Repeated Aerosol Delivery of Carboxyl-terminal Modulator Protein Suppresses Tumor in the Lungs of K-ras^{LA1} Mice

Soon-Kyung Hwang¹, Hwang-Tae Lim^{1,2}, Arash Minai-Tehrani¹, Eun-Sun Lee¹, Jongmin Park³, Seung Bum Park³, George R. Beck, Jr.⁴, and Myung-Haing Cho^{1,2}

¹Laboratory of Toxicology, College of Veterinary Medicine, ²Nano Systems Institute–National Core Research Center, and ³Department of Chemistry, College of Natural Science, Seoul National University, Seoul, Korea; and ⁴Division of Endocrinology, Metabolism and Lipids, Emory University School of Medicine, Atlanta, Georgia

Rationale: Difficulties in achieving long-term survival of patients with lung cancer treated with conventional therapies suggest that novel approaches are required. Recent advances in aerosol-mediated gene delivery have provided the possibility of an alternative for the safe and effective treatment of lung cancer.

Objectives: To investigate the repeated effect of carboxyl-terminal modulator protein (CTMP) on multistage lung tumorigenesis. In this study, we addressed this question by studying the effects of lentivirus-based CTMP in the lungs of 9- and 13-week-old K-ras^{LA1} mice, a model of lung cancer.

Methods: An aerosol of lentivirus-based CTMP was delivered into 9- and 13-week-old K-ras^{LA1} mice, a model of lung cancer, through a nose-only inhalation system twice a week for 4 weeks. The effects of CTMP on lung cancer progression and Akt-related signals were evaluated.

Measurements and Main Results: Long-term repeated delivery of CTMP effectively reduced tumor progression in the lungs at different stages of development. Lentiviral-CTMP inhibited protein synthesis and cell cycle and altered Akt signaling pathway in the lungs of 9-week-old K-ras^{LA1} mice, and increased apoptosis was observed in the lungs of 13-week-old K-ras^{LA1} mice.

Conclusions: Long-term repeated viral delivery of CTMP may provide a useful tool for designing lung tumor treatment.

Keywords: lentivirus; carboxyl-terminal modulator protein; lung cancer; K-ras^{LA1} mice; aerosol gene delivery

Lung cancer is the most common cause of cancer-related deaths worldwide. The cure rate for lung cancer remains the lowest among all malignancies. Multiple options for the treatment of lung cancer have been described, including surgery, chemotherapy, and radiation, but the therapeutic effect is typically transient and mostly absent with advanced disease (1). Therefore, new approaches to the treatment of lung cancer are needed. Therapeutic gene delivery to the lungs is an attractive strategy to correct a variety of pulmonary dysfunctions, such as lung cancer.

Different approaches for pulmonary gene delivery, such as intravenous injection and nasal or intratracheal instillation,

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Difficulties in achieving long-term survival of lung cancer patients require novel approaches for safe and effective treatment.

What This Study Adds to the Field

Long-term repeated viral delivery of carboxyl-terminal modulator protein may provide a useful tool for designing lung tumor treatment.

have been reported in various animal models (2, 3). However, these strategies are invasive or the methods may not be suitable for effective delivery of the gene of interest throughout pulmonary tissues. In contrast, aerosol delivery targets the lungs specifically and represents a noninvasive alternative for targeting genes to the lung. The delivery of genes via aerosol holds promise for the treatment of a broad spectrum of pulmonary disorders and offers numerous advantages over more invasive modes of delivery (4).

A variety of viral vectors have been developed for gene delivery to various organs, such as retrovirus, adenovirus, and adeno-associated virus. Among these vectors, those derived from retroviruses, such as lentiviral vectors, appear to be promising vehicles for gene delivery into respiratory epithelial cells by virtue of their ability to infect nondividing cells and mediate long-term persistence of transgene expression (5, 6). In this regard, we have already demonstrated that lentivirus-derived vector is an effective tool for aerosol delivery and the aerosol delivery efficiency of lentivirus-derived vector was confirmed by green fluorescence protein (GFP) expression (7).

