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## New Compound ChIA-F Induces Autophagy-dependent Anti-cancer Effect via Upregulating Sestrin-2 in Human Bladder Cancer

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### Abstract

ChIA-F is a novel conformation-derivative of Cheliensisin A, styryl-lactone isolates that show potent anti-tumor potential *in vivo* and *in vitro*. However, the anti-cancer activity and its potential mechanisms underlying ChIA-F action have never been explored. In the present study, we evaluated the potency of ChIA-F on autophagy-mediated anchorage-independent growth inhibition in human high-grade invasive bladder cancer (BC) cells. We found that ChIA-F treatment significantly inhibited anchorage-independent growth of human BC cells by inducing autophagy in a Sestrin-2 (SESN2)-dependent fashion. Our results revealed that ChIA-F treatment specifically induced SESN2 expression via increasing its transcription and mRNA stability. On one hand, ChIA-F treatment markedly attenuated Dicer protein abundance, in turn abolishing miR-27a maturation and further relieving miR-27a binding directly to SESN2 mRNA 3'UTR, thereby promoting SESN2 mRNA stabilization. On the other hand, ChIA-F treatment promoted Sp1 abundance and consequently mediated SESN2 transcription. These results demonstrate that its activation of the autophagic pathway through specifically promoting SESN2 expression mediates

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### 6. Conflicts of Interest Statement

Neither this paper nor any similar paper has been or will be submitted to or published in any other scientific journal. All authors are aware and agree to the content of the paper and to their being listed as an author on the manuscript. There is no conflict of interest or competing financial interests for all authors.

the anti-cancer effect of ChIA-F, which offers insights into the novel anti-cancer effect of ChIA-F on BC, as well as providing therapeutic alternatives against human BC.

## Keywords

Anchorage-independent growth; Cheliensisine A-fluoride (ChIA-F); Macro-autophagy (Autophagy); miR-27a, Sestrin-2

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## 1. Introduction

Bladder cancer (BC) is the most common malignant tumor in the urinary tract. It is the sixth most common cancer in the US with an estimated 74,000 new cases and 16,000 deaths, accounting for about 5% of all new cancers and about 3% of all deaths in the USA in the year 2015 [1]. Although various therapies have been developed to treat cancer patients, natural products have been a well-spring source of anticancer drugs and drug leads for decades [2–5]. Cheliensisin A (Chel A), a styryl-lactone isolated from *Goniothalamus cheliensis* Hu, has been shown to have anticancer activities both *in vitro* and *in vivo* [6]. However, its poor water solubility has precluded Chel A as a potential drug candidate [7, 8]. Given the pharmaceutical potential of Chel A and its unresolved water solubility problems in further drug development, a structure-activity study of Chel A has been carried out to search for druggable candidates for the development of a new ChleA derivative with strong anti-cancer potency. ChIA-F has been identified as being appropriate for this purpose [8]. Although ChIA-F possesses better water solubility and chemical stability, which makes it as a potential drug candidate [8], its anti-cancer activity and the molecular mechanisms underlying its biological effects have never been explored.

Autophagy (macro-autophagy) is an intercellular homeostatic process that is important for cellular digesting protein aggregates, nutrient deposits, dysfunctional organelles, eliminating toxic protein inclusion and regulating signal transduction machinery by selective degradation of damaged molecules [9]. Given that autophagy is a highly conserved self-degradative and catabolic process that targets cellular contents to the lysosomal compartment for degradation and possesses an essential function in cellular stress responses and survival [10], the emerging role of autophagy in cancer cells is one of a double-edged sword [11–16]. On one hand, autophagy enables tumor cells to tolerate stress including a hypoxic microenvironment, starvation, and probably some forms of therapy [17]. On the other hand, autophagy plays an important role in damage mitigation in response to stress that can limit tumorigenesis by either clearing away damaged proteins and organelles, or by maintaining energy homeostasis through intracellular recycling, which ultimately can prevent the genome damage that drives tumorigenesis [18–21]. Sestrin2/SESN2, also known as Hi95, SES2 or SEST2, includes a member of the sestrin family of PA26-related proteins and functions as a regulator of cell growth and survival in cell response to different stress conditions and senescence [22, 23]. Emerging evidence indicates that SESN2 is involved in autophagic responses because it can bind to p62 and promotes autophagic degradation of p62 [9, 24]. SESN2 has been reported to act as a novel p53-targeted gene that participates in autophagy induction [25]. Our most recent study reveals that SESN2 is remarkably down-

regulated in clinical human BC tissues in comparison to their paired adjacent normal bladder tissues and induction of SESN2 level in cancer cells mediate the anti-cancer activity of ISO in human BC cells, demonstrating that SESN2 acts as a tumor suppressor in human BC cells [26]. Our study also revealed that SESN2's upregulation *via* JNK/c-Jun-dependent transcription is crucial for autophagic induction and the anti-cancer effect of the anti-cancer compound isorhapontigenin (ISO) [26]. The interesting observation made by Maiuri *et al.* also indicates that SESN2 acts as a new positive regulator of autophagy in p53-proficient but not in p53-deficient cells [25]. In our present studies, by using loss-of-function studies, we demonstrated that ChIA-F treatment resulted in SESN2 induction through both activation of Sp1-dependent transcription and inhibition of miR-27a-mediated mRNA degradation, and that the SESN2 upregulation led to an autophagic induction and anti-cancer activity of ChIA-F in human BC cells.

## 2. Materials and Methods

### 2.1 Plasmids and Reagents.

GFP-LC3 and the c-Jun dominant negative mutant (PRC-CMV/TAM67) were described in our previous studies [26, 27]. shRNA constructs against human SESN2 (V2LHS-117405), Sp1 and ELAVL1 were purchased from Open Biosystems (Pittsburg, PA, USA). The miR-27a overexpression construct was obtained from Addgene (Cambridge, MA, USA). Plasmids were prepared by the Plasmid Preparation/Extraction Maxi kit from QIAGEN (Valencia, CA, USA). The antibodies specific against phospho-JUN Ser63 (2361S), total c-JUN (9165S), E2F1(3742S), BECN1 (3495S), total NFκB RELA (4764S), phospho-NFκB RELA S536 (3033S), LC3B(2775S), GAPDH(5174S), Atg5(12994S), Atg7(8558P), Dicer(5362P), were purchased from Cell Signaling Technology (Beverly, MA, USA); The antibodies specific for SESN2 (sc-292558), Sp1 (sc-H225) and ELAVL1(sc-5261), were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA), The antibody against HNRNPD/AUF1 (ARP40238\_T100) was bought from Aviva (San Diego, CA, USA). The antibodies specific against ACTB (A5441) and NCL (N2662) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Bafilomycin A1 (sc-201550) was purchased from Santa Cruz Biotechnology. Chloroquine (CQ) was acquired from Frontier Scientific (Logan, UT), while actinomycin D (Act D) (50–76-0) was bought from Fisher Scientific (Pittsburgh, PA, USA).

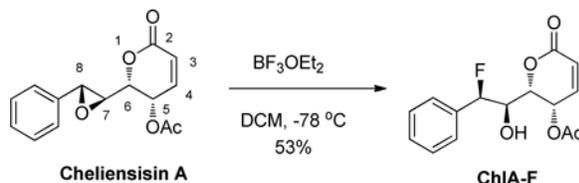
### 2.2 General information for chemical procedures.

ChIA-F with purity over 99% was prepared according to the developed protocol (see Patent ZL201310034985.5 and PCT/CN2014/071751). All the reactions were performed under argon atmosphere using flame-dried glassware unless otherwise noted. Dichloromethane (DCM) was treated as anhydrous solvents prior to use. All reagents were commercial available and used without further purification unless indicated otherwise. Flash Chromatographies were performed with 300–400 mesh silica gels. Thin layer chromatographies (TLC) were carried out on GF254 plates (0.25 mm layer thickness). Visualization of the developed chromatogram was performed by fluorescence quenching or by ceric ammonium molybdate. Yields reported were for isolated, spectroscopically pure compounds. High-resolution mass spectra (HRMS) were taken on a VG Auto Spec-3000 or

on a Finnigan MAT 90 instrument. Optical rotations were measured with a Horiba SEPA-300 polarimeter.  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  experiments were performed on a Bruker AM-300 NMR spectrometer at ambient temperature. Chemical shifts were given in  $\delta$  with TMS as internal reference.

**Preparation of cheliensisin A:** Cheliensisin A was isolated as white crystal with purity more than 99.0% from *Goniothalamus cheliensis* according to the previously reported procedure [28].

**Procedures for preparation of ChIA-F:** (7S,8R)-7-Hydroxyl-8-fluoro cheliensisin A



To a solution of cheliensisine A (548 mg, 2 mmol) in DCM (5 mL) at  $-78^\circ\text{C}$  under  $\text{N}_2$  was added a solution of  $\text{BF}_3\cdot\text{OEt}_2$  (202  $\mu\text{L}$ ) in DCM (3 mL) dropwise. The mixture was stirred at this temperature for 0.5 hour until no starting material was detected. The mixture was quenched by a saturated aqueous  $\text{NaHCO}_3$  solution. The aqueous phase was extracted with dichloromethane (30 mL $\times$ 3). The combined organic layers were washed with brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petrol ether = 1:1.5) to afford ChIA-F as a white foam (312 mg, 53%). High-resolution electrospray ionisation mass spectrometry (m/z): calculated for  $\text{C}_{15}\text{H}_{15}\text{FO}_5\text{Na}$   $[\text{M}+\text{Na}]^+$ , 317.0801, found 317.0795.

