Original Article

In vitro Study of Nucleostemin Gene as a Potential Therapeutic Target for Human Lung Carcinoma*

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Abstract

Objective Nucleostemin (NS) is a GTP-conjugated protein located in the nucleoli of stem cells and some cancer cells, and maintains cell self-renewal. We aimed to evaluate NS as a potential target for lung carcinoma gene therapy by investigating NS gene expression and its effect on A549 cell proliferation.

Methods NS mRNA and protein expression in A549, HepG2, SMMC-7721, HeLa, and U251 cells was analyzed by RT-PCR and western blotting following transfection of NS siRNAs and negative control siRNA (NC). The effect on cell proliferation was also analyzed by MTT assays.

Results NS mRNA and protein were both expressed in A549 cells and four other tumor cell lines; the relative expression levels were similar in all five cell lines. The three pairs of NS siRNA, either transfected alone or cotransfected into A549 cells, could effectively inhibit the expression of NS mRNA and protein. Moreover, the interference ratio showed an obvious concentration-dependent relationship. NS siRNA treatment resulted in significant inhibition of A549 cell proliferation by 35.7%.

Conclusion NS gene was not only highly expressed but also played an important role in A549 cell proliferation. Thus, targeting of NS may be a promising novel strategy for the treatment of lung carcinoma.

Key words: Nucleostemin; RNA interference; A549 cells; Tumor gene therapy

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INTRODUCTION

Nucleostemin (NS) was initially described by Tsai & McKay in 2002[1]. Located at 3p21.1, the NS gene (*GNL3*) encodes a GTP-binding protein, which is highly expressed in central nervous system stem/progenitor cells, as well as in hematopoietic stem cells, but not in their differentiated progeny[1]. NS expression has also been demonstrated in certain somatic cells, such as human BMSCs and several cancer cell lines[2-6]. NS may be an imperative protein for regulating the proliferation of stem cells and cancer cells, and in maintenance of their undifferentiated features. Moreover, NS is a special cell cycle regulatory factor required for cells to pass the G2/M checkpoint[1].

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Lung carcinoma is one of the most common malignant tumors worldwide, and both its morbidity and mortality rank first in the world. At present, clinical treatment of lung carcinoma mainly include surgery, radiotherapy and chemotherapy, but the prognosis is very poor; approximately 80% of patients die in the first year following diagnosis. Recent research has revealed that the development and progression of tumors are related to many genes and pathways, thus tumor gene therapy is a hot spot of medical research. As a result, various targets for lung carcinoma gene therapy have been elucidated, such as vascular endothelial growth factor (VEGF)[7-8], p53[9-10], and epidermal growth factor receptor (EGFR)[11-12], but it is still unknown whether NS may be a potential therapeutic target for lung carcinoma.

Our study investigated the expression of NS in A549 cells, and compared in four additional cell lines. RNAi technology was applied to silence NS gene expression, and A549 cell proliferation was subsequently analyzed. On this basis, the feasibility of NS as a potential target for lung carcinoma gene therapy was assessed.

MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma A549 cells, hepatoma HepG2 and SMMC-7721 cells, cervical cancer HeLa cells and neurogliocytoma U251 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Yuan Heng, China), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% humidified CO_2.

RNA Extraction and RT-PCR

Transfected tumor cells were harvested using Trizol reagent (Sagon Biotech, China) 48 h after transfection, and total RNA was extracted from tumor cells. Reverse transcription was carried out on 1 μg of total RNA in a final volume of 20 μL, using dT18 primer and Revert Aid™M-MuLV Reverse Transcriptase (Fermentas, Lithuania) according to the manufacturer's protocol. Reverse transcription reactions lacking reverse transcriptase served as negative controls. PCR primers are detailed in Table 1. Expression of β-actin (a housekeeping gene) was monitored in all semi-quantitative RT-PCR experiments as an internal control. PCRs were performed using 2 μL of cDNA with 10 μL of 2×Taq Master Mix (SinoBio, China) in 20 μL reaction volumes. PCR conditions were as follows: 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, for 30 cycles, then 72 °C for 5 min. PCR products were visualized by Goldview (Sbsbio, China) staining on 1.5% agarose gels and examined using a gel image analysis system (Biorad GelDoc XR, USA).