The serine–threonine kinase Akt (protein kinase B) is an important regulator of cell survival and cell proliferation. Akt signaling has been implicated in cancer progression through its ability to regulate cellular proliferation, inhibit apoptosis, and modulate protein translation (9, 10). Akt signaling has been found to be involved in the survival and proliferation of a variety of tumor cells (8). Akt activity is regulated by dual phosphorylation of regulatory residues, Thr308 in the activation loop of the catalytic domain and Ser473 in the regulatory domain (11). Amplification of genes encoding Akt isoforms have been found in many tumors (12). Carboxyl-terminal modulator protein (CTMP) acts as a negative regulator of Akt by binding to Akt and subsequently inhibiting its phosphorylation at threonine 308 and serine 473 (13). Thus, enhancement of CTMP may suppress Akt and its related signaling pathways.

Lung cancer progression (from adenoma to adenocarcinoma) is the most important step in the progression of lung

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Correspondence and requests for reprints should be addressed to Myung-Haing Cho, D.V.M., Ph.D., Laboratory of Toxicology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea. E-mail: mchotox@snu.ac.kr

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carcinogenesis. Adenocarcinoma, a subtype of non-small cell lung cancer (NSCLC), is the single most common form of lung cancer, making up ~40% of cases. Activating mutations in the *K-ras* protooncogene are found in ~30% of NSCLC (14). Moreover, *K-ras* gene mutation enhances motility of lung adenocarcinoma cells via Akt activation and radioresistance of *K-ras* mutated human tumor is mediated by the Akt pathway (15). Primary lung tumors in these mice have morphologic, histologic, and molecular features similar to human lung adenocarcinoma (16). In this study, *K-ras*^{LA1} mice, a laboratory animal model of NSCLC, was used to determine the *in vivo* effects of repeated aerosol-delivered CTMP on lung tumorigenesis. In *K-ras*^{LA1} mice, oncogenic alleles of *K-ras* can be activated on a spontaneous recombination event and mice carrying these mutations are highly predisposed to a range of tumor types from hyperplasia/dysplasia to carcinoma, similar to human NSCLC early-onset lung cancer (17).

In this study we investigated the long-term effects of CTMP overexpression on lung tumor development. Here, we report that repeated aerosol delivery of lentivirus-CTMP significantly suppresses tumor growth at different stages of development in the lungs of *K-ras*^{LA1} mice through altering Akt-related signals.

Our results support the notion that lentivirus may act as an effective carrier, and that repeated aerosol delivery of CTMP tumor suppressor gene may be a promising approach for lung cancer therapy.

METHODS

Materials

Monoclonal antibodies against Akt1 and phospho-Akt at Ser473 were generated via a general method described elsewhere (7). Antibodies against phospho-Akt at Thr308, PDK1, cyclinD1, cyclin-dependent kinase (CDK)-2, CDK4, proliferation cell nuclear antigen (PCNA), p70S6K, phospho-p70S6K (Thr389), p21, p27, 4E-BP1, Apaf-1, cytochrome c, CD31 (platelet endothelial cell adhesion molecule-1), vascular endothelial growth factor (VEGF), matrix metalloproteinase protein-2 (MMP-2), Bax, Bad, Bcl-xL and Bcl-2 antibodies were derived from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH antibody was obtained from BD Biotechnology (San Jose, CA). Mammalian target of rapamycin (mTOR), phospho-mTOR (Ser2889), and phospho-4EBP1 (Thr69) and caspase-3 antibody were obtained from Cell Signaling (Beverly, MA). Polyclonal CTMP antibody was prepared using a general method described elsewhere. pEntry 1A-TOPO vector, pLenti4/CMV/V5-DEST vector kits, and lentiviral expression plasmids were purchased from Invitrogen (Carlsbad, CA).

Construct of Lentivirus-CTMP

Lentivirus-CTMP was prepared by amplifying the Flag-CTMP (a kind gift of Dr. Brian Hemmings, FMI, Switzerland) with the primers (forward primer sequence 5'-ACGCGTCGACATGCTGAGGAGCTGCGCC-3' and reverse primer sequence 5'-CCGCTCGAGTGTCA GACTTTTAGCAGGATTC-3'). The PCR products were subcloned between Sall and XhoI sites of the entry vector, pEntry 1A-TOPO vector, using the pEntry directional TOPO cloning kit (Invitrogen).

