BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Tumor-Promoting Activity of Long Noncoding RNA *LINC00466* in Lung Adenocarcinoma via *miR-144*—Regulated *HOXA10* Axis

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Previous investigations have implicated long noncoding RNAs in lung adenocarcinoma, which is an aggressive disease with poor prognosis and high mortality. Through the alteration of lung adenocarcinoma—related long noncoding RNA and miRNA based on microarray analysis, our aim was to understand the role of *LINC00466* and *miR-144* in lung adenocarcinoma progression. The relationship among *LINC00466*, *miR-144*, and *HOXA10* was also verified. Moreover, to examine whether the *LINC00466/miR-144/HOXA10* axis contributed to the cellular processes in lung adenocarcinoma, A549 and XWLC-05 cells were transduced with siRNA *LINC00466*, siRNA *HOXA10*, or *miR-144* mimic plasmids. Highly expressed *LINC00466* and *HOXA10* and lowly expressed *miR-144* were eventually revealed in lung adenocarcinoma tissues. *HOXA10* was down-regulated in response to the overexpression of *miR-144*, whereas inhibition of *LINC00466* decreased its binding to *miR-144*, thereby up-regulating *miR-144*, which, in turn, halted the lung adenocarcinoma progression. *LINC00466* silencing or *miR-144* up-regulation exerted an inhibitory role in the tumorigenicity, invasion, migration, and proliferation, and it also promoted apoptosis of lung adenocarcinoma cells. Furthermore, tumor formation was inhibited by knockdown of *LINC00466* or overexpression of *miR-144*. Taken together, *LINC00466* could restrain the *miR-144* expression to up-regulate *HOXA10* and, therefore, promote lung adenocarcinoma. (*Am J Pathol* 2019, 189: 2154–2170; <https://doi.org/10.1016/j.ajpath.2019.06.014>)

Lung cancer remains one of the top causes of worldwide mortality. To illustrate, >1.2 million new cases are diagnosed each year.¹ Lung adenocarcinoma represents a type of non—small-cell lung carcinoma. This is known as a form of lung cancer with specific etiology, molecular changes, and morphologic features.^{2,3} Cigarette use is one major risk factor for lung adenocarcinoma, although approximately 10% to 15% of lung adenocarcinomas are diagnosed in individuals without a history of smoking,⁴ which may be caused by environmental exposures and genetic susceptibility.^{5,6} Although progress has been made in treating lung cancer, the overall survival rate continues to be relatively low. The major issues in lung cancer treatment include fast onset of chemoresistance and predominantly late diagnosis.⁷

Long noncoding RNAs (lncRNAs) may contribute to the pathogenesis of different cancers, including lung adenocarcinoma.⁸ Genes related to different cell proliferation and

developmental pathways in lung adenocarcinoma were tracked by CpG sites; some of them were related to long noncoding RNAs, including *LINC00466*,⁹ indicating a potential correlation between lung adenocarcinoma and *LINC00466*. *LINC00466*, as one pivot regulator of autism spectrum disorder, is involved in different developmental, neural, or brain-related biological progress.¹⁰ Many miRNAs, including *miR-212*, *miR-451*, and *miR-221*, were involved in lung cancer through different cellular processes.^{11–13} *miR-144* exerts a fundamental function in different carcinomas (eg, colorectal cancer, gastric cancer, and hepatocellular carcinoma).^{14–16} *miR-144* also correlates with lung cancer progression, which is down-regulated in

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Table 1 Primer Sequence for RT-qPCR

Primer	Sequence
LINC00466	F: 5'-AGCCCTTCCGTCTTGTTACG-3' R: 5'-GCTTCCCAACTGCAGGTTTC-3'
miR-144	F: 5'-GCTGGGATATCATCATATACTG-3' R: 5'-CGGACTAGTACATCATATACTG-3'
HOXA10	F: 5'-GCCCCTTCCGAGAGCAGCAAAG-3' R: 5'-AGGTGGACGCTGCGGCTAATCTCTA-3'
GAPDH	F: 5'-CGTATTGGGCGCTGGTCACCAG-3' R: 5'-GTTGTCATGGATGACCTTGGCCAG-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'

F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HOXA10, homeobox A10; R, reverse; RT-qPCR, quantitative RT-PCR.

highly polluted regions of non-small-cell lung carcinoma.¹⁷ Homeobox A10 (*HOXA10*), a member of the HOX family, has been highlighted to regulate cell differentiation, maturation, development, and proliferation. *HOXA10* has been implicated in cancers, including gastric cancer, prostate carcinoma, and colorectal cancer.^{18–20} An increasing amount of evidence proposes that *HOXA10* could be targeted by different miRNAs to regulate cancer

development.^{20–22} Our aim was to study the role of *HOXA10* and *LINC00466* in lung adenocarcinoma.

