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LncNORAD interference inhibits tumor growth and lung cancer cell proliferation, invasion and migration by down-regulating CXCR4 to suppress RhoA/ROCK signaling pathway

Y. WU[1], Q.-W. SHEN[2], Y.-X. NIU[1], X.-Y. CHEN[1], H.-W. LIU[3], X.-Y. SHEN[1]

[1]Department of Thoracic Surgery, Shanghai Key Laboratory of Clinical Geriatric Medicine, HuaDong Hospital, FuDan University, Shanghai, China
[2]Department of Radiation Oncology, Shanghai Huadong Hospital, Fudan University, Shanghai, China
[3]Department of Respiratory Medicine, Shanghai Fengxian District Central Hospital, Shanghai, China

Yun Wu and Qianwen Shen contributed equally

Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) accounts for the majority of lung cancer, with an unfavorable prognosis of 5-year survival rates. It is of great clinical significance to further search for more efficacious and novel targets for diagnosis and therapeutic strategies. This study aimed at clarifying the role of long non-coding RNA (lncRNA) NORAD in proliferation, invasion and migration and tumor growth of NSCLC.

MATERIALS AND METHODS: In this study, mRNA levels of lncRNA NORAD were examined by RT-PCR. CCK-8 assay was applied to test cell viability. Furthermore, wound healing assay and transwell assay were performed to detect the migration and invasion of A549 cells, respectively. Immunohistochemistry was applied to assess the levels of CXC chemokine receptor (CXCR) 4 and CXC chemokine ligand (CXCL) 12. Mice models of NSCLC in vivo were exploited to further examine the potential role of NORAD in tumor growth. Key proteins related to Ras homolog gene family member A (RhoA) GTPase/Rho-associated kinase (RhoA/ROCK) pathway were determined by Western blot.

RESULTS: NORAD has elevated the levels in NSCLC tissues and cells. NORAD interference dramatically inhibited tumor growth and suppressed A549 cell proliferation, migration and invasion by downregulating CXCR4 and CXCL12 expression. RhoA/ROCK signaling pathway was activated in NSCLC.

CONCLUSIONS: This study revealed that the downregulation of lncRNA NORAD could slow down the progression of NSCLC by regulating CXCR4 and RhoA/ROCK signaling pathway.

Key Words:
LncRNA NORAD, CXCR4, RhoA/ROCK pathway, Lung cancer.

Introduction

Nowadays, lung cancer has become one of the most common causes of cancer death, among which non-small cell lung cancer accounts for the majority[1]. Global cancer statistics show that approximately 2.1 million new cases and 1.8 million lung cancer-related deaths have been reported annually in 185 countries[2]. Therapies of lung cancer include surgery, platinum-based chemotherapy, radiotherapy, neoadjuvant chemotherapy (NCT), etc.[3]. Unfortunately, most cases of lung cancer are typically discovered and diagnosed at a distant stage[4]. Despite of the progress in researches of lung cancer, the patients still have an unfavorable prognosis with the 5-year survival rates of 5%[4,5]. Therefore, it is of great clinical significance to further explore the molecular mechanisms of lung cancer, and search for more efficacious and novel targets to develop early diagnosis and therapeutic strategies.

CXCR4, a stromal-derived-factor-1 (also termed CXCL12) specific chemokine receptor, has been reported to play an essential role in over 30 types of malignancies[6]. CXCR4 was found to be highly expressed in NSCLC tissues and implicated in cancer progression[7]. Researchers[8] indicated that CXCR4 and CXCL12 could regulate cell anti-apoptosis and promote local invasion and distant metastasis of lung cancer. Moreover, CXCL12/CXCR4 axis was demonstrated to suppress cisplatin-induced apoptosis of lung cancer cells[9]. Hence, CXCL12/CXCR4 is a significant way to affect the development of lung cancer.

Corresponding Authors: Xiaoyong Shen, MD; e-mail: Shenxy2019@yeat.net
Hongwei Liu, MD; e-mail: HongweiZPP@126.com

RhoA has been known to be a member of small GTPase protein of Rho family and is associated with actin cytoskeleton regulation[10]. Notably, RhoA/ROCK has been proved to be a critical signaling pathway in cancer progression[11]. Wang et al[12] presented that activating RhoA/ROCK signaling pathway could promote ovarian cancer cell migration and invasion. In addition, RhoA/ROCK pathway could participate in the progress of VM formation and limit invasive growth in NSCLC[13,14]. Lin et al[15] showed that CXCR4 could promote the activation of RhoA GTPase and the phosphorylation of ROCK1. These researches suggested that CXCR4 could facilitate cancer progression by adjusting the RhoA/ROCK pathway.

