**Supplementary information**

# Optimized thyroid transcription factor-1 core promoter-driven microRNA-7 expression effectively inhibits the growth of human non-small-cell lung cancer cells

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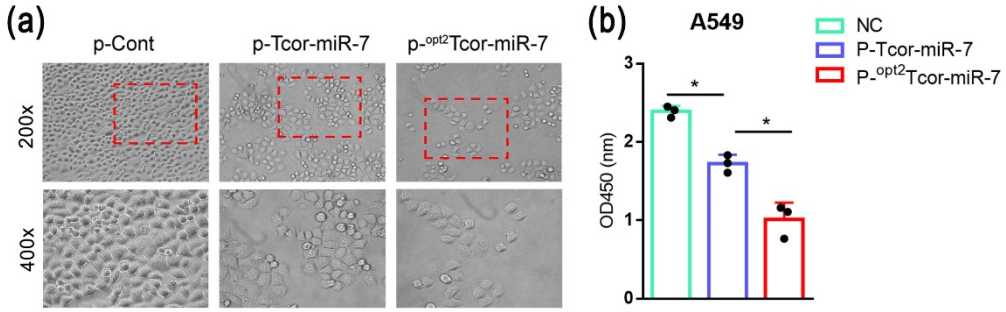


**Fig. S1 $^{opt}$TTF-1 promoter-driven miR-7 expression effectively suppresses the growth of human NSCLC cells *in vitro*. (a, b) Human NSCLC A549 cells were transiently transfected with p-Cont, p-Tcor-miR-7 or p-$^{opt2}$Tcor-miR-7 *in vitro*. Cells were harvested after 48 h. The morphology of cells was observed by microscopy, and the proliferation of A549 cells was measured by CCK-8 assay (magnification 200× or 400×). $^{*}$*P*<0.05.**

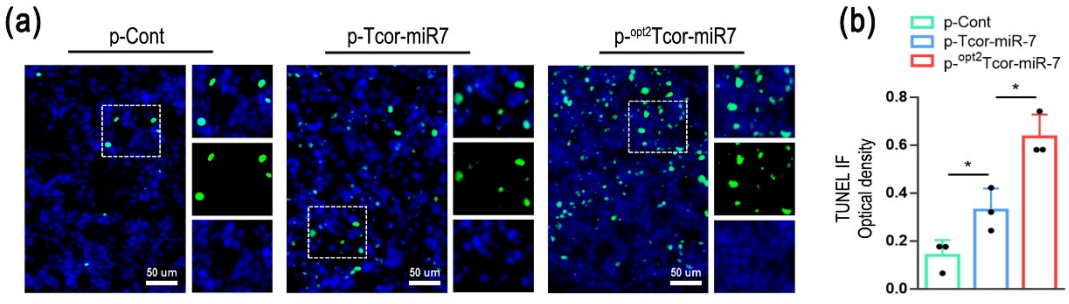


**Fig. S2 $^{opt}$TTF-1 promoter-driven miR-7 expression induces the apoptosis of NSCLC cells *in vivo*. (a, b) Human NSCLC 95D cells xenografts were established in the left flank of female BALB/c nude mice (*n*=5). Once the tumor formation was complete, the plasmid of p-Cont (100 mg), p-Tcor-miR-7 (100 mg) or p-$^{opt2}$Tcor-miR-7 (100 mg) were respectively injected through the tail vein in nude mice once every 3 d, for a total of five times. At the end of the experiment, all mice were sacrificed and the tumor tissues were obtained. The cell apoptosis (TUNEL) in tumor tissues were analyzed by immunofluorescence assay and calculated (scale bars, 50 μm). $^{*}$*P*<0.05.**

