

# MicroRNA-194 restrains the cell progression of non-small cell lung cancer by targeting human nuclear distribution protein C

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**Abstract.** NSCLC accounts for over 80% of all lung cancers and is associated with poor prognosis. Human nuclear distribution C (hNUDC) was predicted to be the target gene of microRNA-194 (miR-194). The present study was designed to demonstrate the mechanism of miR-194 in the regulation of non-small cell lung cancer (NSCLC) via targeting the hNUDC. The hNUDC expression was found to strongly be increased while the miR-194 decreased significantly in the NSCLC cell lines when compared with the healthy controls. Moreover, the luciferase report confirmed the targeting reaction between miR-194 and hNUDC. After transfection with miR-194 mimic into NSCLC cells, we found that the miR-194 overexpression resulted in abnormal nuclear division, decreased cell proliferation and inhibited the expression of hNUDC and Mpl/ERK pathway proteins. Furthermore, the hNUDC overexpression affected the suppression effect of miR-194 in 95D cells, indicating that miR-194 suppresses tumor cell process by inhibiting the hNUDC expression. In brief, the present study suggests that the upregulation of miR-194 affects the hNUDC expression, leading to a downregulated expression of Mpl/ERK pathway proteins, and suppresses the mitosis and proliferation of NSCLC cells. These results offer a potential therapeutic strategy for the treatment of lung cancer.

## Introduction

Lung cancer is one of the most common malignancies globally, with 1.6 million new cases being diagnosed annually and is also the leading cause of cancer deaths worldwide (1).

Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers accompanied by poor prognosis (2). Despite recent treatment advances, immunotherapy in particular, offers promising treatment alternatives that could help fight disease mortality with minimal impact on normal tissues (3), the chances of survival of NSCLC remain bleak, and novel therapeutic approaches are required.

The human homolog of filamentous fungus *Aspergillus nidulans* NUDC (nuclear distribution C), called hNUDC, is structurally based on the similarity of its C-terminus to that of the fungal NUDC from *Aspergillus nidulans* forms (4). NUDC is necessary for nuclei movement following mitosis as well as normal colony growth, which is highly conserved structurally and functionally throughout most evolution (5). The hNUDC has been reported to be involved in mitosis, cytokinesis (6), neuronal migration (7) and hematopoietic cell growth in humans (5). However, the hNUDC function in NSCLC cells has not yet been investigated.

Thrombopoietin receptor (Mpl) is a class I cytokine receptor, belonging to the hematopoietic super family of receptors. hNUDC is confirmed to be the second natural ligand for Mpl (after thrombopoietin) and binds to the extracellular domain of the Mpl (8), thus, inducing a sustained activation of the extracellular signal-regulated protein kinases-1 and -2 (ERK1/2), resulting in megakaryocytic proliferation and differentiation (9). The extracellular signal regulated kinase (ERK1/2), also called the mitogen-activated protein kinase (MAPK), reportedly promotes cell survival and chemotherapeutic resistance in NSCLC cell lines (10). ERK signaling pathway was suggested to play a role in the hNUDC overexpression-induced apoptosis (11). Other reports recorded that nuclear and cytoplasmic ERK1/2 activation positively correlated with advanced and aggressive NSCLC tumors (12). These reports suggested that hNUDC may affect the ERK1/2 pathway and regulate the processing of NSCLC cells.

miRNAs have been identified as classical oncogenes or tumor suppressor genes (13,14). In lung cancer, miR-let-7c (15), miR-506 (16) and miR-34a (17) have been identified as tumor suppressors, whereas miR-21 (18), miR-155 (19) and miR-31 (20) were found to be carcinogenesis promoters. Mature miR-194 is involved in both pri-miR-194-2/192 and pri-miR-194-1/215 clusters (14), and has been suggested to be a putative tumor suppressor in liver (21) and ovarian tissues (22). The miR-194 overexpression in these cancer cells suppresses cell migration, invasion and metastasis. The overexpression of

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**Abbreviations:** hNUDC, human nuclear distribution C; miR-194, microRNA-194; Mpl, thrombopoietin receptor; ERK1/2, extracellular signal-regulated protein kinases-1 and -2; 3'-UTR, 3'-untranslated region; miRNAs, microRNAs

**Key words:** non-small cell lung cancer, human nuclear distribution C, microRNA-194, Mpl/ERK pathway

the miR-194 in lung cancer cell lines has also been reported to suppress metastasis of lung cancer cells (14).

This study helped to identify miR-194 action in the context of non-small cell lung cancer. We first found hNUDC overexpression in NSCLC cell lines and NSCLC patients when compared to healthy controls. Besides, miR-194 was predicted to target hNUDC, which regulated the ERK1/2 pathway. Taken together, our results suggest that miR-194 may provide novel insight into the process of NSCLC via targeting hNUDC.

## Materials and methods

**Patients.** Twenty-six patients (12 males and 14 females) with non-small cell lung cancer and paired non-tumor lung tissues were consecutively included in this study. Both tumor and non-tumor samples were confirmed by the pathological examinations. Patients were recruited from the Respiratory Department of Cangzhou Central Hospital. The study was approved by the ethics committee of our institution. Informed consent was signed by the participants.

**Cell culture.** First, 95C and 95D cells were subcloned from the PLA-801 human giant-cell lung carcinoma cell line, but they had different metastatic potentials (23). Human non-small cell lung cancer cells HCC827, A549, NCI-H460 and human lung fibroblast (NHLF) cell lines obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), were cultivated in modified Eagle's medium (MEM; Invitrogen, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum (FBS; Invitrogen) and 1% antibiotic-antimycotic (Invitrogen), while the NSCLC cell lines 95C and 95D were grown in MEM supplemented with 10% FBS and 1% antibiotic-antimycotic. All the cells were incubated at 37°C in a humidified 21% O<sub>2</sub>, 5% CO<sub>2</sub> atmosphere.