Materials and Methods

Ethics Statement

The Animal Ethics Committee of the Second Hospital Affiliated to Jilin University (Changchun, China) approved the experimentation and animal use in this study. All efforts were made in a humane way to relieve suffering of the animals.

Lung Adenocarcinoma Cell Line Selection

Lung adenocarcinoma cell lines (GLC-82, H1299, XWLC-05, A549, and SPCA-1) and normal lung cell line HBE were purchased from the cell bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The five lung adenocarcinoma cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY). The normal lung cell line HBE was cultured in RPMI 1640 culture medium containing 10% neonatal FBS (Yuanmu Biotech Co, Ltd, Shanghai,

Figure 1 *LINC00466* binds to *miR-144*; *HOXA10* is a target gene of *miR-144*. **A:** The binding sites of *miR-144* and *LINC00466*. **B:** The luciferase activity of *LINC00466* wt/mut in cells transfected with the *miR-144* mimic/negative control (NC) mimic. **C:** The binding sites of *miR-144* and *HOXA10*. **D:** The luciferase activity of *HOXA10* wt/mut in cells transfected with *miR-144* mimic/NC mimic. The measurement data were analyzed by nonpaired *t*-test. The experiment was repeated three times. Data are expressed as means $\pm$ SD (**B** and **D**). **P* < 0.05 versus cells treated with NC mimic. Pct, percentage; UTR, untranslated region.

Figure 2 A549 and XWLC-05 cells are selected for later use. **A:** *miR-144* expression in lung adenocarcinoma tissues and normal lung tissues analyzed from The Cancer Genome Atlas database. **B:** The expression of *LINC00466*, *HOXA10*, and *miR-144* in lung adenocarcinoma cell lines (GLC-82, H1299, XWLC-05, A549, and SPCA-1) and normal lung cell line (HBE), detected by quantitative RT-PCR. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (**A** and **B**). $n = 46$ normal (**A**); $n = 521$ tumor (**A**). **** $P < 0.0001$ versus normal lung tissues; $^{\dagger}P < 0.05$ versus HBE cell line; $^{\dagger\dagger}P < 0.05$ versus GLC-82, H1299, XWLC-05, and SPCA-1 cell lines.

China). With the culture medium renewed every 24 hours, the cells were grown in monolayer. When cells reached 80% to 90% confluence, the process of cell passage was performed. The cell line exhibiting lowest *miR-144* expression was screened out with the use of quantitative RT-PCR.

Plasmid Construction and Cell Treatment

In accordance with the sequence of *LINC00466* and *miR-144* from the National Center for Biotechnology Information, the plasmids of blank vector, *LINC00466*, *LINC00466* negative control, siRNA (si)-*LINC00466* (the plasmid connected with a segment of silencing *LINC00466* sequence), negative control blank (the blank plasmid), siRNA negative control (the plasmid connected with a segment of disorder sequence), *miR-144* mimic, and si-*HOXA10* (the plasmid connected with a segment of silencing *HOXA10* sequence) were provided by Shanghai Sangon Biotech Company (Shanghai, China). These plasmids were then respectively delivered into the A549 cells using Lipofectamine 2000 (11668-019; Invitrogen, Carlsbad, CA).

Extraction and Quantitative Analysis of RNA

From A549 and XWLC-05 cells, the total RNA was isolated with an miRNeasy Mini Kit (217004; Qiagen, Hilden, Germany). Then, with the use of the Primescript RT Reagent Kit (RRO36A; Takara Biotechnology Co Ltd, Dalian, China), the extracted RNAs were reverse transcribed into cDNA. Real-time PCR was conducted using an SYBR Premix Ex Taq II reagent kit (RR820A; Takara Biotechnology Co Ltd) on an ABI7500 real-time quantitative PCR system (7500; ABI Company, Oyster Bay, NY). With glyceraldehyde-3-phosphate dehydrogenase regarded as an internal reference, relative quantification was used to calculate the fold changes. The primers provided by Takara Biotechnology Co Ltd are listed in Table 1.

Western Blot Analysis

Cells during the logarithmic phase were lysed using radioimmunoprecipitation assay lysis buffer that contained protease inhibitors (Roche, Mannheim, Germany). Consequently, after 10% SDS-PAGE separation, the cell lysates were transferred onto a polyvinylidene difluoride membrane. This was then incubated with 5% skim milk for blocking purpose. After that, diluted primary antibodies (Abcam Inc., Cambridge, MA) against human *HOXA10* (dilution 1:1000; ab191470), B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2)—associated X protein (dilution 1:2000; ab32503), and Bcl-2 (dilution 1:1000; ab32124) were used to incubate, followed by addition of horseradish peroxidase-conjugated mouse IgG secondary antibody (dilution 1:2000; ab97051; Shanghai Yanhui Biotechnology Co., Ltd., Shanghai, China). After this part of the procedure, the membranes were treated with enhanced chemiluminescence reagent (ECL808-25; Biomiga, San Diego, CA), which developed by the X-ray film (Shanghai Westang Bio-Tech Co., Ltd., Shanghai, China), with glyceraldehyde-3-phosphate dehydrogenase set as the internal control.

