Semaphorin 5A suppresses the proliferation and migration of lung adenocarcinoma cells

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Abstract. Semaphorin 5A (SEMA5A), a member of the semaphorin family, plays an important role in axonal guidance. Previously, the authors identified another possible role of SEMA5A as a prognostic biomarker for non-smoking women with lung adenocarcinoma in Taiwan, and this phenomenon has been validated in other ethnic groups. However, the functional significance of SEMA5A in lung adenocarcinoma remains unclear. Therefore, we assessed the function of SEMA5A in three lung adenocarcinoma cell lines in this study. Kaplan-Meier Plotter for lung cancer was conducted for survival analyses. Reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis were performed to investigate the expression and post-translational regulation of SEMA5A in lung adenocarcinoma cell lines. A pre-designed PyroMark CpG assay and 5-aza-2’-deoxycytidine treatment were used to measure the methylation levels of SEMA5A. The biological functions of lung adenocarcinoma cells overexpressing SEMA5A were investigated by microarrays, and validated both in vitro (proliferation, colony formation and migration assays) and in vivo (tumor xenografts) experiments. The results revealed that the hypermethylation of SEMA5A and the cleavage of the extracellular domain of SEMA5A were responsible for the downregulation of the SEMA5A levels in lung adenocarcinoma cells (A549 and H1299) as compared to the normal controls. Functional analysis of SEMA5A-regulated genes revealed that they were involved in cellular growth and proliferation. The overexpression of SEMA5A in A549 and H1299 cells significantly decreased the proliferation (P<0.01), colony formation (P<0.001) and migratory ability (P<0.01) of the cells. The suppressive effects of SEMA5A on the proliferative and migratory ability of the cells were also observed in both in vitro and in vivo experiments using brain metastatic Bm7 lung adenocarcinoma cells. On the whole, the findings of this study suggest a suppressive role for SEMA5A in lung adenocarcinoma involving the inhibition of the proliferation and migration of lung transformed cells.

Introduction

Lung carcinoma, which is caused by both genetic and environmental factors, is the leading cause (18%) of cancer-related mortality worldwide (1,2). Non-small cell lung cancer (NSCLC), such as large cell carcinoma, squamous cell carcinoma and adenocarcinoma, constitutes >80% of all lung cancer cases (3). The 5-year overall survival rate of NSCLC is poor despite great advances being made in diagnosis and treatment (4). Although cigarette smoking is the major risk factor for lung cancer, numerous genes have been reported to participate in lung carcinogenesis (5-7).

Semaphorin family proteins, which contain a conserved N-terminal Sema domain of 400-500 amino acids (8), comprise 8 classes with membrane-anchored and cleaved extracellular domain forms (9). The cleaved extracellular domain, such as SEMA3E (10), is modified from the...
membrane-anchored forms by proteolytic effects (11,12). This proteolytic process is executed by the A disintegrin and metalloprotease (ADAM) family. For example, SEMA3C can be cleaved by ADAMTS1 (13) and SEMA5B can be cleaved by ADAM17 (14).

Initially, the semaphorin family proteins were discovered to regulate axon growth and neuronal migration (15,16). Recently, several studies have found that semaphorins are involved in cardiac/skeletal development (17), the immune response (18), the regulation of angiogenesis (19) and tumor growth, as well as metastasis (20). Class 3 semaphorins, such as SEMA3B and SEMA3F have been reported to be regulated by DNA methylation (21,22) and are related to various carcinogenic processes in the lungs, for example angiogenesis and metastasis (15,23-25) and tumor suppressor activities (26-28). However, whether other classes of the semaphorin family are subject to methylation regulation or are involved in carcinogenic processes in the lungs remains unclear.

Previously, a comprehensive analysis of the gene expression signature performed by the authors in non-smoking women with lung adenocarcinoma revealed that the downregulation of SEMA5A was associated with a poor overall survival (29). SEMA5A belonging to class V of the semaphorin family, is an integral membrane protein containing the Sema domain, 7 thrombospondin type-1 repeats and a short cytoplasmic domain (23,30). SEMA5A has been reported to have both a membrane-bound (8,31) and cleaved extracellular domain (32). Sheddases, which are the members of the ADAM protein families, are known to be majorly involved in ectodomain shedding by cleaving the extracellular portions of transmembrane proteins (33). However, the role of sheddase responsible for releasing the ectodomain from membrane-bound SEMA5A has yet to be identified.

In addition, as regards the function of SEMA5A, it was implicated as a susceptible gene related to Cri-du-chat syndrome (34) and autism (35). SEMA5A has also been reported to promote angiogenesis by increasing endothelial cell proliferation and decreasing apoptosis (36), and to have high tumorigenic and metastatic potential in pancreatic and gastric tumors (37-39). On the other hand, some studies have demonstrated that SEMA5A also plays the role of a tumor suppressor. For instance, SEMA5A maintains the epithelial phenotype of malignant pancreatic cancer cells (40), and inhibits glioma cell motility through the RhoGDIα-mediated inactivation of RAC1 GTPase (41) and the actin cytoskeleton (42). However, little is known about the mechanisms and functional role of the downregulation of SEMA5A in lung adenocarcinoma cells. Therefore, this study aimed to elucidate the mechanisms associated with low endogenous expression levels of SEMA5A in lung adenocarcinoma and to determine functional roles in lung carcinogenesis.

