miR-99a regulates ROS-mediated invasion and migration of lung adenocarcinoma cells by targeting NOX4

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Abstract. miR-99a is frequently downregulated in various types of human malignancies including lung adenocarcinoma. Recent studies have reported that miR-99a regulates cell growth and cell cycle progression by targeting mTOR, AKT1 and FGFR3. However, the underlying mechanisms involved in the modulation of invasion and migration by miR-99a remain elusive. In this study, we analyzed the relationship between the expression of miR-99a and clinical stage or metastasis in 90 matched lung adenocarcinoma and adjacent non-tumor lung tissues. Downregulation of miR-99a was significantly associated with advanced stage and tumor metastasis in lung adenocarcinoma patients, and it was found to be a poor prognostic factor in lung adenocarcinoma. Furthermore, functional experiments found that overexpression of miR-99a inhibited the proliferation, migration and invasion of lung adenocarcinoma A549 and Calu3 cells *in vitro*. We then identified NOX4 as a target gene of miR-99a and NOX4 mediated the inhibition of invasion and migration of lung adenocarcinoma cells by miR-99a. By targeting NOX4-mediated ROS production, miR-99a regulated the invasion and migration of lung adenocarcinoma cells. Moreover, overexpression of miR-99a significantly inhibited tumor growth *in vivo*. Immunohistochemical staining analysis of the mouse tumor tissues revealed that NOX4 levels were downregulated in the miR-99a treatment group, confirming the *in vitro* data of NOX4 as a direct target gene of miR-99a. Taken together, these data indicate for the first time that miR-99a directly regulates the invasion and migration in lung adenocarcinoma by targeting NOX4 and that overexpression of miR-99a may become a therapeutic strategy for lung adenocarcinoma.

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Introduction

Lung cancer is a leading cause of cancer-related death worldwide, and more than 85% of cases are non-small cell lung cancers (NSCLCs) (1). Adenocarcinoma is the most common pathological type of NSCLC. Despite the availability of treatment, including surgery, radiotherapy, chemotherapy and targeted therapy, the prognosis of these patients is dismal, and the predicted 5-year survival rate of advanced NSCLC is still less than 15% (2). Similar to other solid cancers, metastatic spread of NSCLC is the primary cause of death for these patients (3). Thus, elucidation of the molecular mechanisms that control NSCLC metastasis is urgently needed.

Several studies have demonstrated that the level of reactive oxygen species (ROS) is increased in malignant tumors, including cervical, pancreatic, breast and lung cancer (4-8). These findings show that ROS play an important role in tumorigenesis, progression and metastasis. Reduction of the ROS level exhibits anticancer effects (9). However, how lung adenocarcinoma cells control the generation of ROS in order to mediate a malignant function remains unclear.

MicroRNAs (miRNAs) are a class of endogenous, small (~22-nt) non-coding single-stranded RNA molecules that negatively regulate the expression of their target genes. Growing evidence indicates that aberrant expression of miRNAs plays an important role in the development of lung cancer (10-15). Recent studies have shown that deregulation of miR-99a is frequently found in lung cancer (16-18). Furthermore, Chen *et al* reported that miR-99a inhibited proliferation, migration and invasion of non-small cell lung cancer cells by targeting insulin-like growth factor 1 receptor (IGF-1R) (19).

In the present study, we demonstrated that miR-99a plays an important role in the regulation of cellular invasion and migration by directly targeting NOX4 *ex vivo* and *in vivo*. By targeting NOX4, overexpression of miR-99a decreased the capabilities of cellular invasion and migration in a ROS-dependent manner. Moreover, miR-99a directly interacts with the 3'UTR of NOX4 to repress NOX4 expression. These findings indicate that NOX4 is a bona fide target of miR-99a and suggest that overexpression of miR-99a could be a critical therapeutic strategy for lung adenocarcinoma.

Materials and methods

Patients and tissue specimens. A total of 90 paired samples of human lung adenocarcinoma and their matched adjacent non-cancerous tissues were collected at the time of surgery between March 2007 and April 2013 at the Department of Surgery in Tianjin Medical University Cancer Institute and Hospital. Matched normal tissues were obtained 2-cm distant from the tumor margin, and were further confirmed by pathologists. Upon resection, all specimens were immediately frozen in liquid nitrogen and stored at -80˚C. All patients were initially treated patients. The use of the tissue samples for all experiments was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Cell lines and cell culture. Human lung adenocarcinoma cell lines A549 and Calu3 were obtained from the China Academia Sinica Cell Repository (Shanghai, China). The cells were cultured according to a routine cultivation method (20).

Reagents, plasmids, oligonucleotides and transfection. N-acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich. Plasmid containing NOX4 lacking the 3'UTR was purchased from GenePharma Co., Ltd. (Shanghai, China). The pGL3-NOX4-3'UTR-Luc reporter was created by ligation of the PCR-amplified 3'UTR of NOX4 into the *Eco*RI/*Eco*RV sites of the modified pGL3 vector (Promega, Madison, WI, USA). The primers for PCR amplification of the NOX4-3'UTR were: NOX4-3'UTR forward, 5'-CGCCTCCCGGGTTTGCA CCACTCTCCTGCCTCAGCCTCCTG-3' and reverse, 5'-ATCATTTTTATTGTCTCAAGAAGAACTTAATAGCA ATTAG-3'. The oligonucleotides for miR-99a and anti-miR-99a were purchased from GenePharma Co., Ltd. The sequences were 2'-OMe-anti-miR-99a (anti-miR-99a) 5'-CACAAGAUCG GAUCUACGGGUU-3' and miR-99a mimics (miR-99a), 5'-AACCCGUAGAUCCGAUCUUGUG-3'. miR-99a or anti-miR-99a (200 pmol) was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Scrambled oligonucleotides were used as control. The scrambled sequence was 5'-UUCUUCGAACGUGUCACGUTT-3'.

RNA isolation and quantitative real-time polymerase chain reaction. As described in detail previously (21), by using TRIzol reagent (Invitrogen) to isolate total RNA, total RNA (0.05 mg) was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). Comparative real-time polymerase chain reaction (PCR) was performed as previously reported (21). Normalization was performed by simultaneous quantification of small endogenous nuclear RNAU6 (RNU6B), and relative expression was calculated by the comparative CT method.

Invasion assay. As described in detail previously (22), the invasive potential of the transfected cells was evaluated by measuring the number of cells invading the Matrigel-coated Transwell chambers (Becton Dickinson).

