

Knockdown of *COPA*, Identified by Loss-of-Function Screen, Induces Apoptosis and Suppresses Tumor Growth in Mesothelioma Mouse Model

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ABSTRACT

Malignant mesothelioma is a highly aggressive tumor arising from serosal surfaces of the pleura and is triggered by past exposure to asbestos. Currently, there is no widely accepted treatment for mesothelioma. Development of effective drug treatments for human cancers requires identification of therapeutic molecular targets. We therefore conducted a large-scale functional screening of mesothelioma cells using a genome-wide small interfering RNA library. We determined that knockdown of 39 genes suppressed mesothelioma cell proliferation. At least seven of the 39 genes—*COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A*—would be involved in anti-apoptotic function. In particular, the *COPA* protein was highly expressed in some mesothelioma cell lines but not in a pleural mesothelial cell line. *COPA* knockdown induced apoptosis and suppressed tumor growth in a mesothelioma mouse model. Therefore, *COPA* may have the potential of a therapeutic target and a new diagnostic marker of mesothelioma.

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Introduction

Malignant mesothelioma is a highly aggressive tumor arising from the serosal surfaces of the pleura, peritoneum, and pericardium [1,2]. About 75% of all cases involve the pleura, and the remaining involve the peritoneum or pericardium. Mesothelioma is associated with previous asbestos exposure with a latency of 30–40 years. The three main categories of mesothelioma are epithelioid (50–70% of all cases), sarcomatoid (7–20%), and mixed/biphasic (20–30%). This tumor was once rare, but the incidence is expected to increase worldwide over the next several decades as a result of widespread asbestos exposure, both occupational and environmental, in many countries [2–4].

Current treatments include some form of surgery, which may be combined with chemotherapy and/or radiation. However, the prognosis of patients with this multimodality therapy remains poor, with typical post-diagnosis survival being 8–18 months [1,2]. Significantly, traditional chemotherapy has yielded poor response rates (typically <15–20%) [1,2]. Thus, the lack of a highly effective therapeutic regimen for mesothelioma underscores the importance of finding new and more effective treatments.

To develop effective drugs for treatment of human cancer, it is important to identify therapeutic target molecules. Small interfering RNAs (siRNA) have been widely used in mammalian cells to define the

functional roles of individual genes, particularly in disease. In addition, the development of whole-genome siRNA libraries and use of high throughput loss-of-function screens have allowed systematic detection of genes required for disease processes such as cancer [5–7]. We previously established a high-throughput screening procedure [8,9] and performed a large-scale screening to identify radiation-susceptible genes [9]. In the present study, we screened potential drug target molecules in mesothelioma cells using the high-throughput screening assay with a genome-wide siRNA library, containing small double-stranded RNAs targeted to more than 8,500 human genes, and conducted *in vitro* and *in vivo* functional analysis of several genes identified by this screening.

Results

Primary screening

The siRNA library used in this study contained 8,589 siRNAs consisting of nine sub-libraries: ion binding, ion channel, kinase, membrane transporter, nucleic acid binding, phosphatase, receptor, transcription factor, and transporter (Table 1) in a 96-well format. We performed primary screening by transfecting each siRNA individually into human malignant mesothelioma cells 211H [10] and measured the remaining viable cells at four days after transfection as determined by a sulforhodamine B-based cell proliferation assay [8,9]. Following gene-specific siRNA transfection, we identified 383 genes for which <50% viable cells remained compared with mock-

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Table 1

Summary of genes in the siRNA library and results of the first screen using MSTO-211H mesothelioma cell line.

Library name	Number of genes in library	Genes showing reduced viability	
		<20% ^a	2–500% ^a
Ion binding	1,479	5	23
Ion channel	349	0	2
Kinase	800	5	24
Membrane Transporter	695	0	12
Nucleic acid binding	1,951	39	117
Phosphatase	599	0	3
Receptor	1,425	16	69
Transcription factor	995	7	36
Transporter	296	6	19
Total	8,589	78	305

^a % viable cells compared with mock transfected cells.

transfected cells (Table 1 and Supplementary Table 1). Of the 383 genes, 78 showed viability of <20%. These 78 most-effective genes had the following functional distribution: ion binding (5), kinase (5), nucleic acid binding (39), receptor (16), transcription factor (7), and transporter (6) (Table 1 and Supplementary Table 1).

