Isorhapontigenin (ISO) Inhibits Invasive Bladder Cancer Formation *In Vivo* and Human Bladder Cancer Invasion *In Vitro* by Targeting STAT1/FOXO1 Axis

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**Abstract**

Although our most recent studies have identified Isorhapontigenin (ISO), a novel derivative of stilbene that isolated from a Chinese herb *Gnetum cleistostachyum*, for its inhibition of human bladder cancer growth, nothing is known whether ISO possesses an inhibitory effect on bladder cancer invasion. Thus, we addressed this important question in current study and discovered that ISO treatment could inhibit mouse-invasive bladder cancer development following bladder carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) exposure *in vivo*. We also found that ISO suppressed human bladder cancer cell invasion accompanied by upregulation of the forkhead box class O 1 (FOXO1) mRNA transcription *in vitro*. Accordingly, FOXO1 was profoundly downregulated in human bladder cancer tissues and was negatively correlated with bladder cancer invasion. Forced expression of FOXO1 specifically suppressed high-grade human bladder cancer cell invasion, whereas knockdown of FOXO1 promoted noninvasive bladder cancer cells becoming invasive bladder cancer cells. Moreover, knockout of FOXO1 significantly increased bladder cancer cell invasion and abolished the ISO inhibition of invasion in human bladder cancer cells. Further studies showed that the inhibition of Signal transducer and activator of transcription 1 (STAT1) phosphorylation at Tyr701 was crucial for ISO upregulation of FOXO1 transcription. Furthermore, this study revealed that metalloproteinase-2 (MMP-2) was a FOXO1 downstream effector, which was also supported by data obtained from mouse model of ISO inhibition BBN-induced mouse-invasive bladder cancer formation. These findings not only provide a novel insight into the understanding of mechanism of bladder cancer’s propensity to invasion, but also identify a new role and mechanisms underlying the natural compound ISO that specifically suppresses such bladder cancer invasion through targeting the STAT1–FOXO1–MMP-2 axis.

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**Introduction**

Bladder cancer is the sixth most common cancer in the United States and the number one cause of death in patients with urinary system malignancies. The incidence of bladder cancer has steadily risen in recent decades. It is estimated that more than 74,690 Americans will be diagnosed with bladder cancer and more than 15,580 will die of this disease in 2014 (1). Approximately 20% to 30% of bladder cancers are muscle-invasive, 50% of these patients die from metastasis within 2 years of diagnosis, and the 5-year survival rate for metastatic bladder cancer is only 6% (2). The remaining 70% to 80% of bladder cancers are diagnosed as non-muscular invasion, in which about 20% progress to muscle-invasive bladder cancer and have a 43% relative lower 5-year survival rate (2). In addition, bladder cancer is one of the most costly cancers as a result of necessary lifetime monitoring and treatment. For these reasons, the identification of new natural compounds that specifically suppress human bladder cancer invasion and defining key molecules that are responsible for mediating human bladder cancer invasion and metastasis are of extremely importance for improving the clinical outcome of patients with this disease. Isorhapontigenin (ISO) is a novel derivative of stilbene and is isolated and purified from a Chinese herb *Gnetum cleistostachyum* (3–5), which has been used for centuries as treatment for several cancers including bladder cancers. Recent work by our group has demonstrated that ISO treatment induces cell-cycle arrest at G0–G1 phase and inhibits anchor-independent cell growth through inhibiting cyclin D1 expression in both RT4 human noninvasive bladder cancer cells and UMCIC3 human-invasive bladder cancer cells (4). Our studies have also found that ISO exhibits the anticancer...
activity accompanied by downregulating X-linked inhibitor of apoptosis protein (XIAP), thus inducing apoptosis in T24T human-invasive bladder cancer cells in a relative high dose (5). Nevertheless, nothing is known whether ISO is able to inhibit bladder cancer invasion at noncytotoxic doses.

For head box O (FOXO) proteins, which include FOXO1, FOXO3a, FOXO4, and FOXO6 in human, primarily function as transcription factors in nucleus and act as tumor suppressors (6, 7). FOXO proteins are reported to upregulate negative regulators of the G1–S transition of the cell cycle, such as p27\(\text{KIP1}\), p21\(\text{WAF1}\), and p130, and repress positive regulators, such as cyclin D1 and D2 (6). Overexpression of FOXO proteins also enhances G2/M arrest (8). Besides, activation of FOXOs triggers apoptosis through binding to the promoters of the proapoptotic gene, FasL and Bim, and inducing their expression (9–11). Moreover, FOXO1 has been found to inhibit prostate cancer cell migration and invasion through binding to Runt-domain containing protein Runx2 and repress its transcriptional activity (12). Expression of a constitutive nuclear active form of FOXO1 significantly inhibited matrix metalloproteinase-9 (MMP-9) activation induced by EGF and prevented cell invasion in glioblastoma cells (13). Although the mRNA and protein expression level of FOXO1 are found to be downregulated in high-grade and invasive bladder cancer, nothing is known about the roles, mechanisms, and the upstream regulators/downtstream effectors of FOXO1 in human bladder cancer invasion (14). Therefore, we addressed these questions and explored the potential FOXO1-related inhibitory effects of ISO on human bladder cancer invasion in the present study both in vitro and in vivo.

