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Research Article

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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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Abstract: Targeted gene therapy has become a promising approach for lung cancer treatment. In our previous work, we reported that the targeted expression of microRNA-7 (miR-7) operated by thyroid transcription factor-1 (TTF-1) promoter inhibited the growth of human lung cancer cells in vitro and in vivo; however, the intervention efficiency needed to be further improved. In this study, we identified the core promoter of TTF-1 (from −1299 bp to −871 bp) by 5' deletion assay and screened out the putative transcription factors nuclear factor-1 (NF-1) and activator protein-1 (AP-1). Further analysis revealed that the expression level of NF-1, but not AP-1, was positively connected with the activation of TTF-1 core promoter in human non-small-cell lung cancer (NSCLC) cells. Moreover, the silencing of NF-1 could reduce the expression level of miR-7 operated by TTF-1 core promoter. Of note, we optimized four distinct sequences to form additional NF-1-binding sites (TGGCA) in the sequence of TTF-1 core promoter (termed as ^{opt}TTF-1 promoter), and verified the binding efficiency of NF-1 on the ^{opt}TTF-1 promoter by electrophoretic mobility shift assay (EMSA). As expected, the ^{opt}TTF-1 promoter could more effectively drive miR-7 expression and inhibit the growth of human NSCLC cells in vitro, accompanied by a reduced transduction of NADH dehydrogenase (ubiquinone) 1α subcomplex 4 (NDUFA4)/protein kinase B (Akt) pathway. Consistently, ^{opt}TTF-1 promoter-driven miR-7 expression could also effectively abrogate the growth and metastasis of tumor cells in a murine xenograft model of human NSCLC. Finally, no significant changes were detected in the biological indicators or the histology of some important tissues and organs, including heart, liver, and spleen. On the whole, our study revealed that the optimized TTF-1 promoter could more effectively operate miR-7 to influence the growth of human NSCLC cells, providing a new basis for the development of microRNA-based targeting gene therapy against clinical lung cancer.

Key words: Lung cancer; Thyroid transcription factor-1 (TTF-1); Promoter; MicroRNA-7 (miR-7); Nuclear factor-1 (NF-1)

1 Introduction

Lung cancer is a malignant tumor with the second highest incidence in the world. Although chemotherapy, radiotherapy, and surgery have made significant advances in recent years, the overall survival rate of patients with lung cancer is still very low (Chan and Coward, 2013; Billiet et al., 2016; Nasim et al., 2019). Based on the improvement of biomedical techniques, some prolific genetic alterations and novel molecular changes in lung cancer were verified, including epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), c-ros oncogene 1 (*ROS1*), B-Raf proto-oncogene (*BRAF*), and neuro trophin receptor kinase (*NTRK*), which aid the development of the pathological diagnosis and classification of lung cancers, as well as the therapeutic strategies of clinical lung cancer (Wilson et al., 2017; Zhuang et al., 2019; Dziadziuszko

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et al., 2021; Umakanthan and Bukelo, 2021). Furthermore, a variety of new anticancer therapies have been applied to compensate for the deficiencies of traditional treatments, such as gene therapy that is expected to provide an alternative treatment effect and minimize the side effects in lung cancer patients (Toloza et al., 2006; Lara-Guerra and Roth, 2016; Zhao X et al., 2021; Du et al., 2022). However, because of a series of shortcomings, such as the lack of sustained and stable expression of genes of interest in vivo and the weak targeting of such genes, its application in clinical lung cancer needs to be further explored. Notably, accumulating evidence has shown that targeted gene expression technology, as a key strategy of gene therapy, enables these therapeutic genes to be accurately expressed in tumor cells through specific promoters and unique delivery systems, thereby improving the intervention effect on lung cancer (Wiggins et al., 2010; Rai et al., 2011; Amreddy et al., 2017; Lee et al., 2019).

A promoter is the upstream regulatory element of a specific genetic region, which starts transcription by combining with certain specific transcription factors, and its activity directly affects the expression level of genes. In the progression of various tumors, sequence mutation, methylation level change, or the regulation of some trans-acting elements reduces the promoter activity, affecting the expression of functional genes (Zhang BY et al., 2013; Zhang EB et al., 2014; Zhao et al., 2015; Chen et al., 2022). Therefore, researchers' aim is to find a promoter with high activity, or increase the promoter activity to improve the dilemma of genes of interest. Importantly, previous studies showed that tumor-specific promoters have high regulatory activity and targeting specificity to drive the expression of the genes of interest while reducing the damage to normal tissues (Fukazawa et al., 2007; Sher et al., 2009; Xie et al., 2009). In addition, some studies confirmed that promoter activity could be improved by screening the core promoter, adding enhancement elements, or adding transcription factor-binding sequences, and eventually enhanced the expression of its target genes (Linnerth-Petrik et al., 2012; Danino et al., 2015; Zhang et al., 2015; Even et al., 2017). Therefore, the means to enhance promoter activity is not only crucial for targeted gene expression, but is also a vital issue for the clinical gene therapy of lung cancer.

In our previous study, we constructed a eukaryotic vector of thyroid transcription factor-1 (TTF-1) promoter-operated microRNA-7 (miR-7) expression, and found that the TTF-1 promoter could regulate miR-7 expression and inhibit the growth of human lung cancer cells both in vivo and in vitro, indicating that TTF-1 promoter-operated miR-7 expression may be one of the promising strategies for the targeted gene therapy of lung cancer (Lei et al., 2017). However, this intervention effect in lung cancer needs to be further improved. In this study, we screened the TTF-1 core promoter by a 5' deletion assay and further identified the key transcription factor that binds to the core sequence of TTF-1 promoter. Then, we optimized the core sequence of TTF-1 promoter to form more binding sites of key transcription factors. Finally, we observed the effect of miR-7 expression operated by optimized TTF-1 core promoter on the growth and metastasis of human lung cancer cells in vitro and in vivo, and explored the related molecular mechanism, which might provide a preliminary experimental basis for promoting the development of new clinical lung cancer gene therapy strategies.

2 Results

2.1 Screening and optimization of TTF-1 core promoter

The schematic of the research program was shown in Fig. 1a. Firstly, 5' deletion assay and bioinformatic analysis were performed to screen out the TTF-1 core promoter and putative transcription factor (Fig. 1a, upper). Secondly, the site mutation technique was employed to optimize the TTF-1 core promoter, and then the efficiency of optimized TTF-1 core promoter-driven miR-7 expression was estimated in a murine xenograft model of human lung cancer (Fig. 1a, lower). As shown in Fig. 1b, the 5' deletion assay was designed in the distinct TTF-1 promoter with different lengths. The data showed that, compared with the p-Cont (pEGFP-N1-basic) group, the expression of miR-7 in the p-T1229-miR-7 (pEGFP-N1-basic-TTF-1 promoter with 1229 bp-miR-7) group was the most upregulated (P<0.01), but not significantly increased in the p-T1612-miR-7 (pEGFP-N1-basic-TTF-1 promoter with 1612 bp-miR-7) with a longer promoter sequence or p-T871-miR-7 (pEGFP-N1-basic-TTF-1 promoter with 871 bp-miR-7) with a shorter promoter