Materials and methods

Cells and cell culture. Cancerous lung cell lines (CL1-0, CL1-5, A549, and H1299) (gifts from Dr Pan-Chyr Yang) and normal cells (BEAS-2B) (a gift from Dr Pan-Chyr Yang) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific) with 1% streptomycin/penicillin (Biological Industries) and 10% fetal bovine serum (FBS; Biological Industries). Brain metastatic lung adenocarcinoma Bm7 cells (generated in the authors’ laboratory) were cultured in DME/F12 plus 10% FBS (43). The cultured plates were maintained at 37°C in a humidified atmosphere with 5% CO2. Another normal lung cell line (MRC-5) and human bronchial epithelial cells (16HBE) (a gift from Dr. Kuo-Ting Chang) were grown in Eagle's Minimum Essential Medium (Gibco; Thermo Fisher Scientific) under the same conditions.

Cell line authentication. Cell experiments were performed on cells that were passaged <20 times, and were routinely tested for mycoplasma using the PCR Mycoplasma Detection kit (ABM Inc., Vancouver, Canada). The identity of the cell lines was authenticated by short-tandem repeat (STR) analysis (Mission Biotech Inc., Taipei, Taiwan) in February, 2018.

Endogenous expression of SEMA5A. To quantify the transcriptional expression of SEMA5A in different cellular models, total RNA was isolated using TRIzol reagent (Ambion) and purified by precipitation with isopropanol (Sigma-Aldrich). A NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) was used to assess the purity and quantity of the RNA. A high Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) was used to synthesize the cDNA from 1 µg of total RNA of each cell line. The final cDNA products were used as the templates for expression analysis using quantitative PCR (qPCR).

qPCR. The quality and quantity of the RNA were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). A total of 1 µg of total RNA from each cell line was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The reaction was carried using thermal cycling conditions, including annealing at 25°C for 5 min, an extension temperature at 42°C for 1 h and inactivation temperature at 70°C for 15 min. The final cDNA products were used as the templates for subsequent qPCR with the following thermal cycling conditions. Denaturation temperature at 95°C for 15 sec, anneal/extend temperature: 60°C for 1 min for 40 cycles. RT-qPCR was performed using SYBR-Green (Roche) on an ABI 7900 system (Life Technologies; Thermo Fisher Scientific) according to standard protocols. All individual experiments were carried out in triplicate. Relative quantification, ΔΔCq (cycle threshold) (44), was applied using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control and empty group as a reference control.

Impact of methylation on gene expression. As a number of tumor suppressor genes are inactivated via hypermethylation within the promoter region (45,46), this study examined the role of methylation in regulating the expression of SEMA5A. The A549, H1299 and BEAS-2B cells were seeded on a 6-well plate, and after 24 h, the cells were treated with 5 or 10 µM of 5-aza-2'-deoxycytidine (5-aza; Sigma-Aldrich). As a number of CpG sites were applied using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control and empty group as a reference control.
The second strand of cDNA was synthesized by employing an Illumina TotalPre RNA Amplification kit (Ambion). Specific 5’-GAT GCA GGG ATG ATG TTC-3’). Protein levels were measured using a microtiter plate reader (BioTek) at 570 nm. The absorbance of the A549 and H1299 cells was then measured using a microtiter plate reader (BioTek) at 570 nm.

Analysis of cell proliferation. A total of 3,000 lung adenocarcinoma cells (A549 & H1299) were seeded in 96-well plates in triplicate and incubated for 12 h at 37℃ in a CO₂ incubator. The day after seeding, the cells were transfected with pZeoSV2-SEMA5A-Flag plasmid or empty vectors. After transfection, the cells proliferative activity was measured by MTT assay. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 48 h following transfection concentration as MTT assay. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 48 h following transfection. The cell membrane was damaged by 0.1% Triton X-100 (Mallinckrodt Specialty Chemicals Co) and blocked with 5% milk, the membranes were incubated with anti-SEMA5A (1:1,000, Cat. no. PA5-26066, Thermo Fisher Scientific), anti-GAPDH antibody (1:20,000, Cat. no. GTX100118, GeneTex), or anti-BSA antibody (1:5,000, Cat. no. GTX79812, GeneTex). Following incubation overnight at 4℃, the membranes were then incubated with horseradish peroxidase-conjugated anti-IgG (1:5,000, Cat. no. GTX213110, GeneTex) at room temperature for 1 h, and the blots were developed with the chemiluminescent western blotting reagent (Millipore). Western blot images were further analyzed using Gel-Pro Analyzer v6.3 software (Meyer Instruments) to obtain the optical density values of SEMA5A and GAPDH antibodies.