Immunofluorescence

Cells cultured on a coverslip were fixed with 4% paraformaldehyde, blocked with 2% FBS for 20 minutes, and incubated with 2.5 mL phycoerythrin-conjugated CD133 antibody at a temperature of 4°C for approximately 30 minutes. The coverslip was sealed using glycerol and then examined under a fluorescence microscope.

Flow Cytometry

After the processes of centrifugation and fixation, 100 μ L of the cell suspension was stained with RNAase-contained propidium iodide (50 μ g) alone for 30 minutes for the cell cycle measurement or with both of annexin V—fluorescein isothiocyanate (10 μ L) and propidium iodide (5 μ L) for 15

Figure 3 *LINC00466* and *HOXA10* are highly expressed in lung adenocarcinoma. **A:** *LINC00466* expression in lung adenocarcinoma and normal lung tissues analyzed from The Cancer Genome Atlas (TCGA) database. **B:** *HOXA10* expression in lung adenocarcinoma and normal lung tissues analyzed from TCGA database. **C:** *LINC00466* was predicted to be mainly located in the cytoplasm through website prediction (<http://lncatlas.org.eu>, last accessed May 28, 2019). **D:** Subcellular localization of *LINC00466* in A549 cells detected by fluorescence *in situ* hybridization assay. $n = 59$ normal (**A** and **B**); $n = 535$ tumor (**A** and **B**). **** $P < 0.0001$ versus normal lung tissues. Scale bars = 25 μm (**D**). Original magnification, $\times 400$ (**D**). CN RCI, the ratio of cytoplasmic to nuclear expression of lncRNA in A549 cells; FPKM, fragments per kilobase of exon model per million mapped fragments; HUVEC, human umbilical vein endothelial cell; NC, negative control.

Figure 4 *LINC00466* directly binds to *miR-144*. **A:** *LINC00466* enrichment detected by RNA pull-down assay. The negative control (NC)-bio-Probe group: A549 cells transfected with NC-bio-Probe; the wt-bio-*miR-144* group: A549 cells transfected with 50 nmol/L biotinylated wt-bio-*miR-144*; the mut-bio-*miR-144* group: A549 cells transfected with 50 nmol/L biotinylated mut-bio-*miR-144*. The above data were analyzed by one-way analysis of variance. **B:** *miR-144* enrichment detected by RNA-binding protein immunoprecipitation. The measurement data were analyzed by nonpaired independent-sample *t*-test. The experiment was repeated three times. Data are expressed as means $\pm$ SD (**A** and **B**). **P* < 0.05 versus cells treated with NC-bio-Probe; †*P* < 0.05 versus cells treated with mut-bio-*miR-144*; ‡*P* < 0.05 versus cells treated with IgG. AGO2, Argonaute 2.

minutes in a dim-lit room for the cell apoptosis measurement. The cell cycle and cell apoptosis were studied using a flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Fluorescence *in Situ* Hybridization Assay

The fluorescence *in situ* hybridization assay was conducted by using the Ribo lncRNA fluorescence *in situ* hybridization Probe Mix (red; RiboBio Co, Ltd, Guangzhou, China). Briefly, the cells in the 24-well plates (6×10^4 cells/well) were treated with 250 μ L prehybridization solution at a relatively high temperature of 42°C for 1 hour. After this process, 250 μ L hybridization solution containing 300 ng/

mL probe was used for cell incubation. The next day, the cell staining was performed with diluted DAPI staining solution for approximately 5 minutes. After sealing with an antifluorescence quencher, the results were observed with the use of the fluorescence microscope (Olympus, Tokyo, Japan).

Bioinformatics Website and Dual-Luciferase Reporter Gene Assay

After the prediction of binding sites of *LINC00466* and *miR-144* as well as *miR-144* and *HOXA10* online (Thomas Jefferson University, <https://cm.jefferson.edu/rna22/Interactive>, last accessed May 28, 2019), a dual-luciferase reporter gene assay was further used for verification. The pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) was used to construct pmirGLO-*LINC00466*

Figure 5 Overexpressing *LINC00466* inhibits *miR-144* expression and promotes *HOXA10* expression. The control group, A549 cells without transfection; the blank vector group, A549 cells transfected with blank plasmid; the *LINC00466* group, A549 cells transfected with *LINC00466* plasmid; the siRNA negative control (si-NC) group, A549 cells transfected with si-NC plasmid; the si-*LINC00466* group, A549 cells transfected with si-*LINC00466*; the NC group, A549 cells transfected with NC plasmid; the *miR-144* mimic group, A549 cells transfected with *miR-144* mimic; the si-*HOXA10* group, A549 cells transfected with si-*HOXA10*. **A:** Expression of *LINC00466*, *miR-144*, and *HOXA10* in A549 cells overexpressing or silencing *LINC00466*, detected by quantitative RT-PCR (RT-qPCR). **B:** Expression of *LINC00466*, *miR-144*, and *HOXA10* in A549 cells transfected with *miR-144* mimic, detected by RT-qPCR. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (**A** and **B**). **P* < 0.05 versus A549 cells without transfection; †*P* < 0.05 versus A549 cells transfected with blank vector, si-NC, or NC plasmids.

