transfections. Increased target expression was confirmed by western blotting. (B) MTT assay was performed to evaluate cell viability. EV was used to evaluate if the EV alone (without inserted gene cassette) affected the transfection results. Values in bar graphs represent the mean ± SD of triplicate independent experiments. *P<0.05 vs. Con. EV, empty vector; Con, control; COL6A1/2, collagen type VI-α1/2.

Gelatinase activity was visualized as a white zone on a dark-blue field.

Nuclear extracts and EMSA assays. Nuclear extracts were prepared using a Nuclear Extraction kit (Promega Corporation) according to the manufacturer's protocol. Briefly, cells were harvested by centrifugation at 3,000 x g for 3 min at 25˚C and resuspended in buffer (50 mM KCl, 0.5% NP-40, 25 mM HEPES pH 7.8, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM DTT and 1 mM PMSF in distilled water). After incubation for 30 min at 4˚C, the nuclear pellet was harvested by centrifugation at 10,000 x g for 1 min at 4˚C and extracted using high salt buffer (500 mM KCl, 25 mM HEPES pH 7.8, 10% glycerol, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM DTT and 1 mM PMSF in distilled water) for 30 min at 4˚C. After centrifugation, the supernatant containing the nuclear extract was obtained. The total amount of nuclear protein was quantified using a BCA protein assay reagent kit (Thermo Fisher Scientific, Inc.). The nuclear extract (5 μg) was incubated at 25˚C for 20 min with 5 fmol ($2x10^4$ cpm) of end-labeled (^{32}P ATP) oligonucleotide in binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 5% glycerol and 2 μg poly dI/dC·poly dI/dC) in a 10-μl reaction volume. The oligonucleotide sequences were as follows: Activator protein 1 (AP-1), 5'-CGCTTGATG AGTCAGCCGGAA-3' and 3'-GCGAACTACTCAGTCGGC CTT-5'; NF-κB, 5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5'; and specificity protein 1 (SP-1), 5'-ATTCGATCGGGGCGGGGCGAGC-3' and 3'-TAAGCTAGCCCCGCCCCGCTCG-5'. Subsequently, the reaction products were mixed with 1 μl loading buffer (250 mM Tris-HCl pH 7.5, 0.2% bromophenol blue and 40% glycerol) and electrophoresed on a 4% polyacrylamide gel at 4˚C. The gel was dried and exposed to X-ray film overnight. The density of the blots was measured using ImageJ software v1.50i (National Institutes of Health). The Hela nuclear extract was used as positive control. Unlabeled AP-1, NF-κB and SP-1 oligonucleotides were used as competitors.

Construction of the protein-protein interaction (PPI) network. To identify interactions between COL6A1, COL6A2 and genes regulating cancer growth and progression in BCa, a PPI network was constructed and analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (https://string-db.org/; v11.0b). The PPI networks analyzed by STRING were based on the functional associations and STRING implements well-known classification systems, such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (27). A PPI score of 0.4 (medium confidence) was used for the analysis. Some important features (derived from GO terms and KEGG pathways) associated with BCa were selected and presented. False discovery rate (FDR)<0.05 and P<0.05 were considered to be statistically significant.

Statistical analysis. The continuous variables were expressed as the mean ± SD. The normality of the data was estimated by One-Sample Kolmogorov-Smirnov test and the statistical differences between three groups (NMIBC, MIBC and controls) were tested by Kruskal-Wallis test followed by Bonferroni's post-hoc test. The groups were compared in terms of non-continuous variables using the χ^2 test. Gene expression values were natural log-transformed and median-centered across samples. The Kruskal-Wallis test was used to examine *COL6A1* and *miR-6124* expression in NMIBC, MIBC and control tissues. For the comparison of *COL6A2* and *miR-4651* expression, one-way ANOVA was used since the datasets followed the normal distribution. Both tests were followed by the Bonferroni correction. The Mann-Whitney U test was used to examine COL6A1 expression in patients with NMIBC with stage Ta and T1. Correlations between *COL6A1* and *COL6A2* expression, and *miR-6124* and *miR-4651* expression were examined by calculating non-parametric Spearman's correlation coefficients. For the *in vitro* tests, triplicate independent reads were measured for all analyses and data were presented as the mean ± SD. All data were evaluated in a blinded manner using factorial ANOVA and Fisher's least significant difference test. Statistical analysis was performed using SPSS v20.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

COL6A1 and COL6A2 mRNA expression in BCa tissues. *COL6A1* and *COL6A2* mRNA expression in both NMIBC and MIBC tissues was significantly lower than that in controls (normal tissue surrounding BCa) (P<0.0001); additionally, there was a significant difference between MIBC and NMIBC tissues (P<0.05; Fig. 1A). By contrast, *miR-6124* and *miR-4651* expression, which interact with *COL6A1* and *COL6A2*, respectively, in both NMIBC and MIBC was significantly higher than in controls (P<0.05; Fig. 1B). Analysis of non-parametric Spearman's correlation coefficients revealed a negative correlation between *miR-6124* and *COL6A1* expression, and between *miR-4651* and *COL6A2* expression (Fig. 1C). In particular, patients with NMIBC with stage T1 disease exhibited lower

