miR-208a-3p promotes NSCLC proliferation and migration by suppressing lncRNA-MEG3 expression

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Abstract

Background The disordered expression of maternally expressed gene 3 (MEG3) has been observed in non-small-cell lung cancer (NSCLC). However, the molecular mechanism accounting for this abnormal expression is not fully understood.

Methods MEG3 expression was detected by qRT-PCR in 51 cases of NSCLC and adjacent normal tissues. Then, the relationship between MEG3 and miR-208a-3p was assessed in vitro by cell viability assay, cell migration assay, protein extraction and western blot analysis.

Resoults We observed that MEG3 expression was decreased in NSCLC tissues. And MEG3 expression was negatively related to lymph node metastasis and differentiation. Moreover, MEG3 expression is regulated by miR-208a-3p expression by overexpression and knockout experiments. Furthermore, we focused on the underlying mechanism of MEG3 downregulation. We found that the overexpression of miR-208a-3p reduced the level of MEG3 expression based on computational predictions and in vitro assays. Using CCK-8 and transwell migration assays, we found that the overexpression of miR-208a-3p can increased proliferation and apoptosis in NSCLC cells. Moreover, the depletion of MEG3 rescued the proliferation and migration induced by *miR-208a-3p* knockdown.

Conclusion Taken together, the results of this study reveal that miR-208a-3p promotes NSCLC tumorigenesis by negatively regulating MEG3 expression and functions as an oncogenic miRNA in NSCLC.

Introduction

Lung cancer is the most frequently diagnosed malignancy and the leading cause of cancer-related death worldwide. A total of 2,206,771 new lung cancer cases and 1,796,144 deaths were estimated globally in 2020[1], with non-small-cell lung cancer (NSCLC) and small-cell lung cancer accounting for 85% and 15% of the incidences, respectively. Recent advances in immunotherapy and targeted therapy are promising, but the prognosis of advanced NSCLC patients remains unsatisfactory[2]. Thus, a better understanding of the complicated molecular mechanisms and the identification of novel targets of this disease may help develop effective strategies to prolong the survival of patients.

Recently, the human genome project identified that more than 90% of the transcriptome is actively transcribed into noncoding RNAs, including microRNAs and long noncoding RNAs (lncRNAs)[3]. LncRNAs have attracted increasing interest and have been proven to be involved in a wide range of cellular processes, such as cell proliferation, differentiation, development, invasion, migration and apoptosis[4]. For example, HOTAIR is upregulated in primary breast cancer, and its expression level is a powerful predictor of eventual metastasis and death[5]. Nkerorema Djodji Damas et al demonstrated that the lnc-*SNHG5* could interact with SPATS2 and impair STAU1-mediated mRNA destabilization in colorectal cancer cells[6]. Lnc-*SPRY4-IT1* is significantly upregulated in lung adenocarcinoma tissues and promotes

tumor migration and invasion[7]. MALAT1 promotes aggressive renal cell carcinoma through Ezh2 and interacts with miR-205[8].

Maternally expressed gene 3 (MEG3), an imprinted gene located within chromosome 14q32, is normally expressed in many normal human tissues[9], while the disordered expression of MEG3 has been demonstrated in various tumor types and tumor cell lines, such as hepatocellular cancer[10], cervical carcinoma[11], meningioma[12], nasopharyngeal carcinoma[13] and NSCLC[14]. Furthermore, Lu KH et al investigated a significant association between MEG3 downregulation and advanced pathological stage and tumor size in NSCLC and found that MEG3 could inhibit the proliferation of and induce the apoptosis of NSCLC cells[15]. Deletion of the Meg3 gene in vivo could promote angiogenesis and result in perinatal death in mice[16]. These data indicate that MEG3 functions as a potential tumor suppressor. However, the underlying molecular mechanisms contributing to this dysregulated expression are not entirely known.

MicroRNAs (miRNAs) are small noncoding RNAs approximately 22 nucleotides in length that can negatively control protein-coding genes at the posttranslational level[17], and many other studies have shown that they are also involved in the regulation of lncRNA expression[18]. miR-208a has been reported as an onco-miRNA in diverse cancers[19, 20]. A previous assay revealed that miR-208a can increase cell growth and inhibit cell apoptosis in lung cancer in vitro[21]. Nevertheless, whether it can regulate the function of lncRNA-MEG3 is still unknown.

Therefore, we revealed that miR-208a-3p was upregulated and MEG3 was downregulated in NSCLC tumor tissues compared with matched tissues in this study. We also found that elevated miR-208a-3p could target MEG3 and repress its expression in NSCLC tumor cell lines, and MEG3 exerts its biological functions through the P53 pathway.

Methods

Clinical samples

A total of 51 NSCLC tissues and pair-matched normal adjacent lung tissues were collected from surgically removed specimens of NSCLC patients at Nanjing Hospital Affiliated of Nanjing Medical University between 2012 and 2015. None of the patients had received radiotherapy or chemotherapy before surgery. **The clinical characteristics of the patients are listed in Table 1.** Specimens were snap-frozen in liquid nitrogen and stored at -80°C immediately after resection. This protocol was approved by the Research Ethics Committee of Nanjing Hospital Affiliated of Nanjing Medical University (Nanjing, China), and informed consent was obtained from all patients before the study was initiated.

Cell lines and culture

The normal human bronchial epithelial cell line (16HBE) and NSCLC cell lines (PC9 and H1299) were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). 16HBE, H1975 and H1299 cells

were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from tissues and cultured cells with TRIzol reagent (Invitrogen, Carlsbad, USA). The extracted RNA (1 μg) was reverse transcribed into complementary DNA (cDNA) using a Reverse Transcription Kit (GeneCopoeia, Guangzhou, China) and a miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia for mRNA and miRNA, respectively. RT-qPCR was carried out in triplicate with an All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia,) on a LightCycler@ 96 PCR System (Roche, Switzerland). U6 small nuclear RNA (RNU6) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls for mRNA and miRNA, respectively. Comparative quantification was calculated using the method of 2-ΔΔCT. **The primer sequences are listed in Table 2.**

Cell transfection

H1299 and PC9 cells were selected for transfection. miRNA mimics and the miRNA inhibitor were purchased from Genepharma Company (Shanghai, China). Cells were seeded into 6-well plates and grown to 70% cell confluence on the day of transfection. shMEG3 was synthesized by Invitrogen (Nanjing, China). **Sequences are listed in Table 3.** Plasmid transfection was carried out with X-tremeGENE HP DNA Transfection Reagent (Roche, Germany), and oligonucleotide transfection was conducted using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) to maintain the final concentration of 150 nM following the manufacturer's protocols. Transfected cells were harvested 24 h posttransfection and then used for further experiments.