Figure 6 Cell proliferation is inhibited by *miR-144* and promoted by *LINC00466*. **A:** Proliferation of A549 cells interferes with *LINC00466* detected by cell counting kit-8 (CCK-8) assay. **B:** Quantification of results from **A**. **C:** Proliferation of A549 cells interferes with *miR-144* or *HOXA10* detected by CCK-8 assay. **D:** Quantification of results from **C**. **A** and **C:** Data at different time points were analyzed by repeated analysis of variance. **B** and **D:** Data at different time points were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (**B** and **D**). * $P < 0.05$ versus A549 cells without transfection; $^{\dagger}P < 0.05$ versus A549 cells transfected with blank vector, siRNA negative control (si-NC), or NC plasmids.

wild-type (wt) and mutant-type (mut) vectors. The sequences were designed and synthesized on the basis of the potential binding sequence on the 3'-untranslated region of *HOXA10* and *miR-144*. The *HOXA10*-wt sequence was 5'-UGGAUUUCCUUUAUACUGUG-3'; the *HOXA10*-mut sequence was 5'-UGGUUUAGCAUAUAUGACAG-3' (the underlined parts are not the binding sites of *HOXA10* to *miR-144*, so the base remains unchanged); the *LINC00466*-wt sequence was 5'-CCCCAGTAAAGCTACAAAGGGA TTACCAGCGTT-3'; and the *LINC00466*-mut sequence was 5'-GGGGGTGTAAAGGTTGTATCGCTTTTGCAGGGTT-3'. The wt or mut luciferase plasmids were then delivered into the cells. It was not a concern whether they contained *miR-144*. Afterward, the luciferase activity was evaluated with the use of Genecopoeia's Dual Luciferase Assay Kit (RG027; Beyotime Institute of Biotechnology, Beijing, China) by a Promega Glomax 20/20 luminometer fluorescence detector (E5311; Shaanxi Zhongmei Biotechnology Co., Ltd., Xian, China).

RNA Pull-Down Assay

To continue, the 50 nmol/L biotinylated wt-bio-*miR-144* and mut-bio-*miR-144* were delivered into the A549 cells, which were then lysed. After being precoated with RNase-free bovine serum albumin and yeast tRNA at a temperature of 4°C for 3 hours, the M-280 streptavidin magnetic beads (S3762; Chemical Company, St. Louis, MO) were used for cell incubation. After this, after washing by low-salt buffer (three times) and high-salt buffer (once), the bound RNA was purified to discover the *LINC00466* enrichment through RNA isolation and quantification.

RNA-Binding Protein Immunoprecipitation Assay

The binding ability of *LINC00466* to Argonaute 2 (AGO2) protein was revealed with the use of the RNA-binding protein immunoprecipitation kit (Millipore, Bedford, MA). A part of the cell extract served as the input, and the remaining part was incubated at 4°C overnight with magnetic bead-antibody complex. After the collection of magnetic bead-protein complexes, the RNAs of samples and inputs were extracted for quantitative RT-PCR. The antibodies were AGO2 (diluted 1:50; ab32381; Abcam, Cambridge, UK) and IgG (dilution 1:100; ab109489; Abcam) as a negative control.

Colony Formation Assay

As a result, 800 cells were inoculated to each 75-mm dish (single cells in suspension > 95%). Then, the cells were further incubated for another 9 days, fixed with formaldehyde for 20 minutes, and finally stained with 10% Giemsa for 20 minutes. Subsequently, the numerical amount of cell colonies with >20 cells in each dish was through the use of the anatomic lens. After this, the colony-forming efficiency was calculated.

Sphere Formation Assay

Moreover, the A549 and XWLC-05 cells were cultured in high-glucose Dulbecco's modified Eagle's medium with 10% FBS in 5% CO₂ at 37°C. Next, the cells were inoculated in a 6-well ultralow cell adhesion plate (the

Figure 7 Colony formation is inhibited by silencing *LINC00466* or *HOXA10* or by overexpressing *miR-144*. Colony formation of A549 cells interfered with *LINC00466* (A and C) and *miR-144* (B and D) detected by colony formation assay. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (C and D). * $P < 0.05$ versus A549 cells without transfection; † $P < 0.05$ versus A549 cells transfected with blank vector, siRNA negative control (si-NC), or NC plasmids.

density was <1000 cells/well) in 5% CO_2 at 37°C . After a period of 6 days, the tumor cell sphere was measured to be approximately $50\ \mu\text{m}$ in diameter. The cell sphere was detached with 0.25% trypsin and 0.02% EDTA at 37°C for 2 minutes and triturated to single cells. Afterward, the cells were counted and inoculated once again in a 6-well ultralow plate (BD, Franklin Lakes, NJ).