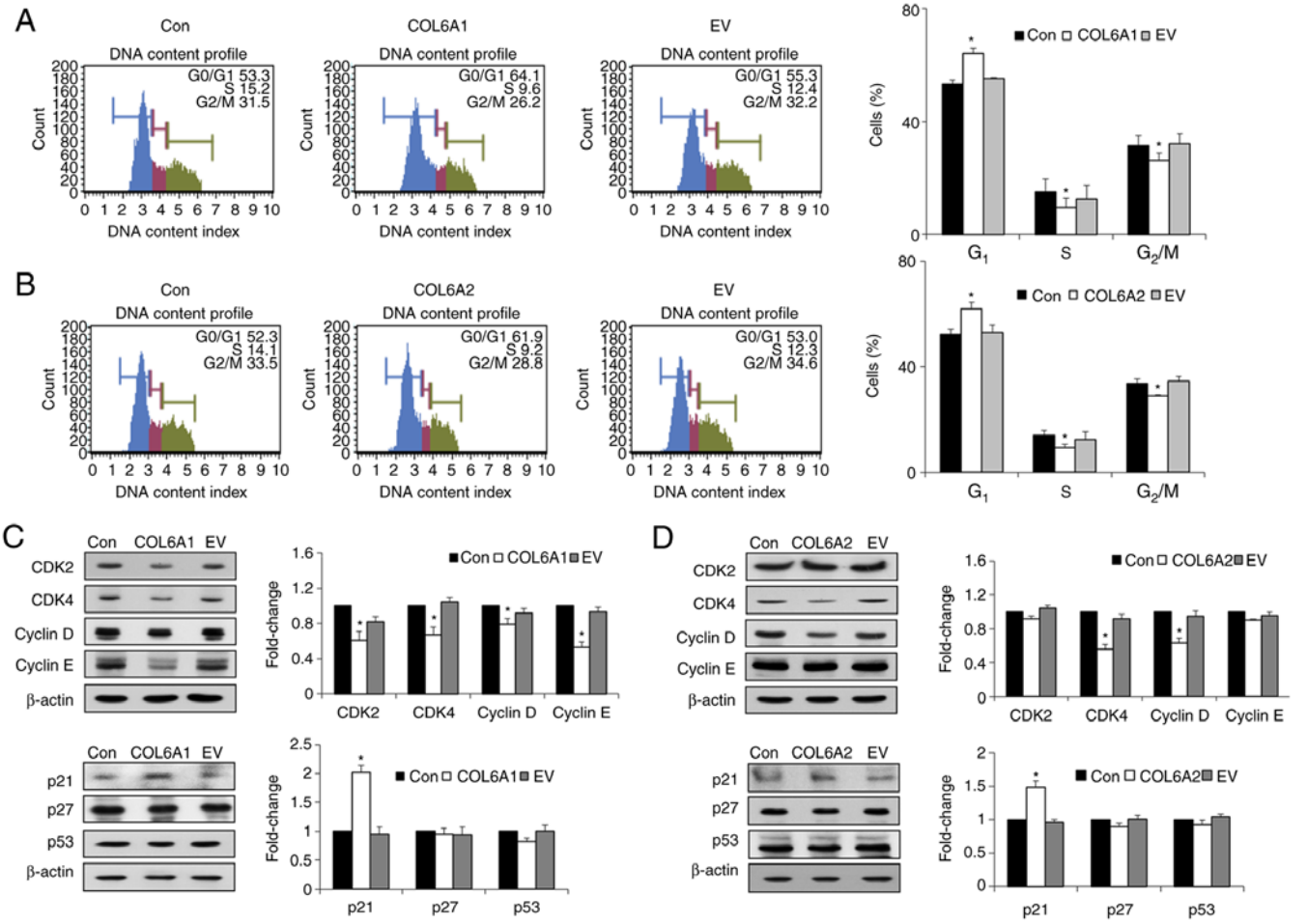

Figure 3. COL6A1 and COL6A2 induce cell cycle arrest of human bladder cancer EJ cells (MGH-U1) at the G_1 phase. Cell cycle distribution and percentage of cells treated with (A) *COL6A1* and (B) *COL6A2* in G_1, S and G_2/M phases were analyzed by flow cytometry. Expression levels of G_1 phase-associated cell cycle regulators (CDK2 and 4, and Cyclin D and E) and negative cell cycle regulators (p21, p27 and p53) were assessed via immunoblotting in cells treated with (C) *COL6A1* and (D) *COL6A2*. EV was used to evaluate if the EV alone (without inserted gene cassette) affected the transfection results. Bar graphs show the relative ratios of each protein (represented as a fold-change compared with the control). Values are expressed as the mean ± SD of three independent experiments. *P<0.05 vs. Con. EV, empty vector; Con, control; COL6A1/2, collagen type VI-α1/2; CDK, cyclin-dependent kinase.

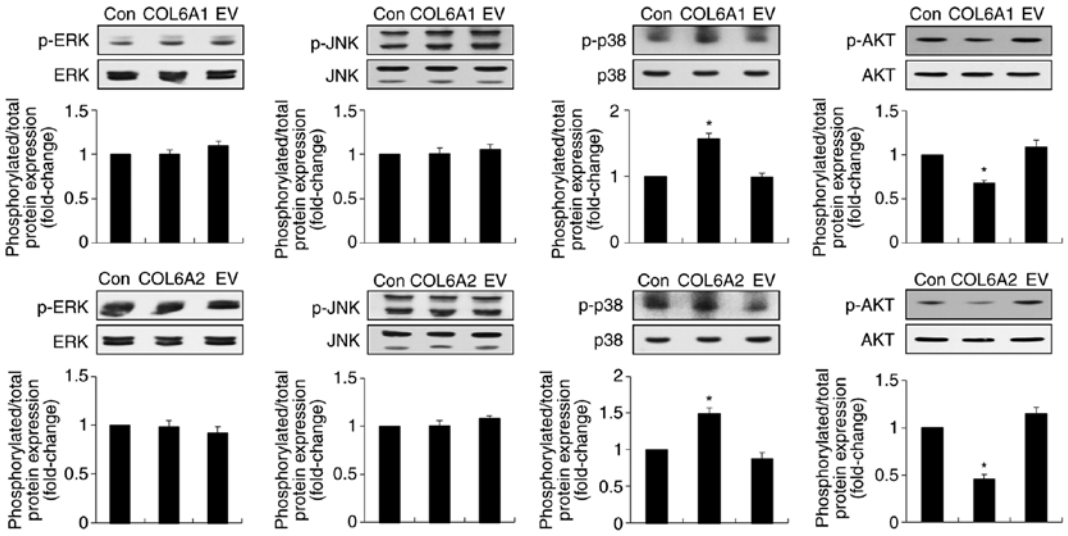

Figure 4. COL6A1 and COL6A2 induce phosphorylation of p38 MAPK and inhibit phosphorylation of AKT in human bladder cancer EJ cells (MGH-U1). Cell lysates were prepared after treatment with *COL6A1* and *COL6A2* for 12 h, and protein expression was measured by immunoblotting with antibodies specific for ERK1/2, JNK, p38MAPK or AKT and their phosphorylated forms. Relative expression of the phosphorylated to unphosphorylated forms was evaluated and expressed as a fold-change compared with the control. Values are expressed as the mean ± SD of three independent experiments. EV was used to evaluate if the EV alone (without inserted gene cassette) affected the transfection results. *P<0.05 vs. Con. EV, empty vector; Con, control; COL6A1/2, collagen type VI-α1/2; p, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

