Honokiol inhibits bladder cancer cell invasion through repressing SRC-3 expression and epithelial-mesenchymal transition

LAN SHEN[1], FANG ZHANG[2], RUIMIN HUANG[3], JUN YAN[1] and BING SHEN[2]

[1]State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center of Nanjing University, Nanjing, Jiangsu 210061; [2]Department of Urology, Shanghai General Hospital, Shanghai Jiaotong University, Shanghai 200080; [3]State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, P.R. China

Abstract. Urinary bladder cancer (UBC) is one of the most common urological cancer types. Muscle invasive bladder cancer possesses high propensity for metastasis with poor prognosis. Honokiol is a lignan isolated from *Magnolia officinalis* with high bioavailability and potent anticancer effects. The results of the present study demonstrated that honokiol significantly inhibited UBC cell migration and invasion in a dose-dependent manner compared with the vehicle-treated control group. In addition, honokiol treatment suppressed epithelial-mesenchymal transition by induction of E-cadherin and repression of N-cadherin. Honokiol was capable of significantly downregulating the expression of cell invasion-associated genes, steroid receptor coactivator-3 (SRC-3), matrix metalloproteinase (MMP)-2 and Twist1. Notably, the inhibition of UBC cell invasion by honokiol was reversed by reintroduction of oncoprotein SRC-3 expression, with the restoration of MMP-2 and Twist1, and reduction of E-cadherin expression. Furthermore, the results of the luciferase assay confirmed that SRC-3 could regulate Twist1 promoter activity. Taken together, the results of the present study suggest that honokiol is a promising agent against UBC cell invasion via downregulation of SRC-3 and its target genes.

Introduction

Urinary bladder cancer (UBC) is one of the most common types of malignant tumor in the United States, with an estimated 58,950 new cases and 11,820 UBC-associated mortalities in 2016 (1). Data between 2005 and 2011 in USA revealed that the 5-year survival rate for localized UBC was ~70%, whereas the rate for patients with UBC with distant lesions was ~5% (1). In China, bladder cancer prevalence ranks the 9th and the 2nd positions for the entire population, and people >65 years, respectively (2). However, current treatments, including chemotherapy and radiotherapy possess limited effects on muscle invasive bladder cancer (>stage 2). Therefore, studies investigating the underlying molecular mechanisms of UBC development and the development of efficacious therapeutic reagents for UBC, particularly for patients with invasive cancer are warranted.

Steroid receptor coactivator-3 (SRC-3) and alias amplification in breast cancer 1 belong to the p160 steroid receptor coactivator family (3). Amplification and/or overexpression of SRC-3 have been implicated in steroid-targeted tissues, including in breast and prostate cancer (4-6), and in non-steroid-targeted tissues, including lung and bladder cancer (7-10). Accumulating evidence indicates that SRC-3 can activate steroid and non-steroid receptors. For example, SRC-3 serves as a co-activator for transcription factors ETS variant 4 (PEA3) and JunB proto-oncogene AP-1 transcription factor subunit, which leads to the upregulation of matrix metalloproteinase (MMP)-2, and -13 in androgen receptor-null PC3 prostate cancer cells (11). Furthermore, SRC-3 facilitates E2F transcription factor 1 (E2F1) to promote the proliferation of breast cancer cells (12). Previous studies, including our previous study, have demonstrated that SRC-3 cooperates with hypoxia-inducible factor 1-α and E2F1, thus promoting the survival and proliferation of UBC cells (9,13). However, whether SRC-3 serves a role in cell migration and invasion of UBC remains unclear.

Honokiol is the major active component derived from the stem and bark of the plant *Magnolia officinalis*, a

Correspondence to: Dr Jun Yan, State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center of Nanjing University, 12 Xuefu Road, Nanjing, Jiangsu 210061, P.R. China
E-mail: yanjun@nju.edu.cn

Dr Bing Shen, Department of Urology, Shanghai General Hospital, Shanghai Jiaotong University, 100 Haining Road, Shanghai 200080, P.R. China
E-mail: shenbingyishi@163.com

traditional Chinese medicine. As one of the major lignans with high bioavailability, honokiol exhibits multiple biological properties, including muscle relaxant, neuroprotective, anti-inflammatory and anticancer effects (14-19). However, whether honokiol exhibits an effect UBC cell migration and invasion remains unclear. The present study demonstrated that honokiol inhibited UBC cell invasion by repressing the process of epithelial-mesenchymal transition (EMT). It was further revealed that honokiol downregulated Twist1 (an EMT-associated transcription factor) and MMP-2 (an enzyme associated with cell invasion) via suppressing SRC-3 expression. However, overexpression of SRC-3 reversed the honokiol-mediated inhibition of UBC cell migration and invasion.