For performing the *in vitro* experiments, ChIA-F was dissolved in Dimethyl sulfoxide (DMSO, Sigma-Aldrich Corporation, USA, 67-68-5) to make a stock concentration at 8 mM. It was the further diluted in DMEM/Ham's F12 medium with final DMSO concentration at 0.1% (v/v) for cell culture experiments. The same amount of DMSO was used as a vehicle control in all experiments.

### 2.3 Cell culture and Transfections

The normal urinary epithelial cell line UROtsa and the different genetic backgrounds human BC cell lines RT4 (p53 wild-type), T24T (p53 mutant) and UMUC3 (p53 mutant) were described in our previous studies[26, 29, 30]. The T24T cell was cultured at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator in a 1:1 mixture of DMEM/Ham's F12 medium supplemented with 5% FBS, 2 mmol/L L-glutamine and a mixture solution of 100 IU penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin; and the UMUC3 cells were cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine and a mix solution of 100 IU penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. UROtsa cells were cultured with 1640 supplemented with 10% FBS and RT4 cells were cultured with McCoy's 5A supplemented with 10% FBS. The cell lines were regularly authenticated every 6-12 months to evaluate viability, recovery, growth, morphology and chemical response, as well as by testing STR loci and gender using the PowerPlex® 16 HS

System provided by Genetica DNA Laboratories (Burlington, NC, USA) as stated in our previous studies [26, 30]. The transfections were carried out with specific plasmid constructs using PolyJet™ DNA *in Vitro* Transfection Reagent (SignaGen Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. The stable transfection selection of SESN2, Sp1, c-Jun, and miR-27a in T24T cells was subjected to puromycin selection for 4 to 6 weeks. The surviving stable transfectants were pooled as stable mass culture as described in our previous studies [26, 30].

#### 2.4 Human bladder cancer tissues

Fourteen pairs of human BC samples and their matched adjacent normal bladder tissues were obtained from patients underwent radical cystectomy in the Department of Urology, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) from 2013 to 2015. All specimens were immediately snap-frozen in liquid nitrogen after surgical resection. Histological and pathological diagnoses were confirmed and the tissues were defined by a certified clinical pathologist according to the 2004 World Health Organization Consensus Classification and Staging System for bladder neoplasms (Table S1). All specimens were obtained with appropriate informed consent from the patients and a supportive grant obtained from the Medical Ethics Committee of China.

#### 2.5 Western Blot.

Cells were extracted with cell lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 1% SDS, and 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>), and protein concentrations were determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The cell extracts were subjected to SDS-PAGE, then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The protein band specifically bound to the primary antibody was detected by Typhoon FLA 7000 (GE Healthcare, Chicago, IL, USA) using an alkaline phosphatase-linked secondary antibody and an enhanced chemifluorescence Western Blot system as described in our previous studies[30].

#### 2.6 RT-PCR and Quantitative Real-Time PCR.

Total RNAs were extracted with TRIzol reagent (Invitrogen, USA) following ChIA-F treatment, according to the manufacturer's instructions, and the cDNAs were synthesized with the Thermo-Script RT-PCR system (Invitrogen). The mRNA amount present in the cells was measured by semi-quantitative RT-PCR. The primers for human *sestrin2* were 5'-CAA CTC TGG GGG CTT TGA GT-3' (Forward) and 5'-ACC TTC TCT GAG TGG CGG AA-3' (Reverse). The primers for human *gapdh* were 5'-AGA AGG CTG GGG CTC ATT TG-3' (Forward) and 5'-AGG GGC CAT CCA CAG TCT TC-3' (Reverse). The PCR products were separated on 2% agarose gels, stained with Ethidium Bromide, and scanned for the images under UV light with Alpha Innotech SP Image system (Alpha Innotech Corporation, San Leandron, CA, USA).

Total microRNAs were extracted using the miRNeasy Mini Kit (QIAGEN, USA), reverse transcription was then performed using the miScript II RT Kit (QIAGEN), and quantitative PCR was performed using miScript PCR Starter Kit (QIAGEN) according to the manufacturer's protocol. U6 was used as the endogenous normalizer. Cycle threshold (CT)

values were determined, and the relative expression of microRNAs was calculated by using the values of  $2^{-\Delta\Delta CT}$ , as described in our publications [30].

## 2.7 Luciferase Reporter Assay.

The cells were transfected with the indicated luciferase reporter in combination with the *pRL-TK* vector (Promega, Fitchburg, WI, USA) as an internal control, and the luciferase activities were determined using a microplate luminometer, as described in previous studies[30].

## 2.8 Anchorage-independent growth assay.

Anchorage-independent growth in soft agar (soft-agar assay) was performed, as described in our earlier studies [26, 30]. Briefly, the  $1 \times 10^4$  cells mixed with Ch1A-F at a final concentration of  $8 \mu\text{mol/L}$  or vehicle control in 2% FBS Basal Medium Eagle (BME) containing 0.33% agar were seeded over the basal layer containing 0.5% agar and 2% FBS/BME in each well of 6-well plates. The plates were incubated in 5% CO<sub>2</sub> in an incubator at 37°C for 3 weeks. Colonies were captured under a microscope, and only colonies with over 32 cells were counted. The results are presented as Mean  $\pm$  SD obtained from three independent experiments.

## 2.9 Confocal laser scanning microscopy.

T24T GFP-LC3 cells were seeded on chambers ( $1 \times 10^4/\text{well}$ ) in a 1:1 mixture of DMEM/Ham's F12 medium supplemented with 5% FBS and cultured at 37°C with 5% CO<sub>2</sub> incubator. When the cell density reaches to the optimal 70~80% confluency, the cells were treated with  $8 \mu\text{M}$  Ch1A-F for the time points as indicated. The cells were washed with pre-warmed 1xPBS once, then subjected to 4% paraformaldehyde for 30min at room temperature. Then the cells were washed with pre-warmed 1xPBS twice and the chamber membrane was permeabilized with 1xPBS containing 0.2% Triton X-100 for 15 min at room temperature, then stained with 0.1 mg/ml DAPI (Sigma-Aldrich Corporation, #9542) for 30 min. The slides were washed 3 times with 1x PBS and mounted with antifade reagent (Molecular Probes, P36930, Eugene, OR, USA). All cellular 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 non-autophagic cells. The percentages of the GFP-LC3-positive cell were calculated based on at least 200 counted cells. The number of GFP-LC3 puncta per cell was counted for at least 50 cells.

## 2.10 Live-cell imaging.

Live-cell imaging was performed using the Lysosome Staining Kit (AAT Bioquest) according to the manufacturer's instructions. Briefly, T24T(RFP-GFP-LC3B) cells were seeded into a 35mm glass bottom culture dish (MatTek Corporation) and cultured in the incubator with 5% CO<sub>2</sub> at 37°C. When the cell density reaches the 70% confluence, the cells were washed once with pre-warmed 1x PBS and then incubated in 1.5 ml of lysosome blue dye-working solution (dilute 3 $\mu\text{l}$  LysoBrite™ Blue into 1.5 ml of Live Cell Staining Buffer according to the manufacture's protocol) containing 2% FBS in an incubator with 5% CO<sub>2</sub>

at 37°C for 2 hours. The culture dishes were washed twice with pre-warmed 1x HBSS (Gibco) and were then filled with 2 ml CO<sub>2</sub>-Independent medium containing 5% FBS and 8 μM of ChIA-F. Cells were imaged overnight every 5 min using the inverted Leica fluorescence microscope (Wetzlar, Germany) at three channels with laser 405, 488 and 594.

### 2.11 Chromatin Immunoprecipitation (ChIP) Assay.

ChIP was performed using the EZ-CHIP kit (Millipore Technologies) according to the manufacturer's instructions and as described in our previous publication [31]. Briefly, T24T cells genomic DNA and the proteins were cross-linked with 1% formaldehyde. The cross-linked cells were pelleted, resuspended in cell lysis buffer, and sonicated to generate 200–500bp chromatin DNA fragments. After centrifugation, the supernatants were diluted 10-fold, then incubated overnight with either anti-Sp1 antibody or the control rabbit IgG at 4°C. The immune complex was captured by protein G-agarose saturated with salmon sperm DNA, then eluted with the elution buffer. Cross-linked DNA-protein was reversed by heating overnight at 65°C. DNA was purified by PCR. To specifically amplify the region containing the putative responsive elements on the human *SESN2* promoter, PCR was performed with the following two pairs of primers: 5'-CATTACCCGAGGCGGACTA-3' (Forward), 5'-CGGTCCAGCCAATCAGAGGT-3' (Reverse) and 5'-ACAGACCTCTGATTGGCT-3' (Forward), 5'-CTCTGACACCAGCAGTT-3' (Reverse). The PCR products were separated on 2% agarose gels and stained with ethidium bromide, the images were scanned under a UV light with Alpha Innotech SP Image system (Alpha Innotech Corporation, San Leandro, CA, USA).

### 2.12 Statistical analysis

The student's T-test was used to determine significant differences and  $p < 0.05$  was considered as a significant difference between the compared groups.