Secondary screening

We synthesized additional siRNAs against the 78 genes identified in the primary screening and built an original sub-library containing 156 siRNAs (two distinct siRNAs for each gene) against these 78 genes in the 96-well format. Knockdown of each gene was achieved by each of two distinct gene-specific siRNAs, and we measured viable cells at 2, 4, and 6 days after transfection. For 39 genes, *COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, *RPL18A*, *OR5211*, *RPA1*, *EIF3C*, *WEE1*, *RPL8*, *RPL3*, *RPL19*, *EIF3A*, *RPS2*, *RPL6*, *SF3B1*, *KISS1R*, *EIF3I*, *LSM2*, *RPL4*, *RPL21*, *POLR2F*, *RPL27A*, *RPL7*, *SF3B4*, *RPL5*, *RPS18*, *RPLP1*, *RPL18*, *KPNB1*, *EIF3E*, *SF3B14*, *RPL11*, *EIF3G*, *RPL35A*, *RPS17*, and *SNRNP200*, both siRNAs reduced cell viability compared with the negative control (Figs. 1A and B, and Supplementary Fig. 1). For 20 other genes, *TBRG1*, *DIS3*, *OR2T27*, *RPS19*, *SETD1A*, *RFX3*, *PHF5A*, *EPS8L3*, *MAX*, *ASCL2*, *LCE3A*, *ZC3H8*, *C9orf98*, *WVVOX*, *ITPKA*, *FCGR3B*, *NDUFAF2*, *SCAND3*, *CAT*, and *XRXG*, only one siRNA reduced cell viability (Supplementary Fig. 1).

Apoptosis assay of mesothelioma cells transfected with siRNAs against seven genes

Based on the greatest negative impact on cell viability of siRNA-mediated knockdown of genes in our screen and their functional categories, we selected seven representative genes, *COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A*, for further analysis of their contribution to apoptosis. On treatment with specific siRNAs, the expression of these seven genes was reduced to <10% compared with the negative control, as assessed by real-time RT-PCR (Fig. 1C). We first stained nuclei of knockdown cells with Hoechst 33342 at 48 h after transfection and observed them under the fluorescence microscope. Apoptosis in cell populations transfected with gene-specific siRNAs targeting the seven selected genes apparently increased compared with cells transfected with the negative control siRNA (data not shown). Using flow cytometry, we then measured annexin V-positive apoptotic cells at 8, 24, and 48 h after transfection (Fig. 2). In the negative control, the apoptotic population was not increased at any time point after transfection. At 24 h after transfection, cells transfected with siRNAs targeting five genes, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A*, contained significantly more apoptotic cells compared with the negative control ($P < 0.01$, Fig. 2B), whereas cells transfected with *COPA* and *COPB2* siRNAs did not. At 48 h after transfection, the apoptotic population in all seven of the gene-knockdown cells was significantly increased compared with the

negative control ($P < 0.01$; Figs. 2A and B). Among cells transfected with the negative control or gene-specific siRNAs, the necrotic population of cells (negative for annexin V and positive for 7-AAD) was not increased at any time point (data not shown).

Functional analysis of *COPA* in mesothelioma cells

Among these 7 genes, because the *COPA* mRNA has been previously reported to be highly expressed in mesothelioma compared with normal tissues (tumor/normal ratio was 2.19) by microarray experiments [12], we focused on further functional analysis of *COPA*. To determine whether *COPA* protein is expressed in mesothelioma, we conducted immunoblotting analysis in four mesothelioma cell lines, 211H, H226, H2052, and H2452, and the pleural mesothelial cell line, MeT-5A. *COPA* protein was highly expressed in 211H, H2052, and H2452 cells, but not in H226 and MeT-5A cells (Fig. 3A). We examined the ability of *COPA* siRNAs to downregulate *COPA* mRNA in mesothelioma and the pleural mesothelial cell lines by real-time RT-PCR. At 24 h after siRNA-transfection, both siRNAs reduced *COPA* mRNA to <10% in 211H cells compared with the negative control siRNA (Fig. 1C). In H2052 and MeT-5A cells, *COPA* mRNAs were reduced to <40% compared with control (Fig. 3B). In H226 and H2452 cells, *COPA* siRNAs were insufficient to reduce *COPA* mRNAs compared with negative control (>60%; Fig. 3B). We then performed cell proliferation assay of *COPA* siRNA-transfected 211H, H2052, and MeT-5A. Both siRNAs (COPAsi1 and COPAsi2) reduced cell viability significantly to <10% (5.4 and 4.6%) and <35% (19.2 and 31.7%) in 211H and H2052, respectively, compared with the negative control, whereas *COPA* siRNA-transfected MeT-5A cells survived >60% (67.1 and 61.2%) compared with control (Fig. 3C).