Wound-healing assay. Transfected cells were seeded into 6-well culture plates. As described in detail previously (22), by using a sterile plastic 100-μl micropipette tip, an artificial homogenous wound was created in the monolayer. Cells that migrated into the wounded area or cells with extended protrusion from the border of the wound were photographed under an inverted microscope (x40 magnification; Olympus, Japan). Three areas were selected from each well at random, and the cells in three wells of each group were quantified in each experiment.

Measurement of the intracellular ROS concentration. Intracellular ROS concentrations were quantified with 2',7',-dichlorofluorescein diacetate (DCFH-DA). After transfection, the culture medium was removed from the cells in a 96-well black plate, and 100 ml of 10 mM DCFH-DA in PBS was added. After incubation at 37˚C in 5% CO_2 for 30 min, the cells were washed with warmed PBS. Then, 100 ml of PBS was added into each well. Fluorescence was measured on a microplate reader using 488 nm excitation and 525 nm emission wavelengths.

Luciferase reporter assay. Cells were cultured in 96-well plates and co-transfected with pGL3-NOX4-3'UTR or pGL3-NOX4-3'UTR mutant vector, and anti-miR-99a, miR-99a or scramble sequences. Following a 72-h incubation, luciferase activity was measured using a Dual-Luciferase reporter system (Promega).

Western blot analysis. The protocol for the western blotting was previously described in detail (22). Membranes were incubated with primary antibodies against NOX4, Akt, pAkt, MMP2, or MMP9 (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with an HRP-conjugated secondary antibody (1:1,000 dilution; Zymed, USA). The membrane was stripped and reprobed with a primary antibody against GAPDH or β-actin (1:1,000 dilution; Santa Cruz Biotechnology).

Nude mouse tumor xenograft model. A549 cells were subcutaneously injected into 5-week-old female nude mice (Cancer Institute of The Chinese Academy of Medical Science). The survival of the mice was monitored every day. The animals received sterile food and water. As tumor implantation was conducted into the right leg, all mice maintained their health until the research was halted. When the tumor volume reached 50 mm^3, the mice were randomly divided into two groups (8 mice/group). The two groups of mice were treated with scrambled oligo or miR-99a by local injection of xenograft tumors in multiple sites. The treatment was performed once every 3 days for 15 days. The tumor volume was measured with a caliper every 2 days, using the formula: Volume = length x $width^2/2$. Twenty-eight days after initiation of the treatment, the mice were sacrificed by CO_2 inhalation followed by cervical

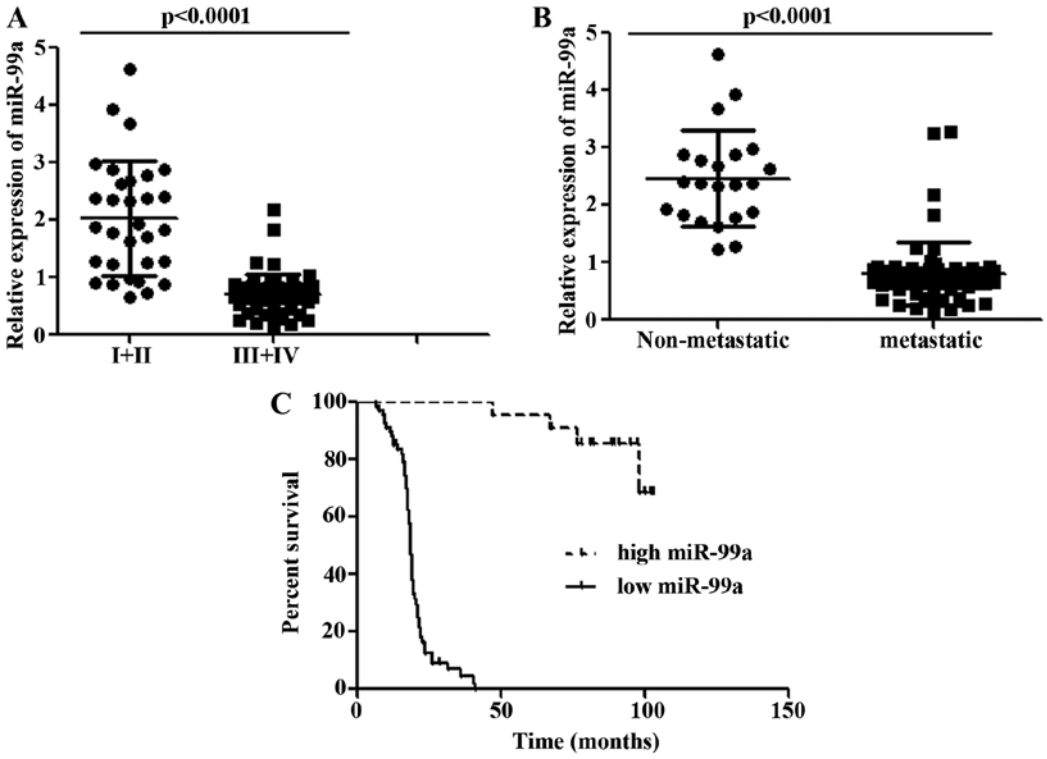

Figure 1. Downregulation of miR-99a expression is a frequent event and a poor prognostic factor in human lung adenocarcinoma. (A) miR-99a was negatively related to the clinical stage of the lung adenocarcinoma cases; lower levels of miR-99a expression were associated with late-stage tumors. (B) miR-99a was negatively related to the metastasis of lung adenocarcinoma cases; lower levels of miR-99a expression were associated with metastatic tumors. (C) Kaplan-Meier analysis showed that lower levels of miR-99a expression were associated with poor overall survival (p<0.0001).

Table I. miR-99a expression and the clinicopathological parameters of the 90 lung adenocarcinoma patients.

Patient characteristics	No. of patients	Expression of miR-99a	P-value
Age (years)			
≥60	61	1.2397±0.1783	0.7921
<60	29	1.1851±0.0987	
Gender			
Male	51	1.0017±0.0872	0.2319
Female	39	1.4766±0.0927	
TNM stage			
Stage I+II	31	2.0200±0.1795	<0.0001
Stage III+IV	59	0.7154±0.0441	
Metastasis[a]			
No	22	2.4617±0.1797	<0.0001
Yes	68	0.8042±0.0652	

[a]Includes lymphatic metastasis. P-value represents the probability from t-test for miR-99a expression between variable subgroups. TNM, tumor node metastasis.

dislocation, and paraffin-embedded tissue sections were used for immunohistochemistry. Animal experiments were approved by the Ethics Committee of the Tianjin Huanhu Hospital.

Statistical analysis. Survival models were used to evaluate the clinical parameters related to overall survival (OS). Survival curves were estimated by the Kaplan-Meier method. Differences with a p-value <0.01 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 5 software.