## 3. Results

### 3.1 ChIA-F induced autophagy concurrent with the inhibition of Anchorage-independent Growth in human BC cells.

The chemical structure of ChIA-F shown in Fig. 1A is a natural product-based druggable compound as a new ChelA derivative [8]. Through structural modifications, ChIA-F has been found to have improved chemical stability and water solubility (Patents ZL201310034985.5 and PCT/CN2014/071751). To evaluate the potential involvement of autophagic responses in ChIA-F-treated human bladder cancer cells, we first evaluated the effects of ChIA-F on the conversion of LC3B I to LC3B II following ChIA-F treatment of normal urothelial cell line UROtsa in comparison to human BC cell lines, including RT4, T24T and UMUC3 [32]. The results indicated that ChIA-F treatment led to a substantial conversion of LC3B I to LC3B II in a time- and dose-dependent manners in human BC cells, whereas same treatment only show a slightly effect on normal UROtsa cells (Figs. 1B & 1C). Further, a well-established tandem GFP-LC3 fusion expression construct was stably transfected into T24T cells to monitor autophagosome formation using confocal laser scanning microscopy. As shown in Figs. 1D-1F, ChIA-F treatment induced formation of autophagosomes in a time-dependent manner. The increase of autophagic flux caused by

ChIA-F was further confirmed by the co-treatment of cells with Bafilomycin A1 (BAF) or Chloroquine (CQ). BAF is an inhibitor that prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes, the late phase of autophagy [33]. CQ inhibits the fusion between autophagosomes and lysosomes, thus preventing the maturation of autophagosomes into autolysosomes, and blocking a late step of macroautophagy [33]. The results showed that co-treatment of cells with BAF or CQ and ChIA-F resulted in a significant increase in the conversion of LC3B I to LC3B II (Fig. 1G) and GFP-LC3 puncta formation (Figs. 1H & 1I), providing evidence that ChIA-F treatment increases the autophagy flux in human BC cells. Consistently, ChIA-F treatment resulted in a significant attenuation of anchorage-independent growth of T24T and RT4 cells, whereas this inhibition by ChIA-F was significantly reversed by co-treatment of cells with BAF (Figs. 1J -1L). Moreover, we performed live-cell imaging to monitor autophagic flux based on the use of a tandem monomeric GFP-RFP-tagged LC3B. GFP is a stably folded protein and relatively resistant to lysosomal proteases. However, the low pH inside the lysosome quenches the fluorescent signal of GFP. In contrast, RFP exhibits more stable fluorescence in acidic compartments, and RFP-LC3 can readily be detected in autolysosomes. By exploiting the lysosomal degradation with these two fluorescent proteins (lysosomal quenching of GFP fluorescence versus lysosomal stability of RFP fluorescence), live-cell imaging is used to morphologically trace with an GFP-RFP- LC3 tandem construct and lysosome blue staining. This method depends on the acidification and degradation capacity of the lysosome. The result showed that RFP-LC3B puncta was degraded in autophagosome over time following ChIA-F treatment (Fig. 1M). These results provide strong evidence indicating an autophagic induction by ChIA-F in human BC cells.

### 3.2 ChIA-F upregulated *SESN2* expression, which, in turn contributes to autophagy induction and anchorage-independent growth inhibition

Autophagy is tightly controlled by different regulators, that include: ATG5/ATG12, ATG7, BECN1, SQSTM1 and *SESN2* [33]. To elucidate the molecular mechanisms that are responsible for ChIA-F-induced autophagy, the potential effect of ChIA-F on autophagy-related protein expression was evaluated in T24T and RT4 cells following ChIA-F treatment. The results show that ChIA-F treatment distinctly increased *SESN2* protein expression, but no observable effect on the expression of other autophagy-related proteins, such as ATG5/ATG12, ATG7, BECN1 and SQSTM1 (Figs. 2A & 2B), suggesting that *SESN2* may be involved in autophagic induction due to ChIA-F treatment. Our most recent study has also reveal that ISO treatment effectively mediates autophagic response in a *SESN2*-dependent manner in human BC cells [26]. We next evaluated whether *SESN2* was responsible for ChIA-F-initiated autophagy induction. To this end, we transfected shRNA that specifically targets human *SESN2* into T24T cells and the stable transfectant T24T(sh*SESN2*) was established and identified, as shown in Fig. 2C. Knockdown of *SESN2* resulted in the attenuation of autophagic responses following ChIA-F treatment in T24T cells (Fig. 2D). These data indicate that *SESN2* does indeed play an essential role in the autophagy process as a result of ChIA-F treatment. Autophagy has been shown to function as a double-edged sword that either protects or suppresses human cancer, depending on the stimuli of autophagy, the stage of cancer, and the downstream mediators or effectors [12, 34–39]. In combination with various conventional chemotherapeutic drugs, induction of autophagy has

emerged as an attractive and promising approach to sensitizing malignancies to chemotherapy in cancer therapy [40, 41]. Therefore, we evaluated the effects of SESN2 on the inhibition of anchorage-independent growth following ChIA-F treatment. The results indicated that *SESN2* knockdown attenuated ChIA-F's inhibition of anchorage-independent growth of T24T cells in comparison to that in the scramble vector transfectant, T24T(Nonsense) (Figs. 2E & 2F). These results provide consistent demonstration of SESN2's contribution to ChIA-F-induced autophagic responses and the inhibition of anchorage-independent growth in T24T cells.

### 3.3 SESN2 upregulation by ChIA-F was due to the promotion of both transcription and mRNA stability

SESN2 could be regulated at multiple levels, including transcription, mRNA stability, translation, and protein degradation. To test whether SESN2 is regulated at the level of either mRNA transcription or mRNA degradation, RT-PCR was employed to determine the effect of ChIA-F on *SESN2* mRNA expression. Consistent with the results observed at the protein level, *SESN2* mRNA expression was markedly upregulated following ChIA-F treatment (Fig. 3A). We next evaluated whether ChIA-F regulated SESN2 expression at the transcription level and/or mRNA stability level. We first transfected the wild-type *SESN2* promoter-driven luciferase reporter into T24T cells [26]. The transfectants were then used to determine ChIA-F on *SESN2* transcription. As expected, ChIA-F treatment indeed led to a significant increase in *SESN2* promoter transcription activity in a time-dependent manner (Fig. 3B). Next, we tried to exclude the possibility of ChIA-F regulation of *SESN2* mRNA stability. Unexpectedly, treatment of T24T cells with ChIA-F in the presence of actinomycin D (Act D) exhibited a reduction of the *SESN2* mRNA degradation rate in comparison to that observed in cells treated with Act D alone (Fig. 3C). Taken together, our results suggest that ChIA-F upregulates SESN2 abundance *via* the promotion of both mRNA transcription and mRNA stability.

### 3.4 Sp1, but not c-Jun, was responsible for ChIA-F-induced SESN2 transcription

Our most recent study reveals that the regulatory effect of ISO on SESN2 expression occurs only at the transcriptional level in a c-Jun/AP-1-dependent manner [26]. Involvement of AP-1 in upregulation of *SESN2* transcription is also found in LPS-treated cells [42]. To evaluate whether c-Jun is involved in ChIA-F-induced SESN2 transcription, the bioinformatics analysis of the *SESN2* promoter was performed and the results showed several potential transcription factor binding sites, including Sp1, AP-1, E2F1, and NF $\kappa$ B in the *SESN2* promoter region as shown in Fig. 3D. To identify the specific transcription factor(s) participating in the modulation of *SESN2* transcription, the effect of ChIA-F on the related transcription factor protein abundance/activation was determined. The results showed that ChIA-F treatment specifically induced expression of Sp1 and c-Jun together with c-Jun phosphorylation at Ser63 and Ser73 (Fig. 3E), whereas it did not show any observable increases in phospho-NF $\kappa$ B (RELA), NF $\kappa$ B (RELA), or E2F1 (Fig. 3E), suggesting that Sp1 and c-Jun might be involved in the transcriptional activation of the *SESN2* promoter that has been treated with ChIA-F. Thus, the stable dominant negative mutant c-Jun transfectant, T24T(TAM67) was used to address the role of c-Jun in ChIA-F-induced SESN2 expression. Unexpectedly, blockage of c-Jun activation by ectopic expression of TAM67 did

not show any observable effect on *SESN2* expression or the conversion of LC3B I to LC3B II followed by ChIA-F treatment (Fig. 3F). In contrast, knockdown of Sp1 in T24T cells abolished the up-regulatory effects of ChIA-F on the expression of *SESN2* and the conversion of LC3B I to LC3B II (Fig. 3G). These results demonstrate that Sp1, but not c-Jun, is particularly crucial for *SESN2* promoter transcription and autophagic induction followed by ChIA-F treatment. To further provide direct evidence showing of Sp1 specifically binding to the *SESN2* promoter region, we performed a chromatin immunoprecipitation (ChIP) assay using an anti-Sp1 antibody. As shown in Fig. 3H, Sp1 did directly bind to the two different putative Sp1 binding sites in the *SESN2* promoter (Fig. 4H). The soft agar assay results indicated that Sp1 knockdown also attenuated ChIA-F's inhibition of anchorage-independent growth of T24T cells in comparison to that of its scramble nonsense transfectant (Figs. 4I & 2J). Taken together, our data demonstrate that ChIA-F induces *SESN2* mRNA transcription *via* a Sp1-dependent and c-Jun-independent fashion, which is distinctly different from the molecular mechanism reported in our recent study, revealing that c-Jun is required for ISO-induced *SESN2* transcription in human BC cells [26].

