**The SNAI2-ELF3-AS1 feedback loop drives gastric cancer metastasis and regulates ELF3** **expression at transcriptional and post-transcriptional levels**

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**Supplementary Materials and Methods**

**Microarray data analysis of GSE62254 and pan-cancer analysis in TCGA**

Public gastric cancer microarray gene profiling dataset (GSE62254) used in this study was downloaded from the Gene Expression Omnibus (GEO) in the NCBI web server. The survival analysis of ELF3-AS1 was conducted by using GEPIA web server. RNA-Seq data of 407 gastric cancer samples and the correlated clinical information of 443 gastric cancer samples were downloaded from the Cancer Genome Atlas (TCGA). Expression level of each gene was calculated from log2 of its upper quartile FPKM (FPKM-UQ) value. The survival analysis of pan-cancer was conducted using the KM-plot web server.

**Wound healing assay, Cell invasion assay** **and Cell proliferation assay**

For wound healing assay, a sterile 100 µl pipette tip was used to longitudinally scratch a constant-diameter stripe in the confluent monolayer. The medium and cell debris were aspirated away and replaced with 2 ml of fresh medium. Photographs were taken at 0 and 48 hours after wounding. For statistical analysis, five to ten randomly selected fields along each wound were marked, and the area of the wound was measured, and the average was calculated as the wound area of this wound.

For transwell assay, the upper chamber was placed into a 24-well plate containing 500 μL of medium containing 20% FBS. A cell suspension (200 μL) was added to the upper chamber of the Transwell module and incubated for 16 hours at 37 oC in 5% CO2. The Transwell module was then washed twice with PBS and fixed with pre-chilled methanol for 10 minutes at -20 oC. The upper chamber of the Transwell module was washed twice with PBS. The cells remaining on the top surface of the upper chamber were removed with a wet cotton swab. The upper chamber was then washed 3 times with PBS and air dried at the inverted position. The chamber membrane was stained with a 0.1% crystal violet staining solution at 500 μL per well for 30 minutes at 37 oC, washed 3 times, and air dried.

For cell proliferation assays, cells were reseeded in 96-well plates at 2,000 cells/well in a final volume of 100 μL and cultured for 4 days. The GC cell proliferation activities were determined with CCK-8 assay every 24 hours. Subsequently, 10 μL of CCK-8 solution (Biosharp, China) were added into each well and incubated for 2 hours. Optical density was measured at a wavelength of 490 nm by an automatic microplate reader (Bio Tek, USA). Triplicate wells were assayed for each experiment, and three independent experiments were performed. Data were expressed as the OD490 mean ± S.D.

**RNA isolation and Quantitative RT-PCR**

Total RNA was extracted using Trizol reagent (Invitrogen, USA). Reverse transcription was performed to obtain cDNA by using the PrimeScriptTM RT reagent Kit (Perfect Real Time, Takara). The qPCR protocol was using One Step TB Green PrimeScriptTM RT-PCR Kit II (Takara) according to the manufacturer’s instructions. The qPCR analysis was conducted on Bio-Rad CFX Manager 3.1 real-time PCR system. All the primers listed in Table S1 were synthesized by Wcgene Biotech (Shanghai, China). RNU6B (U6) and ACTB were used as internal controls. Each gene was run in triplicate. Relative fold changes of gene expression were calculated using the comparative ΔΔCt method.

**Dual luciferase reporter assay**

The wildtype promoters were amplified by PCR and ligated into the pEZX-FR01-dual luciferase reporter vector (GeneCopoeia, USA). The mutant ELF3-AS1 promoters were constructed from wildtype promoters by using seamless cloning to replace CAGGTG with GGGAAA (Yeasen Biotech, China). GC cells were seeded into 12-well-tissue plates 24h before transfection, and then co-transfected with 5ng siRNA and 1mg plasmid using the Lipofectamine 2000 Reagent (Invitrogen), according to the manufacturer’s instructions. After another 48h, cells were assayed using the Dual-Luciferase reporter assay system kit (GeneCopoeia, USA). All experiments were performed in triplicate and data were pooled from three independent experiments.

**Flow cytometry assay**

After 48h transfected with siRNAs and corresponding negative control siRNAs, SGC7901 and AGS cells were collected and performed cell cycle assay and cell apoptosis assay in accordance with the manufacture's protocol (BB-4104, BestBio, China). Flow cytometry assays were performed on the CytoFLEX machine (Beckman, USA). The cell cycle and cell apoptosis distribution were quantified using the CytExpert software.

**Nuclear and cytoplasmic RNA isolation**

GC cells were harvested for isolation of cytoplasm and nuclear RNA using the nuclear-cytoplasmic separation kit (BB-36021-2, BestBio, China) according to the manufacturer’s instructions. The obtained cytoplasmic components and cytoplasmic components were added to 500 ul of Trizol, and then extracted RNA using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. For Quantitative RT-PCR, 1 ug of cytoplasm RNA and 1ug of nuclear RNA were reversed to obtain cDNA using the PrimeScript TM RT reagent Kit (Perfect Real Time, Takara). Relative fold changes of gene expression were calculated using the comparative ΔCt method.