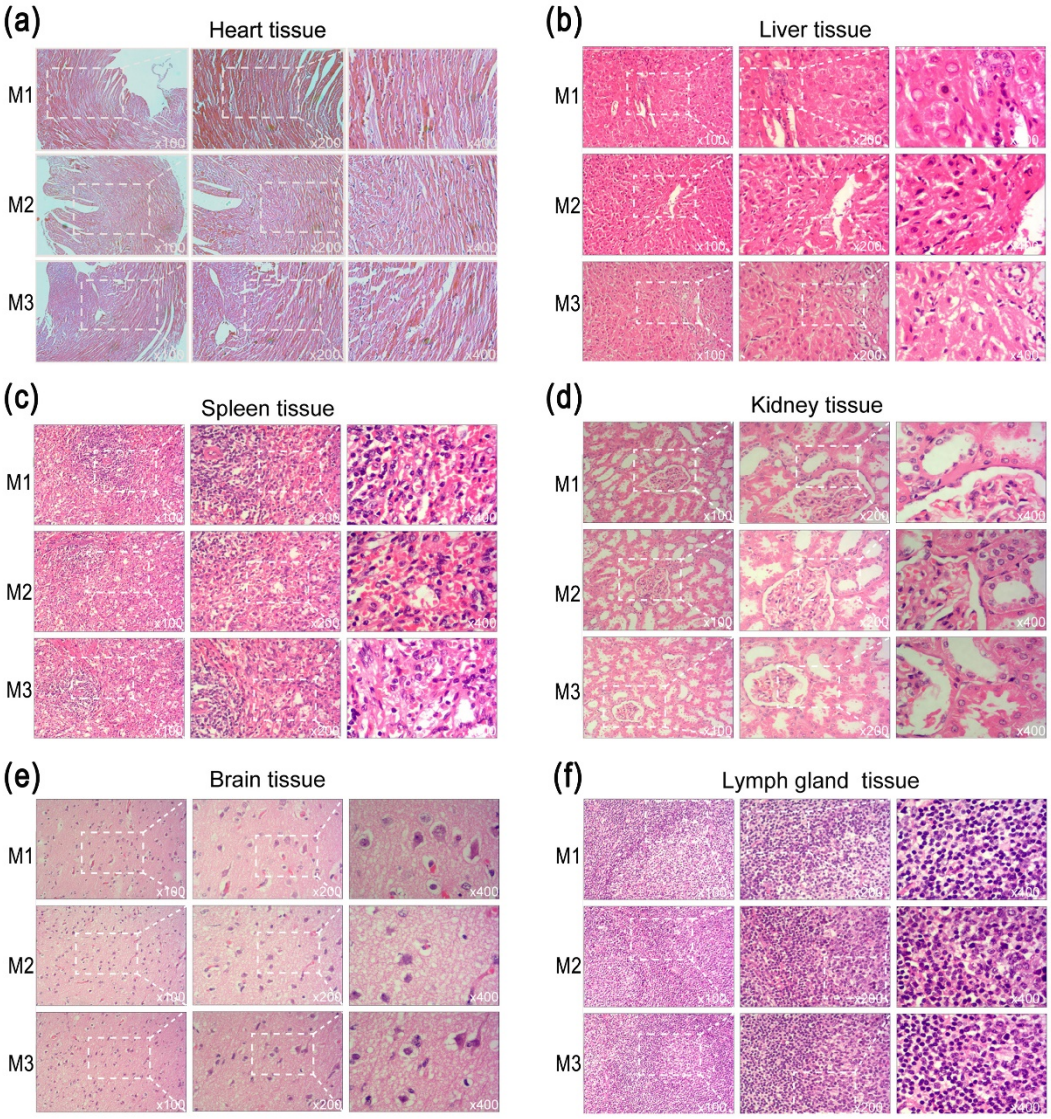


**Fig. S3 The influence of $^{opt}$TTF-1 promoter-driven miR-7 expression on the major organs and tissues of murine tumor-bearing model. Human NSCLC 95D cells xenografts were established in the left flank of female BALB/c nude mice (*n*=5). Once the tumor formation was complete, the plasmid of p-Cont (100 mg), p-Tcor-miR-7 (100 mg) or p-$^{opt2}$Tcor-miR-7 (100 mg) were respectively injected through the tail vein in nude mice once every 3 d, for a total of five times. At the end of the experiment, all mice were sacrificed and the tumor tissues were obtained. (a-f) The major organs or tissues were stained with hematoxylin and eosin (HE)-staining (magnification 100×, 200× or 400×).**