Cell viability assay

In vitro cell proliferation was monitored by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). In brief, cells were plated into 96-well culture plates at a density of 2×10^3 cells/well 24 h after transfection with the miR-208a mimics, miR-208a inhibitor, miR-208a inhibitor+ shMEG3, or matched transfected controls. Then, 10 μl of CCK-8 solution was added to each well and incubated for 4 h. Cell vitality was measured spectrophotometrically at 450 nm every 24 h following the manufacturer's protocol. Each experiment was performed in quintuplicate and repeated three times.

Cell migration assay

Cell migration assays were carried out using transwell inserts with a pore size of 8 μm (Corning, VA, USA). Twenty-four hours after transfection, 4×10^4 cells were resuspended in 300 μl of RPMI-1640 medium (H1299) or DMEM (PC9) containing 2% FBS and seeded into the top chamber of the transwell, and medium containing 10% FBS was added to the bottom chamber as a chemoattractant. Following 24 h of incubation, cells remaining on the upper membrane surface were wiped off using cotton wool, and

the penetrated cells that had invaded into the filter surface were fixed with methanol, stained with 0.2% crystal violet solution and photographed. Migration was assessed by counting the number of stained cells.

Protein extraction and Western blot analysis

Protein samples were extracted using RIPA buffer containing complete protease inhibitor cocktail (Cell Signaling Technology). The protein concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). Protein lysates were quantified and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% defatted milk/Tris-buffered saline (20 mM Tris-HCl, pH=7.4 and 150 mM NaCl supplemented with 0.1% Tween-20; Tris-buffered saline supplemented with Tween-20, TBST) at room temperature for 1 h and incubated with anti-human P53 and GAPDH antibodies at 4°C overnight. On the next day, the membranes were washed with PBS-Tween-20 (PBS-T) three times and further incubated with horseradish peroxidase-conjugated anti-rabbit IgG at room temperature for 1 h. After washing, the signal was visualized through a chemiluminescent detection system (Pierce ECL Substrate Western Blot Detection System, Thermo, Rockford, IL, USA) and then exposed in a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). All the antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). GAPDH served as the internal standard.

Statistical analysis

Data analysis was performed using GraphPad Prism 9 software. The results are presented as the mean ± standard deviation (SD) based on at least three repeats. The statistical significance of the results between each group was determined using Student's *t*-test or one-way analysis of variance (ANOVA). Differences were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

MEG3 expression is inversely correlated with miR-208a-3p levels in NSCLC tissues and cell lines.

First, we performed qRT-PCR to analyze miR-208a-3p and MEG3 levels in 51 cases of NSCLC cancer tissues and matched adjacent normal tissues. As shown in Fig. 1A and 1B, miR-208a-3p was noticeably upregulated and MEG3 was downregulated in cancer tissues compared with their adjacent normal counterparts. Then, the expression of miR-208a-3p and MEG3 was evaluated in NSCLC cell lines (PC9, H1299 and H1975). It was also found that miR-208a-3p expression was higher and MEG3 expression was lower in NSCLC cells than in 16HBE cells (Fig. 1C and 1D). Subsequent regression analysis showed that MEG3 expression was inversely correlated with miR-208a-3p levels in NSCLC tissues (Fig. 1E).

Next, we explored the relationship between MEG3 expression and the clinicopathological features of NSCLC. As shown in Table 1, MEG3 expression levels were positively correlated with differentiation and lymph node metastasis. However, there were no relationships between MEG3 expression and other parameters, such as age, sex, histological type or cancer location. These data indicate that the negative correlation between miR-208a-3p and MEG3 may be implicated in the progression of NSCLC.

MEG3 is negatively regulated by miR-208a-3p.

As we found that miR-208a-3p functions as the predicted target miRNA of MEG3 using bioinformatics *(miRANDA)*, we next focused on studying the role of miR-208a-3p in regulating MEG3. First, we assessed changes in MEG3 expression after the overexpression or knockdown of miR-208a-3p. Fig. 2A and 2B show that the transfection efficiency in PC9 and H1299 cells was estimated by fluorescein (FAM) fluorescence microscopy. The results showed that the miR-208a-3p expression level was significantly decreased and the MEG3 expression level was increased in cells transfected with miR-208a-3p mimics compared with control cells (Fig. 2C and 2E). In contrast, the knockdown of miR-208a-3p resulted in a significant upregulation of MEG3 expression in PC9 and H1299 cells (Fig. 2D and 2F). Interestingly, as shown in Fig. 2G and 2H, MEG3 overexpression or knockdown had no significant effect on the expression of miR-208a-3p. Based on computational predictions and in vitro assays, we found that miR-208a-3p inversely regulated MEG3 expression, but they may not form a reciprocal repression feedback loop in PC9 and H1299 cells.

miR-208a-3p promotes NSCLC cell proliferation and migration by negatively regulating MEG3.

The gain-of-function and loss-of-function analyses in PC-9 and H1299 cells were conducted as models of NSCLC. Cell proliferation was promoted in cells treated with the miR-208a-3p mimics compared with control cells, whereas cell proliferation was inhibited when cells were treated with the miR-208a-3p inhibitor (Fig. 3A,3B).

Transwell migration assays also showed that the number of tumor cells migrating from the chamber after treatment with the miR-208a-3p mimics was significantly greater than that in the control group. The number of tumor cells migrating from the chamber after treatment with the miR-208a-3p inhibitor was substantially less than that after treatment with inhibitor NC (negative control), demonstrating that miR-208a-3p promoted NSCLC cell migration in PC9 and H1299 cells (Fig. 3C, 3D).

Furthmore, we cotransfected the miR-208a-3p inhibitor with the shMEG3 plasmid into H1299 and PC-9 cells. The results showed that the expression of MEG3 was highly promoted after transfection with the miR-208a-3p inhibitor or after cotransfection with the miR-208a-3p inhibitor and ctrl-MEG3, but it was knocked down after cotransfection with the miR-208a-3p inhibitor and shMEG3 (Fig.4A). The growth of both H1299 and PC9 cells was inhibited after transfection with the miR-208a-3p inhibitor or after cotransfection with the miR-208a-3p inhibitor and ctrl-MEG3 compared to after transfection with inhibitor NC, but this inhibitory effect was partly restored after cotransfection with miR-208a-3p inhibitor and shMEG3 (Fig. 4B, 4C). Similar results were observed in migration assays. The migrated cells were

significantly reduced with the miR-208a-3p inhibitor or upon cotransfection with miR-208a-3p inhibitor and ctrl-MEG3; this inhibitory effect of the miR-208a-3p inhibitor was partially reversed by shMEG3 again Fig. 4D, 4E.

