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Supplemental Information

Promoter Methylation-Regulated miR-145-5p Inhibits Laryngeal Squamous Cell Carcinoma Progression by Targeting *FSCN1*

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Figure S1

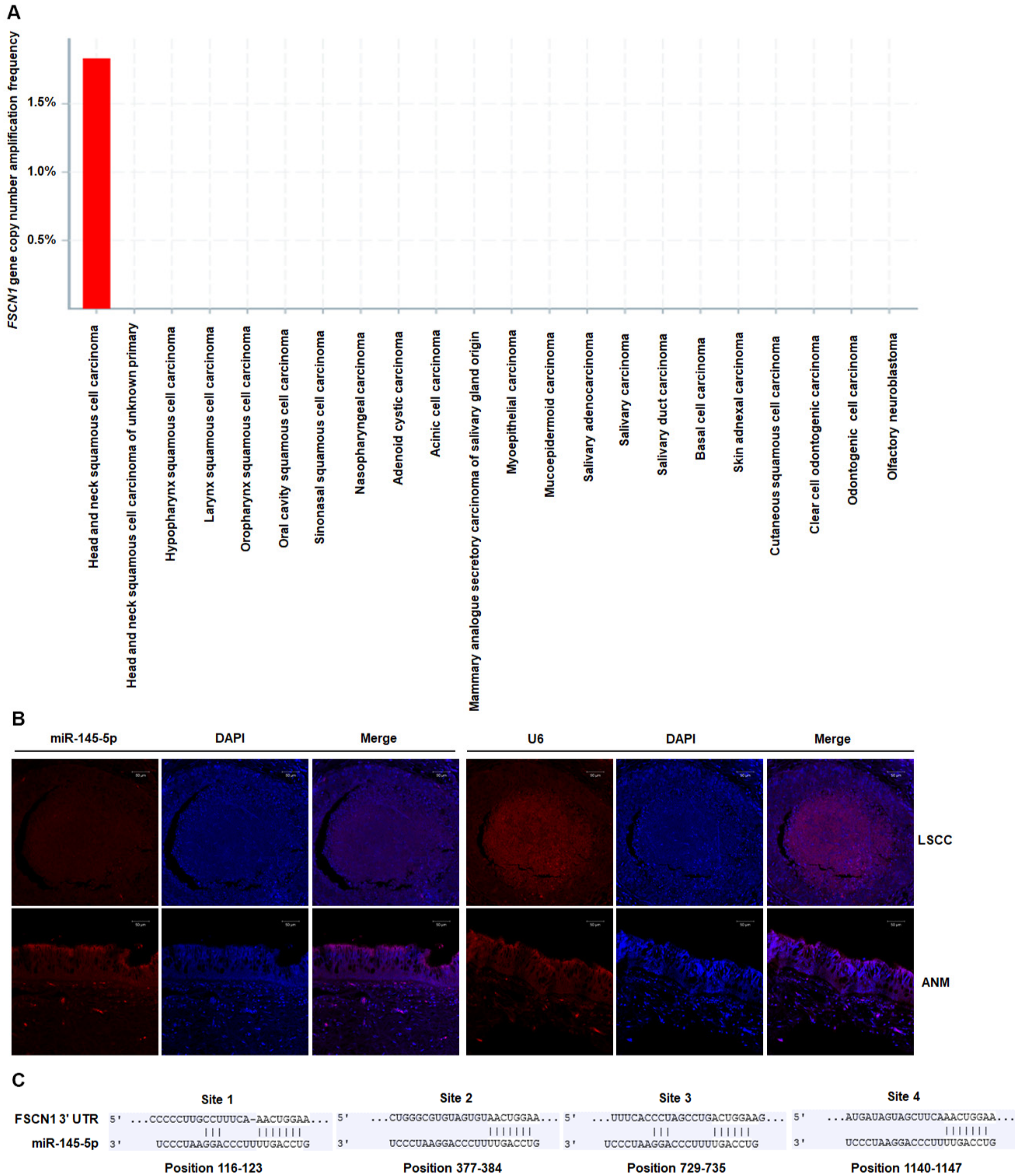
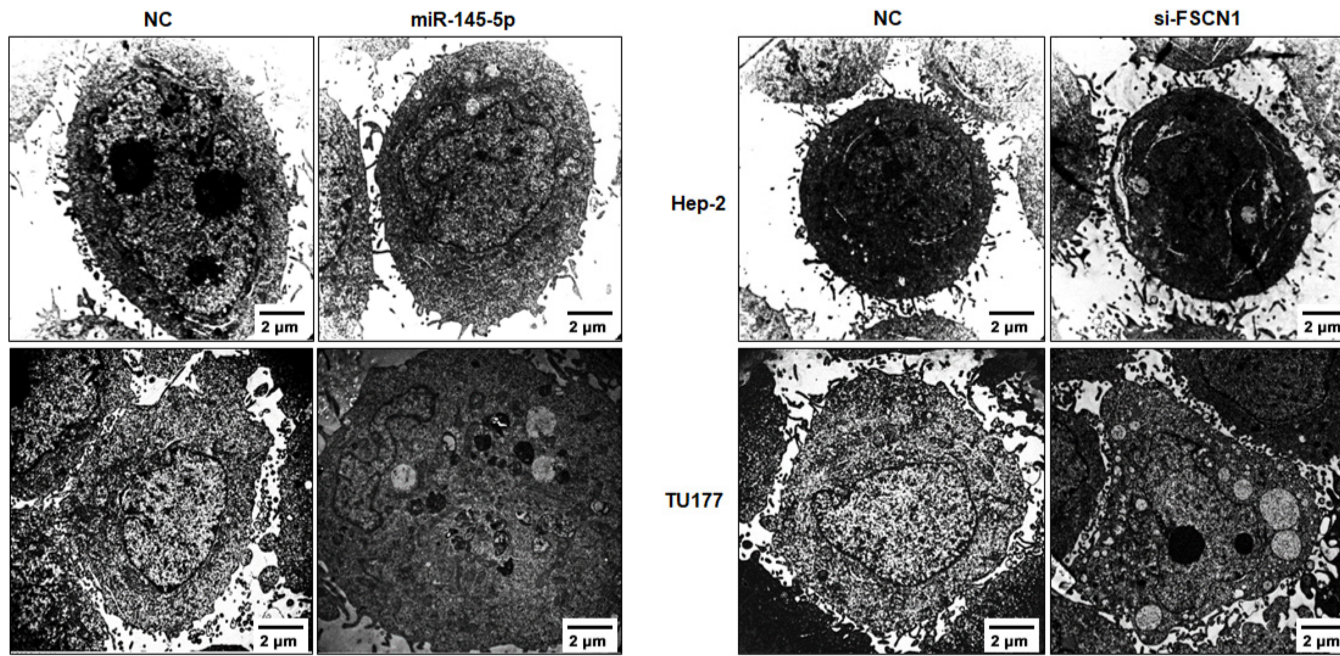
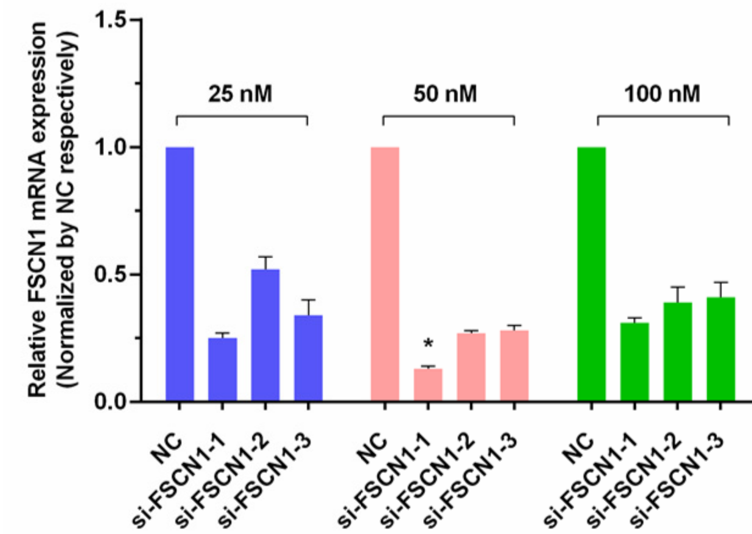


