Sphere formation assay

For the sphere formation assay, we deposited cells into 6-well plates (Corning, NYC, NY, USA) containing serum-free Dulbecco's Modified Eagle Medium with 10 ng/mL fibroblast growth factor 2 (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin and 0.4% Bovine Serum Albumin (BSA). After a 10-day incubation period, we counted the spheres formed using a microscope. Experiments were performed in triplicate (N=3 in each group).

Cell Apoptosis Analysis

The Annexin-V-FITC Apoptosis Detection kit was used to measure apoptosis. After 24 hours starvation treatment, cells were washed with PBS and resuspended in 1×binding buffer at a concentration of 1×10^6 cells/ml. Subsequently, 5 μl of Annexin-V and 5 μl of PI were added to 100 μl of the cell suspension, and the mixture was incubated for 15 min in the dark. The analyses were performed using a flow cytometry. The experiments were repeated at least 3 times (N=3 in each group).

Wound healing assay

We carried out the wound healing assay by first seeding cells in 6-well plates and leaving them for 24 hours. After the cells had reached 80~90% confluency, a 10-μl pipette tip was used to make a straight line in the cell monolayer. Phosphate-buffered saline (PBS) was used to remove cell debris and the wounded cells were subjected to culture in fresh medium. At 0 and 48 hours, images were obtained, and the distance of the wound was measured. The cell migration rate was calculated as the distance of the wound at 0 hours – the distance of the wound after 48 hours)/the distance of the wound at 0 hours × 100%. The experiments were repeated at least 3 times. Experiments were performed in triplicate (N=3 in each group).

Transwell invasion assay

We performed assays to assess the invasive ability of cells by using Matrigel and Transwells (BD Biosciences, San Jose, CA, USA) to construct invasion chambers for the separation of cells with high and low invasion ability. After seeding cells in Matrigel and 100 μl serum-free RPMI-1640 at a density of 1×10^5 in 24-well Transwell plates with a 8-μm pore size polycarbonate filter membrane (Corning, NYC, NY, USA). Medium supplemented with 10% FBS was placed in the lower chamber of the Transwell. After 48 hours' incubation, we used methanol to fix the cells on the membrane's lower surface and then subject them to staining with 1% toluidine blue. After staining photographs of the membrane were taken under a microscope, and the number of invading cells was recorded. Experiments were performed in triplicate (N=3 in each group).

In vivo tumorigenicity experiment

After the mice construction, we monitored tumor growth at 3-day intervals. There were 5 mice in each group (N=5 in each group). Each experiment was performed in triplicate. For each animal, three different investigators were involved as follows: a first investigator administered different cells into the flanks of mice via subcutaneous injection based on the randomization criteria. This investigator was the only person aware of the construction group allocation. A second investigator was responsible for monitoring tumor growth at 3-day intervals. Finally, when the tumor size reached $1cm^3$, a third investigator sacrificed the mice and collected all the tumors from mice. The following formula was applied to calculate the tumor volume (V): V=3.14×L×W×H/6 (L: length, W: width, H: height). The Ethics Committee for Animal Experiments of the Tianjin Medical University Cancer Hospital and Institute granted its approval for the animal experimental protocol used in this research, and all experiments were carried out in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western blot

Radioimmunoprecipitation assay buffer was used for the extraction of total protein. We subjected total protein (an approximate amount of 30 μg) to electrophoresis in a 12% sodium dodecylsulfate polyacrylamide gel before transferring the separated proteins to polyvinylidene difluoride membranes. After being blocked in 5% BSA for

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1 hour at room temperature, the membranes were subjected to incubation overnight with the primary antibody at 4 °C at dilution ratios from 1:1,000 to 1:2,000. We bought rabbit anti-human Lin28A, Lin28B, GAPDH, ERK, p-ERK, JNK, p-JNK, P38, and p-P38 mABs from Cell Signaling Technology (CST, Danvers, MA, USA). The membranes were subjected to three 5-minute washes with tris-buffered saline with 0.05% Tween 20 (TBST), followed by 1-hour incubation with horseradish peroxidase–conjugated secondary antibody (1:4,000; Danvers, MA, USA) at room temperature. After 5 more 10-minute washes with TBST, we added enhanced chemiluminescence reagent to the membranes for the development of specific bands, and then used film for band visualization. Proteins were detected with the Chemi-Doc XRS Detection System (Bio-Rad, Hercules, CA, USA). Relative band density was analyzed with GAPDH/Actin serving as an internal reference.