Immunohistochemistry. The A549 (3,000 cells) or H1299 (3,000 cells) cells were seeded on silane coated micro slides (Muto pure chemicals Co.) with the same cell density and transfection concentration as MTT assay. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 48 h following transfection. The cell membrane was damaged by 0.1% Triton X-100 (Mallinckrodt Specialty Chemicals Co) and blocked with 2% bovine serum albumin (Sigma-Aldrich). Ki-67 primary antibody (1:400, #9129, Cell Signaling Technology) and anti-rabbit IgG secondary antibody (1:800, #8889, Cell Signaling Technology) were used for Ki-67 detection at a wavelength of 550/580 nm. The incubation conditions for the primary antibody were 4℃ overnight, and for the secondary antibody they were 2 h at room temperature. Cell nuclei were stained with Hoechst 33342 (#B2261, 1 µg/ml, Sigma-Aldrich) and detected at wavelength of 360/460 nm. The incubation conditions for Hoechst 33342 were 30 min at room temperature. Images were acquired using the Zeiss AxioImager A1 system (Carl Zeiss).

Clonogenic assay. The A549 (300 cells), H1299 (300 cells) and Bm7 (300 cells) cells were first seeded in 6-well plates for 24 h, and transfected with pZeoSV2-SEMA5A-Flag plasmid or empty vectors. After 2 weeks, the cells were fixed using DNA polymerase and RNAase H to simultaneously degrade the RNA and synthesize the second strand of cDNA. Following cleanup, in vitro transcription was conducted to synthesize biotinylated complementary RNA (cRNA). Following amplification, the cRNA was hybridized to Illumina Human HT-12 v4 BeadChips (Illumina) for 16 h. Following hybridization, the BeadChip was washed and stained with streptavidin-Cy3 dye. The intensity of the beads' fluorescence was detected by HiScan SQ (Illumina), and the results were analyzed using BeadStudio v2011.1 software.

Overexpression of SEMA5A in lung adenocarcinoma cells. Full-length SEMA5A cDNA (3,225 bp) tagged with a Flag epitope was inserted into a pZeoSV2* viral expression vector with the NorI and AxiSI restriction enzymes (Addgene). The pZeoSV2*-SEMA5A-Flag plasmid was transiently transfected into the A549 and H1299 cell lines using TransIT-2020 transfection reagent (MirusBio) according to the manufacturer’s instructions. The empty pZeoSV2* viral expression vector was used as a control. All sequences were verified by Sanger sequencing (First Core Laboratory, College of Medicine, National Taiwan University). mRNA levels were quantified by RT-qPCR using SEMA5A-specific primers (forward, 5’-GTC TATACTTACTGCAGCG-3’ and reverse, 5’-GTTAAA TGCCCTGTAGGCCCT-3’) and GAPDH-specific primers (forward, 5’-TGACCACTACCTGCTTAG-3’ and reverse, 5’-GATGCAGGGATGTAGTTC-3’). Protein levels were examined by western blot analysis.

Isolation and amplification of total RNA for gene expression profiling. The mRNA detection and analysis were performed as previously described (47). Briefly, the cDNA was synthesized from total RNA primed with T7 Oligo(dT) and amplified using an Illumina TotalPre RNA Amplification kit (Ambion). The second strand of cDNA was synthesized by employing the optical density values of SEMA5A and GAPDH antibodies.

Cleavage of SEMA5A by ADAM17. To examine the proteolytic effects of ADAM17 on SEMA5A, the H1299 cells were transfected with SEMA5A overexpression plasmid and treated with active recombinant ADAM17 (BioVision). The amount of administering ADAM17 was 0.66 µg for 6-cm dish. Cell lysates and media were collected at 2 days following transfection. Bovine serum albumin (BSA) was added externally to the culture media as the spike-in control, and all proteins in the medium were concentrated using a 10K Acrodisc syringe filter (Pall Life Sciences).

Western blot analysis. Total cell lysates of A549 and H1299 cells transfected with pZeoSV2*-SEMA5A-Flag plasmid or empty vector were prepared. Proteins (30 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). After blocking with 5% milk, the membranes were incubated with anti-SEMA5A (1:1,000, Cat. no. PA5-26066, Thermo Fisher Scientific), anti-GAPDH antibody (1:20,000, Cat. no. GTX100118, GeneTex), or anti-BSA antibody (1:5,000, Cat. no. GTX79812, GeneTex). Following incubation overnight at 4℃, the membranes were then incubated with horseradish peroxidase-conjugated anti-IgG (1:5,000, Cat. no. GTX213110, GeneTex) at room temperature for 1 h, and the blots were developed with the chemiluminescent western blotting reagent (Millipore). Western blot images were further analyzed using Gel-Pro Analyzer v6.3 software (Meyer Instruments) to obtain the optical density values of SEMA5A and GAPDH antibodies.

Clonogenic assay. The A549 (300 cells), H1299 (300 cells) and Bm7 (300 cells) cells were first seeded in 6-well plates for 24 h, and transfected with pZeoSV2*-SEMA5A-Flag plasmid or empty vectors. After 2 weeks, the cells were fixed using DNA polymerase and RNAase H to simultaneously degrade the RNA and synthesize the second strand of cDNA. Following cleanup, in vitro transcription was conducted to synthesize biotinylated complementary RNA (cRNA). Following amplification, the cRNA was hybridized to Illumina Human HT-12 v4 BeadChips (Illumina) for 16 h. Following hybridization, the BeadChip was washed and stained with streptavidin-Cy3 dye. The intensity of the beads' fluorescence was detected by HiScan SQ (Illumina), and the results were analyzed using BeadStudio v2011.1 software.