Materials and methods

Human bladder cancer cell line and reagents. The human bladder cancer J82 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences Cell Bank (Shanghai, China) and maintained in RPMI-1640 medium (cat no. 31800-022; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS-12A; Capricorn Scientific GmbH, Ebsdorfergrund, Germany). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. HonoPure (98% honokiol; EcoNugenics, Santa Rosa, CA, USA) was dissolved in dimethyl sulfoxide and further diluted with RPMI-1640 medium immediately prior to use. For each protocol, cells treated with DMSO vehicle were used as the negative control.

Luciferase assay. Luciferase assays were performed using a Dual-Luciferase Reporter Assay System kit (cat no. 1910; Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The Twist1 gene promoter was inserted into the pGL3-basic vector (Promega Corporation) to generate the 100 ng Twist1 reporter plasmid (Twist1-Luc) (20). Subsequent to the J82 cells reaching 60% confluency in 24-well plates, Twist1-Luc plasmid was co-transfected into cells with 100 ng SRC-3 expression plasmid, which was constructed by the insertion of the open reading frame of the human SRC-3 gene into pCMV10-3xFLAG (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) (9). Honokiol at various concentrations (0-4.8 μg/ml) were added 1 day following plasmid transfection. After 24 h of incubation at 37°C, the cells were lysed for use in the luciferase assay. A total of 100 μl 1X Passive Lysis Buffer (Promega Corporation) were used to lyse the cells, and then they were tested for luciferase activity according to the protocol. *Renilla* luciferase activity was used for normalization.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using TRIzol reagent (cat no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.). RT was conducted with random primers in the Takara PrimeScript™ RT reagent system (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The expression levels of genes were measured using SYBR-Green-based qPCR (Takara Biotechnology Co., Ltd.). The thermocycler protocol was 95°C for 10 sec, then

95°C for 5 sec, 60°C for 31 sec for 40 cycles from step 2 to step 3. The formula $2^{-\Delta\Delta Cq}$ (Cq cycle threshold) was used to determine the expression levels of target genes normalized by β-actin (21). qPCR was performed in triplicate for each sample. The primer sequences used were as follows: SRC-3 forward, 5'-GGGACTAAGCAACAGGTGTTT-3' and reverse, 5'-TTTGGCCCACCCATACTTGAG-3'; MMP-2 forward, 5'-CCGTCGCCCATCATCAAGTT-3' and reverse, 5'-CTGTCTGGGGCAGTCCAAAG-3'; Twist1 forward, 5'-TGGAGGACCTGGTAGAGGAA-3' and reverse, 5'-GTC CGCAGTCTTACGAGGAG-3'; β-actin forward, 5'-CAT GTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTT AATGTCACGCACGA-3'.

Western blotting. Cells were lysed in RIPA buffer containing a phosphatase inhibitor cocktail I (Sigma-Aldrich; Merck KGaA) and a protease inhibitor cocktail mini-tablet (Roche Diagnostics, Indianapolis, IN, USA). Subsequently, Bradford regent was used to determine protein concentration, and 20 μg protein/lane were separated using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked by 5% non-fat milk at room temperature for 1 h. Primary antibodies directed against E-cadherin (cat no. BS1098; 1:1,000; Bioworld Technology, Inc., St. Louis Park, MN, USA), N-cadherin (cat no. 22018-1-AP; 1:1,000; ProteinTech Group, Inc., Chicago, IL, USA), SRC-3 (cat no. 611104; 1:1,000; BD Biosciences, San Jose, CA, USA), MMP-2 (cat no. 29090; 1:1,000), Twist1 (cat no. 21642; 1:1,000) (both from Signalway Antibody, College Park, MA, USA), and β-actin (cat no. 05-0079; 1:1,000; AbMax Biotechnology Co., Ltd., Beijing, China) were incubated with the membrane overnight at 4°C. Subsequent to washing three times with 1X PBST [1 ml Tween-20 diluted in 1,000 ml 1X PBS (140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO4, 10 mM Na_2HPO_4)], corresponding mouse and rabbit secondary antibodies conjugated with horseradish peroxidase (cat nos. 7076 and 7074; Cell Signaling Technology, Inc., Danvers, MA, USA) were then used at room temperature for 2 h. The western blots were visualized using enhanced chemiluminescence reagents (cat no. 180-501; Tanon Science and Technology Co., Ltd., Shanghai, China).