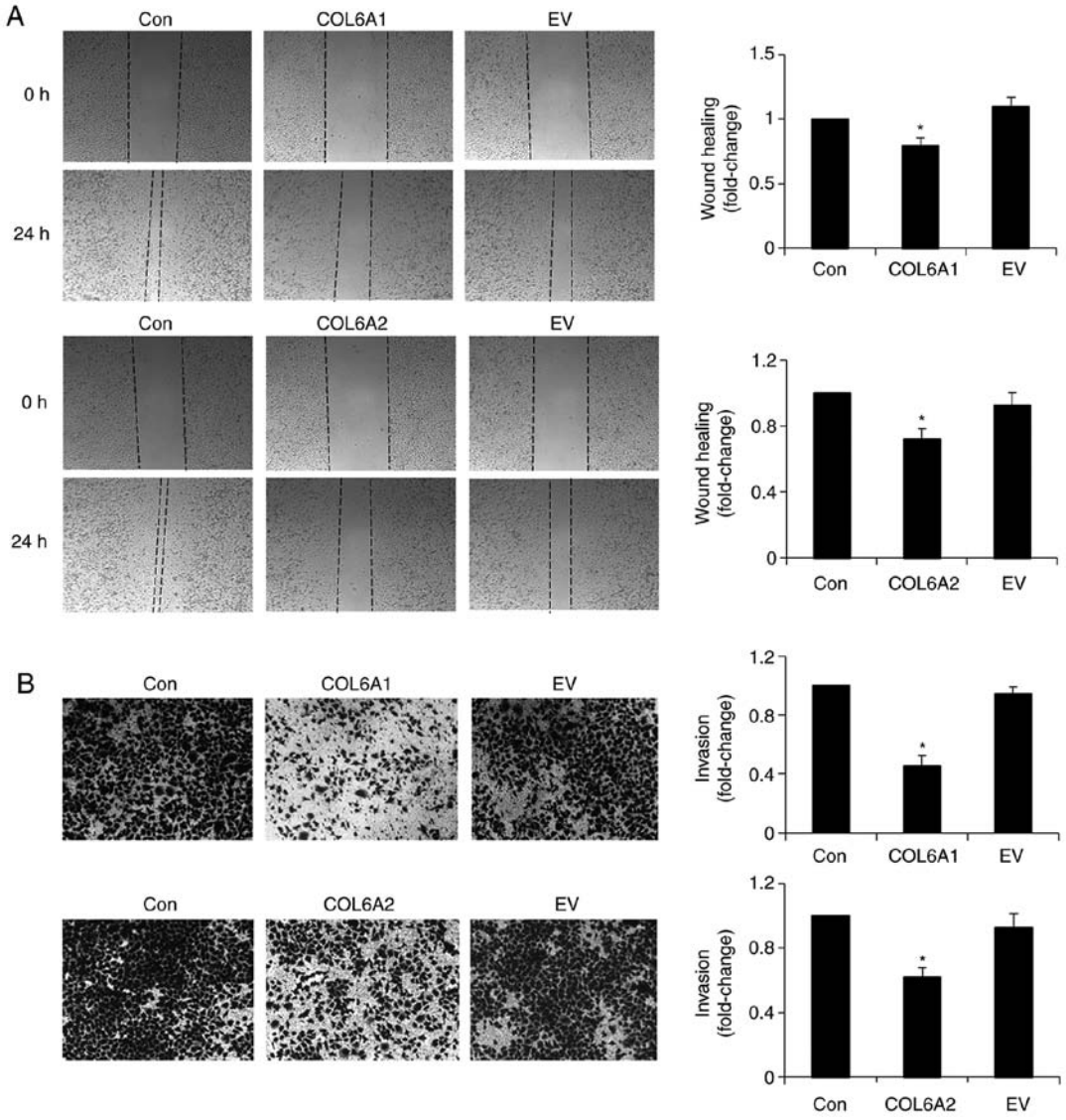

Figure 5. COL6A1 and COL6A2 inhibit the migration and invasion of human BCa EJ cells (MGH-U1). (A) Wound healing assays of cells transfected with *COL6A1* and *COL6A2*. The number of cells migrating into the wound surface was evaluated as the width of the remaining wounded area relative to the initial wounded area (magnification, x40). (B) Invasiveness of BCa cells was assessed using a Transwell invasion assay (magnification, x40). The bar graphs indicate the number of migrating or invading cells (expressed as fold-changes relative to the control). Values represent the mean ± SD from three separate experiments. EV was used to evaluate if the EV alone (without inserted gene cassette) affected the transfection results. *P<0.05 vs. Con. EV, empty vector; Con, control; COL6A1/2, collagen type VI-α1/2; BCa, bladder cancer.

COL6A1 expression than those with stage Ta disease (P<0.05; Fig. S1). No additional associations were identified between *COL6A1* and *COL6A2* mRNA expression and clinicopathological characteristics (data not shown).

COL6A1 and COL6A2 inhibit the proliferation of human BCa EJ cells (MGH-U1). The expression levels of *COL6A1* and *COL6A2* in transfected EJ cells (MGH-U1) were significantly higher than those in the control and EV groups (P<0.05; Fig. 2A). In addition, *COL6A1*- and *COL6A2*-treated cells exhibited lower cell viability than untreated cells (P<0.05; Fig. 2B). These results demonstrated that *COL6A1* and *COL6A2* inhibited the proliferation of human BCa EJ cells (MGH-U1).

COL6A1 and COL6A2 trigger G_1 cell cycle arrest in human BCa EJ cells (MGH-U1). Cell cycle analysis revealed that *COL6A1* and *COL6A2* induced significant accumulation of cells at the G_1 phase (Fig. 3A and B). In addition, treatment with *COL6A1* and *COL6A2* significantly decreased the percentage of cells in both S and G_2/M phases (P<0.05; Fig. 3A and B). To better understand the cell cycle-associated effects of COL6A1 and COL6A2, the expression levels of major regulatory proteins that control progression of the G_1 phase were examined. Treatment with *COL6A1* significantly decreased the expression levels of CDK2, CDK4, cyclin E and cyclin D (P<0.05; Fig. 3C), while *COL6A2* significantly decreased the expression levels of CDK4 and cyclin D compared with the control group (P<0.05; Fig. 3D). Furthermore, the cyclin kinase inhibitor (CKI) $p21^{WAF1}$ was upregulated by both *COL6A1*- and *COL6A2*-treated human BCa EJ cells (MGH-U1) compared with untreated controls (P<0.05; Fig. 3C and D). There were no differences between treated and control cells with respect to the expression levels of other CKIs, such as $p27^{KIP1}$ and