Cell Viability Detection

The cells were cultured with Dulbecco's modified Eagle's medium that contained 10% FBS in 96-well plates (1×10^4 cells/mL) with 5% CO_2 at a temperature of 37°C . After a period of 24 to 72 hours, each well of the plate was incubated with 10 μL of cell counting kit-8 solution (Sigma-Aldrich, St. Louis, MO) for approximately 2 hours. The OD

at the wavelength of 450 nm was obtained through a multimode microplate reader (NYW-96M; Peking Nuoya wei Instrument Co., Ltd., Beijing, China).

Scratch Test

Furthermore, when cell confluence reached around a percentage of 90%, scratches were generated using sterile pipette tips. After that, the cells were cultured with a serum-free medium. The migration distance was measured with the use of the Image-Pro Plus Analysis software version 7.0 (Media Cybernetics, Inc., Rockville, MD).

Transwell Assay

The upper side of a transwell filter chamber was covered by Matrigel (50 μL ; 356234; BD). The

Figure 8 Knocking down *LINC00466* or *HOXA10* or up-regulating *miR-144* inhibits sphere formation. Sphere formation of A549 cells interferes with *LINC00466* (A and C) and *miR-144* (B and D) detected by sphere formation assay. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (C and D). * $P < 0.05$ versus A549 cells without transfection; $^{\dagger}P < 0.05$ versus A549 cells transfected with blank vector, siRNA negative control (si-NC), or NC plasmids. Scale bars = 100 μ m (A and B). Original magnification, $\times 100$ (A and B).

transfected cells (1×10^5 cells/mL) were seeded at 37°C into the apical chamber of the transwell filter and incubated in a serum-free medium for exactly 24 hours (the basolateral chamber was added with medium containing 10% FBS). The invasive cells on the lower side of the filter were fixed with 5% glutaraldehyde, stained with 0.1% crystal violet, and counted under a microscope. The number of cells passing through matrix gum in each group served as an index to determine their invasive ability.

In Vivo Tumor Formation Assay

The anesthetized nude mice were subcutaneously inoculated with the A549 cells that were already transfected with *LINC00466*, *si-LINC00466*, *miR-144* mimic, or *si-HOXA10* plasmids (1×10^6 cells/200 μ L) at the back of their right

hind leg. Moreover, the mice were observed periodically every 4 days. After this, their tumor volume was calculated by the following equation: $(\text{length} \times \text{width})^2/2$.

Specifically, on the 25th day, the mice were euthanized with the tumors collected, with three tumor samples collected in each group.

Statistical Analysis

All data in this study were processed by SPSS 21.0 (IBM Corp., Armonk, NY). Measurement data were expressed as means $\pm$ SD. A *t*-test was performed to analyze the comparison between two groups. For the normally distributed data with homogeneity of variance, the non-paired independent-sample *t*-test was implemented. The normality test of data was performed using the Kolmogorov-Smirnov method, and one-way analysis of

Figure 9 The expression of *HOXA10* and B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) is decreased, whereas the expression of Bcl-2-associated X protein (Bax) is increased, by silencing *LINC00466* or overexpressing *miR-144*. The expression of *HOXA10*, Bax, and Bcl-2 at protein level in A549 cells interferes with *LINC00466* (A and B) and *miR-144* (C and D), detected by Western blot analysis. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (B and D). * $P < 0.05$ versus A549 cells without transfection; † $P < 0.05$ versus A549 cells transfected with blank vector, siRNA negative control (si-NC), or NC plasmids. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

variance was used to compare data among multiple groups with Tukey post hoc test when the data were normally distributed. The cell viability at different time points among different groups was compared by using repeated-measurement analysis of variance. $P < 0.05$ was considered significant.

Results

LINC00466 Binds to *miR-144*, which Targets *HOXA10*

The cells transfected with *miR-144* mimic exhibited lower luciferase activity of *LINC00466*-wt ($P < 0.05$), whereas they exhibited no difference in luciferase activity of *LINC00466*-mut ($P > 0.05$) (Figure 1, A and B), suggesting that *LINC00466* could exclusively bind to *miR-144*. Cells transfected with *miR-144* mimic depicted decreased luciferase activity of *HOXA10*-wt ($P < 0.05$), but displayed no difference in the luciferase activity of *HOXA10*-mut

($P > 0.05$), suggesting a targeting relationship between *HOXA10* and *miR-144* (Figure 1, C and D).

