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Co-targeting of EGFR by co-expressed miRNA-193a-3p/−5p in lung cancer

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Abstract

MicroRNAs (miRNAs) function as important oncogenes or tumor suppressors in each stage of cancer development. While previous studies focused on the individual miRNA and its specific target gene, the cooperative functions of miRNA-3p and miRNA-5p (miRNA-3p/−5p) pairs that frequently co-exist in cells remain largely unclear. In this study, we explored the co-expression and co-targeting of the miRNA-3p/−5p pairs in lung cancer. We identified miRNA-193a-3p and miRNA-193a-5p (miRNA-193a-3p/−5p) as typical co-reduced miRNA-3p/−5p pairs in NSCLC and predicted EGFR as the co-target of miRNA-193a-3p/−5p. In agreement with this, inverse expression between miRNA-193a-3p/−5p and EGFR was detected in NSCLC tissues. Furthermore, in vitro experiments validated that miRNA-193a-3p/−5p effectively suppressed migration and proliferation in lung cancer cells. Finally, data from xenograft tumor model provided in vivo evidence for miRNA-193a-3p/−5p as tumor-suppressive miRNAs by downregulating EGFR expression. In summary, our results highlight a critical role for co-expressed miRNA-193a-3p/−5p in the co-targeting of EGFR during lung tumorigenesis.

Keywords: Non-small cell lung cancer, miRNA-193a-3p, miRNA-193a-5p, Migration, Proliferation

Introduction

Lung cancer is the most common human cancer with highest morbidity and mortality, and most (80%) cases are non-small cell lung cancer (NSCLC). NSCLC is mainly treated surgically together with chemotherapy and/or radiotherapy. A panel of well-known oncogenes drives NSCLC, including EGFR, KRAS and ALK [1]. Among all these oncogenes, EGFR (ERBB1 or HER1) is well studied. EGFR is an effective target of tyrosine kinase inhibitors (TKIs) and treatment with TKIs is the first-line therapy for patients having activating mutations. Unfortunately, these therapeutic agents have limited effects on numerous patients, which may be due to the primary and secondary drug resistance. Novel drugs, which efficiently overcome and prevent resistance in NSCLC patients, are urgently needed.

In mammals, microRNAs (miRNAs), a type of small non-coding RNAs of 21–25 nt in length, bind to mRNA 3′-UTR and thereby inhibit protein expression or cause mRNA degradation [2]. During miRNA biogenesis, primary miRNA (pri-miRNA) is transcribed by RNA polymerase II and cleaved to 60–110 nt hairpin precursor miRNA (pre-miRNA) by Drosha. Pre-miRNA is then exported by Exportin5 to the cytoplasm, where pre-miRNA is cleaved by Dicer to produce a double-stranded miRNA/miRNA* duplex approximately 22 nt in length [3, 4]. Subsequently, the functional strand, known as miRNA, is processed to mature form and followed by loading into the RNA-induced silencing complex (RISC). Meanwhile, the complementary strand is destined to be degraded [5]. However, latest studies demonstrate that miRNA* sequences can also be selected for maturation [6, 7]. Sometimes, the 5′- and 3′- arms of pre-miRNA can co-express and serve as different mature miRNAs. To avoid confusion, the mature miRNAs generated from the 5′- and 3′- arms of pre-miRNA are termed as miRNA-3p or miRNA-5p, respectively. Currently, there are increasing reports that

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show the co-existence of miRNA-3p and miRNA-5p (miRNA-3p/−5p) [8, 9]. The biological function of miRNA-3p/−5p co-expression deserves deeper investigation.

In this study, we investigated whether miRNA-3p/−5p possess overlapping targets and whether they work in combination to accomplish their functions. We explored co-expression and co-targeting of miRNA-3p/−5p pairs in lung cancer cells. EGFR was identified as the co-target of co-expressed miRNA-193a-3p and miRNA-193a-5p (miRNA-193a-3p/−5p). We found the direct suppression of EGFR expression by miRNA-193a-3p/−5p in vitro and identified the anti-tumor role of miRNA-193a-3p/−5p in NSCLC development in vivo.

Results

Co-reduction of miRNA-193a-3p/−5p in cancer tissues

miRNA-193a-3p/−5p were previously observed to be co-downregulated in human osteosarcoma and lung cancer [10, 11]. To investigate the potential co-function of miRNA-3p/−5p pairs, we first selected miRNA-193a-3p/−5p as a representative miRNA pairs and measured the expression patterns of miRNA-193a-3p/−5p in 12 pairs of NSCLC tissues and normal adjacent tissues (NATs). Both miRNA-193a-3p/−5p were consistently reduced in tumors in comparison with NATs from the same patients (Fig. 1a). The results strongly suggest concerted co-reduction of miRNA-193a-3p/−5p pairs during lung tumorigenesis.

Co-targeting of EGFR by miRNA-193a-3p/−5p

A list of co-targets of miRNA-193a-3p/−5p pairs was predicted using the RNAhybrid algorithm [12]. Although miRNA-193a-3p/−5p display no sequence homology and therefore share few predicted targets, our bioinformatics algorithm identified EGFR as a common target. The putative binding sites between miRNA-193a-3p/−5p and EGFR 3′-UTR are showed in Fig. 1b. EGFR 3′-UTR contains one binding site each for miRNA-193a-3p/−5p, and the two binding sites are non-overlapping. The minimum free energy values were − 25.1 and − 28.0 kcal/mol for hybrid between miRNA-193a-3p and EGFR and for hybrid between miRNA-193a-5p and EGFR, respectively, and the values were well within the range of free energy values associated with functional miRNA-target pairs.

Luciferase reporter assays were employed to confirm the binding ability between miRNA-193a-3p/−5p and EGFR. Mimics and antisenses were transfected into cells to overexpress and knockdown miRNA-193a-3p/−5p, respectively, and a negative control (scrambled mimic or antisense) was transfected simultaneously. As anticipated, the luciferase activity was significantly inhibited by overexpression of miRNA-193a-3p/−5p, whereas knockdown of miRNA-193a-3p/−5p increased luciferase activity (Fig. 1c). Interestingly, while reduction of luciferase activity was strengthened when miRNA-193a-3p/−5p mimics were used in combination, a reinforced luciferase activity was not detected in miRNA-193a-3p/−5p antisenses-co-transfected cells (Fig. 1c). The results implied that, although miRNA-193a-3p/−5p can co-target the EGFR transcript despite having different sequences, they have no synergistic effect on EGFR levels. Moreover, we introduced point mutations to the EGFR 3′-UTR in the luciferase reporter plasmid. For mutated plasmid, luciferase activity was not influenced by induction of miRNA-193a-3p/−5p (Fig. 1c).