RNA extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and quantitative Real-time PCR (qPCR) analysis

A TRIzol kit (Invitrogen, Grand Island, NY, USA) was employed for the extraction of total RNA in adherence with the instructions supplied by the manufacturer. Electrophoresis was used to determine the RNA's integrity. Spectrophotometry was adopted for the analysis of RNA concentration and purity. RNA samples were stored at –80 ℃. For synthesis of complementary DNA, we used equal quantities of total RNA (1 μg) in a 20-μL reverse transcriptase reaction mixture (Takara, Tokyo, Japan) For qPCR analysis, SYBR Premix Ex Taq™ (Takara, Tokyo, Japan) was used to detect amplified complementary DNA on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used are shown in *Table S1*. β-actin and HsnRNA U6 (GeneCopoeia, Guangzhou, China) served used as internal controls for mRNA expression and miRNA expression, respectively. For each sample, experiments were performed 3 times (N=3 in each group), and the ΔCT method was adopted. The relativemRNA and miRNA levels in each sample were calculated as $2^{-\Delta CT}$ ($\Delta CT = CT_{\text{target gene}} - CT_{\text{reference gene}}$.).

Dual-luciferase reporter assay

The interplay between Lin28A/B and let-7c was assessed by dual-luciferase reporter assay. The sequence of either Lin28A or Lin28B was subcloned into the psiCheck2.0 basic plasmid (Hanbio Co., Ltd., Shanghai, China). The mutant sequences of Lin28A/B (the mutant sides were located at the potential binding sites of Lin28A/B and let-7c) were also subcloned into the psiCheck2.0 basic plasmid (MUTA). Then, the well-grown HEK-293T cells were co-transfected with Lin28A/B/MUTA and miR-NC/let-7c mimic. Two days after transfection, the relative luciferase activities of Lin28A/B and MUTA were examined using the dual-luciferase reporter assay system.

Drug resistance assay

In the Cell Counting Kit-8 (CCK-8) assay to detect half inhibitory concentration (IC50), we applied and compared the effect of varied doses of cisplatin. Cells were seeded at a density of 1×10^4 per well in a 96-well plate, and varied doses of cisplatin mentioned were added. The cell proliferation was measured using CCK-8 assay after 48h. The cell survival rate was calculated according to the following equation: cell survival rate = OD value of experimental group/OD value of the blank control group × 100%. Cell inhibition rate = 1 - cell survival rate. The linear regression was used to analyze the correlation between cisplatin concentration and cell inhibition rate, and calculated the IC50 values of different cells. The experiments were repeated at least 3 times (N=3 in each group).

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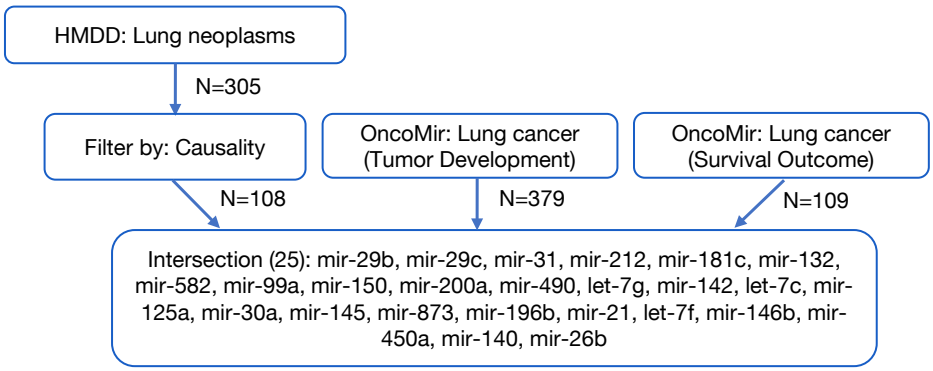

Figure S1 The workflow for filtering the most critical microRNAs (miRNAs) involved in lung cancer development in online databases.