The A549 and XWLC-05 Cell Lines Are Chosen

Initially, the expression data of genes and miRNAs related to lung adenocarcinoma from The Cancer Genome Atlas database were analyzed on the basis of the R software, which showed that *miR-144* expression was lower in lung adenocarcinoma relative to that in a normal lung (Figure 2A). Next, the expression of *miR-144*, *LINC00466*, and *HOXA10* in different lung adenocarcinoma cell lines (GLC-82, H1299, XWLC-05, A549, and SPCA-1) and normal lung cell line (HBE) was measured. *miR-144* expression was significantly lower, and expression of *LINC00466* and *HOXA10* was higher, in the five lung adenocarcinoma cell lines relative to those in normal lung cell line HBE ($P < 0.05$), with the most significant change involving the expression level of *miR-144*,

Figure 10 Cells are arrested at the G₀/G₁ phase, and cell apoptosis is induced by silencing *LINC00466*. Cell cycle analysis (A and B) and cell apoptosis rates (C and D) of A549 cells interferes with *LINC00466*, detected by flow cytometry. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (B and D). * $P < 0.05$ versus A549 cells without transfection; $^{\dagger}P < 0.05$ versus A549 cells transfected with blank vector or siRNA negative control (si-NC) plasmids. FITC, fluorescein isothiocyanate; PI, propidium iodide.

LINC00466, and *HOXA10* found in the A549 and XWLC-05 cell lines (Figure 2B). Therefore, the A549 and XWLC-05 cell lines were used in the following experiment.

LINC00466 Regulates *HOXA10* through Competitively Binding to *miR-144*

Microarray-based gene expression analysis showed high expression of *LINC00466* and *HOXA10* in lung adenocarcinoma (Figure 3, A and B). Website prediction is available at IncAtlas (<http://lncatlas.crg.eu>, last accessed May 28, 2019), and fluorescence *in situ* hybridization assay further

presented that *LINC00466* was mainly located in the cytoplasm (Figure 3, C and D). Furthermore, RNA pull down, together with the RNA-binding protein immunoprecipitation assay, was performed to determine the binding ability of *LINC00466* to *miR-144* and *LINC00466* to AGO2, respectively. The results indicated that *LINC00466* enrichment was increased in wt-*miR-144* when compared with mut-*miR-144* ($P < 0.05$) (Figure 4A), revealing that *miR-144* directly binds to *LINC00466*. When compared with IgG, the binding of *LINC00466* with AGO2 was significantly increased, suggesting that *LINC00466* could bind to AGO2 ($P < 0.05$) (Figure 4B) and suggesting that *LINC00466* could competitively bind to *miR-144* through binding to AGO2.

Figure 11 Cells are arrested at the G₀/G₁ phase, and cell apoptosis is caused by elevating *miR-144*. Cell cycle analysis (A and B) and cell apoptosis rates (C and D) of A549 cells interfere with *miR-144*, detected by flow cytometry. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (B and D). * $P < 0.05$ versus A549 cells without transfection; $^{\dagger}P < 0.05$ versus A549 cells transfected with siRNA negative control (si-NC) or NC plasmids. FITC, fluorescein isothiocyanate; PI, propidium iodide.

Moreover, the targeting relationship among *LINC00466*, *miR-144*, and *HOXA10* was broadened and confirmed. After overexpressing *LINC00466*, A549 and XWLC-05 cells revealed down-regulation of *miR-144* but up-regulation of *LINC00466* and *HOXA10*, whereas those transfected with si-*LINC00466* showed the opposite results ($P < 0.05$) (Figure 5A). Moreover, the A549 and XWLC-05 cells after *miR-144* mimic treatment showed decreased *LINC00466* and *HOXA10* and increased *miR-144* ($P < 0.05$). Furthermore, cells after si-*HOXA10* treatment presented down-regulated *HOXA10* expression ($P < 0.05$) while having no evident change in the expression level of *LINC00466* and *miR-144* ($P > 0.05$)

(Figure 5B). Thus, overexpressing *LINC00466* has the potential to inhibit *miR-144* expression and promote *HOXA10* expression, while simultaneously overexpressing *miR-144* could inhibit the expression of *LINC00466* and *HOXA10*.

Silencing *LINC00466* and *HOXA10* or Overexpressing *miR-144* Inhibits Cell Proliferation, Colony Formation, and Sphere Formation in Lung Adenocarcinoma

The functions of *LINC00466*, *HOXA10*, and *miR-144* in lung adenocarcinoma A549 cells were detected through cell counting kit-8 (Figure 6), colony formation (Figure 7), and

Figure 12 Cell migration and invasion are inhibited by silencing *LINC00466*. Cell migration (A and C) and cell invasion (B and D) of A549 cells interfered with *LINC00466*, detected by scratch test (A and C) and transwell assay (B and D). **A:** Dashed lines denote the edge of areas without cells, and the distance between two dashed white lines is the scratch width. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (C and D). ^{*} $P < 0.05$ versus A549 cells without transfection; [†] $P < 0.05$ versus A549 cells transfected with blank vector or siRNA negative control (si-NC) plasmids. Scale bars = 50 μ m (B). Original magnification: $\times 40$ (A); $\times 200$ (B).