Figure S2





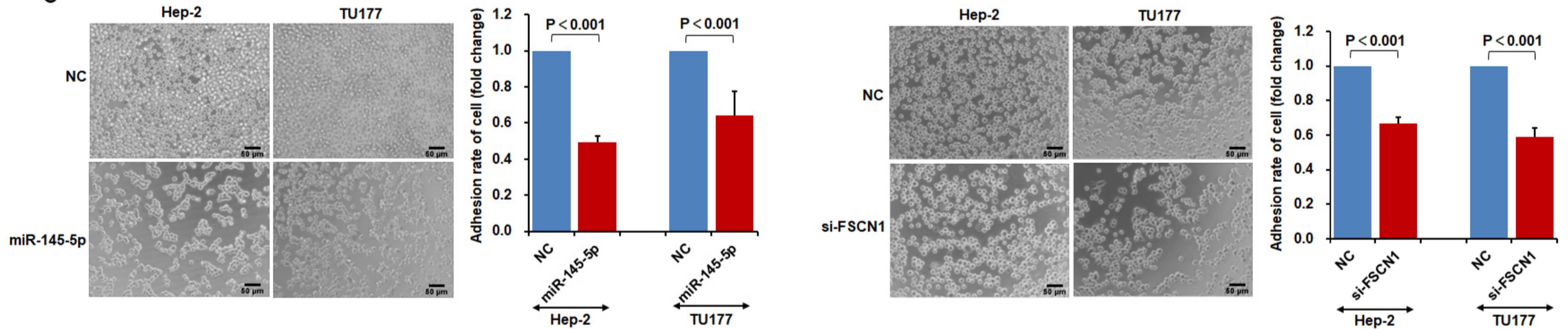


Figure S3

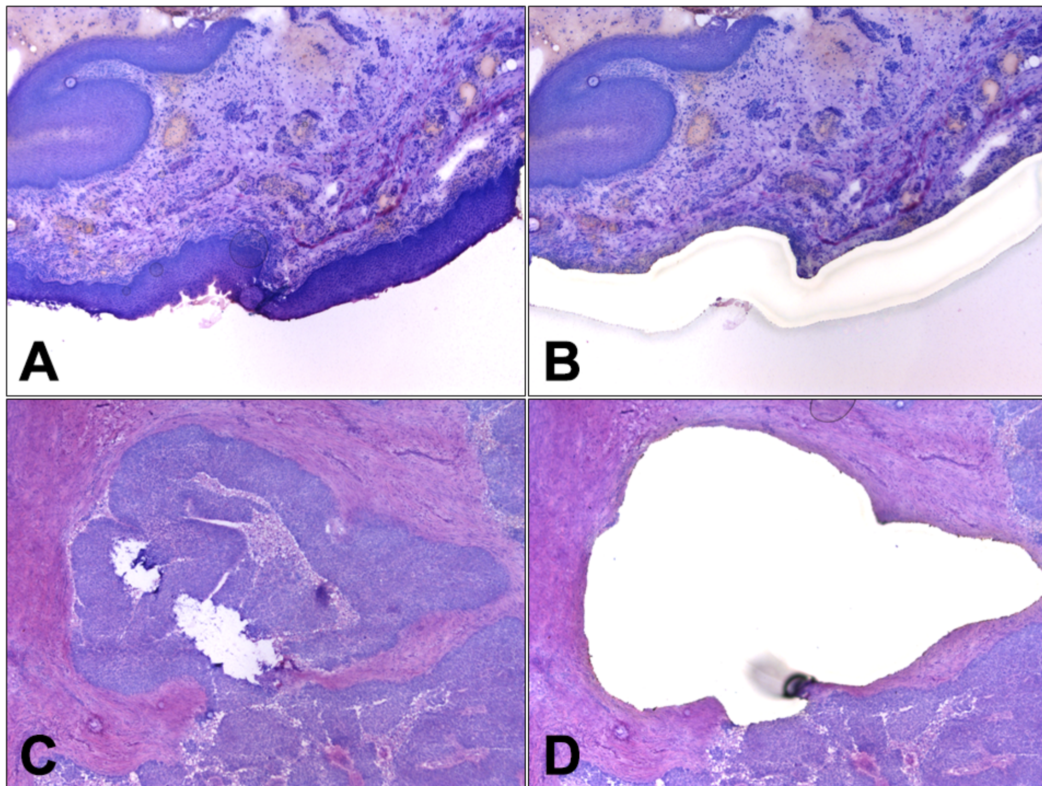
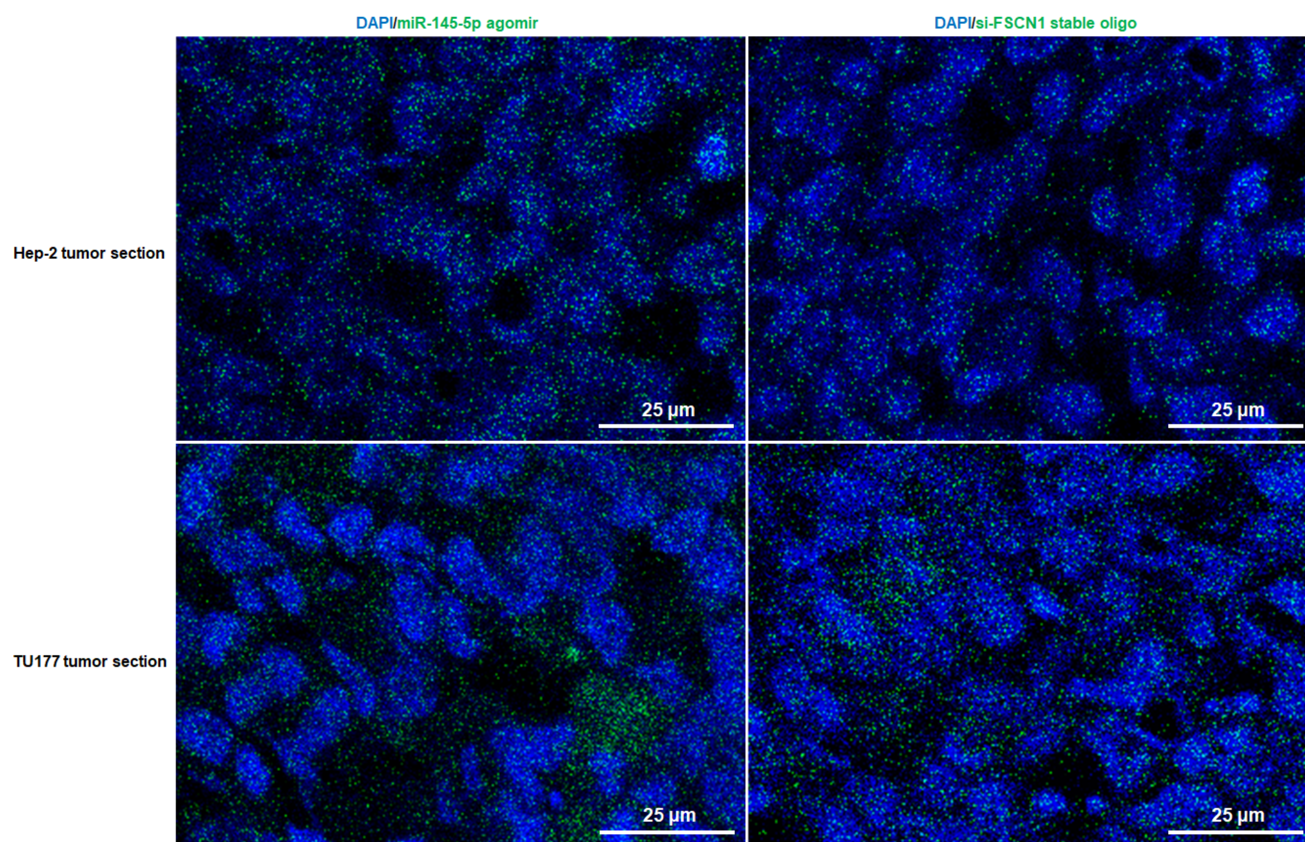


Figure S4



Supplemental legends

Figure S1. **(A)** Copy number variation analysis of *FSCN1* gene in 1685 head and neck cancer samples (including LSCC) from TCGA dataset performed with cBioPortal v1.13.1 (http://www.cbioportal.org/index.do). **(B)** Fluorescence *in situ* hybridization of miR-145-5p in LSCC and ANM tissue. Expression of U6 RNA was an internal control. **(C)** Complementary binding sites of miR-145-5p on 3'-UTR of FSCN1.