Inverse relationship between miRNA-193a-3p/−5p and EGFR in cancer tissues

We further investigated the expression pattern of EGFR in tumors to see if EGFR is negatively correlated with miRNA-193a-3p/−5p in NSCLC. We measured EGFR protein and mRNA expression levels in the same 12 pairs of NSCLC and NAT tissues. EGFR protein levels were uniformly increased in tumors (Fig. 1d), while the alteration of EGFR mRNA levels was irregular (Fig. 1e). We then explored the relationships between miRNA-193a-3p/−5p and the EGFR protein or mRNA levels. miRNA-193a-3p/−5p had more strictly negative correlation with the EGFR protein levels than the mRNA levels in NSCLC tissues (Fig. 1f).

miRNA-193a-3p/−5p co-target EGFR in vitro

Next, we overexpressed or knocked down miRNA-193a-3p/−5p in lung cancer cells and examined the EGFR protein and mRNA levels. As expected, mimics significantly increased miRNA-193a-3p/−5p expression levels when antisenses dramatically decreased their levels in H1975 and A549 cells (Additional file 1: Figure S1A). Consequently, the EGFR protein expression were suppressed by miRNA-193a-3p/−5p mimics (Fig. 2a), whereas miRNA-193a-3p/−5p antisenses increased EGFR protein expression in cancer cells (Fig. 2b). In contrast, EGFR mRNA levels were not obviously affected by overexpression or knockdown of miRNA-193a-3p/−5p (Fig. 2c, d). Based on the results above, miRNA-193a-3p/−5p may negatively regulate EGFR expression by repressing mRNA translation. However, co-treatment of cells with both miRNA-193a-3p/−5p mimics did not enhance the suppressive effect on EGFR protein expression when compared to miRNA-193a-3p/−5p mimic alone (Fig. 2a), and the greater increase in expression of EGFR protein did not occur when miRNA-193a-3p/−5p antisenses were used simultaneously (Fig. 2b). These data imply that miRNA-193a-3p/−5p have no synergistic effect on EGFR levels.

Moreover, we constructed lentiviruses to overexpress miRNA-193a-3p/−5p and infected H1975 and A549 cells with these lentiviruses to produce functional intracellular miRNA-193a-3p/−5p. When H1975 and A549 cells were infected with lentiviruses overexpressing miRNA-193a-3p

