

BASIC RESEARCH PAPER

SESN2/sestrin 2 induction-mediated autophagy and inhibitory effect of isorhapontigenin (ISO) on human bladder cancers

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ABSTRACT

Isorhapontigenin (ISO) is a new derivative of stilbene isolated from the Chinese herb *Gnetum cleistostachyum*. Our recent studies have revealed that ISO treatment at doses ranging from 20 to 80 μM triggers apoptosis in multiple human cancer cell lines. In the present study, we evaluated the potential effect of ISO on autophagy induction. We found that ISO treatment at sublethal doses induced autophagy effectively in human bladder cancer cells, which contributed to the inhibition of anchorage-independent growth of cancer cells. In addition, our studies revealed that ISO-mediated autophagy induction occurred in a SESN2 (sestrin 2)-dependent and BECN1 (Beclin 1, autophagy related)-independent manner. Furthermore, we identified that ISO treatment induced SESN2 expression via a MAPK8/JNK1 (mitogen-activated protein kinase 8)/JUN-dependent mechanism, in which ISO triggered MAPK8-dependent JUN activation and facilitated the binding of JUN to a consensus AP-1 binding site in the *SESN2* promoter region, thereby led to a significant transcriptional induction of *SESN2*. Importantly, we found that *SESN2* expression was dramatically downregulated or even lost in human bladder cancer tissues as compared to their paired adjacent normal tissues. Collectively, our results demonstrate that ISO treatment induces autophagy and inhibits bladder cancer growth through MAPK8-JUN-dependent transcriptional induction of *SESN2*, which provides a novel mechanistic insight into understanding the inhibitory effect of ISO on bladder cancers and suggests that ISO might act as a promising preventive and/or therapeutic drug against human bladder cancer.

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Introduction

Bladder cancer is a common urological cancer that ranks as the sixth most common cancer in the United States following breast cancer, lung cancer, prostate cancer, colon cancer and lymphoma.¹ It is the third most common cancer in men, and the eleventh most common cancer in women.¹ There were an estimated 74,000 new cases diagnosed with bladder cancer in 2015; and 16,000 of these patients died from this disease in the United States according to a report from the National Cancer Institute. The majority of human bladder cancer is transitional cell carcinomas derived from the uroepithelium, which accounts for more than 90% of bladder carcinomas.² The depth of invasion of the bladder wall is closely associated with the clinical treatment of bladder cancers.³ Since high-grade invasive bladder cancer can progress to a life-threatening metastases and a 5-year overall survival in patients with lymph node-only disease was 20.9%,⁴ the discovery and evaluation of a new alternative medication will be of tremendous importance for reducing the mortality of this disease.

The Chinese herb *Gnetum cleistostachyum* has been used for treatment of human bladder cancers for centuries, but its bioactive components and anticancer mechanisms have been

barely explored.⁵ Isorhapontigenin (ISO) is a new derivative of a stilbene compound isolated from this herbal medicine with multiple anticancer activities demonstrated in our previous studies.^{5–7} We found that ISO at low dosages (5–10 μM) effectively induces G₀/G₁ growth arrest through an inhibition of the key cell-cycle regulatory protein CCND1 (cyclin D1) expression,^{5,6} whereas it causes an apoptotic response of cancer cells by inhibiting transcription of the anti-apoptotic *XIAP* (X-linked inhibitor of apoptosis) gene at relatively high dosages (20–60 μM).⁷ Our most recent studies demonstrate that in vivo ISO (150 mg/kg/d) can markedly inhibit xenograft tumor growth in nude mice injected with high invasive human bladder cancer T24T cells ($p < 0.01$, $n = 6$).⁸ All these studies suggest that ISO may be a promising agent for the prevention and/or treatment of human bladder cancer.

Macroautophagy/autophagy is a highly evolutionarily conserved catabolic process characterized by the formation of phagophores, a double-membrane compartment that sequesters long-lived cytoplasmic proteins and organelles; the phagophore matures into an autophagosome allowing cargo degradation by further fusing with a lysosome.⁹ There is emerging evidence for

the role of autophagy during development and differentiation,¹⁰ and in diseases such as cancer.¹¹ In combination with various conventional chemotherapeutic drugs, inhibition of autophagy has emerged as an attractive and promising approach to sensitize malignancies to chemotherapy in cancer therapy, which is based on the fundamental premise that autophagy plays a protective role in the anticancer action of these chemotherapeutic agents.¹² However, a recent report has also shown that there are at least 4 functional forms of autophagy that may occur in response to chemotherapeutic drugs in a context (cancer cell line and agent)-specific manner: cytoprotective, cytostatic, cytotoxic and nonprotective.¹² Therefore, it is necessary and essential to recognize the functional type of autophagy induced by chemotherapeutic drugs and to further determine the underlying mechanism prior to their application in clinical trials.

SESN2 (*sestrin 2*) encodes a member of the sestrin family of PA26-related proteins, which plays major roles in suppression of oxidative stress and regulation of adenosine 5'-monophosphate-activated protein kinase (AMPK)-MTOR (mechanistic target of rapamycin [serine/threonine kinase]) signaling.^{13,14} The *SESN2* protein may be involved in complex regulation of cell viability in response to different stress conditions.¹⁵ Ectopic *SESN2* expression could inhibit cell growth and proliferation, whereas it also results in protection from apoptotic cell death induced by oxygen- and/or glucose-deprivation or H₂O₂ treatment in a breast cancer cell line.¹⁶ In the present study, we found that *SESN2* was dramatically downregulated in human bladder cancer tissues. We also found that ISO treatment effectively induced autophagy in an *SESN2*-dependent manner, and such ISO-induced autophagy was required for the ISO inhibition of anchorage-independent cancer cell growth, demonstrating that autophagy induced by ISO is cytotoxic to human bladder cancer cells. Moreover, we determined that JUN was a direct transcriptional factor that was responsible for the induction of *SESN2* expression by ISO treatment.

Results

ISO effectively induced autophagy in human cancer cells

The *in vitro* anticancer activity of ISO has been well documented by our previous studies showing that treatment of ISO effectively inhibits anchorage-independent growth of human bladder and colon cancer cells via modulating the expression of CCND1 and XIAP. However, potential effect of ISO treatment on autophagy and the role of autophagy in the anticancer activity of ISO have never been explored. To evaluate the potential effect of ISO on autophagy, we therefore treated several human cancer cell lines including human bladder cancer UMUC3 and T24T cells, as well as human cervical cancer HeLa cells with various concentrations of ISO for 24 h. As shown in Fig. 1A, ISO treatment resulted in a significant increase in the conversion of LC3 from LC3-I to LC3-II, indicating that ISO effectively induces autophagy in these cancer cells in a dose-dependent manner.

To further demonstrate autophagy induction by ISO treatment, we established stable UMUC3 cells transfected with a *GFP-LC3* plasmid, encoding a fusion protein that would allow us to observe the formation of autophagosomes.¹⁷ Following the treatment of UMUC3 (*GFP-LC3*) cells with various

concentrations of ISO for 24 h, the percentage of GFP-LC3 puncta-positive cells and numbers of GFP-LC3 puncta per cell increased along with the doses of ISO treatment (Fig. 1B and C), again revealing that ISO treatment led to the formation of autophagosomes and the induction of autophagy in a dose-dependent manner. In addition, autophagy flux was determined by treatment with bafilomycin A₁ (BAF; a vacuolar-type H⁺-ATPase inhibitor that blocks autophagosome and lysosome fusion). ISO treatment led to further increase of LC3-II (Fig. 1D) and GFP-LC3 puncta levels (Fig. 1E and F) in the presence of BAF, suggesting that ISO increases the autophagy flux level.

