TUMORIGENESIS AND NEOPLASTIC PROGRESSION

The Crosstalk between lncRNA-SNHG7/miRNA-181/cbx7 Modulates Malignant Character in Lung Adenocarcinoma

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Lung adenocarcinoma (LUAD) is a malignant tumor with poor patient survival and high patient mortality. Long noncoding RNA is profoundly involved in the tumorigenesis of LUAD. The present study explores the effect of small nucleolar RNA host gene 7 (SNHG7) on the progression of LUAD and its underlying mechanisms. SNHG7 was found to be down-regulated in LUAD tissues compared with normal tissues. Altered SNHG7 expression induced changes in cell proliferation and migration both *in vitro* and *in vivo*. Mechanistically, it was found that SNHG7 interacted with microRNA mir-181 and sequentially up-regulated cbx7. cbx7, which suppresses the Wnt/ β -catenin pathway in LUAD, was found to be a direct target of mir-181. Taken together, loss of SNHG7 in LUAD up-regulated mir-181 and then down-regulated the tumor suppressor cbx7. (*Am J Pathol* 2020, 190: 1343–1354; <https://doi.org/10.1016/j.ajpath.2020.02.011>)

Lung adenocarcinoma (LUAD) is one of the most lethal malignant tumors worldwide.^{1–3} Despite developments in surgical and chemotherapeutic treatments, the prognosis of patients with LUAD is still poor due to metastasis and recurrence. However, the mechanisms that induce metastasis are still unclear. Therefore, it is necessary to investigate new therapeutic targets or prognostic markers related to the tumorigenesis and metastasis of LUAD.

Long noncoding RNAs (lncRNAs) are non-protein-coding RNAs, which are longer than 200 nucleic acids. Accumulating evidence has indicated the involvement of lncRNA in tumor proliferation and metastasis. Aberrantly expressed lncRNAs were observed in different types of cancers. For example, HOTAIR is seen in hepatocellular carcinoma, PVT1 in colorectal cancer, and H19 in gastric cancer.^{4–6} lncRNA plays its critical role through various mechanisms such as lncRNA–miRNA interaction, lncRNA–protein interaction, and epigenetic modulation. In lncRNA–miRNA interaction, lncRNA acts as a competing

endogenous RNA (ceRNA) and sponges miRNA.⁷ In other words, lncRNA and mRNA affect each other through competitively combining with miRNA response elements (MREs). In this process, lncRNA regulates the level of target mRNA through sequestering miRNA. Zhuang et al⁸ reported that the lncRNA XIST inhibited hepatocellular carcinoma by sequestering mir-92. The interaction between XIST and mir-92 increased smad7, a target of mir-92.⁸ HOTAIR promoted renal cell carcinoma progression via regulating mir-138–dependent EZH2.⁹

The lncRNA small nucleolar RNA host gene 7 (SNHG7) has been reported as an oncogenic lncRNA in multiple types of tumors such as hepatocellular carcinoma, breast cancer,

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and colorectal cancer.¹⁰ SNHG7 was also found to promote malignant transformation in LUAD.¹¹ However, in this current study, the authors found an anti-tumor effect of SNHG7 in LUAD. In this study, SNHG7 was found to be down-regulated in LUAD tissues as compared with non-tumor tissues. Patients with high SNHG7 expression had a better prognosis. SNHG7 inhibited cell proliferation and migration in LUAD cells *in vitro* and *in vivo*. Further investigation revealed SNHG7 plays a role as an endogenous sponge of mir-181. mir-181 promotes LUAD cell proliferation and migration by targeting cbx7. SNHG7 abrogates the role of mir-181 in promoting LUAD proliferation and metastasis. Hence, the role of the SNHG7/mir-181/cbx7 pathway in LUAD proliferation and migration was investigated.

Materials and Methods

Tissues and Database

The high-throughput data of LUAD patients were obtained from the Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov>). A total of 112 pairs of tumor and adjacent nontumor tissues were used in this study. All samples were collected from LUAD patients who underwent surgery in the Zhejiang Provincial People’s Hospital. Written informed consent was obtained from all patients. The present study was approved by the ethics committee of the Zhejiang Provincial People’s Hospital.

Cell Culture

All human LUAD cell lines used in the present study were obtained from ATCC (Manassas, VA). These cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C with 5% CO₂.

Colony Formation and Cell Migration

Colony formation and cell migration assays were performed as previously described.¹² All experiments were performed in triplicate.