Materials and Methods

Plasmids, antibodies, and other reagents

The FLAG-tagged human FOXO1 expression construct, Fasl promotor, and IGF/FGF-1 (3×IRIS) promotor luciferase reporters were constructed and used in previously studies (15). The shRNA sets for human FOXO1 were purchased from Open Biosystems (Thermo Fisher Scientific). The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system–specific targeting FOXO1 was purchased from Applied Biological Materials (ABM) Inc. Human FOXO1 promotor luciferase reporter was cloned into the pGL3 luciferase assay vector and was kindly provided by Dr. Jean-Baptiste Demoulin (De Duve Institute, Catholic University of Louvain, BE-1200 Brussels, Belgium; ref. 16). A mutation of Signal transducer and activator of transcription 1 (STAT1)–binding site in the FOXO1 promotor was created using site-directed mutagenesis by the overlap extension PCR method with mutagenic primers 5′-ACAGAAMACACTCGA-GAAGCGCCATCCAATAATAGAGATCCAAA-3′ (sense) and 5′-TTTGATGTCTCAATTTATGAGTTGAATGCCCTCTCAGGTTTTTGTG-3′ (antisense), and flanking primers 5′-ATTGAGTAGATATCCCTCG-CGCGCCGCC-3′ (forward) and 5′-TGCGGCATCTTGCCGTGCTG-TGCCGTTGACAT-3′ (reverse). GFP–STAT1 Y701F was obtained from Addgene. Human MMP-2 expression construct was kindly provided by Dr. Jian Cao (Department of Medicine, School of Medicine, State University of New York at Stony Brook, Stony Brook, NY; ref. 17). Human MMP-2 promotor luciferase reporter was a gift from Dr. Ety N. Benveniste (Department of Cell Biology, The University of Alabama at Birmingham, Birmingham, Alabama; ref. 18). The antibodies against FOXO1, p-FOXO1, FOXO4, PARP, STAT1, p-STAT1, STAT3, p-STAT3, and CREB were commercialized from Cell Signaling Technology. The antibodies against MMP-2, SOCS1, and GAPDH were bought from Santa Cruz Biotechnology. ISO with purity greater than 99.9% was purchased from Higher Biotech. ISO was dissolved in dimethyl sulfoxide (DMSO; Sigma) to make a 20 mmol/L stock solution, and the same concentration of DMSO was also made and used as a vehicle control in all experiments.

Animal experiments

The C57BL/6J male mice at age of 5 to 6 weeks were randomly divided into three groups, 12 mice in each group, including vehicle-treated control group, N-butyl-N{(4-hydroxybutyl) nitrosamine (BBN)-treated group, and BBN combined with ISO-treated group. Mice in BBN-treated group received BBN (0.05%) in drinking water for 20 weeks, whereas mice in BBN combined with ISO-treated group received BBN and ISO (150 mg/kg/day) in drinking water for 20 weeks. ISO was given to the mice in drinking water on the day of initial exposure to BBN and continued throughout the tumor induction period. Mouse bladder tissues were excised and fixed overnight in 4% paraformaldehyde at 4°C. Fixed tissues were processed for paraffin embedding, and the serial 5-μm-thick sections were then stained by hematoxylin and eosin (H&E) staining.

Cell culture and transfection

Human-invasive bladder cancer cell line UMUC3 and noninvasive bladder cancer cell line RT4 were provided by Dr. Xue-Ru Wu (Departments of Urology and Pathology, New York University School of Medicine, New York, NY) in 2010, and were described and used in our previous studies (4, 19). The human metastatic bladder cancer cell line T24T, which is a lineage-related lung metastatic variant of invasive bladder cancer cell line T24 (20–22), was kindly provided by Dr. Dan Theodorescu (Departments of Urology, University of Virginia, Charlottesville, VA; ref. 20) in 2010. All the cell lines were subjected to DNA tests and authenticated in our previous studies (4). The cell lines were regularly authenticated on the basis of viability, recovery, growth, morphology, and chemical response as well and were most recently confirmed 4 to 6 months before use by using a short tandem repeat method. UMUC3 cells were maintained in DMEM supplemented with 10% FBS (HyClone), 1% penicillin/streptomycin, and 2 mmol/L L-glutamine. Life Technologies). T24T cells were cultured in DMEM/Ham’s F-12 (1:1 volume) mixed medium supplemented with 5% FBS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine. RT4 cells were maintained in McCoy’s 5A supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine. Transfections were carried out using PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories) according to the manufacturer’s instructions. The transfected cells were then respectively selected with G418, hygromycin, or puromycin (Life Technologies) for 4 to 6 weeks. Surviving cells were pooled as stable mass transfectants as described in our previous studies (23, 24).

Human tissue specimens

Ninety-eight pairs of primary bladder cancer samples and their paired adjacent normal bladder tissues were obtained from patients who underwent radical cystectomy at Department of Urology of the Union Hospital of Tongji Medical College ( Wuhan, China) between 2012 and 2015. All specimens were
immediately snap-frozen in liquid nitrogen after surgical removal. Histologic and pathologic diagnoses were confirmed, and the specimens were classified by a certified clinical pathologist according to the 2004 World Health Organization Consensus Classification and Staging System for bladder neoplasms. All specimens were obtained with appropriate informed consent from the patients and a supportive grant obtained from the Medical Ethics Committee of China.

**ATP cell viability assay**

Cell viability was measured utilizing the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega Corp.) according to the manufacturer’s instructions as described in our previous studies (25). Briefly, cells were plated in 96-well plates at a density of 10,000 cells per well and allowed to adhere overnight. The cell culture medium was then replaced with 0.1% FBS DMEM and cultured for 12 hours. After ISO treatment for the indicated time and doses, 50 μL of CellTiter-Glo assay reagent was added to each well. The contents were mixed on an orbital shaker for 2 minutes to induce cell lysis and then incubated at room temperature for 10 minutes to stabilize the luminescent signal. Results were read on a microplate luminometer LB 96V (Berthold GmbH & Co. KG). Cell viability (%) was defined as the relative absorbance of treated samples versus that of the untreated control. All experiments were performed with six wells for each experiment and repeated at least three times.