Long non-coding RNA (lncRNA) is composed of more than 200 nucleotides with no significant protein-coding capacity[16]. LncRNAs play an important role in various pathological processes including different types of malignancies[17]. Recently, a new lncRNA was identified and termed Noncoding RNA activated by DNA damage (NORAD)[18]. He et al[19] showed that elevating the expression of NORAD could promote cancer cell growth, invasion and migration. Besides, lncRNA NORAD could affect the proliferation and metastasis of lung cancer[20,21]. Furthermore, silencing NORAD reduced RhoA and ROCK1 expression in gastric cancer[22]. However, the effects of lncRNA NORAD on CXCR4 and RhoA/ROCK pathway in lung cancer have not yet been fully elucidated. Therefore, the present study aims to assess the levels of lncRNA NORAD in NSCLC and evaluate whether lncRNA NORAD can exert influence on lung cancer progression by regulating CXCR4 and RhoA/ROCK pathway.

Materials and Methods

Cell Culture and Transfection

Human NSCLC cell lines A549 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) supplemented with 10% of fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. A total of $1x10^5$ A549 cells were seeded into 24-well plates, and once the cells achieved 85% confluence, they were transfected with lncRNA-NORAD siRNA using Lipofactamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Tumor Xenograft Experiment

4-weeks-old, athymic nude BALBC/c male mice were provided by SLAC Laboratory Animal Co., Ltd (Shanghai, China). A549 cells were subcutaneously injected into the mice with inoculation dosage of 1×10^7 cells. The tumor sizes were measured every 3 days. After 27 days, the mice were killed in order to attain the tumors for analysis. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Dunwill Medical Techology. (IACUC Issue No:201812003).

Cell Viability Assay

Cell Counting Kit-8 (CCK-8) assay was performed to determine the effect of lncRNA-NORAD interference on proliferation, invasion and migration of lung cancer cells. The CCK-8 assay was performed at 24 h intervals to generate the cell growth curve in line with the manufacturer's protocol. The cells during logarithmic growth phase were digested, collected, counted, and prepared into the cell suspension with an appropriate density of 5×10^4 cells/ml. 100 μl of cell suspension containing were added into each well in 96-well plates. 10 μl CCK-8 solution per well (Beyotime, Shanghai, China) were added 24, 48, 72, and 96 h later and incubation was continued for another 4 h in the incubator. The cells were cultured for 4 h. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure OD values at 450 nm.

RNA Isolation and Quantitative Real-time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from A459 cells or clinical tissue samples in accordance with the manufacturer's protocol. For the evaluation of mRNA, the total cDNA was reversed transcribed from isolated RNA using a RNA PCR kit (TaKaRa, Otsu, Shiga, Japan) and quantitative Real Time-PCR was carried out with the SYBR premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. The expression levels of lncRNA-NORAD were detected by qPCR on the ABI Biosystems (Applied Biosystems, Foster City, CA, USA). The relative expression level of lncRNA-NORAD was normalized to internal control GAPDH, and the $2^{-\Delta\Delta Ct}$ method was adopted to calculate the relative quantities.

Wound Healing Assay

The transfected cell lines were seeded into 6-well culture plates and grew until around 80%

confluence. A 10 μL pipette tip was used to generate a linear wound by scraping the cell monolayer. The cell debris was washed with PBS. The wound images for each well were recorded using a Leica microscope in the same position at 0 h, 24 h.

Transwell Assay

Transwell assay was carried out using transwell chambers (Corning, Costar, CA, USA). The transfected cell lines (cultured with FBS free medium) were placed in the upper chamber of each insert, and the medium with 10% FBS was added to the lower chamber. After incubation for 24 h at 37°C, the cells remaining in the upper chamber were removed using a cotton swab. Then, the invaded cells were fixed with 4% paraformaldehyde. After staining with 0.1% crystal violet dye (Solarbio, Shanghai, China) for 10 min, the cells were washed with PBS. Evaluation of invasive capacity was performed by counting the number of cells penetrating the Matrigel under an inverted microscope (Leica, Germany).

Western Blot Analysis

Protein extractions were performed using radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) in strict accordance with the instructions of the kit. Protein concentrations were measured by BCA method in line with the manufacturer's protocol (Beyotime, Shanghai, China). Equal amounts of protein per lane from each sample were separated by 10% SDS-PAGE according to different molecular weights and then transferred onto polyvinylidene difluoride (PVDF; Beyotime, Shanghai, China) membranes. After transmembrane, the PVDF membranes were blocked with skimmed milk or BSA solution (5%) for 1 h at room temperature. After that, the membranes were incubated with primary antibodies overnight at 4°C as follows: CXCR4 (Abcam, Cambridge, CMA, USA, ab181020, 1:10000), CXCL12 (Abcam, Cambridge, MA, USA, ab25117, 1:1000), RHOA (Abcam, Cambridge, MA, USA, ab86297, 1:1000), ROCK1 (Abcam, Cambridge, MA, USA, ab97592, 1:3000), ROCK2 (Abcam, Cambridge, MA, USA, ab125025, 1:20000), LIMK1 (Abcam, Cambridge, MA, USA, ab81046, 1:1000), LIMK2 (Abcam, Cambridge, MA, USA, ab97766, 1:2000), P-CFL (Abcam, Cambridge, MA, USA, ab12866, 1:500), CFL (Abcam, Cambridge, MA, USA, ab42824, 1:1000), GAPDH (Abcam, Cambridge, MA, USA, ab181602, 1:10000). Membranes were washed with PBS for three times, 10 mins each time, and further incubated with IgG-HRP secondary antibody (Abcam, Cambridge, MA, USA, ab97023, 1:20000) at room temperature for 4 h. Enhanced chemiluminescence (ECL; Sigma-Aldrich, St. Louis, MO, USA) was dropped on the membranes to develop signals, and relative expression level of each protein was normalized to endogenous control GAPDH Image J software.