**qRT-PCR.** The total RNA was extracted from TRIzol reagent (Invitrogen) following the manufacturer's instructions. For miR-194 detection, reverse transcription was performed using One Step PrimeScript miRNA cDNA Synthesis kit (Takara, Dalian, China), following the primers: 5'-UGUACAGCAACUCCAUGUGGA-3' (sense); common antisense primer, 5'-GACTGTTCTCTCTTCCTC-3'. For mRNA detection of hNUDC, the cDNA was generated using M-MLV reverse transcriptase (Clontech Laboratories, Palo Alto, CA, USA), and amplified following the primers: 5'-AGACCTGCCCAA TTACCGC-3' (sense); 5'-GCTCCCCATCAATGATCGCT-3' (antisense). To analyze the gene expression, the qRT-PCR mixture system containing the cDNA templates, primers and SYBR-Green qPCR Master Mix were subjected to qRT-PCR quantification according to the standard methods.  $\beta$ -actin and U6 SnRNA were used as the internal control of the mRNA or miRNA, respectively. The human  $\beta$ -actin primers: 5'-GATCATGTTTGAGACCTTC-3' (sense); 5'-GGCATACCCCTCGTAGATG-3' (antisense), the U6 primers: 5'-GCTTCGGCAGCACATATACTAAAAT-3' (sense); 5'-CGCTTCACGAATTTGCGTGTCTAT-3' (antisense). Relative gene expression was quantified by 2<sup>- $\Delta\Delta C_t$</sup>  method.

**Western blot analysis.** A total of 25  $\mu$ g proteins were loaded and separated via sodium dodecyl sulfate-polyacrylamide gel

electrophoresis and then electrotransferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were then blocked in 2.5% non-fat milk for 1 h at 37°C. After washing with Tris-buffered saline with Tween, the membranes were incubated with primary antibodies against Mpl, ERK1/2, c-myc,  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p-CRBE (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Then, the peroxidase-conjugated secondary antibody (Wuhan Boster Biological Technology, Ltd., Wuhan, China) diluted in 1:1,000 was added and incubated for 1 h at room temperature. The immunoreactive protein bands were then visualized using an enhanced chemiluminescence detection system (Amersham).

**Dual-luciferase reporter assay.** The target gene was predicted by TargetScan (<http://www.targetscan.org/>). The 3'-UTR fragment of hNUDC mRNA containing the target sequence (CUGUUAC) of miR-194 was amplified by RT-PCR. The fragment, designated hNUDC 3'-UTR, was inserted into the pMIR-REPORT<sup>TM</sup> luciferase reporter vector (*Mlu*I and *Hind*III restriction enzyme sites; Ambion, Austin, TX, USA). Another expressing vector was also constructed by the insertion of a mutated hNUDC 3'-UTR in which the target sequence of miR-194 was mutated into CUGUAAAC using the QuickChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA). The recombinant reporter vectors with normal and mutated hNUDC 3'-UTR were co-transfected with miR-194 into 95D cells using the TransMessenger<sup>TM</sup> Transfection reagent (Qiagen, Hilden, Germany). The luciferase assay was performed according to the manufacturer's protocol. The relative luciferase activities were normalized to that of the control cells.

**Transfection assay.** miRNA mimics and inhibitors, specific for miR-194 (Invitrogen), were used to increase and silence miR-194 expression in 95C and 95D cell lines, respectively. Two hundred pmoles of miR-194 mimics, miR-194 mimic control, miR-194 inhibitor, and inhibitor control (Ambion) were transfected into 3x10<sup>6</sup> 95C and 95D cells for 48 h by electroporation using a Nucleofector instrument, respectively. After transfection, the cells were allowed to recover by incubating them for 4 h at 37°C. The experiment was replicated thrice for data calculations.

**Giemsa staining.** For Giemsa staining, incubation of the 1x10<sup>4</sup> 95C and 95D cells was done on 35 mm of the cell petri dishes with a coverslip in each dish. After 48 h, the coverslips were immobilized using 100% methyl alcohol and then air dried. The cells were then treated with Giemsa staining solution and the cellular morphology was microscopically observed (Leica AF6000; Leica Microsystems, Wetzlar, Germany).

**MTT assay.** Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Shortly afterwards, cells were transfected according to the above description and were seeded in 96-well plates at 6x10<sup>3</sup> cells/well. The surviving fractions were determined at 0, 24, 48, 72, 96 and 120 h. Thereafter, the old medium was discarded and fresh medium containing MTT (5 mg/ml MTT in PBS; Shanghai Sangon Biological Engineering Technology,

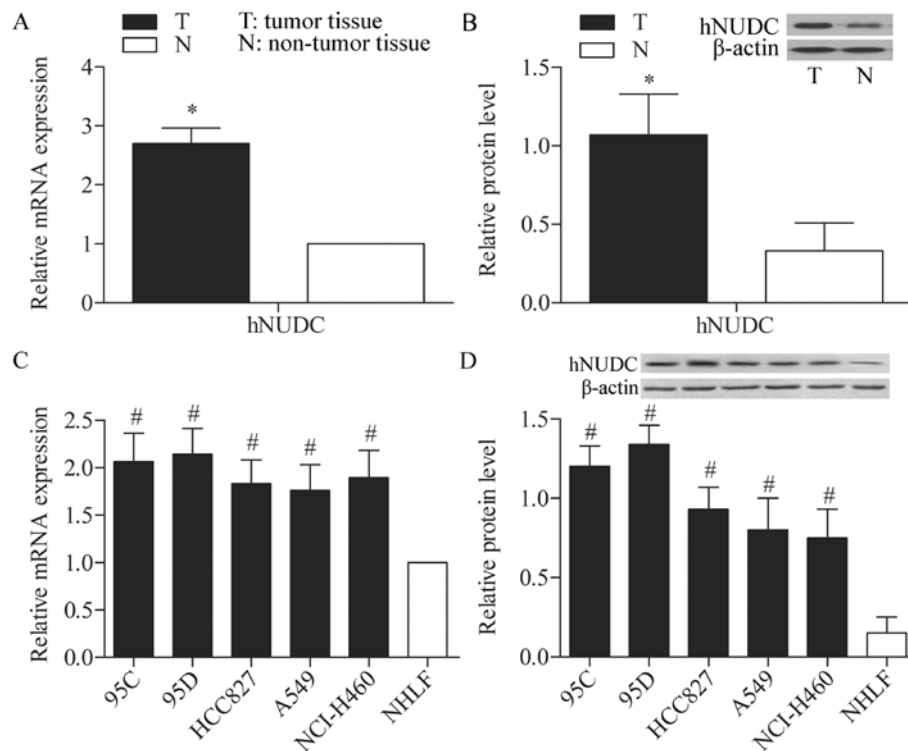


Figure 1. Inverse level of hNUDC in NSCLC and adjacent non-tumor lung tissues. The inverse level was also detected in the NSCLC cells (95C, 95D, HCC827, A549, NCI-H460) and NHLF cells. (A) The mRNA expression levels of hNUDC in the NSCLC and adjacent non-tumor lung tissues were measured by quantitative real-time PCR (qRT-PCR); (B) the protein expression levels of hNUDC in NSCLC and adjacent non-tumor lung tissues were measured by western blot analysis; (C) the mRNA expression of hNUDC in NSCLC cells (95C, 95D, HCC827, A549 and NCI-H460) and NHLF cells was assessed by qRT-PCR assays; (D) western blotting assay was used to confirm the hNUDC expression profile in the cell lines mentioned above. The relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to  $\beta$ -actin; data are presented as the mean  $\pm$  SD of three experiments. \* $P < 0.05$  vs. normal cases, # $P < 0.05$  vs. the NHLF cell lines.