RNA Extraction and qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was then reverse transcribed to cDNA using a reverse transcription kit (Promega, Madison, WI). The quantitative PCR (qPCR) reaction was performed using the SYBR Green Mix (Applied Biosystems, Foster City, CA), and a miRNA cDNA Synthesis kit (TaKaRa Bio, Otsu, Japan) was used for the detection of miRNAs. *GAPDH* and *U6* were used as endogenous controls for mRNA and miRNA detection, respectively. All primers used in the present study are listed in Table 1. All experiments were performed in triplicate.

Table 1 Primers for Real-Time PCR

Construct	Sequence
<i>U6</i>	5′-CTCGCTTCGGCAGCACA-3′
<i>GAPDH</i>	F: 5′-CTGGGCTACACTGAGCACC-3′ R: 5′-AAGTGGTCTGTTGAGGGCAATG-3′
<i>Cbx7</i>	F: 5′-GCGTGCGGAAGGGTAAAGT-3′ R: 5′-GCTTGGGTTTCGGACCTCTC-3′
<i>SNHG7</i>	F: 5′-TTGCTGGCGTCTCGGTTAAT-3′ R: 5′-GGAAGTCCATCACAGGCGAA-3′
<i>mir-181</i>	5′-AACATTCAACGCTGTCGGTGAGT-3′
<i>SNHG7 (BSP)</i>	F: 5′-GTTTTGATTTAGGT AAGAGGTGAGG-3′ R: 5′-TCAAACCATAAACCAT TCTCAATAC-3′
<i>SNHG7</i> , primers for DNA methylation detection	F: 5′-TTTATGAGGGAGGGG TAGTAATATC-3′ R: 5′-AAAACGAAACCTAAA AATAAAACG-3′
<i>SNHG7</i> shRNA	5′-GGTCAATCCTCCAATGTAA-3′
<i>cbx7</i> shRNA	5′-GGGTAAAGTCGAGTATCTG-3′
Scramble shRNA	5′-TTCTCCGAACGTGTACAGT-3′

F, forward; R, reverse.

Bisulfate Sequencing

Genomic DNA was extracted and treated according to the guidelines of the Epi-kit (Qiagen, Hilden, Germany). The bisulfate-treated DNA was then amplified using PCR (Table 1). Afterward, the PCR products were subcloned into the vector. Subsequently, 10 clones of each group were randomly chosen and sent for sequencing. The sequencing results were analyzed using an online tool (Quantification Tool for Methylation Analysis, <http://quma.cdb.riken.jp>, last accessed September 9, 2019).

Detection of DNA Methylation Content

An EpiMark 5-mC analysis kit (New England Biolabs, Ipswich, MA) was used for the detection of 5-mC. Genomic DNA was incubated with T4 Phage b-glucosyltransferase, according to the manufacturer’s instructions. The DNA was then digested with the HpaII and MspI enzymes. Afterward, the digested products were amplified by PCR. The primers used are listed in Table 1. The 5-mC content was calculated according to a previously described method.¹³

Western Blots

Western blots were performed as previously described.¹² Antibodies used in our study are listed in Table 2.

Co-Immunoprecipitation

The cell line was cultured in 6-well plates. The cells were then harvested, and the co-immunoprecipitation was performed as previously described.¹²

Table 2 Antibodies for Western Blot

Antibody	Concentration	Specificity	Company
Cbx7	1:1000	Rabbit	GeneTex (Irvine, CA)
β-catenin	1:1000	Rabbit	CST (Danvers, MA)
cyclin D1	1:1000	Rabbit	CST
c-myc	1:1000	Rabbit	CST
TCF4	1:1000	Rabbit	CST
E-cadherin	1:1000	Rabbit	CST
N-cadherin	1:1000	Rabbit	CST
MMP7	1:1000	Rabbit	CST
c-jun	1:1000	Rabbit	CST
Vimentin	1:1000	Rabbit	CST
ZEB1	1:1000	Rabbit	GeneTex
ZEB2	1:1000	Rabbit	GeneTex
fra-1	1:1000	Rabbit	CST
GAPDH	1:1000	Rabbit	CST
GFP antibody	20 μl	Rabbit	GeneTex

CST, Cell Signaling Technology.