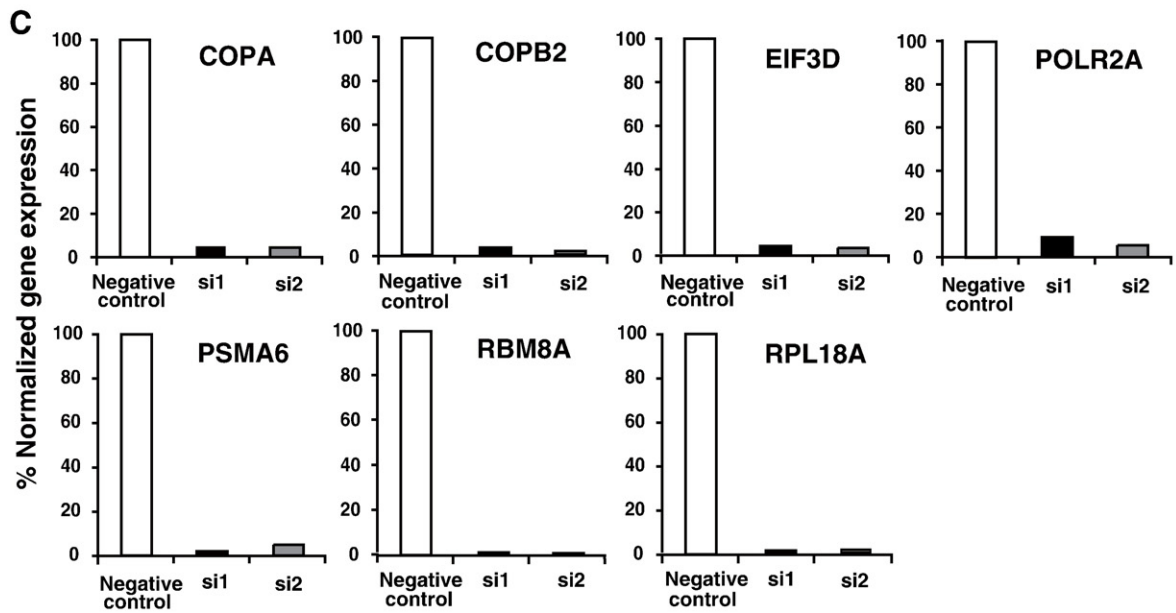
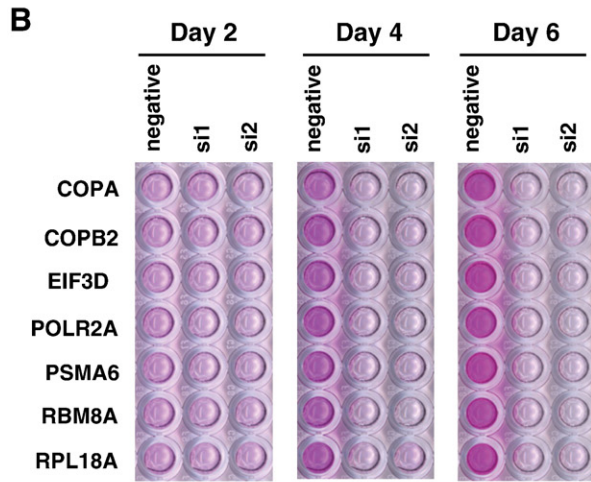
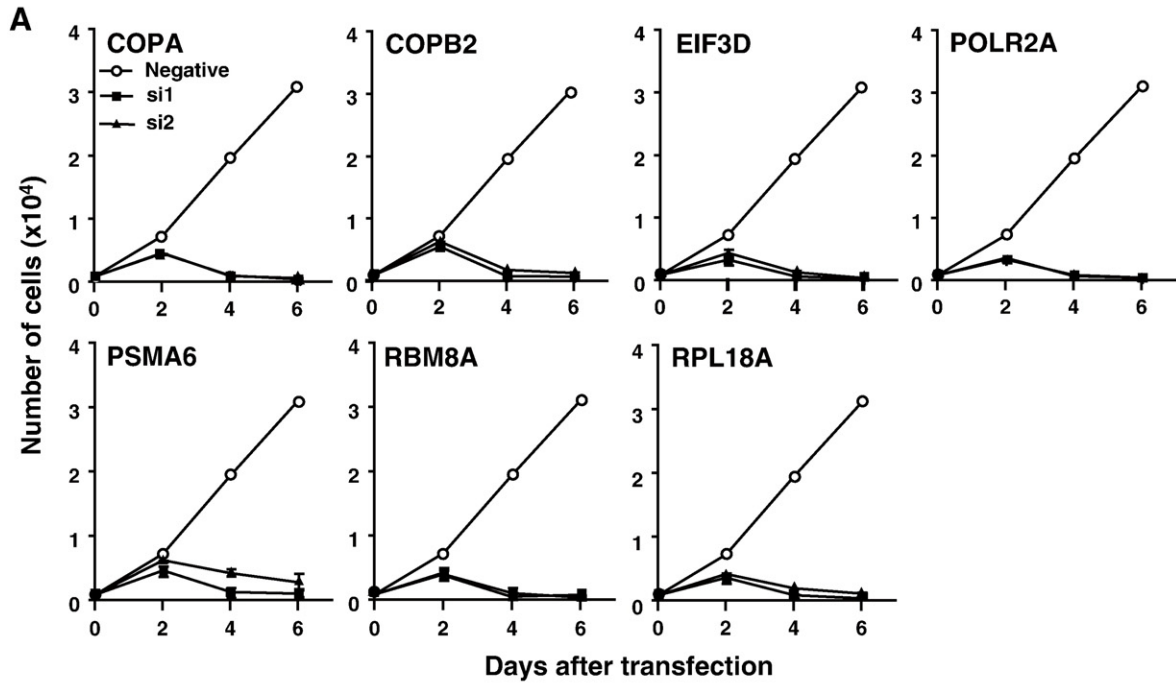
COPA siRNA treatment in a mesothelioma mouse model

To examine whether *COPA* siRNAs suppress tumor growth in a mesothelioma mouse model, we first conducted treatment experiments with the pretreatment protocol in which 211H cells transfected with *COPA* or negative control siRNAs were subcutaneously injected into nude mice. Fig. 4A shows that negative control siRNA-transfected cells formed tumors that grew linearly with time, whereas 211H cells transfected with both *COPA* siRNAs formed tumors with significant reductions in tumor growth compared with the negative control ($P < 0.01$). Second, we conducted a local injection protocol in nude mice bearing 211H xenografts. The untreated 211H cells were subcutaneously inoculated into nude mice, and *COPA* or negative control siRNAs were injected around xenografts twice. The treatment of both *COPA* siRNAs caused significant inhibition in tumor growth compared with the negative control siRNA ($P < 0.05$; Fig. 4B).

