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MicroRNA-29a suppresses the growth, migration, and invasion of lung adenocarcinoma cells by targeting carcinoembryonic antigen-related cell adhesion molecule 6

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ABSTRACT

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is an important regulator of cell adhesion, invasion, and metastasis. The aim of this study was to evaluate the functional roles of CEACAM6 in lung adenocarcinoma and to identify miRNAs that inhibit the growth, migration, and invasion of lung adenocarcinoma cells by targeting *CEACAM6*. CEACAM6 expression is associated with poor prognosis of patients with lung adenocarcinoma, and CEACAM6 has important functional roles in controlling the growth, migration, and invasion of lung adenocarcinoma cells in vitro and in vivo. Furthermore, miR-29a can suppress the growth, migration, and invasion of lung adenocarcinoma cells by targeting *CEACAM6*. Therefore, miR-29a/CEACAM6 axis represents a potential therapeutic target for treatment of lung adenocarcinoma.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for 80–85% of cases [1]. Despite improvements in early diagnosis and recent advances in treatment, the prognosis for patients with NSCLC remains dismal and the overall 5 year survival rate remains a disappointing 15% [2]. Although surgery is a potential curative strategy, a considerable number of patients with NSCLC are initially diagnosed with unresectable, locally advanced, or metastatic disease, and the recurrence rate is high even in early stage groups [3]. Invasion and metastasis, the hallmarks of cancer, are the primary cause of patient mortality during NSCLC progression. Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels, and metastasis refers to the spreading of cancer cells to other parts of the body. A number of cell adhesion molecules play roles as critical regulators of these processes [4].

The carcinoembryonic antigen-related cell adhesion molecule (CEACAM) protein family has been implicated in various intercellular adhesion and signaling processes that mediate cellular growth and differentiation [5,6]. CEACAM6 is a single-chain glycosylphosphoinositol-anchored immunoglobulin-like glycoprotein that mediates homotypic and heterotypic cell–cell interactions with other CEACAM family molecules through integrin receptors [6–8]. CEACAM6 is involved in cell adhesion, migration, invasion, and the metastatic behavior of tumor cells [6,9]. It is overexpressed in a wide variety of carcinomas, including NSCLC and pancreatic, colon, breast, gastric, and hepatocellular carcinomas [10–17]. For NSCLC, lung adenocarcinomas express higher levels of CEACAM6 protein than other histologic subtypes and CEACAM6 expression is associated with poor clinical outcome of the disease [10,17]; however, the molecular mechanisms that underlie its activities in lung adenocarcinoma have not yet been elucidated. Regulators of CEACAM6 expression, such as microRNAs (miRNAs), a family of endogenous small (approximately 22 nucleotides in length) non-coding and functional RNAs that regulate the expression of many genes [18], may have a profound effect on lung adenocarcinoma progression. Aberrant expression of miRNAs occurs in many types

of cancer and several miRNAs function as tumor suppressor genes or oncogenes [19].

The aim of this study was to evaluate the biological roles of CEACAM6 in lung adenocarcinoma growth, migration, and invasion in vitro and in vivo, and to identify miRNAs that inhibit these processes by targeting *CEACAM6*.

2. Materials and methods

2.1. Tissue samples

Lung adenocarcinoma tissue samples were obtained from 51 patients undergoing a curative resection at Chungbuk National University Hospital (Cheongju, South Korea) between January 2003 and December 2011. The following clinical and pathological characteristics were reviewed: age, sex, histological grading according to the World Health Organization's system, and pathologic staging according to the 7th edition of the tumor-node-metastasis cancer staging system of the American Joint Committee on Cancer [2]. This study was reviewed and approved by the Institutional Review Board of Chungbuk National University Hospital.

2.2. Tissue microarray and immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were used to construct a lung adenocarcinoma tissue microarray (TMA). Immunohistochemical (IHC) analyses of 4 μm paraffin sections of the TMA blocks were performed. Fully automated immunostaining was achieved using a BenchMark XT autostainer (Ventana Medical Systems Inc., Tucson, AZ, USA). The sections were incubated with a monoclonal mouse anti-human CEACAM6 antibody (9A6, dilution 1:4000; Santa Cruz Biotechnology Inc., Dallas, TX, USA) for 32 min, followed by Ventana Universal HRP Multimer for 8 min at 37 °C. CEACAM6 expression was scored as follows: score 0, no staining; score 1+, <10% of tumor cells expressing CEACAM6; score 2+, 10–50% of tumor cells expressing CEACAM6; and score 3+, >50% of tumor cells expressing CEACAM6. Scores of 0 and 1+ were classified as low expression of CEACAM6 and scores of 2+ and 3+ were classified as high expression.