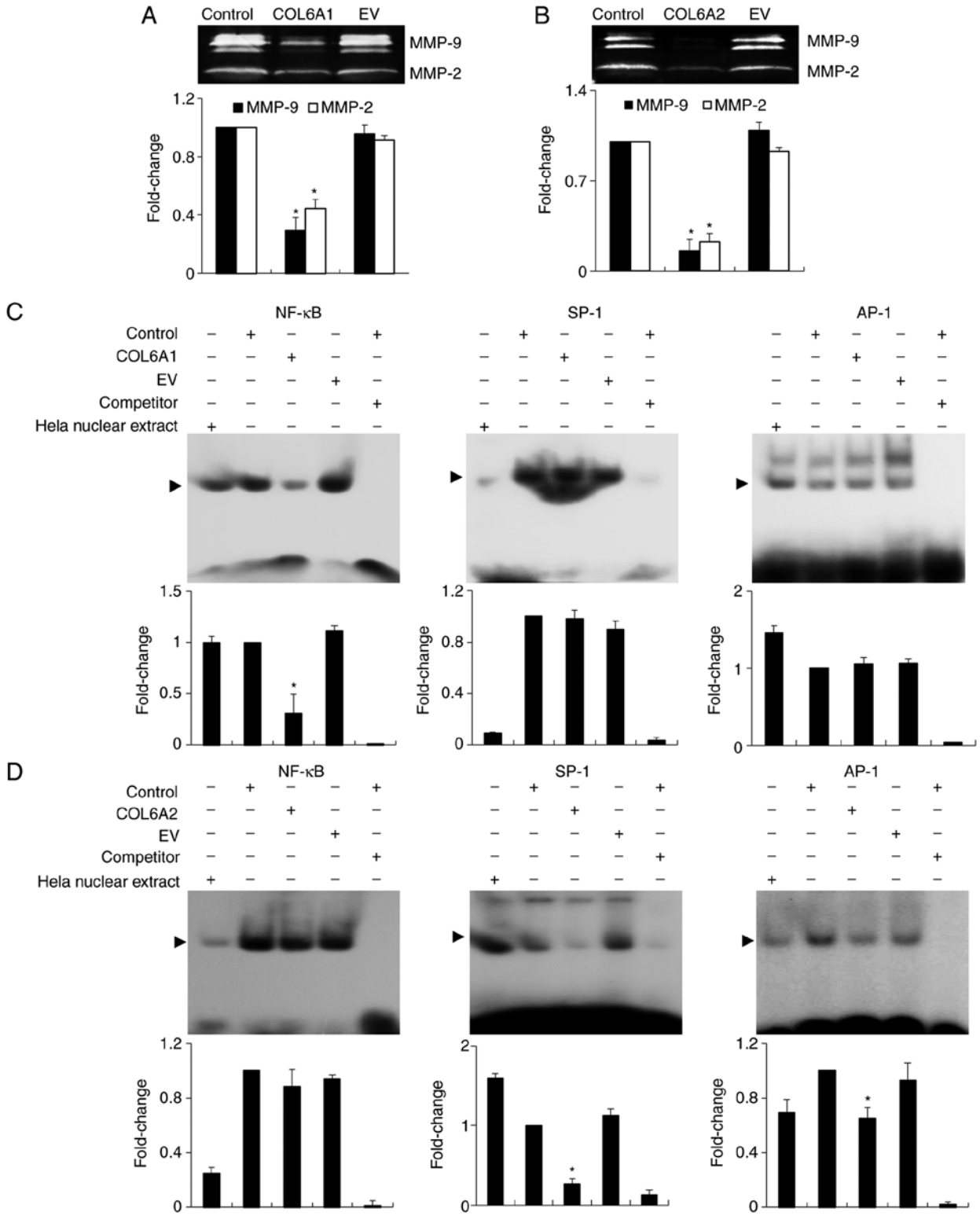

Figure 6. COL6A1 and COL6A2 suppress MMP-2 and MMP-9 activity by repressing the transcription factors NF-κB, AP-1 and SP-1 in human bladder cancer EJ cells (MGH-U1). MMP-2 and MMP-9 activity in cells incubated with (A) *COL6A1* and (B) *COL6A2* were measured using a gelatin zymography assay. NF-κB, AP-1 and SP-1 motifs were used to assess transcriptional binding activity in cells treated with (C) *COL6A1* and (D) *COL6A2* using radiolabeled oligonucleotide probes. Bar graphs show fold-changes relative to the control. Results are presented as the mean ± SD of three independent experiments. EV was used to evaluate if the EV alone (without inserted gene cassette) affected the transfection results. *P<0.05 vs. Con. EV, empty vector; Con, control; COL6A1/2, collagen type VI-α1/2; MMP, matrix metalloproteinase; AP-1, activator protein 1; SP-1, specificity protein 1.

p53 (Fig. 3C and D). Thus, the current results indicated that COL6A1 and COL6A2 induced the arrest of BCa cells at the G_1 phase via the $p21^{WAF1}$-CDK2/cyclin E, $p21^{WAF1}$-CDK4/cyclin D axes and the $p21^{WAF1}$-CDK4/cyclin D axis, respectively.

COL6A1 and COL6A2 induce phosphorylation of p38 MAPK and inhibit phosphorylation of AKT in human BCa EJ cells (MGH-U1). BCa progression involves phosphorylation of early signaling pathway molecules, such as MAPKs,