### 3.5 Downregulation of miR-27a contributed to *SESN2* mRNA stabilization, autophagic response and anchorage-independent growth inhibition following treatment with ChIA-F

Several RNA-binding proteins, including NCL, HNRNPD and ELAVL1, have been reported to bind to their targeted mRNA and regulate their mRNA stability [43–47]. To test the potential contribution of these RNA-binding proteins to ChIA-F upregulation of *SESN2* mRNA stability, the effects of ChIA-F on the expression of NCL, HNRNPD and ELAVL1 were evaluated. As shown in Fig. 4A, ChIA-F treatment led to upregulation of ELAVL1 protein, whereas it did not show an observable effect on the expression of either NCL or HNRNPD. To determine the role of ELAVL1 in the regulation of *SESN2* mRNA stability in T24T cells, shRNA specifically targeting human *ELAVL1* was employed to knockdown *ELAVL1* expression in T24T cells (Fig. 4B). Much to our surprise, knockdown of *ELAVL1* had no observable effect on ChIA-F-induced *SESN2* expression (Fig. 4C). Given that microRNAs (miRNAs) have been reported to regulate their target gene expression by either suppressing protein translation and/or regulating mRNA degradation usually through imperfect complementary base pairing to the target gene mRNA 3'UTR [48, 49], we tested the potential effect of ChIA-F on *SESN2* mRNA 3'UTR activity. The results indicated that ChIA-F treatment promoted *SESN2* mRNA 3'UTR activity (Fig. 4D). In light of this finding, we further used the TargetScan database to analyze potential miRNA binding sites in the 3'UTR region of *SESN2* mRNA. miR-27a is one of the most matched miRNAs that is likely to target the *SESN2* mRNA 3'UTR region (Fig. 4E). We next used real-time PCR to evaluate the possibility that ChIA-F treatment alters miR-27a expression in T24T cells. As expected, ChIA-F treatment resulted in a dramatic reduction of miR-27a in T24T cells (Fig. 4F), suggesting that miR-27a downregulation might be involved in ChIA-F stabilizing *SESN2* mRNA. Furthermore, we compared the expression of miR-27a in 14 pairs of human bladder cancer tissues and matched adjacent normal bladder tissues. As shown in Fig. 4G, the real-time PCR result of miR-27a expression was greatly upregulated in human bladder cancer tissues as compared to the corresponding adjacent normal bladder tissues. Moreover, we determined miR-27a expression levels and its target gene *SESN2* mRNA levels in the

same 14 pairs of human BC tissues and matched adjacent normal bladder tissues (Fig. S2). The correlation of miR-27a and SESN2 mRNA levels in these human tissues were analyzed and the results indicated that there was a negative pathophysiological association between these two molecules as shown in Fig. 4H.

To determine the role of miR-27a in the regulation of ChIA-F-induced SESN2 expression, miR-27a expressing construct was stably transfected into T24T cells and the stable transfectant was identified as shown in Fig. 5A. Ectopic expression of miR-27a resulted in a remarkable inhibition of SESN2 expression, autophagy induction, as well as reversing the inhibition of anchorage-independent growth following ChIA-F treatment in T24T cells (Figs. 5B-5D). To determine whether miR-27a inhibition was due to specific targeting of miR-27a to 3'UTR of *SESN2* mRNA, we constructed wild-type (WT) *SESN2* mRNA 3'UTR luciferase reporter and we point mutated the miR-27a binding site in WT *SESN2* mRNA 3'UTR luciferase reporter as displayed in Fig. 5E. Both WT and mutant of *SESN2* mRNA 3'UTR luciferase reporter were stably transfected into T24T cells, respectively, and the stable transfectants were used to test whether the miR-27a binding site in *SESN2* mRNA 3'UTR luciferase reporter is critical for ChIA-F inhibition of *SESN2* mRNA 3'UTR activity. As shown in Fig. 5F, ChIA-F treatment profoundly promoted WT *SESN2* mRNA 3'UTR activity; whereas mutation of miR-27a binding site impaired *SESN2* mRNA 3'UTR activity followed ChIA-F treatment, indicating that miR-27a binding site is required for ChIA-F inhibition of *SESN2* mRNA 3'UTR activity. Given these results showing that ChIA-F is able to stabilize *SESN2* mRNA, we evaluated the effect of ectopic expression of miR-27a on the stability of *SESN2* mRNA. Consistent with the role of miR-27a in SESN2 protein expression, miR-27a overexpression dramatically reduced *SESN2* mRNA stability in T24T cells in comparison to scramble nonsense transfectant following ChIA-F treatment (Fig. 5G). Collectively, our results clearly indicate that ChIA-F treatment inhibits miR-27a expression, which results in the reduction of miR-27a binding to *SESN2* mRNA 3'UTR and the subsequent increased stabilization of *SESN2* mRNA, thereby promoting autophagy and further mediating the inhibition of anchorage-independent growth of human BC cells.

### 3.6 ChIA-F treatment attenuated Dicer and in turn reduced miR-27a maturation and expression

In order to characterize the molecular mechanism underlying the modulation of miR-27a expression by ChIA-F treatment, the miR-27a promoter-driven luciferase reporter was transfected into T24T cells and the miR-27a promoter activity in the transfectant was determined in the cells treated with ChIA-F. As seen in Fig. 6A, ChIA-F treatment had no significant effect on miR-27a promoter activity, excluding the possibility of ChIA-F inhibiting miR-27a transcription. It is known that miRNAs possess differential stability in human cells [50], the potential effect of ChIA-F on miR-27a stability was therefore determined. T24T cells were treated with ChIA-F with or without Act D (20 µg/ml) for the indicated time periods and Real-time PCR was employed to evaluate miR-27a abundance. The result show that in comparison to miR-27a levels observed in cells treated with Act D alone, the cells treated with the combination of Act D and ChIA-F did not show observable changes in the miR-27a degradation rate (Fig. 6B). These results led us to explore the possibility of ChIA-F modulation of pre-mir-27a maturation. We compared levels of pre-

mir-27a and miR-27a expression in T24T cells treated with ChIA-F. As expected, pre-mir-27a was remarkably induced in ChIA-F-treated T24T cells, whereas matured miR-27a was significantly downregulated in the same ChIA-F-treated cells (Fig. 6C), demonstrating that ChIA-F exhibited an inhibitory effect on pre-mir-27a maturation. Pre-miRNA maturation needs successive enzymatic digestions of precursor miRNAs to produce pri-miRNAs by RNase-III-type enzyme Drosha, then pre-miRNAs by Dicer [51, 52]. We next tested whether Dicer was involved in ChIA-F attenuation of pre-mir-27a maturation. The results showed that Dicer protein was almost completely abolished without affecting its mRNA levels in the same T24T cells treated with ChIA-F (Figs. 6D & 6E). These results demonstrate that attenuation of pre-mir-27a maturation by ChIA-F might be through inhibition of Dicer protein abundance in human BC cells.

#### 4. Discussion

ChIA-F is a new modified conformation-derivative of ChelA that has never been reported before in its biological effect. In our current study, we tested the effect of ChIA-F treatment on autophagy induction in human normal bladder cell line UROtsa vs. human BC cell lines (RT4, T24T and UMUC3), and in human BC cell lines with WT-p53 vs. mutated-p53. We found that ChIA-F treatment significantly induced autophagy and subsequently inhibited anchorage-independent growth in human BC cells, while it only showed slightly effect on autophagic induction in UROtsa cells. These results suggest that ChIA-F have anti-cancer activity with relative cancer cell selectivity. We also found that ChIA-F treatment inhibited human BC cells with both WT-p53 and mutated-p53 cells, revealing that ChIA-F mainly targets p53-independent pathways, which is distinct from its parental compound ChelA (Patent PCT/CN2014/071751). This notion is also supported by the results showing that ChelA treatment induces autophagy in human BC cells [53] with no observable effect on SESN2 protein expression in T24T cells (Fig. S1B). It is also very interesting to note that SESN2 expression were similarly up-regulated by ChIA-F in both RT4 (WT-p53) and T24T (mutated-p53) cells, although high basal level of SESN2 is observed in RT4 (Fig. 2A). Our further studies showed that ChIA-F treatment specifically induced SESN2 expression *via* increasing its transcription and mRNA stability. On one hand, ChIA-F attenuates Dicer protein abundance, in turn abolishing miR-27a maturation and further relieving miR-27a binding directly to SESN2 mRNA 3'UTR, thereby promoting SESN2 mRNA stabilization. On the other hand, ChIA-F treatment promoted Sp1 abundance and consequently mediated SESN2 transcription. These findings are also distinct from previous report that p53 is a transcription factor for regulating SESN2 transcription [54]. The increase of SESN2 expression in turn leads to cell autophagy and anchorage-independent growth inhibition by ChIA-F. Our results not only clarify the role of autophagy in the anti-cancer activity of ChIA-F, but also further provide significant molecular insights into the understanding of autophagy induction upon ChIA-F treatment.