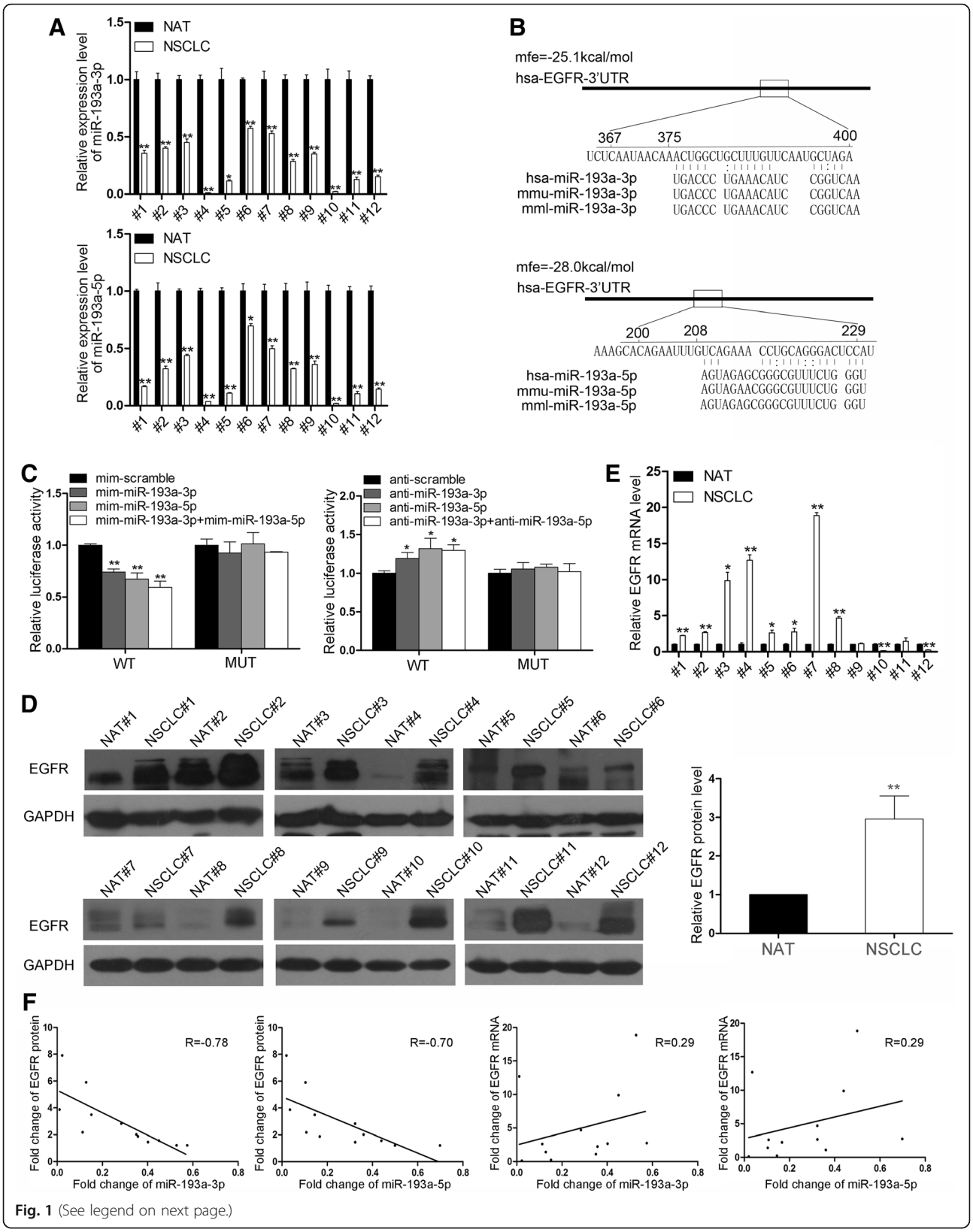

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Inverse correlation of miRNA-193a-3p/−5p and EGFR protein expression levels in NSCLC tissues. **a** The relative expression levels of miRNA-193a-3p/−5p in 12 pairs of NSCLC and NAT samples. **b** Schematic description of the hypothetical duplexes between miRNA-193a-3p/−5p (bottom) and EGFR mRNA (top). The predicted free energy values were indicated. The conservation of miRNAs across species, including *Homo sapiens* (hsa), *Mus musculus* (mmu) and *Macaca mulatta* (mml), are displayed. **c** The relative luciferase activities that showing the binding abilities between miRNA-193a-3p/−5p and EGFR 3'UTR. WT: wild type; MUT: mutant. **d** EGFR protein levels in 12 pairs of NSCLC and NAT samples were analyzed by western blotting assays. Left panel: representative images; right panel: quantitative analysis. **e** EGFR mRNA levels in 12 pairs of NSCLC and NAT samples were analyzed by qRT-PCR. **f** The expression levels between miRNA-193a-3p/−5p and EGFR protein (left two panels) and between miRNA-193a-3p/−5p and EGFR mRNA (right two panels) in NSCLC tissues were analyzed by Pearson's correlation scatter plot.
*$P < 0.05$, **$P < 0.01$

or miRNA-193a-5p, the cellular levels of miRNA-193a-3p/−5p were about 3–5 fold higher than the basal levels (Additional file 1: Figure S1B). Infecting H1975 and A549 cells with miRNA-193a-3p/−5p overexpressing lentiviruses also reduced EGFR protein expression levels (Fig. 2e) but had no effect on EGFR mRNA levels (Fig. 2f) in H1975 and A549 cells. Furthermore, co-treatment with both miRNA-193a-3p/−5p overexpressing lentiviruses did not suppress the EGFR protein levels to a greater extent than either miRNA-193a-3p/−5p overexpressing lentivirus did alone (Fig. 2e). Thus, miRNA-193a-3p/−5p can co-target EGFR, but their cooperative effect is not greater than that of a single miRNA-193a-3p or miRNA-193a-5p.

miRNA-193a-3p/−5p depress the proliferation and migration of lung cancer cells

To explore if miRNA-193a-3p/−5p have an effect on NSCLC tumorigenesis, we transfected H1975 and A549 cells with mimics and antisenses of miRNA-193a-3p/−5p and assessed the cell migration capability by transwell assays and cell proliferation capability by CCK8 assays. Transwell assays revealed that overexpression of

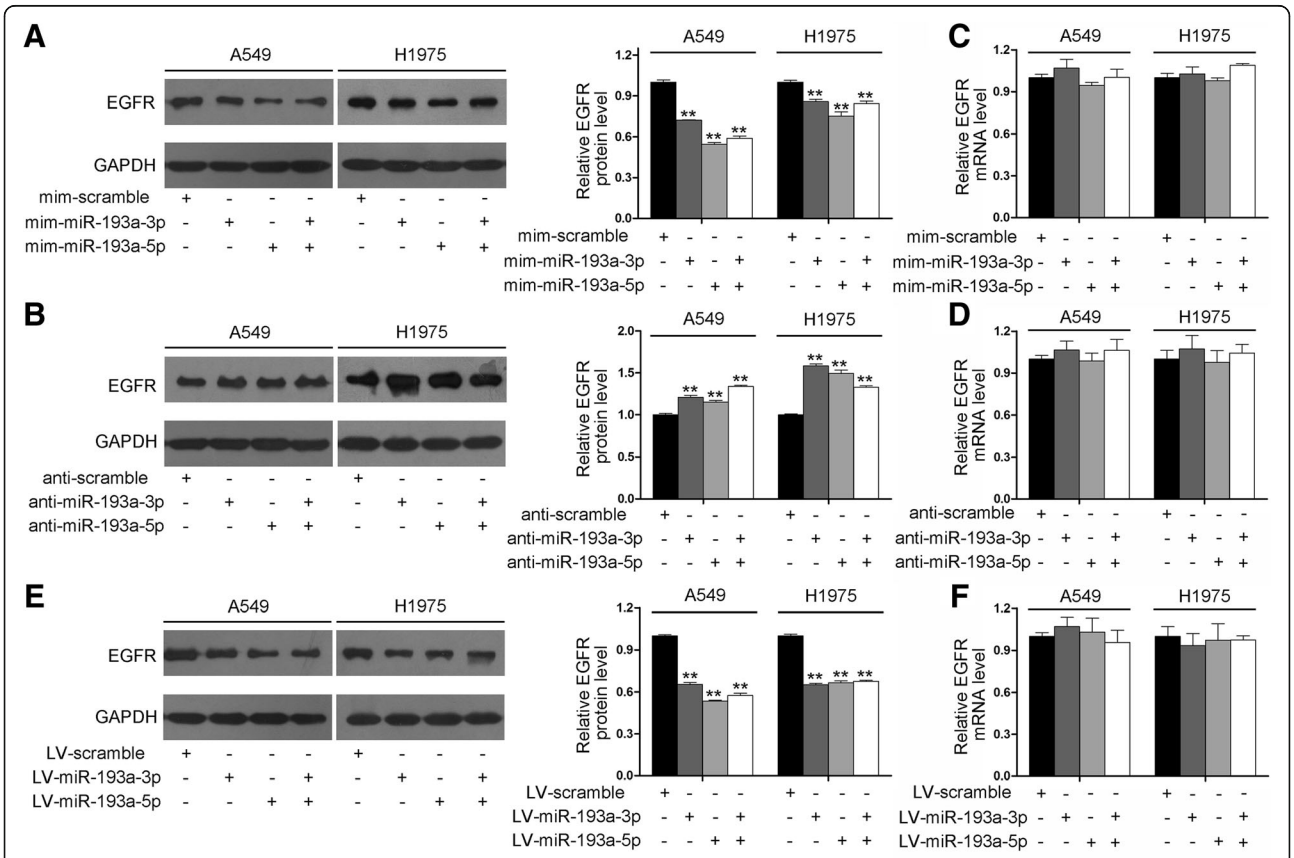

Fig. 2 miRNA-193a-3p/−5p co-target EGFR in H1975 and A549 cells. **a** and **b** Western blotting analysis of EGFR protein levels in H1975 and A549 cells after transfection. Left panel: representative images; right panel: quantitative analysis. **c** and **d** qRT-PCR analysis of EGFR mRNA levels in H1975 and A549 cells after transfection. **e** Western blotting analysis of EGFR protein levels in H1975 and A549 cells after infection with lentivirus. Left panel: representative images; right panel: quantitative analysis. **f** qRT-PCR analysis of EGFR mRNA levels in H1975 and A549 cells after infection with lentivirus. **$P < 0.01$