**In vitro cellular migration and invasion assays**

In vitro migration and invasion assays were conducted by using Transwell chamber (for migration assay) or Transwell precoated Matrigel chamber (for invasion assay) according to the manufacturer’s protocol (BD Biosciences) as described previously (26, 27). Briefly, 700 μL of medium containing 10% FBS (for UMUC3 and T24T cells) or 40% FBS (for RT4 cells) was added to the lower chambers, whereas homogeneous single-cell suspensions (5 × 10⁴ cells/well) in 0.1% FBS medium with or without ISO as indicated were added to the upper chambers. The Transwell plates were incubated in 5% CO₂ incubator at 37°C for 4 hours, and thereafter were washed by PBS, fixed with 4% formaldehyde, and stained with Giemsa stain. The nonmigration or noninvading cells were scrapped off the top of chamber. The migration and invasion rates were quantified by counting the migration and invaded cells at least three random fields under a light microscope (Olympus).

**Western blotting**

Western blot assay was assessed as previously described (28). Briefly, cells were plated in 6-well plates and cultured in normal FBS medium until 70% to 80% confluence. The cells were then cultured in 0.1% FBS medium for 12 hours, followed by treatment with different doses of ISO for the indicated time. The cells were washed once with ice-cold PBS, and cell lysates were prepared with a lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 1% SDS, and 1 mmol/L Na₂VO₄]. An equal amount (80 μg) of total protein from each cell lysate was subjected to Western blot with the indicated antibody as described in previous studies (25, 26). Immunoreactive bands were detected by using the alkaline phosphatase-linked secondary antibody and ECF Western blotting system (Amersham Biosciences). The images were acquired by using Typhoon FLA 7000 imager (GE Healthcare).

**Nuclear extract preparation**

Nuclear extracts were prepared as previously described (23). UMUC3 cells were seeded into 10-cm culture dishes at 70% to 80% confluence, cultured in 0.1% FBS medium for 12 hours, and then treated either with vehicle (0.1% DMSO) or with 10 μmol/L of ISO for 12 hours. The nuclear proteins were extracted by the Nuclear/Cytosol Fractionation Kit (BioVison Technologies) following the manufacturer’s protocols. Equal Protein concentrations were measured by a protein quantification assay kit (Bio-Rad). Nuclear extracts were stored at −80°C before they were used.

**Reverse transcription PCR and qRT-PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen Corp.), and cDNAs were synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp.). A pair of oligonucleotides (Forward: 5’-GATG AITCFTGACG-TGTTGTC-3’ and Reverse: 5’-CAGGGCTGCTTTATAAICTCTG-3’). The flag-foxo1 fragments were amplified by primers 5’-ACAAGGAGCGACTGACAAAGGG-3’ (Forward) and 5’-GGCCG-AGTTGGAATCTGCTAAA-3’ (Reverse). The human mmp-2 cDNA fragments were amplified by 5’-CAAGTGGAAGAGACGACATGATGTC-3’ (Forward) and 5’-CCA AAGTTGATCATGATGTCG-3’ (Reverse). The human mmp-9 cDNA fragments were amplified by 5’-GGGACG-CAGACATCGTCAC-3’ (Forward) and 5’-TGGTATCATGCTCCG-3’ (Reverse). The PCR products were separated on 2% agarose gels and stained with ethidium bromide. The images were visualized and scanned with UV lights with FluorChem SP imaging system (Alpha Innotech Inc.) as described previously (29). The Quantitative reverse transcription PCR (qRT-PCR) analysis was carried out using the SYBR Green PCR Kit (Qiagen) and the 7900HT Fast real-time PCR system (Applied Biosystems).

**Luciferase assay**

As described in our previous studies (4, 5), dual-luciferase reporter assay was performed by using the luciferase assay system (Promega Corp.). Briefly, Human Fasl, promoter, IGFBR-1 (3×1RS) promoter, FOXO1 promoter, and MMP-2 promoter luciferase reporters were transfected into the indicated human bladder cancer cells, respectively. After ISO treatment, cells were extracted with passive lysis buffer [25 mmol/L Tris-phosphate (pH 7.8), 2 mmol/L EDTA, 1% Triton X-100, and 10% glycerol]. The luciferase activity was measured with a microplate luminometer LB 96V (Berthold GmbH & Co. KG). The Renilla luciferase signal was normalized to the internal firefly luciferase transfection control.

**Immunohistochemistry paraffin of mice bladder tissues**

Mice bladder tissues were immunostained by antibodies specific against FOXO1 (Cell Signaling Technology) and MMP-2 (Santa Cruz Biotechnology), respectively. The resultant immunostaining images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss). Protein expression levels were analyzed by calculating the
integrated optical density per stained area (IOD/area) using Image-Pro Plus version 6.0 (Media Cybernetics). Briefly, the IHC-stained sections were evaluated at 400-fold magnifications, at least 5 representative staining fields of each section were analyzed to calculate the optical density based on typical photographs that had been captured.

Statistical methods
Associations between categorical variables were assessed using the χ² test. The Student t test was utilized to compare continuous variables, summarized as mean ± SD, between different groups. Paired t test was performed to compare the difference between paired tissues using real-time PCR analyze. P < 0.05 was considered statistically.