Results

Downregulation of miR-99a expression is related with advanced stage and metastasis of lung adenocarcinoma. Several studies have shown that miR-99a expression is downregulated in various types of tumors (16-18,23-25). We used RT-PCR to evaluate miR-99a expression in tumor and adjacent normal tissues of lung adenocarcinoma patients. The clinicopathological characteristics of the 90 lung adenocarcinoma patients are summarized in Table I. Among the total 90 lung adenocarcinoma patients, miR-99a was downregulated in tumor tissues of 61 patients. We found a statistically significant association between the expression of miR-99a and lung adenocarcinoma clinical stage as well as metastasis. Notably, lower levels of miR-99a expression were associated with late-stage tumors (stage III and IV) compared with early-stage tumors (stage I and II) (0.7154±0.0441 vs. 2.020±0.1795, respectively; P<0.0001) (Fig. 1A and Table I). In addition, lower levels of miR-99a expression were associated with metastatic tumors compared with the level in non-metastatic tumors (0.8042±0.0652 vs. 2.4617±0.1792, respectively; P<0.0001) (Fig. 1B and Table I). By analyzing the clinical data

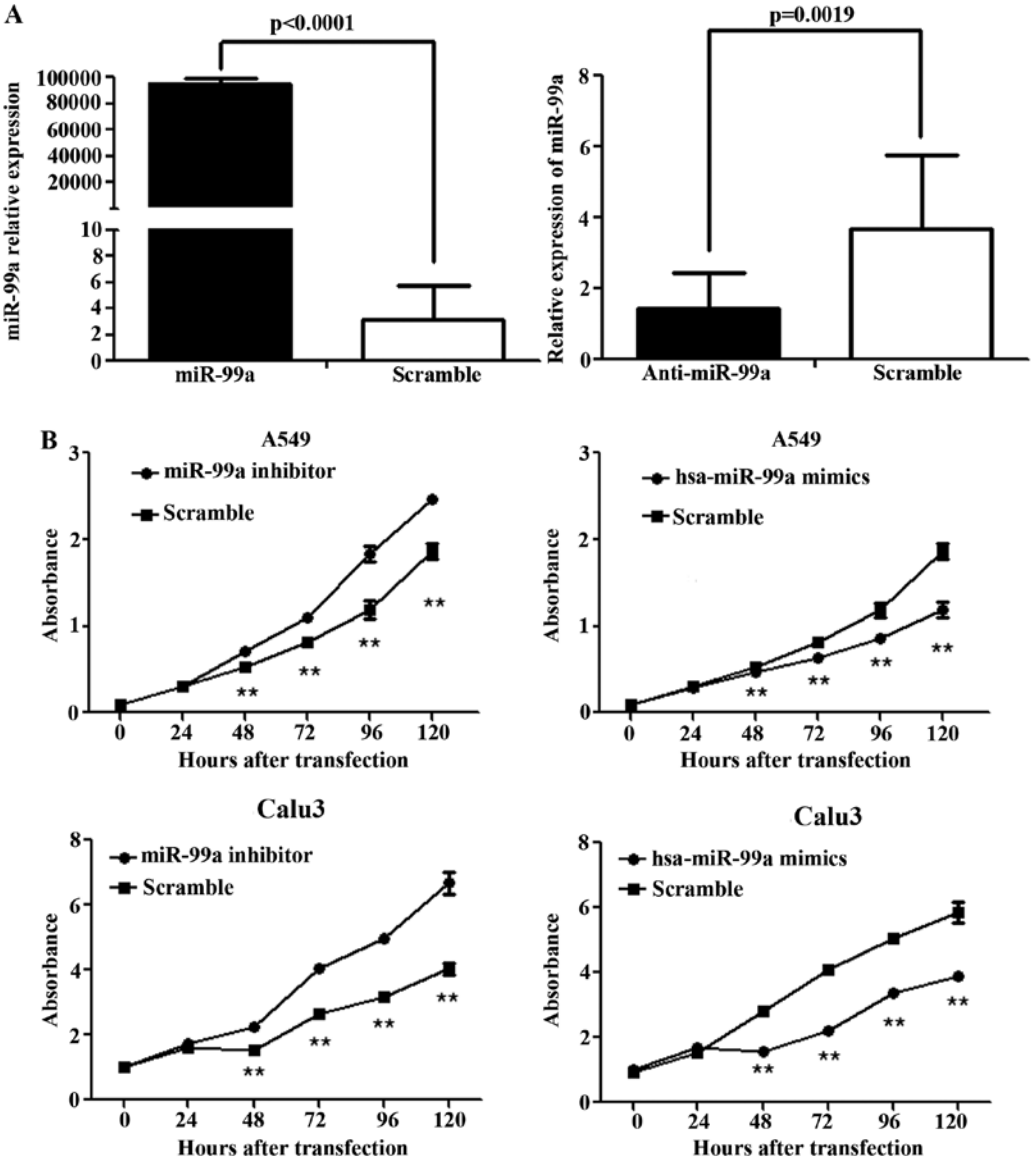

Figure 2. miR-99a inhibits proliferation, invasion and migration of lung adenocarcinoma cell. (A) miR-99a mimic significantly increased miR-99a expression, and anti-miR-99a significantly decreased miR-99a expression. (B) MTT assays showed that miR-99a overexpression significantly inhibited proliferation of lung adenocarcinoma cells ($^{**}p<0.01$).

from all patients, overall survival of the patients in the high miR-99a expression group was longer than that in the patients with low miR-99a expression (84.49±3.195 vs. 19.01±0.7722, P<0.0001). Kaplan-Meier analysis showed that downregulation of miR-99a expression was associated with poor overall survival (P<0.0001) (Fig. 1C). These data suggest that downregulation of miR-99a expression is a frequent event and a poor prognostic factor in human lung adenocarcinoma.

miR-99a inhibits proliferation, invasion and migration of lung adenocarcinoma. Our clinical data demonstrated that miR-99a was downregulated in the lung adenocarcinoma tissues. Next, we aimed to ascertain the mechanism of how miR-99a regulates the lung adenocarcinoma cell phenotype. Previous studies have reported that miR-99a regulates cell cycle progression and cell proliferation by targeting mTOR, AKT1 and FGFR3 (16,24,25). However, its role in invasion and migration has not been fully elucidated. To examine whether miR-99a affects lung adenocarcinoma cell migration and invasion, we performed gain-of-function and loss-of-function analyses by overexpressing or suppressing miR-99a with the miR-99a mimic or anti-miR-99a (Fig. 2A). MTT assays showed that miR-99a overexpression significantly inhibited the proliferation of lung adenocarcinoma cells (Fig. 2B). Interestingly, the Transwell assay revealed that overexpression of miR-99a significantly decreased cell invasion potential of the A549 and Calu3 cells compared with the control cells. Moreover, suppression of miR-99a increased cell invasion (Fig. 2C). Consistent with the Transwell assay results, overexpression of miR-99a significantly inhibited A549 and Calu3 cell migration, whereas suppression of miR-99a increased cell migration as shown in the wound healing assays (Fig. 2D). These findings indicate that miR-99a plays an important role in the invasion and migration of lung adenocarcinoma cells.