Constructs

The *cbx7* and *SNHG7* overexpression plasmids were purchased from Vigene Bioscience (Jinan, China) and GenePharma (Shanghai, China), respectively. The lentivirus used for *mir-181* overexpression and knockdown was purchased from Genechem (Shanghai, China). The shRNA sequences (Table 1) were packed in lentiviruses that were used to stably knockdown *SNHG7* and *cbx7* in the LUAD cell lines.

Luciferase Assay

mir-181 mimics or *mir-NC*, pGL3 plasmids with wild-type or mutant *cbx7* 3' untranslated region (3'-UTR), and a Renilla vector were cotransfected into cells. After 48 hours, luciferase activity was determined using a dual-luciferase reporter assay system (Promega). Renilla luciferase activity was used as an endogenous control. All experiments were performed at least three times.

In Situ Hybridization

LUAD tissues and adjacent nontumor tissues were dewaxed. Tissues with prehybridization solution were then hybridized at 65°C for 1 hour with 500 ng/mL *SNHG7* probe hybridization solution (Dako, Carpinteria, CA). Tissues were then cultured in a wet box at 55°C for 2 hours, followed by washing. Next, tissues were put into nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 1 hour. Finally, double distilled water was used to terminate the reaction. The following steps were performed according to the Ribo lncRNA Probe Mix (RiboBio, Guangzhou, China).

RIP

The plasmids pcDNA-SNHG7-MS2, pcDNA-SNHG7-mut-MS2, pcDNA-H19-MS2, and pcDNA-MS2 were

constructed and then transfected into LUAD cells. Cells were collected for RNA immunoprecipitation (RIP) using green fluorescent protein (GFP) antibody and a RNA immunoprecipitation kit (EMD Millipore, Burlington, MA). *mir-181* level was detected by qPCR.

For argonaute 2 (Ago2)-based RIP assay, cells were harvested and lysed using buffer in a RIP kit (EMD Millipore). The cell lysate was then incubated with magnetic beads coated with anti-Ago2 antibody (EMD Millipore). The lysate was treated with proteinase K. Finally, immunoprecipitated RNA was purified and detected by qPCR.

RNA Pulldown

Biotinylated miRNAs were transfected into LUAD cells. After 72 hours, cells were collected. Cell lysate was incubated with streptavidin-coated magnetic beads (Pierce Biotechnology, Rockford, IL). The enriched RNAs were then purified by TRIzol and subjected to qPCR.

The probe complementary to *SNHG7* was biotin-labeled. LUAD cells were transfected with *SNHG7*, *SNHG7*-mut, and *H19*. After 72 hours, cell lysates were incubated with biotin-labeled probe and streptavidin magnetic beads. The enriched miRNAs were then purified by TRIzol and subjected to qPCR.

Animal Model

Six-week-old male BALB/c nude mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and kept in specific pathogen-free conditions. The *SNHG7* knockdown and *mir-181* overexpressing PC9 cell lines were subcutaneously injected into the mice at a concentration of $2 \times 10^6/100 \mu\text{L}$ per mouse. All mice were euthanized at the end of the experiment. The tumor grafts from the mice were fixed. Tumors were measured and recorded every 5 days, and the tumor volumes were calculated as previously described.¹⁴ The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the Zhejiang Provincial People's Hospital.

Statistical Analysis

The statistical analysis was performed using SPSS software version 19.0 (IBM Corporation, Armonk, NY). The Pearson correlation coefficient and the χ^2 test were used to analyze the relationships. Other experimental data were analyzed via *t*-test. Values were considered statistically significant when $P < 0.05$.

Results

SNHG7 Is Down-Regulated in LUAD and Associated with Poor Survival

Integrated analysis of the TCGA and Genotype-Tissue Expression (GTEx) databases showed that lncRNA

Figure 1 Small nucleolar RNA host gene 7 (SNHG7) is down-regulated in lung adenocarcinoma (LUAD) and associated with poor survival. **A:** SNHG7 is down-regulated in LUAD in the Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. **Black lines** in graphs stand for mean expression. **B:** SNHG7 expression in clinical specimen is shown. **Black lines** indicate the mean expression. **C:** *In situ* hybridization (ISH) of tumor and nontumor tissues. **D:** Kaplan-Meier analysis of LUAD patients with SNHG7 high expression (highExp) and low expression (lowExp). **E:** SNHG7 expression in cell lines. $n = 483$ normal (**A**); $n = 347$ tumor (**A**); $n = 112$ normal and tumor (**B**). $*P < 0.05$, $**P < 0.01$. Original magnification, $\times 200$ (**C**). DFS, disease free survival; N, normal; OS, overall survival; T, tumor.