Results
ISO treatment inhibited BBN-induced mouse-invasive bladder cancer formation in vivo and human bladder cancer invasion in vitro
ISO has been shown to inhibit growth and induce apoptosis in human bladder cancer cells in our recent studies (4, 5). BBN is a well-characterized bladder carcinogen for its induction of 100% invasive bladder cancer in mouse model (30). To explore whether ISO exhibit bladder cancer invasion, we first employed BBN-induced invasive bladder cancer mouse model and examined the effects of ISO on BBN-induced mouse-invasive bladder cancer formation. As shown in Table 1 and Fig. 1A, none of the vehicle-treated control mice developed bladder cancer, whereas

Table 1. Effect of ISO treatment on BBN-induced mouse-invasive bladder cancer formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Carcinogen</th>
<th>Treatment</th>
<th>Number (%) of papillomas</th>
<th>Number (%) of noninvasive bladder cancer</th>
<th>Number (%) of invasive bladder cancer</th>
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<tr>
<td>1</td>
<td>12</td>
<td>Vehicle only</td>
<td>Vehicle only</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>BBN</td>
<td>Vehicle only</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>12 (100) a</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>BBN ISO</td>
<td>ISO</td>
<td>7 (58.3)</td>
<td>3 (25)</td>
<td>2 (16.7) b</td>
</tr>
</tbody>
</table>

aSignificant difference between vehicle control group and BBN-treated group (P < 0.05).
bSignificant difference between BBN-treated group and BBN combined with ISO-treated group (P < 0.05).

Figure 1.
ISO inhibited bladder cancer invasion in vivo and in vitro. A, mice were divided into vehicle-treated control group (n = 12), BBN-treated group (n = 12), and BBN combined with ISO-treated group (n = 12). H&E staining was performed, and the representative images of each group were shown. Human UMUC3 (B) and T24T (C) bladder cancer cells were cultured in chamber or precoated Matrigel chamber and treated with medium containing either vehicle or indicated concentration of ISO for 24 hours. The cells were then fixed and stained. The invasion and migration rates were quantified by counting the relative migrated (Transwell) and invaded cells at least three random fields under a light microscope. D, UMUC3 cells were treated with medium containing either vehicle or 10 µmol/L ISO up to 36 hours for cell viability assessment by ATPase assay. E, T24T cells were treated with medium containing either vehicle or 20 µmol/L ISO for the indicated time. Cell viability was also evaluated by ATPase assay. Results are the mean ± SD of triplicates. Symbol “*” indicates a significant difference between vehicle- and ISO-treated group (P < 0.05).
BBN induced 100% (12/12) high-grade muscle-invasive bladder cancers formation. Interestingly, only 16.7% (2/12) of the BBN-treated mice developed high-grade muscle-invasive bladder cancer while ISO was administrated, with 7 cases of papillomas and 3 cases of low-grade non–muscle-invasive bladder cancers, demonstrating a novel biologic activity of ISO as an efficient drug that targets at stage of invasive bladder cancer development in vivo (P < 0.05). Subsequently, two human-invasive bladder cancer cell lines UMUC3 and T24T were employed for Transwell cancer cell invasion assay in the presence of relative low dosages, 10 µmol/L and 20 µmol/L, of ISO treatment, according to the dosages applied in cell proliferation analyses described previously (4, 5). As shown in Fig. 1B and C, the relative invasion rates of UMUC3 and T24T cells were reduced by 63.1% and 61.2% after 10 µmol/L and 20 µmol/L ISO treatment, respectively, in comparison with the vehicle control, whereas ISO did not show observed effects on the bladder cancer cell migration under same experimental conditions. To further exclude any possible involvement of cellular toxicity, the cells were treated with the same dosages of ISO as shown in Fig. 1D and E for different time points from 6 hours to 36 hours, and cell survival rates were analyzed by ATPase assay. As expected, the selected dosages of ISO did not show any observable cellular toxicity to either UMUC3 (Fig. 1D) or T24T (Fig. 1E) cells at the time point (24 hours) when cell migration and invasion were measured. Taken together, these data, for the first time, demonstrate that ISO is a new natural compound that can specifically inhibit mouse-invasive bladder cancer development in vivo following BBN exposure and suppress bladder cancer cell invasion in vitro.

ISO induced FOXO1 protein expression and FOXO1-dependent transcriptional activity in human bladder cancer cells

FOXO1 has been proposed as a potential prognostic marker for bladder cancer since an increased foxo1 mRNA expression is associated with the reduced bladder cancer disease progression and a significantly prolonged survival of the patients (14). To elucidate the mechanisms underlying ISO inhibitory effect on human bladder cancer, we investigated whether ISO could regulate the expression of FOXO1 in human-invasive bladder cancer cell lines, UMUC3, and T24T. As shown in Fig. 2A, ISO treatment induced FOXO1 protein expression in a dose-dependent manner in UMUC3 cells. Treatment of UMUC3 cells with 10 µmol/L ISO also resulted in a gradual induction in FOXO1 protein level over various time points (Fig. 2B). Consistently, similar FOXO1 protein expression profile was also observed in the metastatic human bladder cancer T24T cells upon ISO treatment (Fig. 2C). It was noted that ISO treatments at dose of 10 µmol/L for UMUC3 cells and 20 µmol/L for T24T cells could markedly induce FOXO1 protein expression (Fig. 2A–C) with a profoundly specific inhibition of cancer cell invasion without affecting their migration (Fig. 1C and D). To unravel the function of ISO-induced FOXO1 protein, we determine FOXO1 protein location and its dependent transcriptional activation in ISO-treated cells. As shown in Fig. 2D, FOXO1 protein was almost all located in nuclear fraction, while it was not observable in cytoplasmic fraction. Consistent with its location, ISO treatment remarkably enhanced FOXO1-dependent IGFBG-1 promoter and Fasl promoter transcription activities (Fig. 2E and F), strongly demonstrating that ISO-induced FOXO1 proteins are not only located in nuclear, but also functional in human bladder cancer cells.