Shanghai, China) was added and incubated for an additional 4 h. Then, cell viability was measured with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 470 nm. Each experiment was performed in triplicate.

**Cell cycle analysis.** Cell cycle analysis was determined by flow cytometry (BD Biosciences, San Jose, CA, USA). In short, 95C and 95D cells at  $1 \times 10^6$  cells/well were cultured in 6-well plates and transfected with 50 nM of the miR-194 mimics, miR-194 inhibitor or their respective control RNA for 48 h. The cells were then harvested and fixed in 70% ice-cold ethanol for 24 h, followed by staining with propidium iodide (PI). The different cell cycle phases were analyzed with the FACSCalibur instrument using CellQuest software (Becton-Dickinson, Mountain View, CA, USA).

**hNUDC overexpression.** The hNUDC overexpression was achieved by PCR amplification using hNUDC cDNA as a template, and the hNUDC expressing vector was constructed by inserting the hNUDC cDNA into pcDNA 3.1 vector. The recombinant plasmid and other agents were co-transfected into  $3 \times 10^6$  95D cells using a Nucleofector instrument. Forty-eight hours later, subsequent experiments were performed on the cells. The experiment was replicated thrice for data calculations.

**NSCLC xenografts.** Nine NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME, USA) (male; body weight,

20–22 g; age, 8 weeks old) were purchased from the Institute of Zoology, Chinese Academy of Medical Sciences. 95D cells were transfected with miR-194 or negative control miRNA (NC pre-miR<sup>TM</sup>; Ambion) following same transfection conditions. Cells ( $5 \times 10^6$ ) 95D transfected with miR-194 were injected subcutaneously into the right flank of NOD/SCID mice ( $n=9$ ). Cells transfected with negative control miRNA were injected into the left flank of NOD/SCID mice ( $n=9$ ). The tumor volumes were measured daily after the injection, and all the rats were assigned to euthanasia at the end of measurements (on day 27). All animal experiments were performed in accordance with current prescribed guidelines and under a protocol approved by the Institutional Animal Care and Use Committee.

**Statistical analysis.** All results are presented as mean  $\pm$  SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni test. The difference was considered statistically significant at  $P < 0.05$ .

## Results

**Inverse level of hNUDC in NSCLC and adjacent non-tumor lung tissues.** The level of hNUDC expression was detected in NSCLC and adjacent non-tumor lung tissues via qPCR and western blot analysis. The results indicated that the level of hNUDC expression was significantly higher in tumor tissues compared with matched non-tumor tissues ( $P < 0.05$ ) (Fig. 1A

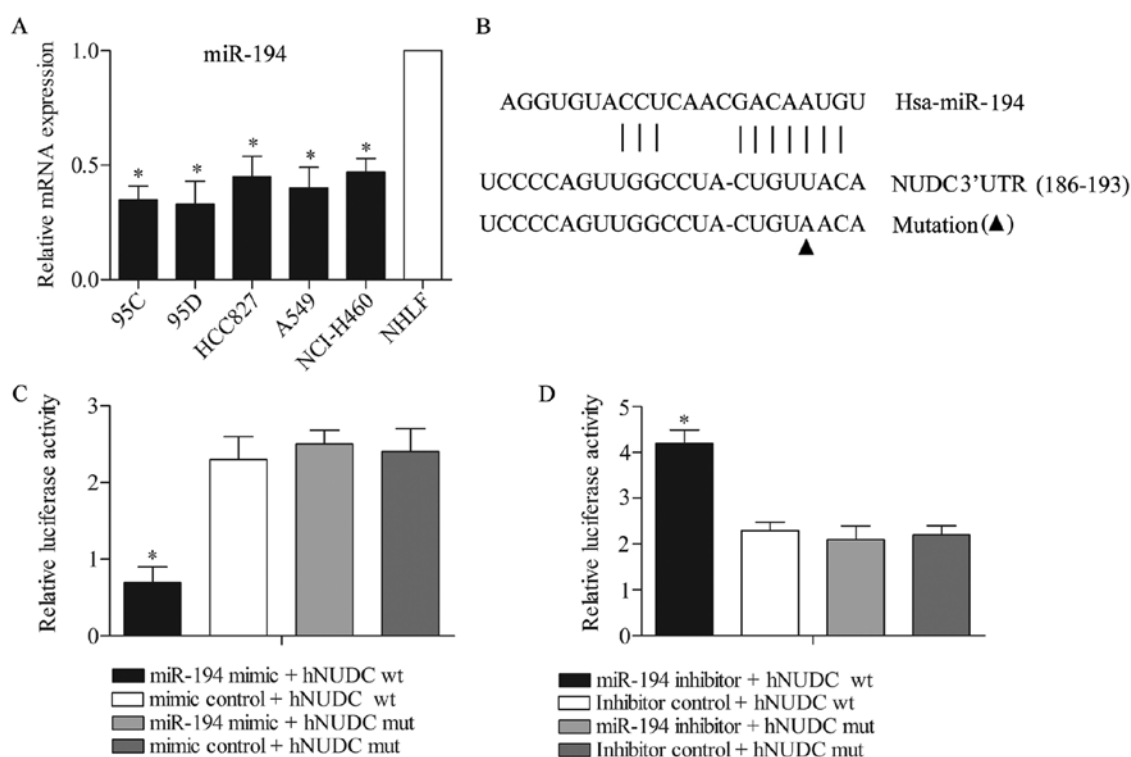


Figure 2. The target relationship between miR-194 and hNUDC. The target gene was predicted by TargetScan database and identified by luciferase activity report. (A) The level of miR-194 expression was detected in 95C, 95D, HCC827, A549 and NCI-H460 and NHLF cell lines by qPCR; (B) the wild and the mutant hNUDC 3'-UTR contained the target sequence of miR-194 was predicted by TargetScan; (C) the 95D cells were transfected with miR-194 mimic or mimic control luciferase reporter vectors containing the wild-type (wt) or mutant (mut) hNUDC 3'-UTR; (E) a similar luciferase assay was performed in 95D cells treated with the miR-194 inhibitor or control. Luciferase activity was demonstrated as firefly luciferase normalized to *Renilla* luciferase. Data are presented as the mean  $\pm$  SD of 3 experiments. \* $P < 0.05$  vs. the control group.

and B). The level of hNUDC expression was also detected in 95C, 95D, HCC827, A549, NCI-H460 and NHLF cell lines by qPCR. The results indicated that the mRNA expression level of the hNUDC was significantly higher in the NSCLC cells (95C, 95D, HCC827, A549 and NCI-H460) compared with NHLF cells ( $P < 0.05$ ) (Fig. 1C). The levels of the hNUDC protein expression in the three cell lines examined by western blot analysis were consistent with the mRNA expression levels (Fig. 1D). The results suggested that the levels of hNUDC expression were significantly elevated in the NSCLC patients and NSCLC cells compared with normal control.