Immunohistochemistry

All slides were deparaffinized with xylene and rehydrated with ethanol. Endogenous peroxidase activity was blocked with 5% H_2O_2 for 15 mins. After heat-induced antigen retrieval (98°C, 25 min, pH 6.0, in citrate buffer), the sections were incubated in blocking solution (1% BSA, 10% normal goat serum, 0.1% Triton X-100 in PBS) for 1 h at room temperature. The primary antibodies against CXCR4 (Abcam, Cambridge, MA, USA, ab1670, 1:150) and CXCL12 (Abcam, Cambridge, MA, USA, ab25117, 1:200) were used on the sections. After being incubated at 4°C overnight, the sections were exposed for 1 h at room temperature to Goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) and goat anti-mouse Alexa Fluor 594 (Invitrogen, Carlsbad, MA, USA). Staining is revealed by 3, 3'-diaminobenzidine and counterstained with hematoxylin. Images were taken with a light microscope (Leica).

Statistical Analysis

Statistical analysis was performed with SPSS 21.0 software (IBM Corp, Armonk, NY, USA). All the experiments were repeated at least three times. A two-tailed Student's *t*-test was applied to the analysis of two groups and difference between multiple groups was analyzed by one-way analysis of variance followed by the Scheffe post-hoc test. The experiment data are presented as mean ± standard deviation. Statistical data were presented with Graph-Pad prism software (La Jolla, CA, USA). A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

NORAD Has Higher Expression in NSCLC Tissues and Cells

To explore whether NORAD was associated with NSCLC, RT-qPCR was applied to determine the expression levels of NORAD in NSCLC

tissues and cells. NORAD was highly expressed in tumor tissues compared with adjacent normal tissues in patients with NSCLC (Figure 1A). Furthermore, NORAD expression levels were detected in the three NSCLC cells A549, SPC-A1 and SK-MES-1, and a normal human bronchial epithelial cell line 16HBE. The results confirmed that NORAD expression levels were higher in NSCLC cells compared with that in 16HBE cells, and NORAD expression level in A549 was the most evident among them (Figure 1B). Therefore, lncRNA NORAD is likely involved in the pathogenesis of NSCLC.

NORAD Interference Suppressed Cell Proliferation, Migration and Invasion in NSCLC Cells

To evaluate the effects of NORAD expression on NSCLC cell proliferation, two siRNA oligonucleotides against NORAD were used to interfere NORAD in A549 cells. NORAD was efficiently interfered following transfection with sh-NORAD-1 or sh-NORAD-2 into A549 cells and the effect of sh-NORAD-1 was the most evident among them (Figure 2A). CCK-8 assay demonstrated that the cell proliferation ability was significantly suppressed by NORAD interference in A549 cells, compared with sh-NC group (Figure 2B). In addition, wound healing assay and transwell assay were respectively exploited for the determination of migratory and invasive abilities. Downregulation of NORAD remarkably suppressed cell migration and invasion (Figure 2C, 2D).

NORAD Interference Suppressed A549 Cell Proliferation, Migration and Invasion by Downregulating CXCR4 and CXCL12 Expression

NORAD interference significantly decreased expression level of both CXCR4 and CXCL12 in A549 cell line, which indicated the critical roles of CXCR4 and CXCL12 in NSCLC (Figure 3A). CCK-8 assay demonstrated that the cell proliferation ability was significantly suppressed by NORAD interference in A549 cells, which was reversed by CXCR4 treatment (Figure 3B). Wound healing assay proved that NORAD interference suppressed A549 cell migration, which was reversed by CXCR4 treatment. Furthermore, the transwell assay showed that suppressed A549 cell invasion, which was reversed by (Figure 3D). Similarly, the suppressive effects of NORAD interference on cell migration and invasion was abolished by CXCR4 treatment (Figure 3C, 3D). These results indicated that NORAD may serve as an upstream regulator of CXCR4 and CXCL12 in NSCLC cells.