SNHG7 is down-regulated in tumor tissues as compared with normal tissues (Figure 1A and Supplemental Figure S1). To validate this result, SNHG7 expression was analyzed by qPCR in 112 pairs of tumor tissues and adjacent nontumor tissues that were obtained from LUAD patients. SNHG7 is significantly down-regulated in tumor tissues compared with nontumor tissues (Figure 1B). *In situ* hybridization also showed down-regulated SNHG7 in tumor tissues (Figure 1C). Kaplan-Meier analysis using data from the TCGA database showed that SNHG7 high expression indicated better prognosis in LUAD patients

(Figure 1D). Finally, SNHG7 expression was also detected in LUAD cell lines. H3235 has the lowest expression of SNHG7, whereas PC9 has the highest expression of SNHG7 (Figure 1E).

SNHG7 Is a Direct Target of mir-181

lncRNA always plays a role as a ceRNA by sponging miRNA.¹⁵ Through bioinformatics analysis, mir-181 was chosen as a target of lncRNA SNHG7 based on its involvement in carcinogenesis in LUAD.² To validate this

Figure 2 Small nucleolar RNA host gene 7 (SNHG7) is a direct target of microRNA mir-181. **A:** Relative luciferase activity of wild-type (WT) and mutant (mut) SNHG7 3'-untranslated region (UTR). The vertical lines in the top panel indicate the interaction between bases. **B:** mir-181 expression after SNHG7 knockdown and overexpression. **C:** Construct of SNHG7 transcripts combined with MS2 binding site. **D:** mir-181 enrichment was detected by MS2-based RNA immunoprecipitation (RIP). **E:** mir-181 enrichment was detected by RNA pull-down through biotin-labeled SNHG7 probe. **F:** SNHG7 enrichment was detected by RNA pull-down through biotin-labeled mir-181. **G:** SNHG7 and mir-181 enrichment was detected by argonaute 2 (Ago2)-based RIP. **H:** Correlation between SNHG7 and mir-181 in clinical samples. Red line indicates the regression line. $**P < 0.01$ versus mimics-NC; $††P < 0.01$ versus MS2-SNHG7-mut (mir-181); $§§P < 0.01$ versus SNHG7-mut (mir-181); $¶¶P < 0.01$ versus biot-N.C. Ctl, control; KD, knockdown.

prediction, luciferase assays were performed. Only wild-type SNHG7 could bind mir-181, but not SNHG7 with mutations in its MRE (Figure 2A). SNHG7 knockdown increased cellular mir-181 expression, whereas SNHG7 overexpression down-regulated mir-181 level (Figure 2B). To validate the direct binding between SNHG7 and mir-181, SNHG7 transcripts combined with MS2-binding sites were constructed (Figure 2C). This construct was cotransfected into LUAD cells with MS2bp-GFP plasmid, and then GFP antibody was used for immunoprecipitation. SNHG7 significantly enriched mir-181 (Figure 2D). The interaction

between SNHG7 and mir-181 was further validated by biotin-labeled SNHG7 probe (Figure 2E). Biotin-labeled mir-181 was used in inverse pull-down to explore whether mir-181 could pull down SNHG7. Only wild-type mir-181 could pull down SNHG7 (Figure 2F). Ago2 was reported to act as a bridge between lncRNA and miRNA.¹⁶ Ago2-RIP was used to test the binding between lncRNA and miRNA.¹⁷ In this study also, the Ago2-RIP assay was used. SNHG7 and mir-181 were enriched by Ago2-RIP as compared with IgG (Figure 2G). Lastly, a negative correlation was found between SNHG7 and mir-181 in LUAD

samples (Figure 2H). These results indicated the direct binding between SNHG7 and mir-181.