**hNUDC is targeted by miR-194.** Assuming that the hNUDC level was increased in the NSCLC cell lines (especially in 95D), in order to obtain the biological role of hNUDC in the NSCLC cells, it was of interest to identify the microRNA that could target hNUDC. Predicted by the bioinformatic software programs TargetScan, we found that hNUDC was targeted by miR-194. The level of miR-194 expression was detected in 95C, 95D, HCC827, A549, NCI-H460 and NHLF cell lines by qPCR. The results indicated that the mRNA level of miR-194 expression was significantly lower in NSCLC cells (95C, 95D, HCC827, A549 and NCI-H460) compared with NHLF cells ( $P < 0.05$ ) (Fig. 2A). The potential binding target sites of miR-194 were found in the 3'-UTR of hNUDC gene (Fig. 2B). To experimentally confirm that hNUDC was an authentic target of miR-194 in the 95D cells, the plasmid pMIR-REPORT-hNUDC-wt or pMIR-REPORT-hNUDC-mut was transfected

into 95D cells together with miR-194 mimics or mimic control. After 48 h of transfection, the results showed that the luciferase activity in the hNUDC-wt with miR-194 mimics transfection group was significantly reduced compared with the other three groups (Fig. 2C). Consistently, the luciferase reporter vectors of the hNUDC-wt and hNUDC-mut were co-transfected with the miR-194 inhibitors or inhibitor controls into 95D cells. The results showed that the luciferase activity in the hNUDC-wt with miR-194 inhibitor co-transfection group was increased significantly compared with the other three groups (Fig. 2D). The data mentioned above demonstrated that hNUDC is a genuine target of miR-194.

**miR-194 causes abnormal mitosis of NSCLC cells via targeting hNUDC.** As hNUDC is associated with human nuclear migration, we examined whether the overexpression or inhibition of miR-194 was capable of affecting cell mitosis by targeting hNUDC. miR-194 mimic was used to amplify the miR-194 expression, whereas a synthetic inhibitor specific for miR-194 was employed to suppress the expression of endogenous miR-194 in NSCLC cell lines. The efficiency of this miR-194 mimic or inhibitor was confirmed by qPCR assay (Fig. 3A and B), and 95C and 95D cells were transfected with miR-194 mimic, mimic control, miR-194 inhibitor and inhibitor control, separately, while the mRNA and protein level of hNUDC expression were examined by qRT-PCR (Fig. 3C and D) and western blot analysis (Fig. 3E and F), respectively. The quantified relative protein expression is summarized in