RhoA/ROCK Signaling Pathway Was Activated in NSCLC Cells

To determine whether NORAD interference suppressed A549 cell proliferation, migration and

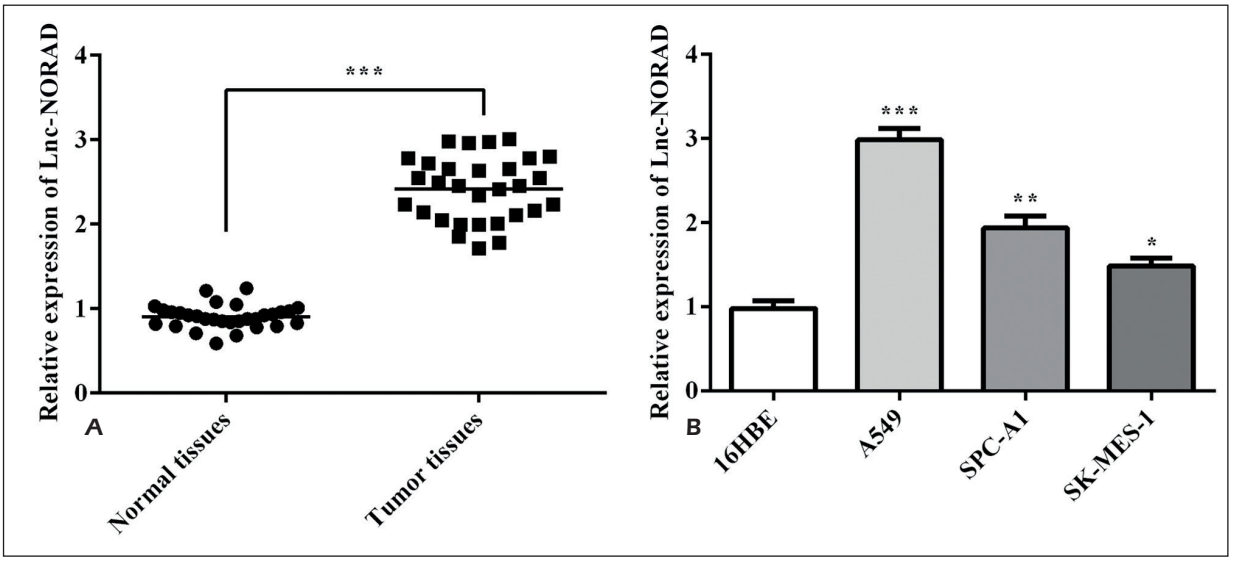

Figure 1. LncNORAD has higher expression in NSCLC tissues and cells. **A**, RT-qPCR analysis of LncNORAD expression in tumor tissues and adjacent normal tissues in patients with NSCLC. **B**, RT-qPCR analysis of LncNORAD expression in the three NSCLC cell lines A549, SPC-A1 and SK-MES-1, and the normal human bronchial epithelial cell line 16HBE. *p<0.05, **p<0.01, ***p<0.001 *vs.* control group.