These results suggest that the downregulation of MEG3 induced by the upregulation of miR-208a-3p could promote the proliferation and migration of NSCLC cells.

miR-208a-3p-induced MEG3 downregulation promotes NSCLC progression by targeting p53.

Since Lu KH et al demonstrated that MEG3 can suppress proliferation and promote migration by upregulating p53 expression in NSCLC cells [16], we further investigated whether miR-208a-3p could promote cell progression by regulating p53.

Western blot analysis showed that H1299 cells did not express p53 protein when transfected with inhibitor NC, and p53 was expressed when transfected with miR-208a-3p inhibitor alone or in combination with ctrl-MEG3. However, upon cotransfection with miR-208a-3p inhibitor and shMEG3, p53 protein was lost again. PC9 cells showed similar results (Fig.5). In summary, miR-208a-3p downregulated MEG3 and promoted NSCLC cell proliferation and migration by targeting p53.

miR-208a-3p regulates MEG3 and promotes NSCLC progression by targeting p53 in vivo.

Subsequently, we evaluated the biological effects of miR-208a-3p and MEG3 on the progression of NSCLC cells in a NSCLC xenograft mouse model. We found a significant size reduction in the size and weight of the tumors in the group with miR-208a-3p-downregulation, but the inhibitory effect was eliminated when the miR-208a-3p-downexpression vector was cotransfected with shMEG3. Additionally, MEG3 and p53 overexpression attenuated the progressive effect of miR-208a-3p (Fig. 6).

Discussion

An increasing number of studies have shown that MEG3 acts as a tumor suppressing lncRNA and has been found to be substantially decreased in multiple human cancers, such as neuroblastomas, hepatocellular cancers[22], gastric cancer[23] and gliomas[24], and its loss of expression has also been found in cancer cells from the breast, liver, and meninges. In our research, we found that MEG3 was downregulated in 51 pairs of NSCLC lung cancer tissues and cell lines compared with adjacent tissues and normal cell lines, consistent with the results from Lu KH et al, whose reports demonstrated that MEG3 acts as a tumor suppressor[15]. Previous studies have identified that MEG3 levels were associated with advanced pathologic stage and tumor size, and patients with lower MEG3 expression had a worse prognosis. Similarly, MEG3 expression was correlated with differentiation and lymph node metastasis in our study.

Multiple mechanisms contribute to the downregulation of MEG3 expression in tumors according to numerous publications reported, including gene deletion, promoter

hypermethylation, and hypermethylation of the intergenic differentially methylated region. However, whether there is any other mechanism responsible for its downregulation is not well known. Recently, an increasing number of studies have shown potential interactions between lncRNAs and miRNAs. On the one hand, some lncRNAs can act as endogenous sponges or decay miRNAs[25], some give rise to specific miRNAs. On the other hand, several miRNAs have been reported to inhibit the expression of lncRNAs by binding to specific sites[26]. Bioinformatics analysis was used, and we hypothesized that miR-208a-3p, which has been reported to be highly expressed in several malignancies and promotes proliferation in NSCLC cell lines, may serve as a regulatory miRNA. We next explored the interplay between miR-208a-3p and MEG3 in NSCLC tissues. We found that miR-208a-3p could negatively regulate MEG3 expression in NSCLC tissues and cell lines. Data from human tissues strongly support a significant inverse correlation between these two noncoding RNAs. After the knockdown of MEG3 with the miR-208a-3p mimic, cell proliferation and migration were significantly increased in NSCLC cells. However, the upregulation of MEG3 expression by the miR-208a-3p inhibitor could contribute to the decreased proliferation and migration abilities in NSCLC cells.

Further studies showed that the MEG3 overexpression plasmid could rescue the increased proliferation and migration abilities, and the MEG3 knockdown plasmid attenuated the decreased proliferation and migration in PC9 and H1299 cell lines. These findings provide additional evidence for such a regulatory network. Interestingly, MEG3 overexpression or knockdown had no significant effect on the expression level of miR-208a-3p, indicating that miR-208a-3p may negatively regulate the expression of MEG3, but they may not form a reciprocal repression feedback loop. Here, we report for the first time that MEG3 downregulated by miR-208a-3p can promote NSCLC cell proliferation and migration. However, the exact molecular mechanisms of the interaction between MEG3 and miR-208a-3p require further study.

As a tumor suppressor, MEG3 may play a role through several different mechanisms. It could downregulate the level of MDM2 an E3 ubiquitin ligase, which can promote the degradation of p53 protein by a ubiquitin-proteasome pathway. MEG3 can also enhance the binding of p53 to the promoter of GDF15, an inhibitor of cell proliferation[27]. Furthermore, MEG3 could suppress the expression of TGF-β-induced EMT-related genes, including CDH1 and the ZEB and miR-200 families, by means of recruiting JARID2 and EZH2, and histone H3K27 methylation on the regulatory regions for transcriptional repression indicates that MEG3 may be associated with the epigenetic regulation of the EMT process of NSCLC cells [(15)]. Gordon *et al.* reported that in embryonic brains of MEG3 KO mice, several genes involved in the angiogenesis pathway, including vascular endothelial growth factor alpha (VEGFA) and its type I receptor (VEFGR1), were upregulated, and the formation of cortical microvessels was significantly increased[28]. Moreover, recent research has also documented that MEG3 plays a critical role in the cisplatin resistance of human lung adenocarcinoma[29]. In our study, we found that P53 protein was dramatically increased in cells treated with the miR-208a-3p inhibitor or the miR-208a-3p inhibitor +shMEG3. This result indicates that miR-208a-3p may act as an oncogene, at least partially through silencing MEG3 and promoting P53 protein levels. However, more experiments are needed to elucidate

whether any other molecular mechanisms among miR-208a-3p, MEG3 and p53 are involved in the progression of NSCLC cells.