**Supplementary Tables**

**Supplementary Table S1:** The information of siRNAs and primers used in this study.

|  |  |
| --- | --- |
|  Gene Name | Sequence (5→`3`) |
| siRNA |
| siELF3-AS1#1 | GGCUGACCUGAGUCAGAAATT | UUUCUGACUCAGGUCAGCCTT |
| siELF3-AS1#2 | GGAGAGGAGUUACUAGGUUTT | AACCUAGUAACUCCUCUCCTT |
| siELF3-AS1#3 | GCCAGAGAAUUGGCUACAATT | UUGUAGCCAAUUCUCUGGCTT |
| siELF3#1 | GCUGCAACCUGUGAGAUUATT | UAAUCUCACAGGUUGCAGCTT |
| siELF3#2 | CCUCUGCAAUUGUGCCCUUTT | AAGGGCACAAUUGCAGAGGTT |
| siELF3#3 | CCAUGAGGUACUACUACAATT | UUGUAGUAGUACCUCAUGGTT |
| si-ILF2#1 | CUUUGUACCACAUAUCCCATT  | UGGGAUAUGUGGUACAAAGTT  |
| si-ILF2#2 | GAACUCCAUUUGGAUAUCATT | UGAUAUCCAAAUGGAGUUCTT |
| si-ILF3#1 | GACCGAAAUUUGCUGCUAATT | UUAGCAGCAAAUUUCGGUCTT |
| si-ILF3#2 | GGAGGUUGAUGGCAAUUCATT | UGAAUUGCCAUCAACCUCCTT |
| qRT-PCR | Forward primer | Reverse primer |
| SNAI2 | GCATTTGCAGACAGGTCAAA | TCCTCATGTTTGTGCAGGAG |
| SNAI1 | AGCGAGCTGCAGGACTCTAA | GGACAGAGTCCCAGATGAGC |
| ILF3-Total | CCCCAGAGGACGACAGTAAA | CTCCTTACACAGCAGCACCA |
| NF110 | CCTTGTCTCACCACCAACCT | CCAGAAGCTCCCAACTATGC |
| NF90 | CGGAGTCATTCTGGCTCTCT | CGCAAAATCTTGCAAGTCAA |
| ILF2 | AACAGTGCCACCCAATCTTC | CCAGGAAAACGAATCCTCAA |
| ELF3 | GAAGTGACGTGGACCTGGAT | CTTCTTGCCCTCGAGACAGT |
| ACTIN | ATCGTCCACCGCAAATGCTTCTA | AGCCATGCCAATCTCATCTTGTT |
| P21 | ttagcagcggaacaaggagt | gccgagagaaaacagtccag |
| P53 | ATGGAGGAGCCGCAGTCAGATC | CCATTGTTCAATATCGTCCGGG |
| CDK6 | AACACCCTTGGTGGCTTATG | TTTCCTTGGAGAAGCAGAGC |
| CASP7 | CACCTATCCTGCCCTCACAT | TTATGGGCCAGGCTTACATC |
| MALAT1 | AAAGCAAGGTCTCCCCACAAG | GGTCTGTGCTAGATCAAAAGGCA |
| GAPDH | TCACCAGGGCTGCTTTTA | AAGGTCATCCCTGAGCTGAA |
| ELF3-201 | ATTGTGTTTCGGGCTGAGTC | CCAGGTATGCAGGTGTGTTG |
| ELF3-AS1 | CGGCTCTGCTTGAAAGTTCT | CTGACTGAACCCAAGCCATT |
| CHIP-primer | Forward primer | Reverse primer |
| ELF3-AS1\_P | tcccccatttgtctaacagg | CCTACCCACAGGTAGCCTCA |
| miRNA qPCR | Forward primer | Reverse primer |
| U6 | CTCGCTTCGGCAGCACA | AACGCTTCACGAATTTGCGT |
| miR-33a | gggGTGCATTGTAGTTGCA | CAGTGCGTGTCGTGGAGT |
| miR-33b | gggGTGCATTGCTGTTGCA | CAGTGCGTGTCGTGGAGT |
| miR-203a | gggGTGAAATGTTTAGGACC | CAGTGCGTGTCGTGGAGT |