Table 1. Primer Sequences of Target Genes and Amplified Product Length

Target Gene	Primer Sequences	PCR Product Length (bp)
NS (NM-206825)	forward:5' -TCCGAAGTCCAGCAAGTATTG-3' (1235-1255) reverse:5' -AATGAGGCACCTGTCCACTC-3' (1447-1467)	233
β-actin (NM-001101)	forward:5' -TCCTGTGGCATCCACGAAACT-3' (884-904) reverse:5' -GAAGCATTTGCGGTGGACGAT-3' (1178-1198)	315

Sequence of NS siRNA and Transfection

Three pairs of double-stranded siRNA corresponding to NS (NS-siRNA), and a pair of negative control siRNAs (NC-siRNA) with no complementary target sequence within the human genome, were synthesized by RiboBio Co., Ltd (Guangzhou, China), as shown in Table 2.

For transfection, A549 cells were seeded in six-well plates at a density of 4×10^4 cells per well in DMEM supplemented without antibiotics. The following day, when cultures were 30%-50% confluent, the supplementation for 1.5 mL Opti-MEM Reduced Serum Medium (Gibco, USA) was changed 1 h before transfection. Five microliters of Lipofectamine 2000 (Invitrogen, USA) was added to 250 μL Opti-MEM Reduced Serum Medium, mixed gently, and incubated at room temperature for 5 min. In parallel, 5, 8, and 12 μL siRNA (20 μmol/L) were each added

Table 2. Sequences of NS-siRNA

	Sequences of NS-siRNA
NS-siRNA-1 (NM_206825, 1743-1760)	Sense: 5'-CCAGGAAACUGUUGAUGAAdTdT-3' Antisense: 3'-dTdTGGUCCUUUGACAACUACUU-5'
NS-siRNA-2 (NM_206825, 1310-1327)	Sense: 5'-GACAGGUAGUACUGAAAUAdTdT-3' Antisense: 3'-dTdTCUGUCCAUCAUGACUUUAU-5'
NS-siRNA-3 (NM_206825, 569-586)	Sense: 5'-CAUCAAAUGUGGAACCUAUdTdT-3' Antisense: 3'-dTdTGUAGUUUACACCUUGGAUA-5'

to 250 μL Opti-MEM Reduced Serum Medium, mixed with the diluted oligomer and Lipofectamine 2000, and incubated at room temperature for 20 min before addition to the cells at a final concentration of 50, 80, and 120 nmol/L. Meanwhile, NS-siRNA-1, NS-siRNA-2 and NS-siRNA-3 were mixed with the same volume of Opti-MEM to achieve final concentrations of 50, 80 and 120 nmol/L, and then cotransfected into A549 cells. The transfected cells were cultured at 37 °C with 5% CO_2; transfection medium was replaced with fresh medium supplemented 10% FBS at 4-6 h post-transfection, and fed daily with fresh medium. A blank control group (A549 cells without treatment) and a negative control group (A549 cells transfected with NC-siRNA) were concurrently established.

Western Blotting

Tumor cells and transfected cells were harvested with 0.25% trypsin at 72 h post-transfection, and washed twice with phosphate buffered solution (PBS). Proteins were extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) supplemented with phenylmethylsulfonyl fluoride (PMSF, final concentration 1 mmol/L; Amerisco, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gel) and were blotted on polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were blocked for 2 h with 5% bovine serum albumin (BSA; Sanland, USA) in Tris-buffered-solution (TBS) with gentle shaking.

Membranes were incubated with anti-NS (1:200) or anti-β-actin (1:200) primary antibody (Santa Cruz, USA) in TBS containing 5% BSA. After overnight incubation at 4 °C with gentle shaking, membranes were washed for 10 min three times with TBS containing 0.1% Tween-20 (TBST). Membranes were incubated with rabbit or mouse horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz, USA) for 2 h at room temperature, and then washed as described previously. Blots were visualized with 3,3'-diaminobenzidine (DAB; Zhongshan, China). When bands reached the desired intensity (2-5 min), membranes were washed briefly in water, followed with TBST. Finally, the blots were analyzed using a gel image analysis system (Biorad GelDoc XR, USA) and Quantity One 1-D software.

Cell Growth Curve and Proliferation Inhibition Analysis

Proliferation of A549 cells was investigated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] assays. Single cell suspensions were prepared in DMEM supplemented with 10% FBS, and then seeded in 96-well plates at a density of 5×10^3 cells/well in 200 μL. Cells were seeded in triplicate wells for each group, and were transfected with 120 nmol/L NS-siRNA-2, as described earlier. At the end of each incubation period, 20 μL MTT (0.5 mg/mL; Amresco, USA) was added, and cells were incubated for 4 h under standard cell culture conditions. Then the liquid was removed, and 200 μL DMSO (Amresco, USA) was added to each well. Absorbance was measured with an ELISA plate reader at 570 nm. Values represented the means of triplicate wells, from which cell growth curves and proliferation inhibition ratios were determined.