After scanning, the intensity data of Illumina BeadChips were analyzed using Partek v7.0 software (Partek). Background-adjusted signals were normalized by a quantile normalization algorithm. Student’s t-tests and Bonferroni P-value adjustment were utilized to identify differentially expressed genes. Principle component analysis (PCA) was utilized to evaluate the similarity of the gene expression profiles. Hierarchical clustering analysis and the Genesis program were used to generate visual representation of expression profiles. All data have been deposited at the Gene Expression Omnibus (GEO, GSE114578). Furthermore, ingenuity pathway analysis (Ingenuity Systems Inc.) was applied to comprehend the biological functions and signaling pathways of differentially expressed genes.

Analysis of cell proliferation. A total of 3,000 lung adenocarcinoma cells (A549 & H1299) were seeded in 96-well plates in triplicate and incubated for 12 h at 37℃ in a CO₂ incubator. The day after seeding, the cells were transfected with pZeoSV2*-SEMA5A-Flag plasmid or empty vectors. Following transfection, the cells proliferative activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (EMD Biosciences) assay at 24, 48 and 72 h, respectively. The absorbance of the A549 and H1299 cells was then measured using a microtiter plate reader (BioTek) at 570 nm.

The second strand of cDNA was synthesized by employing an Illumina TotalPre RNA Amplification kit (Ambion). The second strand of cDNA was synthesized by employing
3:1 methanol-acetic acid and stained using 0.1% crystal violet (Sigma-Aldrich) for 10 min at room temperature. Finally, the stained plates were dried and used for image acquisition using microscopic (x100 magnification) evaluation. Colonies containing >50 cells under a stereomicroscope were counted.

**Gap closure assay.** Following 24 h of transfection, 2x10⁴ cells of A549 and H1299 were seeded into 24-well plates with a sterile culture insert and grown to ~90% confluence in RPMI-1640 medium containing 10% FBS and changed to 2% FBS when removing the cassettes. The culture inserts were removed and an image of the gap at 0 h was captured. The cells were further incubated at 37°C in a CO₂ incubator for 36 h, and images were captured at 24 and 36 h to measure the progress of gap closure.

**Cell migration.** Migration assays were carried out using a 24-well Transwell unit (Corning, Inc.). The upper chamber of the Transwell unit was loaded with 4x10⁵ cells/well in 0.2 ml serum-free RPMI-1640 medium, and the lower chambers were loaded with 0.6 ml RPMI-1640 containing 10% FBS as a chemoattractant. The A549 and H1299 cells were then incubated for 24 h at 37°C. A methanol-acetic acid (3:1) mixture was then added to the lower chamber to fix the cells for 20 min at room temperature followed by staining with 0.1% crystal violet for a further 20 min at room temperature. Cells on the upper side of the membrane surface were removed by scraping with a cotton swab, and the cells that passed through the filter were de-stained using 10% acetic acid. The absorbance was measured at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instruments). Cells transfected with empty vector were used as the controls.

**Time-lapse migration assay.** The experiment was performed as previously described (43). Briefly, cells were cultured on dishes coated with collagen (10 µg/ml) overnight and then cultured in serum-free conditioned medium. Cell movement was detected under A-Plan objectives (X5; 0.55 NA) using an inverted microscope (Axio Observer Z1, Zeiss) in 37°C chambers. Images were collected from CCD video cameras (AxioCam MRm, Zeiss) at 20-min intervals for a total of 16 h using MetaMorph software (Molecular Devices Corp.). The accumulated distance was measured by tracking each cell nuclei for 30 individual cancer cells in each group using the Track Point function of NIH ImageJ v1.43 software.

**Lung cancer animal model.** The SEMA5A-Flag plasmid was transiently transfected into Bm7 lung cancer cells with stable luciferase expression. The following day, the Bm7 cells (2x10⁴ cells in 50 µl of PBS) were mixed with 50 µl of Matrigel and then implanted into 6-8 week-old male SCID mice (weighing 20-25 g; BioLASCO) by the subcutaneous route at two separate sites (right and left side) of the back (each group had 4 mice; 2 groups). Mice were housed in specific pathogen-free rooms with one group in one cage at room temperature (20-23°C), 40-60% relative humidity and under a 12-h light-dark cycle, with free access to food and water. No cachexia and ascites in mice were observed in this study. As Bm7 lung tumors had luciferase, the tumor volume was detected using an IVIS Spectrum Imaging system (Xenogen).

Prior to image acquisition, the mice were subsequently administered D-luciferin via intraperitoneal injection and photons emitted from the mice were detected using an imaging system. Tumor size and distribution *in vivo* were quantified as photons/second. The maximum length and width exhibited by a single subcutaneous tumor in this study did not exceed 1.5 cm. The maximum tumor volume calculated by signal was 500 mm³ using the formula, (length x width²)/2. Anesthesia was induced and maintained with 2.5% isoflurane (Panion & BF Biotech Inc.) in 100% oxygen in an anesthetic chamber when mice were monitored by an IVIS Spectrum Imaging system. The mice were sacrificed by using carbon dioxide (CO₂) at a displacement rate from 10 to 30% of the chamber volume per minute for mouse euthanasia and death was verified by physical methods following euthanasia on day 21, including no heartbeat, no pupillary response to light and no respiratory pattern.