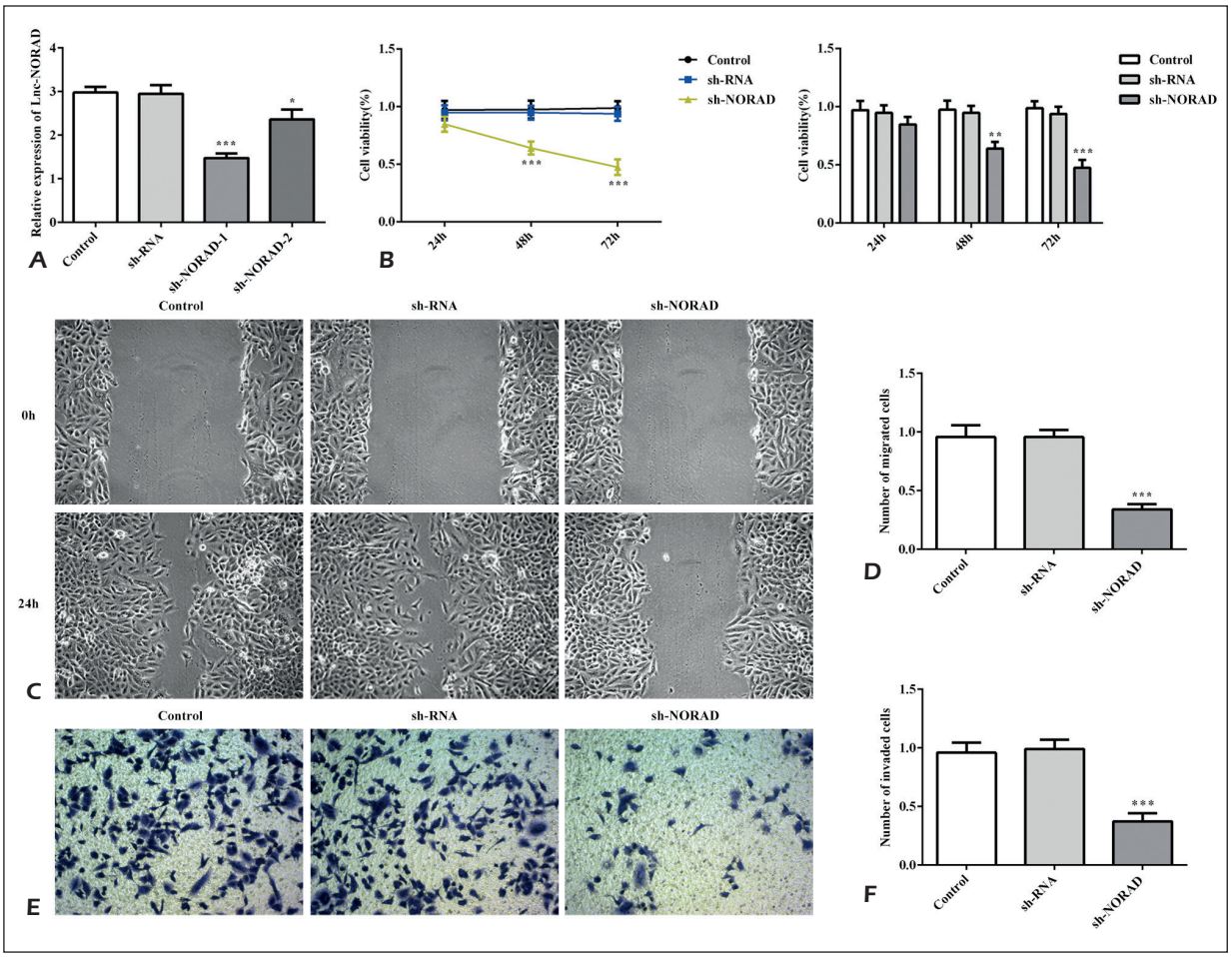

Figure 2. LncNORAD interference suppressed cell proliferation, migration and invasion in NSCLC cells. **A**, A549 cells were transfected with sh-NC, sh-NORAD-1 and sh-NORAD-2 and RT-qPCR was applied to determine the expression levels of Lnc-NORAD. **B**, Cell proliferation was determined using CCK8 assays. **C-D**, Wound healing assays were performed to determine cell migration (magnification 100×). **E-F**, Transwell assays were performed to determine cell invasion. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* control group (magnification 100×).

invasion *via* RhoA/ROCK pathway in NSCLC cells, we detected some crucial proteins related to RhoA/ROCK pathway by Western blot assay. The protein levels of RHOA, ROCK1, ROCK2, LIMK1, LIMK2 and P-CFL were significantly downregulated by NORAD interference compared to the control group, which was reversed by CXCR4 treatment (Figure 4).

NORAD Interference Inhibited Tumor Growth by Downregulating CXCR4 Expression in vivo Model of Non-small Cell Lung Cancer

The *in vivo* model of non-small cell lung cancer was applied to further examine the potential role of NORAD. Monitoring of tumor growth for approximately 4 weeks post-injection revealed a significant reduction in growth (as determined by measurement of tumor volume) in sh-NORAD group compared with the control group, which was reversed by CXCR4 treatment (Figure 5A-5C). In addition, the resected tissues from the agomir-treated xenograft tumors were analyzed to verify CXCR4 and CXCL12 expression using IHC. The IHC results showed a significant loss of CXCR4 and CXCL12 expression in sh-NORAD group compared with the control group, which was reversed by CXCR4 treatment (Figure 5D). The results described above were consistent with the results in *vitro*. All these data indicated that NORAD interference could inhibit tumor growth by downregulating CXCR4 and CXCL12 expression *in vivo*.