2.3. Western blot analysis

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk and then incubated with a mouse anti-CEACAM6 monoclonal antibody (9A6, Santa Cruz Biotechnology Inc.) or a mouse anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, USA) as a loading control. The protein complexes were detected with enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

2.4. Cell proliferation assay

Cell proliferation assays were performed using the Cell Proliferation Kit II (XTT) (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. The cells were seeded onto 96-well plates at a density of 1×10^4 cells/well and incubated for the indicated number of hours. The XTT labeling mixture was prepared by mixing 50 volumes of 1 mg/ml sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate with 1 volume of 0.383 mg/ml N-methyldibenzopyrazine methyl sulfate. A 50 μl aliquot of XTT labeling mixture was subsequently added to each well and the cells were incubated for 2 h at 37 °C. Absorbance was measured at 495 and 650 nm was used as a reference wavelength.

2.5. Cell migration and invasion assays

Cell migration analyses were performed using wound healing assays. The cells were plated into low 35 mm μ-dishes containing culture inserts (Ibidi, Martinsried, Germany). The inserts were removed with sterile forceps to create a wound field of approximately 500 μm when the cells were confluent. The cells were then allowed to migrate in an incubator. At 0 and 24 h, three fields of the injury area were photographed with a light microscope at 100× magnification. For each image, the area that was uncovered by cells was determined by analysis with Image Pro Plus 4.5.1 software. For the invasion assays, 10^5 cells in serum-free medium were placed into the top chamber of each insert coated with 150 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After incubation at 37 °C for 24 h, cells adhering to the lower membrane were stained with 0.1% crystal violet in 20% methanol, imaged, and then ten different fields of each filter were manually counted at 200× magnification.

2.6. Luciferase reporter assay

Luciferase reporter assays were performed using the Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions. Transfections were performed in 48-well plates using Lipofectamine 2000 reagent (Invitrogen). At 48 h post-transfection, the culture medium was removed, the cells were washed twice with cold PBS, and then 50 μl of Passive Lysis Buffer (Promega) were added to each well. A 10 μl aliquot of the lysate was analyzed using a GloMax microplate luminometer (Promega). *Renilla* luciferase activity was measured and normalized to that of firefly luciferase, which was used as an internal control.

2.7. In vivo tumor xenograft experiments

Control cells or A549 cells that were stably transfected with the indicated *CEACAM6*-specific shRNA or miR-29a expressing vector were subcutaneously injected into the right flank of 6-week-old BALB/c athymic nude mice (seven mice per group, 9×10^6 cells/mouse). Tumor growth was determined every 2 or 3 days for 4 weeks by measuring the length (L), width (W), and height (H) with calipers and using the formula $V = (L \times W \times H) \times 0.5$. All animal work was performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Korea Research Institute of Bioscience and Biotechnology.

2.8. Statistical analysis

All data are representative of experiments that were repeated at least three times with similar results on each separate occasion. The data are represented as the mean ± standard deviation. Student *t*-tests were used for comparisons between groups and the Chi-square test was used for categorical variables. Survival curves were estimated using the Kaplan–Meier method and compared using the log rank test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. CEACAM6 expression is associated with the overall survival of lung adenocarcinoma patients

IHC staining of human lung adenocarcinoma specimens revealed that CEACAM6 was localized mainly to the cytoplasm of tumor cells (Fig. 1A). Staining was absent or sporadic in non-neoplastic lung tissue, which was used as negative control
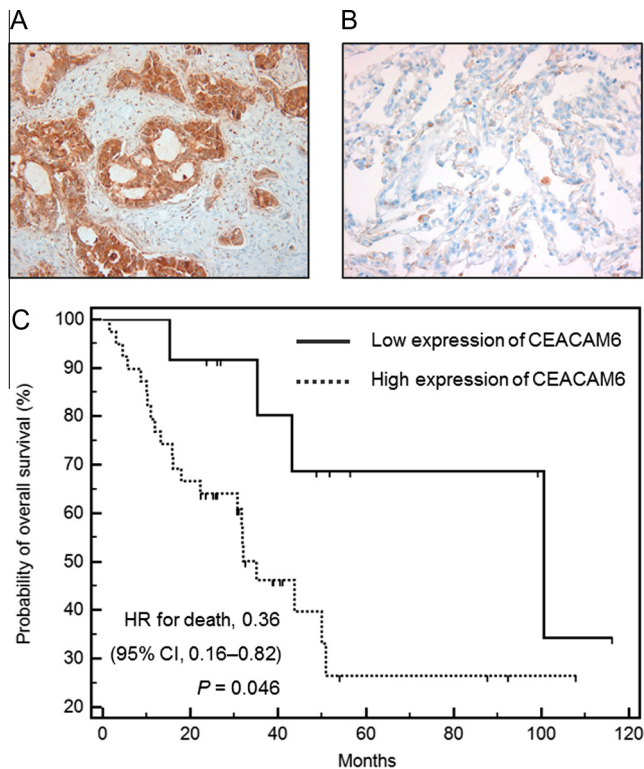
Fig. 1. CEACAM6 expression in lung adenocarcinoma specimens and its relationship to overall survival. (A) Immunohistochemical analysis of a representative lung adenocarcinoma specimen showing positive staining for CEACAM6 (magnification, 200×). (B) Immunohistochemical analysis of a representative normal lung tissue specimen showing negative staining for CEACAM6 (magnification, 400×). (C) Kaplan–Meier estimates of overall survival classified according to the CEACAM6 expression level identified by IHC staining (low expression, scores of 0 and 1+; high expression, scores of 2+ and 3+). HR, hazard ratio; CI, confidence interval.