This animal model followed protocols approved by the Institutional Animal Care and Use Committee of China Medical University and Hospital (animal protocol no. 2016-102). Suitable humane endpoints were included in the approved animal experiments. In this study, the mouse experiment was terminated by euthanasia (using carbon dioxide) on day 21 or animals that reached humane endpoints when the tumor size was near 1,000 mm³ or unexpected circumstances, such as illnesses (infection, difficulty breathing, etc.) and injuries (necrosis or bleeding in tumors). The mice were sacrificed by using carbon dioxide (CO₂) at a displacement rate from 10 to 30% of the chamber volume per minute for mouse euthanasia and death was verified by methods, including no heartbeat, no pupillary response to light, and no respiratory pattern for at least 5 min.

**Kaplan-Meier survival analysis.** Kaplan-Meier survival analyses were conducted using Kaplan-Meier Plotter for lung cancer (48,49), an online platform (http://www.kmplot.com/lung), using SEMA5A probe (Affy ID: 229427_at) and adenocarcinoma in histology restriction. In total, Kaplan-Meier Plotter analyzed 1,715 patients for survival analysis, which included 1,120 patients in 7 GEO datasets (GSE4573, GSE14814, GSE8894, GSE19188, GSE3141, GSE31210, GSE29013 and GSE37745), 462 patients in the caArray, and 133 patients in The Cancer Genome Atlas (TCGA). The log rank test was used to determine differences in the survival rate between the high and low expression groups.

**Statistical analysis.** Data are expressed as the means ± standard deviation (SD) of at least 3 independent experiments. Student's t-tests and Bonferroni P-value adjustment were utilized to identify differentially expressed genes. P-values <0.05 were considered to indicate statistically significant differences.

**Results**

**Association of SEMA5A expression in lung cancer tissues with a poor overall survival.** In previous genomic studies, it was found that the downregulation of SEMA5A in non-smoking women with lung adenocarcinoma was associated with a poor overall survival (29,50,51). To validate this phenomenon in a Caucasian population, another publicly available dataset
was examined (52). As shown in Fig. 1A, SEMA5A was also significantly (P<0.01) downregulated in cancer tissues as compared to adjacent normal control tissues. Furthermore, the survival of lung adenocarcinoma patients in the new dataset for >15 years was examined using Kaplan-Meier plotter (www.kmplot.com/lung) (49). In total, 1,715 patients were used for survival analysis, which included 1,120 patients in 7 GEO datasets (GSE4573, GSE14814, GSE8894, GSE19188, GSE3141, GSE31210, GSE29013 and GSE37745), 462 patients in the caArray, and 133 patients in The Cancer Genome Atlas (TCGA). Patients were divided into the high and low expression groups based on the median expression value of SEMA5A. The results of Kaplan-Meier survival analysis revealed that the lung adenocarcinoma patients with higher expression levels of SEMA5A had a lower risk of death (hazard ratio, 0.65), i.e., a higher overall survival probability (Fig. 1B), indicating the potential utility of SEMA5A as a prognostic marker for patients with lung adenocarcinoma.

Mechanisms responsible for the lower SEMA5A expression in lung adenocarcinoma cells. Since SEMA5A was downregulated in non-smoking female lung adenocarcinoma patients (29), the endogenous expression levels of SEMA5A were then examined in 4 lung adenocarcinoma cell lines (CL1-5, CL1-0, A549 and H1299), 2 normal lung cell lines (MRC-5 and 16HBE). The results of RT-qPCR revealed that SEMA5A was significantly (P<0.01) downregulated in all lung adenocarcinoma cell lines, whereas they were not altered in the BEAS-2B normal cells (Fig. 2C). Moreover, pyrosequencing analysis was performed to identify the specific CpG sites of methylation in SEMA5A. In total, 5 CpG sites in the 5’ untranslated region of SEMA5A were examined (Fig. 2D top panel). The 4 CpG sites in the A549 cells and 3 CpG sites in the H1299 cells revealed a significantly higher methylation percentage as compared to the normal BEAS-2B cells (Fig. 2D bottom panel), indicating that methylation plays a role in regulating the expression of SEMA5A in lung adenocarcinoma cells.

Subsequently, SEMA5A was transiently overexpressed in the A549 and H1299 cells. As shown in Fig. 3A, the mRNA levels of SEMA5A in the A549 and H1299 cells following transfection were significantly (P<0.01) increased. Western blot analysis also validated that the SEMA5A protein levels increased in both the A549 and H1299 cells upon transfection with SEMA5A expression plasmid (Fig. 3B). Since a previous study demonstrated that the mature form of SEMA5B was proteolytically processed by ADAM17 (14), this study also examined whether SEMA5A can be cleaved by ADAM17. H1299 cells overexpressing SEMA5A were treated with active recombinant ADAM17. As shown in Fig. 3C and D, the amount of SEMA5A in the cell lysate from the A549 (Fig. 3C) and H1299 (Fig. 3D) cells was lower in the presence of ADAM17. By contrast, the amount of SEMA5A in the medium increased (Fig. 3C and D), suggesting that membrane-bound SEMA5A was released into the medium in the presence of ADAM17.