Wound healing assay. Cells were seeded at a density of $5x10^5$ cells/well into 35-mm dishes and treated with 0, 2.4 or 4.8 μg/ml honokiol. After 24 h, a wound scratch was made with a 100 μl pipette tip on cell monolayer and images were captured after 24 h to estimate the area occupied by migratory cells. Cells were maintained at 37°C throughout the protocol.

Transwell invasion assay. Following treatment with different concentrations of honokiol, $1x10^5$ J82 cells were diluted in 500 μl serum-free RPMI-1640 medium and inoculated in the upper Transwell chamber coated with growth factor-reduced Matrigel. RPMI-1640 medium containing 10% FBS was added to the lower chamber as a chemoattractant. Following 16 h, cells on upper surface of the membrane were removed using a Q-tip, and invaded cells were fixed with 4% formaldehyde for 10 min at room temperature followed by 0.5% crystal violet staining (Sigma-Aldrich; Merck KGaA) for another 10 min at room temperature. The numbers of invaded cells were counted
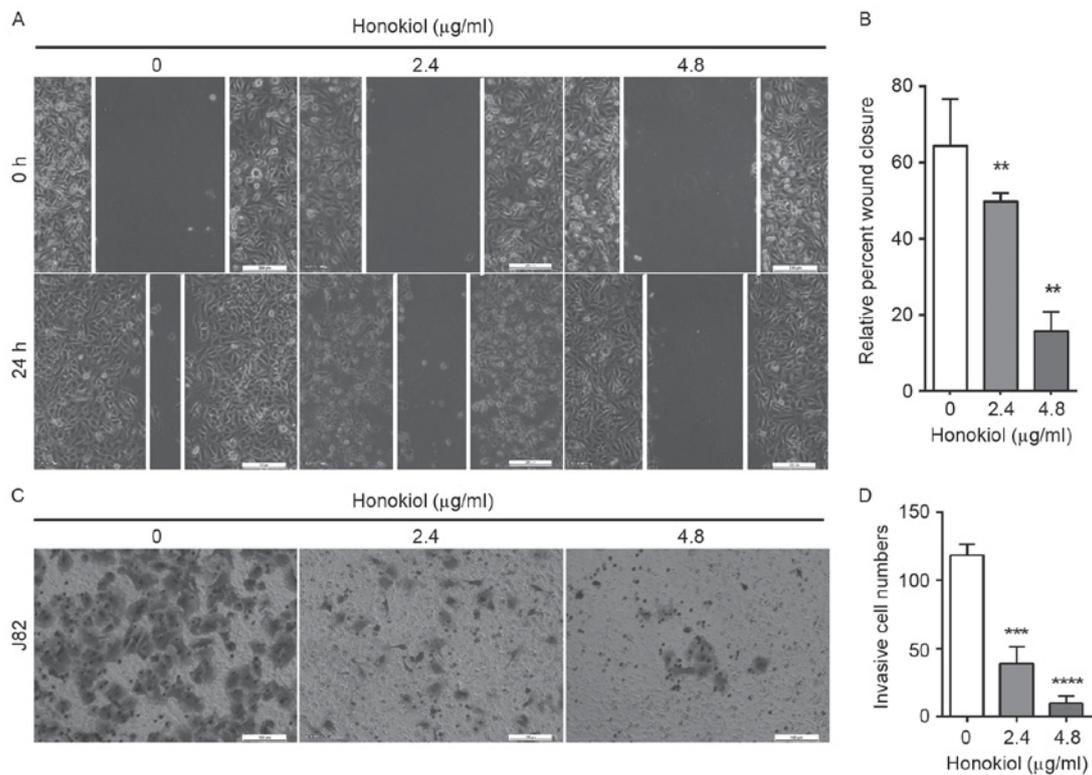
Figure 1. Honokiol inhibits UBC cell migration and invasion. (A) Representative image and (B) quantification of the wound healing assay results, which revealed that honokiol (2.4 and 4.8 µg/ml) significantly inhibited the migration capacity of J82 cells following 24 h of treatment in a dose-dependent manner. Scale bar, 200 µm. (C) Representative image and (D) quantification of the Transwell assay results demonstrated that honokiol (2.4 and 4.8 µg/ml) repressed J82 cells invasion capacity following 16 h of treatment in a dose-dependent manner. Scale bar, 100 µm. **P<0.01, ***P<0.001, ****P<0.0001. Data are represented as the mean ± standard deviation following experiments performed in triplicate.