## Materials and methods

### 1. Construction of eukaryotic vector

The gene of miR-7 was amplified by PCR from DNA derived from human 95D cells using forward primer (5'-CGACGCGTAAGAGAGAAATGAGCCACTTGC) and reverse primer (5'-CCCAAGCTTCCTGCCACAGTGGGGGATG), and then subcloned into Kpn1 and Bam H1 sites of pEGFP-N1 eukaryotic vector to generate pEGFP-N1-miR-7 vector (called p-miR-7). Next, in order to construct pEGFP-N1-basic-TTF-1-promoter-miR-7 (called p-T-miR-7) vector, the promoter region of TTF-1 was amplified from DNA derived from human 95D cells and subcloned into Ase1 and Kpn1 sites of p-miR-7 vector using forward primer (5'-CGGGGTACCTGTTT CGGCAACTAC) and reverse primer (5-CGACGCGTCCTTCTGGGTCCTT). In addition, we also constructed the corresponding vectors containing different lengths of TTF-1 promoter (p-T-miR-7, p-T1612-miR-7, p-T1229-miR-7, p-T871-miR-7) according to the above methods. For our optimized sequence, two or four distinct sequences in TTF-1 core promoter were optimized to form more NF-1 binding sites by DNA sequence synthesis technology. Then, the related vectors (including p-$^{opt1}$Tcor-miR-7 and p-$^{opt2}$Tcor-miR-7) were synthesized according to the above methods. The recombinant plasmid was cleaved with restriction enzyme and the target fragment was verified by DNA sequencing. Endofree plasmid giant kit (QIAGEN) was used to extract endotoxin-free plasmids. Then, according to the manufacturer's instructions, Lipofectamine-3000 (Invitrogen) was used in subsequent experiments to instantly transfer the relative plasmid to the cultured cells.

### 2. Cell culture

Human lung cancer 95D cells were purchased from Chinese Academy of Life Sciences, the other cell lines including human lung cancer A549 cells, human normal bronchial epithelial BEAS-2B cells, human breast cancer MB231 cells and human colon cancer SW620 cells were purchased from American Type Culture Collection (ATCC). All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% dual antibodies (penicillin and streptomycin) at 37 °C, 5% $CO_2$.

### 3. Cell transfection

Cells were seeded at 60%–80% confluence, and transfected with the designated vector using Lipofectamine 3000 transfection kit (Thermo). All steps were performed according to the manufacturer’s instructions.

### 4. Real-Time PCR (qPCR) assay

The primers were purchased from Shanghai Sangon Biological Engineering, and the other reagents were obtained from TAKARA Bio. Next, RT-PCR and qPCR were performed according to the manufacturer’s instructions. The primer sequences can be seen in Table S1. Data were analyzed using $2^{-\Delta\Delta Ct}$ for relative expression of these indicated genes.

**Table S1 Real-time PCR primers sequences**

| Gene | Sequence (5'→3') |
|---|---|
| CDK1 forward | AGCGAGCAAGAAGGAGCGTG |
| CDK1 reverse | TCGTCGCGAGTGCAAACTGC |
| CDK2 forward | GTCTCGCTTAGTAGCTTCGA |
| CDK2 reverse | AGTGCTCTAGGCGCGTATG |
| CDK4 forward | GCTGGTGACGGTCACGCCT |
| CDK4 reverse | TACTGCGTTGATGCGACTG |
| CDK6 forward | TAGCCTGTACTTCGTCGAAGC |
| CDK6 reverse | TGTCGGTCAGACTGTGAATC |
| CXCR4 forward | AGTCGCCTTCTACTCCACG |
| CXCR4 reverse | CGTCAGGCTGTGGATGGCTA |
| E-cadherin forward | TGCTAGTGCTCCTCTGGCTC |
| E-cadherin reverse | ATCCTGTCCGCATCCGTCTT |
| MMP-2 forward | ATCCCCAACCTTTACCACG |
| MMP-2 reverse | TCAGAACCGACCCTACAA |
| MMP-3 forward | TGCTCGCCTACAGTTACCTG |
| MMP-3 reverse | GCATCTGCTCGTCCCAGTC |
| MMP-9 forward | TCGTCGCCATCACTGTCATG |
| MMP-9 reverse | GCCTCTACTTGTGGCTCTG |
| miR-7 forward | CGGCGGTGGAAGACTAGTGATT |
| miR-7 reverse | ATCCAGTGCAGGGTCCGAGG |
| U6 forward | AGAGAAGATTAGCATGGCCCCTG |
| U6 reverse | ATCCAGTGCAGGGTCCGAGG |
| GAPDH forward | TGCACCACCAACTGCTTAGC |
| GAPDH reverse | GCATGGACTGTGGTCATGAG |