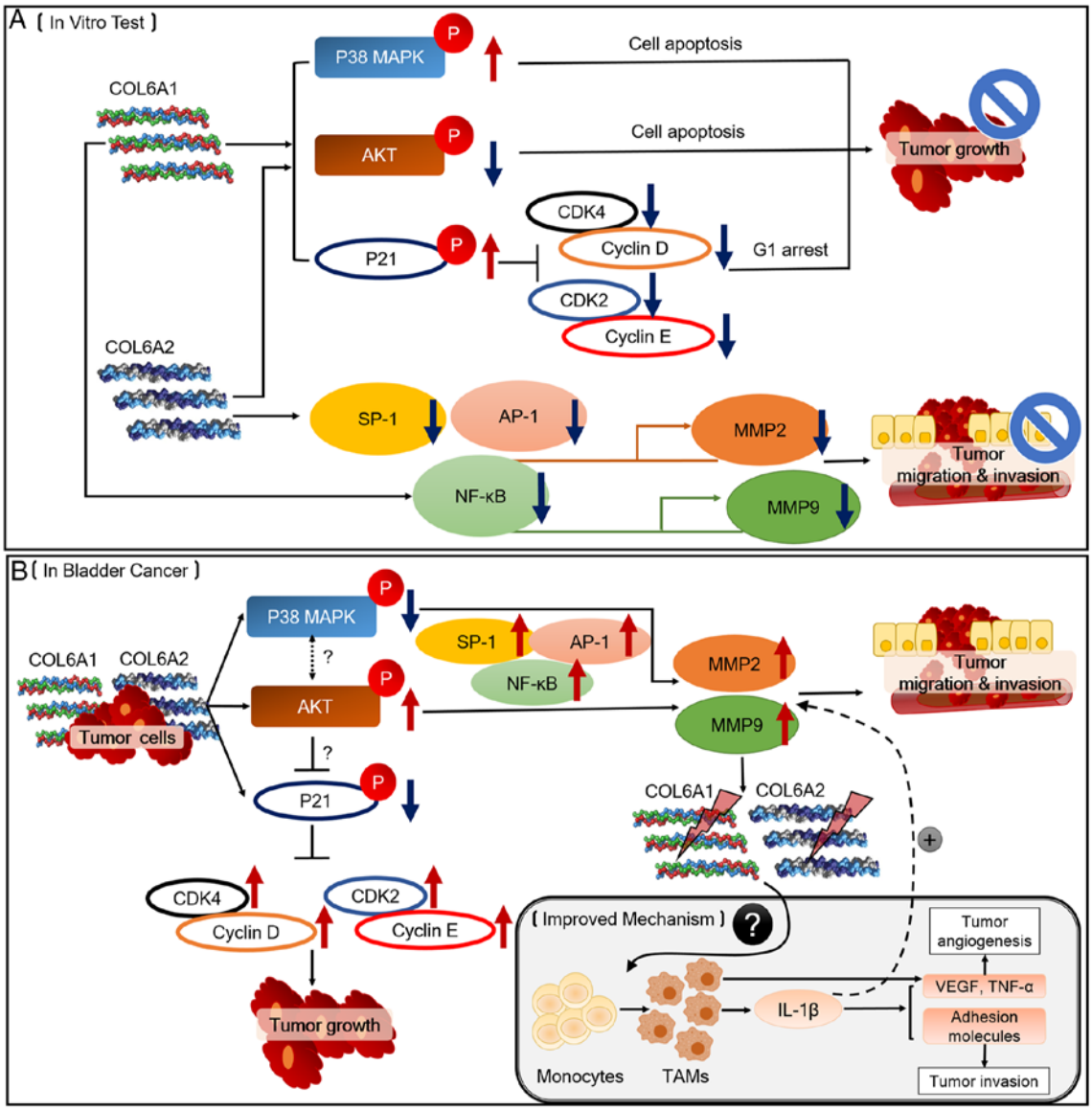

Figure 7. Effects of COL6A1 and COL6A2 on BCa. (A) Schematic diagram depicting the proposed inhibitory mechanisms of COL6A1 and COL6A2 in BCa cells. (B) Proposed mechanisms by which COL6A1 and COL6A2 may affect BCa pathogenesis and progression, as well as the mechanisms regulating the tumor microenvironment. BCa, bladder cancer; COL6A1/2, collagen type VI-α1/2; MMP, matrix metalloproteinase; AP-1, activator protein 1; SP-1, specificity protein 1; CDK, cyclin-dependent kinase; TAMs, tumor-associated macrophages.

ERK, c-JNK, p38 MAPK and AKT (28). To investigate whether COL6A1 and COL6A2 affect the levels of the phosphorylated forms of MAPKs and AKT, immunoblot analysis was performed. The results revealed that both COL6A1 and COL6A2 significantly induced phosphorylation of p38 MAPK in human BCa EJ cells (MGH-U1) ($P<0.05$), but did not alter phosphorylation of ERK and JNK (Fig. 4). By contrast, phosphorylation of AKT was significantly inhibited in both *COL6A1*- and *COL6A2*-treated human BCa EJ cells (MGH-U1) ($P<0.05$; Fig. 4). These results suggested that COL6A1 and COL6A2 may inhibit BCa cell proliferation by upregulating p38 MAPK phosphorylation and downregulating AKT phosphorylation.

COL6A1 and COL6A2 inhibit migration and invasion of human BCa EJ cells (MGH-U1). Wound healing migration was assessed by examining movement of cells into a wounded area. Compared with the control group, treatment with *COL6A1* and *COL6A2* significantly suppressed migration of human BCa EJ cells (MGH-U1) into the wounded area ($P<0.05$; Fig. 5A). The invasive potential is shown in Fig. 5B. The number of *COL6A1*- and *COL6A2*-treated cells that invaded through the Transwell basement membrane was lower than that of the control group ($P<0.05$; Fig. 5B).

COL6A1 and COL6A2 suppress the activity of MMP-2 and MMP-9 by repressing the transcription factors NF-κB, AP-1 and SP-1 in human BCa EJ cells (MGH-U1). The enzymatic activity of MMP-2 and MMP-9, both of which are essential for degradation of the ECM and subsequent migration and invasion of tumor cells, was analyzed in human BCa EJ cells (MGH-U1) by gelatin zymography. The proteolytic activities of both MMP-2 and MMP-9 in *COL6A1*- and *COL6A2*-treated cells were significantly lower than those in

untreated cells (control group) (P<0.05; Fig. 6A and B). Next, the transcriptional activity of MMP-2 and MMP-9 was examined by measuring the binding activities of the transcriptional responsive elements NF-κB, *AP-1* and SP-1 via EMSA. As shown in Fig. 6C, the binding activity of NF-κB was significantly inhibited in the presence of COL6A1, while COL6A2 significantly inhibited the binding activity of AP-1 and SP-1 (P<0.05; Fig. 6D).

Association between COL6A1, COL6A2 and genes regulating cancer cell proliferation and migration in BCa. Interactions between COL6A1, COL6A2, p38 MAPK, AKT, MMP-2, MMP-9 and IL-1β were depicted using the STRING web tool (Fig. S2). COL6A1 regulated both MMP-2 and MMP-9, which interacted with IL-1β, p38 MAPK and AKT, whereas COL6A2 only regulated MMP-2. Subsequently, BCa-associated features, including extracellular matrix organization (FDR, 0.0002), positive regulation of cell migration (FDR, 0.00047), negative regulation of apoptotic signaling (FDR, 0.0011), pathways involved in bladder cancer (FDR, $6.93x10^{-6}$) and pathways involved in cancer (FDR, 0.00012) were extracted from GO terms and KEGG pathways, each of them depicted in different colors in Fig. S2.