in five randomly chosen fields under a light microscope at x20 magnification.

Cell viability assay. J82 cells were seeded into 96-well plates at a density of $1x10^4$ cells/well. Honokiol at various concentrations (0-4.8 µg/ml) were added 1 day after cell inoculation. Following treatment with honokiol for 16 h, cells were washed with PBS and 5 mg/ml MTT was added for 3 h at 37˚C. Subsequently, 100 µl DMSO/well was loaded to dissolve the formazan crystals. Plates were incubated at 37˚C for 15 min. Absorbance at 490 nm was examined using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) and absorbance at 680 nm was used as reference.

Immunofluorescence staining. Cells growing on the coverslips in 24-well plates were fixed by 4% paraformaldehyde for 15 min and washed with PBS three times. After blocking with 5% BSA in PBS for 60 min, the coverslips were incubated in the primary antibodies against E-cadherin (Bioworld Technology) and N-cadherin (ProteinTech Group, Inc.) were used overnight at 4˚C. Fluorescein-conjugated secondary antibodies were applied, followed by DAPI counterstaining.

Statistical analysis. Each experiment was repeated three times. Data are represented as the mean ± standard deviation following experiments performed in triplicate. The significant difference between control and experimental groups was analyzed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference. All of the statistical analyses were performed with Graphpad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Honokiol inhibits UBC cell migration and invasion. Patients with bladder cancer with metastatic lesions have poor prognosis. Thus, an investigation into whether honokiol, an anticancer traditional medicine, has any effects on bladder cancer cell migration and invasion was performed. The highly metastatic human bladder cancer J82 cell line was chosen for the present study. J82 cells were treated with different concentrations of honokiol (0, 2.4 and 4.8 µg/ml). The results from the wound healing assay demonstrated that honokiol was capable of inhibiting the migratory capacity of J82 cells in a dose-dependent manner (Fig. 1A). Following 24 h treatment with honokiol at 2.4 and 4.8 µg/ml, the wound closures were significantly reduced by 23, and 75%, respectively, compared with that in the vehicle-treated