miRNA-193a-3p/−5p dramatically reduced the number of migrated H1975 and A549 cells, whereas knockdown of miRNA-193a-3p/−5p increased cell migration (Fig. 3a, b). Likewise, H1975 and A549 cells infected with miRNA-193a-3p/−5p overexpression lentivirus displayed decreased migration ability (Fig. 3c). Furthermore, overexpression of miRNA-193a-3p/−5p led to significant suppression of cell proliferation in H1975 and A549 cells, whereas miRNA-193a-3p/−5p inhibition promoted cell growth (Fig. 4a, b). Similarly, H1975 and A549 cells infected with miRNA-193a-3p/−5p overexpression lentivirus exhibited decreased proliferation ability (Fig. 4c). Overall, the data indicate that miRNA-193a-3p/−5p have negative effects on lung cancer cell proliferation/migration and therefore function as tumor suppressors during tumorigenesis. Although miRNA-193a-3p/−5p can inhibit

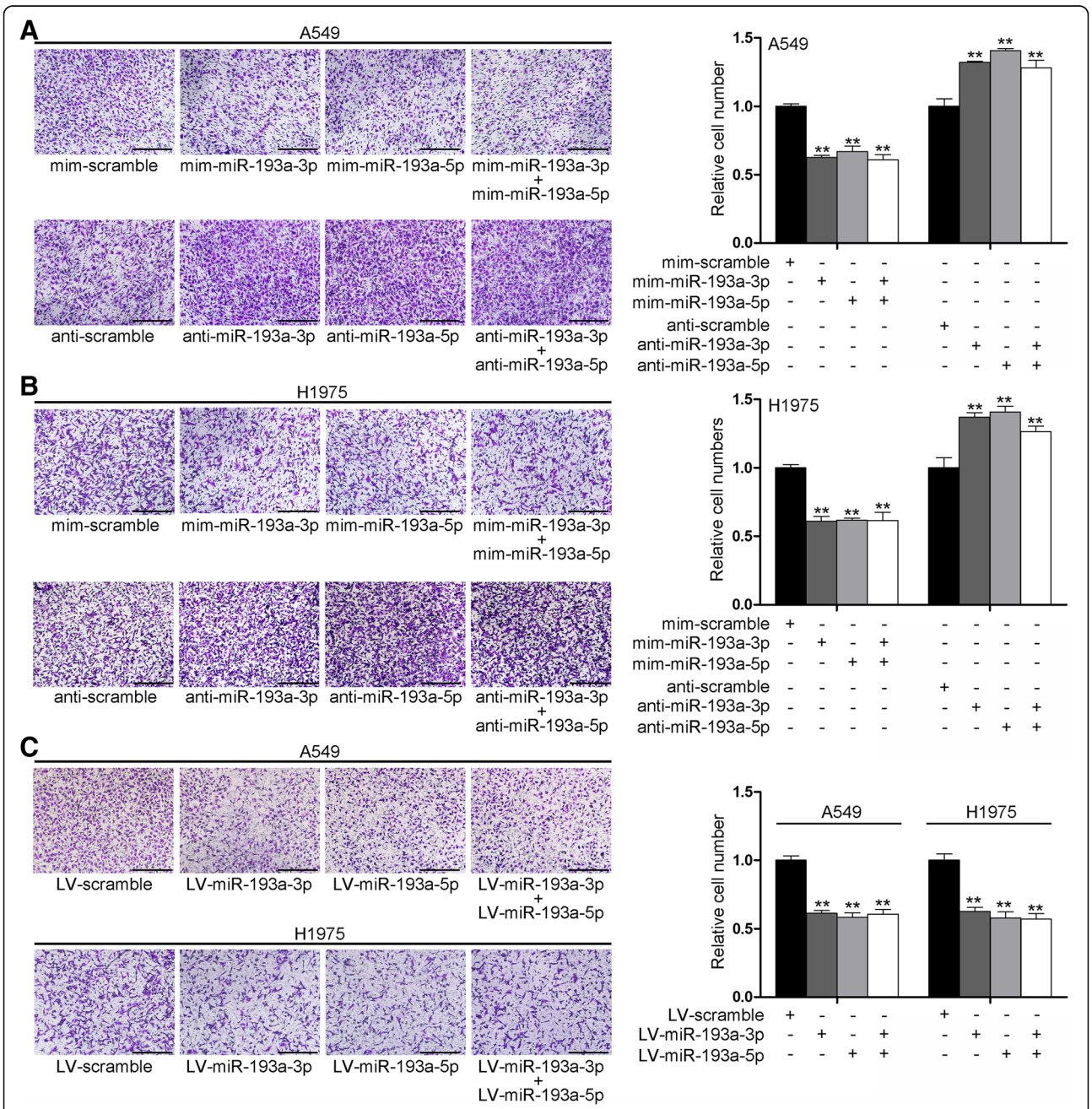

Fig. 3 miRNA-193a-3p/−5p regulate cell migration in H1975 and A549 cells. **a** and **b** Transwell analysis of migrated A549 (**a**) and H1975 (**b**) cells after transfection. Left panel: representative images; right panel: cell numbers counted in each field. **c** Transwell analysis of migrated H1975 and A549 cells after infection with lentivirus. Left panel: representative images; right panel: cell numbers counted in each field. Scale bar: 500 μm. Magnification: × 100. $^{**}P < 0.01$