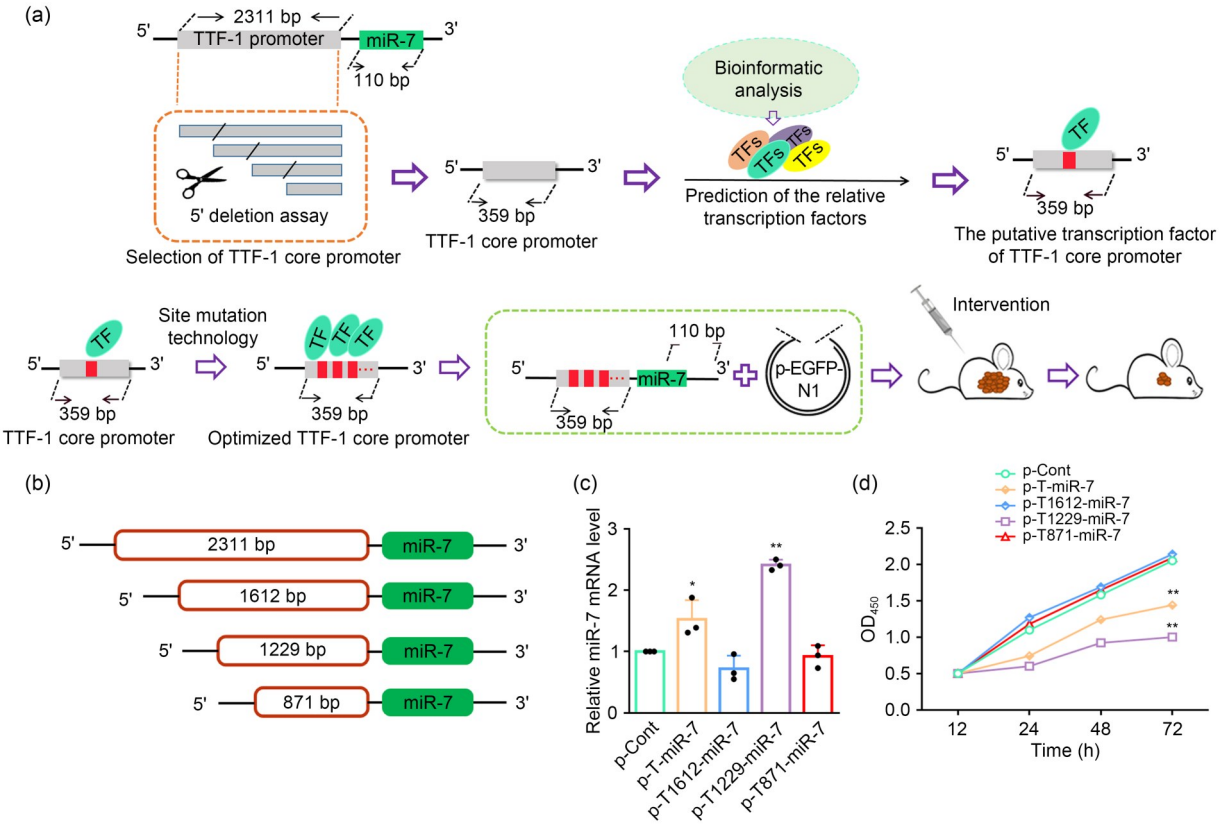


Fig. 1 Screening of TTF-1 core promoter. (a) The whole research program was shown: firstly, the TTF-1 core promoter and putative transcription factor were screened out by 5' deletion assay and bioinformatic analysis (upper); secondly, the site mutation technique was used to optimize the TTF-1 core promoter, and then the anticancer efficiency of optimized TTF-1 core promoter-driven miR-7 expression was estimated in a murine xenograft model of human lung cancer (lower). (b) Different lengths of TTF-1 promoter. (c, d) Human lung cancer 95D cells were transiently transfected with p-Cont (10 μg), p-T-miR-7 (10 μg), p-T1612-miR-7 (10 μg), p-T1229-miR-7 (10 μg), or p-T871-miR-7 (10 μg) in vitro: (c) cells were harvested after 48 h and the relative expression of miR-7 was examined by qPCR; (d) the proliferation of 95D cells was detected after 12, 24, 48, and 72 h by CCK-8 assay. Data are expressed as mean±SEM (n=3). $^{*}P$<0.05, $^{**}P$<0.01, vs. p-Cont. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; qPCR: quantitative real-time polymerase chain reaction; CCK-8: cell counting kit-8; TF: transcription factor; mRNA: messenger RNA; OD_{450}: optical density at 450 nm; p-Cont: pEGFP-N1-basic; p-T-miR-7: pEGFP-N1-basic-TTF-1 promoter with 2311 bp-miR-7; p-T1612-miR-7: pEGFP-N1-basic-TTF-1 promoter with 1612 bp-miR-7; p-T1229-miR-7: pEGFP-N1-basic-TTF-1 promoter with 1229 bp-miR-7; p-T871-miR-7: pEGFP-N1-basic-TTF-1 promoter with 871 bp-miR-7; SEM: standard error of the mean.

sequence transfection group (P>0.05; Fig. 1c). Moreover, cell counting kit-8 (CCK-8) analysis further showed that the proliferation of human lung cancer 95D cells in the p-T1229-miR-7 transfection group was strongly inhibited (P<0.01; Fig. 1d), but the inhibition effect in the p-T1612-miR-7 or p-T871-miR-7 transfection group was not significantly changed (P>0.05; Fig. 1d). These results indicated that the sequence from −1229 bp to −871 bp (359 bp in total) was the core sequence of the TTF-1 promoter.

Then, the SignalScan, MatrixCatch, and Patch biological databases were used to predict and analyze the putative transcription factors on the core sequence of the TTF-1 promoter. Two transcription factors, nuclear factor-1 (NF-1) and activator protein-1 (AP-1), were screened out by cross comparison, whose binding sites were TGGCA and CCAAG, respectively (Fig. 2a). Next, to explore the relationship among the activity of TTF-1 core promoter regulating miR-7 expression and potential transcription factors, the related vectors were transiently transfected into five different cell lines in vitro. The results showed that, among the five cell lines, the expression levels of miR-7 in human non-small-cell lung cancer (NSCLC) 95D and A549 cells were higher (P<0.01; Fig. 2b). Unexpectedly, the expression of transcription factor NF-1, but not AP-1, in 95D and A549 cells was also significantly higher than that in other cell lines (P<0.05; Fig. 2c), displaying a positive