sphere formation (Figure 8) assays. The results showed that in A549 cells, overexpression of *LINC00466* promoted cell proliferation, colony formation, and sphere formation. However, it also silenced *LINC00466*, which induced the opposite results ($P < 0.05$). Nonetheless, after *miR-144* mimic or si-*HOXA10* treatment, the A549 cells showed inhibited cell proliferation, colony formation, and sphere formation ($P < 0.05$). Furthermore, the above experiments were repeated in XWLC-05 cells, the results of which were similar to those found in A549 cells (Supplemental Figures S1, S2, and S3). The aforementioned results demonstrated that cell proliferation, colony formation, and sphere

formation were inhibited by silencing *LINC00466* and *HOXA10* or overexpressing *miR-144*.

Depletion of *LINC00466* and *HOXA10* or Overexpression of *miR-144* Promotes Cell Apoptosis in Lung Adenocarcinoma

The functions of *LINC00466*, *HOXA10*, and *miR-144* in apoptosis of lung adenocarcinoma A549 cells were discovered through Western blot analysis (Figure 9) and flow cytometry (Figures 10 and 11). After overexpressing *LINC00466*, the expression of Bcl-2-associated X protein,

Figure 13 Cell migration and invasion are repressed by overexpressing *miR-144*. Cell migration (**A** and **B**) and cell invasion (**C** and **D**) of A549 cells interfere with *miR-144*, detected by scratch test (**A** and **B**) and transwell assay (**C** and **D**). **A:** Dashed lines denote the edge of areas without cells, and the distance between two dashed white lines is the scratch width. The measurement data were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as means $\pm$ SD (**C** and **D**). * $P < 0.05$ versus A549 cells without transfection; † $P < 0.05$ versus A549 cells transfected with siRNA negative control (si-NC) or NC plasmids. Scale bars = 50 μ m (**B**). Original magnification: $\times 40$ (**A**); $\times 200$ (**B**).

the proportion at G₀/G₁ phase, and cell apoptosis rate in A549 cells were reduced, whereas the expression of *HOXA10* and Bcl-2 and proportion at S phase in A549 cells were increased. Meanwhile, A549 cells transfected with si-*LINC00466* exhibited the opposite results ($P < 0.05$). In addition, in the A549 cells after the treatment of *miR-144* mimic or si-*HOXA10*, the expression of Bcl-2—associated X protein, the proportion at G₀/G₁ phase, and cell apoptosis rate were higher, whereas the expression of *HOXA10* and Bcl-2 and proportion at S phase were lower ($P < 0.05$). To elaborate, the aforementioned experiments were conducted in XWLC-05 cells again, the results of which were consistent with A549 cells (Supplemental Figures S4, S5,

and S6). Thus, silencing *LINC00466* or *HOXA10* or even just overexpressing *miR-144* enhanced apoptosis of lung adenocarcinoma cells.

Silencing *LINC00466* and *HOXA10* or Overexpressing *miR-144* Arrests Cell Migration and Invasion in Lung Adenocarcinoma

The effects of *LINC00466*, *HOXA10*, and *miR-144* in lung adenocarcinoma A549 cells were found through scratch test and transwell assay. Overexpressing *LINC00466* stimulated cell migration and invasion; however, silencing *LINC00466* in A549 cells also inhibited cell migration and invasion

Figure 14 Tumor formation and growth are inhibited by silencing *LINC00466* or overexpressing *miR-144*. **A–F:** Tumor volume of A549 cells interferes with *LINC00466* (**A** and **B**) and *miR-144* (**C** and **D**), detected by tumor formation in nude mice. Tumor growth of A549 cells interferes with *LINC00466* (**E**) and *miR-144* (**F**), detected by tumor formation in nude mice. **A** and **C:** Data at different time points were analyzed by repeated analysis of variance. **B** and **D:** Data at different time points were analyzed by one-way analysis of variance. The experiment was repeated three times. Data are expressed as mean $\pm$ SD (**B** and **D**). * $P < 0.05$ versus the nude mice injected with A549 cells without transfection; $^{\dagger}P < 0.05$ versus the nude mice injected with A549 cells transfected with blank vector, siRNA negative control (si-NC), or NC plasmids.

($P < 0.05$) (Figure 12). In addition, A549 cells after *miR-144* mimic treatment or si-*HOXA10* treatment showed repressed cell migration and invasion ($P < 0.05$) (Figure 13). Furthermore, the results of XWLC-05 cells coincided with A549 cells (Supplemental Figures S7 and S8). Therefore, absence of *LINC00466* and *HOXA10* or presence of *miR-144* repressed lung adenocarcinoma cell migration and invasion.