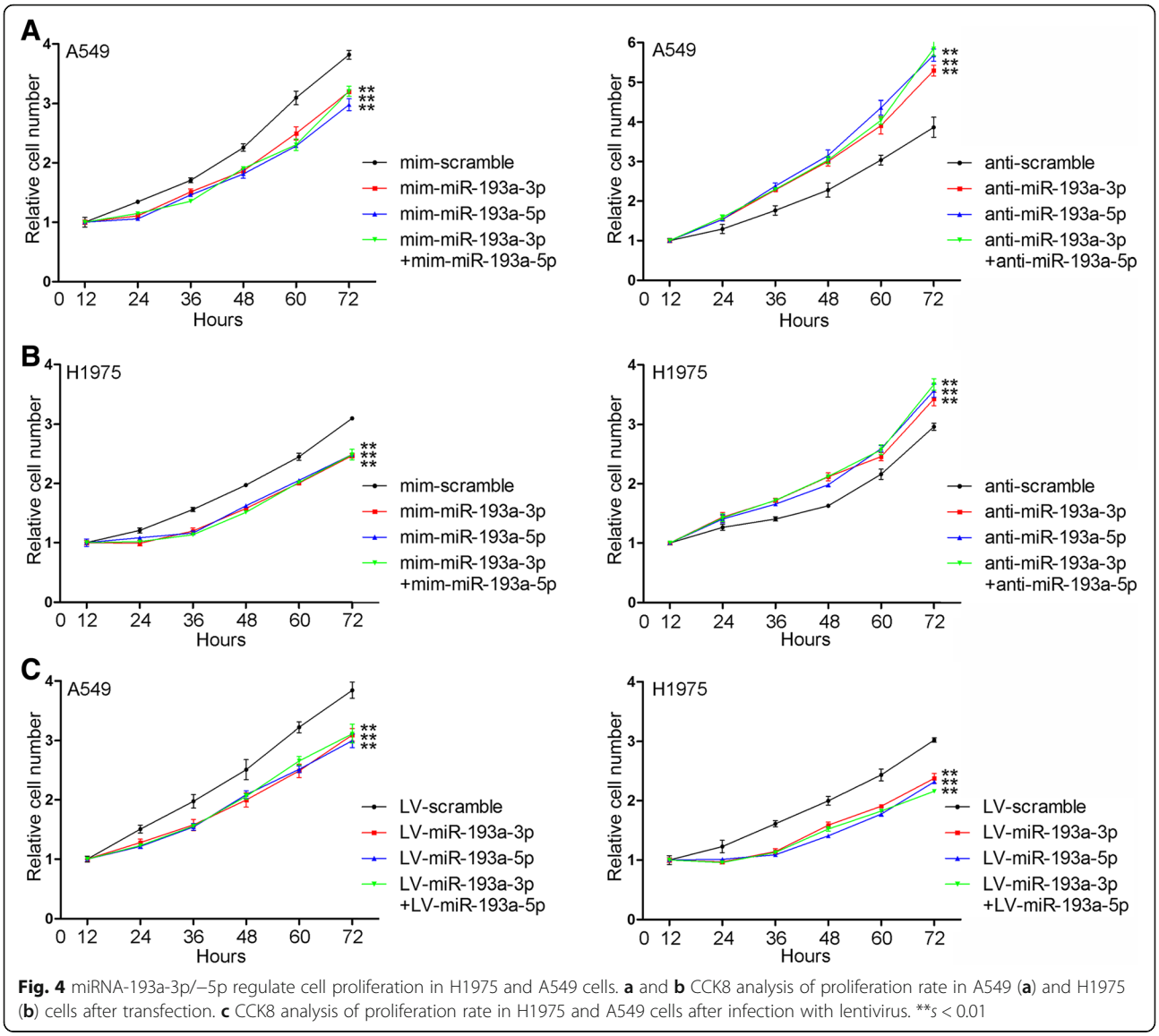

Fig. 4 miRNA-193a-3p/−5p regulate cell proliferation in H1975 and A549 cells. **a** and **b** CCK8 analysis of proliferation rate in A549 (**a**) and H1975 (**b**) cells after transfection. **c** CCK8 analysis of proliferation rate in H1975 and A549 cells after infection with lentivirus. **s < 0.01

cell migration and proliferation seperately, the simultaneous overexpression of miRNA-193a-3p/−5p with mimics or lentiviruses did not show a synergistic effect on cell migration and proliferation (Figs. 3, and 4).

EGFR plays a fundamental role in each stage of tumorigenesis in many cancer types, enhancing cell growth, inhibiting cell apoptosis and contributing to angiogenesis [13–21]. For better understanding of the EGFR pathway in lung cancer, we examined the consequences of EGFR inhibition in lung cancer cells by siRNA assays. EGFR protein levels were obviously lower following treatment with EGFR siRNA compared to scrambled siRNAs in lung cancer cells (Fig. 5a). Moreover, down-expression of EGFR significantly reduced the proliferation rate in H1975 and A549 cells (Fig. 5b), suggesting that miRNA-193a-3p/−5p may inhibit cell proliferation via downregulating EGFR.

miRNA-193a-3p/−5p inhibit tumor growth in vivo by targeting EGFR

Subsequently, we investigated if miRNA-193a-3p/−5p may have an effect on tumor growth in a mouse model implanted with lung cancer cells. H1975 cells were infected with lentivirus overexpressing miRNA-193a-3p/−5p or control lentivirus and injected subcutaneously into nude mice. The miRNA-193a-3p/−5p expression levels in H1975 cells after lentiviral infection were determined (Additional file 1: Figure S1B). Compared to the control group, the tumors in miRNA-193a-3p or miRNA-193a-5p-overexpressing group grew much faster (Fig. 6a). Mice were then sacrificed and tumor size/weight were measured. As expected, tumors from the miRNA-193a-3p or miRNA-193a-5p-overexpressing group were lighter and smaller (Fig. 6b-d). Tumors from the miRNA-193a-3p or miRNA-193a-5p-overexpressing group had an increase