Identification and function of SEMA5A-regulated genes using microarray. In order to investigate the functional role of SEMA5A, the functions of SEMA5A-regulated genes were first investigated. Total RNA was extracted 24 h following the transfection of SEMA5A plasmid into the A549 cells. Differentially expressed genes regulated by SEMA5A were identified by Illumina Human HT-12 v4 Bead Chips. The criteria for the selection of differentially expressed genes consisted of a fold change ≥3 and significant differences (P<0.05). In total, 350 differentially expressed probes were
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Figure 2. Lower expression levels and hypermethylation of SEMA5A observed in lung adenocarcinoma cells. (A) Endogenous expression levels of SEMA5A in lung adenocarcinoma cells. Total mRNA was extracted from lung adenocarcinoma cells (CL1-5, CL1-0, A549 and H1299) and normal controls (MRC-5, BEAS-2B and 16HBE). The endogenous expression levels of SEMA5A were measured by RT-qPCR. GAPDH was used as a loading control. Bars represent the means ± SD of 3 independent experiments; *P<0.01 as compared to MRC-5 cells; †P<0.01 as compared to BEAS-2B cells; †P<0.01 as compared to 16HBE cells. (B) Western blot analysis of endogenous expression levels of SEMA5A in lung adenocarcinoma cells and normal controls. GAPDH was used as an internal control. (C) Relative expression of SEMA5A mRNA in cells treated with 5 and 10 µM of the methylation inhibitor, 5-aza-2-deoxycytidine (5-aza), for 3 days. Relative expression levels of SEMA5A were normalized against the untreated group of each cell line. (D) Methylation levels of SEMA5A. Upper panel, scheme of CpG sites in the 5'-untranslated region of SEMA5A for pyrosequencing. CpG sites were numbered as shown in parentheses according to genome assembly version GRCh37/hg19. Lower panel, quantification of DNA methylation of SEMA5A. Genomic DNA was extracted from the A549, H1299 and BEAS-2B cells and treated with bisulfide to examine the methylation percentage of CpG sites by pyrosequencing; *P≤0.05 as compared to BEAS-2B cells. SEMA5A, semaphorin 5A.

Figure 3. SEMA5A is cleaved by ADAM17. (A) Relative expression levels of SEMA5A in A549 and H1299 cells overexpressing SEMA5A were measured by RT-qPCR. GAPDH was used as a loading control. Bars represent the means ± SD of 3 independent experiments. *P<0.01 as compared to the empty control vector. (B) Western blot analysis of SEMA5A in A549 and H1299 cells overexpressing SEMA5A. GAPDH was used as an internal control. (C and D) Western blot analysis of SEMA5A in the cell lysate and the medium following treatment with ADAM17. (C) A549 and (D) H1299 cells were transfected with SEMA5A and treated with active recombinant ADAM17 (0.66 µg/6-cm dish). GAPDH was used as an internal control. Bovine serum albumin (BSA) was used as the spike-in control. SEMA5A, semaphorin 5A; ADAM17, A disintegrin and metalloprotease 17.
identified. The volcano plot illustrated in Fig. 4A represents 350 differentially expressed probes (a.k.a. genes), with 296 probes upregulated (red points) and 54 ones downregulated (green points) (Fig. 4A and Table SI). Genes that did not meet the criteria of differentially expressed genes were shown in gray (Fig. 4A). Principal component analysis (PCA) of A549 cells overexpressing SEMA5A, PCA was plotted using the expression of differentially expressed probes following quantile normalization. Each dot represents each sample.

A heatmap with hierarchical clustering, a common method of visualizing the relative intensity of gene expression by arranging genes together based on the similarity of their expression levels, is presented in Fig. 4C. The relative intensities of the 296 upregulated probes are shown in red, and those of the 54 downregulated ones are shown in green.

Furthermore, network analyses were performed by ingenuity pathway analysis. The dashed lines indicate indirect interactions between molecules as supported by information in the Ingenuity knowledge base. Red areas denote genes that were upregulated in cells overexpressing SEMA5A and green areas indicate downregulated genes. SEMA5A, semaphorin 5A.
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Figure 5. SEMA5A suppresses the proliferation and migration of lung adenocarcinoma cells. (A and B) MTT assays of (A) A549 and (B) H1299 cells overexpressing SEMA5A vs. empty vector. Proliferation assays were performed by the addition of MTT at different time points using 3,000 cells transfected with SEMA5A expression vector or empty control vector. The absorbance values indicate the proliferation status of the cells. (C and D) Immunohistochemistry assay of Ki-67 in (C) A549 and (D) H1299 cells overexpressing SEMA5A. Ki-67: A cellular marker for proliferation. Scale bar 100 µm. (E and F) Colony formation assays of (E) A549 and (F) H1299 cells overexpressing SEMA5A. (G and H) Representative pictures and quantification of gap closure assays of (G) A549 and (H) H1299 cells overexpressing SEMA5A. Images were acquired at 0 and 48 h. Scale bar, 100 µm. (I and J) Transwell migration assays of (I) A549 and (J) H1299 cells overexpressing SEMA5A. The absorbance values of migratory cells were normalized against the empty vector group. Bars represent the means ± SD of 3 independent experiments; *P<0.01 vs. empty vector. SEMA5A, semaphorin 5A.
growth and proliferation, as shown in Fig. 4E, which was later validated by further functional analysis.