Silencing of *LINC00466* and *HOXA10* or Elevation of *miR-144* Inhibits Tumor Formation and Growth in Nude Mice

Consequently, the functions of *LINC00466*, *HOXA10*, and *miR-144* in lung adenocarcinoma tumor formation and

growth were validated in nude mice (Figure 14). Overexpressing *LINC00466* promoted tumor growth, whereas silencing *LINC00466* lowered the chances of tumor growth ($P < 0.05$). Meanwhile, nude mice after the treatment of *miR-144* mimic or si-*HOXA10* presented repressed tumor growth ($P < 0.05$). Thereby, depletion of *LINC00466* and *HOXA10* or elevation of *miR-144* could induce inhibition of tumor formation and growth of lung adenocarcinoma *in vivo*.

Discussion

Lung adenocarcinoma is a malignant tumor known to have high mortality. Special treatment for lung adenocarcinoma is urgently needed.^{3,5} LncRNAs were indicated to be critical regulators in cellular development and human diseases

Figure 15 The mechanism of *LINC00466* regulating proliferation, invasion, apoptosis, and migration in lung adenocarcinoma A549 cells. High expression of *LINC00466* is found in lung adenocarcinoma. Down-regulation of *LINC00466* can enhance the expression of *miR-144* by reducing the binding with *miR-144*, which would further inhibit the expression of *HOXA10* and, therefore, suppress migration, proliferation, and invasion and promote apoptosis of lung adenocarcinoma cells.

through the modulation of gene expression.²³ In this experiment, the main purpose was to verify the mechanism of *LINC00466* in lung adenocarcinoma progression.

In lung adenocarcinoma, *LINC00466* and *HOXA10* expression was found to be elevated and *miR-144* expression was found to be depleted. A few lncRNAs were highly expressed in lung adenocarcinoma, including *LINC00152*, *LINC01207*, and *LINC01614*, which advanced tumor progression.^{24–26} Lung cancers correlate with frequent and substantial changes in *HOX* gene expression.²⁷ Several *HOX* family members, such as *HOXB9*, *HOXA9*, and *HOXA10*, displayed high expression in lung cancer tissues and cell lines, which functioned as negative prognostic factors in lung cancer.²⁷ Consistent with this study, *HOXA10* was also highly expressed in human lung tumor tissues and cells. This has the potential to promote lung cancer development.²⁸ *HOXA10* antisense RNA was also up-regulated in lung adenocarcinoma.²⁹ Moreover, a study also indicated that relative to paired adjacent normal tissues, lung cancer tissues exhibited a lower *miR-144* expression.³⁰ Furthermore, *miR-144* was down-regulated in whole blood in lung adenocarcinoma³¹ as a positive regulator.

Another fundamental discovery was that *LINC00466* has the ability to bind to *miR-144*, which directly targeted *HOXA10*. *LINC00466* overexpression inhibited *miR-144* expression and promoted *HOXA10* expression. A study that investigated the lncRNA-miRNA-mRNA network in breast cancer showed that *LINC00466* may down-regulate *miR-144* in breast cancer.³² *HOXA10* is targeted by several miRNAs in different cancers: *HOXA10* is targeted by miR-135a in epithelial ovarian cancer, by *miR-494* in oral cancer, and by *miR-218* in hepatocellular carcinoma.^{22,33,34}

Furthermore, silencing *LINC00466* or *HOXA10* or over-expressing *miR-144* inhibited tumorigenesis, cell proliferation, migration, and invasion and promoted cell apoptosis of lung adenocarcinoma cells. *LINC00152* and *LINC01207* were highly expressed in lung adenocarcinoma, and knockdown of *LINC00152* or *LINC01207* suppressed cell growth and induced cell apoptosis in lung adenocarcinoma.^{25,35} LncRNA *HOTAIR* was a significant factor in lung cancer diagnosis and treatment, which had the ability to promote survival, invasion, proliferation, metastasis, and drug resistance of lung cancer cells.³⁶ Overexpression of *miR-144* was also capable of inducing cell apoptosis and inhibiting cell growth and tumorigenesis in non-small-cell lung carcinoma through down-regulation of zinc finger X-chromosomal.³⁷ Another study also revealed that *miR-144-5p* promotes the radiosensitivity of non-small-cell lung cancer cells via targeting activating transcription factor 2.³⁸ Silencing *HOXA10-AS* was found to attenuate lung adenocarcinoma progression through repression of proliferation, migration, and epithelial-mesenchymal transition as well as promotion of apoptosis in lung adenocarcinoma cells.²⁹ Therefore, *LINC00466*, *HOXA10*, or *miR-144* could be considered as potential therapeutic target for lung adenocarcinoma.

LINC00466 knockdown may exhibit an attenuated ability on the *HOXA10* expression by promoting *miR-144* expression, which regulates cell proliferation, invasion, migration, and tumorigenicity in lung adenocarcinoma (Figure 15). This present study provides evidence for the cross talk between *miR-144*, *LINC00466*, and *HOXA10*, which sheds new light on the treatment of lung adenocarcinoma.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.06.014>.

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