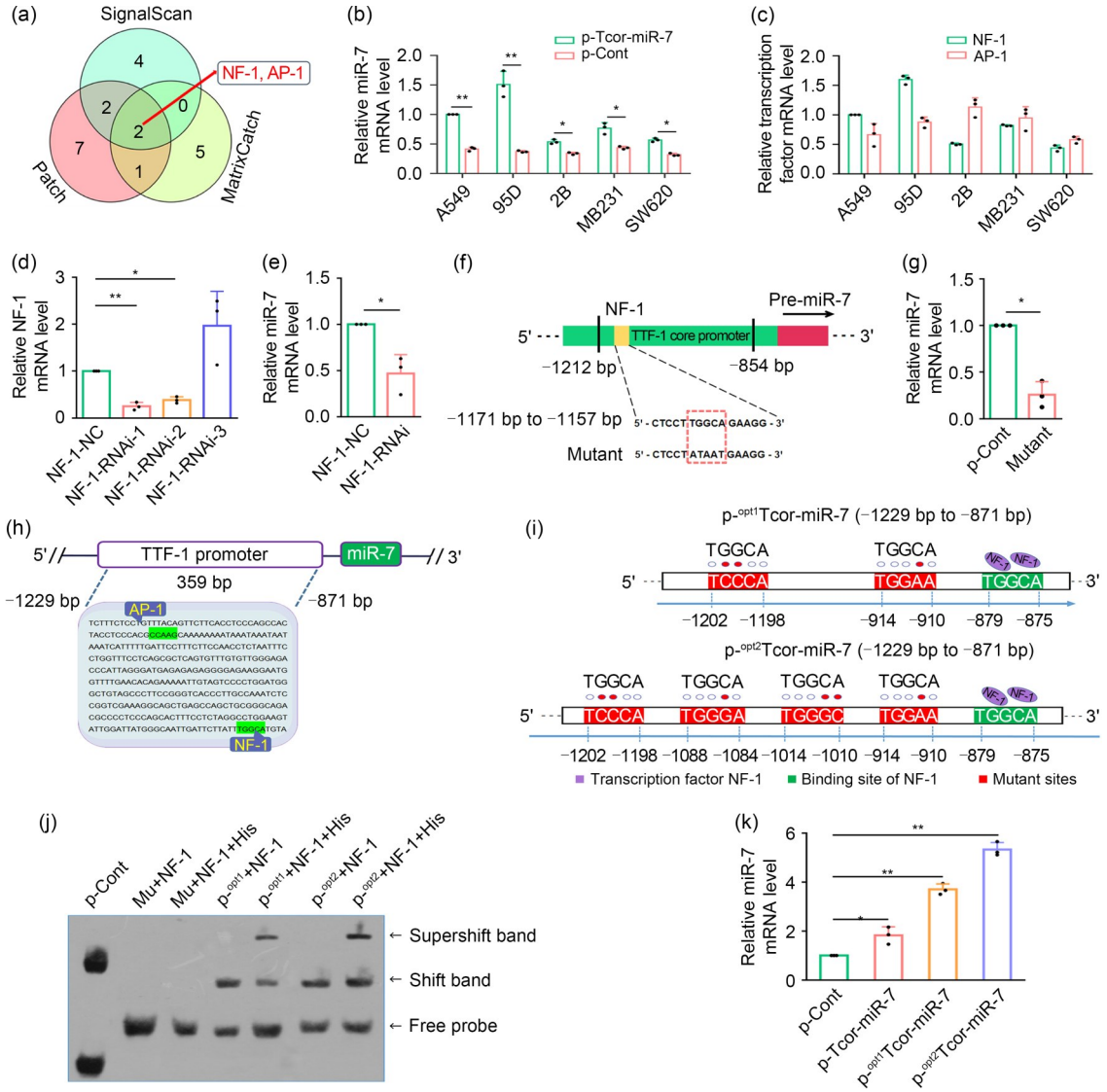


Fig. 2 Optimization of TTF-1 core promoter. (a) SignalScan, MatrixCatch, and Patch biological databases were used to predict the transcription factors binding to the core sequence of TTF-1 promoter. (b, c) Human lung cancer 95D cells, A549 cells, human normal bronchial epithelial cell 2B cells, human breast cancer MB231 cells, and human colon cancer SW620 cells were transiently transfected with p-Cont or p-Tcor-miR-7 in vitro, and 48 h later cells were collected: (b) the expression of miR-7 was examined by qPCR assay; (c) relative transcription factor mRNA levels of A549, 95D, 2B, MB231, and SW620 cells transfected with p-Tcor-miR-7 were examined by qPCR assay. (d, e) Human lung cancer 95D cells were transiently transfected with NF-1 RNAi plasmids containing different sequences, and 48 h later cells were harvested: (d) the NF-1 mRNA level was examined by qPCR; (e) the miR-7 mRNA expression was analyzed by qPCR. (f, g) Human lung cancer 95D cells were transiently transfected with eukaryotic vector encoding the original sequence or mutant sequence of the potential binding sites in the promoter sequence of TTF-1 (f) to drive miR-7 expression; 48 h later, cells were collected, and the expression of miR-7 mRNA was analyzed by qPCR (g). (h) TTF-1 promoter core sequence. (i) The schematic diagrams of optimized TTF-1 promoter sequences (p-^{opt1}Tcor-miR-7: two NF-1-binding sites were created; p-^{opt2}Tcor-miR-7: four NF-1-binding sites were created). (j) After the incubation of modified probes with purified proteins, EMSA was used to detect the level of NF-1 protein binding to the core sequence of TTF-1 promoter (Mu probe: 0 NF-1-binding site; p-^{opt1} probe: three NF-1-binding sites; p-^{opt2} probe: five NF-1-binding sites; His: anti-6*His tag monoclonal antibody). (k) Human lung cancer 95D cells were transiently transfected with p-Cont, p-Tcor-miR-7, p-^{opt1}Tcor-miR-7, or p-^{opt2}Tcor-miR-7 in vitro; cells were harvested after 48 h and the relative expression level of miR-7 mRNA was examined by qPCR. Data are expressed as mean±SEM (n=3). * P<0.05, ** P<0.01. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; qPCR: quantitative real-time polymerase chain reaction; mRNA: messenger RNA; NF-1: nuclear factor-1; RNAi: RNA interference; EMSA: electrophoretic mobility shift assay; AP-1: activator protein-1; p-Cont: pEGFP-N1-basic; p-Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter-miR-7; p-^{opt2}Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7; SEM: standard error of the mean.

connection with the miR-7 expression pattern. Next, to further verify whether NF-1 was the key transcription factor regulating the activity of TTF-1 core promoter in NSCLC cells, we used RNA interference (RNAi) to reduce the expression of NF-1 in human NSCLC 95D cells. The results showed that RNAi-1 in different RNAi sequences inhibited NF-1 messenger RNA (mRNA) most significantly (P<0.01; Fig. 2d). After downregulation of NF-1 by RNAi-1 sequence, the expression of miR-7 was also downregulated (P< 0.05; Fig. 2e). Meanwhile, we mutated the binding site of NF-1 and obtained a similar phenomenon (P< 0.05; Figs. 2f and 2g). These results suggested that NF-1 is a key transcription factor that binds to the core sequence of TTF-1 promoter to regulate the expression of miR-7 in human NSCLC cells.

Next, considering NF-1 as one of the key factors in regulating the activation of TTF-1 core promoter, we envisioned whether the activity of TTF-1 core promoter could be enhanced by increasing the NF-1-binding sites to recruit more NF-1. Therefore, based on above bioinformatic analysis, two or four distinct sequences without any putative transcription factor-binding sites in the TTF-1 core promoter were optimized to form more NF-1-binding sites (TGGCA) (Figs. 2h and 2i). Interestingly, compared with that in the control group, the electrophoretic mobility shift assay (EMSA) showed that more NF-1 transcription factors were bound to the p-^{opt1}Tcor-miR-7 (pEGFP-N1-basic-TTF-1 core promoter with 3 NF-1-bounding sites-miR-7) group with two artificial NF-1-binding sites (Fig. 2j). Of note, more NF-1 transcription factors were bound to the core sequence of the p-^{opt2}Tcor-miR-7 (pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7) group with four artificial NF-1-binding sites compared with other groups (Fig. 2j). Finally, we further confirmed that the expression of miR-7 driven by the TTF-1 core promoter in the p-^{opt2}Tcor-miR-7 transfection group was upregulated to the greatest extent (P<0.01; Fig. 2k). Combining these data demonstrated that miR-7 could be more effectively operated by the optimized TTF-1 core promoter.

2.2 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the growth and metastasis of human NSCLC cells in vitro

In order to observe the effects of optimized TTF-1 promoter-driven miR-7 expression on human NSCLC in vitro, relative vectors were transiently transfected into human NSCLC cells. The results showed that, compared with the p-Tcor-miR-7 (pEGFP-N1-basic-TTF-1 core promoter-miR-7) transfection group, the cell growth and clone formation ability of human NSCLC cells in the p-^{opt2}Tcor-miR-7 (termed as ^{opt}TTF-1 promoter) transfection group were dramatically suppressed (P<0.05; Figs. 3a–3d and S1). Consistently, the expression of cell growth-related molecules cyclin-dependent kinase 1 (CDK1), CDK2, CDK4, and CDK6 was significantly decreased (P<0.01; Fig. 3e). In addition, the expression of metastasis-related molecules, including matrix metalloproteinase 2 (MMP2), MMP3, MMP9, C-X-C chemokine receptor type 4 (CXCR4), and E-cadherin, was also dramatically suppressed (P<0.01; Fig. 3f), accompanied by an attenuation of migration and invasion ability (P<0.05; Figs. 3g–3j). Further analysis showed that the proportions of G0/G1 and G2/M phases in the p-^{opt2}Tcor-miR-7 group were significantly upregulated, while the cell proportion of S phase was significantly downregulated (P< 0.05; Figs. 3k and 3l), indicating that optimized TTF-1 promoter-driven miR-7 expression blocked the cycle progression of human NSCLC 95D cells. The above data suggested that ^{opt}TTF-1 promoter-driven miR-7 expression was more effective in decreasing the growth and metastasis of human NSCLC cells in vitro.

2.3 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the transduction of NDUFA4 pathway in vitro

Many studies have evidenced that miR-7 could regulate biological processes such as tumor growth and metastasis by protein kinase B (Akt) and extracellular signal-regulated kinase (Erk) signaling pathways (Liu et al., 2014; Cao et al., 2016). In our previous work, we also found that miR-7 could affect the growth of human lung cancer cells through the target molecule NADH dehydrogenase (ubiquinone) 1α subcomplex 4 (NDUFA4) and related signaling pathways, such as Akt and Erk (Lei et al., 2017). Therefore, to further investigate the molecular mechanism of human NSCLC cell growth affected by ^{opt}TTF-1 promoter-driven miR-7 expression, the relative protein expression levels were analyzed. We found no difference in the expression of Akt or Erk among these groups (P>0.05; Figs. 4a and 4b); however, the expression of NDUFA4 in the p-^{opt2}Tcor-miR-7 transfection group