Conclusions

In conclusion, we identified the lncRNA MEG3 as a novel target of miR-208a-3p, and this negative regulation showed a significant impact on P53 protein expression, which contributed to the carcinogenesis of NSCLC. As a result, this posttranscriptional regulation has a significant impact on the proliferation, invasion, and metastasis of NSCLC cells. Our results highlight a new interaction between miRNAs and the lncRNA MEG3 and support that miRNAs are involved in the regulation of lncRNAs.

Abbreviations

LncRNAs:long noncoding RNAs;MEG3:maternally expressed gene 3;NC:negative control;NSCLC: non-small-cell lung cancer;SDS-PAGE:sodium dodecyl sulfate-polyacrylamide gel electrophoresis;SD:standard deviation; VEGFA:vascular endothelial growth factor alpha (VEGFA);VEGFR1:vascular endothelial growth factor type I receptor.

Declarations

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Authors' contributions

LY and QL contributed equally to this work. WG and YQ designed the research; LY, QL, HZ, SF, WG and YQ performed the research; LY and QL analyzed the data, and wrote the paper; SF, WG, and YQ is the supervision.

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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 supplementary information files.

Ethics approval and consent to participate

We have supplied a statement that said that a local ethics committee ruled that no formal ethics approval was required in this particular case.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F: **Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries**. *CA: a cancer journal for clinicians*2021, **71**(3):209-249.

2. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2019**. *CA: a cancer journal for clinicians*2019, **69**(1):7-34.

3. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F*et al*: **Landscape of transcription in human cells**. *Nature*2012, **489**(7414):101-108.

4. Ali T, Grote P: **Beyond the RNA-dependent function of LncRNA genes**. *eLife*2020, **9**.

5. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL*et al*: **Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis**. *Nature*2010, **464**(7291):1071-1076.

6. Damas ND, Marcatti M, Côme C, Christensen LL, Nielsen MM, Baumgartner R, Gylling HM, Maglieri G, Rundsten CF, Seemann SE*et al*: **SNHG5 promotes colorectal cancer cell survival by counteracting STAU1-mediated mRNA destabilization**. *Nature communications*2016, **7**:13875.

7. Zhang X, Chi Q, Zhao Z: **Up-regulation of long non-coding RNA SPRY4-IT1 promotes tumor cell migration and invasion in lung adenocarcinoma**. *Oncotarget*2017, **8**(31):51058-51065.

8. Hirata H, Hinoda Y, Shahryari V, Deng G, Nakajima K, Tabatabai ZL, Ishii N, Dahiya R: **Long Noncoding RNA MALAT1 Promotes Aggressive Renal Cell Carcinoma through Ezh2 and Interacts with miR-205**. *Cancer Res*2015, **75**(7):1322-1331.

9. Miyoshi N, Wagatsuma H, Wakana S, Shiroishi T, Nomura M, Aisaka K, Kohda T, Surani MA, Kaneko-Ishino T, Ishino F: **Identification of an imprinted gene, Meg3/Gtl2 and its human homologue MEG3, first mapped on mouse distal chromosome 12 and human chromosome 14q**. *Genes to cells : devoted to molecular & cellular mechanisms*2000, **5**(3):211-220.

10. Braconi C, Kogure T, Valeri N, Huang N, Nuovo G, Costinean S, Negrini M, Miotto E, Croce CM, Patel T: **microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer**. *Oncogene*2011, **30**(47):4750-4756.

11. Zhang J, Lin Z, Gao Y, Yao T: **Downregulation of long noncoding RNA MEG3 is associated with poor prognosis and promoter hypermethylation in cervical cancer**. *Journal of experimental & clinical cancer research : CR*2017, **36**(1):5.

12. Zhang X, Gejman R, Mahta A, Zhong Y, Rice KA, Zhou Y, Cheunsuchon P, Louis DN, Klibanski A: **Maternally expressed gene 3, an imprinted noncoding RNA gene, is associated with meningioma pathogenesis and progression**. *Cancer Res*2010, **70**(6):2350-2358.

13. Chak WP, Lung RW, Tong JH, Chan SY, Lun SW, Tsao SW, Lo KW, To KF: **Downregulation of long non-coding RNA MEG3 in nasopharyngeal carcinoma**. *Molecular carcinogenesis*2017, **56**(3):1041-1054.

14. Terashima M, Tange S, Ishimura A, Suzuki T: **MEG3 Long Noncoding RNA Contributes to the Epigenetic Regulation of Epithelial-Mesenchymal Transition in Lung Cancer Cell Lines**. *The Journal of biological chemistry*2017, **292**(1):82-99.

15. Lu KH, Li W, Liu XH, Sun M, Zhang ML, Wu WQ, Xie WP, Hou YY: **Long non-coding RNA MEG3 inhibits NSCLC cells proliferation and induces apoptosis by affecting p53 expression**. *BMC cancer*2013, **13**:461.

16. Zhou Y, Zhang X, Klibanski A: **MEG3 noncoding RNA: a tumor suppressor**. *Journal of molecular endocrinology*2012, **48**(3):R45-53.

17. Galagali H, Kim JK: **The multifaceted roles of microRNAs in differentiation**. *Current opinion in cell biology*2020, **67**:118-140.

18. Goodall GJ, Wickramasinghe VO: **RNA in cancer**. *Nature reviews Cancer*2021, **21**(1):22-36.

19. Yin K, Liu M, Zhang M, Wang F, Fen M, Liu Z, Yuan Y, Gao S, Yang L, Zhang W*et al*: **miR-208a-3p suppresses cell apoptosis by targeting PDCD4 in gastric cancer**. *Oncotarget*2016, **7**(41):67321-67332.

20. Sun X, Jiang S, Liu J, Wang H, Zhang Y, Tang SC, Wang J, Du N, Xu C, Wang C*et al*: **MiR-208a stimulates the cocktail of SOX2 and β-catenin to inhibit the let-7 induction of self-renewal repression of**

breast cancer stem cells and formed miR208a/let-7 feedback loop via LIN28 and DICER1. *Oncotarget*2015, **6**(32):32944-32954.

21. Tang Y, Cui Y, Li Z, Jiao Z, Zhang Y, He Y, Chen G, Zhou Q, Wang W, Zhou X*et al*: **Radiation-induced miR-208a increases the proliferation and radioresistance by targeting p21 in human lung cancer cells**. *Journal of experimental & clinical cancer research : CR*2016, **35**:7.