Statistical Analyses

All experiments were replicated three times, and statistical analyses were executed by SPSS10.0 software. *P* values of <0.05 were considered to be statistically significant.

RESULTS

NS mRNA and Protein are Highly Expressed in A549 Lung Carcinoma Cells

NS mRNA and protein were both highly expressed in A549 lung carcinoma cells (Figure 1); the relative expression were 63.28%±3.50% and 94.04%±3.80%, respectively.

NS-siRNAs Could Effectively Silence NS mRNA Expression

Three pairs of NS-siRNAs were independently introduced into A549 cells at 50, 80, and 120 nmol/L. Mixed siRNAs, as mentioned earlier, were simultaneously cotransfected into A549 cells. NS mRNA expression in A549 cells was assessed after NS-siRNA treatment, using RT-PCR analysis (Figure 2). Compared with the control groups, the electrophoresis strips of NS-siRNA transfection groups were less bright (Figure 2 a,b,c). The inhibition ratios of NS mRNA under three transfection conditions are shown in Figure 2d. Statistical analyses showed that there was statistical significance ($P<0.05$) in terms of NS mRNA expression between all NS-siRNA transfection groups and A549 blank control group. Thus, all three pairs of NS-siRNA could interfere with NS mRNA expression when transfected alone and cotransfected; the interference rates at 120 nmol/L siRNA were 64.67%±4.10%, 70.16%±3.59%, 67.53%±0.76%, and