cell group (Fig. 1B). Invasion capacity of UBC cells was measured using Transwell assays. The cells invading into the lower chambers were significantly decreased upon treatment with honokiol for 16 h compared with the vehicle-treated control group (Fig. 1C and D). The numbers of the invading cells significantly reduced by 67 and 92% upon 2.4, and 4.8 µg/ml honokiol treatment, respectively (Fig. 1D). In order to confirm that the honokiol-induced decrease in migration and invasion ability was not merely due to the
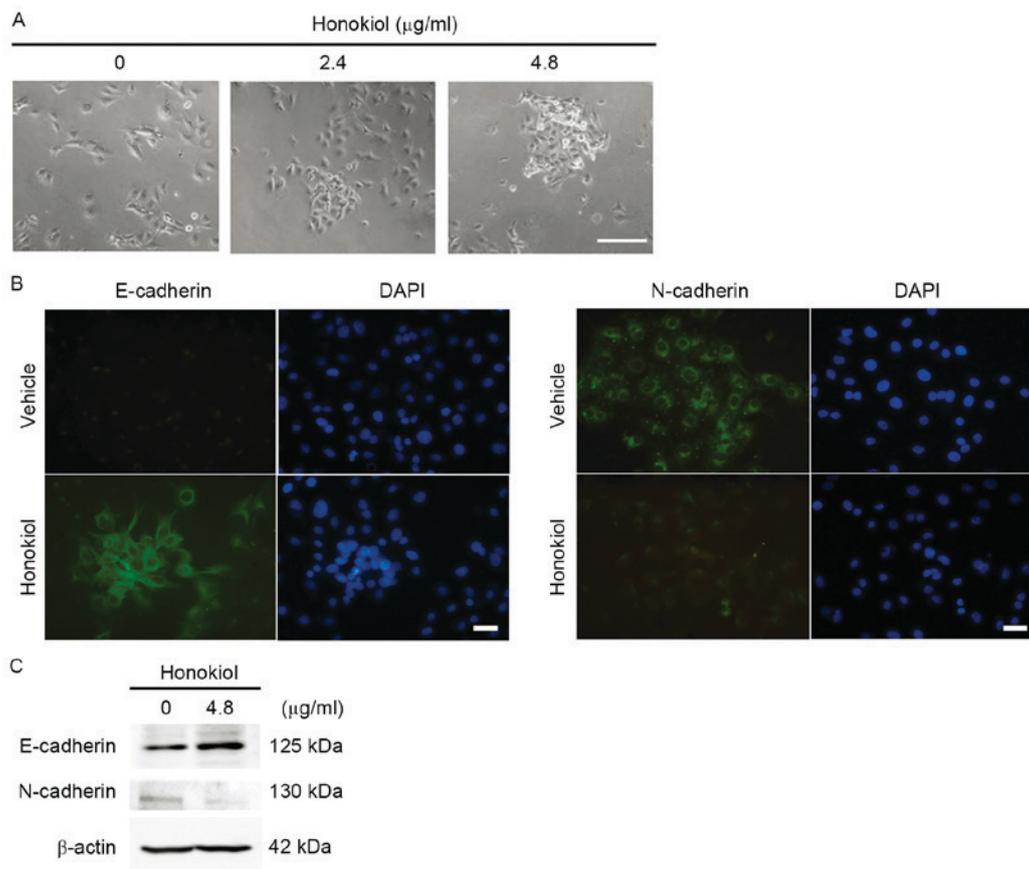
Figure 2. Honokiol suppresses epithelial-mesenchymal transition of bladder cancer cells. (A) Morphologic changes of J82 cells treated with honokiol for 24 h. DMSO was used as vehicle control. Scale bar, 200 μm. (B) Honokiol treatment increased E-cadherin expression and decreased N-cadherin expression as demonstrated through the immunofluorescence staining assay. Scale bar, 20 μm. (C) E- and N-cadherin protein expression determined using a western blotting assay. β-actin was used as a loading control.

decrease of cell number associated with honokiol-induced cell growth arrest, a MTT assay was applied to determine UBC cell viability. J82 cell viability was significantly decreased by 17 and 33% when treated with 2.4, and 4.8 μg/ml honokiol for 16 h, respectively, compared with the vehicle control group (data not shown). The inhibition on cell viability observed was less compared with the effects on cell invasion demonstrated using the Transwell assay. These data suggest that honokiol can inhibit UBC cell migration and invasion.

Honokiol suppresses EMT of bladder cancer cells. Since EMT has been implicated in cancer cell invasion, whether EMT could be suppressed by honokiol treatment (4.8 μg/ml) was investigated in J82 cells. Morphological changes, including cell-cell adhesion, were observed upon honokiol treatment (Fig. 2A). Loss of E-cadherin and gain of N-cadherin are considered to be the fundamental events of EMT (22-24). Immunofluorescence staining assays revealed increased expression of E-cadherin and the decreased expression of N-cadherin (Fig. 2B), which were further confirmed by the western blotting assay (Fig. 2C). These results suggest that honokiol suppresses EMT of UBC cells via regulating the expression levels of E- and N-cadherin.