FOXO1 was downregulated in human bladder cancer tissues and acted as an effective suppressor of invasion of human bladder cancer cells

To find out the clinical relevance of therapeutic effect of ISO in human bladder cancer, we evaluated FOXO1 expression in 98 pairs of bladder cancer tissues and their adjacent normal bladder tissues surgically removed from patients who were diagnosed with bladder cancers. As shown in Fig. 3A, a profound reduction of foxo1 mRNA expression was observed in human bladder cancer tissues with an overall average 2.4-fold lower relative foxo1 mRNA level in comparison with their adjacent normal bladder tissues (P < 0.001). Moreover, a significant negative relationship was observed between foxo1 expression and bladder cancer grade, invasion, as well as the
FOXO1 was downregulated in human bladder cancer tissues and acted as a regulator of human bladder cancer cell invasion. Total RNA and protein lysates were prepared from human normal (N) and paired cancerous (T) tissues among 98 patients diagnosed with bladder cancers, and subjected to qRT-PCR and Western blotting analyses for determining FOXO1 mRNA (A) and protein (B) expression profiles, respectively. Flag-FOXO1 expression construct was used to stably transfect into UMUC3 and T24T cells, respectively. The stable transfectants, UMUC3(Flag-FOXO1) and T24T(Flag-FOXO1), were identified by Western blotting (C) and then used for determination of their abilities of cell invasion and migration as compared with their vector control transfectants (D & E) as described in the "Materials and Methods" section. F, RT4 cells were stably transfected with nonsense shRNA or two FOXO1 shRNA constructs (shFOXO1-4 and shFOXO1-6), respectively, and the knockdown efficiency of FOXO1 protein was evaluated by Western blot. G, FOXO1 stably knockdown transfectants, RT4(shFOXO1-4) and RT4(shFOXO1-6), as well as Nonsense transfectant RT4(Nonsense) were then used for determination of their migration and invasion abilities. Results are the mean ± SD of triplets. Symbol *** indicates a significant difference between vector control and FOXO1 overexpression group (P < 0.05). Symbol ** indicates a significant difference between Nonsense transfectant and shRNA transfectants (P < 0.05).

Table 2. Correlation between foxo1 mRNA level and clinicopathologic factors

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Total</th>
<th>Low</th>
<th>High</th>
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<th>P value</th>
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<tr>
<td>Gender</td>
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<td>79</td>
<td>60</td>
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<td></td>
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<td>81</td>
<td>59</td>
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<td>0.548</td>
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<tr>
<td>Histologic grade</td>
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<td>19</td>
<td>13</td>
<td></td>
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<tr>
<td></td>
<td>High</td>
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<td>54</td>
<td>12</td>
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<tr>
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<td>T1, T2, T3</td>
<td>39</td>
<td>22</td>
<td>17</td>
<td></td>
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<tr>
<td>Tumor size</td>
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<td>9</td>
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<td>0.570</td>
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<tr>
<td></td>
<td>≥3.0 cm</td>
<td>58</td>
<td>42</td>
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<td>Tumor multiplicity</td>
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<td>Multifocal</td>
<td>73</td>
<td>54</td>
<td>19</td>
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<td>Lymph nodes metastasis</td>
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<td>45</td>
<td>20</td>
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<td>0.008*</td>
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<tr>
<td></td>
<td>Present</td>
<td>33</td>
<td>30</td>
<td>3</td>
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</table>

*P < 0.05.

Figure 3.

FOXO1 protein to bladder cancer invasion, UMUC3 and T24T cells were stably transfected with exogenous Flag-FOXO1. As shown in Fig. 3C–E, ectopic expression of FOXO1 significantly attenuated the cell invasion with no significant inhibition of cell migration in both high-grade UMUC3 and T24T bladder cancer cells. On the other hand, knockdown of FOXO1 (shFOXO1-4 and shFOXO1-6; Fig. 3F) increased invasiveness without affecting migration of noninvasive RT4 bladder cancer cells (Fig. 3G). These results are consistent to demonstrate that downregulation of FOXO1 expression plays an important role in the bladder cancer invasion, and the FOXO1 induction by ISO treatment might be crucial for its inhibitory effect on human bladder cancer invasion.

ISO upregulated FOXO1 expression by increasing its mRNA transcription in human bladder cancer cells

It is well-known that FOXO1 expression can be regulated through its nuclear exportation and degradation (6). To address whether ISO treatment alternates FOXO1 protein degradation, both transfectants of UMUC3(vector) and UMUC3(Flag-FOXO1) were employed. As shown in Fig. 4A and B, ISO treatment only increased endogenous FOXO1 protein expression in UMUC3(vector) cells, whereas it did not affect exogenous
ISO Inhibits Bladder Cancer Invasion by Inducing FOXO1