IHC staining score; overall, 39 (76.5%) and 12 (23.5%) specimens showed high (score of 2+ or 3+) and low (score of 0 or 1+) CEACAM6 expression, respectively. The level of CEACAM6 expression was not significantly correlated with age, sex, differentiation, or pathologic stage (Supplementary Table 1). However, patients with low CEACAM6 expression had significantly longer overall survival times than those with high CEACAM6 expression. The median overall survival times for the low and high expression group were 100.7 months (95% confidence interval, 42.2–100.7) and 35.1 months (95% confidence interval, 22.2–50.9), respectively (hazard ratio for death, 0.36; 95% confidence interval, 0.16–0.82; $P = 0.046$) (Fig. 1C).

3.2. CEACAM6 enhances the proliferation, migration, and invasion of lung adenocarcinoma cells in vitro

To examine the biological function of CEACAM6 in lung adenocarcinoma, two different *CEACAM6*-targeting shRNAs (B12 and G1) were designed and transfected into A549 cells. The G1 shRNA reduced the expression of CEACAM6 more effectively than the B12 shRNA (Fig. 2A) and was used in further analyses. Knockdown of CEACAM6 suppressed the proliferation (Fig. 2B), migration (Fig. 2C), and invasion (Fig. 2D) of the A549 cells significantly. To verify these results, the effect of overexpression of exogenous CEACAM6 on the proliferation, migration, and invasion of lung adenocarcinoma cells in vitro was also examined. In support of the shRNA studies, overexpression of CEACAM6, which was confirmed by Western blot analysis (Fig. 2E), increased the proliferation (Fig. 2F), migration (Fig. 2G), and invasion (Fig. 2H) of the A549 cells significantly.

3.3. Knockdown of CEACAM6 inhibits the growth of lung adenocarcinoma tumors in vivo

To confirm the results of the in vitro study, A549 cells expressing the *CEACAM6*-specific shRNA (or empty vector as a control) were subcutaneously injected into the right flank of nude mice, which were sacrificed 4 weeks after injection. Tumor outgrowth and size were reduced in CEACAM6-deficient tumor-bearing mice, but control mice displayed visible tumors 10 days after injection
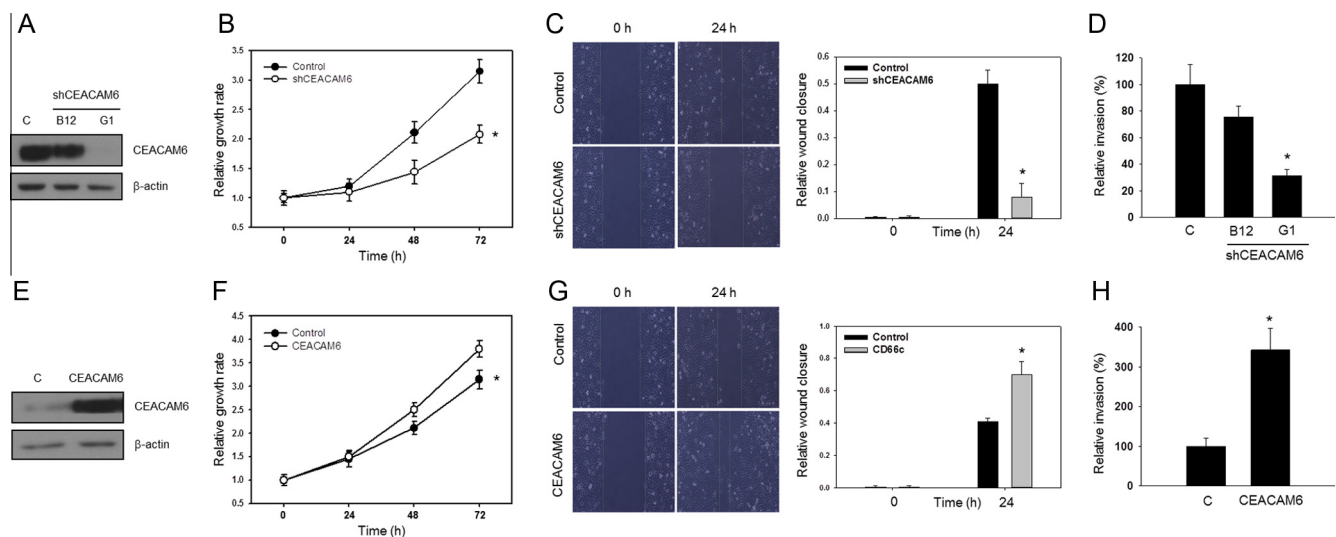
(Fig. 1B). Positive expression of CEACAM6 was identified in 44 (86.3%) of the 51 lung adenocarcinoma specimens examined. The tumor specimens were divided into two groups according to the