SNHG7 Inhibits Cell Proliferation and Migration through Competitively Binding to mir-181

Gene ontology analysis was performed on differently expressed genes between SNHG7-high and SNHG7-low groups in LUAD samples from the TCGA. Cell proliferation and adhesion were involved in these differently expressed genes (Figure 3A). Colony formation and transwell assays were then performed. SNHG7 knockdown increased cell proliferation and cell migration, and this trend was reversed by mir-181 knockdown (Figure 3, B and C). mir-181 could rescue the suppression of cell proliferation and cell migration induced by SNHG7 overexpression (Figure 3, B and C). As the epithelial-mesenchymal transition (EMT) process and Wnt/ β -catenin pathway was activated in various kinds of tumors, cell proliferation and migration were promoted. Thus, this study investigated the regulation of SNHG7 on the EMT and the Wnt/ β -catenin pathway. SNHG7 knockdown increased the EMT process and activated Wnt/ β -catenin downstream genes (Figure 3D). SNHG7 overexpression showed the opposite effect on EMT and Wnt/ β -catenin pathway. These results indicated that SNHG7 inhibits cell proliferation and migration through competitively binding to mir-181.

mir-181 Directly Targets cbx7

Based on predictions from MiRanda, TargetScan, and PicTar, cbx7 was selected on the basis of its involvement in carcinogenesis in LUAD. To validate that mir-181 directly targets cbx7, dual-luciferase assays were performed. The luciferase activity of the wild-type cbx7 3'-UTR was significantly decreased by mir-181, whereas the activity of the mutant cbx7 3'-UTR was not changed by mir-181 (Figure 4A). Additionally, mir-181 can complement the seed region of cbx7, and the MRE of SNHG7 was found to be the same as the cbx7 seed region (Figure 4B). To further validate the correlation of mir-181 and cbx7, the expression of mir-181 and cbx7 in tumor tissues and adjacent nontumor tissues was detected. The expression of mir-181 was negatively correlated with cbx7 (Figure 4C). The results of the Western blots showed that mir-181 promoted the EMT process, whereas cbx7 reversed this trend (Figure 4D). Colony formation and migration assays also showed that mir-181 overexpression promoted cell proliferation and migration, but cbx7 showed the opposite effect (Figure 4, E and F).

Down-Regulation of cbx7 Is Correlated with LUAD Progression

LUAD sequencing data from the TCGA database was analyzed. cbx7 was down-regulated in LUAD compared

with adjacent nontumor tissues (Figure 5A and Supplemental Figure S2). Kaplan-Meier analysis of TCGA database showed that low expression of cbx7 indicated poorer survival in LUAD patients (Figure 5B). Cbx7 expression was also validated in clinical samples using qPCR (Figure 5C). Colony formation and transwell assays were then performed. Compared with the control group, cbx7 knockdown increased cell proliferation and migration (Figure 5, D and E). In addition, cbx7 overexpression decreased colony numbers and cell migration.

cbx7 Suppresses Wnt/ β -Catenin Pathway in LUAD

The Wnt/ β -catenin pathway was activated in various kinds of tumors and promoted cell proliferation and migration. Therefore, the regulation of cbx7 on the Wnt/ β -catenin pathway was investigated. First, a canonical top-flash reporter system was used to detect the activity of the Wnt/ β -catenin pathway. The cbx7 knockdown activated the Wnt pathway, whereas cbx7 overexpression suppressed Wnt pathway activity (Figure 6A). Furthermore, direct downstream genes of the Wnt/ β -catenin pathway such as *c-myc*, *cyclin D1*, *c-jun*, *fra-1*, and *MMP-7* were also detected. These genes were activated by the cbx7 knockdown, and cbx7 overexpression suppressed the expression of these genes (Figure 6B). It was also found that cbx7 knockdown promoted EMT, whereas cbx7 overexpression inhibited EMT (Figure 6C). The authors then explored the mechanism of how cbx7 suppressed the Wnt/ β -catenin pathway. Kim et al¹⁸ reported that cbx7 inhibited the Wnt/ β -catenin pathway through DKK-1. However, in this study, the authors observed a direct interaction between cbx7, TCF4, and β -catenin (Figure 6D), and cbx7 decreased the endogenous interaction between TCF4 and β -catenin (Figure 6E). Taken together, these results suggest that cbx7 may inhibit Wnt/ β -catenin pathway by inhibiting the formation of TCF4 and β -catenin complex in LUAD.

lncRNA-SNHG7 Is Regulated by Methylation of Promoter

To explore the mechanism that induced down-regulated SNHG7 in LUAD, the SNHG7 promoter was analyzed. The UCSC Genome Browser showed that CpG islands were enriched in the SNHG7 promoter (Figure 7A). Methylation data from TCGA also showed a hypermethylation status in the SNHG7 promoter in tumor samples as compared with normal samples (Figure 7A). Furthermore, bisulfate sequencing showed that compared with SNHG7 high-expression samples, SNHG7 low-expression samples had more methylated CpG sites in their promoters (Figure 7B). In cell lines, 5-aza, a reagent that inhibits DNA methyltransferases (DNMTs) and induces hypomethylation in gene promoters, was used. 5-aza clearly reduced DNA methylation in the SNHG7 promoter (Figure 7C). These results