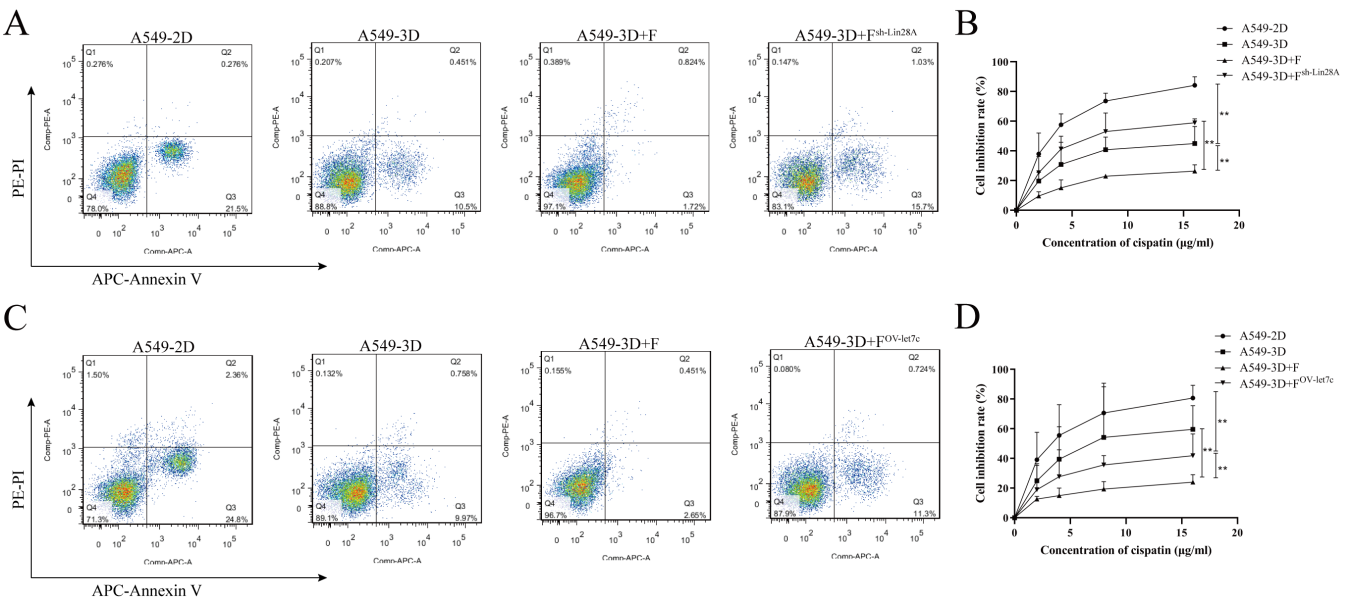

Figure S2 Knockdown of Lin28A or over-expression of let-7c weakened the resistance to apoptosis and chemotherapy drugs of lung cancer stem cells (LCSCs). (A) Results of Annexin-V apoptosis assays among A549 cells from the 2D culture (A549-2D cells), A549 cells from the 3D culture (A549-3D cells), A549 cells from the 3D culture with growth factors (A549-3D+F cells), and A549 cells over-expressing let-7c from the 3D culture with growth factors (A549-3D+$F^{OV\text{-}let7c}$ cells). (B) A549 cells from 4 groups were treated with several doses of cisplatin. The linear regression was drawn by drug concentration (X axis) and cell growth inhibition rate (Y axis) and half inhibitory concentration (IC50) was obtained. (C) Results of Annexin-V apoptosis assays among A549-2D, A549-3D, A549-3D+F, and A549 cells knockdown of Lin28A from the 3D culture with growth factors (A549-3D+$F^{sh\text{-}Lin28A}$ cells). (D) A549 cells from 4 groups were treated with several doses of cisplatin, followed by calculated IC50.