To determine the physiological relevance of ISO-induced autophagy in ISO-mediated inhibition of anchorage-independent cancer cell growth, a soft-agar assay was performed using UMUC3 cells treated with ISO alone or in combination with BAF. As seen in Fig. 1G and H, the presence of BAF obviously abolished the inhibitory effects of ISO on anchorage-independent growth of UMUC3 cells. These results demonstrate that ISO is able to induce autophagy effectively, which might be associated with its inhibition of anchorage-independent growth of human bladder cancer cells.

SESN2, but not BECN1, was crucial for autophagy induction and inhibition of anchorage-independent growth of human bladder cancer cells by ISO

BECN1 is a critical upstream regulator of autophagy, and heterozygous disruption of *BECN1* causes a high incidence of spontaneous tumors,^{18,19} and *SESN2* has also been reported to participate in serum deprivation-induced autophagy and cell death in cancer cells.²⁰ Thus, we determined whether *BECN1* and/or *SESN2* were involved in ISO-associated autophagy. Interestingly, ISO treatment increased expression of both *BECN1* and *SESN2* at the autophagy induction doses (2.5~10 μM; Fig. 2A). To evaluate the roles for *BECN1* and *SESN2* in ISO-induced autophagy, we employed short-hairpin RNAs (shRNAs) targeting human *BECN1* or *SESN2* to knock down expression of endogenous *BECN1* or *SESN2* in UMUC3 cells. The corresponding stable transfectants were established and identified as shown in Fig. 2B and D. Unexpectedly, knockdown of *BECN1* expression had no observable effects on ISO-induced conversion of LC3 from LC3-I to LC3-II (Fig. 2C). However, *SESN2* depletion significantly attenuated ISO-mediated induction of LC3-II (Fig. 2E). These data suggested that ISO-induced autophagy occurred in a *SESN2*-dependent and *BECN1*-independent manner. Furthermore, we assessed the effects of *SESN2* depletion on ISO-mediated inhibition of anchorage-independent growth, and found that *SESN2* knockdown pronouncedly abolished the inhibitory effects of ISO on anchorage-independent growth of human bladder cancer cells (Fig. 2F and G). Collectively, these results suggest that induction of *SESN2*, but not *BECN1*, plays an essential role in ISO-mediated autophagy induction and anchorage-independent growth inhibition.

ISO increased SESN2 transcription via a JUN-dependent mechanism

In order to characterize the molecular mechanism underlying the modulation of *SESN2* expression by ISO treatment, we first

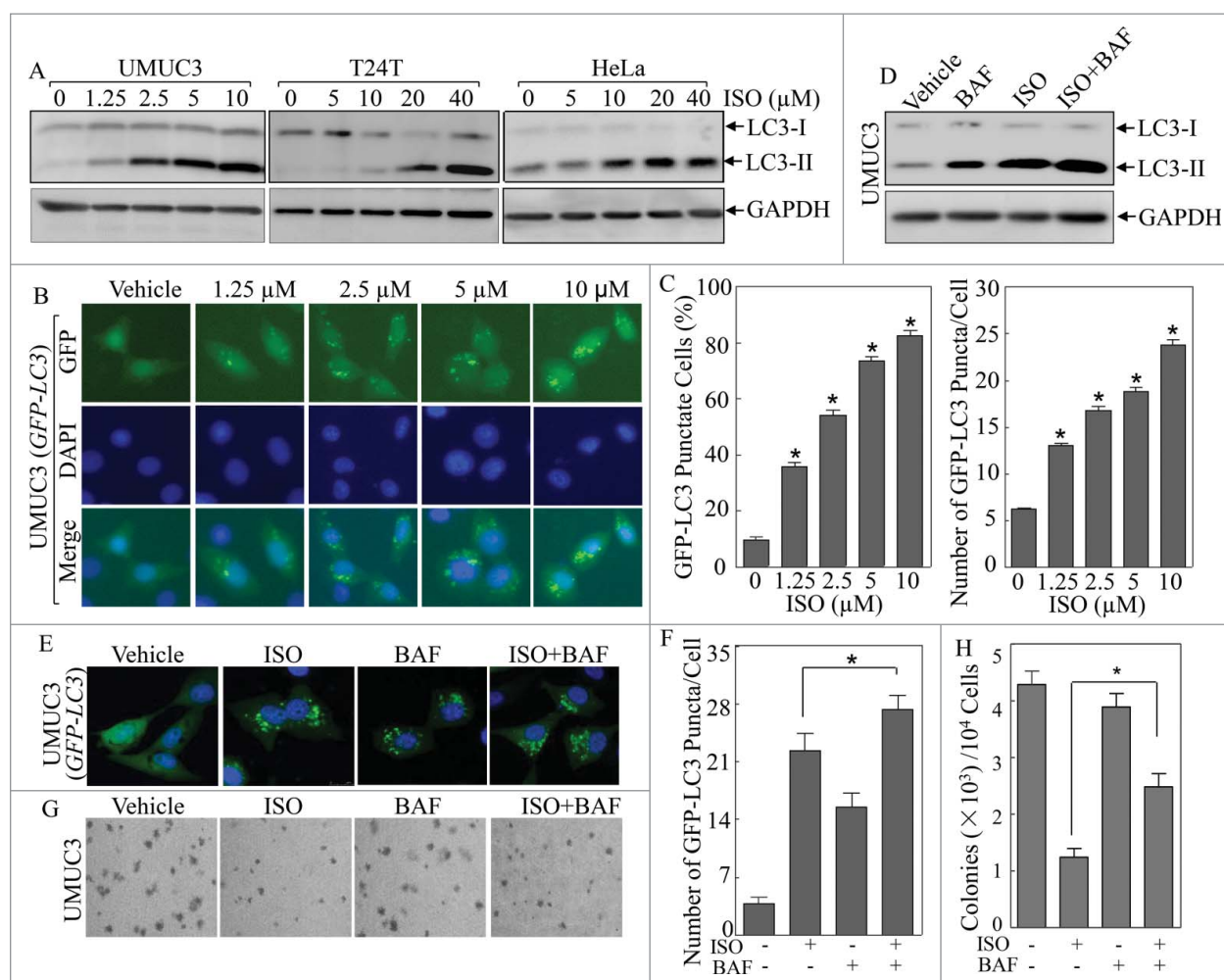


Figure 1. ISO induced autophagy in human cancer cells. (A) Human bladder cancer UMUC3 and T24T cells and human cervical cancer HeLa cells, were treated with ISO at the indicated doses for 24 h. The cells were extracted and cell lysates were subjected to western blotting assay by using the indicated antibodies. (B) The *GFP-LC3* construct was stably transfected into UMUC3 cells, and the transfectants were treated with various doses of ISO for 24 h. LC3 puncta formation was observed and images were captured using fluorescence microscopy. (C) Percentage of cells with GFP-LC3 puncta (left) and number of puncta per positive cell (right) were calculated and presented as described in Materials and Methods. The symbol (*) indicates a significant increase from the vehicle control ($p < 0.05$). (D) UMUC3 cells were treated with ISO ($10 \mu\text{M}$), together with or without 5 nM BAF for 24 h and the protein levels of LC3 were assessed by western blotting. (E) UMUC3 cells were transfected with the *GFP-LC3* construct and the transfectants were treated with ISO ($10 \mu\text{M}$), with or without 5 nM BAF for 24 h. The representative images of GFP-LC3 puncta were captured using a confocal fluorescence microscope. (F) Number of puncta per GFP-LC3-positive cell was calculated and presented as described in Materials and Methods. The symbol (*) indicates a significant increase from the ISO treatment group ($p < 0.05$). (G) Representative images of colonies of UMUC3 treated with $10 \mu\text{M}$ ISO, 1 nM BAF or both in soft-agar assay were captured as described in Materials and Methods. (H) Colonies shown in (G) were counted under a microscope with more than 32 cells of each colony. The results are presented as colonies per 10^4 cells, and the bars show mean \pm SD from 3 independent experiments. The symbol (*) indicates a significant increase from the ISO treatment group ($p < 0.05$).