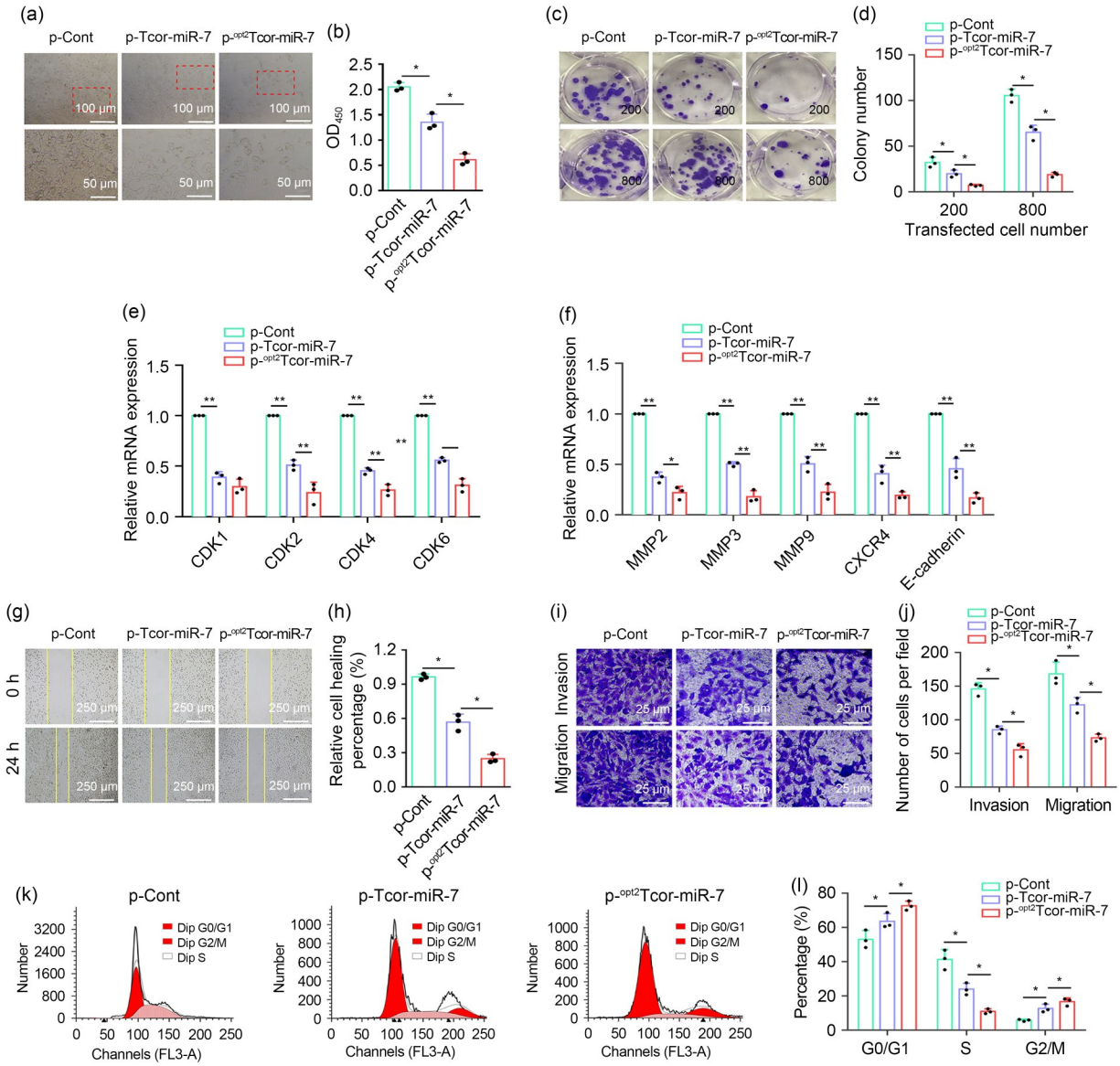


Fig. 3 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the growth and metastasis of human NSCLC cells in vitro. Human NSCLC cells were transiently transfected with p-Cont, p-Tcor-miR-7, or p-^{opt2}Tcor-miR-7 in vitro. Cells were harvested after 48 h. (a) The treated cells were imaged by light microscopy. (b) The proliferation ability of 95D cells was measured by CCK-8 assay. (c, d) Colony formation assay was performed by inoculating 200 or 800 transfected cells on a 24-well plate (one representative vision out of three was shown), and the colony numbers were calculated. (e) The relative mRNA levels of cell growth-related molecules (CDK1, CDK2, CDK4, and CDK6) in 95D cells were examined by qPCR assay. (f) The relative mRNA levels of cell metastasis-related molecules (MMP2, MMP3, MMP9, CXCR4, and E-cadherin) in 95D cells were examined by qPCR assay. (g, h) The migration ability of cells was determined by scratch assay, and the cell number was calculated; the yellow line indicates the border of scratch wound. (i, j) The cell migration and invasion abilities of 95D cells were determined by transwell assay, and the number of migrated or invaded cells was calculated. (k, l) Human NSCLC 95D 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 and incubated with PI for 24 h, and then the cell cycles of 95D cells were detected by FACS (one representative data out of three was presented). Data are expressed as mean±SEM (n=3). * P<0.05, ** P< 0.01. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; NSCLC: non-small-cell lung cancer; CCK-8: cell counting kit-8; mRNA: messenger RNA; CDK: cyclin-dependent kinase; qPCR: quantitative real-time polymerase chain reaction; CXCR4: C-X-C chemokine receptor type 4; MMP: matrix metalloproteinase; PI: propidium iodide; FACS: fluorescence-activated cell sorting; OD_{450}: optical density at 450 nm; p-Cont: pEGFP-N1-basic; p-Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter-miR-7; p-^{opt2}Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7; SEM: standard error of the mean.