NOX4 is a target of miR-99a. To determine the mechanism involved in the regulation of invasion and migration by

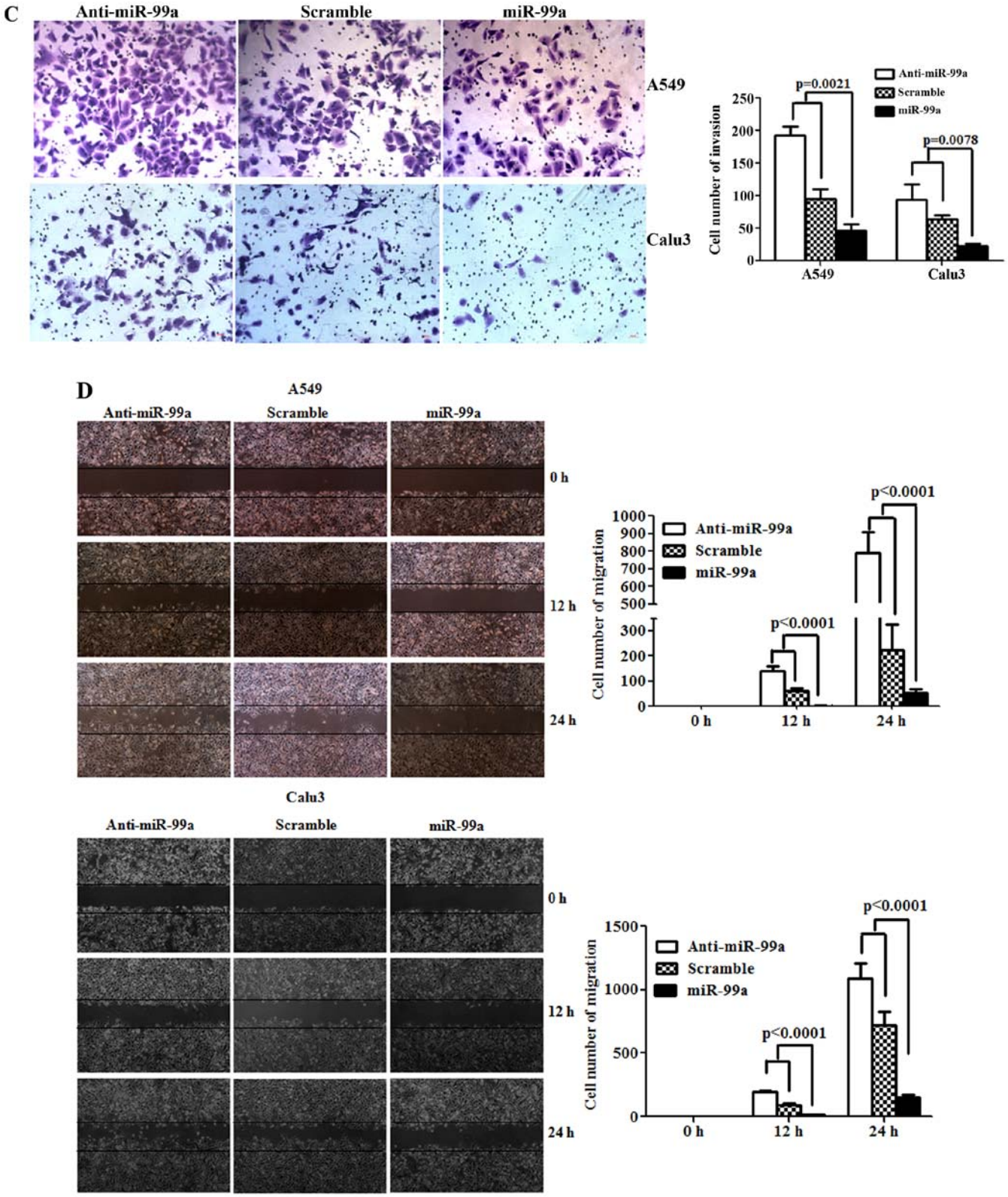

Figure 2. Continued. miR-99a inhibits the proliferation, invasion and migration of lung adenocarcinoma cells. (C and D) Transwell and wound healing assays showed that miR-99a overexpression significantly inhibited the invasion and migration of lung adenocarcinoma cells.

miR-99a, we performed an miRNA target search using TargetScan (www.targetscan.org) and identified a 'seed sequence' of miR-99a that matches the 3'UTR of the NOX4 gene (Fig. 3A). To determine whether NOX4 is a target gene of miR-99a, we generated a pGL3-NOX4-3'UTR luciferase reporter vector and pGL3-NOX4-3'UTR mutant plasmid, in which the seed sequence of miR-99a was mutated. We transfected the luciferase vectors into miR-99a-knockdown or ectopically miR-99a-expressing A549 cells. Luciferase results showed that knockdown of miR-99a significantly enhanced

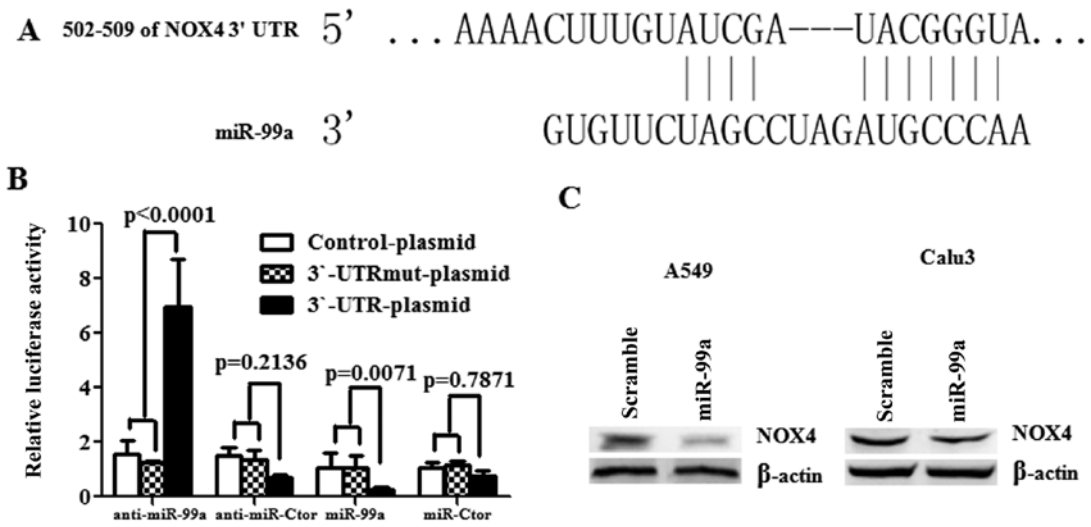

Figure 3. NOX4 is a target of miR-99a. (A) Using bioinformatic analysis, we identified that NOX4 was a potential target gene of miR-99a. (B) Luciferase reporter assay confirmed that NOX4 is a target gene of miR-99a. (C) Western blotting showed upregulation of NOX4 expression in cells with suppression of miR-99a, whereas downregulated NOX4 expression was observed in cells overexpressing miR-99a compared with the controls.