22. Zhuo H, Tang J, Lin Z, Jiang R, Zhang X, Ji J, Wang P, Sun B: **The aberrant expression of MEG3 regulated by UHRF1 predicts the prognosis of hepatocellular carcinoma**. *Molecular carcinogenesis*2016, **55**(2):209-219.

23. Peng W, Si S, Zhang Q, Li C, Zhao F, Wang F, Yu J, Ma R: **Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate gastric cancer progression**. *Journal of experimental & clinical cancer research : CR*2015, **34**(1):79.

24. Zhang X, Zhou Y, Mehta KR, Danila DC, Scolavino S, Johnson SR, Klibanski A: **A pituitary-derived MEG3 isoform functions as a growth suppressor in tumor cells**. *The Journal of clinical endocrinology and metabolism*2003, **88**(11):5119-5126.

25. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I: **A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA**. *Cell*2011, **147**(2):358-369.

26. Han Y, Liu Y, Zhang H, Wang T, Diao R, Jiang Z, Gui Y, Cai Z: **Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long non-coding RNA MALAT1**. *FEBS letters*2013, **587**(23):3875-3882.

27. Zhou Y, Zhong Y, Wang Y, Zhang X, Batista DL, Gejman R, Ansell PJ, Zhao J, Weng C, Klibanski A: **Activation of p53 by MEG3 non-coding RNA**. *The Journal of biological chemistry*2007, **282**(34):24731-24742.

28. Gordon FE, Nutt CL, Cheunsuchon P, Nakayama Y, Provencher KA, Rice KA, Zhou Y, Zhang X, Klibanski A: **Increased expression of angiogenic genes in the brains of mouse meg3-null embryos**. *Endocrinology*2010, **151**(6):2443-2452.

29. Liu J, Wan L, Lu K, Sun M, Pan X, Zhang P, Lu B, Liu G, Wang Z: **The Long Noncoding RNA MEG3 Contributes to Cisplatin Resistance of Human Lung Adenocarcinoma**. *PloS one*2015, **10**(5):e0114586.

Tables

Table 1. Correlation between MEG3 expression and clinic-pathologic characteristics.

Characteristic	Cases	Percentage (%)	*P* value
Age (years)			0.28
<60	14	27.45	
>=60	37	72.56	
Sex			0.47
Male	40	78.43	
Female	11	21.56	
Smoking			0.56
Yes	36	70.59	
No	15	29.41	
Differentiation			0.02*
Low + Middle	30	58.82	
High	21	41.18	
Lymph node metastasis			< 0.01**
Yes	37	72.55	
No	14	27.45	
TNM stage			< 0.01**
I/II	17	45.10	
III	34	54.90	

Table 2. Specific primers of target and control genes.

Name	Sequence
miR-208a forward primer	
miR-208a reverse primer	
U6 forward primer	5'-ATTGGAACGATACAGAGAAGATT-3'
U6 reverse primer	5'-GGAACGCTTCACGAATTTG-3'
MEG3 forward primer	5'-CCTCTCCATGCTGAGCTGCT-3'
MEG3 reverse primer	5'-TGTTGGTGGGATCCAGGAAA-3'
GAPDH forward primer	5'-GTCAACGGATTTGGTCTGTATT-3'
GAPDH reverse primer	5'-AGTCTTCTGGGTGGCAGTGAT-3'

Table 3. Sequences of miR-208a mimics, the miR-208a inhibitor, shMEG3 and negative controls.

Name		Sequence
miRNA-208a-5p mimics	Sense	5'-GAGCUUUUGGCCCGGGUUAUAC-3'
	Antisense	5'-AUAACCCGGGCCAAAAGCUCUU-3'
miRNA mimics NC-FAM	Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense	5'-ACGUGACACGUUCGGAGAATT-3'
miRNA-208a-3p inhibitor		5'-GUAUAACCCGGGCCAAAAGCUC-3'
miRNA inhibitor NC-FAM		5'-CAGUACUUUUGUGUAGUACAA-3'