checked the alteration of *SESN2* mRNA expression in UMUC3 upon ISO treatment by using RT-PCR (Fig. 3A). Consistent with the effects of ISO on *SESN2* protein expression, ISO treatment increased *SESN2* mRNA expression in a dose-dependent manner, suggesting that the regulatory effect of ISO on *SESN2* expression may occur at the transcriptional level. As expected, ISO treatment indeed led to a ~ 2 to 3-fold increase in the activity of a *SESN2* promoter-driven luciferase reporter (Fig. 3B). We then performed a bioinformatics search for putative transcription factors that are potentially responsible for the induction of *SESN2* transcription by ISO, and found that there were several potential binding sites for transcription factors in the *SESN2* proximal promoter region including AP-1, NFKB, E2F1 and SP1 (Fig. 3C). Therefore, we examined the expression alterations of those potential transcription factors.

As shown in Fig. 3D, ISO treatment had no observable effects on the expression levels of phospho-NFKB RELA, NFKB RELA,

SP1 or E2F1, but profoundly increased JUN phosphorylation at Ser63, suggesting that JUN might be involved in the transcriptional induction of *SESN2* by ISO treatment. To test this notion, we blocked JUN activation in UMUC3 cells by using a well-characterized JUN dominant negative mutant, TAM67 (Fig. 3E),²¹ and treated these stable transfectants with $10 \mu\text{M}$ ISO for 12 or 24 h. As expected, ectopic expression of TAM67 successfully inhibited the induction of endogenous AP-1-dependent transcriptional activity by ISO (Fig. 3F), and further abolished the ISO-mediated transcriptional induction of *SESN2* promoter activity (Fig. 3G). Moreover, blockade of endogenous JUN activation by TAM67 also significantly attenuated the increased effects of ISO on *SESN2* mRNA levels (Fig. 3H). These results strongly demonstrate that JUN-AP-1 plays an essential role in ISO-mediated induction of *SESN2* expression.

To provide direct evidence for the involvement of JUN in ISO-induced *SESN2* expression, we performed a chromatin

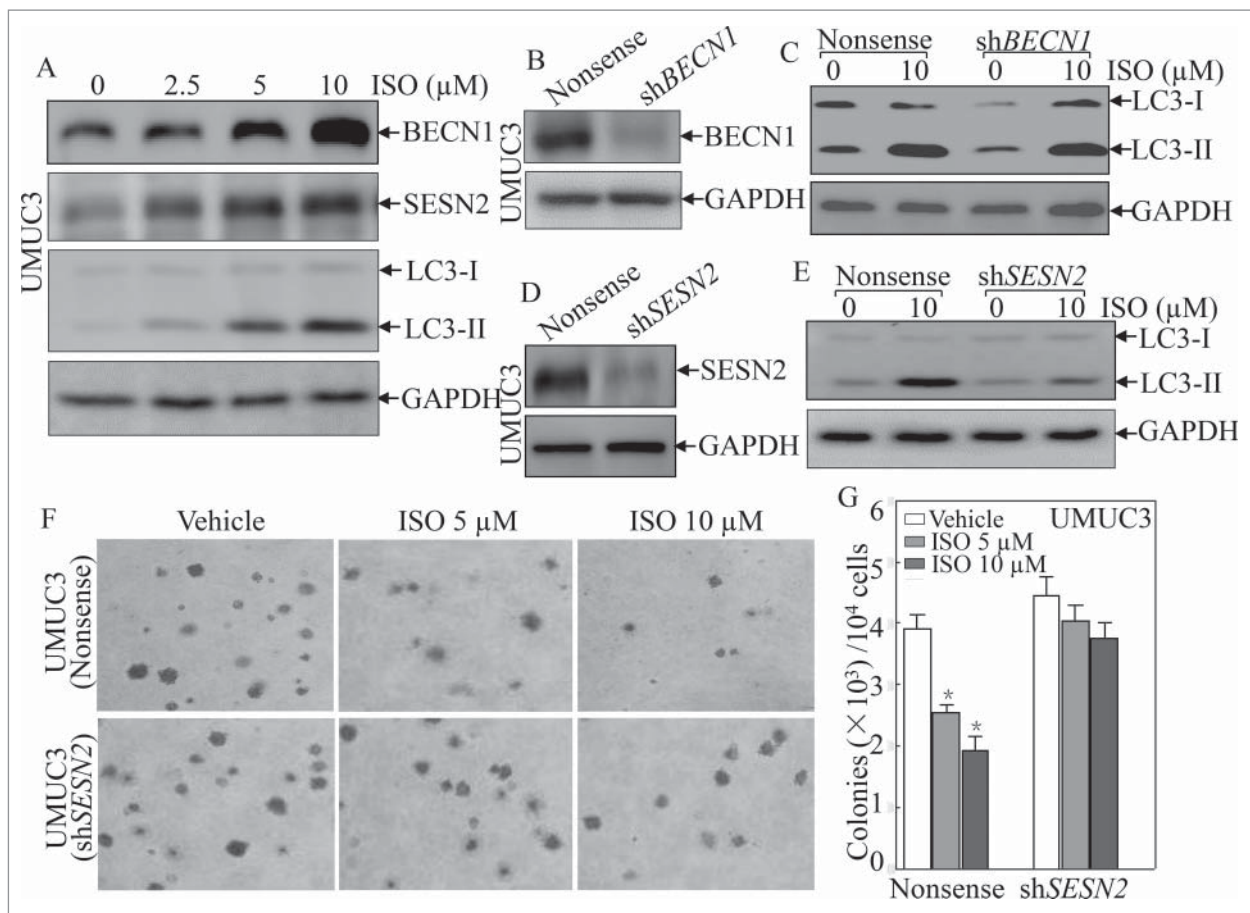


Figure 2. SESN2 was required for autophagic induction and anchorage-independent growth inhibition by ISO in human bladder cancer UMUC3 cells. (A) UMUC3 cells were treated with various concentrations of ISO for 24 h. The cells were extracted and cell lysates were subjected to western blotting to detect the expression of BECN1, SESN2 and LC3. (B and D) shRNA *BECN1* (B) and shRNA *SESN2* (D) were stably transfected into UMUC3 cells, and the stable transfectants were identified. (C and E) The indicated stable transfectants were subjected to ISO treatment for 24 h for determination of LC3 in ISO-induced autophagy in UMUC3 cells. (F) Representative images of colonies of UMUC3 (shSESN2) and UMUC3 (Nonsense) cells in soft-agar assay in the presence or absence of various concentrations of ISO were captured using a microscope. (G) Colonies shown in (F) were counted under a microscope with more than 32 cells of each colony. The results are presented as colonies per 10^4 cells, and the bars shows mean \pm SD from 3 independent experiments. The symbol (*) indicates a significant decrease from vehicle control ($p < 0.05$).

immunoprecipitation (ChIP) assay using anti-JUN antibody, and found that JUN directly bound to the putative AP-1 binding site in the *SESN2* promoter even in the absence of ISO treatment (Fig. 3I). More importantly, ISO treatment further resulted in a remarkable increase in the binding of JUN to the AP-1 site in the *SESN2* promoter (Fig. 3I). Taken together, our results reveal that ISO treatment increases SESN2 expression via a JUN-AP-1-dependent mechanism, in which JUN is activated upon ISO exposure and further upregulates *SESN2* transcription by directly binding to the putative AP-1 site in the *SESN2* promoter.