Preparation of Lentivirus-CTMP and Titration

A total of 5×10^6 293T cells were seeded in 10-cm diameter dishes 24 hours prior to transfection in Dulbecco's modified Eagle medium (WelGENE, Daegu, Korea) with 10% fetal bovine serum and penicillin (100 IU/ml), in a 5% CO₂ incubator. A total of 12 µg of plasmid DNA was used for the transfection of one dish: 3 µg of expression plasmid, and 9 µg of transfer vector plasmid viral power packaging plasmids (pLP1, pLP2, pLP/VSV-G). An equal volume of lipofectamine reagent was added to this DNA mixture and the complexes were incubated for 20 minutes at room temperature prior to transfection. After 24 hours, the medium (10 ml) was replaced with Dulbecco's modified Eagle medium. The viral supernatant was collected after

48 hours. The viral concentration was determined using an HIV-1 p24 ELISA kit (Perkin Elmer, Boston, MA).

In Vivo Aerosol Delivery of Lentivirus-CTMP

Experiments were performed on C57BL/6 (three mice/group), 9-week-old male and 13-week-old male *K-ras*^{LA1} mice (eight mice/group). The breeding mice were obtained from Human Cancer Consortium-National Cancer Institute (Frederick, MD) and kept in the laboratory animal facility with temperature and relative humidity maintained at $23 \pm 2^\circ\text{C}$ and $50 \pm 20\%$, respectively, under a 12-hour light/dark cycle. All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-070516-2). For the effects of CTMP against lung cancer development, the *K-ras*^{LA1} mice were divided into three groups. The control group was not treated with anything and the other two groups were exposed to aerosol containing lentivirus-CTMP or lentivirus expression vector alone. The *K-ras*^{LA1} mice were placed in a nose-only exposure chamber and exposed to aerosol containing lentivirus-CTMP solution (50 ml) containing 40 ng/ml of lentivirus-CTMP twice a week for a total of 4 weeks (7). At the end of the study, the mice were killed and the lungs were collected. During the autopsy procedure, the neoplastic lesions of lung surfaces were carefully counted and the lesion diameter was measured with the aid of digital calipers under microscope as described by Singh and colleagues (18). The volume of tumors with diameter greater than 1.5 mm was calculated based on formulas described elsewhere (19). Simultaneously, the lungs were perfused and fixed in 10% neutral-buffered formalin for histopathological examination and immunohistochemistry (IHC). Remaining lungs were stored at -70°C for further analysis. For delivery efficiency and analysis of the effects of CTMP on normal lung, C57BL/6 mice were exposed to aerosol containing two different concentrations of lentivirus-GFP or CTMP for 30 minutes. Two days after exposure the mice were killed and lung samples were collected for further experiments (see Figure E1 in the online supplement).

Western Blot Analysis

After measuring the protein concentration of the homogenized lysates using a Bradford kit (Bio-Rad, Hercules, CA), equal amounts (50 µg) of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia, Cambridge, UK). The membranes were blocked for 1 hour in Tris-buffered saline + Tween 20 containing 5% skim milk, and immunoblotting was done by incubating overnight at 4°C , and then with secondary antibodies conjugated to horseradish peroxidase for 3 hours at room temperature or overnight at 4°C . After washing, the bands of interest were pictured by luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan), and quantification of Western blot analysis was done by using MultiGauge version 2.02 program (Fujifilm).

Histopathological Analysis and IHC

The lung tissues were fixed in 10% neutral buffered formalin. Formalin-fixed, paraffin-embedded tissue sections were cut at 5 µm and transferred to plus slides (Fisher Scientific, Pittsburgh, PA). For histological analysis, the tissue sections were stained with hematoxylin and eosin. For IHC, the tissue section were deparaffinized in xylene and rehydrated through alcohol gradients, then washed and incubated in 3% hydrogen peroxide (AppliChem, Darmstadt, Germany) for 30 minutes to quench endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS), the tissue sections were incubated with 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature to block the unspecific binding sites. Primary antibodies were applied on tissue sections overnight at 4°C . The following day, the tissue sections were washed and incubated with secondary horseradish peroxidase-conjugated antibodies (1:50) for 2 hours at room temperature. After washing, tissue sections were counterstained with Mayer's hematoxylin (DAKO, Carpinteria, CA) and washed with xylene. Cover slips were mounted using Permount (Fisher Scientific), and the slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY). Quantification of staining intensity of CTMP, phospho-p70S6K, caspase-3, VEGF, CD31, MMP-2, and PCNA was performed using In Studio version 3.01 program (Pixera, San Jose, CA). Staining intensity was assessed by counting the number of positive cells in randomly selected fields viewed with appropriate magnification of objective lens.