Fig. 2. CEACAM6 enhances the proliferation, migration, and invasion of lung adenocarcinoma cells in vitro. (A) Western blot analyses of CEACAM6 in the two different *CEACAM6*-targeting shRNAs (B12 and G1). The expression level of β-actin was used as an internal loading control. C, control. (B) The proliferation of control A549 cells and A549 cells transfected with the G1 *CEACAM6*-specific shRNA. (C) Wound healing assays to evaluate the migration of control and G1 shRNA-expressing A549 cells. (D) Invasion assays of control, B12 shRNA-expressing, and G1 shRNA-expressing A549 cells. (E) Western blot analyses of CEACAM6 in the control (C) and CEACAM6-expressing cells. (F–H) The proliferation (F), migration (G), and invasion (H) of control A549 cells and A549 cells overexpressing exogenous CEACAM6. Statistical analyses were performed by Student *t*-tests and the data are represented as the mean ± S.D. *$P < 0.05$.
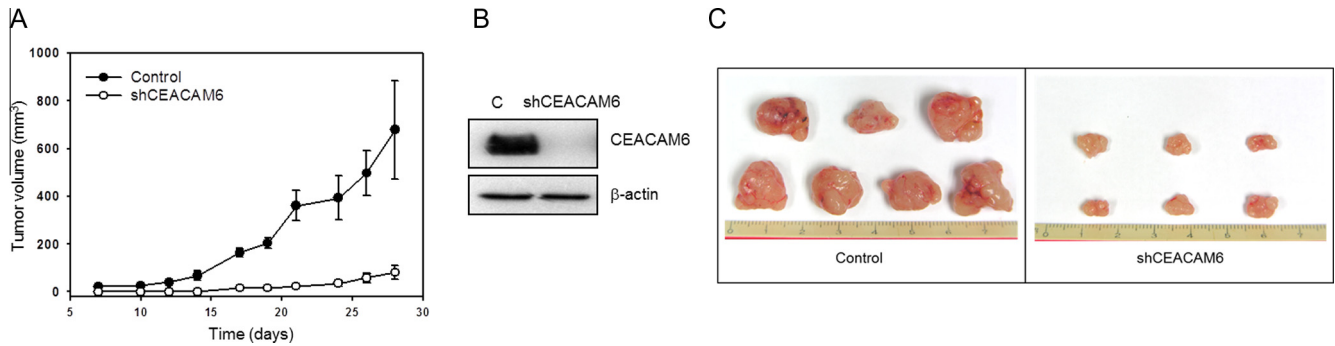
Fig. 3. Knockdown of CEACAM6 inhibits the growth of lung adenocarcinoma tumors in vivo. (A) Tumor growth in mice subcutaneously injected with A549 cells expressing the *CEACAM6*-specific shRNA or empty vector as a control. (B) Western blot analysis of CEACAM6 expression in the control (C) and *CEACAM6*-specific shRNA-expressing A549 tumors. The expression level of β-actin was used as an internal loading control. (C) Representative image of tumors from nude mice 4 weeks after injection of 9×10^6 control or *CEACAM6*-specific shRNA-expressing A549 cells.

and tumor growth in these mice was exponential for the 4 weeks observation period (Fig. 3A–C). These results indicate that knockdown of CEACAM6 in lung adenocarcinoma cells can inhibit tumor growth in vivo.