Discussion

Cancer is not only a disease caused by tumor cells, but also a disease of imbalance. Thus, the role of the TME is critical. Collagen is one of the main ECM proteins, and its degradation and/or re-deposition affects the TME by regulating ECM remodeling to promote tumor progression (29). The traditional view of collagen is that it acts as a passive barrier to tumor cells (29). However, a number of studies have indicated that several types of cancer exhibit high expression levels of COL6 (19,20,29,30); it should be noted that the traditional role of COL6 is to block tumor cells in the TME (31). The present study revealed that *COL6A1* and *COL6A2* expression was downregulated in BCa tissues. During tumorigenesis, COL6 acts directly on tumor cells through several pathways, including upregulation of transcription factors, growth factors, protein kinases and angiogenic factors, and through activation of the AKT/glycogen synthase kinase-3β/β-catenin-T-cell factor/lymphoid enhancer factor signaling pathway (29). Indirectly, COL6 promotes tumor growth and progression by cleaving the ETP peptide, which induces epithelial-mesenchymal transition and fibrosis via the TGF-β-dependent signaling pathway (29). By contrast, ETP in the TME initiates tumor inflammation by recruiting macrophages and increasing TNF-α and interleukin (IL)-6 expression, or by promoting tumor angiogenesis by recruiting endothelial cells and upregulating the expression levels of CD31, VEGF receptor 2 and hypoxia-inducible factor 1α (29). COL6 comprises three major polypeptide chains (α1, 2 and 3), which are encoded by distinct genes (*COL6A1*, *COL6A2* and *COL6A3*, respectively). Recent database-dependent studies have identified COL6A1, COL6A2 and COL6A3 as prognostic biomarkers for BCa (19,20,30). However, the mechanism underlying the involvement of COL6 in BCa tumorigenesis or progression is unclear.

One of the main hallmarks of cancer is a dysregulated cell cycle, which leads to abnormal cell proliferation and division (32,33). $p21^{WAF1}$ protein is an established CDK inhibitor that plays an important role in cell cycle arrest at the G_1 phase by inhibiting DNA replication via its interaction with proliferating cell nuclear antigen (34). The CDK4-cyclin D complex is responsible for early G_1 phase progression, while the CDK2-cyclin E complex is thought to act at late G_1 phase; the activity of both complexes is inhibited by $p21^{WAF1}$ (35). The present study demonstrated that COL6A1 and COL6A2 exert a strong inhibitory effect on the proliferation of BCa cells by inducing cell cycle arrest. COL6A1 and COL6A2 increased $p21^{WAF1}$ expression, followed by suppression of CDK4-cyclin D and CDK2-cyclin E complexes and, finally, arrest of BCa cells at the G_1 phase. The phosphorylation states of MAPKs, such as ERK1/2, JNK, p38 MAPK, and AKT, are crucial to the mechanisms that regulate cell cycle progression and cell proliferation (36,37). p38 MAPK serves a dual role in regulating cell survival or cell death depending on the stimulus (38). AKT plays an anti-apoptotic role, and suppression of AKT may induce phosphorylation of p21, which in turn inhibits cell growth and proliferation, and activates pro-apoptotic pathways (39). The present study revealed that both COL6A1 and COL6A2 induce phosphorylation of p38 MAPK in BCa cells; however, they impeded activation of AKT. Phosphorylation of ERK and JNK was unchanged. Thus, COL6A1 and COL6A2 may regulate BCa cell growth and proliferation by activating p38 MAPK phosphorylation and by preventing AKT phosphorylation, leading to p21 upregulation and decreased expression of CDK-cyclin complexes, resulting in cell cycle arrest at the G_1 phase. In addition, migration and invasion of BCa cells treated with COL6A1 and COL6A2 was inhibited, suggesting that COL6A1 and COL6A2 prevent BCa metastasis. MMPs, such as MMP-2 and MMP-9, accelerate proteolytic degradation of the ECM, thereby enabling migration and invasion of cancer cells, which in turn promotes tumor metastasis (8,9). Thus, MMP-2 and MMP-9 expression, and their transcription factors NF-κB, AP-1 and SP-1 were evaluated in *COL6A1*- and *COL6A2*-treated BCa cells. COL6A1 inhibited both MMP-2 and MMP-9 by regulating NF-κB; COL6A2 inhibited MMP-2 and MMP-9 by decreasing the expression of AP-1 and SP-1. This indicates that COL6A1 and COL6A2 may inhibit BCa metastasis.

Several studies have revealed an association between classical signaling pathways involved in major tumor events, such as cancer cell proliferation, invasion and migration (40-42). Active p38 MAPK signaling regulates BCa cell migration or invasion by regulating MMP-2/-9 activity (40). MMP-2 triggers VEGF-induced angiogenesis in lung tumors by activating the PI3K/AKT signaling pathway (41), while PI3K/AKT downregulates p38 MAPK (42). Accordingly, a search using STRING in the present study identified interactions between COL6A1, COL6A2, p38 MAPK, AKT, MMP-2, MMP-9 and IL-1β, which is a potent pro-inflammatory cytokine that interacts with MMPs to regulate collagen synthesis (43). It was demonstrated that COL6A1 regulated both MMP-2 and MMP-9, which interacted with IL-1β, p38 MAPK and AKT, whereas COL6A2 only regulated MMP-2.

The antitumor effects of COL6A1 and COL6A2 on BCa cells are summarized in Fig. 7. Fig. 7A outlines the potential roles of COL6A1 and COL6A2 as BCa tumor suppressors, and Fig. 7B outlines the proposed mechanisms by which COL6A1 and COL6A2 may be involved in BCa pathogenesis and progression, as well as the mechanisms associated with the TME. During BCa

occurrence and development, the ECM undergoes remodeling via collagen degradation to further coordinate the behavior of cancer cells. Degraded COL6A1 and COL6A2 may then inhibit p38 MAPK and p21, whereas they may activate AKT signaling. According to previous studies (37-39), de-phosphorylation of p38 MAPK induces AKT phosphorylation, which inhibits p21 phosphorylation. These events trigger tumor growth, migration and invasion by activating MMPs induced by SP-1, AP-1 and NF-κB. Furthermore, increased expression levels of MMPs may decrease the expression levels of COL6A1 and COL6A2. MMP-derived collagen fragments may recruit monocytes and trigger them to differentiate into tumor-associated macrophages (TAMs) that secret factors such as IL-1β, which are responsible for tumor progression (31). Further studies should focus on the roles of COL6A1 or COL6A2 on TAMs during BCa progression.