In Situ Detection of Apoptosis

We used a terminal deoxynucleotidyl transferase-mediated dUDP-nick-end labeling (TUNEL) kit (Roche, Basel, Switzerland) to identify apoptotic cells in formalin-fixed, paraffin-embedded lung sections, as previously described. Apoptotic cells within lungs were visualized at 1,000 $\times$ magnification and scored by counting positive cells in five individual lungs for each lung sample.

Data Analysis

Quantification of Western blot analysis was conducted using Multi-Gauge version 2.02 programs (Fujifilm). All data are given as mean $\pm$ SE, and statistical differences among treatment groups were analyzed via Student *t* test (Graphpad Software, San Diego, CA). The statistical analysis was performed separately for 9- and 13-week-old *K-ras*^{LA1} mice. *P* < 0.05 was considered significant and *P* < 0.01 highly significant compared with the corresponding control values.

RESULTS

Repeated Aerosol Delivery of Lentivirus-CTMP Suppressed Multistage Lung Tumorigenesis in the Lung of *K-ras*^{LA1} Mice

To determine whether repeated aerosol delivery of lentivirus-CTMP might affect multistage lung tumorigenesis, CTMP or control was delivered twice a week for one month to *K-ras*^{LA1} mice. Pathological analysis was performed in the lungs and results revealed CTMP delivery suppressed lung tumor mass in the lungs of 9- and 13-week-old mice (Figure 1A). The suppression of lung tumor formation was further confirmed by histological examination (Figure 1B). The nodules that developed in the lungs of control (CON) and vector control (VEC) groups at 9 weeks of age were in general alveolar/bronchial adenocarcinoma, whereas only lung adenomas were observed in the CTMP-treated group (CTMP) in which progression to adenocarcinoma was inhibited (Table 1). The low-grade adenocarcinomas were observed in the lungs of 13-week-old CTMP-treated mice compared with control and vector control (Table 2). Furthermore, the number and volume of tumors were significantly decreased by lentiviral-CTMP delivery in the lungs of 9- and 13-week-old mice as shown in Tables 1 and 2. In summary, these findings demonstrated that aerosol delivery of CTMP suppressed tumor progression to lung adenocarcinomas. These findings support the idea that repeated aerosol delivery of CTMP can suppress tumor growth and development.

Repeated Aerosol Delivery of Lentivirus-CTMP Inhibited Akt Activity Multistage Lung Tumorigenesis in the *K-ras*^{LA1} Mice

In a recent study on cell lines from NSCLC, it was observed that constitutive activation of the Akt/PI3K pathway promotes cell survival, and its genetic or pharmacological modulation alters the response to different therapeutic approaches (20). Previously we reported that a single aerosol delivery of lentivirus-CTMP to the *K-ras* null mice was sufficient to alter signals important for the Akt pathway (7). To investigate whether repeated aerosol delivery of lentivirus-CTMP would alter the Akt activity in the lungs of 9- and 13-week-old *K-ras*^{LA1} mice, Western blot analysis was performed to quantify protein expression levels of representative Akt signals. Our result showed that repeated aerosol delivery of lentivirus-CTMP significantly increased CTMP protein level (Figure 2A). Furthermore, IHC analysis demonstrated that aerosol-delivered lentivirus-CTMP increased the expression of CTMP in the lungs of 9- and 13-week-old *K-ras*^{LA1} mice (Figure 2E). Measuring the relative intensity of staining for CTMP further supported this finding (Figure 2F). Lentivirus-CTMP delivery decreased PDK1 expression level and inhibited Akt1 activity through suppression of Akt1 phosphorylation at Ser473 and Thr308 (Figures 2A and 2B). Quantification of bands-of-

Figure 1. Tumor pathology of the lungs in *K-ras*^{LA1} mice. (A) Pathological examination of 9- and 13-week-old *K-ras*^{LA1} mice lungs were exposed to aerosols containing lentivirus-carboxyl-terminal modulator protein (CTMP) twice a week for total of 4 weeks. (B) Histological examination of 9- and 13-week-old *K-ras*^{LA1} mice lungs (upper panel, 9 weeks; lower panel, 13 weeks). White arrows and red circles indicate the adenocarcinoma in the lungs of *K-ras*^{LA1} mice ($\times 100$, bars represent 100 μ m). CON = control; CTMP = CTMP-delivered group; VEC = vector control.

interest clearly confirmed the result of Western blot analysis (Figures 2C and 2D).