## 5. Cell proliferation assay

Human NSCLC cells were seeded in 96-well plates at $1\times10^4$/well and transiently transfected with p-Tcor-miR-7 plasmid, p-$^{opt2}$Tcor-miR-7 plasmid, or p-Cont plasmid. At the indicated time points, cells in each well were treated with diluted CCK8 reagent (1:9) and incubated for 2 h at 37 °C in the dark. The absorbance was measured with a spectrophotometer at 450 nm. For colony-formation assay, cells were transfected as described above. Next, cells of 200/well and 800/well were seeded in 6-well plates and then cultured for 15 days at 37 °C in a humidified atmosphere of 5% $CO_2$. Finally, colonies were stained with crystal violet, and the cell colonies were counted and photographed.

## 6. Wound-Healing assays

Human NSCLC 95D cells were transiently transfected with p-Cont plasmid, p-Tcor-miR-7 plasmid, or p-$^{opt2}$Tcor-miR-7 plasmid in 24-well plate. Then, a single scratch wound was created using a micropipette tip. The first image was obtained with a microscope at 0 h and the second

image was harvested at 48 h post wounding. Observations were expressed as percent wound healing, calculated as follows: [(initial wound area−final wound area)/initial wound area] × 100%.

**7. Animal experiment**

Female nude mice (BALB/C, 4 weeks old) were purchased from iCell Bioscience Inc, Shanghai. License number: SCXK (Beijing) 2019-0010. All animals were kept in the SPF laboratory of Department of Immunology, Zunyi Medical University. For construction of subcutaneous xenograft model, 0.2 mL human NSCLC 95D cells ($7.0\times10^6$) were subcutaneously injected into the right side of nude mice. Once the tumor was formation, the plasmid of p-Cont(100mg), p-Tcor-miR-7(100mg) or p-$^{opt2}$Tcor-miR-7(100mg) were respectively injected through tail vein in nude mice once every three days, for a total of five times. Meanwhile, the volume of tumors was determined accordingly. After 18 days, all mice were killed. The collected mouse eyeball blood was used to detect various biochemical indicators, and the removed tumor tissue and other organs were weighed and then stored in a −80 °C refrigerator for subsequent experiments.

**8. Immunohistochemistry**

The tumor tissues of three groups of mice were fixed with 4% paraformaldehyde and then embedded in paraffin. Next, cut into 5 μm thick slices and dewaxing. The repaired specimens were incubated overnight with rabbit anti-human p-ERK antibody (Cell Signaling Technology; No. 4370) or rabbit anti-human p-AKT antibody (Cell Signaling Technology; No. 4060) at 4 °C. Finally, the DAB kit is used for dyeing according to the manufacturer's instructions.

**9. Immunofluorescence**

The prepared frozen sections or cell climbing sections were incubated with an indicated dilution of Ki-67 antibody (sc-7907, rabbit-anti-mouse, Santa Cruz Biotechnology), TUNEL reaction mixture (11684817910, In situ Cell Death Detection Kit, Roche), p-ERK antibody (Cell Signaling Technology; No. 4370), p-AKT antibody (Cell Signaling Technology; No. 4060) or NDUFA4 antibody (Santa Cruz Biotechnology; No. Sc-517091) over night at 4 °C. The next day, samples were washed in PBS for 3 times, and then the secondary antibody anti-rabbit immunoglobulin labeled with fluorescence was added in the dark at room temperature for 1 h. After washing with PBS for 3 times, the nuclei were stained by DAPI protected from light for 10 min, and evaluated by fluorescence microscopy.