JUN activation was crucial for ISO-mediated autophagy induction and anchorage-independent growth inhibition

To evaluate the role for JUN in ISO inhibition of anchorage-independent growth of human bladder cancer cells, we established stable transfectants by using the dominant mutant of *JUN* (*TAM67*) or shRNA specifically targeting human *JUN*. As shown in Fig. 4A, ectopic expression of *TAM67* significantly abolished the induction of SESN2 protein expression upon ISO treatment. As expected, blockade of endogenous *JUN* activation by *TAM67* also dramatically inhibited the ISO-mediated

conversion of LC3-I to LC3-II (Fig. 4A), suggesting that *TAM67* blocked the autophagy induction by ISO treatment. Additionally, ISO treatment at 5 and 10 μ M led to an obvious reduction in colony formation of vector control cells in soft agar, while ISO treatment at the same doses failed to inhibit anchorage-independent growth in UMUC3 cells stably transfected with *TAM67* (Fig. 4B and C). This notion was greatly supported by the results obtained from utilization of stable transfectants of shRNA specifically targeting human *JUN* (Fig. 4D–F). Collectively, JUN expression and activation are essential for ISO-mediated SESN2 protein expression, autophagy induction and cancer cell anchorage-independent growth inhibition.

MAPK8 is the upstream kinase responsible for JUN-dependent SESN2 induction and SESN2 was downregulated in human bladder cancer tissues

MAPKs²² are rapidly activated in response to various extracellular stimuli including growth factors, cytokines and cellular stresses.²³ Therefore we determined whether a MAPK was the upstream kinase responsible for JUN-dependent SESN2 induction by ISO treatment. As shown in Fig. 5A, activation of

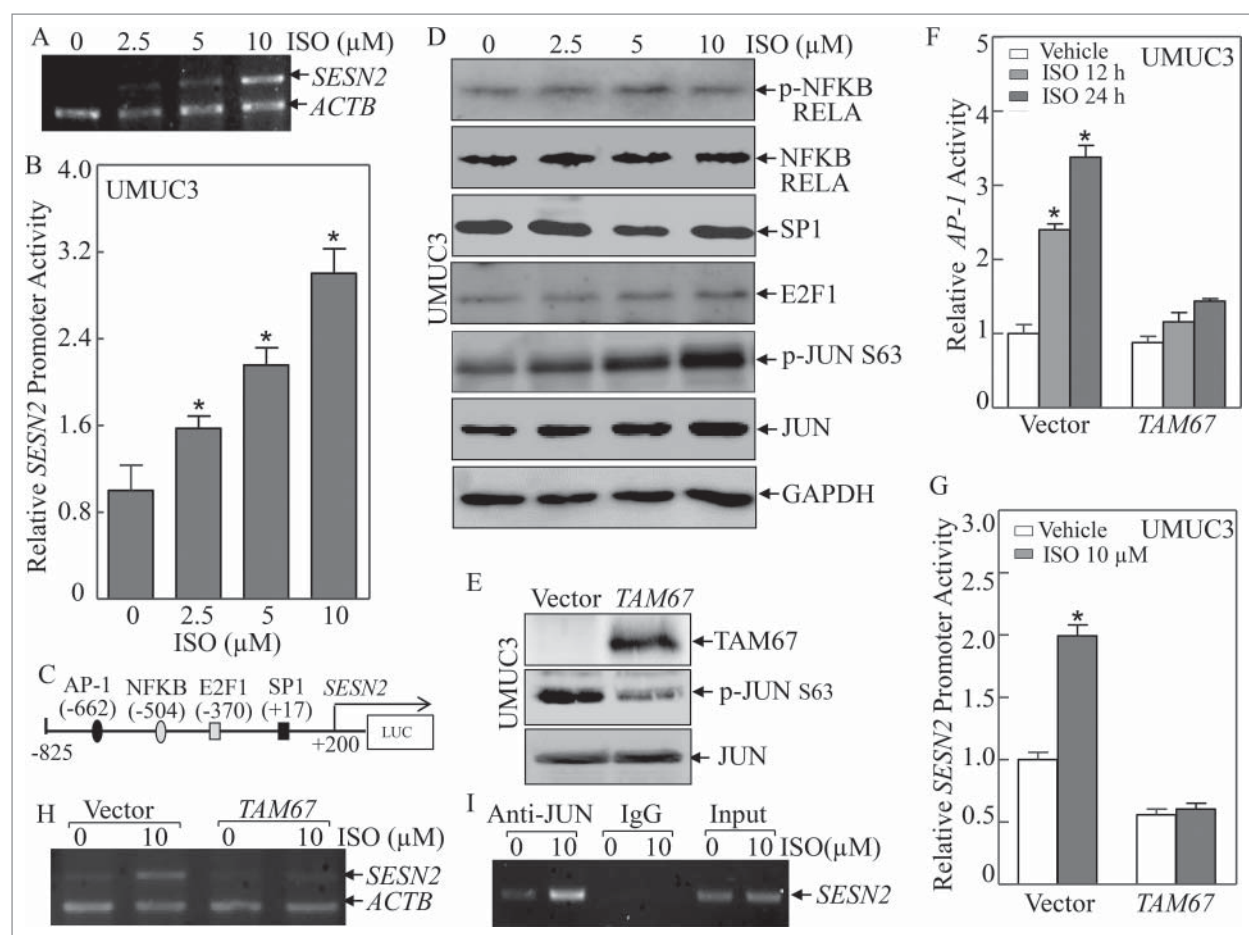


Figure 3. JUN activation and its binding to the AP-1 binding consensus in *SESN2* promoter-mediated *SESN2* transcriptional expression in human bladder cancer cells. (A) UMUC3 cells were treated with various concentrations of ISO for 12 h. RT-PCR was carried out to evaluate the expression of *SESN2* mRNA. (B) The *SESN2* promoter-driven luciferase reporter was transfected into UMUC3 cells. The stable transfectants were subjected to ISO treatment for 12 h to evaluate *SESN2* promoter transcriptional activity. The induction fold was normalized using *pRL-TK* as an internal control. The results are presented as *SESN2* promoter activity relative to vehicle control (relative *SESN2* promoter activity). The bars show mean \pm SD from 3 independent experiments. The symbol (*) indicates a significant increase from the vehicle control ($p < 0.05$). (C) The putative transcription factor consensus binding site in the *SESN2* proximal promoter region was predicted using bioinformatics analysis. (D) The expression levels of potential transcription factors were determined following ISO treatment for 24 h using western blotting. (E) *TAM67* was stably transfected into UMUC3 cells and the stable transfectants were identified by western blotting. (F and G) *AP-1* luciferase reporter (F) or *SESN2* promoter luciferase reporter (G) was transiently transfected into UMUC3 (*TAM67*) and UMUC3 (Vector) cells in combination with *pRL-TK*. The transfectants were treated with 10 μM ISO for 12 or 24 h and luciferase activity was determined and presented as relative *AP-1* activity (F) or relative *SESN2* promoter activity (G). The symbol (*) indicates a significant increase from the vehicle control ($p < 0.05$). (H) RT-PCR was carried out to determine the mRNA changes of *SESN2* following ISO treatment for 12 h in the indicated cells. (I) ChIP was carried out using anti-JUN antibody to detect the interaction of JUN with the *SESN2* promoter following ISO treatment for 12 h.