the activity of the wild-type reporter vector, whereas overexpression of miR-99a decreased its luciferase activity (Fig. 3B). No effects were observed with co-expression of the mutant reporter. Furthermore, western blot analysis showed that NOX4 expression was upregulated in the cells with suppressed miR-99a and downregulated in the cells with overexpression of miR-99a compared with the controls (Fig. 3C). These data demonstrated that NOX4 is a target gene of miR-99a.

Overexpression of NOX4 overrides miR-99a-mediated invasion and migration effects. Having demonstrated that NOX4 is a direct target gene of miR-99a, we next examined the role of NOX4 in the miR-99a-mediated lung adenocarcinoma cell invasion and migration. We co-transfected miR-99a and NOX4 lacking the 3'UTR into the A549 or Calu3 cells. Western blotting determined that miR-99a decreased NOX4 expression and NOX4 lacking the 3'UTR reversed NOX4 expression after transfection of miR-99a (Fig. 4A). Transwell and wound healing assays showed that the cells transfected with NOX4 lacking the 3'UTR significantly reversed cell invasion and migration after transfection of miR-99a (Fig. 4B and C). Thus, these results indicate that NOX4 is a bona fide target gene of miR-99a and mediates miR-99a to regulate the invasion and migration of lung adenocarcinoma cells.

miR-99a regulates lung adenocarcinoma invasion and migration by targeting NOX4-mediated ROS production. Furthermore, we explored the molecular mechanism of miR-99a-mediated invasion and migration by targeting NOX4. NOX4 is a member of the NADPH oxidases, and its primary function is to produce superoxide or hydrogen peroxide, including ROS. Thus, we evaluated ROS levels after overexpression or repression of miR-99a by using DCFH-DA. The results showed that miR-99a significantly inhibited ROS levels, whereas knockdown of miR-99a increased ROS levels (Fig. 5A). Several studies have shown that ROS mediate invasion and migration. Then, western blot results showed that knockdown of miR-99a resulted in significantly increased pAkt, MMP2 and MMP9, while overexpression of miR-99a had the opposite effects (Fig. 5B). Previous studies have shown that MMP2 and MMP9 mediate cell invasion and migration (26,27). To further show that miR-99a modulates cell invasion and migration dependent on ROS levels, the cells were treated with NAC as a ROS scavenger (Fig. 5C). The results showed that NAC significantly attenuated anti-miR-99a-induced cell invasion and migration (Fig. 5D and E). Western blotting showed that NAC also decreased anti-miR-99a-induced pAkt, MMP2 and MMP9 expression (Fig. 5F). Thus, these data revealed that miR-99a regulated invasion and migration by targeting the NOX4-ROS pathway.

miR-99a inhibits lung adenocarcinoma xenograft growth by targeting NOX4. Since miR-99a is frequently downregulated in lung adenocarcinoma and plays an important role in cell survival, we further examined the effects of the overexpression of miR-99a on tumor growth. The results showed that overexpression of miR-99a significantly inhibited tumor growth *in vivo* (Fig. 6A). RT-PCR showed increased expression of miR-99a in the miR-99a treatment group (Fig. 6B). Immunohistochemical staining analysis revealed that NOX4 levels were downregulated in the miR-99a treatment group (Fig. 6C), confirming the *in vitro* data of NOX4 as a direct target gene of miR-99a. Additionally, pAkt, MMP2 and MMP9 expression levels were decreased in sections from the xenograft tumors treated with miR-99a. No change in Akt expression was observed (Fig. 6C). These findings further indicate that miR-99a targets NOX4 and that miR-99a mimics could be a therapeutic means for lung adenocarcinoma intervention.

Discussion

As well as in other solid cancers, metastatic spread is the primary cause of death for NSCLC patients (3). Cancer metastasis is a complex regulated process in which invasion and migration of cancer cells play an important role in determining the