Immunohistochemical staining of 211H xenografted tumors was performed two days after second injection of siRNAs to evaluate apoptosis. We observed a marked increase of apoptotic cells in tumors treated with both *COPA* siRNAs compared with the negative control (Fig. 4C). From quantitative analysis of immunohistochemical staining, both *COPA* siRNAs caused 12- and 10-fold increases of TUNEL-positive cells, respectively, compared with the negative control siRNA ($P < 0.01$; Fig. 4D).

Discussion

We report here for the first time a large-scale loss-of-function screening to identify potential drug target molecules for mesothelioma treatments. We conducted a large-scale functional screening of a mesothelioma cell line, 211H, using a genome-wide siRNA library to identify potential drug target molecules. Of siRNAs to 383 genes that reduced mesothelioma cell viability by at least 50%, siRNAs to 78



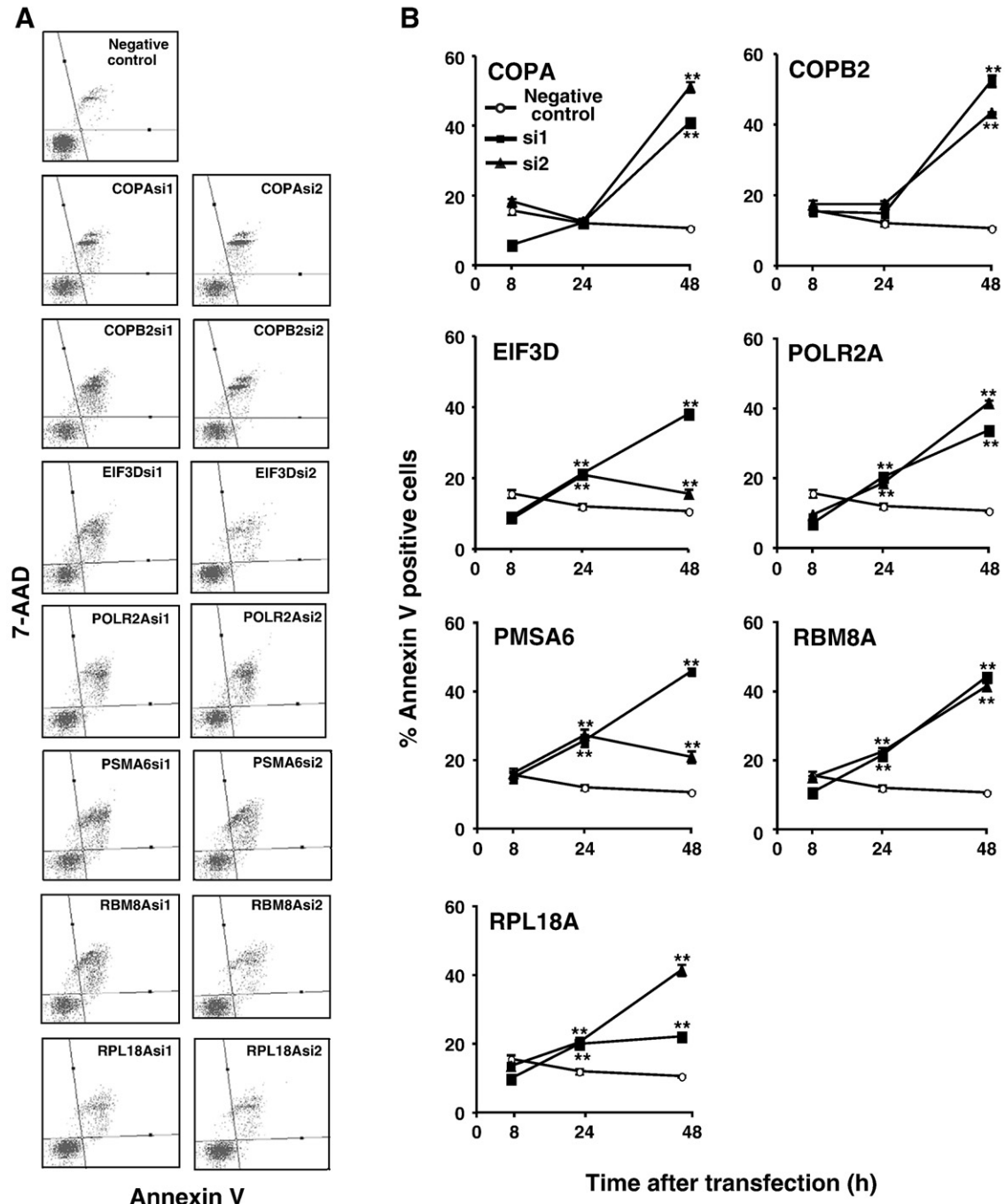


Fig. 2. Apoptotic analysis of 211H cells transfected with seven individual siRNAs against *COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A* and stained with annexin V and 7-amino-actinomycin D (7-AAD, DNA staining) at 8, 24, and 48 h after transfection. Living (annexin V negative and 7-AAD negative), necrotic (annexin V negative and 7-AAD positive), and apoptotic (annexin V positive) cells were determined by a Guava PCA system. (A) Representative flow cytometry dot plots at 48 h after transfection. (B) Mean percentage values of apoptotic cell from three independent experiments, as analyzed by ANOVA with the Student–Newman–Keuls method multiple comparison test (vs. negative control, $^{**}P < 0.01$). Data are presented as means \pm SD from three independent experiments. Open circles represent cells transfected with negative control siRNA, closed squares represent gene-specific siRNA (si1) of each gene, and closed triangles represent gene-specific siRNA (si2) of each gene.

Fig. 1. Cell viability and gene expression analysis of 211H transfected with seven genes, *COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A*. (A) Cell viability of 211H cells transfected with 14 siRNAs against seven genes selected by primary screening shown in Supplementary Table 1 and the negative control siRNA. Cell viability about more 71 genes were shown in Supplementary Fig. 1. Cell viability was measured by sulforhodamine B-based cell proliferation assay at 2, 4, and 6 days after transfection. Data represent mean \pm SD from three independent experiments. Open circles represent cells transfected with negative control siRNA, closed squares represent gene-specific siRNA (si1) for each gene, and closed triangles represent gene-specific siRNA (si2) for each gene. (B) Representative sulforhodamine B-stained cells at 2, 4, and 6 days after transfection. Cells transfected with negative control siRNA or gene-specific siRNAs targeting seven genes. (C) Gene expression analysis of 211H cells transfected with siRNAs. The cDNAs were directly synthesized from cells at 24 h after transfection with either negative control siRNA or gene-specific siRNAs targeting seven genes. Gene expression was determined by real-time RT-PCR with TaqMan probes. The expression level of each target gene was normalized to that of 18S ribosomal RNA. Data represent mean and SD from three independent experiments.