Repeated Aerosol Delivery of Lentivirus-CTMP Suppressed mTOR/p70S6K Pathway in the Lungs of 9-Week-Old *K-ras*^{LA1} Mice

The Akt/mTOR pathway controls cellular protein translation through regulation of p70-kD ribosome protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) phosphorylation and protein translation closely related to cancer cell growth (21). In this study, we investigated the changes in protein expression of mTOR, phospho-mTOR (Ser2889), the p70-kD ribosomal protein S6 kinase (p70S6K), and phospho-p70S6K (Thr389), in the lungs of aerosol-treated mice. Our result showed that aerosol delivery of CTMP significantly decreased mTOR, phospho-mTOR (Ser2889), p70S6K protein, and phospho-p70S6K (Thr389) protein levels with reconfirmation by densitometric analysis (Figures 3A and 3B). Such suppressed phospho-p70S6K at Thr389 protein expression was distinctively demonstrated by

TABLE 1. SUMMARY OF TUMOR INCIDENCE IN THE LUNGS OF K-ras^{LA1} MICE

Group	Number of Mice	Tumor Numbers/Mouse			Adenocarcinoma Incidence	Adenoma	
		Total	>1.5 mm*	<1.5 mm†		++‡	+§
CON	8	25 ± 2.99	8 ± 1.00	17 ± 5.24	3	5	0
VEC	8	29 ± 3.60	9 ± 2.49	20 ± 6.06	4	4	0
CTMP	8	17 ± 1.6	5 ± 1.29¶	12 ± 3.35	0	4	4

Definition of abbreviations: CON = control; CTMP = carboxyl-terminal modulator protein–delivered group; VEC, vector control.

Nine-week-old K-ras^{LA1} mice were exposed to aerosols containing lentivirus-CTMP twice a week for a total of 4 weeks. At the end of the test period, the K-ras^{LA1} mice were killed, the lungs were collected, and the lesions on the surface of lungs were counted. In parallel, the lungs from five mice were fixed in 10% neutral buffered formalin for histological examination. Incidence and multiplicity of lung proliferative lesions were compared.

* Number of tumors of greater than 1.5 mm in diameter.

† Number of tumors of smaller than 1.5 mm in diameter.

‡ Alveolar/bronchial adenoma grade was moderate.

§ Alveolar/bronchial adenoma grade was mild.

|| $P < 0.01$ compared to control.

¶ $P < 0.05$ compared to control.

the relative intensity of staining for phospho-p70S6K at Thr389 (Figures 3C and 3D). Lentivirus-CTMP did not affect the protein level of total 4E-BP1; however, decrease in phospho-4E-BP1 (Thr69) was observed in lungs of K-ras^{LA1} mice (Figures 3A and 3B).

Repeated Aerosol Delivery of Lentivirus-CTMP Significantly Inhibits Proteins Important for Cell Cycle Regulation in the Lungs of 9-Week-Old K-ras^{LA1} Mice

The capability of aerosol-delivered lentivirus-CTMP to regulate cell growth through cell cycle control was examined by Western blot. Repeated aerosol delivery of lentivirus-CTMP significantly increased the protein levels of p27 and p21 (Figure 4A). Densitometric analysis confirmed Western blot results (Figure 4B). Moreover, aerosol delivery of lentivirus-CTMP suppressed the proteins important for cell cycle regulation, such as cyclinD1, CDK4, and CDK2 (Figure 4A), again confirmed by densitometric analysis (Figure 4B).

Repeated Aerosol Delivery of Lentivirus-CTMP Induced Apoptosis in the Lungs of 13-Week-Old K-ras^{LA1} Mice

To investigate whether the loss of cell proliferation capacity is associated with the induction of apoptosis in the lung, we examined the changes in protein expression of apoptosis-related proteins. Our result showed that aerosol delivery of CTMP significantly increased the levels of apoptosis-regulating proteins, such as Bad, Bax, Apaf-1, cytochrome c, and

caspase-3 (Figures 5A and 5B), although aerosol-delivered CTMP decreased Bcl-xL and Bcl-2 protein levels in the lungs of CTMP-delivered mice with densitometric analysis (Figures 5C and 5D). In addition, we measured the number of apoptotic cells by TUNEL assay and IHC analysis of caspase-3. The results revealed distinct apoptosis in the cells from CTMP-delivered lungs compared with control and vector control lungs, as measured by the presence of fragmented DNA, an indication of apoptotic cell death. IHC also clearly confirmed the increase of caspase-3 expression (Figure 5E). The quantitative analysis confirmed the significant increase of TUNEL and caspase-3–positive cells in the CTMP-treated lung compared with control and vector control lungs (Figure 5F).