Autophagy is a multi-step process and numerous signaling pathways have been reported to be involved in the regulation of autophagy either at the early stage or at advanced stages of tumor development [55]. At the early stage of oncogenesis, autophagy is often inhibited; leading to the speculation that autophagy functions as a tumor suppressor by preventing the accumulation of damaged organelles and aggregated proteins [55, 56]. Given that autophagy

is a tumor-suppression mechanism, and that autophagy defects cause a predisposition to cancer and other diseases, the prospect of stimulating autophagy as a disease prevention measure is promising [57]. It has been reported that multiple autophagic pathways are involved in an anti-cancer effect in various cancers. Tamoxifen (TAM), which targets the estrogen receptor, induces autophagic cell death in breast cancer cells [58]. The natural product resveratrol has anti-neoplastic activities and induces autophagy, thus inhibiting cell growth in ovarian cancer [59]. The anti-cancer compound fangchinoline is reported to induce autophagy through the activation of the p53/SESN2/AMPK signaling pathway in human hepatocellular carcinoma cells [54]. Our recent study demonstrates that the natural compound ISO induces autophagic growth inhibition *via* increasing *SESN2* transcription in a c-Jun-dependent manner [26]. The current study illuminates the anti-cancer activity and the molecular mechanisms of ChIA-F in its inhibition of BC cells and defines its remarkable inhibition of anchorage-independent growth through inducing autophagy in a SESN2-dependent fashion. We first found that ChIA-F treatment activated the autophagy process in human BC cells. Using loss-of-function approach, we found that SESN2 is specifically critical for ChIA-F-triggered autophagy. Further studies reveal that ChIA-F promotes both Sp1-mediated *SESN2* mRNA transcription and miR-27a directly binding-mediated stabilization of *SESN2* mRNA. This novel finding indicates that the function of ChIA-F on BC inhibition is distinctly different from the mechanism that our lab identified for ISO's anti-cancer activity, which indicates that ISO treatment only increases *SESN2* transcription *via* c-Jun binding to the putative AP-1 binding site in the *SESN2* promoter region [26]. In the current studies, blockage of c-Jun phosphorylation at Ser63 in T24T cells by ectopic expression of c-Jun dominant negative mutant, TAM67, did not show any observable inhibition of SESN2 expression following ChIA-F although ChIA-F treatment initiates c-Jun phosphorylation at Ser63, revealing that c-Jun activation is not involved in SESN2 transcription by ChIA-F. In contrast to c-Jun, knockdown of Sp1 not only attenuated the up-regulatory effect of ChIA-F on SESN2 protein expression, but also impaired ChIA-F inhibition of anchorage-independent growth of BC cells. Moreover, ectopic expression of miR-27a showed a suppression of *SESN2* mRNA 3'UTR activity and protein expression in T24T cells, whereas mutation of the miR-27a binding site in *SESN2* mRNA 3'UTR luciferase reporter blocked miR-27a inhibition of *SESN2* mRNA 3'UTR activity, indicating that mi-27a specifically targets *SESN2* mRNA 3'UTR for its inhibition of *SESN2* mRNA stability. Sp1 is a ubiquitously expressed transcription factor in various cancer cells and tumor tissues [60]. It may activate and suppress a number of essential oncogenes and tumor suppressors [60–63]. The studies we present here demonstrate that ChIA-F treatment activates Sp1, which is able to bind to two Sp1 binding sites of the *SESN2* promoter region, consequently resulting in transactivation and expression of SESN2, as well as autophagy in human BC cells. Therefore, the current studies define distinct mechanistic cascades that mediate the autophagic induction and anti-cancer activities of ChIA-F.

miR-27a is up-regulated and functions as an oncogenic miRNA in several types of human cancers [64–67]. For examples, miR-27a is reported to play a role in proliferation, migration and invasion of Hepatocellular carcinoma (HCC) cells [64]. It can also act as an androgen-regulated oncogenic miRNA in prostate cancer *via* the increase of expression of AR-regulated genes, thereby promoting prostate cancer cell growth [66]. In the current

study, we found that miR-27a is significantly upregulated in human bladder cancer tissues as compared to the corresponding adjacent normal bladder tissues, whereas SESN2 mRNA levels show a opposite expression in same human BC tissues. Statistically, miR-27a showed a negative correlation with SESN2 mRNA expression in same set of human bladder cancer tissues. Moreover, we also found that miR-27a overexpression is able to inhibit SESN2 expression, autophagy induction and reverse the inhibition of anchorage-independent growth followed ChIA-F treatment in T24T cells *via* its binding to 3'UTR region of *SESN2* mRNA. These results reveal a novel mechanism underlying the oncogenic function of miR-27a in human BC cell biology. This function has never been reported in previous studies. Furthermore, our current studies show that ChIA-F treatment almost completely abolished Dicer protein expression accompanied by attenuating miR-27a maturation and expression without affecting *dicer* mRNA level in T24T cells, indicating that it's most likely that ChIA-F exhibits its anti-cancer activity on human BC cells most likely through its retardation of pre-mir-27a maturation processing into miR-27a by abolished targeting of either Dicer protein translation and/or degradation. Further elucidating the molecular mechanisms underlying ChIA-F's inhibition of Dicer protein expression will provide significant insight into understanding the action of this anti-cancer compound in human BCs.

In summary, our studies demonstrate that ChIA-F exhibits its anti-cancer activity on human bladder cancer *via* promotion of both Sp1-mediated *SESN2* mRNA transcription and miR-27a-mediated stabilization of *SESN2* mRNA, both of which lead to SESN2 protein expression, in turn triggering autophagic responses and subsequently resulting in the inhibition of anchorage-independent growth of human BC cells. The study provides important insights into the understanding of the molecular mechanism(s) that are responsible for the anti-cancer effect of ChIA-F, as well as revealing that elevating SESN-2-mediated autophagy activity might be a promising approach to treat human invasive/metastatic BC patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## 5. Acknowledgments

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## Abbreviations:

<b>Act D</b>	actinomycin D
<b>ATG</b>	autophagy-related
<b>BAF</b>	bafilomycin A1
<b>BC</b>	bladder cancer

<b>ChIA-F</b>	Cheliensisine A-fluoride
<b>CQ</b>	chloroquine
<b>DAPI</b>	40,6-diamidino-2-phenylindole
<b>FBS</b>	fetal bovine serum
<b>GFP</b>	green fluorescent protein
<b>IgG</b>	immunoglobulin G
<b>AP1LC3B/LC3B</b>	microtubule-associated protein 1 light chain 3 beta
<b>miRNA</b>	microRNA
<b>RFP</b>	Red fluorescent protein
<b>RT-PCR</b>	reverse transcription-polymerase chain reaction
<b>SESN2</b>	sestrin2
<b>shRNA</b>	short hairpin RNA
<b>3'-UTR</b>	3'-Untranslated Regions
<b>WT</b>	wild type