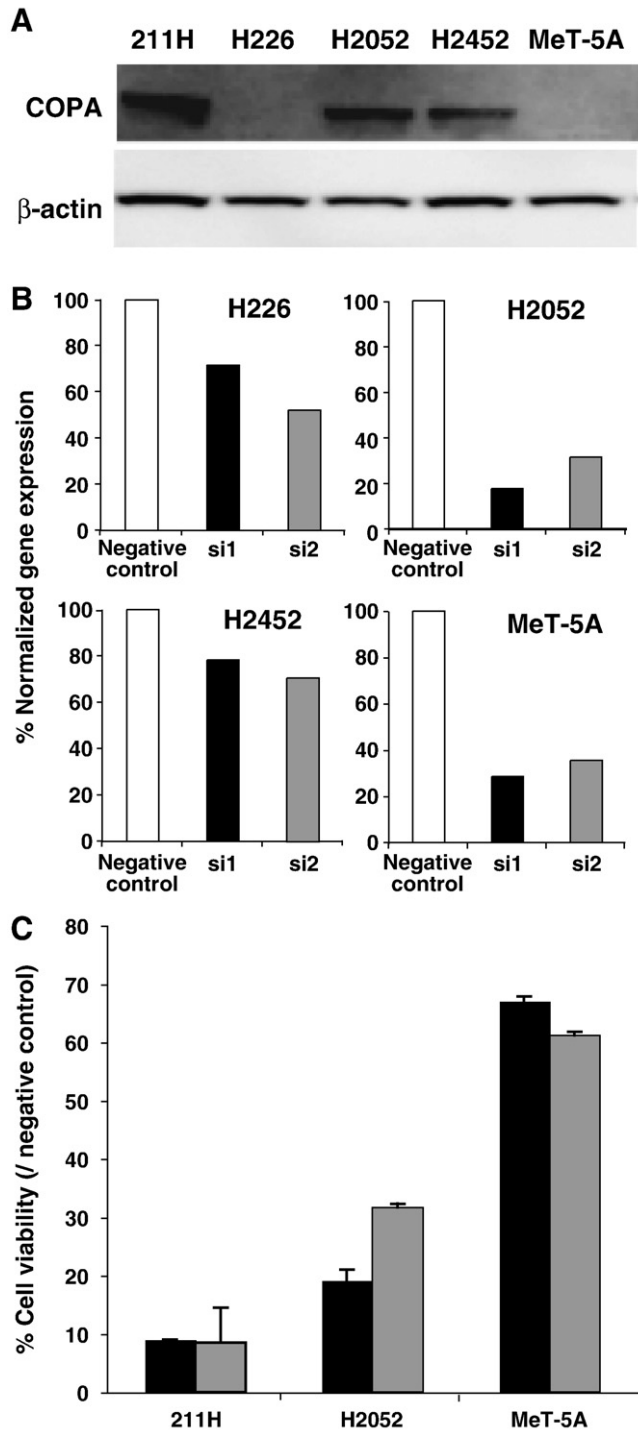


Fig. 3. *COPA* expression in mesothelioma cell lines and cell proliferation of *COPA* siRNA-transfected cells. (A) *COPA* protein expressions were detected by immunoblot analysis in mesothelioma cell lines (211H, H226, H2052, and H2452) and pleural mesothelial cell line (MeT-5A). (B) Expression of *COPA* mRNA was determined by real-time RT-PCR with TaqMan probes. Data represent mean and SD from three independent experiments. (C) Cell proliferation assay of *COPA* siRNA-transfected cells. Data are presented as means and SD from three independent experiments.

genes decreased viability by at least 80%, raising the possibility that these genes regulate cell viability in mesothelioma. To further explore this possibility, we newly synthesized and built an original sub-library containing 156 siRNAs against the above-mentioned 78 genes and assessed the effect of gene silencing on cell viability. Of these 78 genes, we found 39 genes that reduced cell viability at 4 and/or 6 days after transfection with gene-specific siRNAs, suggesting a crucial role for

these genes in mesothelioma cell viability and as potential drug targets for treatment. We focused on further functional studies of these 39 genes (mentioned below). For additional 22 genes, cell viability was reduced by one but not both gene-specific siRNAs. This result cannot conclusively establish whether the lack of significant reduction of viable cells resulted from insufficient knockdown in either siRNA or whether the result reflected an off-target effect [7,11]. To clearly identify which of these genes could be associated with cell viability in mesothelioma will require further investigation using additional siRNAs.

Of the 39 genes detected by this screening, almost all cells transfected with siRNAs died at 4 and/or 6 days after transfection. To determine whether apoptosis caused this cell death, we chose seven representative genes, *COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A*, based on the greatest negative impact on cell viability of siRNA-mediated knockdown of genes in our screen and their functional categories. According to the apoptosis assay of knockdown cells using microscopic and flow cytometric analyses, apoptosis of cell populations targeted with gene-specific siRNAs for each of the seven genes was significantly increased compared with cells with the negative control siRNA. This suggests that these genes can be involved in anti-apoptotic function. Interestingly, *COPA*, *COPB2*, *EIF3D*, and *PBM8A* have not been known to be associated with apoptosis. Thus, further investigation of these genes may lead to the elucidation of new molecular mechanisms of apoptosis.