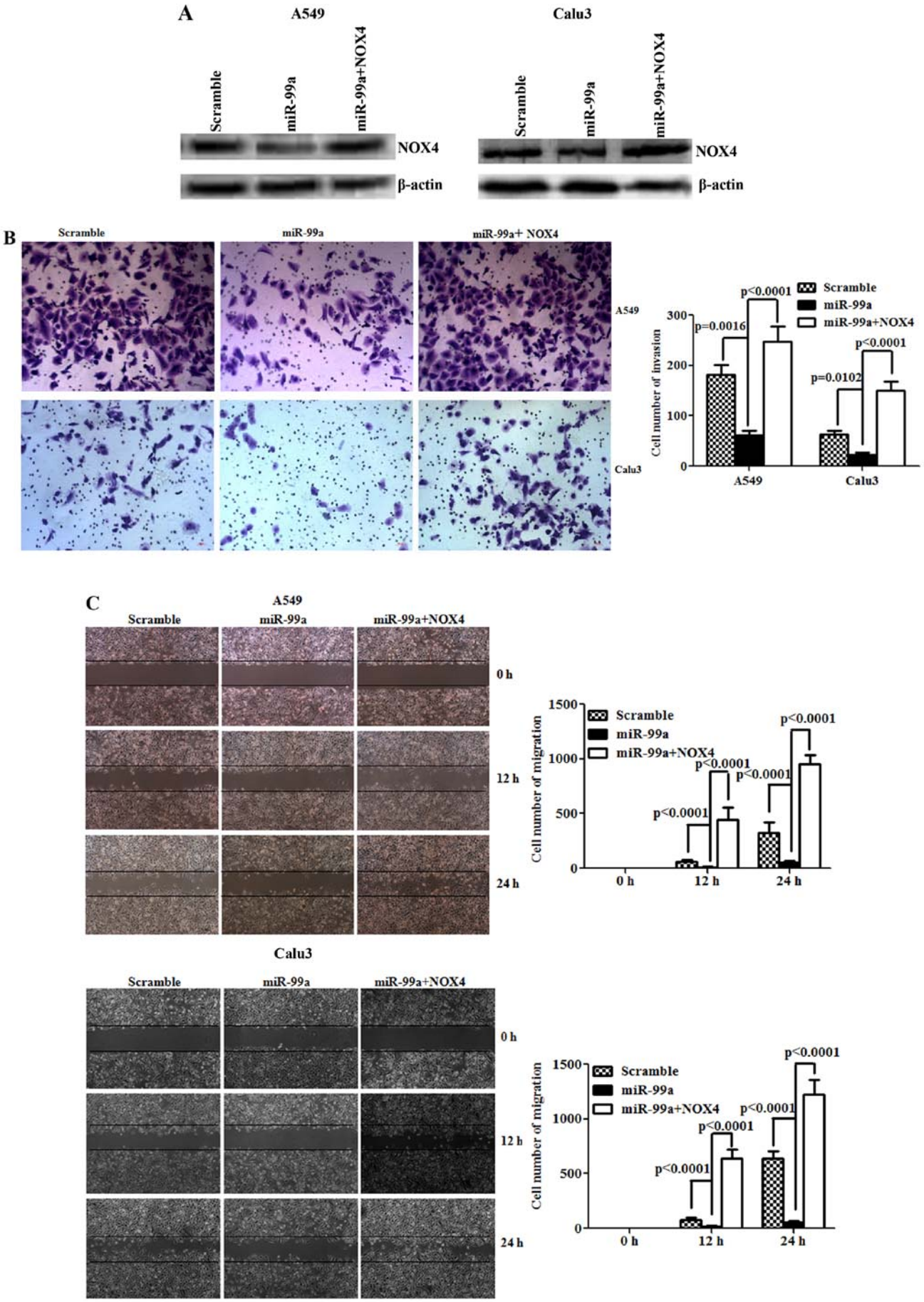

Figure 4. Expression of NOX4 overrides miR-99a invasion and migration function. (A) Western blotting showed that miR-99a decreased NOX4 expression, and expression of NOX4 lacking its 3'UTR reversed the miR-99a-mediated effects on NOX4 expression. (B and C) NOX4 lacking its 3'UTR significantly reversed cell invasion and migration after transfection with miR-99a.

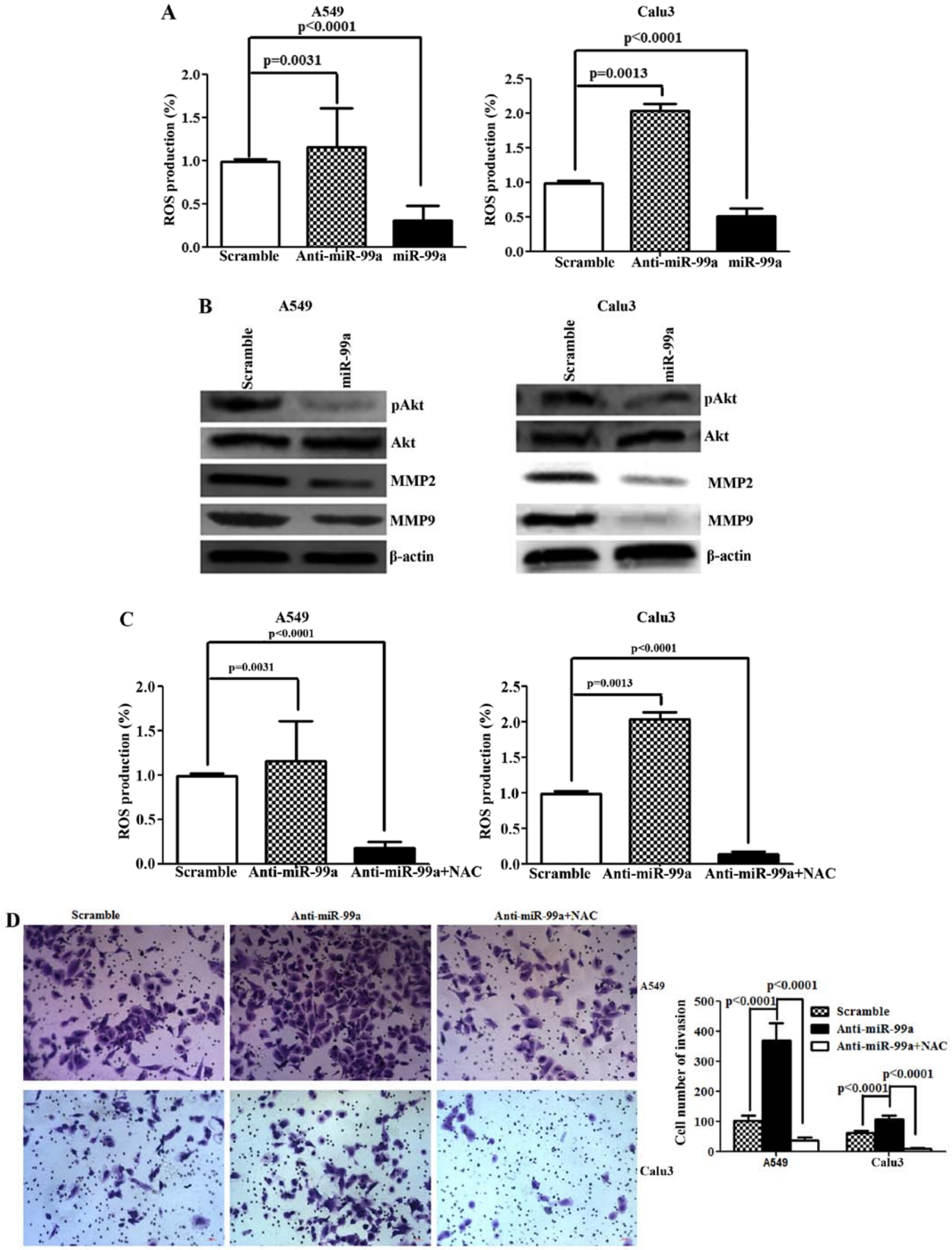

Figure 5. miR-99a regulates lung adenocarcinoma cell invasion and migration by targeting NOX4-mediated ROS production. (A) miR-99a significantly inhibited the ROS level, and knockdown of miR-99a increased the ROS level. (B) Western blotting showed that knockdown of miR-99a resulted in a significant increase in pAkt, MMP2 and MMP9 levels, and upregulation of miR-99a exhibited the opposite effects. (C) NAC reversed the production of ROS after knockdown of miR-99a. (D) NAC significantly attenuated anti-miR-99a-induced-cell invasion.