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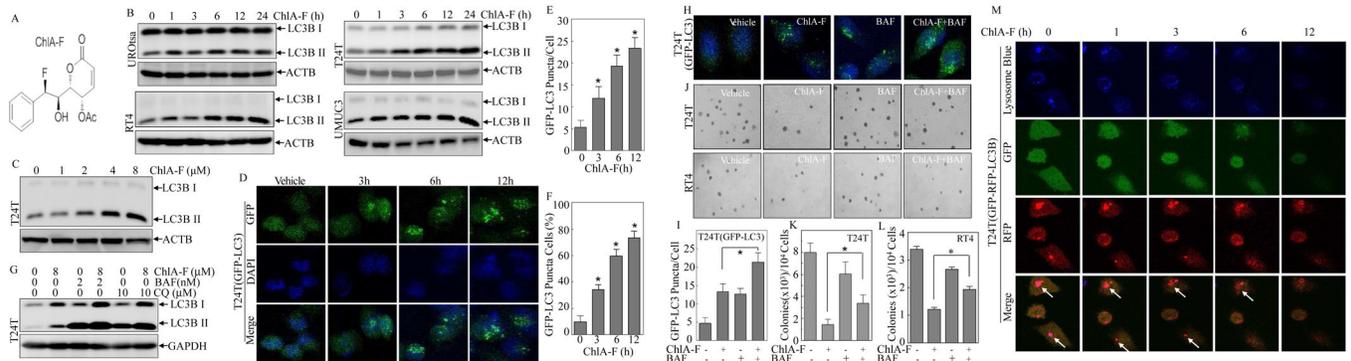
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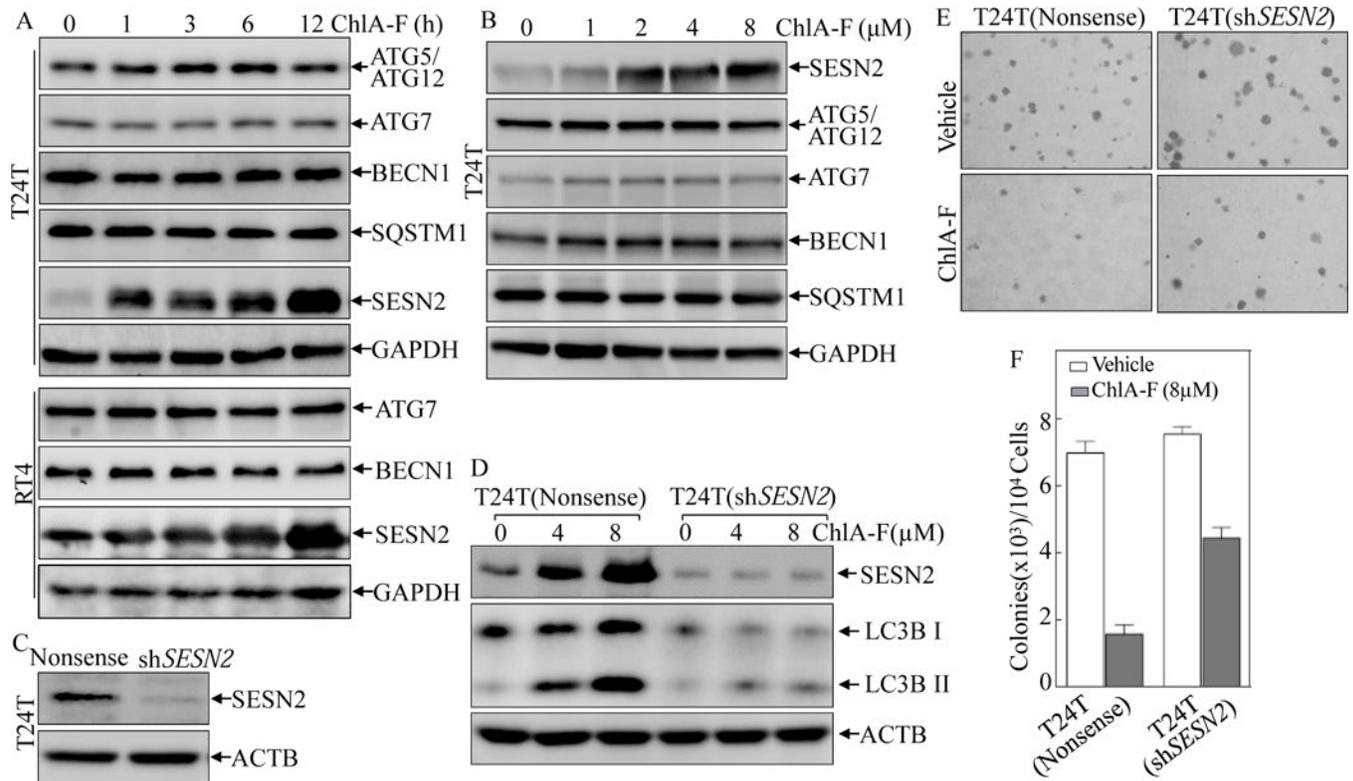
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1. The anti-cancer activity and its potential mechanisms underlying ChIA-F action is first explored.
2. ChIA-F treatment significantly inhibited anchorage-independent growth of human BC cells by inducing autophagy in a SESN2-dependent fashion.
3. ChIA-F treatment specifically induced SESN2 expression via increasing its transcription and mRNA stability.
4. The study of the anti-cancer effect of ChIA-F on BC provides therapeutic alternatives against human BC.



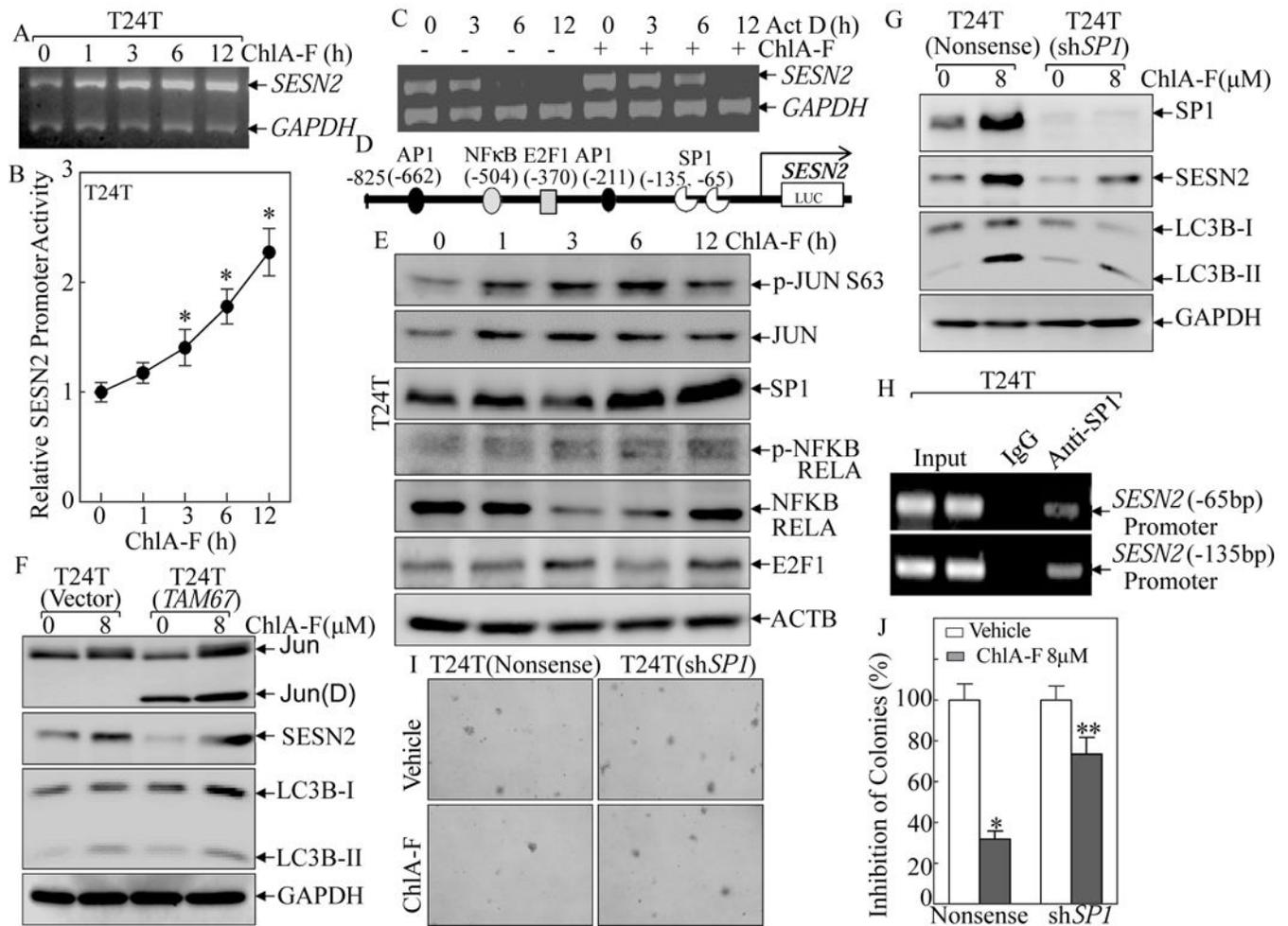
**Fig. 1. ChIA-F induced autophagy and inhibited anchorage-independent growth in human invasive bladder cancer cells.**

(A) The structure of ChIA-F. (B & C) UROtsa, RT4, T24T and UMUC3 cells were treated with  $8\mu\text{M}$  of ChIA-F for the indicated periods (B) or ChIA-F at the indicated concentrations for 12h (C). The cell lysates were subjected to Western Blot assay using the indicated antibodies. (D) The *GFP-LC3* construct was stably transfected into T24T cells, and the transfectants were treated with  $8\mu\text{M}$  of ChIA-F for various times. LC3 puncta formation was observed and images were captured using fluorescence microscopy. (E & F) Percentage of cells with GFP-LC3B puncta (E) and number of puncta per positive cell (F) was calculated and presented as described in “Materials and Methods”. (G) T24T cells were treated with ChIA-F ( $8\mu\text{M}$ ), together with or without 2 nM BAF or  $10\mu\text{M}$  CQ for 12 h and the protein levels of LC3B were assessed by Western Blot. (H) T24T cells were transfected with the *GFP-LC3* construct and the transfectants were treated with ChIA-F ( $8\mu\text{M}$ ), with or without 2 nM BAF for 12h. The representative images of GFP-LC3 puncta were captured using a confocal fluorescence microscope. (I) Number of puncta per GFP-LC3-positive cell was calculated and presented as described in “Materials and Methods”. (J) Representative images of colonies of T24T treated with either  $8\mu\text{M}$  ChIA-F or 2nM BAF, or both, in soft-agar assay, were captured as described in “Materials and Methods”. (K & L) Colonies shown in Fig. 1J were counted under an inverted microscope with more than 32 cells of each colony. The results are presented as colonies per  $10^4$  cells. The bars show Mean  $\pm$  SD from three independent experiments. The symbol (\*) indicates a significant increase from the ChIA-F treatment group ( $p < 0.05$ ). (M) T24T(RFP-GFP-LC3B) cells were seeded into 35mm glass dish to perform Live-cell imaging. Image were presented as the indicated time following ChIA-F treatment.