**Supplementary Table S2:** The information of antibodies used in this study.

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| --- | --- | --- | --- | --- | --- |
| Gene Name | Protein Name | Dilution ratio | Cat. No. | Species | Brand |
| ILF2 | NF45 | 1:5000 | 14714-1-AP | R | Proteintech |
| ELF3 | ESE-1 | 1:5000 | HPA003316 | R | SIGMA |
| ACTB | β-actin | 1:10000 | 66009-1-Ig | R | Proteintech |
| TARBP2 | TRBP2 | 1:100 | 15753-1-AP | R | Proteintech |
| ILF3 | NF90/NF110 | 1:5000 | 13099-1-AP | R | Proteintech |
| SNAI2 | Slug | 1:1000 | 12129-1-AP | R | Proteintech |
| YBX1 | YBX1 | 1:1000 | 20339-1-AP | R | Proteintech |
| RBMX | RBMX | 1:1000 | 17994-1-AP | R | Proteintech |
| SNAI1 | Snail | 1:500 | 13099-1-AP | R | Proteintech |

**Supplementary Figures**

**Supplementary Figure S1** Overexpression of SNAI2 and SNAI1 correlates with poor prognosis in GC. (A, B) The expression of SNAI2 and SNAI1 were upregulated in GC according to the pan-cancer analysis of the available TCGA dataset. (C, D) Kaplan–Meier analysis of overall survival time, disease-free time of GC patients from GSE62254 cohort according to the expression of SNAI2 and SNAI1. (E, F): Kaplan–Meier analysis of overall survival time, disease-free time of GC patients from TCGA cohort according to the expression of SNAI2 and SNAI1.

**Supplementary Figure S2:** ELF3-AS1 inhibits GC metastasis *in vitro* and *in vivo*. (A) The percentage of apoptotic cells was determined by flow cytometric analysis (left panel). The statistical results were shown on the left panel. (B, C) The cell proliferation assays were performed in the GC cells that knocked down (B) or overexpressed ELF3-AS1 (C). (D, E) The effects of ELF3-AS1 knockdown on GC cells migration were assessed by wound healing assays in GC cell lines. Scale bars=100μm. (F) The effects of ELF3-AS1 knockdown on GC cells invasion were assessed by transwell assays in two GC cell lines. Scale bars=50μm (G) The cell colony formation assay was performed in the GC cell line that overexpressed ELF3-AS1. (H, I) The wound healing assays and transwell assays were performed in HGC-27 cells to detect the effect of ELF3-AS1 overexpression on GC cells metastasis. Scale bars=100μm (J) The volumes of xenograft tumor from negative control group and the ELF3-AS1 knockdown group were measured. Scale bars=50μm. (K) The weight of xenograft tumor from negative control group and the ELF3-AS1 knockdown group were measured. \*\*, P < 0.01.

**Supplementary Figure S3:** The gene expression profile of ELF3-AS1 knockdown and SNAI2 overexpression are very similar. (A) The heat map reveals clusters of genes altered by ELF3-AS1 knockdown (left panel) and SNAI1/2 overexpression (right panel). The red color indicates genes that are up regulated compared with the control cells, and the green color indicates genes that are down regulated compared with the control cells. (B) The genes significantly regulated by both ELF3-AS1 knockdown (Log2FC>0.7) and SNAI2 overexpression were shown in the heat map. The numbers in the figure represent the expression changes (Log2FC value) after ELF3-AS1 knockdown and SNAI1/2 overexpression. (C) The Venn plot of genes significantly regulated by SNAI2 overexpression and ELF3-AS1 knockdown. (D) The validation of genes that significantly regulated by both SNAI2 and ELF3-AS1 using qPCR method. \*\*, P < 0.01.

**Supplementary Figure S4:** The ILF2/ILF3 complex dynamically regulates mRNA stability of ELF3-AS1 and ELF3. (A) The transcripts abundance of ILF3 in ILF3-silenced samples was detected by RNA-seq. (B) The ILF3 knockdown efficiency was verified in the SGC7901 and AGS cell lines by qRT-PCR analysis. (C, D) The expression of ELF3-AS1 and ELF3 were determined in GC cell lines by qRT-PCR assays. (E) The transcripts abundance of ILF2 in ILF2-silenced samples was detected by RNA-seq. (F) The ILF2 knockdown efficiency was verified in the SGC7901 and AGS cell lines by qRT-PCR analysis. (G, H) The expression of ELF3-AS1 and ELF3 were determined in the ILF2-silenced GC cell lines by qRT-PCR assays. (I) The normalized expression levels (FPKM value) of different transcripts of ELF3 and ELF3-AS1 after silencing ILF2 or ILF3 or overexpressing SNAI2 and SNAI1. (J, K) The expression of NF90 and NF110 were determined by qRT-PCR in ILF2-silenced cell lines. \*\*P < 0.01

**Supplementary Figure S5:** Knockdown of ELF3-AS1 resulted in upregulation of SNAI2 mRNA in A549, Hela and Caki-2 cell lines. The expression levels of SNAI2 and ELF3-AS1 in GSE92250 were normalized to FPKM values (fragments per kilobase of exon per million fragments mapped).

**Supplementary Figure S6:** Survival Analysis of ELF3-AS1 and SNAI2 in pan-cancer. ELF3-AS1 and SNAI2 possessed opposite prognosis in pan-cancer.