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Table S1 Primer sequences for realtime quantitative PCR (RT-qPCR)

Gene/miRNA	Forward primer	Reverse primer
Lin28A	5'-ACAATGGGTGGGGGCTATTC-3'	5'-GTGTGAACCCAAGCCTGAGA-3'
Lin28B	5'-GCCCCTTGGATATTCCAGTC-3'	5'-TGACTCAAGGCCTTTGGAAG-3'
E-cadherin	5'-TGCCCAGAAAATGAAAAAGG-3'	5'-GTGTATGTGGCAATGCGTTC-3'
N-cadherin	5'-ACAGTGGCCACCTACAAAGG-3'	5'-CCGAGATGGGGTTGATAATGC -3'
β-catenin	5'-TGGTGACAGGGAAGACATCA-3'	5'-CCATAGTGAAGGCGAACTGC-3'
Snail	5'-AGCCTGGGTGCCCTCAAGAT-3'	5'-AGGTTGGAGCGGTCAGCGAA-3'
Slug	5'-TGCCTGTCATACCACAACCAGA-3'	5'-GGAGGAGGTGTCAGATGGAGGA -3'
Zeb1	5'-ACCTGCCAACAGACCAGACAGT-3'	5'-ACATCCTGCTTCATCTGCCTGAG-3'
Twist1	5'-ACTTCCTCTACCAGGTCCTCCAG-3'	5-CCTCCATCCTCCAGACCGAGAA-3'
Oct4	5'-TCCACTTTGTATAGCCGCTGG-3'	5'-TGCATACACACAAACACAGCAA-3'
Sox2	5'-GGATAAGTACACGCTGCCCG-3'	5'-ATGTGCGCGTAACTGTCCAT-3'
Nanog	5'-CAATGGTGTGACGCAGGGAT-3'	5'-GACTGGATGTTCTGGGTCTGG-3'
Aldh1	5'-CCGTGGCGTACTATGGATGC-3'	5'-CGCAATGTTTTGATGCAGCCT-3'
miR-29b-5p	5'-CTGGTTTCACATGGTGGCTTAG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-29c-3p	5'-TAGCACCATTTGAAATCGGTTA-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-31-3p	5'-TGCTATGCCAACATATTGCCAT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-212-3p	5'-TAACAGTCTCCAGTCACGGCC-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-181c-5p	5'-AACATTCAACCTGTCGGTGAGT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-132-3p	5'-TAACAGTCTACAGCCATGGTCG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-582-5p	5'-TTACAGTTGTTCAACCAGTTACT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-99a-5p	5'-AACCCGTAGATCCGATCTTGTG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-150-5p	5'-TCTCCCAACCCTTGTACCAGTG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-200a-3p	5'-TAACACTGTCTGGTAACGATGT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-490-3p	5'-CAACCTGGAGGACTCCATGCTG-3'	5'-GAACATGTCTGCGTATCTC-3'
let-7g-3p	5'-CTGTACAGGCCACTGCCTTGC-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-142-3p	5'-TGTAGTGTTTCCTACTTTATGGA-3'	5'-GAACATGTCTGCGTATCTC-3'
let-7c-5p	5'-TGAGGTAGTAGGTTGTATGGTT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-125a-5p	5'-TCCCTGAGACCCTTTAACCTGTGA-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-30a-5p	5'-TGTAAACATCCTCGACTGGAAG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-145-3p	5'-GGATTCCTGGAAATACTGTTCT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-873-5p	5'-GCAGGAACTTGTGAGTCTCCT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-196b-5p	5'-TAGGTAGTTTCCTGTTGTTGGG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-21-5p	5'-TAGCTTATCAGACTGATGTTGA-3'	5'-GAACATGTCTGCGTATCTC-3'
let-7f-3p	5'-CTATACAGTCTACTGTCTTTCC-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-146b-3p	5'-GCCCTGTGGACTCAGTTCTGGT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-450a-5p	5'-TTTTGCGATGTGTTCCTAATAT-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-140-5p	5'-CAGTGGTTTTACCCTATGGTAG-3'	5'-GAACATGTCTGCGTATCTC-3'
miR-26b-3p	5'-CCTGTTCTCCATTACTTGGCT-3'	5'-GAACATGTCTGCGTATCTC-3'
U6	5'-CGCTTCGGCAGCACATATAC-3'	5'-CAGGGGCCATGCTAATCTT-3'
β-actin	5'-TGGTGACAGGGAAGACATCA-3'	5'-CCATAGTGAAGGCGAACTGC-3'

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