Figures

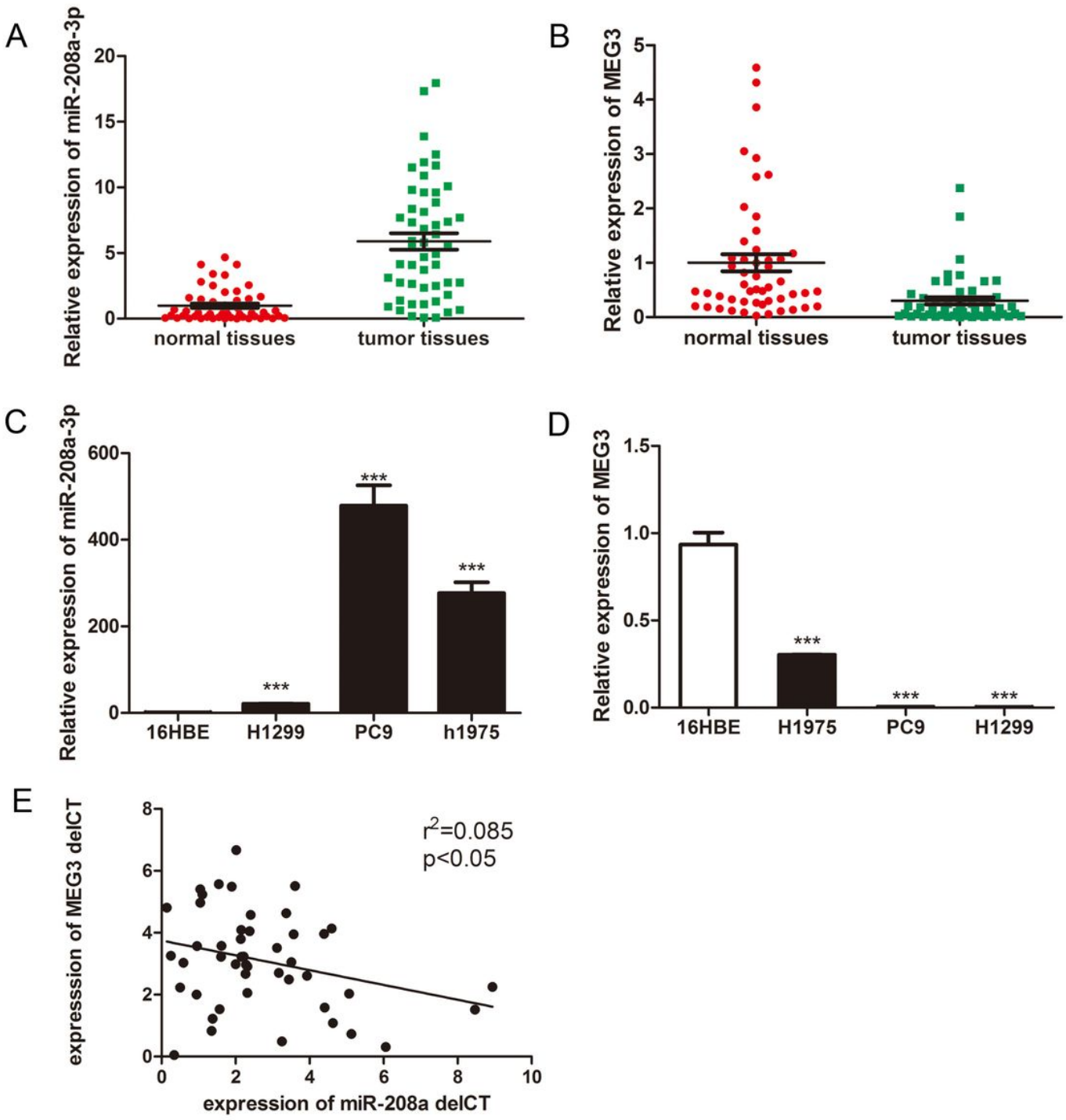

Figure 1

Expression changes in miR-208a-3p and MEG3 in NSCLC tissues and cell lines. (A) qRT-PCR was conducted to determine the expression of miR-208a-3p in 51 pairs of NSCLC tissues and adjacent normal tissues. (B) MEG3 levels were detected using qRT-PCR in 51 pairs of matched NSCLC tissues and adjacent normal tissues. (C) The expression of miR-208a-3p in NSCLC cell lines (H1975, PC9 and H1299) and in the normal bronchial epithelial cell line (16HBE) was evaluated by RT-PCR. (D) The expression of miR-208a-3p in NSCLC cell lines (H1975, PC9 and H1299) and in the normal bronchial epithelial cell line

(16HBE) was evaluated by RT-PCR. (E) Linear regression analysis of the correlation between miR-208a-3p and MEG3 in NSCLC tissues and adjacent normal tissues.

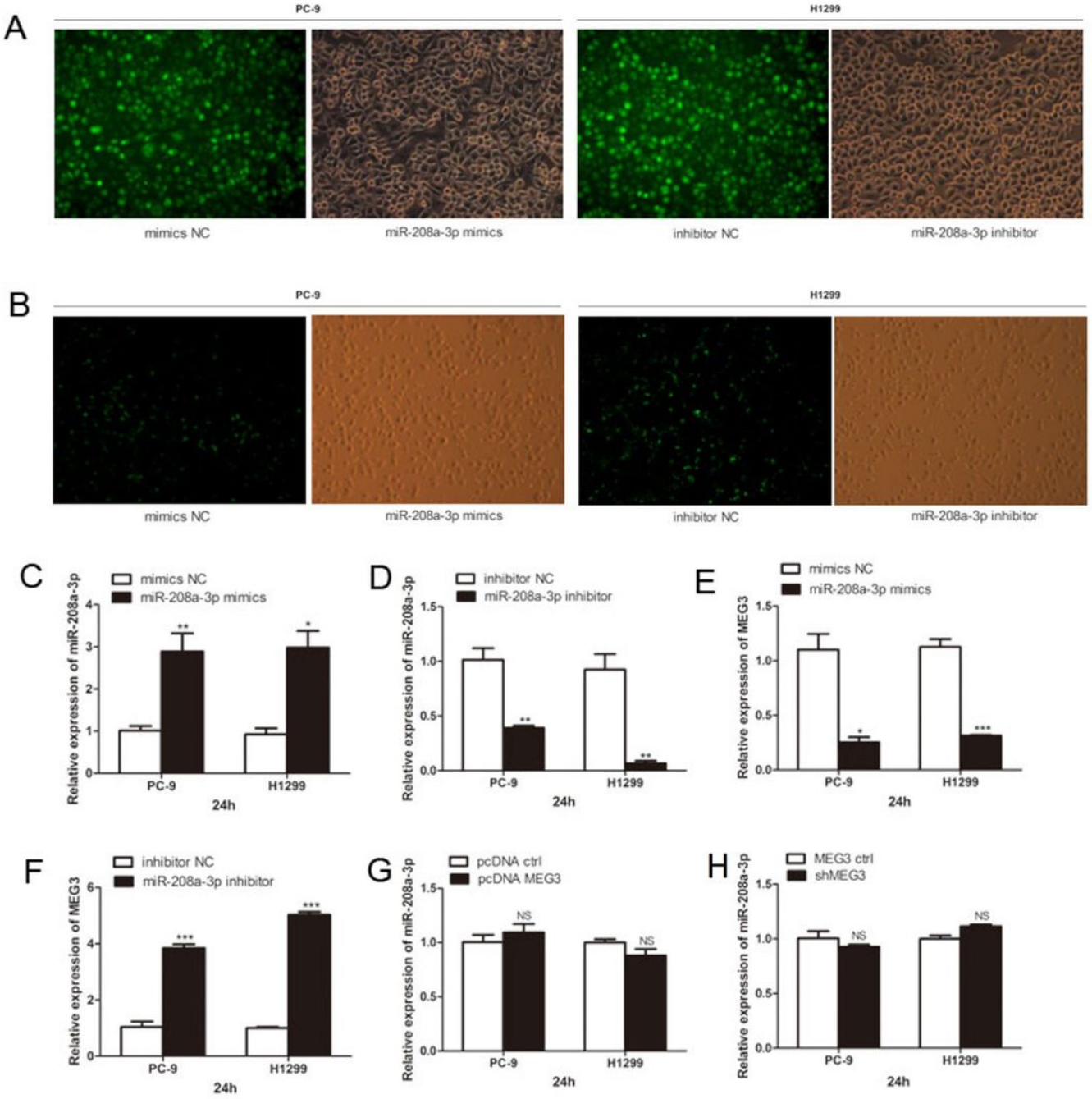

Figure 2

miR-208a-3p negatively regulated MEG3 expression. PC-9 and H1299 cells were transiently transfected with miR-208a-3p mimics, the miR-208a-3p inhibitor or a negative control (NC). (A-B) Transfection efficiency in PC-9 and H1299 cells was estimated by fluorescein (FAM) fluorescence microscopy (*100) 6 h after transfection with miR-208a-3p mimics or the miR-208a-3p inhibitor and is presented as FAM-

expressing cells and differential interference contrast images. (C-D) RT-PCR was used to investigate miR-208a-3p expression after transfection. miR-208a-3p expression was significantly increased (C) or decreased (D) in response to miR-208a-3p mimics or the miR-208a-3p inhibitor, respectively, in PC-9 and H1299 cells compared to their negative controls (mimics NC or inhibitor NC). (E-F) RT-PCR was used to investigate MEG3 expression after transfection. MEG3 expression was significantly decreased (E) or increased (F) in response to miR-208a-3p mimics or the miR-208a-3p inhibitor, respectively, in PC-9 and H1299 cells compared to their negative controls (mimics NC or inhibitor NC). (G-H) MEG3 overexpression (G) or knockdown (H) had no significant effect on the expression level of MEG3.