Figure S2. (A) Hep-2 and TU177 cells were transfected with miR-145-5p mimic or siRNA oligos targeting FSCN1 for 48 h; apoptotic bodies were observed by TEM. **(B)** Screening of FSCN1 siRNA. qPCR analysis of mRNA level of FSCN1 in Hep-2 cells transfected with siRNAs targeting 3 different sequences of FSCN1 mRNA at the indicated concentrations for 48 h. **(C)** Hep-2 and TU177 cells were transfected with miR-145-5p mimic or siRNA oligos targeting FSCN1 for 48 h; cell adhesive ability was measured. Data are mean ± SD of three independent experiments.

Figure S3. Laser capture microdissection (LCM) of LSCC or paired ANM fresh frozen tissues. Representative images of ANM tissue section before **(A)** and after **(B)** laser capture microdissection (LCM). Representative images of LSCC fresh tissue sections before **(C)** and after **(D)** LCM.

Figure S4. Verification of delivery efficiency of RNA oligos injected into xenograft tumors**.** Hep-2 or TU177 cells were subcutaneously injected into the flanks of BALB/C nude mice to generate xenograft tumors. When the tumors had grown to 0.5 mm3, FAM labeled miR-145-5p agomir or FSCN1 siRNA stable oligos were subcutaneously injected into the tumor site as follows: multi-point injection with 0.1 ml oligos (10 nmol), 2 times/week/position and on the first and fourth day per week. Tumors were separated 96 h after injection, then frozen sections were prepared and observed under confocal laser scanning microscopy. Representative images show distribution and level of miR-145-5p agomir or FSCN1 siRNA in tumors. The nuclei were stained with DAPI (blue). The miR-145-5p agomir or FSCN1 siRNA can be seen as green fluorescent dots.

Supplemental Table S1. Prediction of miRNA targeting FSCN1. Potential miRNA of FSCN1 was predicted with miRwalk-2, the information of potential binding site was shown in the Excel file.

Supplemental Table S2. Differentially expressed miRNAs from microarray data of paired LSCC and adjacent normal margin tissues.

Gene Name	P value	Fold Change	Style
hsa-miR-486-5p	0.04	8.2	Down
hsa-miR-204-5p	<0.001	5.02	Down
hsa-miR-139-5p	0.03	3.2	Down
hsa-miR-145-5p	0.02	2.74	Down
hsa-miR-1305	0.02	2.4	Down
hsa-miR-1225-3p	0.03	2.1	Down
hsa-miR-21-5p	0	8.14	Up
hsa-miR-21-3p	0	5.76	Up
hsa-miR-135b-5p	0.02	5.72	Up
hsa-miR-210	0.02	5.66	Up
hsa-miR-130b-3p	<0.001	5.17	Up
hsa-miR-7-5p	0.02	4.62	Up
hsa-miR-222-3p	0.04	3.56	Up
hsa-miR-19a-3p	0.01	3.55	Up
hsa-miR-181b-5p	0.01	3.35	Up
hsa-miR-106b-5p	0.01	3.3	Up
hsa-miR-185-5p	0.03	3.03	Up
hsa-miR-19b-3p	0.02	3.01	Up
hsa-miR-151-3p	0.03	2.98	Up
hsa-miR-362-5p	0.02	2.72	Up
hsa-miR-93-5p	<0.001	2.61	Up
hsa-miR-20a-5p	0.01	2.56	Up
hsa-miR-425-5p	0.01	2.52	Up
hsa-miR-20b-5p	0.02	2.38	Up
hsa-miR-374b-5p	0.04	2.36	Up
hsa-miR-181a-5p	<0.001	2.29	Up
hsa-miR-27a-3p	0.03	2.28	Up
hsa-miR-424-5p	0.04	2.26	Up
hsa-miR-152	0.01	2.18	Up

Supplemental Table S3. Clinical features of 188 LSCC patients.