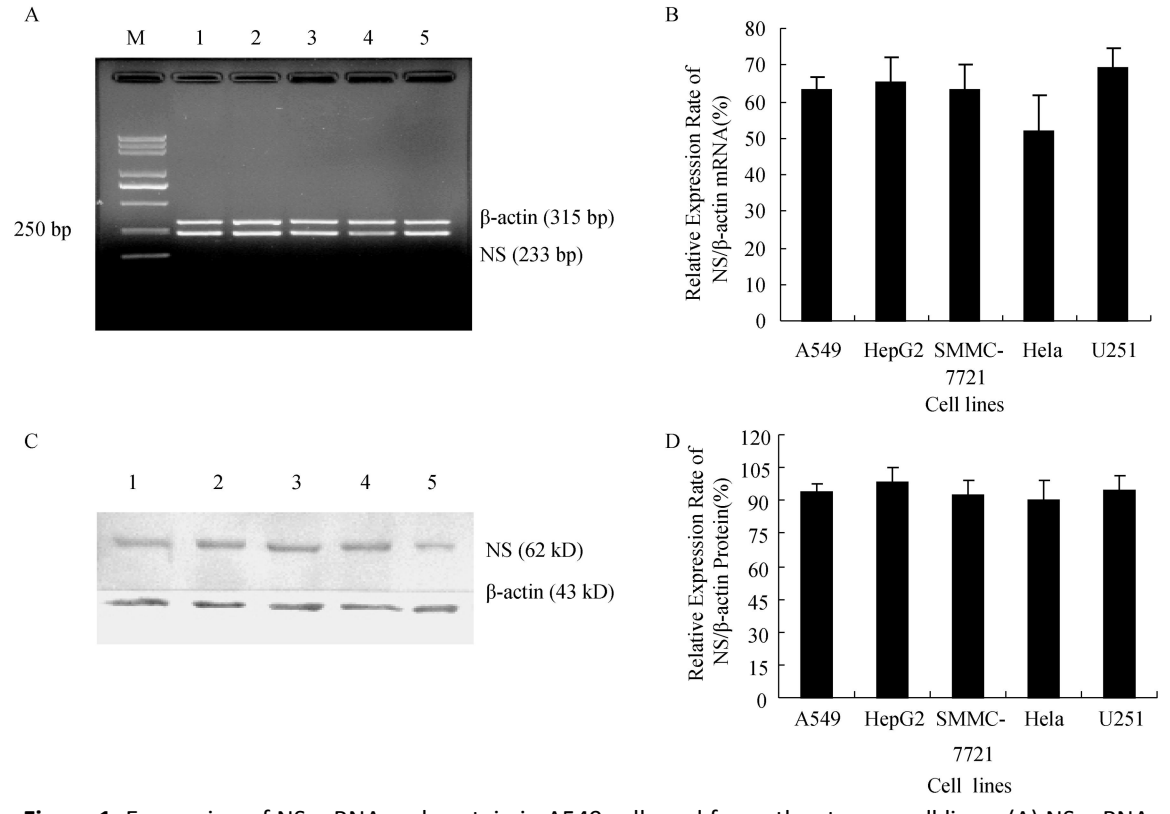

Figure 1. Expression of NS mRNA and protein in A549 cells and four other tumor cell lines. (A) NS mRNA expression. Lanes are labeled as follows: M. DNA Marker; 1. A549; 2. HepG2; 3. SMMC-7721; 4. HeLa; 5. U251. (B) Relative expression rates of NS/β-actin mRNA were 63.28%±3.5%, 65.07%±6.8%, 63.00%±7.1%, 51.63%±9.9%, and 69.32%±5.3%, respectively. (C) Expression of NS protein. Lanes are as follows: 1. A549; 2. HepG2; 3. SMMC-7721; 4. HeLa; 5. U251. (D) Relative expression rates of NS/β-actin protein were 94.04%±3.8%, 98.17%±6.8%, 92.52%±6.4%, 90.15%±8.8%, and 94.36%±7.2%, respectively. Expression of β-actin mRNA and protein was monitored as internal control.

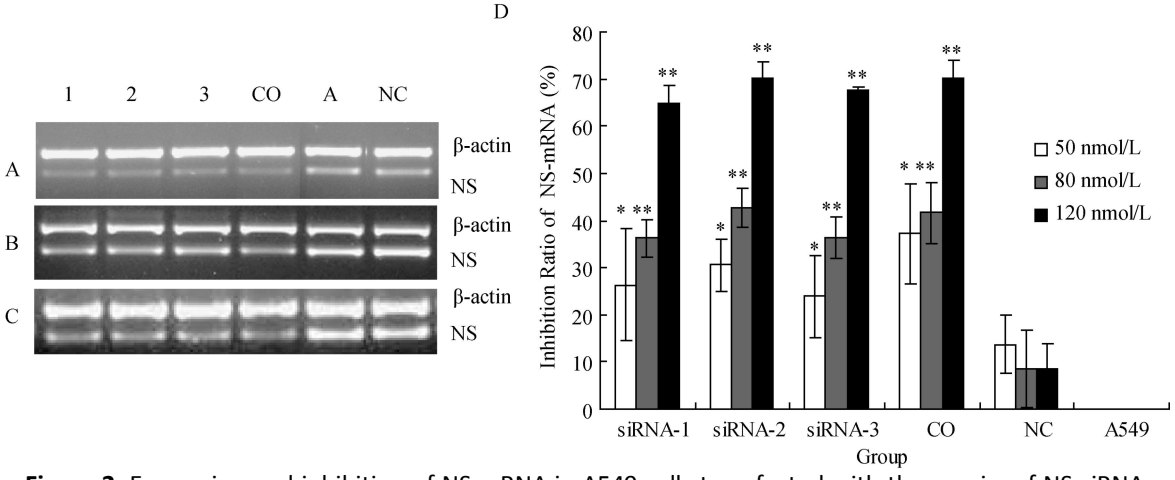

Figure 2. Expression and inhibition of NS mRNA in A549 cells transfected with three pairs of NS-siRNA. (A) 50 nmol/L NS-siRNA, (B) 80 nmol/L NS-siRNA, and (C) 120 nmol/L NS-siRNA. Lanes are labeled as follows: 1. siRNA-1; 2. siRNA-2; 3. siRNA-3; CO, cotransfection with siRNA-1, 2, 3; A, A549; NC, negative control. (D) Inhibition ratio of NS mRNA in A549 cells. Compared with the control group, NS mRNA expression under the three transfection conditions was reduced by approximately 30%, 40%, and 70%, respectively. Statistical analyses by SPSS one-way ANOVA showed that the differences in NS mRNA expression were significant between all NS-siRNA transfection groups and the A549 control group. $^{*}P<0.05$, $^{**}P<0.01$.

70.2%±3.83% for NS-siRNA-1, NS-siRNA-2, NS-siRNA-3, and cotransfection of all three NS-siRNAs, respectively. All three concentrations of siRNA could reduce NS mRNA expression. Furthermore, the inhibition ratio gradually improved with increased transfection concentration of NS-siRNA. Therefore, 120 nmol/L was optimal for the subsequent experiments.