3.4. MiR-29a targets CEACAM6 directly in lung adenocarcinoma

A target prediction program (TargetScanHuman 6.2) was used to identify putative miRNA-binding sites in the 3′ UTR of human *CEACAM6*, and miR-29a and miR-16 were selected as candidates for further analysis. The 3′ UTR of *CEACAM6* contains two putative binding sites for miR-29a (Fig. 4A). To examine the roles of these miRNAs as potent regulators of CEACAM6 expression, A549 cells were transfected with a miR-16 mimic, miR-29a mimic, or a negative vector mimic. Western blot analyses showed that, compared with the negative vector-transformed cells, those overexpressing miR-29a had significantly lower expression levels of endogenous CEACAM6. In support of this finding, transfection of the cells with
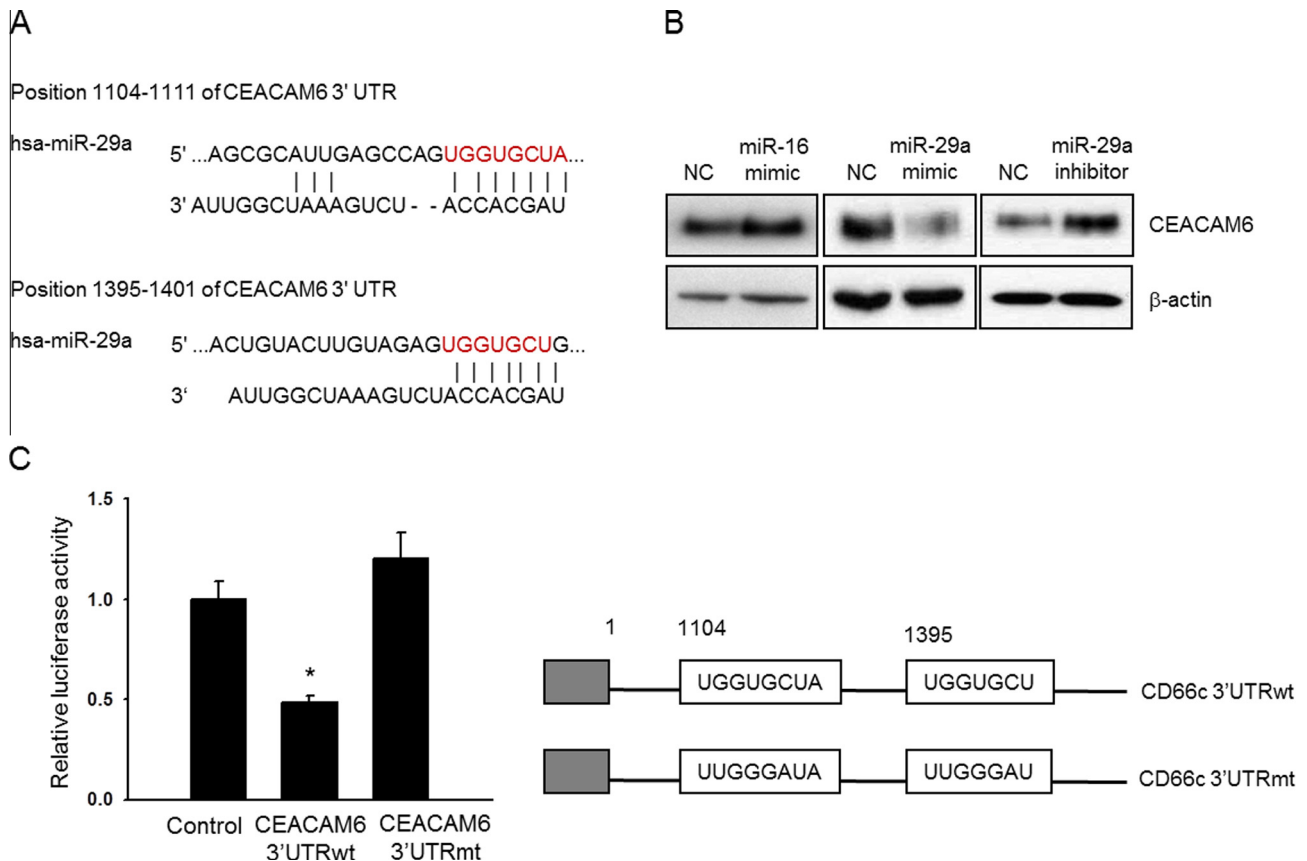

Fig. 4. MiR-29a targets CEACAM6 directly in lung adenocarcinoma cells. (A) Alignment of the miR-29a sequence with that of the 3′ UTR of *CEACAM6* showing the putative binding sites (red). The vertical lines indicate identity and the gaps indicate mismatches. (B) Western blot analyses of CEACAM6 expression 48 h after transfection of A549 cells with a miR-16 mimic, miR-29a mimic, or miR-29a inhibitor. The expression level of β-actin was used as an internal loading control. NC, negative control. (C) Relative luciferase activity assays of A549 cells co-transfected with a reporter vector containing the wild-type (wt) or mutant (mt) 3′ UTR of *CEACAM6* and a miR-29a mimic or vector control. The activity of *Renilla* luciferase was normalized to that of firefly luciferase. Statistical analysis was performed by Student *t*-test and the data are represented as the mean ± S.D. *$P < 0.05$.

a miR-29a inhibitor increased the expression level of CEACAM6 (Fig. 4B). By contrast, the CEACAM6 protein level was not affected by overexpression of miR-16 (Fig. 4B).