Parameters	Number of Cases（%）
Age	
≤60	89（47.3）
＞60	99（52.7）
Sex	
Female	21（11.2）
Male	167（88.8）
Primary cancer site	
Glottic	101（53.7）
Supraglottic	83（44.1）
Subglottic	4（2.1）
Differentiation	
High	72（38.3）
Medium	75（39.9）
Low	41（21.8）
T staging[1]	
T1	53（28.2）
T2	58（30.9）
T3	41（21.8）
T4	36（19.1）
Cervical lymph node metastasis	
N0	142（75.5）
N+	46（24.5）
Distant metastasis	
M0	183（97.3）
M1	5（2.7）
Clinical stage	
I	51（27.1）
II	45（23.9）
III	47（25.0）
IV	45（23.9）
Smoke preoperatively[2]	
No	75（39.9）
Yes	113（60.1）

[1]TNM Staging is referring to the 7th UICC TNM Staging Criteria
[2]Smoker is referring to definition from WHO 1997: at least one cigarette each day continuous or accumulation for six months.

Supplemental Table S4. COX regression analysis of miR-145-5p/FSCN1 expression combination

Prognostic Factor	Regression Coefficient	Standard Error	Wald	*P* value	Relative Risk（*RR*）	*95.0% CI*
miR145(High)/FSCN1(Low)					1.00	
miR145(Low)/FSCN1(Low)	0.260	1.23	0.05	0.833	1.30	0.12～14.42
miR145(High)/FSCN1(High)	2.70	0.79	11.67	***0.001***	14.85	3.16～69.85
miR145(Low)/FSCN1(High)	2.54	0.77	11.01	***0.001***	12.69	2.83～56.91

Supplemental Table S5. COX regression analysis of FSCN1 protein expression.

Prognostic Factor	Regression Coefficient	Standard Error	Wald	*P* value	Relative Risk (RR)	***95.0% CI***
Age（>60）	0.87	0.30	8.518	***0.001***	2.39	1.33～4.29
Neck lymph node metastasis	1.096	0.28	15.28	***<0.001***	2.99	1.73～5.19
Distant metastasis	2.245	0.52	18.85	***0.002***	9.44	3.43～26.01
Smoke preoperatively	0.97	0.35	7.55	***0.016***	2.65	1.32～5.30
FSCN1 high-expressed	2.507	0.64	15.26	***0.001***	12.27	3.49～43.19

Supplemental Table S6. Clinical features of LSCC samples underwent DNA methylation analysis.

Parameters	Number of Cases
Age	
≤60	5
＞60	7
Sex	
Female	1
Male	11
Primary cancer site	
Glottic	2
Supraglottic	8
Transglottic	2
Differentiation	
High	5
Medium	4
Low	3
T staging[1]	
T1	4
T2	1
T3	4
T4	3
Cervical lymph node metastasis	
N0	9
N+	3
Distant metastasis	
M0	12
M1	0
Clinical stage	
I	3
II	1
III	4
IV	4
Smoke preoperatively[2]	
No	0
Yes	12

[1]TNM Staging is referring to the 7th UICC TNM Staging Criteria

[2]Smoker is referring to definition from WHO 1997: at least one cigarette each day continuous or accumulation for six months.

RNA extraction and qPCR analysis

Total RNA was extracted from frozen cancer tissues by use of TRIzol reagent (Invitrogen). For formalin-fixed paraffin-embedded (FFPE) samples, total RNA was extracted by using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion). cDNA was synthesized from total RNA by using the PrimeScript RT Kit (TaKaRa, Dalian, China). qPCR was performed on the ABI 7500 FAST real-time PCR system (Applied Biosystems, Foster City, CA, USA). The procedures for qPCR were 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The $2^{-\Delta\Delta ct}$ method was used to calculate the relative expression level of target genes. U6 RNA and 18S rRNA were served as internal control for miR-145-5p and FSCN1, respectively. The primer sequences used were for Hsa-miR-145 F: ACACTCCAGCTGGGGTCCAGTTTTCCCAGGAA;

Hsa-miR-145 R: CTCAACTGGTGTCGTGGA;

U6-F: CTCGCTTCGGCAGCACA;

U6-R: AACGCTTCACGAATTTGCGT;

FSCN1-F: AGCTGCTACTTTGACATCGA;

FSCN1-R: TCATGAGGAAGAGCTCTGAGT;

18S RNA-F: CCTGGATACCGCAGCTAGGA;

18S RNA-R: GCGGCGCAATACGAATGCCCC.