Figure 3 Small nucleolar RNA host gene 7 (SNHG7) inhibits cell proliferation and migration through competitively binding to microRNA mir-181. **A:** Gene ontology G0 analysis of genes between SNHG7 high group and low group. **Red boxes** indicate the target biological process. **B:** Colony formation in cells with SNHG7 knockdown (KD) or overexpression. **C:** Cell migration in cells with SNHG7 knockdown or overexpression. **D:** Alteration of epithelial-mesenchymal transition (EMT) markers and Wnt/ β -catenin down-stream genes in cells with SNHG7 knockdown and overexpression. $**P < 0.01$. Original magnification, $\times 200$ (C). Ctl, control.

Figure 4 **A:** Relative luciferase activity of wild-type (WT) and mutant (mut) cbx7 3'-untranslated region (UTR). **Vertical lines** indicate the interaction between bases. **B:** mir-181 can complement the seed region of cbx7, which is similar to the small nucleolar RNA host gene 7 (SNHG7) miRNA response element (MRE). **Red boxes** indicate the seed region of cbx7 and the MRE of SNHG7; **vertical lines**, the interaction between bases. **C:** Correlation between SNHG7 and mir-181 expression. **Red line** indicates the regression line. **D:** Alteration of epithelial-mesenchymal transition (EMT) markers in cells with mir-181 knockdown and overexpression. **E and F:** Colony formation and cell migration in cells with mir-181 knockdown (KD) or overexpression. ****** $P < 0.01$. Original magnification, $\times 200$ (F).

Figure 5 Down-regulation of cbx7 is correlated with lung adenocarcinoma (LUAD) progression. **A:** cbx7 is down-regulated in LUAD in the Cancer Genome Atlas (TCGA) database. **B:** Kaplan-Meier curve of LUAD patients with cbx7 high expression (highExp) and low expression (lowExp). **C:** Small nucleolar RNA host gene 7 (SNHG7) expression in clinical samples. **Black lines** indicate the mean expression (**A** and **C**). **D** and **E:** Colony formation and cell migration in cells with cbx7 knockdown (KD) or overexpression. $n = 483$ tumor (**A**); $n = 59$ normal (**A**). * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 200$ (**E**). Ctl, control; DFS, disease free survival; OS, overall survival.

indicate that SNHG7 expression may be regulated by CpG methylation in the promoter.

A xenograft mouse model was used to explore the effects of SNHG7 on tumor formation *in vivo*. Compared with the control group, SNHG7 knockdown and mir-181 overexpression significantly increased tumor volume and tumor weight (Figure 7D). Relative mRNA expression of cbx7, E-cadherin, and N-cadherin in the harvested tumor tissues was analyzed by qPCR (Figure 7E).

Discussion

Accumulating attention has been attracted to lncRNA because of its crucial role in gene regulation and cell process. In the present study, the authors identified a set of differently expressed lncRNAs in LUAD as compared with adjacent nontumor tissues, using the TCGA and GTEx databases. The low expression of SNHG7, a down-regulated lncRNA, is correlated with poor prognosis in LUAD. SNHG7 expression was down-regulated in LUAD tissues and cell lines. The authors also detected a biological effect

of SNHG7 in LUAD cell lines. SNHG7 knockdown, not only increased cell proliferation, but also promoted cell migration. SNHG7 overexpression showed the opposite effect in cell lines. *In vivo*, SNHG7 knockdown increased tumor volume and tumor weight.

The crosstalk between lncRNA and mRNA was mediated by competing MREs. Hu et al¹⁹ showed that the lncRNA HOTAIR promotes osteoarthritis progression through acting as a ceRNA. HOTAIR sponges mir-17 and increases the expression of alpha-1, 2 fucosyltransferase 2 (FUT2). In LUAD, lncRNA-UCA1 exerts oncogenic functions by targeting mir-193.²⁰ In this study, bioinformatics analysis predicted that SNHG7 contains an mir-181 binding site. The following luciferase assay, RIP, and RNA pulldown all demonstrated SNHG7 could significantly enrich mir-181. But SNHG7 with a mutation in MRE could not associate with mir-181. Cellular functional assay revealed that mir-181 overexpression markedly increased cell proliferation and migration through directly targeting cbx7, a critical tumor suppressor. *In vivo*, it was also found that SNHG7 acts as a tumor suppressor and mir-181 promotes tumorigenesis.