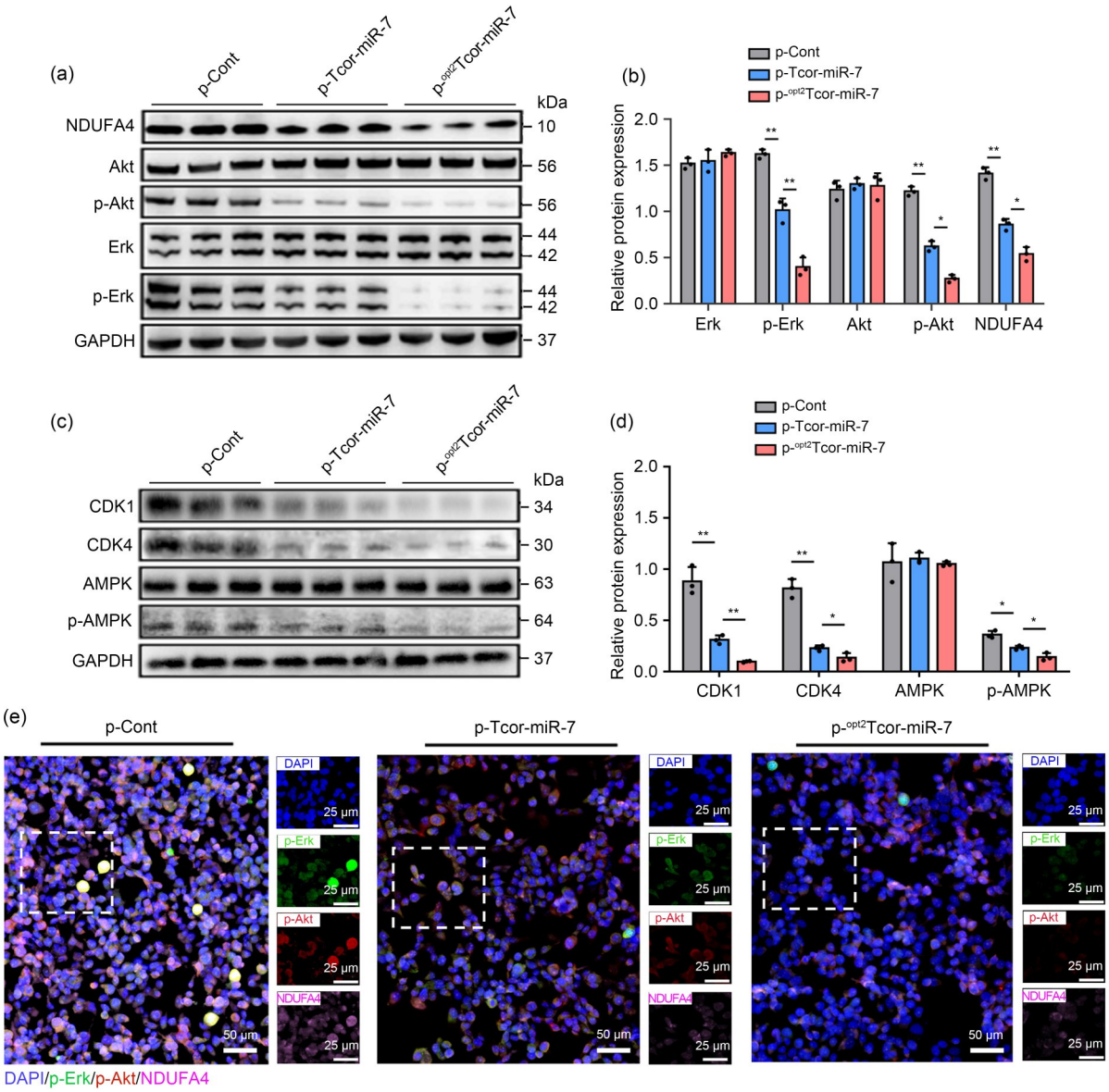


Fig. 4 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the transduction of NDUFA4 pathway in vitro. Human NSCLC 95D cells were transiently transfected with p-Cont (10 μg), p-Tcor-miR-7 (10 μg), or p-^{opt2}Tcor-miR-7 (10 μg) in vitro. Cells were harvested after 48 h. (a, b) The protein expression of Akt, phospho-Akt (p-Akt), Erk, phospho-Erk (p-Erk), and NDUFA4 was detected by western blot assay and calculated. (c, d) The protein expression of CDK1, CDK4, AMPK, and phospho-AMPK (p-AMPK) was detected by western blot assay and calculated. (e) The relative expression of p-Erk, p-Akt, and NDUFA4 was analyzed by immunofluorescence assay. Data are expressed as mean±SEM (n=3). * P<0.05, ** P<0.01. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; NDUFA4: NADH dehydrogenase (ubiquinone) 1α subcomplex 4; NSCLC: non-small-cell lung cancer; Akt: protein kinase B; Erk: extracellular signal-regulated kinase; CDK: cyclin-dependent kinase; AMPK: adenosine 5'-monophosphate (AMP)-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4',6-diamidino-2-phenylindole; p-Cont: pEGFP-N1-basic; p-Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter-miR-7; p-^{opt2}Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7; SEM: standard error of the mean.

was significantly decreased (P<0.05; Figs. 4a and 4b), which was consistent with our previous findings (Lei et al., 2017). As expected, the expression of phospho-Akt (p-Akt) and phospho-Erk (p-Erk) also decreased significantly (P<0.05; Figs. 4a and 4b). Moreover, the energy metabolism and growth-related molecule analyses revealed that the total adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) did not change, but phospho-AMPK (p-AMPK) and growth-related molecules CDK1 and CDK4 were significantly

downregulated (P<0.05; Figs. 4c and 4d). In addition, we confirmed the expression changes of p-Akt, p-Erk, and NDUFA4 in the three groups by immunofluorescence, and similar results were obtained (Fig. 4e). Overall, our data indicated that ^{opt}TTF-1 promoter-driven miR-7 expression decreased the growth of human NSCLC cells in vitro by regulating the expression of NDUFA4 and related pathways.

2.4 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the tumorigenicity of NSCLC in vivo

In order to further explore the intervention effect of ^{opt}TTF-1 promoter-driven miR-7 expression on human NSCLC in vivo, a xenotransplantation model of human NSCLC in nude mice was established (Fulzele et al., 2006; Huang et al., 2017). Once the tumor formation was complete, tumor-bearing nude mice were injected with p-Cont, p-Tcor-miR-7, or p-^{opt2}Tcor-miR-7 recombinant vectors through the tail vein every 3 d, for a total of five times (Fig. 5a). Then, the growth changes of the tumors were observed accordingly. As shown in Figs. 5b and 5c, the tumor growth in the p-^{opt2}Tcor-miR-7 injection group was significantly slower than that in the p-Tcor-miR-7 injection group (P<0.05). Moreover, hematoxylin and eosin (HE) staining showed that the tumor tissues in the p-^{opt2}Tcor-miR-7 injection group showed extensive necrosis, accompanied with decreased tumor weight (P<0.05; Figs. 5d and 5e). In our previous research, we found that TTF-1 promoter-operated miR-7 expression could effectively inhibit the growth of tumors in vivo, with the tumor inhibition rate of about 34% (Lei et al., 2017). Importantly, our data showed that the tumor inhibition rate of the p-^{opt2}Tcor-miR-7 injection group was significantly enhanced, with an inhibition rate increased by 126% (inhibition rate 34% vs. 77%) (P<0.01; Fig. 5f). Moreover, the expression level of miR-7 in tumor tissues in the p-^{opt2}Tcor-miR-7 injection group was significantly higher than that in the p-Tcor-miR-7 injection groups (P<0.05; Fig. 5g). Meanwhile, the immunofluorescence assay further showed that the proliferation ability of tumor cells was seriously weakened (P< 0.05; Figs. 5h and 5i). Conversely, the apoptosis of tumor cells was significantly increased (P<0.05; Fig. S2). Finally, to confirm the effect of ^{opt}TTF-1 promoter-operated miR-7 expression on the growth and metastasis of tumor cells in vivo, the relative molecules in tumor tissues were analyzed. Consistent with these findings in vitro, the expression of growth-related molecules, including CDK2, CDK4, and CDK6, and metastasis-related molecules, including MMP2, MMP3, MMP9, CXCR4, and E-cadherin, was significantly reduced in the p-^{opt2}Tcor-miR-7 injection group (P<0.05; Figs. 5j and 5k). Combining these data demonstrated that the ^{opt}TTF-1 promoter could effectively drive the expression of miR-7 in tumor tissues, which subsequently inhibited the tumorigenicity of NSCLC in vivo.

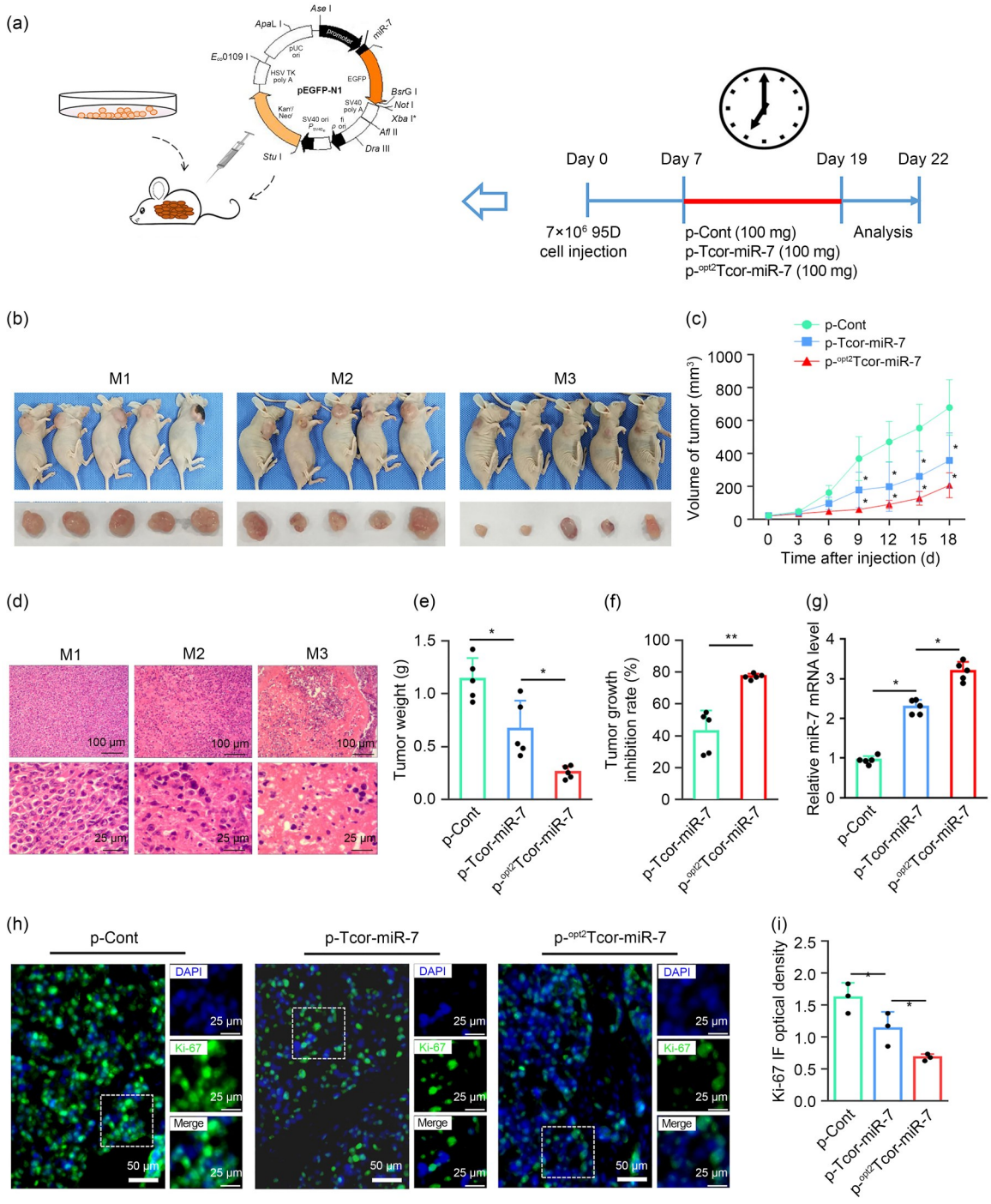
2.5 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the NDUFA4 pathway in vivo