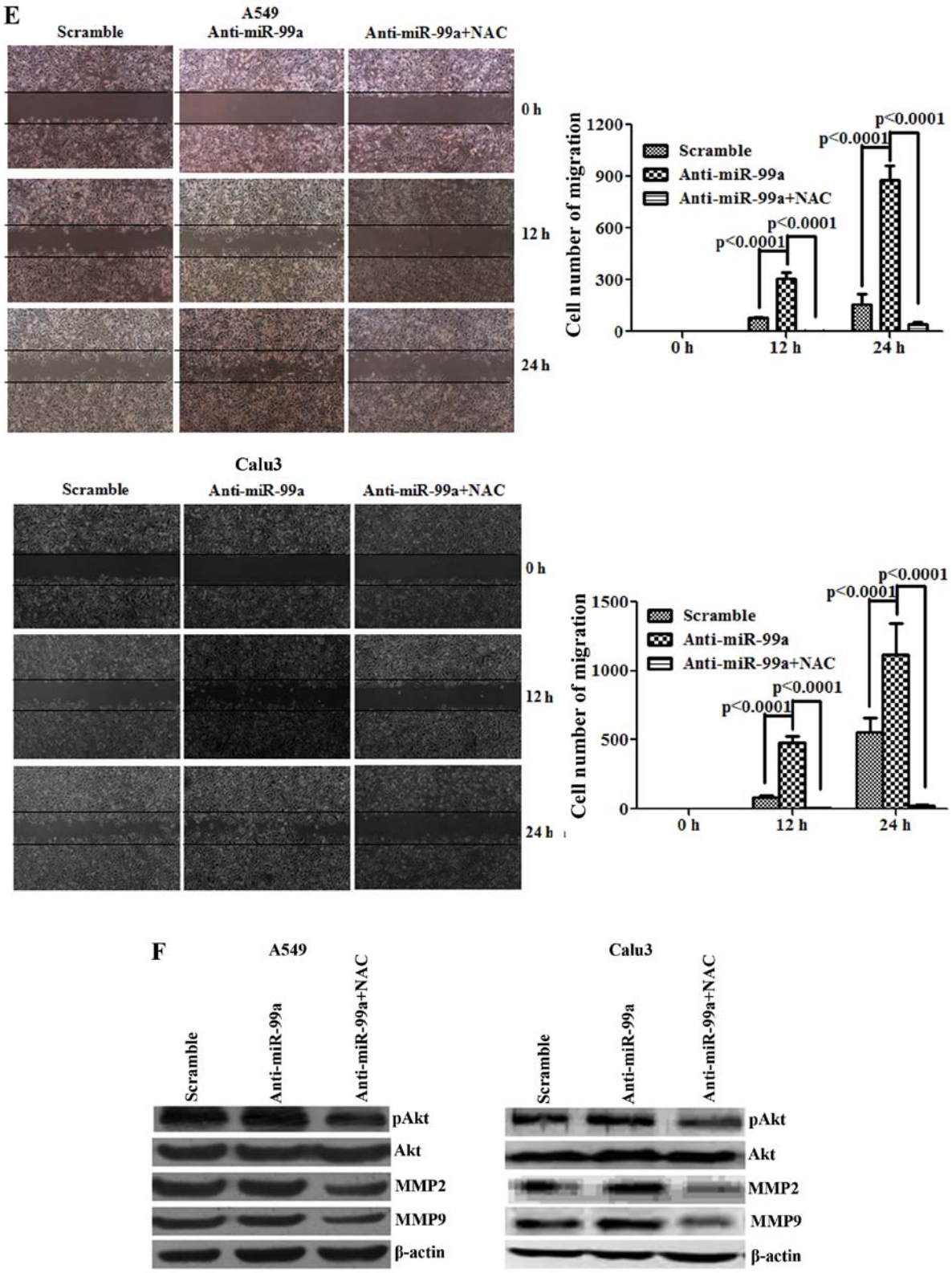

Figure 5. Continued. miR-99a regulates lung adenocarcinoma cell invasion and migration by targeting NOX4-mediated ROS production. (E) NAC significantly attenuated anti-miR-99a-induced cell migration. (F) Western blotting showed that NAC also decreased anti-miR-99a-induced-pAkt, MMP2 and MMP9 expression.

capability of cancer cells to metastasize. Aberrant expression of miRNAs in cancer cells plays an important role in mediating cell invasion and migration by targeting specific target genes (28,29). In our study, we identified that miR-99a was significantly downregulated in lung adenocarcinoma tissues compared with adjacent normal tissues. Moreover, we found a statistically significant association between downregulation of miR-99a and clinical late stage as well as metastasis in lung