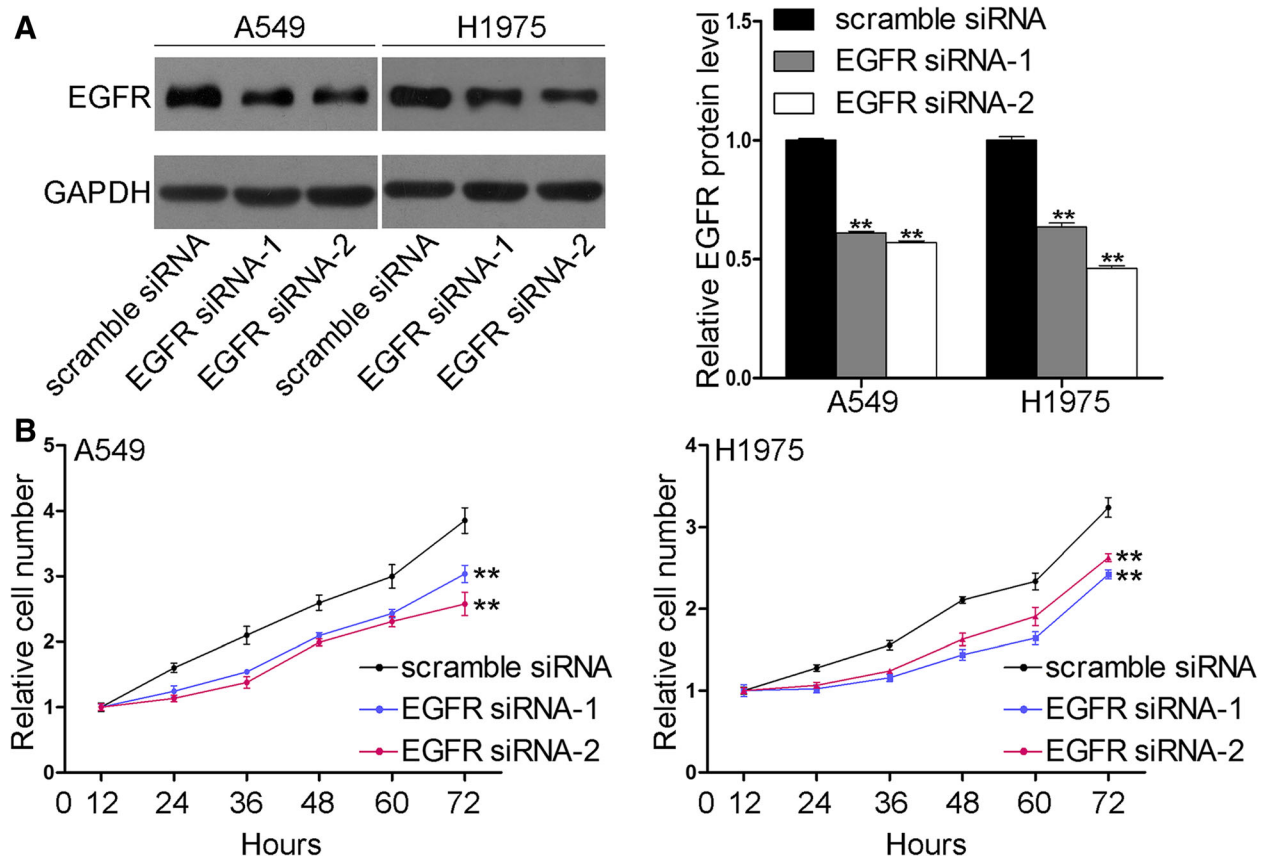

Fig. 5 Effect of EGFR knockdown on cell proliferation in H1975 and A549 cells. **a** The levels of EGFR protein in cancer cells after transfection with scrambled siRNA or EGFR siRNAs were analyzed by western blotting. Left panel: representative images; right panel: quantitative analysis. **b** Proliferation ablities of H1975 and A549 cells after transfection with scrambled siRNA or EGFR siRNAs were analyzed by CCK-8 assays. $^{**}P < 0.01$

in miRNA expression (Fig. 6e) and decrease in EGFR protein expression levels in tumors (Fig. 6f). Finally, we performed immunohistochemical staining assays to examine the PCNA and Ki-67 levels. The staining intensity of Ki-67 and PCNA was decreased in tumors from the miRNA-193a-3p or miRNA-193a-5p-overexpressing group (Fig. 6g), which means the proliferative ability of cancer cells was depressed. These data offer in vivo evidence for the inhibitory effect of miRNA-193a-3p/−5p on lung cancer through the inhibition of EGFR expression.

Discussion

Current research in tumor biology has identified several new prognostic and predictive biomarkers and therapeutic targets for NSCLC and uncovered some dominant oncogenes involved in NSCLC carcinogenesis [22]. EGFR is one of such gene, whose central contribution to the deregulation of NSCLC cell behavior has become clear. During last decades, miRNAs are proved to play important roles in cancers by targeting oncogenes or tumor suppressors. In lung cancer, miR-193a-3p have been demonstrated to regulate ERBB4, KRAS and S6K2, when miR-193a-5p play roles in regulating WT1-E-cadherin axis, mTOR and PIK3R3 [10, 23, 24]. Herein we showed that miRNA-193a-3p/−5p behaved together as tumor-suppressive miRNAs in vitro and in vivo and proved that EGFR is a key target through which miRNA-193a-3p/−5p contribute to cancer development.

Chemotherapy and/or radiotherapy are the main therapeutic agents for NSCLC for decades [25–27]. At present, several targeted drugs have significantly altered the landscape of NSCLC therapy. However, there are still lots of patients that have no response to these therapies or become resistance to treatment. Hence, it remains important to overcome the problems of drug resistance and develop new therapeutic strategies. Considering that miRNA-193a-3p/−5p are upstream regulators of EGFR, it could be possible to upregulate miRNA-193a-3p/−5p for containment of EGFR during tumorigenesis. To date, increasing evidences demonstrated that miRNAs are very promising therapeutic molecules for human cancers [28, 29]. Here we suggest that the replacement treatment with miRNA-193a-3p/−5p may be a potential solution for NSCLC having miRNA-193a-3p/−5p reduction.