Next, a luciferase reporter assay was performed to confirm that CEACAM6 is a direct target of miR-29a. In these experiments, A549 cells were co-transfected with a vector containing the 3′ UTR of CEACAM6 directly downstream of a luciferase reporter, and a vector containing miR-29a or a control miRNA. Compared with that in the control cells, the relative luciferase activity was significantly lower in the cells co-transfected with miR-29a, whereas it was not affected by co-transfection with a mutant CEACAM6 3′ UTR in which the putative miR-29a binding sites were disrupted (Fig. 4C). These results indicate that miR-29a can down-regulate

the expression of CEACAM6 by binding directly to its 3′ UTR, suggesting that CEACAM6 may be a downstream target of miR-29a in lung adenocarcinoma cells.

3.5. MiR-29a suppresses the proliferation, migration, and invasion of lung adenocarcinoma cells in vitro

To investigate the function of miR-29a in lung adenocarcinoma cells, in vitro proliferation, migration, and invasion assays of control A549 cells and A549 cells expressing a miR-29a mimic were performed. Overexpression of miR-29a decreased the proliferation activity (Fig. 5A) and migratory ability (Fig. 5B) of the cells significantly. In the invasion analyses, the transwell assays were
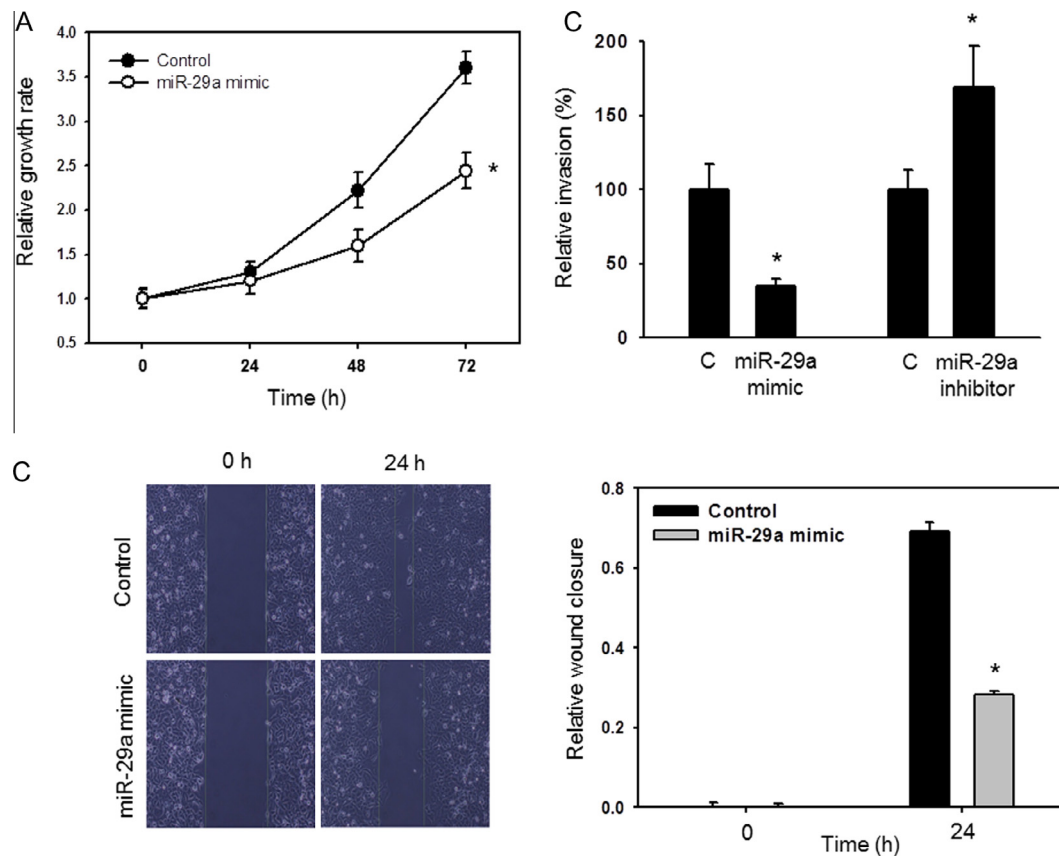