Next, to investigate the changes of related signaling pathways in vivo, the relevant molecules were analyzed in the tumor tissues of tumor-bearing mice. The results showed that, compared with those in the p-Tcor-miR-7 group, the expression levels of NDUFA4, p-Akt, and p-Erk in the p-^{opt2}Tcor-miR-7 group were significantly decreased (P<0.05; Figs. 6a and 6b). Besides, p-AMPK, CDK1, and CDK4 were also significantly downregulated (P<0.05; Figs. 6c and 6d). To confirm these findings, we detected the expression levels of p-Akt and p-Erk in tumor tissues by immunohistochemistry (Figs. 6e and 6f), and the expression of NDUFA4 by immunofluorescence (P<0.05; Figs. 6g and 6h), and obtained similar results. Overall, these findings showed that ^{opt}TTF-1 promoter-driven miR-7 expression affects the tumorigenicity of NSCLC in vivo, which is related to the altered expression of NDUFA4 pathway.

2.6 Ideal targeting efficacy and safety of ^{opt}TTF-1 promoter-driven miR-7 expression

Many studies have shown that the biological changes of organs or tissues have an important reference value for the wide application of targeted gene therapy (Naldini, 2015; Lux and Scharenberg, 2017; Bucher et al., 2021). Therefore, we analyzed the distribution of p-^{opt2}Tcor-miR-7 plasmid copies in the main organs of mice. As shown in Fig. 7a, the p-^{opt2}Tcor-miR-7 plasmid was distributed in all the main organs. Importantly, the distributions of plasmid copy numbers in tumor and lung tissues were significantly higher than those in other major organs (P<0.01; Fig. 7a). Consistently, the expression levels of miR-7 were significantly increased in lung and tumor tissues (P<0.01), but not in other organs (Fig. 7b), indicating rational targeting efficacy.

Furthermore, we observed the potential influence of optimized TTF-1 promoter-driven miR-7 expression on the major organs of the murine tumor-bearing model. The data revealed no significant differences in the weights or indexes of main organs, including heart, liver, spleen, lung, kidney, brain, and intestine, between the three groups (P>0.05; Figs. 7c and 7d). In addition, the results of HE staining showed no obvious pathological changes in these major organs (Fig. S3). Finally, we detected some key biological indexes such as aspartate aminotransferase (AST), alanine transaminase (ALT), and serum total cholesterol (TC) in the sera, which were used to assess the functional changes of various organs. As expected, these biochemical indexes showed no significant changes among these groups (P>0.05; Fig. 7e). Combining these data



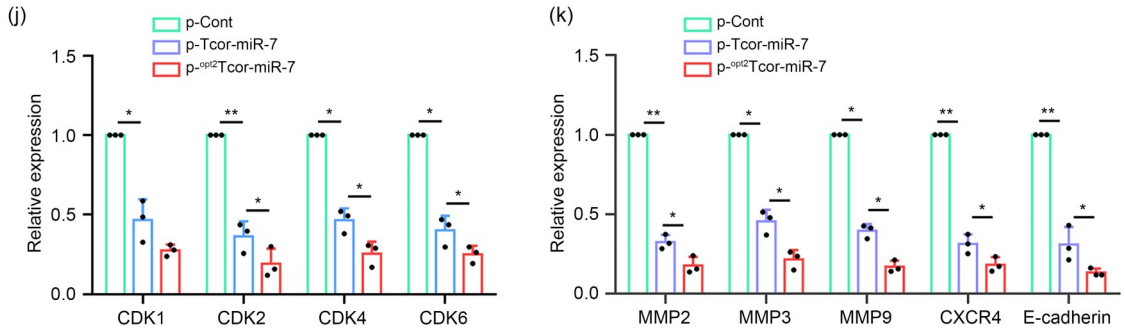


Fig. 5 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on tumorigenicity of NSCLC in vivo. (a) Human NSCLC 95D cell xenografts were established in the left flank of female BALB/c nude mice (n=5). Once the tumor formation was complete, the plasmid of p-Cont (100 mg), p-Tcor-miR-7 (100 mg), or p-^{opt2}Tcor-miR-7 (100 mg) was injected through the tail vein of 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. (b) The mice and tumor tissues of p-Cont (M1), p-Tcor-miR-7 (M2), and p-^{opt2}Tcor-miR-7 (M3) groups were imaged. (c) The growth curve of tumors. (d) The tumor tissues of the M1, M2, and M3 groups were subjected to hematoxylin and eosin (HE) staining, and one representative vision of three independent experiments was shown. (e) The weight of tumors. (f) The tumor growth inhibition rate. (g) The relative expression of miR-7 in tumor tissues was examined by qPCR assay. (h, i) The expression of proliferation antigen (Ki-67) in tumor tissues was analyzed by immunofluorescence (IF) assay and calculated. (j) The relative expression of tumor growth-associated factors in tumor tissues was examined by qPCR assay. (k) The relative expression of tumor metastasis-related molecules in tumor tissues was examined by qPCR assay. Data are expressed as mean±SEM (n=3 or 5). * P<0.05, ** P<0.01. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; NSCLC: non-small-cell lung cancer; qPCR: quantitative real-time polymerase chain reaction; mRNA: messenger RNA; CDK: cyclin-dependent kinase; MMP: matrix metalloproteinase; CXCR4: C-X-C chemokine receptor type 4; p-Cont: pEGFP-N1-basic; p-Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter-miR-7; p-^{opt2}Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7; SEM: standard error of the mean.

suggested that ^{opt}TTF-1 promoter-driven miR-7 expression exhibits an ideal targeting efficacy and safety.

3 Discussion

In recent years, the expression of target genes driven by specific gene promoters has become an important strategy of gene therapy for many kinds of cancers (Powell et al., 2015; Massaro et al., 2020; Pruller et al., 2021). The TTF-1, as a pedigree-specific oncogene, is dominantly expressed in lung cancer, but not in other types of cancer or tissues (Kim et al., 2018; Guan et al., 2021). Importantly, in our previous work, we reported that TTF-1 promoter-operated miR-7 expression could reduce the growth and metastasis of human lung cancer cells, indicating the potential value of TTF-1 promoter in targeted gene therapy against lung cancer (Lei et al., 2017). However, the intervention efficiency needed to be further improved. In the present study, we further identified and optimized the core sequence of TTF-1 promoter. Notably, we could extend our previous findings by demonstrating that the optimized TTF-1 promoter could more effectively drive miR-7 expression and abrogate the growth of human NSCLC cells in vivo with an inhibition rate increased by 126% (inhibition rate 34% vs. 77%), indicating that the reasonable optimization of TTF-1 promoter might be much more valuable for successive application in gene therapy against clinical lung cancer. In addition, it was noticed that one recent study reported TTF-1 as mainly expressed in lung adenocarcinoma (Umakanthan et al., 2021). Therefore, the potential effects of TTF-1 promoter-driven miR-7 expression in other types of lung cancer still need to be further explored, which might be valuable for the clinical application of this approach in lung cancer patients.