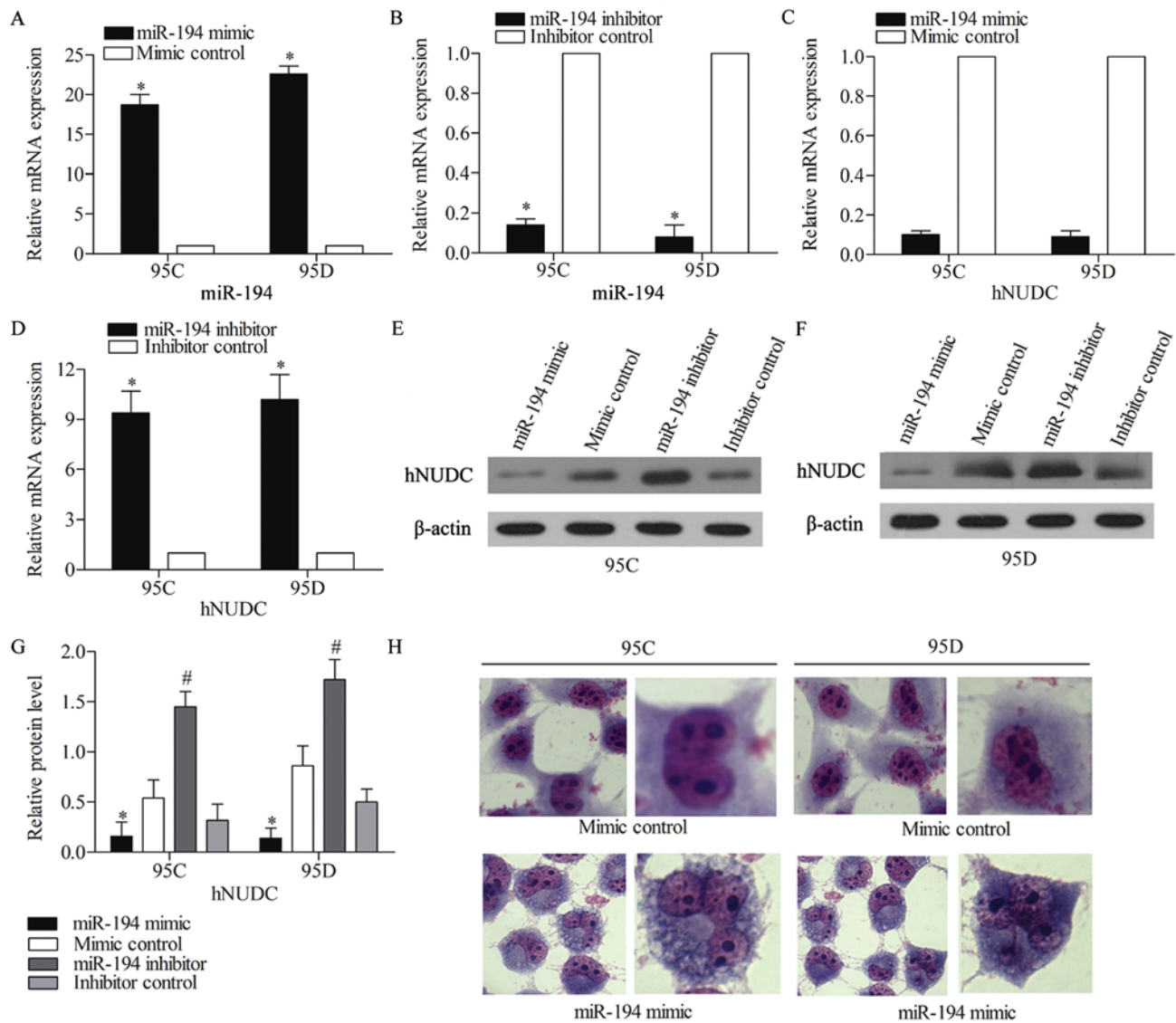


Figure 3. miR-194 induces abnormal mitosis of NSCLC cells via targeting hNUDC. 95C and 95D cells were transfected with miR-194 mimic, mimic control, miR-194 inhibitor or inhibitor control, respectively. (A) The expression levels of miR-194 in 95C and 95D cells co-transfected with miR-194 mimic or mimic control were measured by qPCR; (B) the levels of miR-194 expression in 95C and 95D cells co-transfected with miR-194 inhibitor or inhibitor control were measured by qPCR; (C) the relative mRNA levels of hNUDC in 95C and 95D cells co-transfected with miR-194 mimic or mimic control were measured by qRT-PCR; (D) the mRNA levels of hNUDC in 95C and 95D cells co-transfected with miR-194 inhibitor or inhibitor control were measured by qRT-PCR; western blot analysis was performed to measure the relative protein levels of hNUDC in 95C cells (E) and 95D cells (F); (G) the relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to  $\beta$ -actin; (H) the cell nuclear division of 95C and 95D cells was detected by Giemsa staining assay. The data are presented as the mean  $\pm$  SD of three experiments. \* $P < 0.05$  vs. the mimic control group; # $P < 0.05$  vs. the inhibitor control group.

Fig. 3G. The results showed that both the mRNA and protein levels of hNUDC were significantly downregulated when treated with miR-194 mimic, and increased when treated with miR-194 inhibitor compared with control groups. The Giemsa staining assay showed the nucleus was divided into two in the mimic control group in 95C and 95D cells. However, the nucleus exhibited abnormal division due to the overexpression of miR-194, three nuclei were detected within one cell in 95C cells, and sometimes even four (Fig. 3H). These results indicated that miR-194 affected the NSCLC cell mitosis via regulating the expression of hNUDC.

*The effect of miR-194 on the cell cycle and proliferation of 95C and 95D cells.* To further investigate the impact of miR-194 on the NSCLC cells, we studied whether the miR-194 overexpres-

sion was capable of affecting cell cycle and proliferation. The 95C and 95D cells were transfected with miR-194 mimic and mimic control separately. The analysis of the cell cycle indicated that miR-194 overexpression induced an accumulation of 95C cells in G0/G1 phase (Fig. 4A), and an accumulation of 95D cells in G2/M phase compared with mimic control (Fig. 4B), implying a cell cycle arrest in 95C and 95D cells with the change in the miR-194 levels. The cell cycle distribution in each group in 95C and 95D cells is summarized in Fig. 4C and D. The cell proliferation assay was performed in the cell lines, and miR-194 mimic was observed to strongly suppress the 95C cell growth compared with mimic control group (Fig. 4E). Similar MTT results were obtained in 95D cells, in which the cell proliferation rate was decreased under the treatment of miR-194 mimic (Fig. 4F) compared with mimic

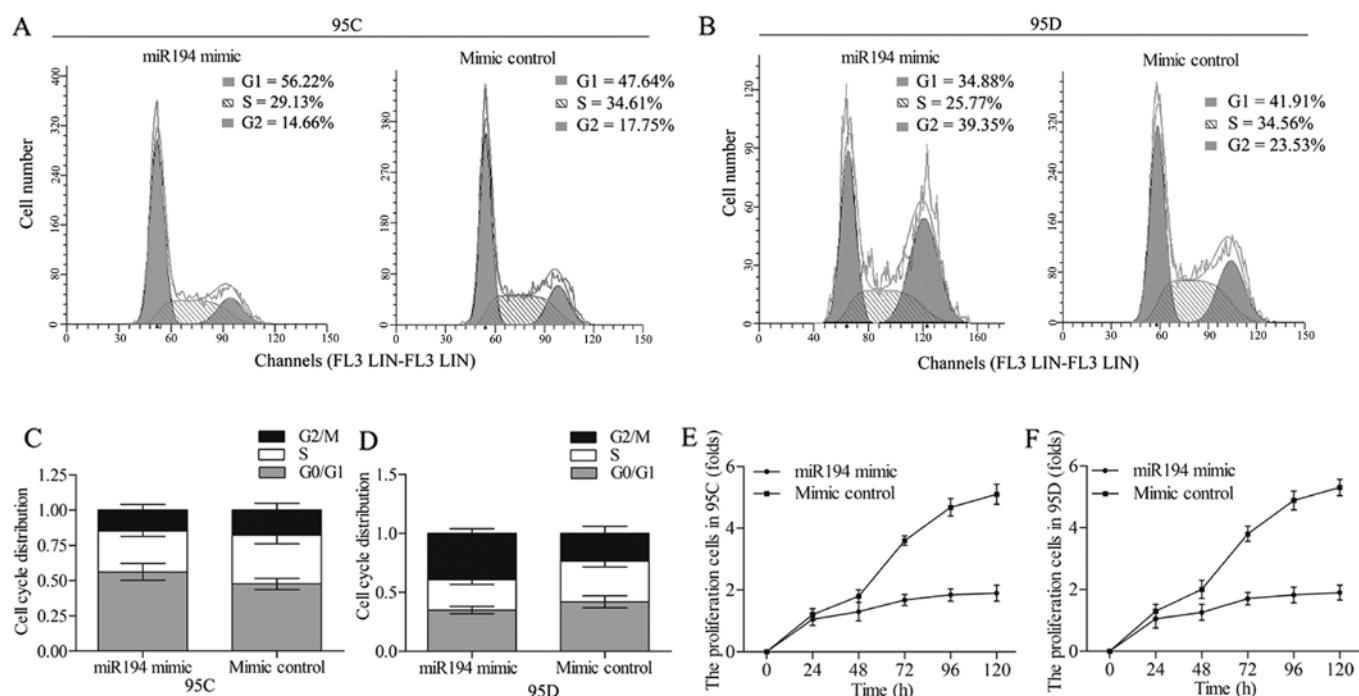


Figure 4. The effect of miR-194 on the cell cycle and proliferation of 95C and 95D cells. 95C and 95D cells were transfected with miR-194 mimic and mimic control, respectively. The cell cycle analysis of 95C (A) and 95D cells (B) with the miR-194 mimic or with mimic control were determined by flow cytometric analysis; the distributions of G0/G1, S and G2/M phases were evaluated in 95C (C) and 95D (D) cells transfected with miR-194 or with mimic control for 48 h. The proliferation of 95C (E) and 95D (F) cells was determined at the time-points indicated by the MTT assays. All the experiments were repeated three times with three replicates.