**Fig. 2. SESN2 induction was required for autophagic response and anchorage-independent growth inhibition by ChlA-F in human invasive bladder cancer cells.**

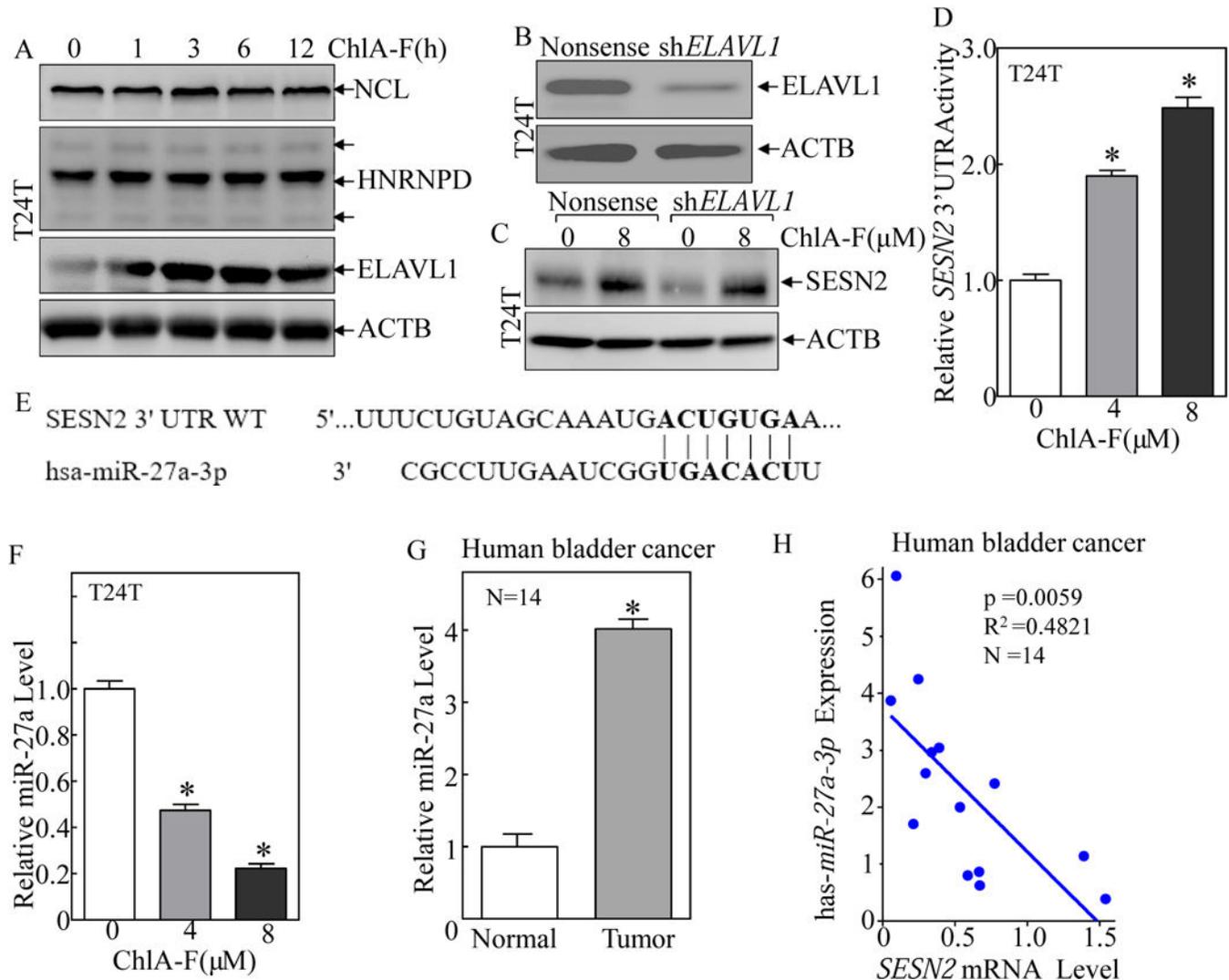
(A & B) treatment of T24T and RT4 cells with ChlA-F at 8 μM for the time points indicated (A) or at the indicated concentrations for 12 h (B). The cell lysates were subjected to Western Blot to evaluate the expression of ATG5/ATG12, ATG7, BECN1, SQSTM1, and SESN2. (C) shRNA *SESN2* were stably transfected into T24T cells and the stable transfectants were identified by Western Blot. (D) The indicated stable transfectants were subjected to Western Blot to determine SESN2 and LC3B levels in T24T cells following ChlA-F treatment for 12 h. (E & F) Representative images of colonies of T24T (sh*SESN2*) and T24T (Nonsense) cells in soft-agar assay in the presence or absence of ChlA-F (8 μM) were captured (E) or counted under an inverted microscope. The results are presented as colonies per 10<sup>4</sup> cells, and the bars show Mean ± SD from three independent experiments (F). The symbol (\*) indicates a significant decrease from vehicle control (p < 0.05).



**Fig. 3. ChIA-F treatment increased both *SESN2* transcription and *SESN2* mRNA stability.**

(A) Total RNAs were isolated from T24T cells treated with or without 8  $\mu$ M ChIA-F for the indicated times. RT-PCR was performed to determine *SESN2* mRNA levels. The *gapdh* mRNA levels were used as loading control. (B) T24T stably co-transfected with *SESN2* promoter-driven luciferase reporter and *pRL-TK* was treated with 8  $\mu$ M ChIA-F for the indicated times to evaluate *SESN2* promoter transcriptional activity. The induction fold was normalized to internal control *pRL-TK*. The results were presented as *SESN2* promoter activity relative to vehicle control (relative *SESN2* promoter activity). The bars indicate a Mean  $\pm$  SD from three independent experiments. The symbol (\*) indicates a significant increase from the vehicle control ( $p < 0.05$ ). (C) T24T cells were co-incubated with ChIA-F (8  $\mu$ M) and Act D (20  $\mu$ g/ml) for the indicated times. Total RNA was isolated and RT-PCR was then performed to determine *SESN2* mRNA levels. The result is representative of three independent experiments. (D) Schematic representation of the putative transcription factor consensus binding sites in the *SESN2* proximal promoter region was predicted using bioinformatics analysis. (E) The protein expressions of potential transcription factors were determined following ChIA-F (8  $\mu$ M) treatment for the indicated periods using Western Blot. (F) TAM67 was stably transfected into T24T cells and the stable transfectants were then

subjected to ChlA-F treatment for 12 h for identification of c-JUN(D) (TAM67) expression and determination of SESN2 and LC3B. (G) T24T Cells stably transfected with nonsense control or shRNA specifically targeting human Sp1 were treated with ChlA-F (8  $\mu$ M) for 12 h, and the cell extracts were subjected to Western Blot as indicated. (H) ChIP assay was carried out using anti-Sp1 antibody to detect the interaction between Sp1 and the *SESN2* promoter. (I & J) Anchorage-independent growth of T24T(Nonsense) and T24T(sh.*Sp1*) cells was determined in soft agar in the absence or presence of ChlA-F. The number of colonies is scored and is presented as colonies per  $10^4$  cells. The symbol (\*) indicates a significant increase in comparison to the nonsense transfectant ( $p < 0.05$ ).



**Fig. 4. ChIA-F treatment promoted *SESN2* mRNA 3'UTR activity accompanied by attenuation of miR-27a expression in human invasive BC cells.**

(A) T24T cells were treated with ChIA-F (8 μM) for the indicated periods. The total cell extracts were subjected to Western Blot analysis of expression of HNRNP, ELAVL1, NCL. (B & C) T24T cells were transfected with an shRNA specifically targeting human *ELAVL1* or nonsense control constructs. The cell extracts from stable transfectants were subjected to Western Blot analysis for determination of ELAVL1 expression (B). The stable transfectants were treated with ChIA-F for 12 h, and the cell extracts were subjected to Western blot analysis for *SESN2* expression (C). (D) *SESN2* 3'UTR luciferase reporter was transiently transfected into T24T cells, and the transfectants were subjected to Western Blot to determine the effect of ChIA-F on *SESN2* mRNA 3'UTR activity in T24T cells following ChIA-F treatment for 12h. The symbol (\*) indicated a significant increase of *SESN2* 3'UTR activity in comparison to the vehicle control ( $P < 0.05$ ). The results are shown as Mean  $\pm$  SD from three independent experiments. (E) The binding site of miR-27a in 3'UTR of human *SESN2* mRNA was analyzed and presented as indicated. (F & G) The relative expression