The *COPA* mRNA has been previously reported to be highly expressed in mesothelioma compared with normal tissues [12]. To examine *COPA* protein expression in mesothelioma cells, we performed immunoblotting analysis for four mesothelioma cell lines (211H, H226, H2052, and H2452) and the pleural mesothelial cell line (MeT-5A). The *COPA* protein is highly expressed in 211H, H2052, and H2452 cells, but not in H226 and MeT-5A cells. This result suggests that *COPA* could be a new therapeutic target for mesotheliomas that highly express *COPA*. To test this possibility, we conducted cell proliferation assay in 211H, H2052, and MeT-5A cells, in which *COPA* siRNAs were sufficient to reduce *COPA* mRNAs for the assay. *COPA* siRNAs markedly reduced cell viability to <10% and <35% in 211H and H2052 cells, respectively, compared with the negative control, whereas MeT-5A cells survived >60%. We treated mesothelioma mouse models using *COPA* siRNAs with both protocols (pretreatment and local injection) and clearly showed that *COPA* siRNAs suppressed tumor growth and induced apoptosis. The pretreatment protocol was more effective than the local injection protocol. Because the transfection efficiency of mesothelioma cells in dishes is expected to be higher than that in mice, this result was probably due to the transfection efficiency of *COPA* siRNAs. If we could find a small inhibitor molecule of *COPA*, it would help clarify this point and be a potential drug compound for treatment of mesotheliomas that highly express *COPA*.

COPA is one of the seven non-clathrin-coated vesicular coat subunits that form the “coatome,” which plays a role in membrane transport between the endoplasmic reticulum and the Golgi apparatus [13,14]. *COPA* and *COPB2* have a tryptophan-aspartic acid (WD)-repeat motif and belong to a large conserved family of WD proteins found in all eukaryotes and implicated in a variety of functions ranging from signal transduction and transcriptional regulation to cell cycle control and apoptosis [15]. This report and our present results suggest that *COPA* and *COPB2* could play a role not only in membrane transport but also in apoptosis. Thus, further study of *COPA* and *COPB2* might help elucidate the molecular mechanism of apoptosis. *COPA* and *COPB2* have been reported to interact with platelet-derived growth factor β -receptor [16], which is a cell surface tyrosine kinase receptor, binds SH2 domain containing proteins, activates cell growth signaling, and is related to angiogenesis [17,18]. Tumor angiogenesis is a critical step in tumor development through which tumors establish independent nutrient and oxygen supply, consequently enhancing tumor