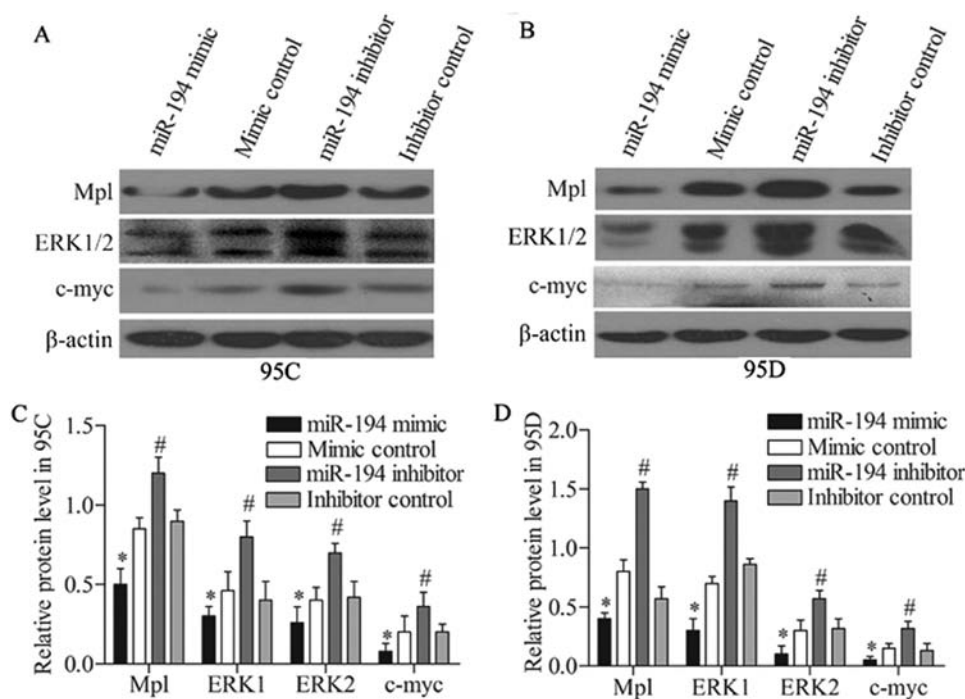


Figure 5. miR-194 regulates Mpl/ERK pathway via targeting hNUDC. The 95C and 95D cells were transfected with the miR-194 mimic, mimic control and miR-194 inhibitor or inhibitor control, separately. (A) The protein expression levels of Mpl/ERK pathway in 95C cells were measured by western blotting; (B) a similar western blotting assay was performed in 95D cells; (C) the relative protein expressions in 95C cells were quantified using Image-Pro Plus 6.0 software and normalized to  $\beta$ -actin; (D) relative protein expressions in 95D cells. Data are presented as the mean  $\pm$  SD of all three experiments. \* $P < 0.05$  vs. mimic control, # $P < 0.05$  vs. inhibitor control.

control. These findings suggest that miR-194 suppresses the cell cycle by decelerating the G0/G1 phase in 95C cells and

the G2/M phase in 95D cells, and inhibits cell proliferation in the NSCLC cells.