Antibodies

FSCN1 (Cat#ab126772) and Cyclin D1 (Cat#ab16663) rabbit monoclonal antibodies were purchased from Abcam (Cambridge, MA); GAPDH mouse monoclonal antibody (Cat#HC301) was purchased from TransGen Biotech. (Beijing, China); Cleaved Caspase-3 (Cat#9664S), E-cadherin (Cat#3195S), N-cadherin (Cat#13116S), Vimentin (Cat#5741S), and Snail (Cat#3879S) rabbit monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA); MMP-2 (Cat#sc-13594) and MMP-9 (Cat#sc-21733) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Western blot analysis

Tissue protein lysates were prepared by use of the Tissue Protein Extraction Reagent and Proteasome Inhibition Mixture (Cwbiotech, Beijing) according to the manufacturer's instructions. Cell protein lysates were prepared by using RIPA buffer (Pierce). Protein concentration was determined by using a BCA kit (Cwbiotech). Protein with 2× SDS loading buffer was boiled for 10 min, underwent SDS-PAGE and was transferred to PVDF membranes (Millipore), which were blocked with 5% nonfat milk, then incubated with primary antibody at 4 °C overnight, then horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were developed with ECL substrate (Millipore).

Generation of reporter constructs

The wild-type FSCN1-3' UTR reporter construct was constructed by amplifying and inserting the 3' UTR of FSCN1 into the psiCHECK-2 vector. Mutated FSCN1-3' UTR sequences were obtained by overlap extension PCR, and resulting fragments were inserted into psiCHECK-2 to generate mutated reporter constructs. All constructs were verified by DNA sequencing.

Luciferase reporter assay

Luciferase assay involved use of the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) following the manufacturer's instructions. Briefly, after 48-h transfection, growth media was removed and cells were washed gently with PBS twice, then 100 μl/well passive lysis buffer was added with gentle rocking for 15 min at room temperature. Cell lysates were collected for luciferase assay.

Immunohistochemistry staining and analysis

Paraffin sections were dewaxed and re-hydrated in ethanol in descending concentrations (100%, 90%, 80%, 70%). For antigen retrieval, samples were processed in an autoclave for 2 min, 15 sec with sodium citrate (pH 6). Endogenous peroxidase activity was blocked by immersing tissue sections in 3% H_2O_2 in methanol (v/v) at room temperature for 10 min, then washing with PBS. Nonspecific background staining was reduced by incubating sections with nonimmune goat serum (Boster Co., Wuhan, China) for 15 min at room temperature. Sections were incubated with antibody for FSCN1 (1:200, Vector Laboratories) overnight in a moist chamber at 4 °C and washed 3 times with PBST for 5 min. Sections were incubated with the secondary antibody from the Max Vision HRP-Polymer anti-Mouse IHC kit (MaxinBio, Fuzhou, China) for 15 min at room temperature, then washed 3 times for 5 min with PBST. The DAB substrate detection system was used (Vector Laboratories). Counterstaining was with hematoxylin, then sections were dehydrated and mounted with coverslips. Two independent pathologists, blinded to the clinical parameters, calculated the immunoreactivity score for FSCN1 expression. Immunohistochemical staining was assessed semiquantitatively by the proportion of tumor cells with positive staining and staining intensity. Staining intensity was scored as 0 (no staining), 1 (weak intensity), 2 (moderate intensity) and 3 (strong intensity). Percentage of positive staining tumor cells in tumor tissue was for score 1, ≤ 10%; 2, 11–50%; 3, 51–80%; and 4, 81-100%. The final assessment was calculated as mean combined score for staining intensity and positive-stained cell percentage, ranging from 1 to 7. Specimens were divided into 3 groups of expression by overall scores: negative (1), low expression (2-5), and high expression (6-7). Negative and low-expression specimens were defined as low expression in statistical analysis.

Cell proliferation assay

Cell proliferation was determined by using the Cell Counting Kit-8 (DOJINDO, Beijing). Moreover, the percentage of cells incorporating EdU was evaluated by using the Cell-Light EdU imaging kit (RiboBio Co., Guangzhou, China).