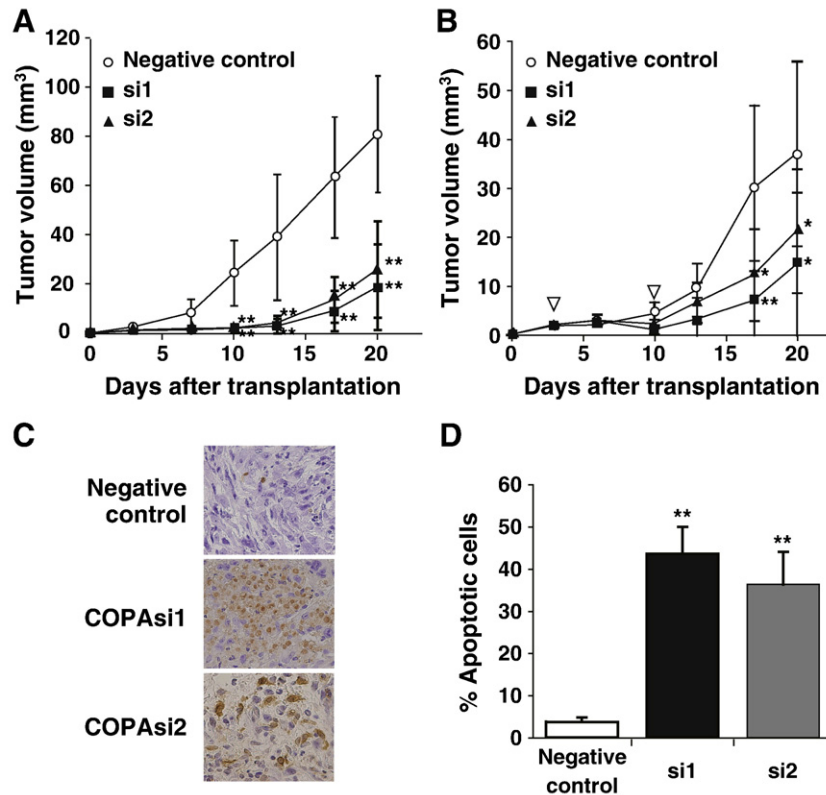


Fig. 4. Therapeutic efficacy of *COPA* siRNA *in vivo*. (A) For the pretreatment protocol, siRNA-transfected 211H cells were injected subcutaneously into nude mice, and tumor diameters were measured at a regular interval for up to 20 days and tumor volume was calculated. Results represent mean \pm SD ($n=5$) compared with negative control (** $P<0.01$). Open circles represent cells transfected with negative control siRNA, closed squares represent gene-specific siRNA (si1), and closed triangles represent gene-specific siRNA (si2). (B) For the local injection protocol, 3 and 10 days after inoculation of 211H cells, 10 nmol of *COPA* siRNAs or negative control siRNA was mixed with atelocollagen, and 100 μ l of each mixture was injected around tumor. Tumor diameters were measured at a regular interval for up to 20 days and tumor volume was calculated. Results represent the mean \pm SD ($n=4$ tumors) compared with negative control (* $P<0.05$, ** $P<0.01$). Open triangles represent siRNA injection. (C) The effect of *COPA* siRNA on apoptosis *in vivo*. Immunohistochemical analysis of 211H tumor xenografts stained with TUNEL (Magnification, 400 \times , Size bar, 50 μ m). (D) The ratio of the apoptotic cells. Data are presented as mean \pm SD compared with negative control (** $P<0.01$).

growth. *COPA* was highly expressed in some mesotheliomas according to our results and the previous report [12]. These findings suggest that *COPA* over-expression could play a role in mesothelioma development through anti-apoptosis and/or angiogenesis. Further studies would be important to explore the correlation between mesothelioma development and potential roles of *COPA* in anti-apoptosis and/or angiogenesis.

In conclusion, we conducted a large-scale functional screening in mesothelioma cells using a genome-wide siRNA library and determined that knockdown of 39 genes suppressed cell proliferation of mesothelioma cells, at least seven of which—*COPA*, *COPB2*, *EIF3D*, *POLR2A*, *PSMA6*, *RBM8A*, and *RPL18A*—are associated with an anti-apoptotic function. Functional characterization of these genes and their role in apoptosis pathways may provide clues to the underlying molecular mechanisms of cell death as well as to the development of novel therapeutic agents for mesothelioma. In particular, *COPA* may hold promise for the development of new therapy for malignant mesothelioma that highly express *COPA*.

Materials and methods

Cell culture

We obtained human malignant mesothelioma cell lines, 211H [10], H226, H2052, and H2452, and the human pleural mesothelial cell line MeT-5A from American Type Culture Collections (Manassas, VA). Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 5% fetal calf serum (JRH Biosciences, Lenexa, KS).

siRNA library

We purchased a human genome-wide siRNA library (siPerfect Library; RNAi Co., Ltd, Tokyo, Japan) containing small double-stranded RNAs against 8,589 human genes. This library consists of nine sub-libraries: ion binding, ion channel, kinase, membrane transporter, nucleic acid binding, phosphatase, receptor, transcription factor, and transporter. We also purchased a negative control siRNA and additional gene-specific siRNAs custom-synthesized by RNAi Co., Ltd.