Functional roles of SEMA5A in lung adenocarcinoma. Based on the function of SEMA5A-regulated genes, the effects of SEMA5A on tumor growth by MTT assays were hence examined. The results revealed a significant decrement in the proliferation of both the A549 and H1299 cells overexpressing SEMA5A (Fig. 5A and B). In agreement with these results, immunohistochemistry assay of Ki-67, a cellular marker for proliferation, also revealed a markedly decreased amount of Ki-67 and cell numbers following transfection with the SEMA5A expression plasmid (Fig. 5C and D). Furthermore, SEMA5A overexpression reduced colony formation in both the A549 (Fig. 5E) and H1299 (Fig. 5F) cells. These results demonstrated the suppressive effects of SEMA5A on the proliferation and colony formation of lung adenocarcinoma cells.

Subsequently, the effects of SEMA5A on the mobility of lung adenocarcinoma cells were investigated by gap closure assay and Transwell migration assays. Both assays revealed that the overexpression of SEMA5A significantly suppressed the migratory abilities of both the A549 (Fig. 5G and I) and H1299 (Fig. 5H and J) cells. Previously, SEMA5A has been shown to impede the motility of human gliomas via the indirect inactivation of RAC1 and FSCN1 (41,42). However, the transcriptional levels of RAC1 and FSCN1 were not altered significantly in this study (Fig. S1).

Lastly, the role of SEMA5A in severe combined immunodeficiency (SCID) mice was examined. Since the growth of tumors in SCID mice using either A549 or H1299 lung cancer cells was not successful in pilot studies by the authors (data not shown), brain metastatic Bm7 lung cancer cells (43) were used to demonstrate the role of SEMA5A in in vivo experiments. Similar results were observed in the in vitro experiments (Fig. 6). SEMA5A overexpression reduced colony formation (Fig. 6A) and cell migration examined by time-lapse video microscopy (Fig. 6B). In the tumor xenograft assays, Bm7 cells overexpressing SEMA5A were injected subcutaneously into the backs of SCID mice to develop subcutaneous tumors. After 13 days, the tumor size was found to be significantly smaller in the SEMA5A group (Fig. 6C). No lymph nodes or distant organ metastases were detected during the experimental period.

Discussion
In a previous genomic study by the authors, SEMA5A was identified as a prognostic biomarker in non-smoking Taiwanese
women with lung adenocarcinoma (29). In this study, it was confirmed that the downregulation of SEMA5A in lung adenocarcinoma tissues was associated with a poor overall survival in different ethnic groups. In addition, lower levels of SEMA5A in lung adenocarcinoma cells were the result of both hypermethylation in the 5'untranslated region at the genetic level and cleavage to the secretory form. In addition, microarray analyses revealed that SEMA5A-regulated genes were involved in growth and proliferation. Finally, in vitro and in vivo analyses revealed the suppressive effects of SEMA5A overexpression on lung adenocarcinoma cell lines in terms of proliferation, colony formation and migration.

Semaphorins represent a large family of proteins, many of which are promising targets for interfering with cancer progression due to their roles in tumor angiogenesis, tumor growth and metastasis (27,53,54). In particular, previous studies have reported the roles of SEMA5A in the development of several types of cancer, such as pancreatic cancer, gastric cancer, ovarian cancer and gliomas (37,41,42,55,56). SEMA5A has been reported to enhance the invasion and metastasis of gastric cancer and pancreatic cancer cells (39,57) and has been shown to be associated with a poor survival in ovarian cancer (56). In contrast to this finding, a previous investigation by the authors revealed that the incidence of lung adenocarcinoma was associated with the downregulation of SEMA5A expression in non-smoking female lung adenocarcinoma patients, and that this down-regulation was associated with a poor overall survival (29). To investigate these seemingly opposing roles of SEMA5A in different types of cancer, this study first examined the endogenous expression levels of SEMA5A in lung adenocarcinoma cell lines. Consistent with the expression pattern in clinical tissues, the expression of SEMA5A was downregulated in lung adenocarcinoma cells as compared to their normal counterparts (Fig. 2A).

Furthermore, the inactivation of several tumor suppressor genes has been shown to be partly due to hypermethylation within the promoter region (45,46). Epigenetically disrupted gene expression can further alter various cancer-related processes, such as cell proliferation, apoptosis and angiogenesis (58,59). The abnormal DNA methylation of genes may be associated with clinical outcomes in lung cancer patients (60). This study found that hypermethylation in the upstream genetic loci was partly responsible for the downregulation of SEMA5A in lung adenocarcinoma cells, as has been previously reported for other tumor suppressor genes (45,46). In this study, when the cells were treated with the methylation inhibitor, 5-aza, the upregulation of SEMA5A was observed only in the cancer cell lines. Pyrosequencing results further identified the methylated CpG sites modulating the expression of SEMA5A in these cell lines, suggesting that aberrant methylation changes result in the inactivation of SEMA5A in lung adenocarcinoma cells (Fig. 2B and C). However, additional studies using larger numbers of tissue samples that contain clinical features are required to validate whether methylation changes of SEMA5A are involved in tumorigenesis.