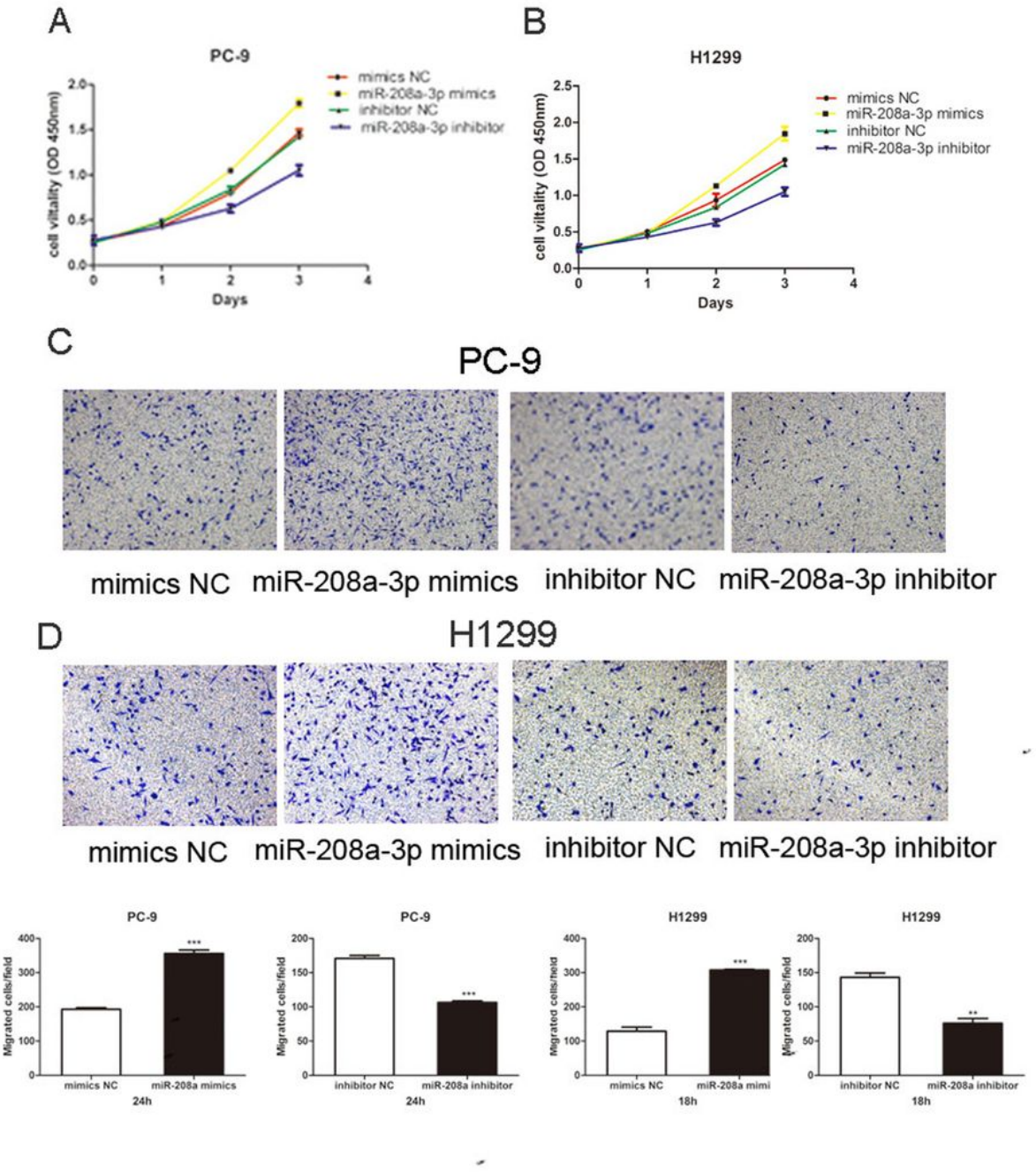

Figure 3

miR-208a-3p promoted NSCLC cell proliferation and migration by negatively regulating MEG3. (A-B) The proliferation of PC-9 (A) and H1299 (B) cells transfected with miR-208a mimics or the miR-208a-3p inhibitor was examined at 24, 48 and 72 h by CCK8 assays. (C-D) Cell migration was analyzed by a transwell chamber assay in cell lines transfected with miR-208a-3p mimics or the miR-208a-3p inhibitor.

Representative images of crystal violet-stained migrated cells were captured 24 h after invasion of PC-9 cells (C) and 18 h after invasion of H1299 cells (D) using an inverted microscope at x100 magnification.