MAPK8 was observed in accordance with JUN activation as well as LC3 conversion. When shRNA to *MAPK8* was introduced to knock down endogenous *MAPK8* expression in UMUC3 cells, it not only blocked JUN activation, but also attenuated *SESN2* induction and LC3-II formation following ISO treatment (Fig. 5B). Similar results were also obtained from human cervical cancer HeLa cells (Fig. 5C). These results suggest that *MAPK8* is crucial for ISO-induced JUN activation and *SESN2*-dependent autophagy. In addition, we also found that knockdown of endogenous *MAPK8* abolished the transcriptional induction of *SESN2* mRNA following ISO treatment (Fig. 5D and E), again supporting the crucial role for *MAPK8* in ISO-mediated transcriptional induction of *SESN2*. More importantly, 5 and 10 μM of ISO treatment led to a significant inhibition of anchorage-independent cell growth in nonsense control UMUC3 cells, but lost this inhibition in *MAPK8* knockdown transfectants (Fig. 6A and B), emphasizing the indispensable role for the *MAPK8*-JUN axis in ISO inhibition of anchorage-independent cell growth of human bladder cancer cells.

We had defined the crucial role for the *MAPK8*-JUN axis in ISO-mediated transcriptional induction of *SESN2* in vitro as mentioned above. However, the expression pattern of *SESN2* in human bladder cancer tissues remained unknown. Therefore, we compared the protein levels of *SESN2* in 10 pairs of human bladder cancer tissues and matched adjacent normal bladder tissues. As shown in Fig. 6C, *SESN2* expression was greatly downregulated or even lost in human bladder cancer tissues as compared to the corresponding adjacent normal bladder tissues. Our results reveal that *SESN2* downregulation is associated with bladder cancer formation, and further suggest that elevation of *SESN2* expression by ISO might be related to its anticancer effects.

Discussion

ISO is a new derivative of stilbene isolated from the Chinese herb *Gnetum cleistostachyum*.⁶ Our recent studies have

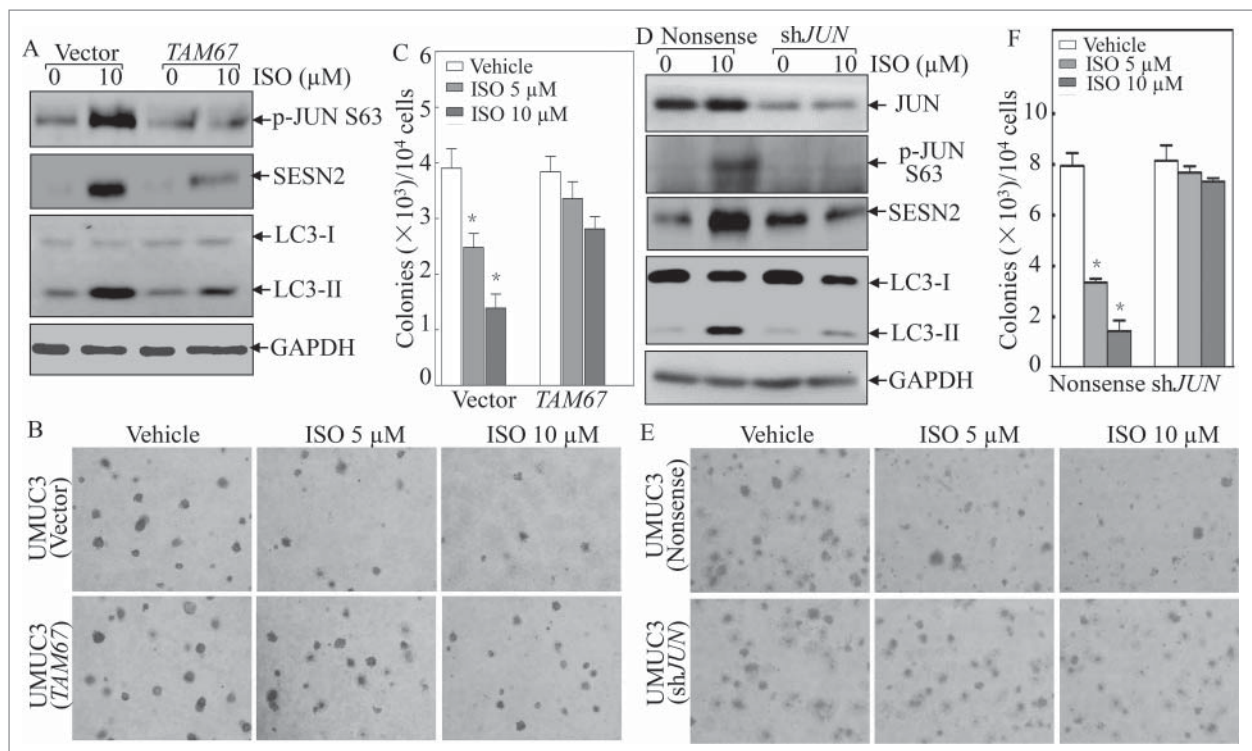


Figure 4. JUN activation was crucial for autophagy induction and anchorage-independent growth inhibition by ISO treatment. (A and D) The stable transfectants, UMUC3 (*TAM67*) vs. UMUC3 (Vector) or UMUC3 (*shJUN*) vs. UMUC3 (Nonsense) cells, were treated with 10 μ M ISO for 24 h. The cells were then extracted and the cell lysates were subjected to western blotting for determination of JUN phosphorylation, SESN2 induction and LC3-II generation as indicated. (B and E) Representative images of anchorage-independent growth of UMUC3 (*TAM67*) vs. UMUC3 (Vector) or UMUC3 (*shJUN*) vs. UMUC3 (Nonsense) in the absence or presence of various concentrations of ISO were visualized and captured using a microscope. (C and F) The colony formation was counted under a microscope with more than 32 cells of each colony, and the results presented as colonies/ 10^4 cells. The bars indicate mean \pm SD from 3 independent experiments. The symbol (*) shows a significant decrease from the vehicle control ($p < 0.05$).

revealed that ISO triggers apoptosis in multiple human cancer cell lines.^{5,7} In the current studies, we found that at sublethal doses ISO treatment induced autophagy via upregulation of SESN2 in a MAPK8-JUN-dependent manner, which further contributed to ISO inhibition of anchorage-independent growth of cancer cells. An interesting observation made from this study is that SESN2 was downregulated in human bladder cancer tissues as compared to the paired adjacent normal human bladder tissues, while ISO treatment induced upregulation of SESN2 expression in bladder epithelial cells. Collectively, our results not only identify the participation of autophagy in the anticancer activity of ISO, but also further provide a novel molecular insight into understanding autophagy induction upon ISO treatment as diagrammed in Fig. 6D.

Autophagy is a critical process that senses intracellular homeostasis changes and helps cells to survive during nutrient depletion, energy insufficiency or other cellular stress conditions.²⁴ Under stress circumstances, autophagy can also target the degradation of larger protein complexes and organelles, such as ribosomes and mitochondria, leading to the inhibition of cell growth.^{25,26} Conventional cytotoxic chemotherapeutic drugs and irradiation induce autophagy, and depending on the extent of induction, duration, and cellular context, autophagy further causes cell growth arrest, senescence and apoptosis.^{27,28} In line with this, our recent studies showed that treatment with relatively higher doses of ISO (20–60 μ M) trigger obvious cell death in T24T cells,⁷ whereas lower doses of ISO treatment (5–20 μ M) induce cell cycle arrest in UMUC3 cells.⁵

In the current study, we further found that ISO treatment activated the autophagy process. Using loss-of-function analyses, we found that SESN2 played an essential role in ISO-induced autophagy. In addition, ISO treatment increased MAPK8-JUN phosphorylation under the same experimental conditions, and introduction of shRNA to *MAPK8*, shRNA to *JUN* or *TAM67*, attenuated SESN2 expression as well as autophagy induction upon ISO treatment. The results obtained from using specific shRNA targeting *MAPK8* and *JUN* or *TAM67* indicate that these render UMUC3 cells resistant to ISO inhibition of anchorage-independent growth. All results demonstrate that autophagy induction plays an important role in mediating inhibitory effect of ISO on anchorage-independent growth of human bladder cancer cells.