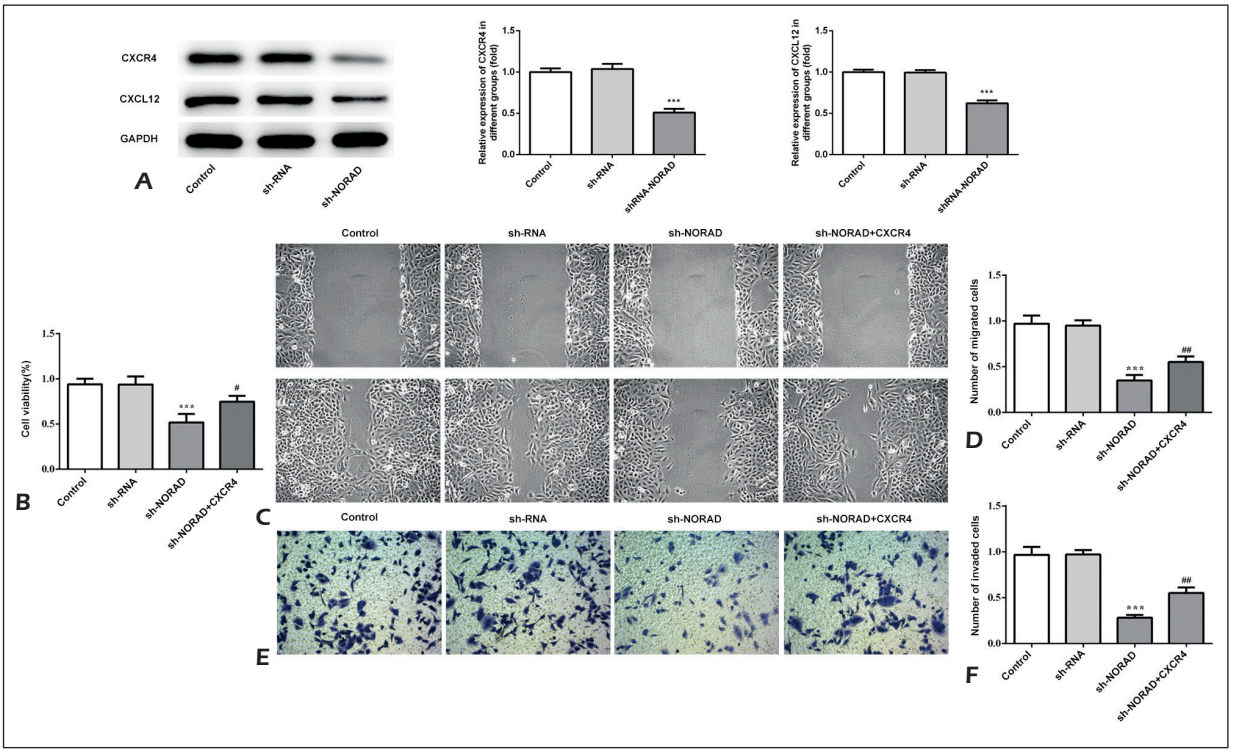

Figure 3. LncNORAD interference suppressed A549 cell proliferation, migration and invasion by downregulating CXCR4 and CXCL12 expression. **A**, The protein levels of CXCR4 and CXCL12 were detected by western blot. **B**, Cell proliferation was determined using CCK8 assays. **C-D**, Wound healing assays were performed to determine cell migration (magnification 100×). **E-F**, Trans-well assays were performed to determine cell invasion. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* sh-NORAD (magnification 100×).

RhoA/ROCK Signaling Pathway Was Activated in vivo Model of Non-small Cell Lung Cancer

To further determine whether NORAD interference suppressed tumor growth *via* RhoA/ROCK pathway *in vivo* model of non-small cell lung cancer, we detected the crucial proteins related to RhoA/ROCK pathway by Western blot assay. The protein levels of RHOA, ROCK1, ROCK2, LIMK1, LIMK2 and P-CFL

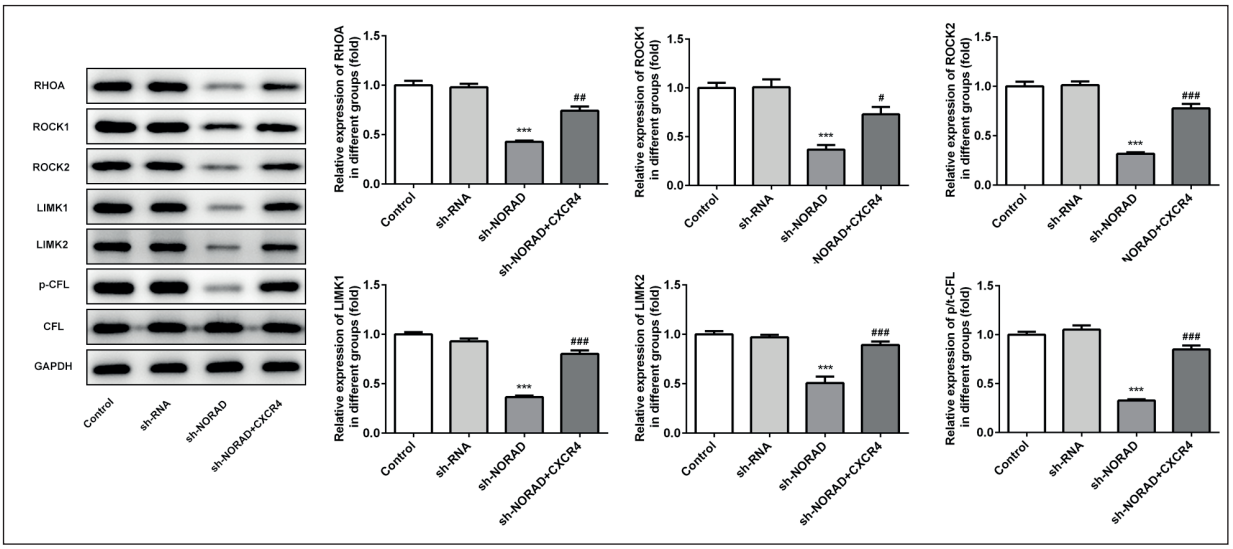

Figure 4. RhoA/ROCK signaling pathway was activated in NSCLC cells. Proteins related to RhoA/ROCK pathways in A549 cells were detected by Western blot. $^{***}p<0.001$ *vs.* control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* sh-NORAD.