However, there is a limitation in the present study. Only one BCa cell line was used, the human BCa EJ cells (MGH-U1). Human BCa EJ cells (MGH-U1) are considered to reflect high grade BCa, and they are widely used in invasive BCa studies (44-48). In the present study, both *COL6A1* and *COL6A2* mRNA expression was significantly lower in NMIBC and MIBC compared with controls, and these genes were more highly expressed in MIBC than in NMIBC. Accordingly, it may be inferred that the human BCa EJ cells (MGH-U1) are a superior option that may be able to cover results of COL6A1 and 2 from both NMIBC and MIBC. Nevertheless, the number of cell lines used in the present study is inadequate. However, to the best of our knowledge, this is the first research paper to excavate the association between COL6 and BCa, which may provide a reference for studying the functions of COL6 in BCa. As the scaffold of the TME, collagens serve an important role in carcinogenesis and cancer progression. Thus, it would be interesting to explore the functions of collagens in BCa. Following the present study, future studies should use more reasonable cohorts (including more cases of bladder carcinoma tissues and cell lines) to verify the detailed mechanisms.

In conclusion, the current study demonstrated a novel mechanism by which COL6A1 and COL6A2 may affect BCa pathogenesis and progression. Along with the present findings, further study of the roles of the *COL6A1* and *COL6A2* mRNA-interacting miRNAs *miR-6124* and *miR-4651* may help determine whether these miRNAs definitely act as oncomiRNAs in BCa.

Acknowledgements

The authors would like to thank Ms. Eun-Ju Shim from the National Biobank of Korea at Chungbuk National University Hospital (Cheongju, South Korea) for the sample preparations and excellent technical assistance.

Funding

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (grant nos. 2020R1F1A1068488 and 2020R1I1A3062508) and by the Osong Medical Innovation Foundation funded by Chungcheongbuk (grant no. 2020017001).

Availability of data and materials

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

Authors' contributions

XMP, IYK, SJY and WJK made contributions to conception and design of the study. XMP and BH performed all experiments. XMP, BH, YJB, JYL, YHC, SKM and EJC contributed to data acquisition and analysis. PJ, SPS, HWK, WTK, YSH and YSL contributed to data interpretation. XMP and BH drafted the manuscript. YHC, IYK, SKM, EJC, SJY and WJK revised the manuscript critically for important intellectual content, and gave final approval of the version to be published. XMP, BH, SKM and WJK assessed and confirmed the authenticity of all the raw data. WJK agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, and provided funding. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board at Chungbuk National University (approval no. GR2010-12-010). The collection and analysis of all samples were approved by the Institutional Review Board at Chungbuk National University, and written informed consent was obtained from each subject. All samples derived from the National Biobank of Korea at Chungbuk National University Hospital (Cheongju, South Korea) were obtained with informed consent under institutional review board-approved protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chavan S, Bray F, Lortet-Tieulent J, Goodman M and Jemal A: International variations in bladder cancer incidence and mortality. Eur Urol 66: 59-73, 2014.
2. Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, Newling DW and Kurth K: Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: A combined analysis of 2596 patients from seven EORTC trials. Eur Urol 49: 466-477, 2006.
3. Shariat SF, Karakiewicz PI, Palapattu GS, Lotan Y, Rogers CG, Amiel GE, Vazina A, Gupta A, Bastian PJ, Sagalowsky AI, *et al*: Outcomes of radical cystectomy for transitional cell carcinoma of the bladder: A contemporary series from the Bladder Cancer Research Consortium. J Urol 176 (6 Pt 1): 2414-2422, 2006.
4. Tilki D, Reich O, Karakiewicz PI, Novara G, Kassouf W, Ergün S, Fradet Y, Ficarra V, Sonpavde G, Stief CG, *et al*: Validation of the AJCC TNM substaging of pT2 bladder cancer: Deep muscle invasion is associated with significantly worse outcome. Eur Urol 58: 112-117, 2010.
5. Cancer Genome Atlas Research Network: Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 507: 315-322, 2014.