Honokiol downregulates expression levels of cancer cell invasion-associated genes. To investigate the mechanism

underlying the inhibition of UBC cell invasion induced by honokiol, the expression levels of genes associated with cell invasion were determined using RT-qPCR and western blotting assays. The results demonstrated that the expression of SRC-3, MMP-2 and Twist1 was significantly downregulated by honokiol at the mRNA (Fig. 3A) and protein (Fig. 3B) level compared with the vehicle-treated control group. The positive association between SRC-3 and MMP-2 is consistent with the fact that MMP-2 is a direct target of SRC-3 gene (11). Twist1 is a basic helix-loop-helix transcription factor and serves an essential role in cancer metastasis (20,25). To examine whether Twist1 is downregulated by honokiol through inhibition of its promoter activity, J82 cells were transfected with a reporter plasmid, firefly luciferase driven by human Twist1 promoter (Twist1-Luc). Following 24 h of transfection, cells with were treated honokiol for another 24 h. As a result, honokiol (2.4 and 4.8 μg/ml) significantly reduced the luciferase activity of Twist1-reporter in a dose-dependent manner (Fig. 3C). Overall, these data indicate that honokiol represses the expression of genes involved in cancer cell invasion, including SRC-3, MMP-2 and Twist1.

Overexpression of SRC-3 inhibits the effects of honokiol on cell migration and invasion. To further investigate whether honokiol inhibits bladder cancer cells migration and invasion through SRC-3, SRC-3 expression was reintroduced into
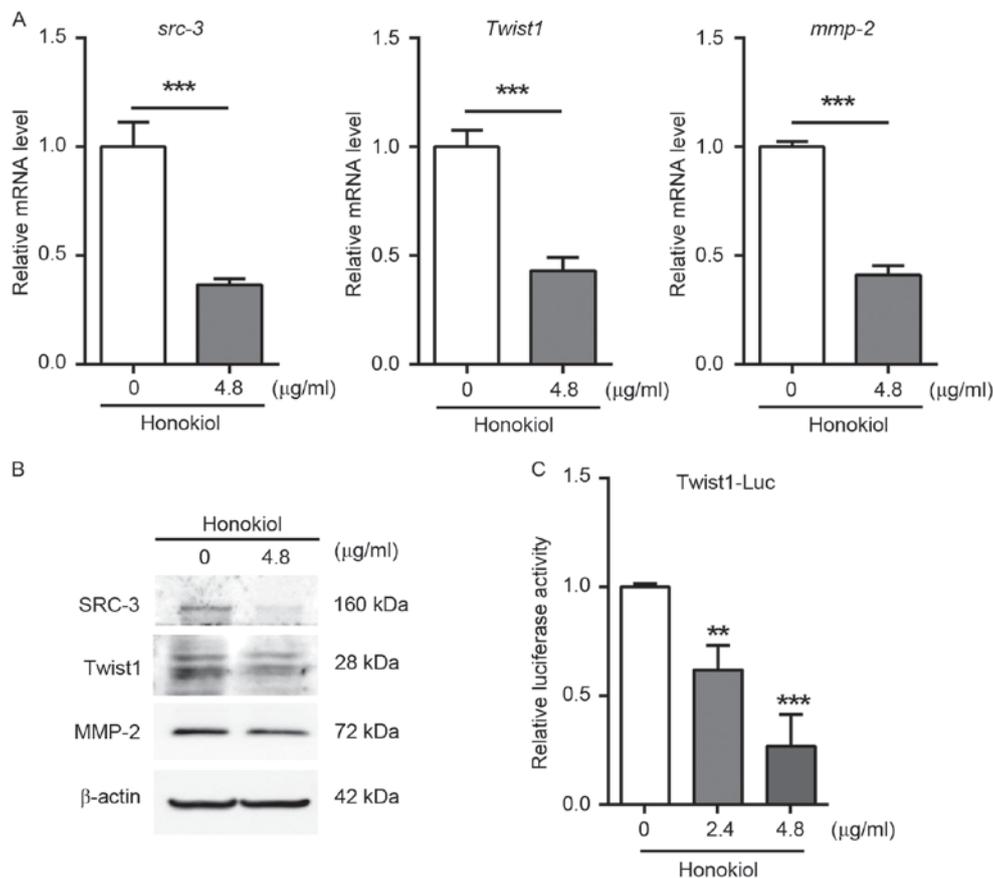
Figure 3. Honokiol reduces genes involved in cancer cell invasion. (A) mRNA and (B) protein expression levels of SRC-3, MMP-2 and Twist1 genes in J82 cells treated with honokiol for 24 h. β-actin was used as a normalization control. (C) Honokiol significantly suppressed Twist1 gene promoter activity in J82 cells as demonstrated through the luciferase reporter assay. **P<0.01, ***P<0.001. Data are represented as the mean ± standard deviation following experiments performed in triplicate. SRC-3, steroid receptor coactivator-3; MMP-2, matrix metalloproteinase 2.