Cell proliferation assay

We seeded 1.5×10^3 cells in each well of 96-well plates and transfected them with siRNA (5 nM) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 4 days, we performed cell proliferation assay using a sulforhodamine B-based Toxicology Assay kit (Sigma) as reported previously [8,9]. We estimated the numbers of cells based on a standard curve obtained by serial dilutions (10^4 to 625 cells).

Real-time quantitative reverse transcriptase-PCR

We seeded 1.5×10^5 cells in a 3-cm dish and transfected them with siRNA (5 nM). After 24 h, we synthesized first-strand cDNAs from knockdown cells using the FastLane Cell cDNA kit (Qiagen, Hilden, Germany). Predesigned and preoptimized TaqMan probes to detect genes of interest and 18S ribosomal RNA were purchased from Applied Biosystems (Foster City, CA). Real-time reverse transcriptase-PCR (RT-PCR) was performed in triplicate using a Premix Ex Taq

reagent (Takara-Bio, Otsu, Japan) on a Mx3000 (Stratagene, La Jolla, CA) real-time PCR instrument. Gene expression levels were normalized to 18S ribosomal RNA expression in each sample.

Apoptosis analysis with Hoechst 33342 staining

We seeded 1.5×10^5 cells in a 6-cm dish and transfected them with siRNAs (10 nM). After 48 h, we added Hoechst 33342 (3 μ l; 1 μ g/ml; Dojindo Laboratories, Kumamoto, Japan) to the culture medium and incubated the cells for 60 min. The culture medium was then removed and replaced with fresh medium. We observed the stained cells under a fluorescence microscope (Olympus, Tokyo, Japan).

Apoptosis analysis with annexin V staining

We transfected 1.5×10^5 cells with siRNAs (10 nM) and harvested them for 8, 24, and 48 h. The cells were stained with annexin V and 7-amino-actinomycin D (7-AAD) using a Guava PCA Nexin kit (Guava Technologies, Hayward, CA) as described previously [9]. We counted 2,500 events and identified living (annexin V negative, 7-AAD negative), apoptotic (annexin V positive, 7-AAD positive and negative), and necrotic cells (annexin V negative, 7-AAD positive) by a Guava PCA system (Guava Technologies). Data were analyzed by ANOVA with the Student–Newman–Keuls method multiple comparison test.

Immunoblot analysis

We lysed cells in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 μ g/ml leupeptin, and 0.2 M 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride. We estimated the protein concentration using the Quick Start Bradford Protein Assay kit (Bio-Rad, Hercules, CA). Cell lysates (40 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes using the iBlot Dry Blotting System (Invitrogen), and probed with anti-COPA polyclonal (Abcam, Cambridge, UK) or anti- β -actin monoclonal (Sigma) antibodies. We detected the primary antibodies using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (GE Healthcare, Little Chalfont, UK) and visualized them by the ECL Plus kit (GE Healthcare).

COPA siRNA treatment in mesothelioma mouse models

We obtained female nude mice (BALB/c-nu/nu, 5–6 weeks old) from CLEA Japan (Tokyo, Japan) and maintained them under specific pathogen-free conditions. For a pretreatment protocol, we transfected 211H cells (2×10^6) with COPA-specific (si1 or si2) or control siRNAs, and 24 h later we implanted the cells subcutaneously into the nude mice under ether anesthesia. The size of subcutaneous tumor was measured twice a week using a caliper. Tumor volume was calculated using the following formula: tumor volume (mm^3) = $(W \times H \times D)/2$, where W is width, H is height, and D is depth in millimeters. For a local injection protocol, we implanted 2×10^6 of 211H cells subcutaneously into nude mice under ether anesthesia. We treated the tumor-bearing nude mice with 10 nmol of COPA-specific (si1 or si2) or control siRNAs mixed at a ratio of 1:1 in atelocollagen (Koken, Tokyo, Japan) by local injections at 3 and 10 days after subcutaneous tumors had grown to approximately 20 mm^3 . We measured the tumor size and calculated the tumor volume. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of our institute.

TUNEL staining of xenografted tumors after treatment

We excised the tumors two days after the last siRNA treatment, fixed them in 10% neutral buffer formalin, and embedded them in paraffin for sectioning and detection of apoptosis. We detected apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) staining using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection kit (Chemicon International, Temecula, CA), according to the manufacturer's protocol. Briefly, we stripped nuclear proteins from DNA by incubation in proteinase K for 15 min at room temperature, blocked endogenous peroxidase with 0.3% H_2O_2 for 15 min, and incubated for 10 s with equilibration buffer. The sections were incubated in terminal deoxynucleotidyl transferase enzyme for 1 h at 37 °C. The reaction was terminated by incubation with stop buffer at room temperature. The sections were incubated with anti-digoxigenin conjugate for 30 min, and the reaction was developed with incubation in peroxidase substrate for 5 min. We quantified the TUNEL-stained cells in at least four randomly selected fields at 400 \times magnification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: [10.1016/j.ygeno.2010.02.002](https://doi.org/10.1016/j.ygeno.2010.02.002).

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