Fig. 5. MiR-29a suppresses the proliferation, migration, and invasion of lung adenocarcinoma cells in vitro. (A, B) The proliferation (A) migration (B) of A549 cells transfected with a miR-29a mimic or a negative control vector. (C) Invasion assays of control (C) cells and cells transfected with a miR-29a inhibitor alone or a miR-29a inhibitor and a miR-29a mimic. Statistical analyses were performed by Student t-tests and the data are represented as the mean ± S.D. $^*P < 0.05$.
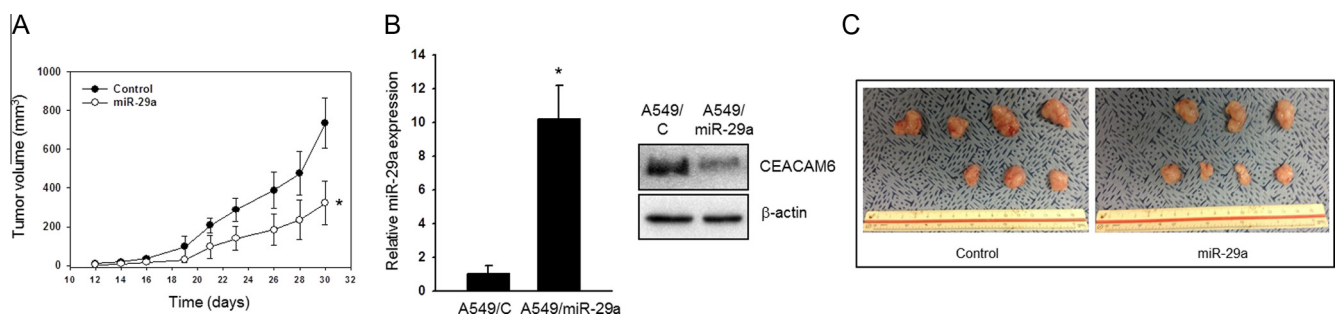

Fig. 6. MiR-29a inhibits the growth of lung adenocarcinoma tumors in vivo. (A) Tumor growth in mice injected with control vector-expressing cells (A549/C) or miR-29a-expressing cells. (B) Real-time RT-PCR analyses of miR-29a and Western blot analyses of CEACAM6 expression in A549 tumors from mice injected with control vector-expressing cells (A549/C) or miR-29a-expressing cells. The expression level of β-actin was used as an internal loading control in both real-time RT-PCR and Western blot analyses. Statistical analyses were performed by Student t-tests and the data are represented as the mean ± S.D. $^*P < 0.05$. (C) Representative images of tumors from nude mice 4 weeks after injection of 9×10^6 control vector-expressing or miR-29a-expressing cells.

performed after transfecting the cells with a miR-29a inhibitor to decrease the expression of the endogenous miRNA. The results showed that overexpression of the miR-29a mimic suppressed the invasion ability of the cells significantly and inhibition of miR-29a enhanced this ability significantly (Fig. 5C). These results indicate that miR-29a may suppress the proliferation, migration, and invasion of lung adenocarcinoma cells by targeting *CEACAM6*.

3.6. MiR-29a inhibits the growth of lung adenocarcinoma tumors in vivo

After transfection with empty vector or a vector expressing miR-29a, a xenograft of A549 cells was subcutaneously injected into the dorsal flank of nude mice and tumor volumes were calculated after the mice developed palpable tumors. At 4 weeks post-injection, the volumes of the xenografts were smaller in mice that received miR-29a-transfected cells than those that received control A549 cells (Fig. 6A–C). These results demonstrate that treatment of lung adenocarcinoma cells with miR-29a can inhibit tumor growth by suppressing CEACAM6 expression in vivo.

4. Discussion

In the present study, CEACAM6 expression is associated with poor prognosis of patients with lung adenocarcinoma, and we demonstrate that CEACAM6 has important functional roles in controlling the growth, migration, and invasion of lung adenocarcinoma cells in vitro and in vivo. Furthermore, the data indicate that miR-29a suppresses lung adenocarcinoma growth, migration, and invasion by targeting *CEACAM6*.