**10. Western blot**

Total protein was extracted from tissues or cells, and separated by 10% SDS/PAGE gel. Next, the interest protein was transferred to a nitrocellulose membrane at 250 mA using a wet transfer system. Membranes were blocked with nonfat milk for 1 h and then incubated the following primary antibodies including NDUFA4 (Santa Cruz Biotechnology; No. Sc-517091), ERK (Cell Signaling Technology; No. 4695), p-ERK (Cell Signaling Technology; No. 4370), AKT (Cell Signaling Technology; No. 4691), p-AKT (Cell Signaling Technology; No. 4060), AMPK (ab207442),

p-AMPK (ab133448), or GAPDH (ab181602) over night at 4 °C. The next day, membranes were washed three times with PBST buffer (PBS with Tween-20) and subsequently incubated with a secondary antibody (Ab)-conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology; No. 7074) at an indicated dilution of 1:2000 for 2 h. Finally, the target proteins were detected and analyzed by chemiluminescence Imaging System (ChemiScope5600, CLINX); the images were cropped for presentation via photoshop.

**11. Electrophoretic mobility shift assay (EMSA)**

Biotin-labeled oligonucleotide probes designed for the core promoter region of the TTF-1 gene were synthesized by Shanghai Sangon Biological Engineering. Probe sequences are shown in Table S2. Next, nuclear extracts were prepared from human lung cancer 95D cells at the indicated time points. Finally, follow the detailed instructions of the EMSA kit manufacturer (20148, Thermo).

**Table S2 EMSA oligonucleotide probe sequences**

| Probe name | Sequence (5'→3') |
|---|---|
| MuTcor: | TCTTTCTCCTGTTTACAGTTCTTCACCTCCCAGCCACTACCTCCCACGCCAAGCAAAAAA AATAAATAAATAATAAATCATTTTTGATTCCTTTCTTCCAACCTCTAATTTCCTGGTTTCC TCAGCGCTCAGTGTTTGTGTTGGGAGACCCATTAGGGATGAGAGAGAGGGGAGAAGGA ATGGTTTTGAACACAGAAAAATTGTAGTCCCCTGGATGGGCTGTAGCCCTTCCGGGTCA CCCTTGCCAAATCTCCGGTCGAAAGGCAGCTGAGCCAGCTGCGGGCAGACGCCCCTCCC AGCACTTTCCTCTAGGCCTGGAAGTATTGGATTATGGGCAATTGATTCTTATTTGGCATG TA |
| $^{opt1}$Tcor: | TCTTTCTCCTGTTTACAGTTCTTCACCTGGCAGCCACTACCTCCCACGCCAAGCAAAAAA AATAAATAAATAATAAATCATTTTTGATTCCTTTCTTCCAACCTCTAATTTCCTGGTTTCC TCAGCGCTCAGTGTTTGTGTTGGGAGACCCATTAGGGATGAGAGAGAGGGGAGAAGGA ATGGTTTTGAACACAGAAAAATTGTAGTCCCCTGGATGGGCTGTAGCCCTTCCGGGTCA CCCTTGCCAAATCTCCGGTCGAAAGGCAGCTGAGCCAGCTGCGGGCAGACGCCCCTCCC AGCACTTTCCTCTAGGCCTGGCAGTATTGGATTATGGGCAATTGATTCTTATTTGGCATG TA |
| $^{opt2}$Tcor: | TCTTTCTCCTGTTTACAGTTCTTCACCTGGCAGCCACTACCTCCCACGCCAAGCAAAAAA AATAAATAAATAATAAATCATTTTTGATTCCTTTCTTCCAACCTCTAATTTCCTGGTTTCC TCAGCGCTCAGTGTTTGTGTTGGCAGACCCATTAGGGATGAGAGAGAGGGGAGAAGGA ATGGTTTTGAACACAGAAAAATTGTAGTCCCCTGGATGGCATGTAGCCCTTCCGGGTCA CCCTTGCCAAATCTCCGGTCGAAAGGCAGCTGAGCCAGCTGCGGGCAGACGCCCCTCCC AGCACTTTCCTCTAGGCCTGGCAGTATTGGATTATGGGCAATTGATTCTTATTTGGCATG TA |

## 12. Statistical analysis

SPSS software was used for statistical analysis. Differences between groups were compared with an unpaired two-tailed *t*-test. A value of $P < 0.05$ was considered to be indicative of statistical significance.