SESN2 is reported to be associated with oxidative stress-induced autophagy.²⁹ TP53/p53 is defined as a major transcriptional regulator of SESN2 under DNA damage and oxidative stress.³⁰ In our current studies, we found that ISO treatment increased SESN2 expression at the transcriptional level demonstrated by using RT-PCR and a SESN2 promoter-driven luciferase reporter. Since UMUC3 cells carry a TP53 F113C missense mutation, which inactivates TP53-dependent transcriptional regulation,³¹ we focused on the TP53-independent transcriptional regulation of SESN2 induction. In our case, AP-1 was identified as the key transcription factor that was activated by ISO. Knockdown of JUN or overexpression of TAM67 impaired SESN2 induction, and the physical binding of JUN to the promoter region of SESN2 was also proven by CHIP assay.

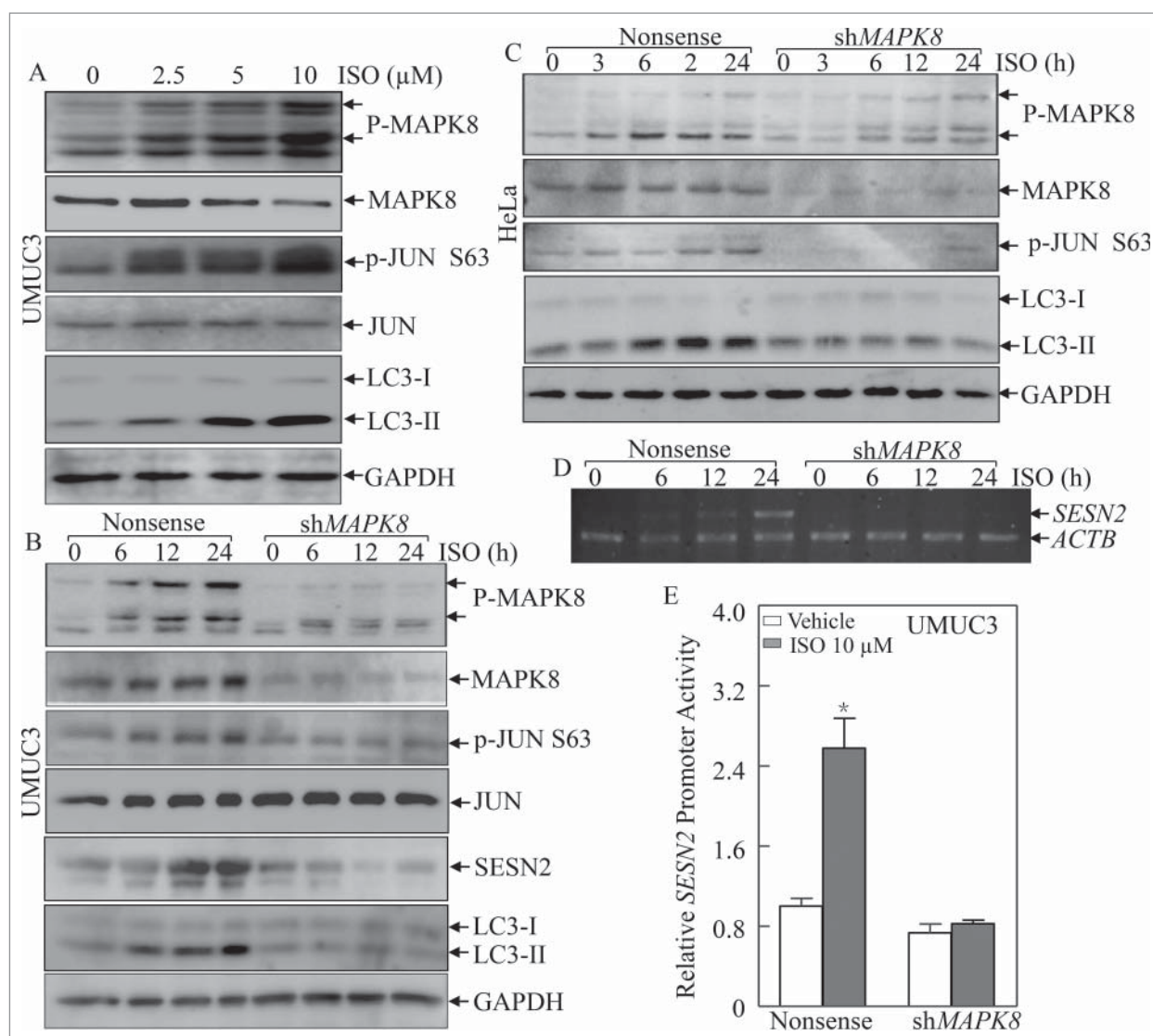


Figure 5. MAPK8 is the upstream kinase mediating for JUN activation and SESN2 expression following ISO treatment. (A) UMUC3 cells were treated with various concentrations of ISO for 24 h. The cells were extracted and cell lysates were subjected to western blotting to determine the activation of JUN and MAPK8 as well as generation of LC3-II. (B and C) shRNA *MAPK8* was stably transfected into UMUC3 cells (B) and HeLa cells (C). Following treatment with ISO (10 μ M) for the indicated time points, proteins were extracted and cell extracts were then subjected to western blotting for evaluation of the changes of JUN, MAPK8, SESN2 and LC3. (D) UMUC3 (shMAPK8) and UMUC3 (Nonsense) transfectants were subjected to ISO treatment and *SESN2* mRNA level was detected by RT-PCR. (E) *SESN2* promoter-driven luciferase reporter together with *pRL-TK* was transiently transfected into UMUC3 (shMAPK8) and UMUC3 (Nonsense) cells. The transfectants were treated with ISO (10 μ M) for 12 h and then analyzed for luciferase activity assay. The bars show mean \pm SD from 3 independent experiments. The symbol (*) indicates a significant difference between the vehicle control and ISO treatment ($p < 0.05$).

Our results demonstrate that ISO treatment results in activation of JUN-AP-1, which in turn upregulates *SESN2* transcription and expression.

MAPK8 is known as a stress-activated protein kinase (SAPK) of the MAPK family, and is initially activated in response to a variety of stress signals and has been implicated in many cellular events including apoptosis and autophagy.^{32,33} It has been reported that the activation of the MAPK8-JUN pathway involves the upregulation of *SESN2* induced by oxidized LDL.¹⁵ Our previous findings revealed that ISO treatment activates the MAPK8-JUN pathway, which plays an important role in suppression of transformation of normal cells to cancerous cells promoted by growth factors.⁶ Our findings here indicate that the MAPK8-JUN pathway contributes to autophagy induction by ISO in UMUC3 cells, suggesting that the

MAPK8-JUN pathway is a new key pathway for *SESN2* induction that can lead to autophagy induction and repression of transformation under stress conditions.⁶

In summary, our current studies reveal that ISO treatment at sublethal dose triggers autophagy of human bladder cancer cells in a MAPK8-JUN-*SESN2*-dependent manner, which contributes to abrogation of cancer cell anchorage-independent growth. The results generated from the current studies, taken together with our most recent data demonstrating ISO inhibition of human bladder tumor growth in xenograft nude mice,⁸ not only improve our understanding of ISO induction of autophagy and the underlying mechanisms of this important biological effect, but also provide us with valuable information for our future investigation of the potential application of ISO in prevention and/or therapy of human bladder cancer.