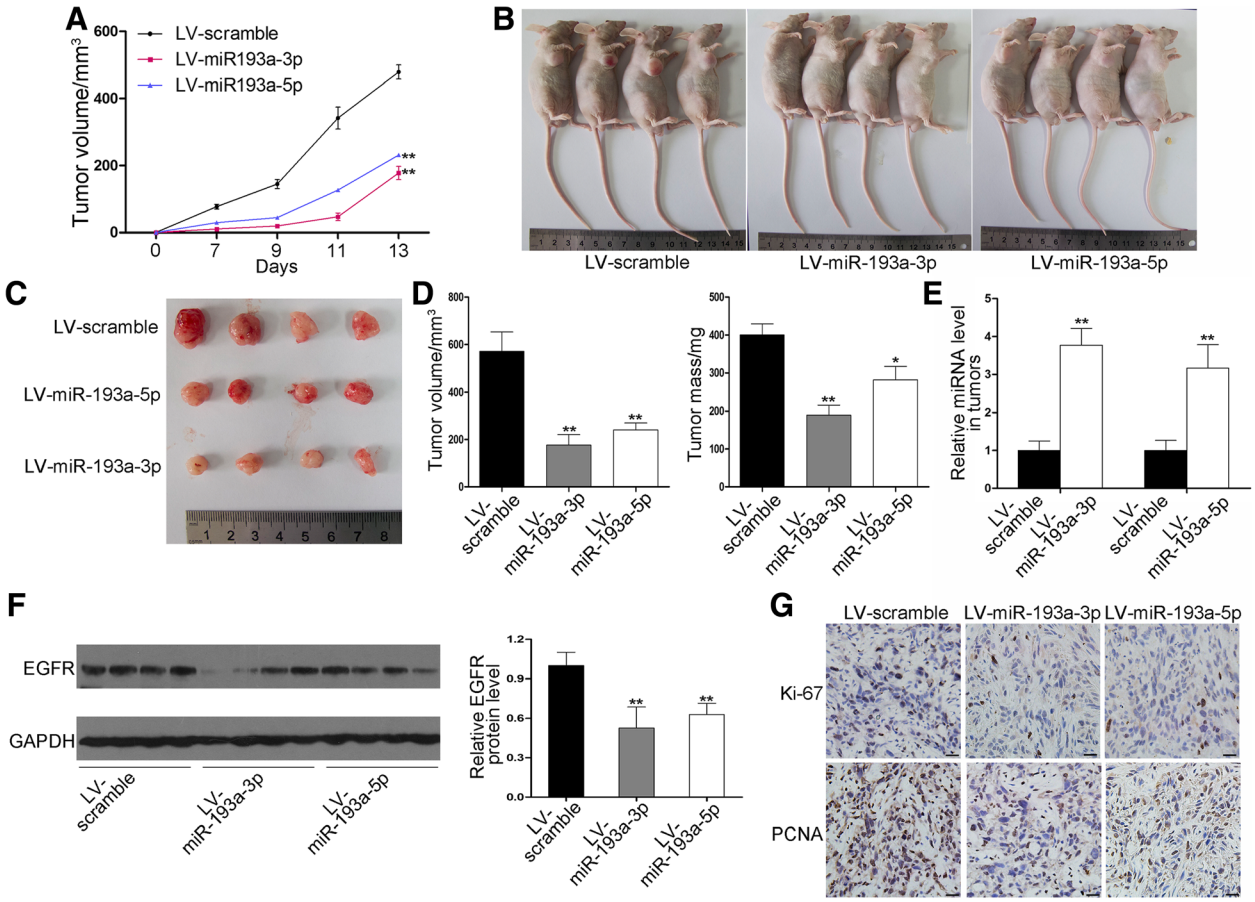

Fig. 6 Effects of miRNA-193a-3p/−5p on the growth of NSCLC xenografts in mice. H1975 cells were infected with lentiviruses overexpressing miRNA-193a-3p or miRNA-193a-5p or control lentivirus and implanted subcutaneously into nude mice. **a** Growth curves of xenograft tumors. **b** Representative images of the implanted mice. **c** Representative images of the dissected tumors. **d** Volume and mass of dissected tumors. **e** Relative miRNA-193a-3p/−5p levels in the tumors were analyzed by qRT-PCR. **f** EGFR protein levels in the tumors were analyzed by western blotting. Left panel: representative images; right panel: quantitative analysis. **g** PCNA and Ki-67 in the tumors were analyzed by Immunohistochemical staining. Scale bar: 25 μm. Magnification: × 400. $^{*}P < 0.05$, $^{**}P < 0.01$

More studies are necessary to explore the possibility of targeting miRNA-193a-3p/−5p for NSCLC therapy and to develop feasible miRNA delivery systems.

During miRNA biogenesis, the same pre-miRNA may generate two miRNA species from the 5′ and 3′ arms. In traditional thought, one miRNA species remains while the other one is degraded. However, more researches have proved the coexistence of two mature miRNA species in cells, albeit in different concentrations. Despite the widespread evidence for the consistency of expression of miRNA-3p/−5p pairs, the reason for this co-expression is not fully understood. However, most studies about miRNAs to date focused on the regulation of a specific gene by a specific miRNA, and the overall cellular functions affected by co-expressed miRNA-3p/−5p pairs remain unknown. Considering the current incomplete understanding of miRNA-3p/−5p pairs and their common targets in cancer, more studies on the biological roles of miRNA-3p/−5p pairs are urgently needed. In the present study, bioinformatic analysis of EGFR 3′-UTR identified two non-overlapping binding sites for miRNA-193a-3p/−5p. In vitro and in vivo data supported the hypothesis of co-targeting of EGFR by miRNA-193a-3p/−5p. Because there are currently very few reports of miRNA-3p/−5p pairs targeting the same genes [10, 11, 30], this study offers new evidence that miRNA-3p/−5p can simultaneously repress a same gene. Although we did not observe a cooperative and synergistic effect of miRNA-193a-3p/−5p on EGFR repression and identify more efficient and potent co-regulation of target gene by miRNA-3p/−5p pairs than by a single miRNA, we propose that miRNA-193a-3p/−5p may work in concert to provide a fail-proof mode to make sure that when a miRNA-3p/miRNA-5p species is not functional, the other one is still functional. In summary, our results suggest the importance of further elucidating possible cooperation of co-expressed miRNA-3p/−5p pairs in cancer pathogenesis.

In summary, this study demonstrates that miRNA-193a-3p/−5p possess tumor-suppressive ability and could inhibit NSCLC carcinogenesis through down-regulating EGFR. Co-downregulation of miRNA-193a-3p/−5p may be a reason for the dysregulation of EGFR function in lung cancer. In future, miRNA-193a-3p/−5p may provide a strategy towards EGFR-targeted lung cancer therapy, especially for those patients with drug resistance.

Conclusion

This study highlights an important role for co-expressed miR-193a-3p/−5p in the regulation of EGFR in lung cancer cells. miR-193a-3p/−5p may act as novel anti-cancer drug that have clinical transformation values in the EGFR-targeted therapy.