Repeated Aerosol Delivery of Lentivirus-CTMP Inhibits Signals Important for Angiogenesis in the Lungs of 13-Week-Old K-ras^{LA1} Mice

Tumor angiogenesis is an important prognostic marker for many solid tumors, including lung cancer, that independently predicts pathologic stages of tumor progression, including malignant potential (18). Tumor angiogenesis can also be used to estimate tumor growth rate (22). Therefore, we examined the changes in protein expression of angiogenesis-related proteins. Our result showed that aerosol delivery of CTMP significantly decreased VEGF, MMP-2, and CD31 protein levels (Figures 6A and 6B). We also demonstrated that lungs from control and vector control

TABLE 2. SUMMARY OF TUMOR INCIDENCE IN THE LUNGS OF K-ras^{LA1} MICE

Group	Number of Mice	Tumor Numbers/Mouse			Tumor Volume mm ³ /mouse	Adenoma	Adenocarcinoma Incidence	
		Total	>1.5 mm*	<1.5 mm†			++‡	+§
CON	8	28 ± 0.53	14 ± 4.04	14 ± 4.8	10.02 ± 3.60	2	4	2
VEC	8	25 ± 0.68	13 ± 2.65	12 ± 1.6	8.56 ± 1.13	3	3	2
CTMP	8	14 ± 0.50	7 ± 1.0¶	7 ± 1.71¶	3.45 ± 0.70	3	1	4

Definition of abbreviations: CON = control; CTMP = carboxyl-terminal modulator protein–delivered group; VEC, vector control.

Thirteen-week-old K-ras^{LA1} mice were exposed to aerosols containing lentivirus-CTMP twice a week for a total of 4 weeks. After 4 weeks, the K-ras^{LA1} mice were killed, the lungs collected, and the lesions were counted. In parallel, the lungs from five mice were fixed in 10% neutral buffered formalin for histological examination. Incidence and multiplicity of lung proliferative lesions were compared.

* Number of tumors of greater than 1.5 mm in diameter.

† Number of tumors of smaller than 1.5 mm in diameter.

‡ Alveolar/bronchial adenoma grade was moderate.

§ Alveolar/bronchial adenoma grade was mild.

|| $P < 0.01$ compared to control.

¶ $P < 0.05$ compared to control.

Figure 2. Western blot analysis of carboxyl-terminal modulator protein (CTMP), Akt1, phospho-Akt (Ser473) and phospho-Akt (Thr389) proteins in the lungs of K-ras^{LA1} mice. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (A and B) Expression of CTMP, Akt1, PDK1, phospho-Akt (Ser473) and phospho-Akt (Thr308) proteins (A, 9 weeks; B, 13 weeks). (C and D) The bands of interest were further analyzed by densitometry (C, 9 weeks; D, 13 weeks). (E) Immunohistochemistry analysis of CTMP in the lung (upper panel, 9 weeks; lower panel, 13 weeks). Dark brown color indicates the expression (magnification, $\times 400$; bar = 20 μm). (F) Comparison of CTMP labeling index (left, 9 weeks; right, 13 weeks). CTMP-positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of diaminobenzidine-positive cell per 100 cells at $\times 400$ magnification. Each bar represents the mean $\pm$ SE (n = 8). * $P < 0.05$ was considered significant and ** $P < 0.01$ highly significant compared with corresponding control values. CON = control; CTMP = CTMP-delivered group; VEC, vector control.

Figure 3. Western blot analysis of Akt signaling pathway. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (A) Expression of mTOR, phospho-mTOR (Ser2448), p70S6K, phospho-p70S6K (Thr389), 4E-BP1 and phospho-4E-BP1 (Thr69) proteins in the lungs of 9-week-old *K-ras^{LA1}* mice. (B) The bands of interest were further analyzed by densitometry. (C) Immunohistochemistry analysis of phospho-p70S6K at Thr389 in the lung. Dark brown color indicates the expression (magnification, $\times 400$; bar = 50 μm). (D) Comparison of phospho-p70S6K at Thr389 labeling index. Phospho-p70S6K at Thr389 positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of diaminobenzidine-positive cells per 100 cells at $\times 400$ magnification. Each bar represents the mean $\pm$ SE (n = 8). * P < 0.05 was considered significant and ** P < 0.01 highly significant compared with corresponding control values. CON = control; CTMP = carboxyl-terminal modulator protein-delivered group; VEC = vector control.