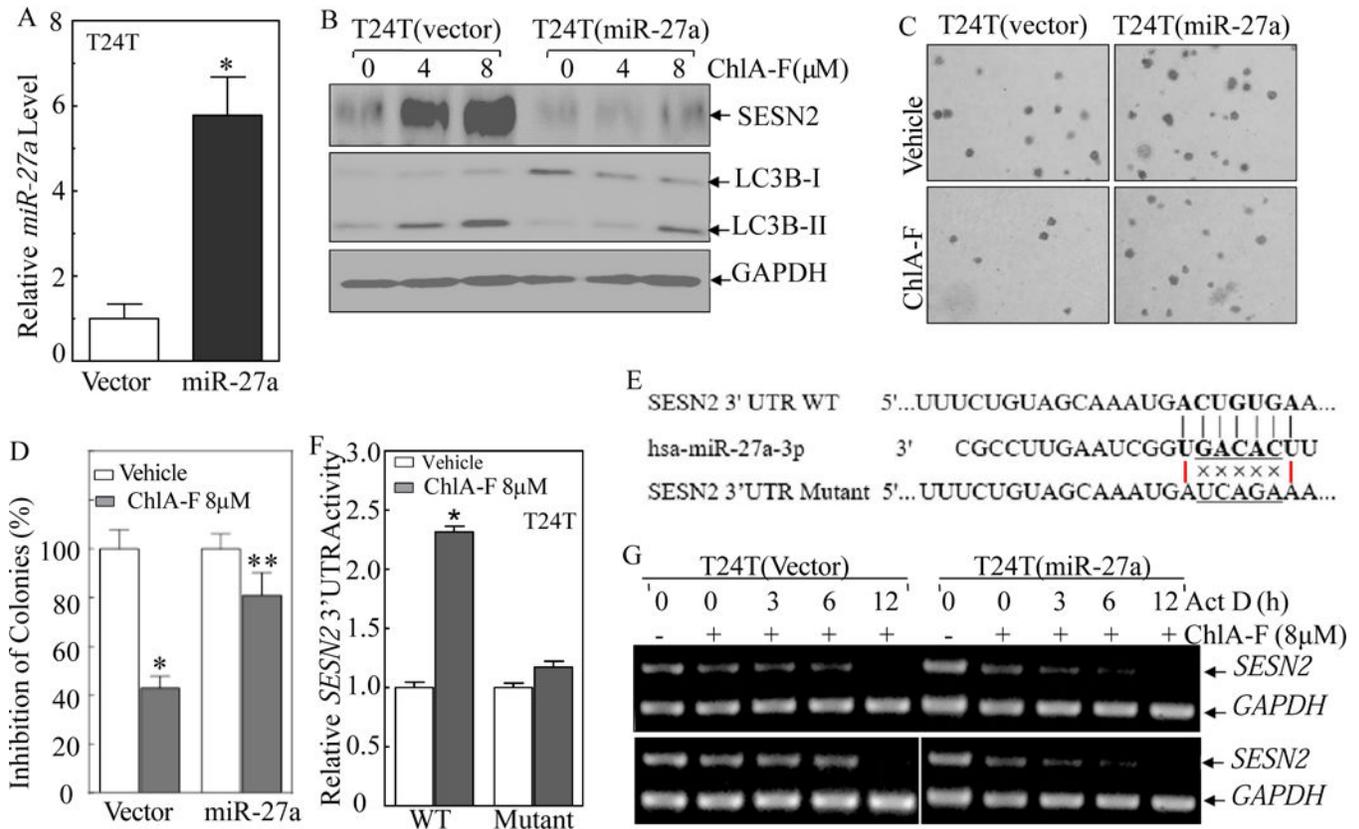
level of miR-27a was evaluated by quantitative real-time PCR in T24T cells following Ch1A-F treatment at different concentrations for 12 h (F) and in human bladder cancer tissues as compared to the corresponding adjacent normal bladder tissues (G). The symbol (\*) indicates a significant increase in comparison to normal bladder tissues. (H) The correlation analysis of pathophysiological association between miR-27a and SESN2 mRNA in human BC tissues.

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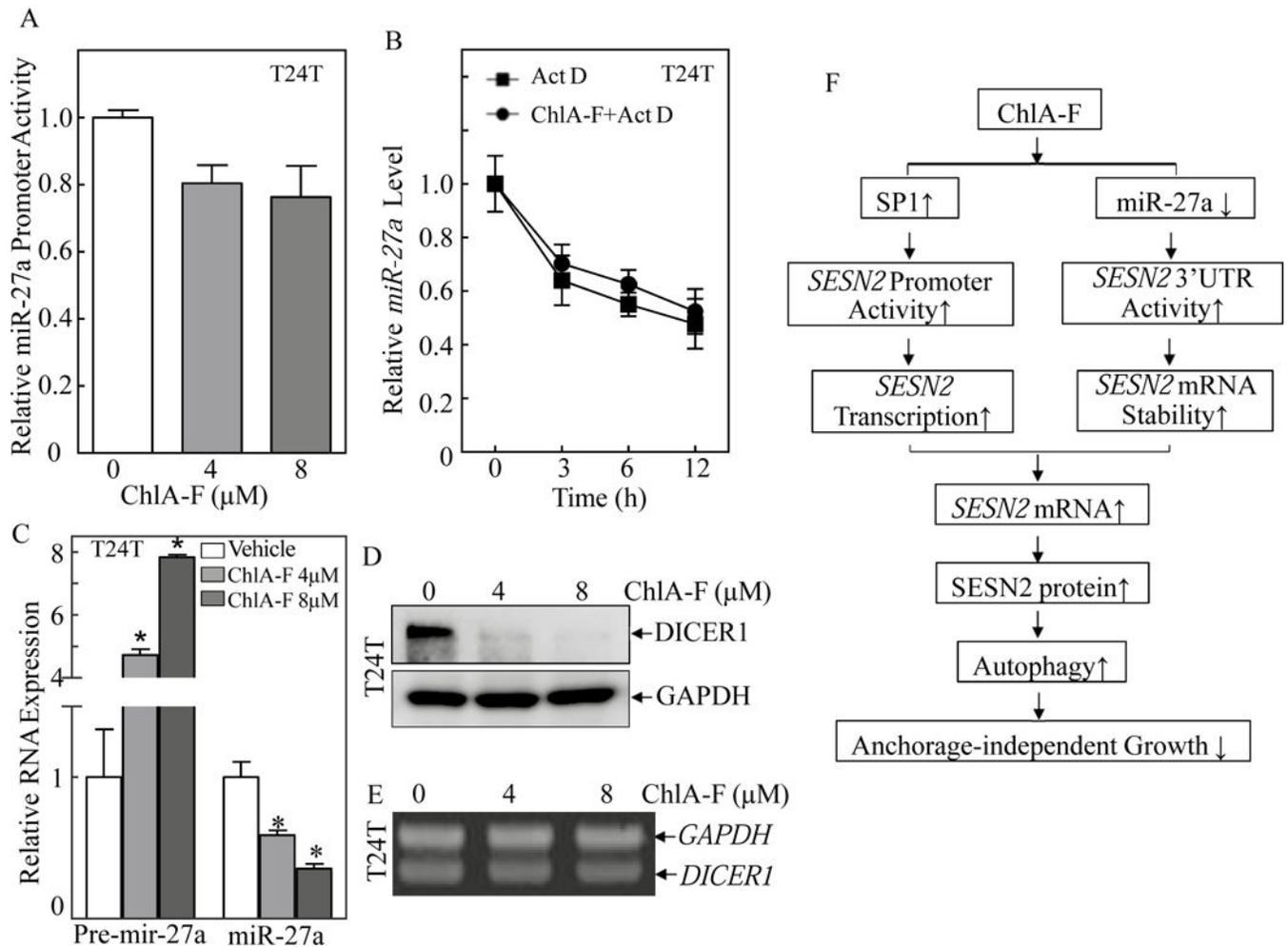
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**Fig. 5. miR-27a downregulation was critical for ChIA-F-induced stabilization of *SESN2* mRNA, autophagy induction, and anchorage-independent growth inhibition in human invasive BC cells.** (A) Overexpression of miR-27a in T24T cells was identified by real-time PCR assay. (B) Ectopic expression of miR-27a reversed ChIA-F-induced *SESN2* abundance and autophagy induction in T24T cells. (C & D) Representative images of colony formation of T24T(miR-27a) and T24T(Nonsense) cells in soft-agar assay in the presence or absence of ChIA-F (8 μM) were captured (C) and scored (D) under an inverted microscope. The results are presented as colonies per 10<sup>4</sup> cells. The bars show Mean ± SD from three independent experiments. The symbol (\*) indicates a significant decrease from vehicle control (p < 0.05) (D). (E) Schematic of the miR-27a binding sites and its mutant of the pMIR-report-*SESN2* 3'UTR luciferase reporter. (F) Attenuation of ChIA-F induction of *SESN2* mRNA 3'UTR activity in the miR-27a binding site mutant of pMIR-report-*SESN2* 3'UTR transfectants. (G) T24T cells were incubated with Act D (20 μg/ml) for the indicated time periods with or without ChIA-F (8 μM). Total RNA was isolated and RT-PCR was then performed to determine *SESN2* mRNA levels. The result are representative of three independent experiments.



**Fig. 6. ChIA-F treatment impaired miR-27a maturation accompanied by specific attenuation of Dicer protein, but not mRNA expression in human invasive BC cells.**

(A) T24T stably transfected with miR-27a promoter-driven luciferase reporter was treated with ChIA-F at different concentrations for 12 h to determine the potential effect of ChIA-F on miR-27a promoter transcriptional activity. (B) T24T cells were co-incubated with ChIA-F (8 μM) and Act D (20 μg/ml) for the indicated time periods. Total RNA was isolated and quantitative real-time PCR was then performed to determine miR-27a levels. The fold change was normalized to internal control *gapdh*. The result is representative of three independent experiments. (C) The relative expression levels of pre-mir27a and miR-27a were evaluated by quantitative real-time PCR in T24T followed by ChIA-F treatment at different concentrations for 12 h. The symbol (\*) indicates a significant change from vehicle control ( $p < 0.05$ ). (D) T24T cells were treated with various concentrations of ChIA-F for 12h. The cell lysates were subjected to Western Blot to detect Dicer protein expression. (E) Total RNA was isolated from the T24T cells treated with the indicated concentrations of ChIA-F for 12 hours. RT-PCR was performed to determine *dicer* mRNA levels. The *gapdh*

mRNA levels were used as loading control. **(F)** The schematic summary of molecular mechanisms underlying anticancer activity of ChIA-F on human bladder cancers.

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