Materials and methods

Human tissues

Paired NSCLC and NAT tissue samples were derived from the Nanjing Drum Tower Hospital Affiliated to Medical School of Nanjing University (Nanjing, China). All patients involved signed an informed consent form. This study was approved by the Ethics Committee of Nanjing University. Additional file 1: Table S1 summarized the patient information.

Cell lines and reagents

A549 and H1975 lung cancer cell lines were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Anti-EGFR antibody was purchased from R&D systems (USA, 1:1000). Anti-GAPDH antibody was purchased from Santa Cruz Biotechnology (CA, USA, 1:2000).

miRNA target prediction

The co-targets of the miRNA-193a-3p/−5p pairs were predicted using the RNAhybrid algorithm [12]. First, only the putative targets shared between miRNA-193a-3p/−5p were selected. Second, hybrid energy should be lower than − 25 kcal/mol. Third, there should be interspecies conservation within the miRNA binding sites. Based on these rules, approximately 100 genes were identified as candidate co-targets of miRNA-193a-3p/−5p pairs.

Quantitative RT-PCR (qRT-PCR) assay

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. miRNA detection was performed with Taqman probes (Applied Biosystems) according to the manufacturer's instructions. U6 snRNA served as the internal control of miRNAs because U6 is a common reference gene in analyzing miRNAs with qRT-PCR assays [31–33]. The relative amount of miRNA expression was calculated with the eq. $2^{-\Delta\Delta CT}$, in which $\Delta\Delta C_T = (C_{T\ miRNA} - C_{T\ U6})_{test\ condition} - (C_{T\ miRNA} - C_{T\ U6})_{control\ condition}$. EGFR mRNA expression levels were determined using the SYBR Green (Invitrogen) method and were normalized to GAPDH. Primers were as follows: 5′-CGAGCCACA TCGCTCAGACA-3′ (GAPDH, sense); 5′-GTGGTGA AGACGCCAGTGGA-3′ (GAPDH, antisense); 5′-TTG CCGCAAAGTGTGTAACG-3′ (EGFR, sense); 5′-GT CACCCCTAAATGCCACCG-3′ (EGFR, antisense).

Cell transfection

EGFR siRNAs and miRNA mimics/antisenses were synthesized by Genepharma (Shanghai, China). Cells (in 6-well plate) were transfected with Lipofectamine 2000 (Invitrogen) when they grew to 70% confluence. For each well, 100 pmol of mimics, antisenses or siRNAs were used. For co-transfection of miRNA-193a-3p/−5p mimics or antisenses, 50 pmol of each miRNA were used. After 48 h, cells were harvested for western blotting and qRT-PCR analysis. SiRNA sequences were: EGFR siRNA-1: 5′-GCAGUCUUAUCUAACUAUG AUGCAA-3′; EGFR siRNA-2: 5′-GCAGUGACU UUCUCAGCAA-3′.

Cell infection

Scrambled lentivirus and lentivirus overexpressing miRNA-193a-3p or miRNA-193a-5p were constructed by Realgene (Nanjing, China). Lentivirus was added to H1975 and A549 cells at an MOI of 3. Cells were harvested for further investigation 48 h post-infection.

Luciferase assay

A 501-bp fragment of the 3'UTR of EGFR Transcript Variant 4, containing the predicted miRNA-193a-3p/−5p binding sites, was amplified and inserted into a p-MIR-report plasmid (Ambion, Austin, TX, USA). Mutant 3'UTR of EGFR Transcript Variant 4 was synthesized and inserted into the same plasmid. The mutant sequences were 396 to 399 for miRNA-193a-3p and 225 to 229 for miRNA-193a-5p. For luciferase assays, 40 pmol of miRNA mimics or scrambled mimics were transfected into 12-well plates along with 0.8 μg of reporter plasmid and 0.8 μg of transfection control β-galactosidase plasmid (Ambion). Luciferase activity was measured with a kit (Promega, Madison, WI, USA) 24 h post-transfection.

Cell proliferation assay

The cell proliferation ability is verified by CCK-8 assays. For each well of 96-well plates, 5×10^3 cells were seeded. Cell proliferation index was assessed at 12, 24, 36, 48, 60 and 72 h.

Cell migration assay

In migration assay, an 8.0 μm Pore Polycarbonate Membranes (Corning, New York, USA) was placed in 24-wells. Then 3×10^4 cells in 100 μl serum-free medium were added to the upper chamber, and 10% FBS-containing medium was added to the lower chamber. Cells were incubated for another 12 h for A549 cells or 24 h for H1975 cells at 37 °C. Cells that migrated from the upper chamber to the bottom of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and visualized under a microscope.

Establishment of tumor xenografts in nude mice

Nude mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All animal care and handling procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. H1975 cells were infected with control lentivirus (LV-scramble) or lentiviruses to express miRNA-193a-3p or miRNA-193a-5p, and 5×10^6 H1975 cells were injected subcutaneously into the mice (3 groups, 10 mice/group). Tumor widths and lengths were measured every 2 days. Then the mice were sacrificed and the xenograft tumors were photographed and weighed. Total RNA and protein were isolated from the tumors for further analysis, and the xenograft tumors were fixed in 4% paraformaldehyde followed by immunohistochemical staining of Ki-67 and PCNA. Images were obtained using the microscope at × 400 magnification (Olympus, Japan).

Statistical analyses

Each assay was repeated independently for at least three times. Data were presented as the mean ± SE. The differences were considered statistically significant at $P < 0.05$ (Student's t-test). * indicates $P < 0.05$; ** indicates $P < 0.01$.

Additional file

Additional file 1: Figure S1. The expression levels of miR-193a-3p and -5p in A549 and H1975 cells after transfection or infection. **Table S1.** Clinical features of lung cancer patients. (DOCX 202 kb)

Abbreviations

3′-UTR: 3′-untranslated region; ALK: Anaplastic lymphoma kinase; EGFR: Epidermal growth factor receptor; KRAS: the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; miRNAs: microRNAs; NSCLC: Non-small cell lung cancer; TKIs: Tyrosine kinase inhibitors

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional file 1.

Authors' contributions

XC and CYZ formulated the hypothesis, commented on this study and edited the manuscript. KZ and HD performed the experiments and AY, ZL, ZF, YH offered some assistance. KZ did data analysis and XW offered some assistance. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nanjing University. All animal care and handling procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Review Board of Nanjing University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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