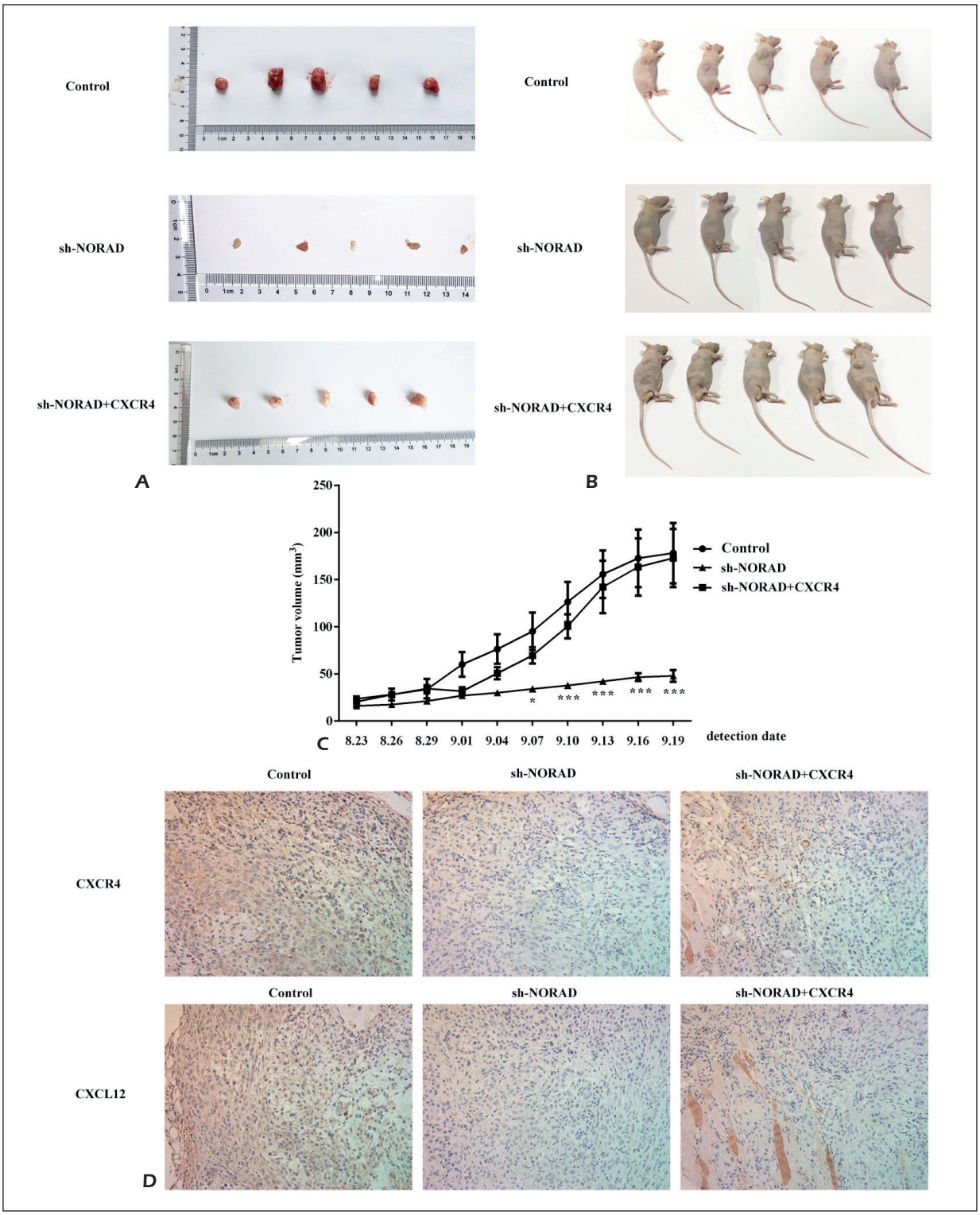

Figure 5. NORAD interference inhibited tumor growth by downregulating CXCR4 expression in vivo model of non-small cell lung cancer. **A**, Photographs of tumor tissues from different groups at 4 week. **B**, Photographs of xenografts of nude mice from different groups at 4 week. **C**, Growth curves of tumor volumes in xenografts of nude mice were determined based on tumor volume measured every 3 days for 4 week. **D**, Expression of CXCR4 and CXCL12 were detected by immunohistochemistry. *$p<0.05$, ***$p<0.001$ *vs.* control group (magnification 200×).

were significantly downregulated by NORAD interference compared to the control group, which was reversed by CXCR4 treatment (Figure 6). The results of Western blot assay *in vivo* model of non-small cell lung cancer were consistent with that *in vitro*. All the results above verified that NORAD interference significantly inhibited tumor growth and suppressed A549 cell proliferation, migration and invasion via RhoA/ROCK pathway.