group have high expression of VEGF, MMP-2, and CD31 as displayed by strong staining intensity with an antibody to VEGF, MMP-2, and CD31, whereas the staining intensity of lung from repeated aerosol-delivered CTMP group shows low expression

Figure 4. Analysis of signal proteins for cell cycle regulation. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (A) Expression of p27, p21, cyclinD1, CDK4, and CDK2 proteins in the lungs of 9-week-old *K-ras^{LA1}* mice. (B) The bands of interest were further analyzed by densitometry. Each bar represents the mean $\pm$ SE (n = 8). * P < 0.05 was considered significant and ** P < 0.01 highly significant compared with corresponding control values. CON = control; CTMP = carboxyl-terminal modulator protein-delivered group; VEC = vector control.

of VEGF, MMP-2, and CD31 (Figure 6C). The relative intensity of staining for VEGF, MMP-2, and CD31 confirmed such patterns, as shown in Figure 6D.

Repeated Aerosol Delivery of Lentivirus-CTMP Suppressed Cell Proliferation in the Lungs of *K-ras^{LA1}* Mice

It has been reported that epithelial cell proliferation in normal and neoplastic lungs can be assessed by quantifying the percentage of tumor cells that are positive for PCNA (23). To investigate the possible mechanisms by which CTMP reduced the number of lung tumors, we analyzed the effect of CTMP on tumor cell proliferation using an antibody to PCNA. Immunohistochemistry revealed that aerosol-delivered lentivirus-CTMP decreased the expression of PCNA in the lung of 9- and 13-week-old *K-ras^{LA1}* mice (Figures 7A and 7B). These results clearly indicated that aerosol-delivered lentivirus-CTMP suppressed cell proliferation in the lungs of treated mice.

DISCUSSION

The lungs are a common site of involvement by primary and metastatic malignant disease. Patients with malignancies in the lung have limited treatment options and are usually not treatable (24). Numerous investigators have studied the potential of delivering various therapeutic agents directly to the lungs and pulmonary lymphatics by nebulization (25). However, there are several barriers to overcome in lung cancer gene therapy. One of the biggest challenges is the design of appropriate vectors. A number of nonviral methods for gene transfer have been developed for lung cancer gene therapy, but nonviral vectors have limitations, such as low transfection efficiency and limited targeting. To overcome these problems, viral vectors were developed. Viral vector systems, including retroviruses, lentivi-

Figure 5. Effect of aerosol-delivered carboxyl-terminal modulator protein (CTMP) on apoptotic proteins. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (A) Expression of Bad, Bax, Bcl-xL, and Bcl-2 proteins. (B) Expression of Apaf-1, cytochrome c, and caspase-3 proteins in the lungs of 13-week-old *K-ras^{LA1}* mice. (C and D) The bands of interest were further analyzed by densitometry (C, 9 weeks; D, 13 weeks). (E) Terminal deoxynucleotidyl transferase-mediated dUDP-nick-end labeling (TUNEL) assay (upper panel, magnification $\times 1,000$; bar = 20 μm) and immunohistochemistry analysis of caspase-3 (lower panel, magnification $\times 400$; bar = 50 μm). (F) Quantitative analysis of TUNEL-positive cells and comparison of caspase-3 labeling index. Each bar represents the mean $\pm$ SE ($n = 8$). * $P < 0.05$ was considered significant and ** $P < 0.01$ highly significant compared with corresponding control values. CON = control; CTMP = CTMP-delivered group; VEC = vector control.

ruses, adenoviruses, and adeno-associated viruses, currently offer the best choice for efficient gene delivery (26). Among them lentiviral vectors have several advantages over other viral systems in that they are less immunogenic and are capable of transducing a wide range of different cell types (27). Our previous work reported that lentiviral vector systems enhanced gene delivery efficiency and showed aerosol gene delivery with lentivirus-CTMP was compatible with *in vivo* gene delivery. Also, our group reported that a single aerosol delivery of lentivirus-CTMP on the *K-ras* null mice was sufficient to alter signals important for Akt pathway regulation (7). However, the inhibitory effect of CTMP on the development and progression

of lung tumor is still unknown. To address this question, the current study was performed to elucidate the effects of repeated aerosol CTMP delivery on lung tumor at different stages of progression and growth. This study emphasizes the importance of developing effective and selective preventive options for lung cancer through extensive *in vivo* research into the therapeutic effects of CTMP.