NS-siRNAs Interfered with NS Protein Expression in A549 Cells

Three pairs of siRNA were transfected into A549 cells at a concentration of 120 nmol/L. Mixed siRNAs (120 nmol/L), as discussed earlier, were cotransfected into A549 cells. NS protein expression after siRNA treatment was assessed by western blot analysis (Figure 3a). Compared with the control groups, NS protein expression in the transfected groups was lower (Figure 3a). NS protein inhibition ratios are shown in Figure 3b. Statistical analyses demonstrated that, compared to the A549 blank control group, NS protein expression of all NS-siRNA transfection groups were statistically significant (P<0.01). The three pairs of NS-siRNA affected NS protein expression in A549 cells after being transfected alone and cotransfected, and the interference rates were 57.23%±3.54%, 68.00%±1.96%, 48.53%±12.06%, and 74.55%±0.94% for NS-siRNA-1, NS-siRNA-2, NS-siRNA-3, and cotransfection of all three NS-siRNAs, respectively.

NS-siRNA-2 (120 nmol/L) Inhibited A549 Cell Proliferation

A549 cells were transfected with 120 nmol/L siRNA-2 in 96-well plates, and cell proliferation was assessed by MTT assays (Figure 4). Cell proliferation

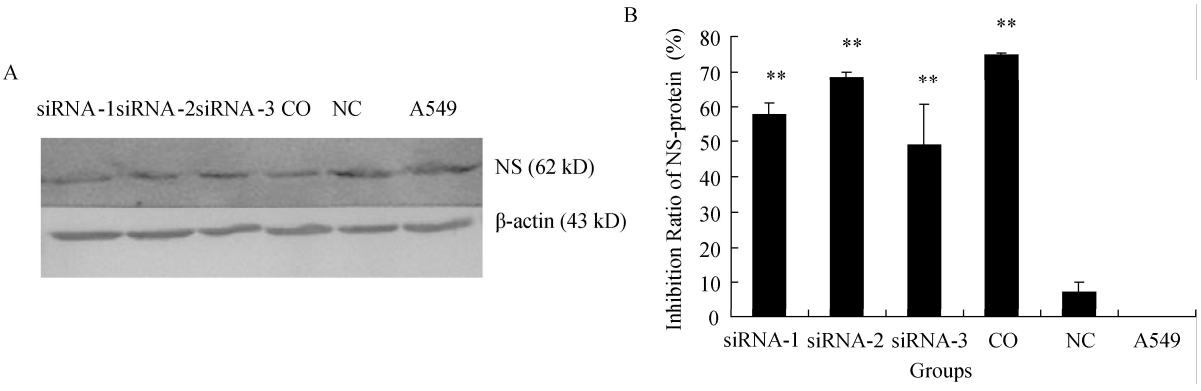

Figure 3. Expression and inhibition ratio of NS-protein in A549 cells transfected with 120 nmol/L siRNAs (A) Expression of NS-protein in transfection groups were reduced compared with control group. CO.co-transfection with siRNA-1,2,3 NC.Negtive Control; (B) Inhibition ratio of NS-protein expression were about 50%-70% compared with A549 control group, Statistics analysis by SPSS one-Way ANOVA showed that the differences of NS-protein expression were significance between all of NS-siRNA transfection groups and A549 blank control group. $^{**}P$<0.01.