Cell adhesion assay

Hep-2 and TU177 cells transfected with miR-145-5p mimic or si-FSCN1 were plated in 96-well plates, then cell adhesion was measured by using the Cell Counting Kit-8 (DOJINDO). The absorbance was measured by using a microplate reader Multiscan MK3 (ThermoFisher Scientific), and background absorbance was corrected by using the CCK-8 solution without cells.

Apoptosis analysis

Apoptosis analysis of Hep-2 and TU177 cells involved use of the Annexin V Apoptosis Detection Kit I (BD, NJ, USA), and apoptosis was analyzed by FACSCalibur flow cytometry (BD, San Jose, CA, USA). Apoptotic cells in LSCC tumor tissues were detected by using the DeadEnd™ Colorimetric TUNEL System (Promega) following the manufacturer’s instructions.

Cell cycle analysis

An amount of 1×10^6 cells was harvested 48 h after transfection and fixed overnight in 70% ice-cold ethanol and 4 ℃. Cells were stained with 50 μg/ml propidium iodide (PI), 100 U/ml RNase A, and 0.2% Triton X-100 for 30 min, then quantified by FACSCalibur flow cytometry (BD), and data were analyzed by using ModFit software.

Transwell migration assay

After transfection, 1×10^6 cells were plated in 100 μl DMEM-F12 medium without serum, then incubated in Transwell plates at 37 °C and 5% CO_2 for 24 h and 48 h; the upper side of the filter membrane was wiped with a cotton swab to remove the cell debris. Cells on the lower side of the insert

filter were stained with crystal violet for 10 min. The absorbance at OD570 was measured by using the microplate reader Multiscan MK3 (ThermoFisher Scientific).

Cell invasion assay

Matrigel was thawed at 4 °C, then 40 µl Matrigel solution (Matrigel : medium=1:3) was added to a precooled Transwell insert and solidified in a 37°C incubator for 2 h to form a thin gel layer. Cells were resuspended with serum-free medium at 1 x 10^5 cells/well. The upper side of the filter membrane was wiped with a cotton swab to remove cell debris, then cells on the lower side of the insert filter were stained with crystal violet for 10 min and washed with PBS once. The absorbance at OD570 was measured by using the microplate reader Multiscan MK3.

Microscopy imaging and staining

Images were captured by using the JSM-6360LV scanning electron microscope (JEOL, Tokyo). F-actin staining was performed using Rhodamine-Phalloidin (Cytoskeleton, Inc., Denver, CO.) according to the manufacturer's instructions. Briefly, fixed cells were incubated with 200 µl Rhodamine-Phalloidin (200 nM), counterstained with DAPI (Sigma) and imaged by using a confocal laser-scanning microscope (Leica TCS SP8).

Laser capture microdissection

Frozen sections of LSCC or paired ANM fresh tissue was stained with Histogene staining solution (Thermo Fisher Scientific, Waltham, MA) and dehydrated in ascensional concentrations ethanol (70%, 80%, 90%, 100%). Laser capture microdissection was performed on Leica LMD6500 laser microdissection Systems (Leica Microsystems CMS GmbH, Wetzlar, Germany).

Primer sequences for DNA methylation of miR-145 promoter

1. Primer sequence for Massarray methylation analysis of miR-145 promoter

F：AGGAAGAGAGAGAGAGGAAGTTGTTAAATTTAGGT-3'

R：CAGTAATACGACTCACTATAGGGAGAAGGCTAATTTAAAACATAATTCATAAACCCT

2. Primer sequences for pyrosequencing methylation analysis of miR-145 promoter

Amplified fragments	Primer name	Nucleotide sequence
Fragment 1	miR-145-F1	TTTGGTAGGAGATTGGGGAATAT
	miR-145-R1	Biotin-CTCTTCTACATCCAACCCCATCTA
	Sequencing Primer 1	AGTTTTGGGGGTGGG
Fragment 2	miR-145-F2	TTTGGTAGGAGATTGGGGAATAT
	miR-145-R2	Biotin-CTCTTCTACATCCAACCCCATCTA
	Sequencing Primer 2	TTATTTTTTTTGAGAGTAATAA
Fragment 3	miR-145-F3	GGGTTGGATGTAGAAGAGAATTT
	miR-145-R3	Biotin-TTTCCAAAAATCCCCATCTTAA
	Sequencing Primer 3	TTAGTTGGTTTTTAGGGATA