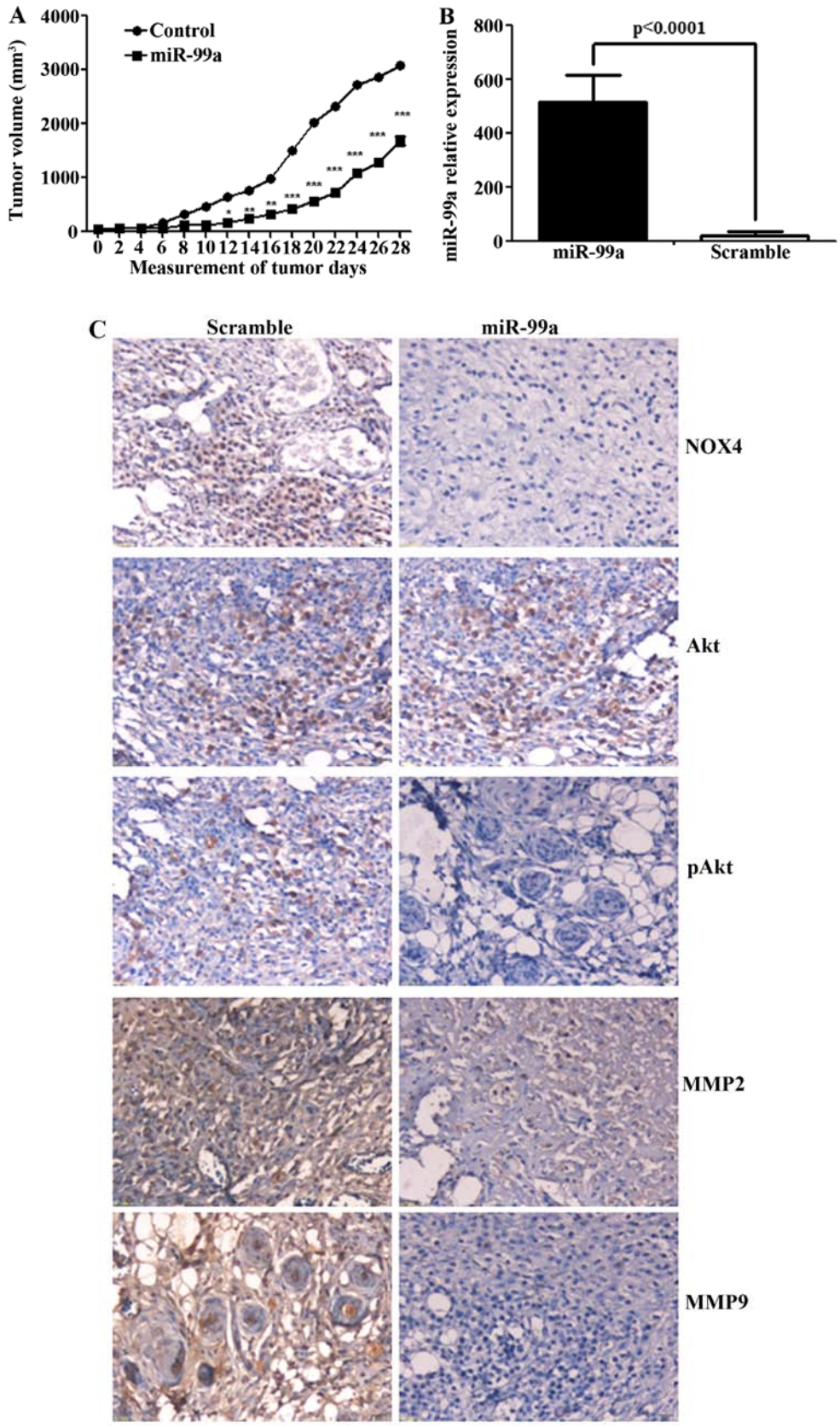

Figure 6. miR-99a inhibits lung adenocarcinoma xenograft growth by targeting NOX4. (A) Overexpression of miR-99a significantly inhibited tumor growth *in vivo*. (B) RT-PCR demonstrated that miR-99a increased expression of miR-99a in the tumor tissues. (C) Immunohistochemical staining analysis revealed that miR-99a downregulated NOX4, pAkt, MMP2 and MMP9 expression *in vivo*.

adenocarcinoma patients. Kaplan-Meier survival analyses showed that downregulation of miR-99a was a poor prognostic factor for lung adenocarcinoma patients. Next, we identified whether miR-99a regulated the invasion and migration of

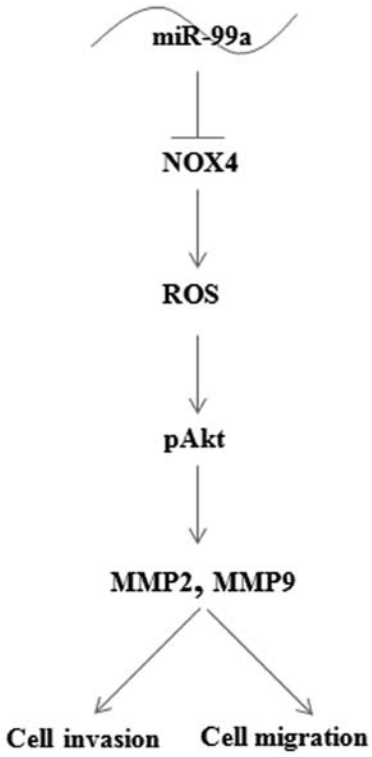

Figure 7. The mechanism of miR-99a. miR-99a inhibits the invasion and migration of lung adenocarcinoma cells by targeting NOX4/ROS.

lung adenocarcinoma. Transwell and wound healing assays showed that miR-99a negatively regulated invasion and migration of lung adenocarcinoma cells. MTT assay confirmed that miR-99a significantly inhibited the proliferation of lung adenocarcinoma cells. Taken together, miR-99a regulated proliferation, invasion and migration of lung adenocarcinoma cells. Downregulation of miR-99a may act as an independent poor prognostic factor for lung adenocarcinoma patients.

To determine the mechanism by which miR-99a regulates invasion and migration in lung adenocarcinoma cells, we performed an miRNA target search using TargetScan and found the 'seed sequence' of miR-99a that matched with the 3'UTR of the NOX4 gene, which was found to regulate the invasion and migration of NSCLC cells (30). Luciferase reporter assay and western blotting showed that NOX4 is a target gene of miR-99a. Furthermore, by co-transfection of miR-99a and NOX4 lacking its 3'UTR, we identified that NOX4 is a direct target gene of miR-99a and modulated miR-99a-mediated invasion and migration of lung adenocarcinoma cells by targeting NOX4.

How does miR-99a regulate the invasion and migration of lung adenocarcinoma cells by targeting NOX4? Some studies have shown that NOX4 regulates invasion and migration of cells by producing ROS (31,32). Thus, we used DCFH-DA to quantify intercellular ROS. The results showed that miR-99a significantly decreased the production of ROS. In addition, repression of miR-99a significantly increased production of ROS. Zha *et al* reported that ROS activates the PI3K/Akt pathway (33). In our study, by western blotting, we demonstrated that miR-99a downregulated the NOX4/ROS/Akt pathway to inhibit MMP2 and MMP9 expression. MMPs are a family of zinc-dependent endopeptidases that degrade all extracellular matrix components, including collagens, fibronectin, laminin and basement membrane proteoglycans. Among the MMPs, much attention has focused on MMP2 and MMP9 in NSCLC (34). MMPs are key proteases modulating invasion and migration of NSCLC. In our study, overexpression of miR-99a downregulated expression of MMP2 and MMP9, and vice versa. In a mouse subcutaneous xenograft model, we identified that miR-99a inhibited the growth of lung adenocarcinoma cells. Moreover, miR-99a downregulated NOX4, pAkt, MMP2 and MMP9 expression. These results were the same as that *in vivo*. Taken together, our data demonstrated that miR-99a inhibited the invasion and migration of lung adenocarcinoma cells by targeting the NOX4-mediated ROS/Akt pathway.

In summary, our data demonstrated that miR-99a was frequently downregulated in lung adenocarcinoma. As Fig. 7 shows, downregulation of miR-99a resulted in an increase in expression of its target gene NOX4. NOX4 increased ROS production to activated Akt, MMP2 and MMP9 expression which mediated the invasion and migration of lung adenocarcinoma cells. Thus, our data suggest that overexpression of miR-99a is a potential therapeutic strategy for preventing lung adenocarcinoma cell invasion and migration.

Acknowledgements

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