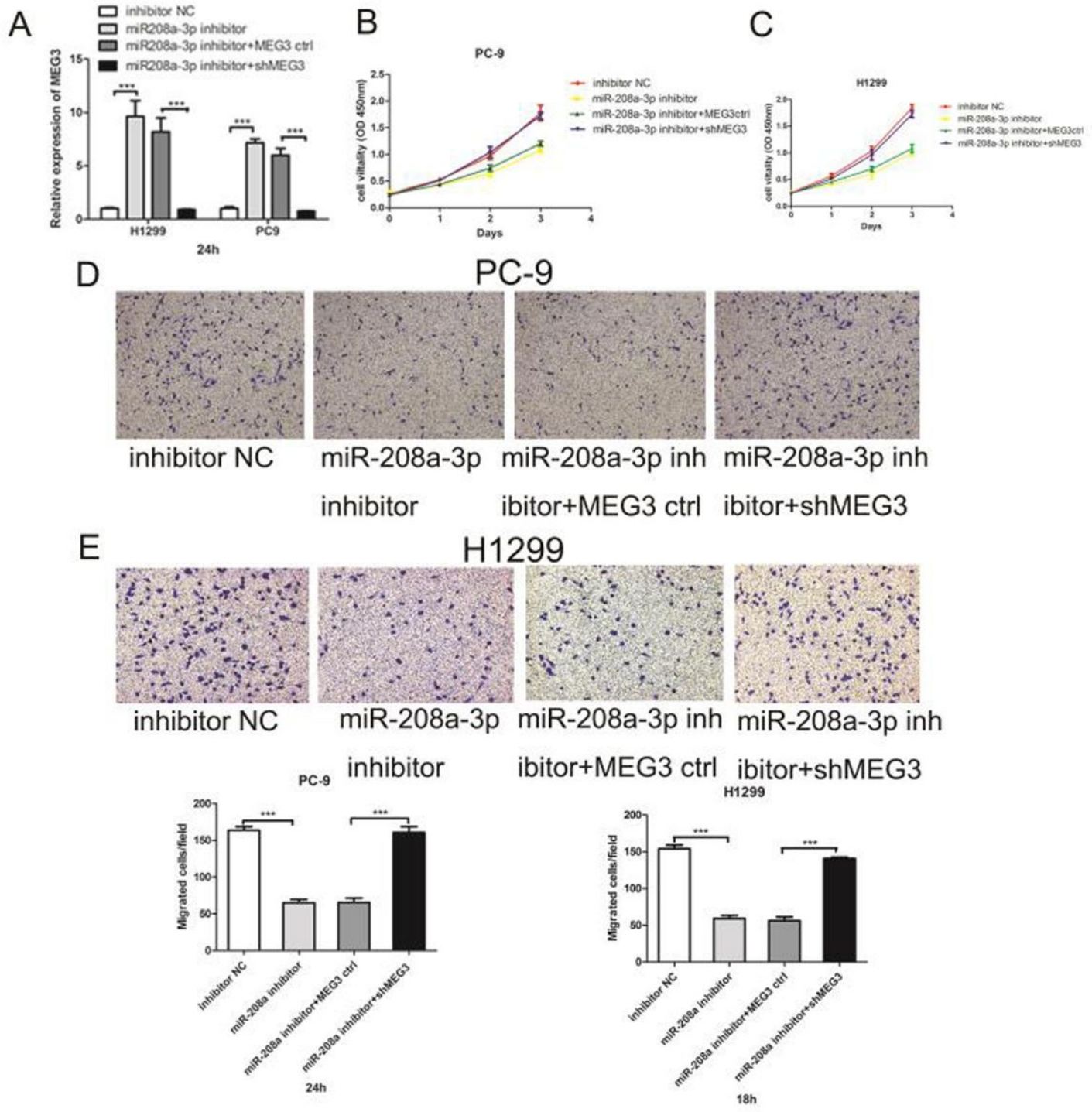

Figure 4

Downregulation of MEG3 rescued the effects of miR-208a-3p downregulation on NSCLC progression. PC-9 and H1299 cells were transfected with the miR-208a-3p inhibitor, inhibitor NC, miR-208a-3p inhibitor+MEG3 ctrl or miR-208a-3p inhibitor+shMEG3. (A) MEG3 miRNA was detected by RT-PCR 24 h after transfection in PC-9 and H1299 cells. (B-C) Cell viability at 24, 48, and 72 h was investigated by the

CCK8 assay in transfected PC-9 and H1299 cells. (C-D) Cell migration capacity was assessed by the transwell migration assay in transfected PC-9 and H1299 cells.

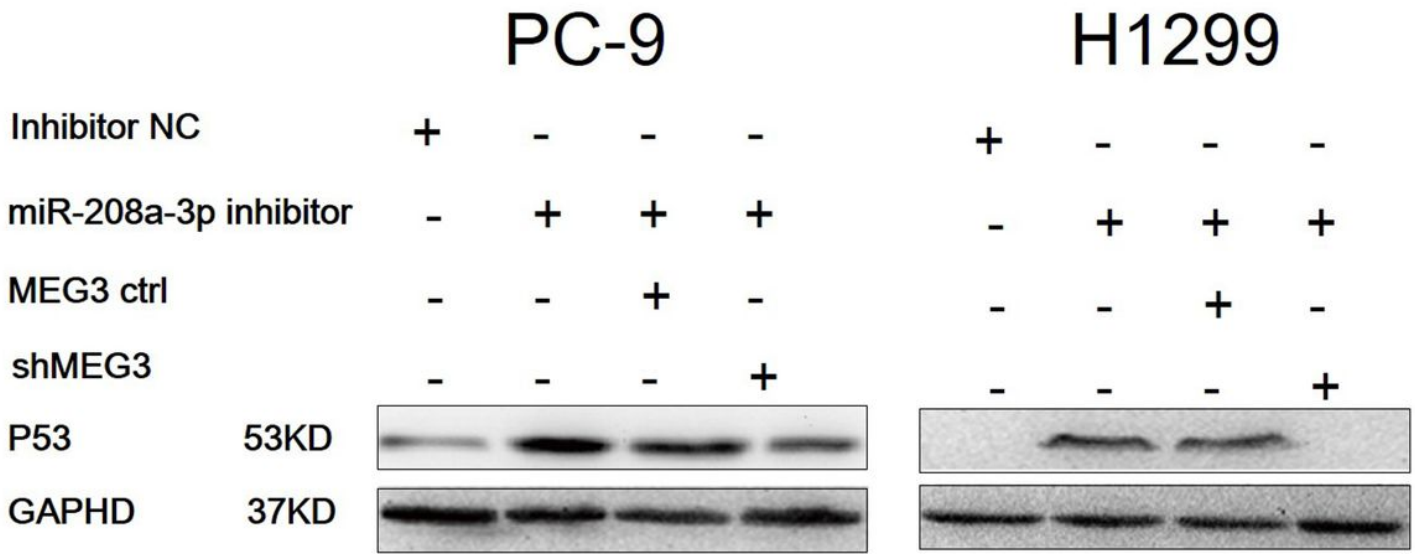

Figure 5

MEG3 affects the levels of P53 in PC-9 and H1299 cells. P53 protein expression in PC-9 and H1299 cells 48 h after transfection with the miR-208a-3p inhibitor, inhibitor NC, miR-208a-3p inhibitor+MEG3 ctrl or miR-208a-3p inhibitor+shMEG3.