The above results demonstrate that ISO inhibition of human bladder cancer invasion was dependent on FOXO1 induction via upregulating FOXO1 gene promoter transcription activity. To identify the transcription factor that is responsible for ISO upregulation of FOXO1 transcription, TFTRANSFAC Transcription Factor Binding Sites Software (Biological Database) was used for bioinformatics analysis of the FOXO1 promoter region. The results revealed that an approximate 800-bp promoter region of the human FOXO1 gene contained the putative DNA-binding sites of various transcription factors, including CREB, FOXO4, STAT1, and STAT3 (Fig. 6A). We next examined the changes in both expression and the nuclear translocation of these factors following ISO treatment for 12 hours. As shown in Fig. 6B, treatment of UMUC3 cells with ISO resulted in a dramatic inhibition of STAT1 phosphorylation at Tyr701 in a dose-dependent manner without affecting total protein expression, whereas there was only a slightly effect on phosphorylation of STAT3 and expression of FOXO4 and CREB. The results obtained from distributions of the transcription factors between nuclear and cytoplasm fractions consistently indicated that ISO treatment mainly targeted STAT1 phosphorylation at Tyr701 (Fig. 6C). To investigate the role of STAT1 phosphorylation in upregulation of FOXO1 by ISO, a dominant-negative mutant form of STAT1(GFP-STAT1 Y701F) was ectopically expressed in UMUC3 cells, and the dominant-negative effect was confirmed by its inhibition of a STAT1-regulated SOCS1 expression (ref. 31; Fig. 6D). The results showed that transfection of FOXO1 expression, excluding the possibility that FOXO1 protein induction by ISO is at protein degradation level. We, therefore, next evaluated the mRNA levels of foxo1 in the same bladder cancer cells. Consistent with the results obtained from protein levels, endogenous foxo1 mRNA expression was markedly upregulated upon ISO treatment in both UMUC3 and T24T cells (Fig. 4C–E), whereas the flag-tagged exogenous flag-foxo1 mRNA was not affected by ISO treatment (Fig. 4F and G). These results suggest that ISO-induced FOXO1 expression may be regulated at mRNA transcription level. To test this notion, the foxo1 promoter–driven luciferase reporter was transfected into UMC3 cells, and foxo1 promoter activity was significantly upregulated in a time-dependent manner upon ISO treatment (Fig. 4H). Taken together, these data strongly reveal that ISO-induced FOXO1 protein expression is regulated at mRNA transcriptional level in human bladder cancer cells.

 FOXO1 expression was required for ISO inhibition of bladder cancer cell invasion

To address the contribution of FOXO1 induction by ISO to its inhibition of bladder cancer invasion, we transfected various FOXO1 shRNAs into UMUC3 and T24T, respectively, and the stable FOXO1 knockdown transfectants were identified by Western blotting as shown in Fig. 5A and B. The stable transfectants, UMUC3(shFOXO1-4), UMUC3(shFOXO1-6), T24T (shFOXO1-4), and T24T(shFOXO1-6), were used to determine invasion and migration abilities as compared with their Non-sense control transfectants, UMUC3(Nonsense), and T24T (Nonsense), respectively. As shown in Fig. 5C and Supplementary Fig. S1A and S1B, knockdown of FOXO1 by its shRNAs (shFOXO1-4 and shFOXO1-6) showed no effects on cell migration, but significantly promoted invasion in both UMUC3 and T24T cells (Fig. 5D; Supplementary Fig. S1A and S1B), which consistently support that FOXO1 protein exhibits specific inhibition of cancer invasion. Importantly, suppression of FOXO1 protein expression markedly reduced ISO inhibition of cell invasion in both UMUC3 and T24T cells (Fig. 5E; Supplementary Fig. S1A and S1B). To further verify the role of FOXO1 in ISO suppression of bladder cancer invasion, CRISPR/Cas9 systems were used to knockout FOXO1 gene in both UMUC3 and T24T cells. The single-clone stable FOXO1 knockout transfectants, UMUC3(KO FOXO1, clone 2) and T24T(KO FOXO1, clone 5), were selected for our studies (Fig. 5F and G). As shown in Fig. 5H and Supplementary Fig. S2A and S2B, bladder cancer migration was not affected upon FOXO1 knockout, whereas the invasion of UMUC3 and T24T cells was further increased (Fig. 5I and Supplementary Fig. S2A and S2B). Moreover, knockdown of FOXO1 successfully diminished the effects of ISO on bladder cancer invasion (Fig. 5J; Supplementary Fig. S2A and S2B). These results greatly demonstrate that FOXO1 protein induction by ISO is crucial for its inhibition of bladder cancer invasion.

ISO treatment enhanced FOXO1 transcription through inhibition of STAT1 phosphorylation at Tyr701
dominant-negative STAT1 markedly enhanced ISO-induced FOXO1 protein expression (Fig. 6D) as well as FOXO1 promoter–driven transcription activity (Fig. 6E), indicating that suppression of STAT1 activity could mimic ISO treatment. In the contrast, mutation of STAT1-binding site effectively diminished the increased FOXO1 promoter activity induced by ISO (Fig. 6F). Thus, these data suggest that phosphorylated STAT1 at Tyr701 can bind to the FOXO1 promoter to inhibit its transcription, and ISO-induced upregulation of FOXO1 transcription is specifically mediated through ISO suppression of STAT1 phosphorylation at Tyr701.

FOXO1 mediated ISO inhibition of bladder cancer invasion through downregulation of MMP-2 expression

Extensive studies have identified MMPs as the key players in cancer cell invasion by degrading cellular matrix components and the basement membrane (32). Elevated MMP-2 levels have been reported to be of independent prognostic value in patients with bladder cancers (33), whereas the other essential MMP member, such as MMP-9 expression, is not correlated with tumor grade, stage, or overall survival of the patients with bladder cancer (34). Our previous study has also confirmed that upregulation of MMP-2, but not MMP-9, participated in mediation of bladder cancer invasion (22). To determine whether MMP-2 is associated with ISO inhibition of human bladder cancer invasion, we evaluated the effect of ISO on MMP-2 protein expression. The results showed that ISO treatment profoundly inhibited MMP-2 protein expression in both UMUC3 and T24T cells (Fig. 7A and B). Moreover, MMP-2 mRNA level and its promoter-driven luciferase reporter activity were also attenuated by ISO treatment (Fig. 7C and D), suggesting that the downregulation of MMP-2 expression by ISO occurred at transcriptional level. Meanwhile, the mRNA level of MMP-9 was not affected upon ISO treatment (Fig. 7C). To test the potential relation of FOXO1 induction to MMP-2 inhibition following ISO treatment in bladder cancer cells, we next evaluated the effects of FOXO1 overexpression and knockdown on MMP-2 expression in T24T cells. As shown in Fig. 7E, enforced expression of FOXO1 impaired MMP-2 expression,