Furthermore, this study demonstrated that SEMA5A can be possibly cleaved by ADAM17, which belongs to the protein family of disintegrins and metalloproteases (Fig. 3C and D). ADAM17 is involved in the release of a soluble ectodomain from membrane-bound pro-proteins. It has also been reported to be upregulated in non-small cell lung cancer (61), and this upregulation of ADAM17 can be caused due to ionizing radiation (62). Moreover, the silencing of ADAM17 has been shown to suppress the migration and invasion of A549 cells in vitro, and tumor growth in vivo (63). Since previous studies have demonstrated that the mature form of SEMA5B is proteolytically processed by ADAM17 (14), and that the cleaved extracellular domain of SEMA5A decreases following the silencing ADAM17 (32), this study assessed the proteolytic effect of recombinant ADAM17 on SEMA5A. The results suggested that the membrane-bound SEMA5A was exported to the medium following cleavage by ADAM17. However, further clarifications are required to conclude whether the endogenous ADAM17 in lung adenocarcinoma cells has enough proteolytic activity to shed membrane-bound SEMA5A.

The extracellular domain of SEMA5A is involved in angiogenesis (36). There is evidence to indicate an increase in proliferation and the upregulation of anti-apoptotic genes (e.g., BCL-2 and BIRC5) following treatment of endothelial cells with the extracellular domain of recombinant SEMA5A (36). In addition, pancreatic cells transfected with the extracellular domain of SEMA5A exhibit a greater metastatic potential and an enhanced endothelial cell proliferative ability (64). These results suggest that the extracellular domain of SEMA5A plays a potential role in carcinogenesis.

However, this study found that the total amount of membrane-bound SEMA5A in lung adenocarcinoma cells was downregulated compared to normal lung cells. The transient overexpression of SEMA5A in lung adenocarcinoma cells had tumor-suppressive effects, such as decreasing cell proliferation, colony formation and migration (Figs. 5 and 6), although not increasing apoptosis (data not shown). Furthermore, lower expression levels of SEMA5A were found to be associated with a worse prognosis (Fig. 1B), which suggested the tumor suppressive role of SEMA5A in lung adenocarcinoma. Consistent with the findings of this study, lower endogenous SEMA5A levels have been found to be associated with increased invasiveness in glioma. Furthermore, SEMA5A has been shown to impede the motility of human gliomas upon its interaction with Plexin-B3 via the indirect inactivation of RalGDPx, and the inactivation of protein kinase C (PKC) to phosphorylate fascin-1 (41,42). However, the transcriptional levels of RAC1 and FSCN1 did not alter significantly in this study (Fig. S1), and whether the levels and activity of these proteins are altered remains to be determined in lung adenocarcinoma cells.

On the contrary, a high expression of SEMA5A protein has been shown to be associated with poor overall survival outcomes in metastatic ovarian cancer (56) and to be associated with progression and metastasis in gastric cancer and pancreatic cancer (39,57). In explaining the discrepancy of the functions of SEMA5A, it was thus speculated that the cleaved extracellular domain and full-length of SEMA5A may carry out opposite functions in different cancer types. The alternative explanation is that receptor-ligand interactions generate simultaneous bidirectional signals (i.e., forward signaling and reverse signaling) with opposite functions (65-67). That is, the full-length of SEMA5A on the membrane may function as both a receptor and ligand to
generate simultaneous forward and reverse signals, whereas the cleaved extracellular domain may only initiate reverse signaling by serving as ligand for receptors on other cells. Therefore, it was hypothesized that these phenomena may mainly be due to different experimental settings and tumor types. Yet, further studies are warranted to explore the function of SEMA5A in different cancer types.

Finally, the functions of SEMA5A in lung adenocarcinoma were investigated by identifying the downregulated related genes using microarrays. Both pathway analysis and network analysis revealed that one function of SEMA5A-regulated genes was cell growth and proliferation (Fig. 4). Among these genes involved in growth and proliferation, a number of genes have been reported with similar functions in other types of cancer. For example, ARRD3 and CASP1 were found to be upregulated in this study. ARRD3 has been reported to suppress breast carcinoma invasion (68). The downregulation of the expression of CASP1 has been shown to result in the proliferation and invasion of breast cancer cells (69). In this study, the functions of SEMA5A were further validated in in vitro (Fig. 5) and in vivo (Fig. 6) experiments, demonstrating that SEMA5A truly plays a tumor-suppressive role in the proliferation and migration of lung adenocarcinoma cells. On the whole, the findings of this study may thus contribute to the development of novel therapeutic regimens for lung adenocarcinoma in the future.

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Availability of data and materials
All data have been deposited in the Gene Expression Omnibus (GEO, GSE114578). The other data and materials are available upon request from the corresponding authors.

Authors’ contributions
PHK, GL, YPS and LCL conceived and designed the experiments. PHK, GL and YPS performed the experiments. PHK, GL, YAC, MHT and EYC analyzed the data. MHT, EYC and LCL contributed reagents, materials and/or analysis tools. PHK, GL, YPS and LCL wrote the manuscript. All authors have reviewed and approved the final manuscript.

Ethics approval and consent to participate
This animal model followed protocols approved by the Institutional Animal Care and Use Committee of China Medical University and Hospital (animal protocol no. 2016-102).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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