Discussion

LncRNAs play a vital role in the progression of various neoplastic diseases, including NSCLC[23,24]. This study revealed that the level of lncRNA NORAD is elevated in tumor tissues and cells compared with that in normal tissues and 16HBE cells. Besides, we proved that NORAD interference could inhibit the proliferation, migration, and invasion of A549 cells and subcutaneous transplanted tumor tissues. Moreover, we evaluated that transfecting sh-NORAD strikingly decreased the expression of CXCR4 and CXCL12. Finally, NORAD interference inhibited the protein expression of RHOA, ROCK1, ROCK2, LIMK1, LIMK2 and p-CFL in RhoA/ROCK pathway, while CXCR4 factor could reverse this effect in NSCLC cells and tissues.

The expression of NORAD raised in various cancers, and NORAD has been characterized as a potential oncogene[25,26]. Our research showed that the expression of NORAD was elevated in NSCLC cells and tissues. Recently, it has been demonstrated[27] that increased expression of NORAD could promote the proliferation, invasion and migration of breast cancer cells. Similarly, our results revealed that NORAD interference could enhance the proliferation, invasion and migration of A549 cells and promote the growth in subcutaneous transplanted tumor tissues. LncRNA LSINCT5 could influence on ovarian cancer by inhibiting CXCL12/CXCR4 signaling axis to affect cells[28]. Our research showed that NORAD interference could significantly disrupt CXCL12/CXCR4 signaling axis, while CXCR4 factor could promote the activation of CXCL12/CXCR4 signaling. Up to now, there is no research about the effect of NORAD on RhoA/ROCK pathway. Zhuang et al[29] proved that lncRNA TUG1 could function on laryngocarcinoma through activating RhoA/ROCK pathway. We found that transfecting sh-NORAD could inhibit the protein expression in RhoA/ROCK pathway, and CXCR4 factor could activate this pathway.

Conclusions

The main finding of this study is that NORAD serves as a novel regulator in the pathological progress of NSCLC by regulating CXCL12/

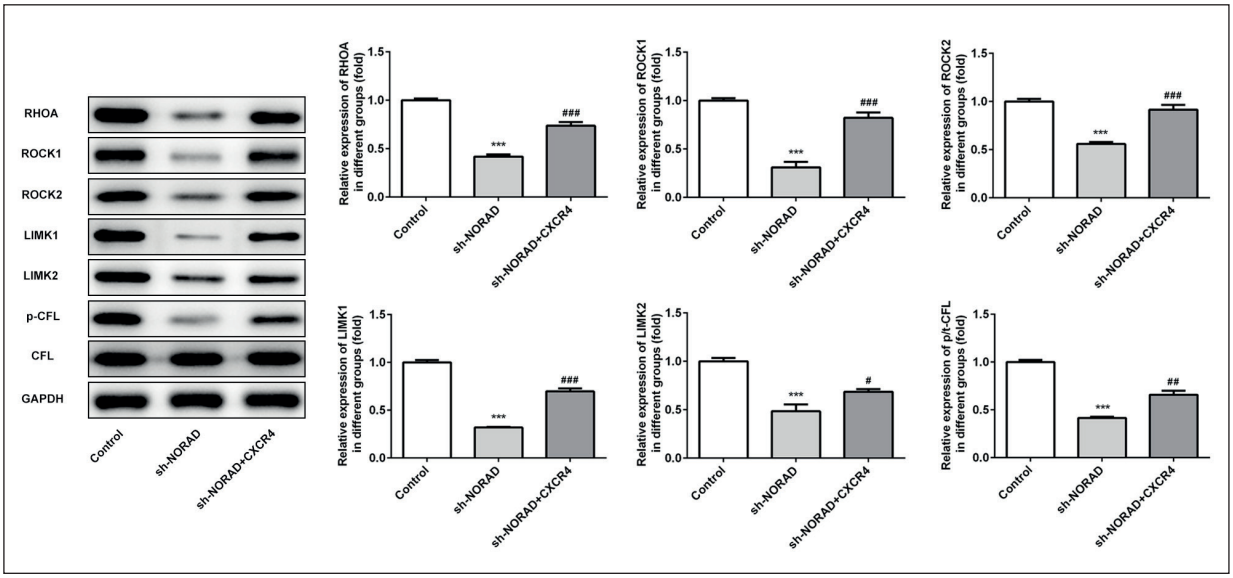

Figure 6. RhoA/ROCK signaling pathway was activated in vivo model of non-small cell lung cancer. Proteins related to RhoA/ROCK pathways in vivo model of non-small cell lung cancer were detected by Western blot. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* sh-NORAD.

CXCR4 and RhoA/ROCK pathways. Increased NORAD expression level can be used to distinguish NSCLC patients from healthy people and it also predicts poor survival of NSCLC patients. Therefore, this study may provide new insights into the possibility of NORAD being a potential therapeutic target in NSCLC.

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Availability of Data and Materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' Contributions

All the authors made substantial contributions to the conception and design of the study, performed the experiments and analyzed the data. All authors read and approved the final version of the manuscript.

Conflict of Interests

The authors declare that they have no conflict of interests.

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