MiR-7 is a unique member of the microRNA (miRNA) family that plays an important role in the progression of various diseases, including lung cancer, through multiple targets (Zhao JJ et al., 2015; Chen et al., 2021; Zhao JJ et al., 2021). For example, miR-7 can inhibit the growth and migration of NSCLC cells in vivo by targeting BCL-2, an important anti-apoptotic gene (Xiong et al., 2011; Du et al.,2021). In addition, Li JR et al. (2014) found that the restoration of miR-7 expression inhibited the tumorigenicity of lung cancer

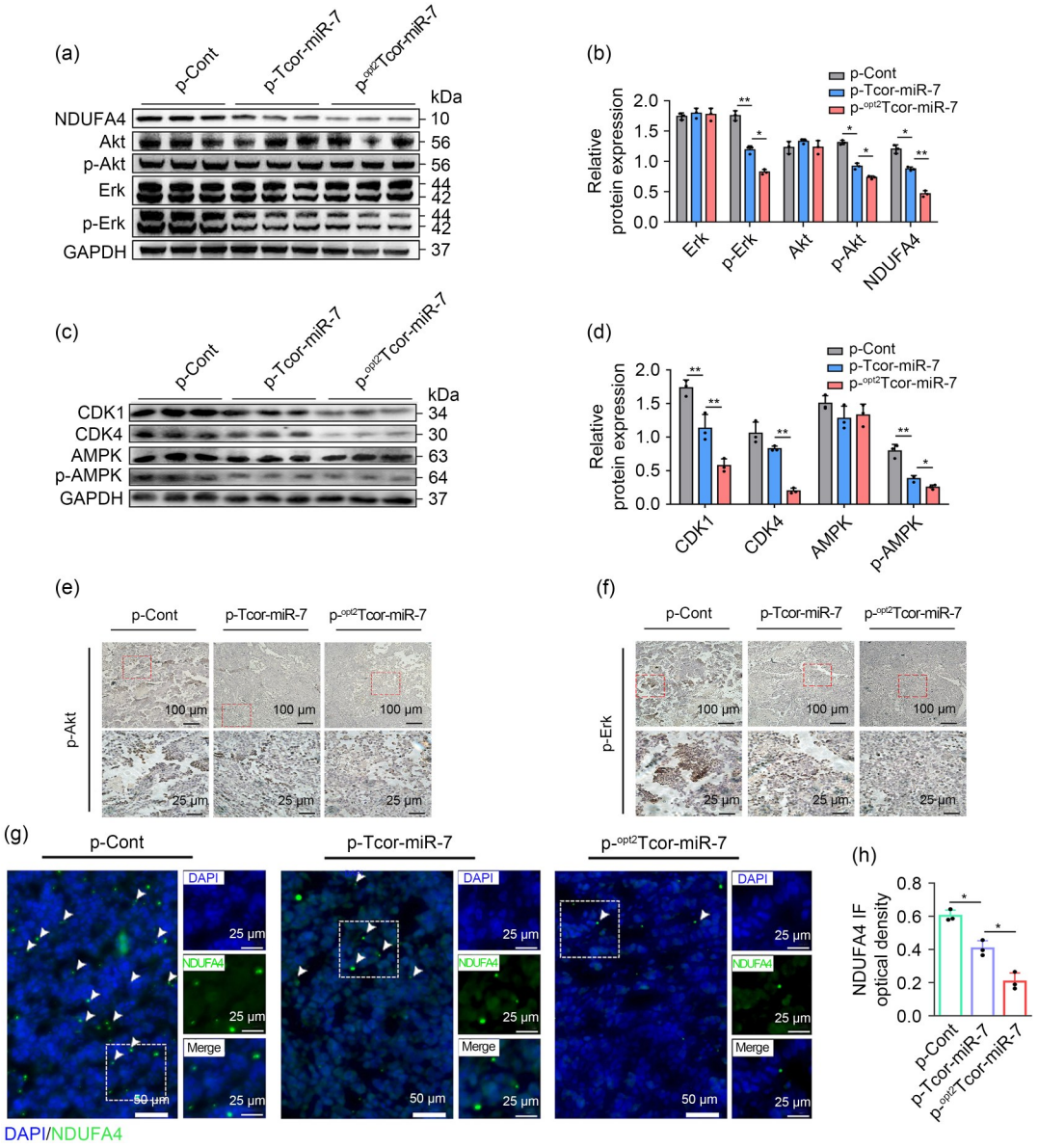


Fig. 6 Effects of ^{opt}TTF-1 promoter-driven miR-7 expression on the NDUFA4 pathway in vivo. Human NSCLC 95D cell xenografts were established in the left flank of female BALB/c nude mice (n=5). Once the tumor formation was complete, the plasmid of p-Cont (100 mg), p-Tcor-miR-7 (100 mg), or p-^{opt2}Tcor-miR-7 (100 mg) was 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, b) The protein expression levels of Akt, phospho-Akt (p-Akt), Erk, phospho-Erk (p-Erk), and NDUFA4 were detected by western blot assay and calculated. (c, d) The protein expression levels of CDK1, CDK4, AMPK, and phospho-AMPK (p-AMPK) were detected by western blot assay and calculated. (e, f) The expression of p-Akt and p-Erk in tumor tissues was analyzed by immunohistochemistry assay, and one representative image out of three was presented. (g, h) The expression of NDUFA4 in tumor tissues was analyzed by immunofluorescence (IF) assay and calculated, in which the white arrows represent some typical visual fields of NDUFA4 IF staining. Data are expressed as mean±SEM (n=3). * P<0.05, ** P<0.01. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; NDUFA4: NADH dehydrogenase (ubiquinone) 1α subcomplex 4; NSCLC: non-small-cell lung cancer; Akt: protein kinase B; Erk: extracellular signal-regulated kinase; CDK: cyclin-dependent kinase; AMPK: adenosine 5'-monophosphate (AMP)-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4', 6-diamidino-2-phenylindole; p-Cont: pEGFP-N1-basic; p-Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter-miR-7; p-^{opt2}Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7; SEM: standard error of the mean.

cells in vivo. Moreover, highly expressed miR-7 targeting the epidermal growth factor EGFR promoted lung tumor regression and attenuated drug resistance in vivo (Rai et al., 2011). Consistently, our previous