Figure 6 **A:** TOPFlash reporter assay in cells with cbx7 knockdown (KD) and overexpression. **B:** Alteration of Wnt/β-catenin downstream genes in cells with cbx7 knockdown and overexpression. **C:** Alteration of epithelial-mesenchymal transition (EMT) markers in cells with cbx7 knockdown (KD) and overexpression. **D** and **E:** Coimmunoprecipitation of cbx7, transcription factor 4 (TCF4), and β-catenin. ** $P < 0.01$. Ctrl, control; IP, immunoprecipitation; WB, Western blot.

Finally, it was demonstrated that SNHG7 inhibits LUAD proliferation and metastasis in a mir-181-dependent way.

In previous studies, SNHG7 was reported to be an oncogene. Li et al¹¹ reported that SNHG7 promotes colorectal cancer progression. She et al²¹ reported that SNHG7 acted as a tumor activator. But in the current study, it was found that SNHG7 inhibited tumorigenesis in LUAD. First, SNHG7 exhibits cellular- and tissue-specific functions in a context-dependent manner, which may explain the contradiction between the antitumor function of SNHG7 observed in this study and its pro-tumor action in Li et al's study.¹¹ Second, to expand the sample size, the TCGA and GTEx databases were used, which could supply more convincing data. Unlike She et al's study,²¹ the current study found that SNHG7 expression was down-regulated in tumor tissues as compared with adjacent nontumor tissues. In the current study, SNHG7 plays a role as a tumor suppressor. These difference between the current study and She et al's report²¹ may be attributed to

sample size. She et al's study used a small sample size ($n = 25$ tumor, $n = 25$ normal), whereas the current study used the TCGA and GTEx databases ($n = 483$ tumor, $n = 347$ normal). Thus, SNHG7 may play different roles depending on the cancer type, and the antitumor effect of SNHG7 in this study was based on a large-sample investigation.

This research also has limitations. First, it was found that the lncRNA SNHG7 was regulated by the methylation status of its promoter. It has been reported that lncRNA could be regulated by transcriptional factors.²² In the UCSC Genome Browser, it was also found transcriptional factor binding sites in the SNHG7 promoter. Thus, whether SNHG7 could be regulated by transcriptional factors needs further investigation. Second, it was found that mir-181 was regulated by SNHG7. But, through bioinformatic analysis, it was found that mir-181 could be regulated by other lncRNAs such as SNHG5, MALAT1, and XIST. It is unknown whether these lncRNAs exert their functions through mir-181 in LUAD.

Figure 7 Long noncoding (lnc) RNA small nucleolar RNA host gene 7 (lncRNA-SNHG7) is regulated by methylation of the promoter. **A:** CpG islands of the SNHG7 promoter in the UCSC Genome Browser (left panel). DNA methylation analysis of SNHG7 in lung adenocarcinoma (LUAD) and corresponding normal tissues generated from the 450 k DNA methylation data in the Cancer Genome Atlas (TCGA) database (right panel). Red box indicates the differentially expressed methylation sites. **B:** Bisulfate sequencing of SNHG7 high-expression and low-expression samples. **C:** Methylated and unmethylated DNA content in the SNHG7 promoter of cell lines treated with 5-aza. **D:** Tumor volume and tumor weight in a xenograft mouse model of SNHG7 knockdown (KD) and microRNA mir-181 overexpression. **E:** Relative mRNA expression of cbx7, E-cadherin, and N-cadherin in the harvested tumor tissues. Black lines indicate the mean expression (D and E); black dots, outliers (D and E). **F:** Schematic presentation of crosstalk between long noncoding RNA (lncRNA)-SNHG7/miRNA-181/cbx7 in LUAD. * $P < 0.05$, ** $P < 0.01$. Ctrl, control.

Collectively, in the present study, crosstalk between lncRNA-SNHG7/miRNA-181/cbx7 was demonstrated. SNHG7 exerts its function as a ceRNA through interacting with mir-181. This process protected cbx7

mRNA from degradation by mir-181 (Figure 7F). The effectiveness and safety of the lncRNA-SNHG7/miRNA-181/cbx7 pathway in LUAD therapy needs further investigation.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.02.011>.

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