6. Lee JY, Yun SJ, Jeong P, Piao XM, Kim YH, Kim J, Subramaniyam S, Byun YJ, Kang HW, Seo SP, *et al*: Identification of differentially expressed miRNAs and miRNA-targeted genes in bladder cancer. Oncotarget 9: 27656-27666, 2018.
7. Piao XM, Jeong P, Kim YH, Byun YJ, Xu Y, Kang HW, Ha YS, Kim WT, Lee JY, Woo SH, *et al*: Urinary cell-free microRNA biomarker could discriminate bladder cancer from benign hematuria. Int J Cancer 144: 380-388, 2019.
8. Joyce JA and Pollard JW: Microenvironmental regulation of metastasis. Nat Rev Cancer 9: 239-252, 2009.
9. Venning FA, Wullkopf L and Erler JT: Targeting ECM disrupts cancer progression. Front Oncol 5: 224, 2015.
10. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, *et al*: Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 139: 891-906, 2009.
11. Arnold SA, Rivera LB, Miller AF, Carbon JG, Dineen SP, Xie Y, Castrillon DH, Sage EH, Puolakkainen P, Bradshaw AD and Brekken RA: Lack of host SPARC enhances vascular function and tumor spread in an orthotopic murine model of pancreatic carcinoma. Dis Models Mech 3: 57-72, 2010.
12. Aitken KJ and Bägli DJ: The bladder extracellular matrix. Part I: Architecture, development and disease. Nat Rev Urol 6: 596-611, 2009.
13. Cescon M, Gattazzo F, Chen P and Bonaldo P: Collagen VI at a glance. J Cell Sci 128: 3525-3531, 2015.
14. Wan F, Wang H, Shen Y, Zhang H, Shi G, Zhu Y, Dai B and Ye D: Upregulation of COL6A1 is predictive of poor prognosis in clear cell renal cell carcinoma patients. Oncotarget 6: 27378-27387, 2015.
15. Zhu YP, Wan FN, Shen YJ, Wang HK, Zhang GM and Ye DW: Reactive stroma component COL6A1 is upregulated in castration-resistant prostate cancer and promotes tumor growth. Oncotarget 6: 14488-14496, 2015.
16. Hou T, Tong C, Kazobinka G, Zhang W, Huang X, Huang Y and Zhang Y: Expression of COL6A1 predicts prognosis in cervical cancer patients. Am J Transl Res 8: 2838-2844, 2016.
17. Chiu KH, Chang YH, Wu YS, Lee SH and Liao PC: Quantitative secretome analysis reveals that COL6A1 is a metastasis-associated protein using stacking gel-aided purification combined with iTRAQ labeling. J Proteome Res 10: 1110-1125, 2011.
18. Stone R II, Sabichi AL, Gill J, Lee IL, Adegboyega P, Dai MS, Loganantharaj R, Trutschl M, Cvek U and Clifford JL: Identification of genes correlated with early-stage bladder cancer progression. Cancer Prev Res (Phila) 3: 776-786, 2010.
19. Shi S and Tian B: Identification of biomarkers associated with progression and prognosis in bladder cancer via co-expression analysis. Cancer Biomark 24: 183-193, 2019.
20. Zhu H, Chen H, Wang J, Zhou L and Liu S: Collagen stiffness promoted non-muscle-invasive bladder cancer progression to muscle-invasive bladder cancer. OncoTargets Ther 12: 3441-3457, 2019.
21. Oosterlinck W, Lobel B, Jakse G, Malmström PU, Stöckle M and Sternberg C; European Association of Urology (EAU) Working Group on Oncological Urology: Guidelines on bladder cancer. Eur Urol 41: 105-112, 2002.
22. Witjes JA, Compérat E, Cowan NC, De Santis M, Gakis G, Lebret T, Ribal MJ, Van der Heijden AG and Sherif A; European Association of Urology: EAU guidelines on muscle-invasive and metastatic bladder cancer: Summary of the 2013 guidelines. Eur Urol 65: 778-792, 2014.
23. Alfred Witjes J, Lebret T, Compérat EM, Cowan NC, De Santis M, Bruins HM, Hernandez V, Espinós EL, Dunn J, Rouanne M, *et al*: Updated 2016 EAU guidelines on muscle-invasive and metastatic bladder cancer. Eur Urol 71: 462-475, 2017.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Yun SJ, Jeong P, Kang HW, Kim YH, Kim EA and Yan C: Urinary MicroRNAs of prostate cancer: Virus-Encoded hsv1-miRH18 and hsv2-miR-H9-5p could be valuable diagnostic markers. Int Neurourol J 19: 74-84, 2015.
26. Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, Xie H, Xu Y and Zeng X: U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun 454: 210-214, 2014.
27. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, *et al*: STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47(D1): D607-D613, 2019.
28. Knowles MA and Hurst CD: Molecular biology of bladder cancer: New insights into pathogenesis and clinical diversity. Nat Rev Cancer 15: 25-41, 2015.
29. Chen P, Cescon M and Bonaldo P: Collagen VI in cancer and its biological mechanisms. Trends Mol Med 19: 410-417, 2013.
30. Jin Z, Yao J, Xie N, Cai L, Qi S, Zhang Z and Li B: Melittin constrains the expression of identified key genes associated with bladder cancer. J Immunol Res 2018: 5038172, 2018.
31. Fang M, Yuan J, Peng C and Li Y: Collagen as a double-edged sword in tumor progression. Tumor Biol 35: 2871-2882, 2014.
32. Otto T and Sicinski P: Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer 17: 93-115, 2017.
33. Jeggo PA, Pearl LH and Carr AM: DNA repair, genome stability and cancer: A historical perspective. Nat Rev Cancer 16: 35-42, 2016.
34. Shamloo B and Usluer S: p21 in cancer research. Cancers 11: 1178, 2019.
35. Donjerkovic D and Scott DW: Regulation of the G1 phase of the mammalian cell cycle. Cell Res 10: 1-16, 2000.
36. Kamiyama M, Naguro I and Ichijo H: In vivo gene manipulation reveals the impact of stress-responsive MAPK pathways on tumor progression. Cancer Sci 106: 785-796, 2015.
37. Vivanco I and Sawyers CL: The phosphatidylinositol 3-kinase-AKT pathway in human cancer. Nat Rev Cancer 2: 489-501, 2002.
38. Koul HK, Pal M and Koul S: Role of p38 MAP kinase signal transduction in solid tumors. Genes Cancer 4: 342-359, 2013.
39. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH and Hung MC: Cytoplasmic localization of p21 Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol 3: 245-252, 2001.
40. Kumar B, Koul S, Petersen J, Khandrika L, Hwa JS, Meacham RB, Wilson S and Koul HK: p38 mitogen-activated protein kinase-driven MAPKAPK2 regulates invasion of bladder cancer by modulation of MMP-2 and MMP-9 activity. Cancer Res 70: 832-841, 2010.
41. Chetty C, Lakka SS, Bhoopathi P and Rao JS: MMP-2 alters VEGF expression via alphaVbeta3 integrin-mediated PI3K/AKT signaling in A549 lung cancer cells. Int J Cancer 127: 1081-1095, 2010.
42. Gianni` M, Kopf E, Bastien J, Oulad-Abdelghani M, Garattini E, Chambon P and Rochette-Egly C: Down-regulation of the phosphatidylinositol 3-kinase/Akt pathway is involved in retinoic acid-induced phosphorylation, degradation, and transcriptional activity of retinoic acid receptor gamma 2. J Biol Chem 277: 24859-24862, 2002.
43. Liu W, Ding I, Chen K, Olschowka J, Xu J, Hu D, Morrow GR and Okunieff P: Interleukin 1beta (IL1B) signaling is a critical component of radiation-induced skin fibrosis. Radiat Res 165: 181-191, 2006.
44. Wang F, Tang J, Li P, Si S, Yu H, Yang X, Tao J, Lv Q, Gu M, Yang H and Wang Z: Chloroquine enhances the radiosensitivity of bladder cancer cells by inhibiting autophagy and activating apoptosis. Cell Physiol Biochem 45: 54-66, 2018.
45. Devanand P, Kim SI, Choi YW, Sheen SS, Yim H, Ryu MS, Kim SJ, Kim WJ and Lim IK: Inhibition of bladder cancer invasion by Sp1-mediated BTG2 expression via inhibition of DNA methyltransferase 1. FEBS J 281: 5581-5601, 2014.
46. Wu D, Tao J, Xu B, Qing W, Li P, Lu Q and Zhang W: Phosphatidylinositol 3-kinase inhibitor LY294002 suppresses proliferation and sensitizes doxorubicin chemotherapy in bladder cancer cells. Urol Int 86: 346-354, 2011.
47. Wahafu W, Gai J, Song L, Ping H, Wang M, Yang F, Niu Y and Xing N: Increased H^2S and its synthases in urothelial cell carcinoma of the bladder, and enhanced cisplatin-induced apoptosis following H^2S inhibition in EJ cells. Oncol Lett 15: 8484-8490, 2018.
48. Silvers CR, Liu YR, Wu CH, Miyamoto H, Messing EM and Lee YF: Identification of extracellular vesicle-borne periostin as a feature of muscle-invasive bladder cancer. Oncotarget 7: 23335-23345, 2016.