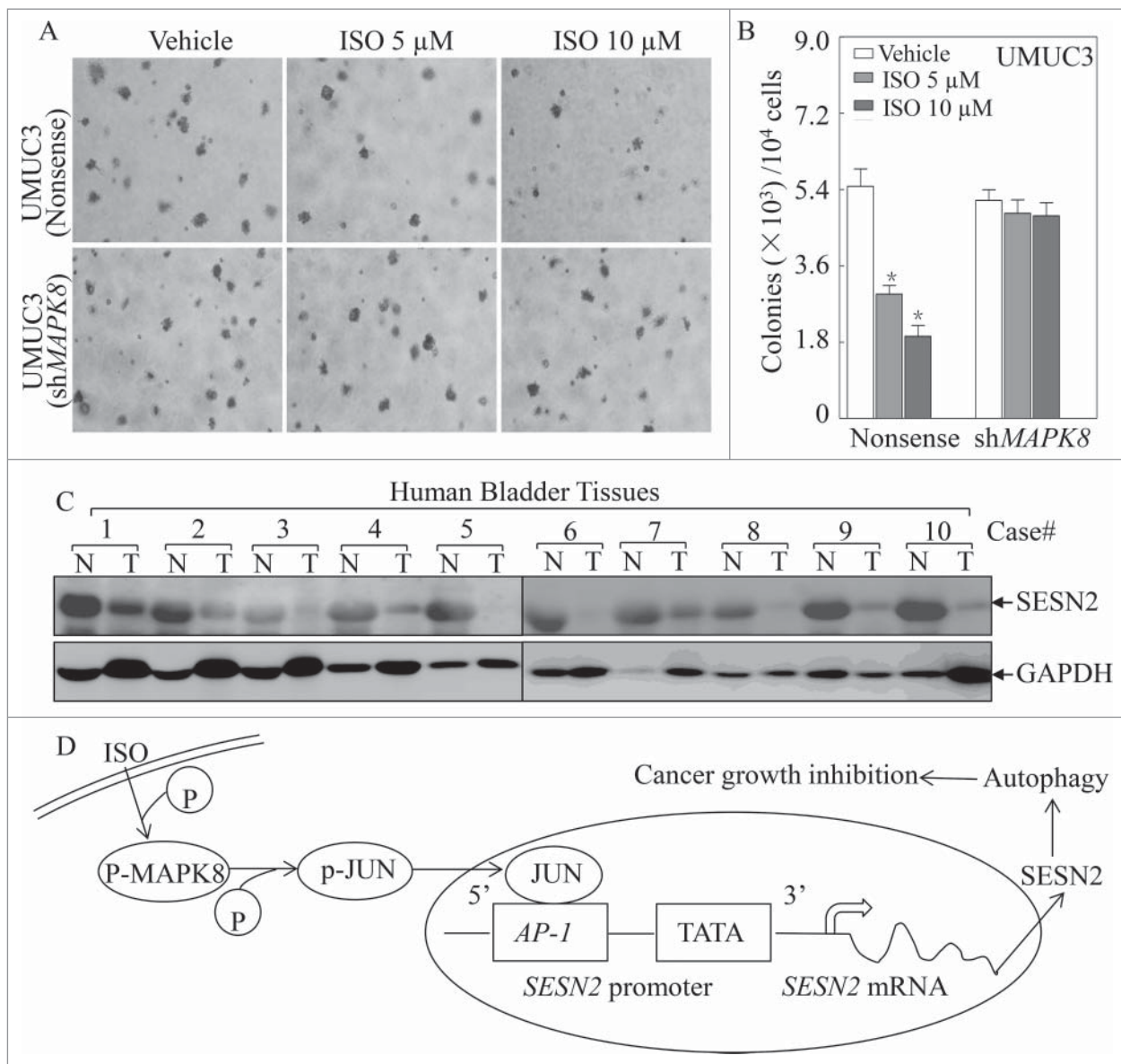


Figure 6. MAPK8 is required for ISO inhibition of anchorage-independent growth in UMUC3 cells and SESN2 was downregulated in human bladder cancer tissues. (A) Representative images of colonies of UMUC3 (shMAPK8) and UMUC3 (Nonsense) cells in a soft-agar assay in the absence or presence of various concentrations of ISO were captured using a microscope. (B) The colony formation was counted under a microscope with more than 32 cells of each colony, and the results presented as colonies/ 10^4 cells. The bars indicate mean \pm SD from 3 independent experiments. The symbol (*) shows a significant decrease from the vehicle control ($p < 0.05$). (C) Clinically freshly collected human bladder cancer samples in comparison with the paired adjacent normal bladder tissue were subjected to western blotting to analyze SESN2 expression. N, normal; T, tumor. (D) The proposed model for ISO induction of SESN2 and autophagy and inhibitory effect on human bladder cancer cells.

Materials and methods

Cell culture and reagents

UMUC3 and HeLa cells as well as their stable transfectants were maintained at 37°C in a 5% CO₂ incubator with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Corporation, D6429) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, S12450), 2 mmol L-glutamine (GIBCO, 25030-081), and 1% penicillin/streptomycin (Life Technologies, 15140163). T24T cells were cultured in DMEM: F12 (1:1; Corning Inc., 10-090-CV) with 5% FBS. The cultures were dissociated with trypsin (GIBCO, 15400-054) and transferred to new 75-cm² culture flasks twice a wk. ISO (Higher Biotech, 10200109) with over 98% purity was dissolved in dimethyl sulfoxide (Sigma-Aldrich Corporation, 67-68-5) to

make a stock concentration at 20 mmol and the same concentration (0.1%, v/v) of dimethyl sulfoxide was used as a negative control in all experiments. BAF (sc-201550) was from Santa Cruz Biotechnology.

Constructs and transfection

GFP-LC3 and its vector control were a kind gift from Dr. Gang Chen (University of Kentucky, Lexington, KY). ShRNA constructs against human *BECN1* (V3LHS-349509), *SESN2* (V2LHS-117405), *JUN/C-JUN* (V2LHS-262965) and *MAPK8/JNK1* (V2LHS-170499) were purchased from Open Biosystems. An expression construct for the *JUN* dominant negative mutant (pcDNA3.1/TAM67) was kindly provided by Dr. Tim G. Bowden (College of Pharmacy, University of Arizona, Tucson, AZ)

and Dr. Matthew Young (The Center for Cancer Research, National Cancer Institute, Frederick, MD). The *AP-1-Luc* plasmid (219074) was purchased from Stratagene. The cells were stably transfected by using PolyJet™ DNA In Vitro Transfection Reagent (SigmaGen Laboratories, SL100468), according to the manufacturer's instruction, as described in our previous studies.^{34,35} The transfections were subjected to selection with either puromycin (Santa Cruz Biotechnology, Sc-108071B) or hygromycin B (Gold Biotechnology, H-270-1) and all surviving transfectants from the selection were pooled as stable mass cultures. These stable transfectants were cultured in selective antibiotic-free medium for at least 2 passages before utilization for experiments.