Figure 5.
Knockdown and knockout of FOXO1 reversed ISO inhibition of bladder cancer cell invasion. A–E, UMUC3 (A) and T24T (B) cells were stably transfected with nonsense shRNA or a set of six various FOXO1 shRNA (shFOXO1) constructs, respectively. The knockdown efficiency of FOXO1 protein was assessed by Western blotting. FOXO1 stably knockdown transfectants, including UMUC3(shFOXO1-1), UMUC3(shFOXO1-4), and T24T(shFOXO1-4), and T24T (shFOXO1-6), as well as Nonsense transfectants were then used for determination of their cell migration (C) and invasion (D and E) in the presence of either vehicle or the indicated concentration of ISO treatment for 24 hours. F–J, CRISPR/Cas9 systems were then applied to knockout FOXO1 gene, and the single-clone stable FOXO1 knockout transfectants, including UMUC3(KO FOXO1, clone5) and T24T(KO FOXO1, clone2), as well as Vector control transfectants were used for determination of their cell migration (H) and invasion (I and J) in the presence of either vehicle or the indicated concentration of ISO treatment for 24 hours. Results are the mean ± SD of triplicates. Symbol * indicates a significant difference between Nonsense transfectant and shRNA transfectants (P < 0.05). Symbol ** indicates a significant difference between scramble vector control and FOXO1 knockout transfectants (P < 0.05). Symbol *** indicates a significant difference between vehicle- and ISO-treated group (P < 0.05).
ISO promoted FOXO1 transcription by inhibition of STAT1 phosphorylation at Tyr701. A, schematic representation of transcription factor binding sites of human FOXO1 gene promoter. B, UMUC3 cells were treated with either vehicle or ISO as the indicated concentrations for 12 hours. Expression of the related transcription factors in the whole-cell lysates was determined by Western blotting, and GAPDH was used as protein loading control. C, the UMUC3 cells were treated with either vehicle or 10 μmol/L of ISO for 12 hours. The cell extracts were used to isolate cell nuclear and cytoplasm fractions and then subjected to Western blotting with the specific antibodies as indicated. PARP and GAPDH were used as markers for nuclear and cytoplasm fractions, respectively. D, UMUC3 cells were stably transfected with dominant-negative STAT1, GFP-STAT1 Y701F, and the stable transfectants were treated with either vehicle or 10 μmol/L ISO for 12 hours. The expression of FOXO1 and SOCS1 was determined by Western blotting. GAPDH was used as protein loading control. E, FOXO1-promoter-driven luciferase reporter was transfected into UMUC3(EGFP) and UMUC3(STAT1 Y701F) cells, and the stable transfectants were then treated with 10 μmol/L of ISO for the indicated times. Luciferase activity was evaluated by the Dual-Luciferase Reporter Assay System. F, UMUC3 cells were stably transfected with FOXO1-promoter-driven luciferase or the STAT1-binding site mutant, and the stable transfectants were treated with 10 μmol/L of ISO for 18 hours. Dual-Luciferase Reporter Assay System was performed to determine the Luciferase activity. Results are the mean ± 5D of triplicates. Symbol "#" indicates a significant difference between UMUC3(EGFP-vector) and UMUC3(STAT1 Y701F) in the same time point upon ISO treatment (P < 0.05). Symbol "##" indicates a significant difference between vehicle control and ISO-treated group (P < 0.05).

Discussion

Muscular invasion of bladder cancer causes 100% death and represents a major therapeutic challenge of this disease. Therefore, an endeavor to identify new anticancer compounds with strong inhibition of cancer invasion and understand the mechanisms underlying their inhibitory effect on bladder cancer invasion is the key step to discover the unmet future medicines against the invasive malignant disease. ISO is a new derivative isolated from a Chinese herb Gnetum Cleistostachyum that has been used to treat bladder cancer for many years (3). Our recent in vivo pharmacokinetic study shows that the concentration of ISO in serum can reach to 47.9 μmol/L without any observed toxicity to experimental mice (4). Here, our results obtained from in vivo animal studies show that ISO treatment inhibits BBN-induced mouse-invasive bladder cancer formation, for first time demonstrating the chemopreventive effects of ISO to the best of our knowledge. We also find that ISO at in vivo–relevant concentrations of 10 to 20 μmol/L specifically represses bladder cancer invasion by promoting FOXO1 transcription through inhibition of STAT1 phosphorylation at Tyr701. Our study also identify that MMP-2 is a downstream target of FOXO1 for its mediation of ISO inhibition of cancer invasion. Our results clearly demonstrate that the natural compound ISO specifically inhibits human bladder cancer invasion through targeting STAT1–FOXO1–MMP-2 axis, which is completely distinct from its induction of cancer cell

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apoptosis and inhibition of cancer cell anchorage-independent growth. Prevention and therapeutic intervention by phytochemicals represents a newer dimension in cancer management. Silymarin, a mixture of flavonoids isolated mainly from milk thistle, has been extensively studied in preclinical models as well as clinically for its efficacy against a variety of cancers including bladder cancer, and it has been shown to effectively inhibit cell proliferation, angiogenesis, epithelial mesenchymal transition, metastasis, as well as promote cell apoptosis (35, 36). Whether ISO exhibits other anticancer effects apart from targeting cell growth, apoptosis, and invasion deserves further investigation.