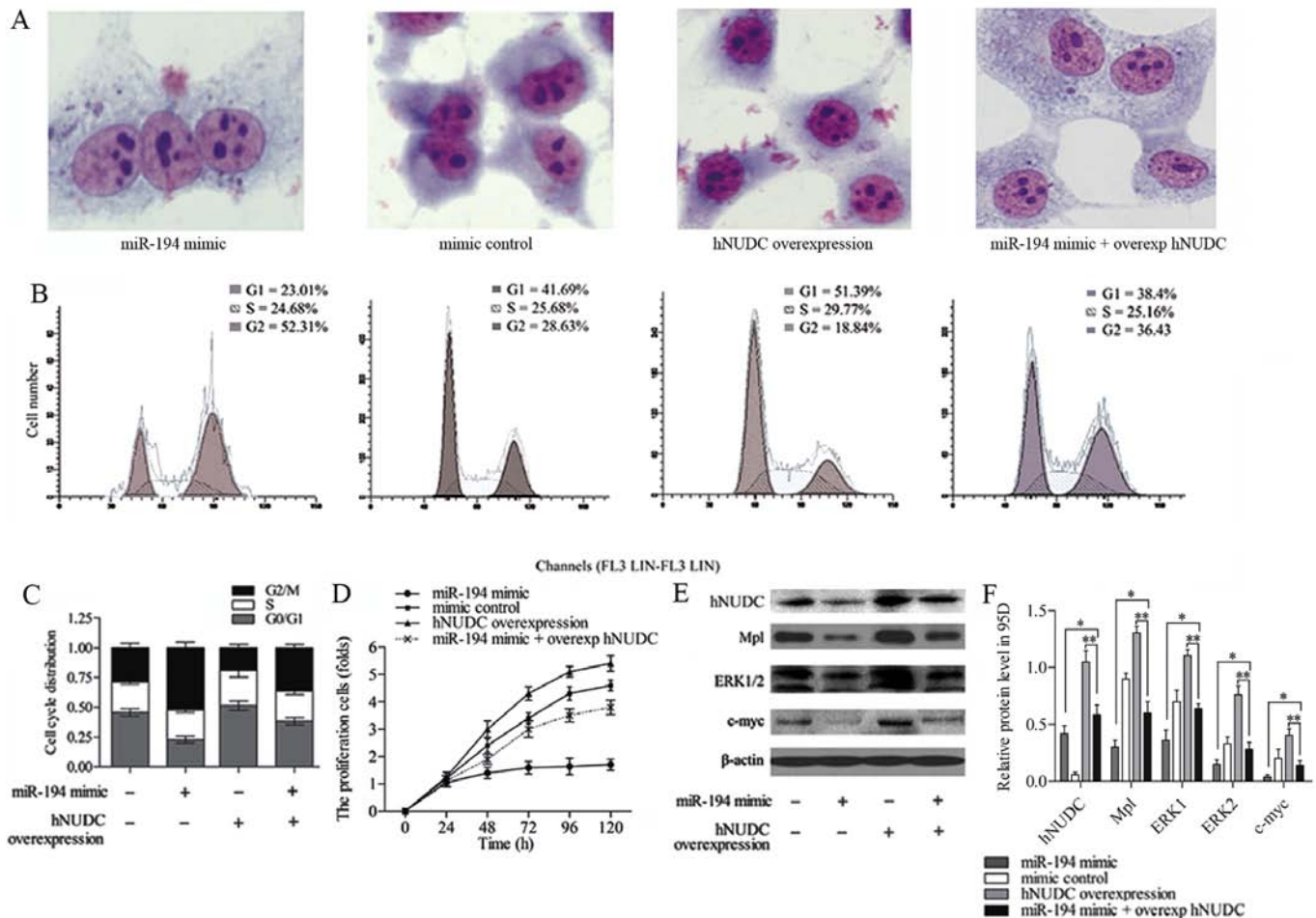


Figure 6. hNUDC restoration is necessary to affect the miR-194 suppression on the NSCLC cells and ERK pathway. Cells were treated with miR-194 mimic or mimic control with or without the overexpression hNUDC vector. (A) The cell nuclear division of 95D cells in each group was detected by Giemsa staining assay; (B) the cell cycle progression was conducted by flow cytometric analysis; (C) the distributions of the G0/G1, S and G2/M phases were evaluated in 95D cells; (D) MTT assay was employed to examine the proliferation rates of 95D cells treated with miR-194 mimic or mimic control with or without the overexpression hNUDC vector; (E) the relative hNUDC, Mpl, ERK1/2 and c-myc expressions were detected via western blotting; (F) the relative protein expressions in 95D cells were quantified using Image-Pro Plus 6.0 software and normalized to  $\beta$ -actin. The data are shown as means  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

*miR-194 inhibits the Mpl/ERK pathway by targeting hNUDC.* hNUDC has been reported to promote cell proliferation and differentiation via activation of the thrombopoietin receptor (Mpl) and the extracellular signal-regulated protein kinases-1 and -2 (ERK1/2) pathway (9). Thus, we detected the effect of miR-194 overexpression and the suppression on the expression of Mpl/ERK pathway, which included Mpl, ERK1/2 and c-myc genes. The protein expression was detected via western blot analysis. The results revealed that miR-194 mimic effectively decreased the expressions of Mpl, ERK1/2 and c-myc in 95C cells compared with mimic control. Whereas, miR-194 inhibitor promoted the expression of these genes compared with mimic control (Fig. 5A). Western blotting assay was also performed in 95D cells, miR-194 mimic intensively restrained the expressions of Mpl, ERK1/2 and c-myc compared with the mimic control group. Stronger expression of these genes was detected when they were subjected to the miR-194 inhibitor treatment compared with inhibitor control (Fig. 5B). The quantified relative protein expressions of Mpl/ERK pathway in 95C and 95D cells is summarized in Fig. 5C and D. The data

mentioned above suggested that miR-194 restrained the Mpl/ERK pathway in the NSCLC cells.

*miR-194 suppression of hNUDC is necessary to influence NSCLC cells and ERK pathway.* Given that hNUDC is the target of miR-194, it was of interest to study whether the hNUDC mediated the effect of miR-194 on the process of NSCLC cells and Mpl/ERK pathway. To determine whether the overexpression of hNUDC counteracted the effect of miR-194 in 95D cells, miR-194 mimic or mimic control with or without the hNUDC overexpression vector were co-transfected into 95D cells. Giemsa staining assay showed the presence of three nuclei within one cell in the miR-194 mimic group, however, cell division was found to be normal after hNUDC was overexpressed. This was because hNUDC is related to nucleus distribution, and the hNUDC overexpression resulted in an increase in migration ability of the nucleus. Cells co-transfected with miR-20b mimic plus overexpression hNUDC vector exhibited relatively abnormal mitosis compared with overexpression hNUDC group (Fig. 6A). Flow cytometric analysis of the cell cycle progression demonstrated

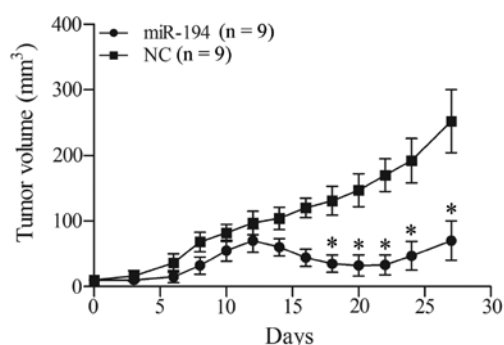


Figure 7. miR-194 suppresses tumor growth of lung cancer xenografts. 95D cells were transfected with miR-194 or negative control miRNA (NC) *ex vivo* and injected subcutaneously into 9 NOD/SCID mice using  $5 \times 10^6$  cells/flank (day 0). 95D cells transfected with negative control miRNA (NC) were injected into the opposite flank of the same animals that also received miR-194 treated cells. Measurements of tumor volumes were taken daily till day 27. Standard deviations are indicated in the graph. \* $P < 0.05$ .

that the G2/M arrest in 95D cells could be obviously detected in the miR-194 mimic group. After co-transfecting the miR-194 mimic and overexpression of hNUDC in 95D cells, the G2/M phase was extended compared with overexpression hNUDC group (Fig. 6B). The cell cycle distribution in each group in 95D cells is summarized in Fig. 6C. The results indicated that the inhibition effect of the cell cycle by miR-194 mimic could be reversed by the hNUDC overexpression.

To further confirm the enhanced expression of hNUDC counteracting the effect of miR-194 mimic in 95D cells, MTT assay indicated that the increasing hNUDC and miR-194 levels could suppress the proliferation rates of 95D cells treated with overexpression of hNUDC alone (Fig. 6D). Western blotting was used to measure the expression of hNUDC, Mpl, ERK1/2 and c-myc (Fig. 6E), and the bands were quantified in Fig. 6F. In the miR-194 mimic plus overexpression hNUDC group, the protein expression of hNUDC, Mpl, ERK1/2 and c-myc was strongly decreased compared with the overexpression hNUDC group ( $P < 0.01$ ), and increased when compared with the miR-194 mimic group ( $P < 0.05$ ). The results illustrated that the hNUDC overexpression was able to offset the effect of miR-194 inhibition on Mpl/ERK pathway.