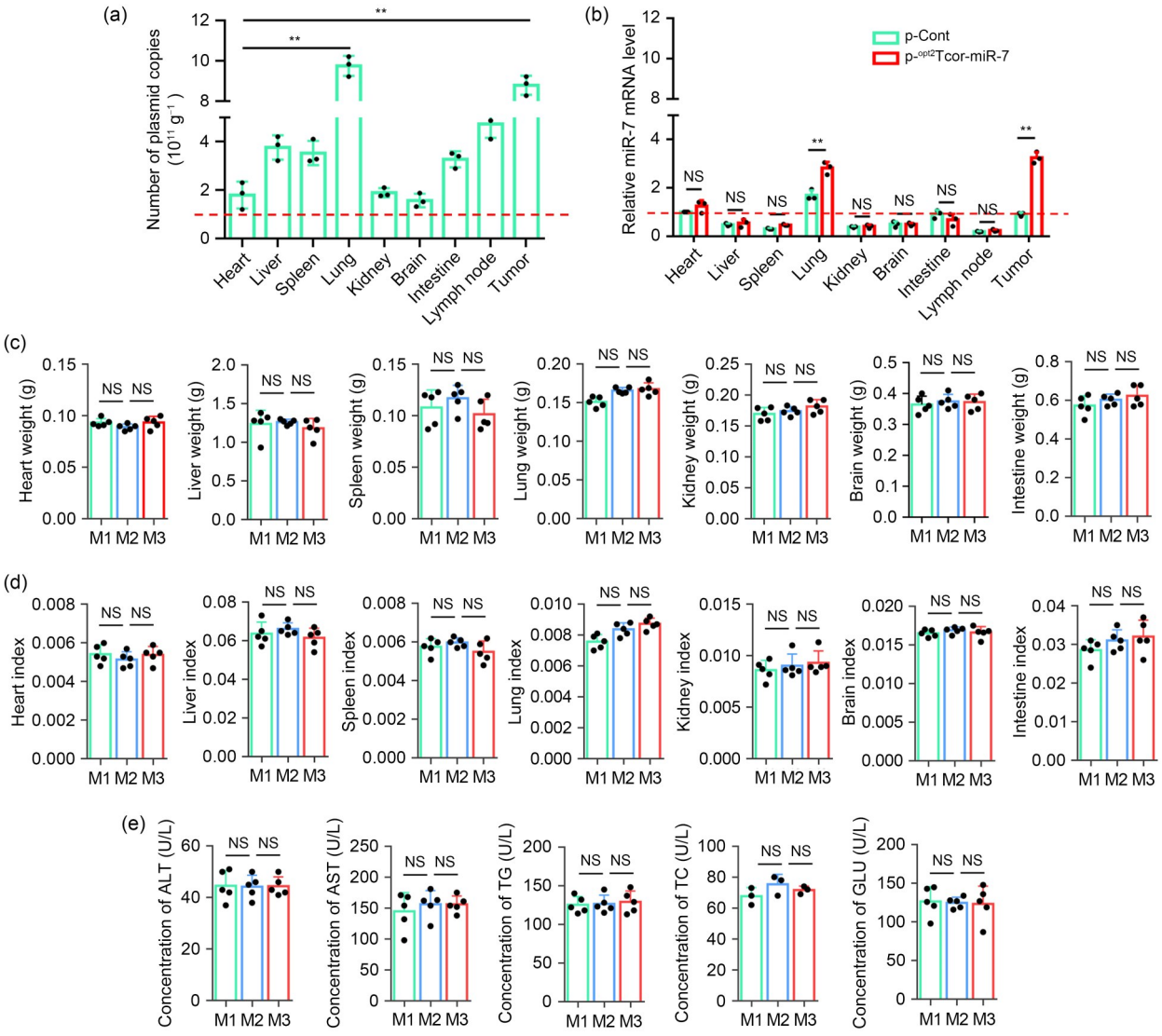


Fig. 7 Ideal targeting efficacy and safety of ^{opt}TTF-1 promoter-driven miR-7 expression. Human NSCLC 95D cell xenografts were established in the left flank of female BALB/c nude mice (n=5). Once the tumor formation was complete, the plasmid of p-Cont (100 mg), p-Tcor-miR-7 (100 mg), or p-^{opt2}Tcor-miR-7 (100 mg) was injected through the tail vein of nude mice once every 3 d for a total of five times. At the end of the experiment, all mice were sacrificed and the major organs were obtained. (a) The distribution of p-^{opt2}Tcor-miR-7 plasmid copies in the nude mice model of human lung cancer was examined by qPCR assay. (b) The relative expression of miR-7 in major organs or tissues was examined by qPCR assay. The main organs of mice were obtained by the above method, which were grouped as M1 (p-Cont), M2 (p-Tcor-miR-7), and M3 (p-^{opt2}Tcor-miR-7). (c) The weight of major organs. (d) The organ index of major organs. (e) The concentrations of ALT, AST, TG, TC, and GLU were separately detected. Data are expressed as mean±SEM (n=3 or 5). * P<0.05, ** P<0.01, ^{NS} P>0.05. TTF-1: thyroid transcription factor-1; miR-7: microRNA-7; NSCLC: non-small-cell lung cancer; qPCR: quantitative real-time polymerase chain reaction; ALT: alanine transaminase; AST: aspartate aminotransferase; TG: triglyceride; TC: total cholesterol; GLU: glucose; p-Cont: pEGFP-N1-basic; p-Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter-miR-7; p-^{opt2}Tcor-miR-7: pEGFP-N1-basic-TTF-1 core promoter with 5 NF-1-bounding sites-miR-7; SEM: standard error of the mean.

work also showed that the overexpression of miR-7 could reduce the growth and metastasis of human lung cancer cells through NDUFA4 (Lei et al., 2017). In this study, we further reported that the optimized TTF-1 promoter-driven miR-7 expression could more effectively reduce the growth and metastasis of human NSCLC cells both in vivo and in vitro, accompanied by a reduced expression of NDUFA4 and altered

transduction of Akt, Erk, and AMPK pathways. Therefore, combined with these works, we further highlighted the important role of miR-7 as a tumor suppressor gene in the development of lung cancer through regulating multiple targets and signaling pathways, which verifies its potential for gene therapy against lung cancer.

As a GTPase-activating protein, NF-1 negatively regulates Ras signal transduction under normal physiological conditions, and its abnormal expression contributes to the development of various cancers (Li C et al., 2014; Philpott et al., 2017; Su et al., 2019). Furthermore, recent studies have shown that the expression of NF-1 in NSCLC is significantly upregulated and enhances the abilities of cell proliferation and migration (Qian et al., 2018). In the present study, we screened out NF-1, but not AP-1, as the putative transcription factor binding to the TTF-1 core promoter, and its expression level was closely related to the activation of TTF-1 promoter-driven miR-7 expression in NSCLC cells. Moreover, the silencing of NF-1 could impair the activation of TTF-1 core promoter. These data indicated that NF-1 is a critical transcription factor binding to the TTF-1 core promoter in human NSCLC. Notably, based on bioinformatic analysis, we replaced several distinct sequences without any putative transcription factor-binding sites with NF-1-binding site for optimization, and successively verified the appreciable binding capacity of the optimized TTF-1 core promoter. Meanwhile, we found that in the TTF-1 core promoter, four NF-1-binding sites showed stronger activity than the two NF-1-binding sites. These data indicated the new value of NF-1 in gene therapy against cancer. However, it must be pointed out that some transcription factors documented can regulate the activity of TTF-1 promoter, such as nuclear factor I (NFI), hepatocyte nuclear factor-3β (HNF-3β), and small mothers against decapentaplegic homolog 2 (Smad2) (Ikeda et al., 1996; Nakazato et al., 2000; Li et al., 2013). Moreover, we also noticed that the activity of TTF-1 promoter was elevated in the absence of the region from −1612 bp to −1229 bp sites, indicating that some other factors might negatively regulate its activity. Therefore, successive research work on the identification of other transcription factors, which were not investigated in current study, and the potential connections among these factors might be rather valuable for the optimization of TTF-1 promoter, ultimately benefiting the development of gene therapy against clinical lung cancer.

The safety of gene therapy strategies, including the distribution of exogenous DNA, the function of vital organs, and histopathological changes, is essential for the potential application of cancer gene therapy (Chandler et al., 2021; Li et al., 2021; Sheikh et al., 2021). In our previous work, we described that the remote subcutaneous injection of TTF-1 promoter-operated miR-7 expression plasmid had no significant effects on tissues or organs (Lei et al., 2017). In the present study, we further observed that the ^{opt}TTF-1 promoter-driven miR-7 expression plasmid was injected through the distal tail vein rather than tissue locally, mainly distributed in lung and tumor tissues, while only trace amounts were found in other major organs. Besides, some studies reported that naked plasmid DNA injected through the tail vein was mainly distributed in liver, but not in lung (Sebestyén et al., 2006; Kizzire et al., 2013). Meanwhile, the expression of miR-7 driven by ^{opt}TTF-1 promoter was mainly upregulated in tumors and lungs, but not in other tissues or organs. We proposed that these phenomena might be related to the different dosages and time courses in distinct experimental settings, as well as the regulatory mechanism of TTF-1 promoter activity including differential intrinsic expression of NF-1 in distinct tissues and organs, which remains to be elucidated. Finally, the function or pathological structure of several major organs was not significantly affected, indicating the valuable application of ^{opt}TTF-1 promoter in successive research work on clinical NSCLC patients.

4 Conclusions

In summary, for the first time, our study revealed that ^{opt}TTF-1 promoter-driven miR-7 expression could significantly inhibit the growth of human NSCLC cells both in vitro and in vivo, which was related to the decreased expression of NDUFA4 and related signaling pathways. Our current study further expands the application prospect of targeted gene expression technology in lung cancer, providing a new basis for the development of miRNA-based targeting gene therapy against clinical lung cancer.

Methods and materials

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Shipeng CHEN and Lian GUAN performed the experiments, analyzed the data, and wrote this paper. Xu ZHAO, Jing YANG, and Longqing CHEN designed the paper. Mengmeng GUO, Juanjuan ZHAO, and Chao CHEN edited the manuscript. Ya ZHOU, Yong HAN, and Lin XU conceived, designed, and wrote the paper. All authors have reviewed and approved the paper, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Shipeng CHEN, Lian GUAN, Xu ZHAO, Jing YANG, Longqing CHEN, Mengmeng GUO, Juanjuan ZHAO, Chao CHEN, Ya ZHOU, Yong HAN, and Lin XU declare that they have no conflict of interest.

All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals of Zunyi Medical University. The experimental procedures were approved by the ethical guidelines of Zunyi Medical University Laboratory Animal Care and Use Committee (permit number 2018016).

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Supplementary information

Figs. S1–S3; Materials and methods