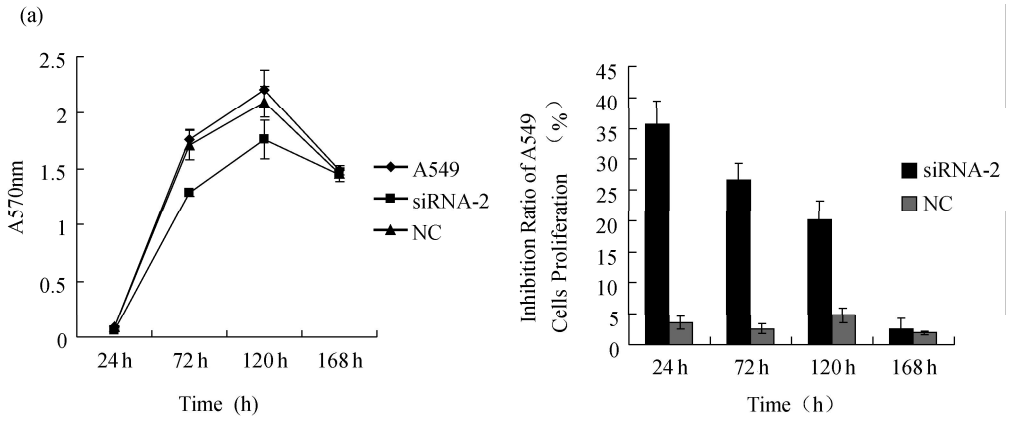

Figure 4. Growth curve and inhibition ratio of A549 cells transfected with 120 nmol/L siRNA-2. (a) Growth curve of A549 cells treated with NS-siRNA-2; the proliferation of NS-siRNA-2 transfected group was decreased. (b) Inhibition ratio of A549 cells treated with NS-siRNA-2 reached 35.7%, and then gradually decreased with time.

of the NC-transfected group and the A549 blank control group were similarly unaffected, but the proliferation of NS-siRNA-2 transfected group was inhibited. The results showed that the proliferation inhibition ratio decreased over time. Most significantly, the inhibition ratio reached 35.7% at 24 h, and then gradually decreased to 26.6% and 20.2% at 72 h and 120 h, respectively.

DISCUSSION

Nucleostemin was discovered by Tsai and Mckay[1] in their study of the genes and proteins expressed by stem cells to regulate proliferation. It was named nucleostemin as it is expressed by embryonic stem cells and is localized to nucleoli.

Expression of nucleostemin was identified in mice embryo stems (ES), mesenchyme stem cells (MSCs)[2], bone marrow stem cells[5], cultured human neural stem cells[13], and human embryonic stem cell s(huES)[14], amongst others. Thus, it is thought that NS protein may be a marker of stem cells. Although expressed in stem cells, NS may also be expressed in some malignant and benign tumor tissues, benign hyperplasia tissues and leukemia cells[3,15-20]. Many studies suggested that NS may have similar functions in stem cells and tumor cells, such as self-renewal, proliferation and differentiation regulation[21-23], and therefore may be important in tumor genesis, development and metastasis.

At present, tumor gene therapy is a hot spot in medicine, and some targeted drugs have been licensed for use, bringing hope to lung cancer patients. However, the question remains as to why tumor metastasis and recurrence occur after clinical treatment. It may be that the tumor stem cells (TSC) theory[24] will provide a definitive answer. The TSC theory is based on the hypothesis that a small number of tumor stem cells exist in almost all tumor types. These undifferentiated cells are capable of infinite proliferation and self-renewal, and have multiplex differentiation potential. It is believed that TSCs are progenitor cells of tumors, and are thus thought to be the drivers of tumor genesis, development, invasion, metastasis and drug resistance.

Since NS may contribute to self-renewal, proliferation, differentiation and cell-cycle progression regulation in stem cells and tumor cells[25-27], various studies have investigated the effect of NS downregulation on the inhibition of tumor cell proliferation[28-32]. As to whether suppression of NS expression can inhibit proliferation of lung tumor cells, thereby curing lung carcinoma, is not yet clear.

In our research, we first examined NS expression in A549 cells and four other tumor cell lines by RT-PCR and Western blotting. Our results showed that NS mRNA and protein were highly expressed in all five tumor cell lines. These results verified the findings of NS expression in several tumor cell lines in earlier studies. Then, we designed and synthesized three pairs of siRNA against NS mRNA, and used an RNAi approach to specifically promote degradation of NS mRNA. We found that all three pairs of NS-siRNA could reduce NS mRNA expression by about 70%, compared to the negative control siRNA-treated group. Downregulation of NS was also mirrored at the protein level, as demonstrated by western blot analysis, and NS protein expression was also reduced by about 70%. Finally, after A549 cells were transfected with 120 nmol/L siRNA-2, we observed a significant reduction in the proliferation of NS-siRNA-treated cells compared with the blank and negative control groups. These results suggested that NS plays an important regulatory role in promoting proliferation of A549 cells, and may be a candidate target in lung tumor therapy. Along with these observations in tumor cells, corresponding results from separated and purified tumor stem cells are desirable in our follow-up studies.

To conclude, NS gene is not only highly expressed, but also plays an important role in the promotion of A549 cell proliferation. Hence, targeting of NS may be a novel promising strategy for the treatment of lung carcinoma.

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