honokiol-treated J82 cells. Empty vector-transfected J82 cells (mock transfectants) were used as a control. In the presence of honokiol (4.8 µg/ml), the ectopic expression of SRC-3 in J82 cells significantly increased the number of invading cells to lower chamber in the Transwell assay compared with that of the mock transfectants (Fig. 4A and B). Furthermore, the ectopic expression of SRC-3 reversed the honokiol-induced changes to E-cadherin, MMP-2 and Twist1 expression (Fig. 4C). Consistently, SRC-3 overexpression almost restored the Twist1-reporter activity under honokiol treatment, suggesting that Twist1 could be a target gene of SRC-3 (Fig. 4D). Taken together, these data suggest that honokiol inhibits UBC cell invasion via repression of EMT and regulation of the expression of cell invasion-associated genes, including SRC-3, MMP-2 and Twist1.

Discussion

Cell invasion is a highly coordinated cellular process, including secretion of MMPs for degradation of extracellular matrix and morphological changes to facilitate EMT. The cadherin switch from E-cadherin to N-cadherin in EMT has been demonstrated to be essential for bladder cancer-associated mortality (24,26). In the present study, it was demonstrated that a low concentration of honokiol (4.8 µg/ml) was capable of inhibiting UBC cell migration and invasion, which was accompanied with the

induction of the epithelial marker E-cadherin, and the reduction of two mesenchymal markers Twist-1 and N-cadherin. Mechanistically, SRC-3, the transcriptional factor coactivator, is indispensable in honokiol-mediated cell invasion inhibition.

SRC-3 is a bona fide oncoprotein in multiple types of solid tumor, including in breast and prostate cancer (5). It was reported that SRC-3 overexpression and amplification occurred in 32.5, and 7.0% human UBC specimens (n=163), respectively (10). The expression levels of SRC-3 in patients with UBC have been suggested to be an independent prognostic marker (10). In addition, data from the present study and other studies indicate that overexpression of SRC-3 is essential for UBC cell survival and proliferation (9,10). Therefore, SRC-3 is an important oncoprotein and serves essential roles in UBC development.

Multiple lines of evidence suggest that different mechanisms are used in SRC-3-mediated cancer cell migration and invasion in a cancer-specific manner (10,11,27-29). An inverse correlation between SRC-3 and E-cadherin has been reported in human pancreatic adenocarcinoma, implying that SRC-3 regulates E-cadherin directly or indirectly (27). By co-activating estrogen receptor α (ERα) in T47D breast cancer cells, SRC-3 also transcriptionally upregulates Snail, which directly represses E-cadherin (28). However, SRC-3 overexpression is not associated with the levels of ERα in UBC tissue samples (10), and urothelial specific ERα-knockout enhanced
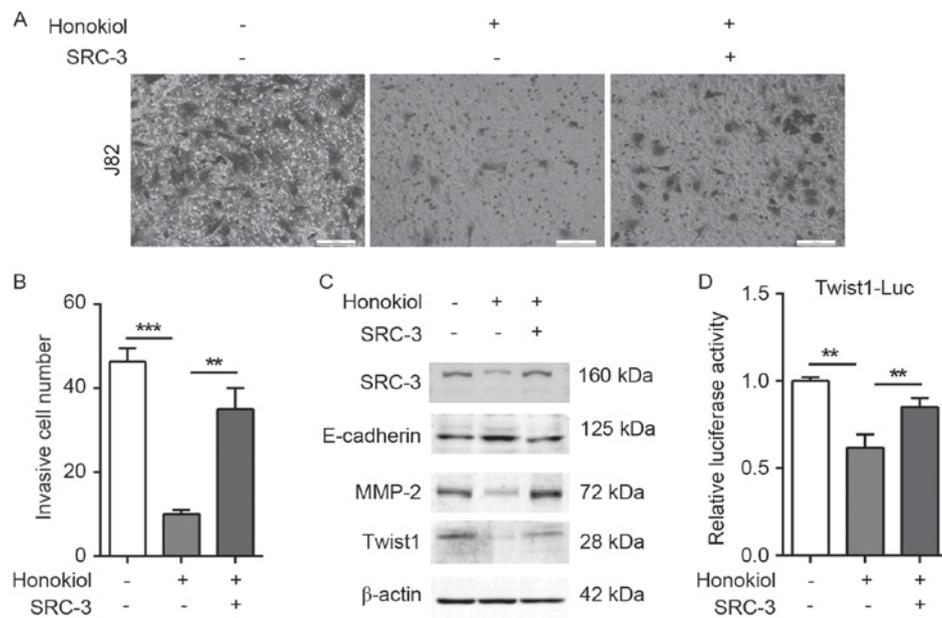
Figure 4. Overexpression of SRC-3 abrogates the effects of honokiol on cell invasion. (A) J82 cells were transfected with SRC-3 expression plasmid (pCMV-3xFLAG-SRC-3) for 24 h, followed by the treatment of honokiol (4.8 μg/ml) for another 24 h. The empty vector was used as a negative control. Transwell inserts were stained with crystal violet. Scale bar, 100 μm. (B) The numbers of invaded cells were counted in five randomized fields under a light microscope and mean values were plotted. (C) Western blotting assay of SRC-3 and invasion/epithelial-mesenchymal transition-associated proteins, MMP-2, E-cadherin, and Twist1. (D) The reduction of Twist1 promoter activity by honokiol treatment was reversed by the forced expression of SRC-3 using luciferase assay. **P<0.01, ***P<0.001. Data are represented as the mean ± standard deviation following experiments performed in triplicate. SRC-3, steroid receptor coactivator-3; MMP-2, matrix metalloproteinase 2.