Fluorescence microscopy

UMUC3 cell transfectants were cultured on cover slides in 10% FBS DMEM medium for 24 h. The cells were exposed to ISO at the indicated dose and time and fixed with 4% paraformaldehyde (Sigma-Aldrich Corporation, 158127) in PBS (135 mM NaCl, 4.7 mM KCl, 10 mM Na₂HPO₄, 2.0 mM NaH₂PO₄, pH 7.4) at room temperature for 10 min, then washed 3 times with PBS (Corning Inc., 55-031-PC). Next, the membrane was permeabilized with PBS containing 0.1% Triton X-100 (Thermo Fisher Scientific Inc., 28314) for 10 min, and then stained with 0.1 mg/ml DAPI (Sigma-Aldrich Corporation, 9542) for 30 min. The slides were washed 3 times with PBS and mounted with antifade reagent (Molecular Probes, P36930). All cell images were captured using an inverted Leica fluorescence microscope (Wetzlar, Germany). Cells with 5 or more intense GFP-LC3 puncta were considered autophagic cells, whereas those with diffuse cytoplasmic GFP-LC3 staining were considered nonautophagic cells. The percentage of GFP-LC3-positive cells were calculated based on at least 200 counted cells. The number of GFP-LC3 puncta per cell was counted for at least 50 cells.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using Trizol reagent (Invitrogen Corporation, 15596-018). Total cDNAs were synthesized by ThermoScript™ RT-PCR system (Invitrogen Corporation, 11146-057). The mRNA amount present in the cells was measured by semiquantitative RT-PCR. The primers for human *SES2* were 5'-AGA GGG CAC AGG AAA GAA-3' and 5'-TCA AGC ATA AAG GAC CAA A-3'; human *ACTB/β-ACTIN* were 5'-CTC GCC TTT GCC GAT CCG CCG-3' and 5'-AGT GGT ACG GCC AGA GGC GTA C-3'. The PCR products were separated on 2% agarose gels, stained with ethidium bromide (Fisher Scientific Corporation, 1239-45-8), and scanned for images under UV light. The results were displayed with an Alpha Innotech SP image system (Alpha Innotech Corporation, San Leandro, CA, USA).

Construction of *SES2* promoter-driven luciferase reporter

A fragment spanning from -825 to +200 relative to the transcription start site of the human *SES2* genomic sequence was produced by PCR with the forward primer 5'-TCA GGT ACC

GAA TTT AGC TTG GAG GTG C-3' and the reverse primer 5'-CCG CTC GAG GCT TTG GTG CTG GAC TCT T-3'. The PCR products were subcloned into the *KpnI* and *XhoI* sites of pGL3-Basic vector (Promega Corporation, E1751) to generate the *SES2* promoter-driven luciferase reporter (*SES2-Luc*). The construct was confirmed by DNA sequencing.

Luciferase reporter assay

AP-1 luciferase reporter (*AP-1-Luc*) was purchased for determining AP-1-dependent transcriptional activity. In this plasmid, the expression of the firefly luciferase gene is controlled by a synthetic promoter that contains direct repeats of the transcription recognition sequences for AP-1. The *SES2-Luc* construct was generated as described above for determining relative *SES2* promoter activity. UMUC3 cells were transfected with an *AP-1-Luc* plasmid or *SES2-Luc* constructs in combination with the *pRL-TK* vector (Promega Corporation, E2241) as an internal control. The dual luciferase assay kit (E1960) was purchased from Promega Corporation. The luciferase activities were determined using a luminometer (Wallac 1420 Victor 2 multilabel counter system) as described in previous studies.^{36,37}

Anchorage-independent growth assay

The soft-agar assay was performed as described previously.^{6,38} Briefly, 2.5 ml of 0.5% agar in basal modified Eagle's medium supplemented with 10% FBS with or without ISO was layered onto each well of 6-well tissue culture plates. 1 × 10⁴ UMUC3 cells or their transfectants were mixed with 1 ml of 0.5% agar (Becton, Dickinson and Company, 214010) in basal modified Eagle's medium supplemented with 10% FBS with or without ISO, and then layered on top of the 0.5% agar layer. The plates were incubated at 37°C in 5% CO₂ for 2 weeks. The colonies with more than 32 cells were scored and the results were presented as colonies/10⁴ cells.

ChIP assay

ChIP was performed using the ChIP kit (Millipore Technologies, 17-295) as described in our previous publication.⁷ Briefly, UMUC3 cells were treated with or without 10 μM ISO for 24 h. Then genomic DNA and the proteins were cross-linked with 1% formaldehyde (Protocol Corporation, 245-684). The cross-linked cells were pelleted, resuspended in lysis buffer, and sonicated to generate 200–500 base pair chromatin DNA fragments. After centrifugation, the supernatant fractions were diluted 10-fold and then incubated with anti-JUN/C-JUN antibody (Santa Cruz Biotechnology, sc-45X) or the control rabbit IgG (Santa Cruz Biotechnology, sc-2027) overnight at 4°C. The immune complex was captured by protein G-agarose (Santa Cruz Biotechnology, C1014) saturated with salmon sperm DNA (Upstate Biotechnology Inc., 0606031838), then eluted with the elution buffer. DNA-protein crosslinking was reversed by heating overnight at 65°C. DNA was purified and subjected to PCR analysis. To specifically amplify the region containing the putative responsive elements on the human *SES2* promoter, PCR was performed with the following pair of primers:

5'-AAA AGG GTC AGA TAA AAC AT-3' and 5'-TTA GTA AAT AGA GAC AGG GT-3'. The PCR products were separated on 2% agarose gels and stained with ethidium bromide; the images were then scanned with a UV light.³⁹

Western blotting assay

The cells were washed twice with ice-cold PBS and collected with cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS [Thermo Fisher Scientific Inc., BP16650], 1 mM Na₃VO₄). The cell extracts were sonicated, denatured by heating at 100°C for 5 min, and protein concentrations were determined by Nano Drop 2000 (Thermo Fisher Scientific Inc., Waltham, MA). Equal aliquots of cell extracts were separated on SDS-polyacrylamide gels. The proteins were then transferred to PVDF membranes, blocked, and probed with one of the antibodies against phospho-JUN S63 (2361S), total-JUN (9165S), phospho-MAPK8 T183/Y185 (4668S), total-MAPK8 (9252S), E2F1 (3742S), BECN1 (3495S), total NFKB RELA (4764S), phospho-NFKB RELA S536 (3033S), LC3 (2775S) or GAPDH (5174S) (Cell Signaling Technology); SESN2 (sc-292558) or SP-1 (sc-59) (Santa Cruz Biotechnology); or ACTB (Sigma-Aldrich Corporation, A5441). Immunoreactive bands were detected using the alkaline phosphatase-linked secondary antibody and ECF western blotting system (Amersham Biosciences, Piscataway, NJ). The images were acquired using a Typhoon FLA 7000 imager (GE Healthcare, Pittsburgh, PA). The results shown are representative of 3 independent experiments.

Patients and bladder cancer tissue specimens

Ten pairs of primary invasive bladder cancer specimens and their paired adjacent normal bladder tissues were obtained from patients who underwent radical cystectomy at the Department of Urology of the Union Hospital of Tongji Medical College (Wuhan, China) between 2012 and 2013. All specimens were immediately snap-frozen in liquid nitrogen after surgical removal. Histological and pathological 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, and the experiments were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Statistical analysis

The significance of the difference between the treated and untreated groups was determined with the Student *t* test. The results are expressed as mean ± SD.

Abbreviations

AMPK	adenosine 5'-monophosphate-activated protein kinase
BAF	bafilomycin A ₁

BECN1	Beclin 1, autophagy related
DAPI	4',6-diamidino-2-phenylindole
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
IgG	immunoglobulin G
ISO	isorhapontigenin
MAP1LC3/LC3	microtubule-associated protein 1 light chain 3
MAPK	mitogen-activated protein kinase
MTOR	mechanistic target of rapamycin (serine/threonine kinase)
PBS	phosphate-buffered saline
RT-PCR	reverse transcription-polymerase chain reaction
SAPK	stress-activated protein kinase
SESN2	sestrin 2
shRNA	short hairpin RNA
XIAP	X-linked inhibitor of apoptosis

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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