**miR-194 reduces tumor growth in xenograft models of lung cancer.** To further explore the tumor suppressor effect of miR-194, we assessed tumor growth of xenografts that were transfected with miR-194 or negative control miRNA was subcutaneous injected into NOD/SCID mice. As shown in Fig. 7, miR-194 inhibited tumor growth of the 95D xenograft. At early time-points, tumors that developed from miR-194-treated cells were smaller compared with their control tumors. At later time-points, tumor growth was resumed but still significantly reduced compared with the control group. The results *in vivo* demonstrated the inhibition effect of miR-194 acting towards cell growth of NSCLC.

## Discussion

NSCLC is the leading cause of cancer death. The hNUDC overexpression has been reported to induce differentiation in megakaryocytes (11). In the present study, the hNUDC expres-

sion is significantly increased in the NSCLC tumor tissues and NSCLC cell lines compared with the matched non-tumor tissues and NHLF cells. To investigate the role of hNUDC in NSCLC, the non-coding region, 3'-UTR of hNUDC was studied. miRNAs are non-coding RNAs that can suppress the expression of the protein-coding genes by binding to the target sequence at the 3'-UTR region of the target gene (24). In the present study, we predicted miR-194 is the targeted miRNA for the hNUDC, which may be involved in the NSCLC pathogenesis.

The strikingly decreased level of miR-194 expression has been reported in many cancer cell lines. In gastric cancer (GC), miR-194 was significantly downregulated and RING box protein1 (RBX1) was upregulated in GC tissues, the upregulation of miR-194 can inhibit proliferation and invasion of GC cells, possibly by targeting RBX1 (25). Moreover, the overexpression of miR-194 regulates osteoblast differentiation through modulating STAT1-mediated Runx2 nuclear translocation (26). Additionally, miR-194 expression was also found to be in a strongly negative association with metastasis in the clinical specimens of NSCLC, as it suppressed the metastasis of NSCLC through regulation of the expression of BMP1 and p27kip1 (14). In the present study, the level of hNUDC was strongly increased in NSCLC cells compared with NHLF cells, and the target connection between hNUDC and miR-194 was predicted by bioinformatic software program TargetScan. Therefore, we chose miR-194 to be the targeted miRNA for hNUDC. The results showed that miR-194 decreased significantly in the NSCLC cells compared with the healthy controls, which was consistent with a prior study by Wu *et al* (14). To verify the targeting reaction between miR-194 and hNUDC, luciferase reporter vectors of the wild-type and mutant hNUDC were constructed. The results confirmed that hNUDC is a target gene for miR-194.

Nuclear migration is essential for growth, development, and cellular function of eukaryotes (27). hNUDC protein plays an important role in nuclear migration (28). The increased hNUDC expression was found to be closely associated with cell malignant hyperplasia in nasopharyngeal carcinoma. A report indicated that anti-NUDC antibody could inhibit the growth of nasopharyngeal carcinoma cell lines (27). From the perspective mentioned above, targeting hNUDC with certain small RNAs offers a novel strategy to prevent the abnormal growth of the NSCLC cells. Therefore, to investigate the role of miR-194 in NSCLC cell process via targeting hNUDC, we detected the effect of miR-194 overexpression on the mitosis, cell proliferation and cell cycle ability of 95C and 95D cells. The results showed that the miR-194 mimic strongly inhibited the hNUDC expression, inducing abnormal nuclear division and decreased cell proliferation rate. Multiple evidence confirms that 95C and 95D cells have relatively low and high metastasis potential (23,29,30). Consistent with these studies, the relatively low level of malignancy of 95C cells renders them more sensitive to the external factors compared with 95D cells. The cell growth was withheld in G1 phase during the treatment of miR-194 mimic compared with mimic control. However, as 95D cells were characterized by rapid rate of DNA synthesis and cell growth, they became accumulated in G2 phase in the treatment with miR-194 mimic. It is reported that cells arrested in G2/M achieved nuclear enlargement and



polyploidization, possibly through failure to complete both mitosis and cytokinesis (31). These results confirmed that miR-194 acted as a negative control in the cell processing in NSCLC cells via targeting hNUDC.

hNUDC has been found to act as a secondary ligand for Mpl involved in regulating proliferation and differentiation of different types of megakaryocytes (32,33). hNUDC plays a key role in the megakaryocytes undergoing endomitosis through the interaction with Mpl (34). Native hNUDC and Mpl were seen around the nuclei and in cytoplasm extensions at all stages of megakaryocytic development (8). A report also indicated that the overexpression of hNUDC activated the ERK1/2 pathway (11), and this activation was profound and prolonged in an Mpl-dependent manner (34). The present study revealed that the miR-194 mimic inhibited the expressions of Mpl/ERK/c-myc pathway proteins by a decrease in hNUDC expression. The downregulation of c-myc is an important event that has been connected to the terminal differentiation and growth arrest of several cell types (35). In order to further confirm the inhibitory effect of miR-194 on the expression of Mpl/ERK pathway proteins and on the cell process of NSCLC cells via targeting hNUDC, hNUDC was overexpressed in 95D cells and the results revealed that the overexpression of hNUDC restored the inhibitory effect of the miR-194 on the mitosis, cell growth and protein expression of Mpl/ERK pathway in 95D cells. These results suggest that miR-194 is a newly identified miRNA that suppresses the expression of hNUDC, at least in NSCLC cells (possibly in other types of human cells). This finding can contribute to a clearer understanding of the regulatory network of Mpl/ERK pathway in human cancers. hNUDC is revealed as a new NSCLC-associated tumor-promoting gene in this study. Notably, this could facilitate the development of a therapeutic strategy targeting hNUDC for lung cancer treatment in studies in the future.

It has been reported that the overexpression of miR-194 suppressed invasion and migration of liver cancer cells in mice (36), indicating the tumor-suppressing role of miR-194 *in vivo*. In this study, the overexpression of miR-194 inhibited tumor growth of the 95D xenograft in NOD/SCID mice at early time-points, however, miR-194 delayed the onset of 95D tumor growth and did not reduce growth of the resulting tumors from day 21 to 27. We speculate that miR-194 induces a growth delay of the transplanted cells that is overcome with time. Multiple administrations or stable expression systems might be necessary to suppress tumor growth more efficiently.

In conclusion, our results demonstrate that the miR-194 overexpression affects the hNUDC expression, resulting in abnormal nuclear division, suppresses cell growth by cell cycle arrest in G1 or G2 phase and downregulates Mpl/ERK pathway proteins in NSCLC cells. The present study is an important step towards understanding the pathogenesis of NSCLC and implicates miR-194 as a potential therapeutic target for NSCLC.

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