Our recent studies have revealed that ISO effectively attenuates transcription factor–specific protein 1 (Sp1) expression and transactivation (4, 5), which results in the decreased bindings of Sp1 to promoter region of its regulated genes, cyclin D1 and XIAP, in turn leading to the downregulation of these gene expressions and consequently resulting in suppression of cancer cell anchorage-independent growth and induction of apoptosis in bladder cancer cells (4, 5). Although our group has reported above mechanism underlying anticancer effects of ISO compound, evaluation of whether ISO is a new agent with strong activity for inhibition of cancer invasion and understanding the potential mechanism involved in its inhibition of bladder cancer invasion are of tremendous importance for ISO potentially therapeutic clinical application. In the present study, we demonstrate that ISO at relevant applicable concentrations shows a great inhibition of bladder cancer invasion in vitro and mouse-invasive bladder cancer development in vivo. Accordingly, treatment of ISO significantly promotes FOXO1 expression, whereas suppression of FOXO1 expression completely abolishes ISO inhibition of bladder cancer invasion in both UMUC3 and T24T cells. Thus, ISO is an effective therapeutic agent for inhibiting bladder cancer invasion, and upregulation of FOXO1 plays a critical role in
Figure 8. ISO reversed the BBN-induced downregulation of FOXO1 and upregulation of MMP-2 in mice. Mice were divided into vehicle-treated control group (n = 12), BBN-treated group (n = 12), and BBN combined with ISO-treated group (n = 12). A, immunohistochemistry paraffin staining by antibodies specific against FOXO1 and MMP-2 was performed. FOXO1 (B) and MMP-2 (C) protein expression levels were analyzed by calculating the integrated IOD/area using Image-Pro Plus version 6.0. Results are the mean ± SD of 12 mice in each group. Symbol # indicates a significant difference between vehicle control group and BBN-treated group (P < 0.05). Symbol ** indicates a significant difference between BBN-treated group and BBN combined with ISO-treated group (P < 0.05).
STAT1 has been shattered by emerging evidence that constitutively activated STAT1 signaling is involved in the resistance to DNA-damaging therapeutic agents of epithelial cancers (45–47). In line with these observations, muscle-invasive bladder cancer tissues were reported to be characterized by constitutively nuclear expression of phosphorylated STAT1 (48). STAT1 has been thought to exhibit a negative regulatory effect on FOXO1 transcription in pancreatic β cells (49); however, this notion has never been explored in any cancer cells. In addition, nothing is known about the mechanism underlying STAT1 regulation of FOXO1 transcription. In the present study, we demonstrate that ISO treatment increases FOXO1 transcription accompanied with repression of STAT1 phosphorylation at Tyr701 in bladder cancer cells. The inhibition of STAT1 activity by overexpression of dominant-negative STAT1 could mimic the biologic effects of ISO treatment, whereas the mutation of STAT1-binding site in FOXO1 promoter region reversed the increased FOXO1 transcription activity induced by ISO treatment. These results reveal a novel function of phosphorylated STAT1 at Tyr701 for its direct binding to FOXO1 promoter resulting in suppression of FOXO1 transcription, which is also the mechanisms for ISO upregulation of FOXO1 transcription and expression, and in turn inhibiting bladder cancer invasion in vitro and mouse-invasive bladder cancer formation in vivo and providing new information for using ISO as a new agent targeting STAT1 and FOXO1 for the bladder cancer therapy.

FOXO proteins could not only act as transcription factors in the nucleus through sequence-specific interaction with DNA-binding sites, but can also cooperate with or titrate away specific transcription factors or cofactors, and subsequently activate or repress the transcription of the downstream genes that lack consensus binding sequences for FOXO factors (50). FOXO1 has been found to physically interact with transcription factor Runx2 and downregulate the transcription of Runx2-targeted endogenous genes, such as the osteopontin (OP), VEGF, and MMP-13, thereby inhibiting prostate cancer cell migration and invasion (12). The present study shows that transcription of MMP-2, a key player in many cancer cell migration, is downregulated upon ISO treatment. Moreover, knockdown of FOXO1 upregulates the basal expression level of MMP-2 and effectively reverses ISO inhibition of MMP-2 expression and bladder cancer invasion as well, suggesting that FOXO1 plays a negative role in regulation of MMP-2 transcription. Accordingly, the inhibition of BBN-induced mouse-invasive bladder cancer formation by ISO is accompanied by upregulation of FOXO1 and downregulation of MMP-2 expression. As MMP-2 promoter lacks consensus binding site for FOXO1, we anticipate that FOXO1 probably represses MMP-2 transcription indirectly through interacting with other specific transcription factors. Further elucidating of this hypothesis is currently undergoing in our laboratory.

In conclusion, we demonstrate that FOXO1 acts as a tumor suppressor by inhibiting bladder cancer invasion, and the novel derivative anticancer drug ISO specifically represses BBN-induced invasive bladder cancer formation in vivo and human bladder cancer invasion in vitro through upregulation of FOXO1 expression at transcription level. Moreover, the STAT1-binding site in FOXO1 promoter is critical for its suppression of FOXO1 transcription, and ISO-induced FOXO1 expression is mediated through dephosphorylation of STAT1 at Tyr701. In addition, downregulation of MMP-2 mRNA transcription by FOXO1 is responsible for ISO inhibition of bladder cancer invasion. These findings not only provide a novel insight into understanding of underlying mechanism of bladder cancer’s propensity to invasion, but also identify natural compound ISO acting as a new agent for inhibition of invasive bladder cancer formation, which could be used for preventive strategies or to prevent recurrence and progress after Transurethral Resection of Bladder Tumor of non-muscle-invasive tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Isorhapontigenin (ISO) Inhibits Invasive Bladder Cancer Formation \textit{In Vivo} and Human Bladder Cancer Invasion \textit{In Vitro} by Targeting STAT1/FOXO1 Axis

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