CEACAM6 inhibits anoikis (apoptosis induced by inadequate adhesion to the surrounding extracellular matrix) and modulates the malignant phenotype of cancer cells [20,21]. Anoikis resistance conferred by CEACAM6 expression results in cell survival under anchorage-independent conditions and is a characteristic associated with tumorigenesis and metastasis. CEACAM6 is expressed at low levels in normal epithelial, endothelial, and hematopoietic cells, including granulocytes and monocytes [13,22]. By contrast, CEACAM6 expression is up-regulated in many epithelial malignancies, including pancreatic, colorectal, breast, gastric, and lung adenocarcinomas, and its overexpression is associated with poor clinical outcomes [10–17]. In pancreatic cancer cells, overexpression of CEACAM6 is associated with anoikis resistance, metastasis, and gemcitabine resistance [11,21,23,24]. Furthermore, overexpression of the protein is correlated with reduced survival and the development of metastasis in colorectal cancer patients [9,14,25], and is a significant predictor of recurrence in breast cancer [26]. Regarding lung adenocarcinoma, CEACAM6 acts as an inducer of cell proliferation in A549 cells and increased serum levels of CEACAM6 are frequently detected in patients with lung adenocarcinoma [16]. Moreover, CEACAM6 expression is significantly associated with adverse clinical outcomes of lung adenocarcinoma patients [17]. In agreement with these findings, CEACAM6 was expressed at a high level in most of the 51 lung adenocarcinoma tissue samples examined here, and elevated expression of the protein was associated with poor prognosis of the patients. Furthermore, overexpression of CEACAM6 functionally enhanced the growth, migration, and invasion of lung adenocarcinoma cells both in vitro and in vivo. RNA interference-mediated knockdown of CEACAM6 decreased the proliferation, migration, and invasion of A549 cells, whereas its overexpression increased the proliferation, migration, and invasion of transfected cells in vitro. These findings are consistent with the results of the in vivo mouse xenograft experiments, in which knockdown of CEACAM6 inhibited the growth of lung adenocarcinoma tumors.

The results presented here indicate that miR-29a acts as a tumor suppressor in lung adenocarcinoma by targeting *CEACAM6* directly. Overexpression of miR-29a suppressed cell proliferation, migration, and invasion in vitro and inhibited the growth of lung adenocarcinoma tumors in vivo. In addition, overexpression of this miRNA induced a significant downregulation of CEACAM6 protein expression and suppressed the activity of a luciferase reporter fused to the 3′ UTR of *CEACAM6*. The human miR-29 family comprises miR-29a, miR-29b, miR-29c, and miR-29d [27,28]. Members of this family have been described as tumor suppressor genes and their expression levels are down-regulated in several cancers [27–32]. Although the detailed functional mechanisms of these miRNAs that are relevant to cancer are poorly understood, a number of previous studies have revealed that they target genes involved in cell proliferation and the cell cycle, cell senescence, differentiation, apoptosis, and metastasis at genetic and epigenetic levels in various types of cancer [27,28]. As a tumor promoter, miR-29 mediates epithelial–mesenchymal transition and promotes metastasis in breast and colon cancers [28,33], indicating that the function and targets of miR-29 are context dependent. MiR-29a expression is reduced in NSCLC, and it has a significant anti-invasive and anti-proliferative effect on lung cancer cells in vitro [34]: this function is likely mediated through direct and indirect post-transcriptional fine tuning of the cellular levels of several proteins. The expression levels of miR-29a and miR-29b in lung cancer are regulated by DNA methyltransferases (DNMTs) 3A and 3B (de novo methyltransferases), which alter the epigenetic modifications of chromatin and inhibit tumor suppressor gene expression. These DNMTs are frequently up-regulated in lung cancer and are associated with poor prognosis [35]. Suppression of DNMT3A and DNMT3B restores the expression of Wnt inhibitory factor-1, which is silenced by promoter methylation in NSCLC [36], and miR-29a, miR-29b, and miR-29c suppress the Wnt signaling pathway through demethylation of Wnt inhibitory factor-1 in NSCLC [36]. Furthermore, miR-29a has a tumor suppressive role in restricting the activity of DNA damage responsive kinases such as Cdc7 in lung cancer cells [37]. Recently, Chen et al. reported that CEACAM6 directly impacts the epithelial–mesenchymal transition, migration, invasion, and metastasis of pancreatic cancer cells, and showed that miR-29a/b/c can regulate *CEACAM6* at the post-transcriptional level in pancreatic cancer [24]. Therefore, miR-29a may act as a tumor suppressor in lung adenocarcinoma and pancreatic cancer by inhibiting the expression of CEACAM6.

In conclusion, the results presented here demonstrate that miR-29a can suppress the growth, migration, and invasion of lung adenocarcinoma cells by targeting *CEACAM6*. The miR-29a/CEACAM6 axis provides a new insight into the pathogenesis of lung adenocarcinoma and represents a potential therapeutic target for treatment of the disease.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.08.023.

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