carcinogen-induced UBC development, suggesting that ERα behaves as a tumor suppressor in UBC (30). Therefore, SRC-3 is likely to induce EMT through transcription factors other than ERα in UBC cells. It has been reported that SRC-1, another member of the p160 family, can induce EMT-associated transcription factor Twist1 by co-activating PEA3 (29). In prostate cancer cells, SRC-3 is capable of upregulating MMP-2 by co-activating the PEA3/activator protein-1 complex (11). In the present study, it was demonstrated that SRC-3 is essential for the expression of MMP-2 and Twist1. Notably, the overexpression of SRC-3 in UBC cells reversed honokiol-mediated invasion repression, and upregulated MMP-2 and EMT-associated marker Twist1 expression. Therefore, it was hypothesized that SRC-3 enhances UBC cell invasion by co-activating transcriptional factors similar to those activated by PEA3, in order to upregulate Twist1 and MMP-2.

Intensive screening for small molecular inhibitors targeting the oncoprotein SRC-3 is ongoing (31-33). Several potential agents from different chemical libraries, including gossypol, bufalin and verrucarin, have been identified, which all induce the instability of SRC-3 protein (31-33). In the current study, it was demonstrated that honokiol is capable of downregulating the mRNA expression of SRC-3. However, the suppressive effect of honokiol on cancer cell migration and invasion may not be limited to target SRC-3. It has been reported that honokiol targets multiple signaling pathways, including KiSS-1 metastasis-suppressor (KISS1)/KISS1 receptor in renal cell carcinoma (18), signal transducer and activator of transcription 3 signaling in breast cancer (34), epidermal growth factor receptor signaling in head and neck squamous cell carcinoma (35), and the inflammation-associated nuclear factor κB pathway in other cancer cells (36,37). Penetration

through the endothelial cell layer is one of the prerequisite steps in metastasis. Joo *et al* demonstrated that by reducing the interaction between cancer and endothelial cells, honokiol suppresses EMT and transendothelial invasion of glioblastoma cells via targeting vascular cell adhesion molecule 1 (38). Taken together, these data suggest that honokiol serves a range of inhibitory roles in cancer cell invasion and metastasis, therefore further *in vivo* studies are warranted to confirm the results presented.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that honokiol inhibits UBC cell migration and invasion via suppression of oncoprotein SRC-3, and two SRC-3 downstream targets, MMP-2 and Twist1. Further clinical trials are required to confirm whether honokiol is a chemotherapeutic agent for patients with UBC, particularly for the muscle invasive subtype.

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