Akt/PKB mediates a variety of biological functions, including cell cycle, protein synthesis, and inhibition of apoptosis (11). Also, Akt1 is implicated in the treatment resistance of NSCLC, suggesting that Akt1 inhibition is a key factor for specific cancer cell death (28). CTMP negatively regulates Akt1 activity by the

Figure 6. Effect of aerosol-delivered carboxyl-terminal modulator protein (CTMP) on lung tumor angiogenesis. Western blot analysis of vascular endothelial growth factor (VEGF), CD31, and matrix metalloproteinase protein (MMP)-2 proteins in the lungs of 13-week-old *K-ras^{LA1}* mice. (A) Expression of VEGF, CD31, and MMP-2 proteins. (B) The bands of interest were further analyzed by densitometry. (C) Immunohistochemical analysis of VEGF, CD31, and MMP-2 in the lung tumors (magnification, $\times 400$; bar = 50 μm). (D) Comparison of VEGF, CD31, and MMP-2 labeling index. VEGF, CD31, and MMP-2-positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of DAB-positive cells per 100 cells at $\times 400$ magnification. Each bar represents the mean $\pm$ SE (n = 8). * $P < 0.05$ was considered significant and ** $P < 0.01$ highly significant compared with corresponding control values. CON = control; CTMP = CTMP-delivered group; VEC = vector control.

binding of CTMP and inhibition of phosphorylation on serine 473 and threonine 308. In fact, our result showed that lentivirus-CTMP delivery induced overexpression of CTMP and suppressed Akt1 activity through inhibition of Akt phosphorylation at Ser473 and Thr308, respectively at 9 weeks and 13 weeks in *K-ras^{LA1}* mice (Figures 2A and 2B). The suppression of Akt1 activity by CTMP overexpression may play a key role in suppressing tumorigenesis at different stages of development (Tables 1 and 2, Figure 1).

A recent report indicated that approximately 90% of NSCLC was associated with activation of the Akt-mTOR-p70S6K pathway, and the activation of this pathway promoted cellular survival and resistance to chemotherapy or γ -irradiation (12). In mammals, Akt can phosphorylate mTOR on Ser2448 to activate this kinase, and subsequently activated mTOR leads to trans-

lation initiation through phosphorylation of p70S6K and 4E-BP1 protein. Overexpression of p70S6K has been found in many tumor cells and results in increased tumor size *in vivo*, whereas inhibition of p70S6K prevents cell proliferation (29). Our result indicated that mTOR, p70S6K, phospho-4EBP1 (Thr69) protein levels were significantly decreased by repeated aerosol delivery of lentivirus-CTMP in the lungs of 9-week-old *K-ras^{LA1}* mice suggesting that CTMP can regulate cell growth and protein translation through Akt-related signals (Figure 3).

The mTOR pathway plays an important role in cell proliferation by coupling cell growth with G1 to S progression. Progression through the cell cycle is regulated by CDKs, whose activity is inhibited by the CDK inhibitors (30). Activation of cyclin/CDK activities is required for cell cycle progression and G1/S transition is a response to growth factor stimulation (31,

Figure 7. Effect of aerosol-delivered lentivirus-carboxyl-terminal modulator protein (CTMP) on cell proliferation. (A) Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) in the lungs of K-ras^{LA1} mice (upper panel, 9 weeks; lower panel, 13 weeks). Dark brown color indicates the PCNA expression (magnification, ×400; bar = 50 μm). (B) Comparison of PCNA labeling index in lungs of K-ras^{LA1} mice (left, 9 weeks; right, 13 weeks). PCNA-positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of DAB-positive cells per 100 cells at ×400 magnification. Each bar represents the mean ± SE (n = 8). *P < 0.05 was considered significant and **P < 0.01 highly significant compared with corresponding control values. CON = control; CTMP = CTMP-delivered group; VEC, vector control.

32). On the other hand, the regulation of CDK inhibitors, such as p21 and p27, is frequently responsible for the inhibition of G1 cell cycle progression and for withdrawal from the cell cycle (33). In this study, the decreased levels of cyclinD1, CDK4, and CDK2, and increased levels of cell cycle inhibitors such as p27 and p21 suggest that CTMP can play a crucial role in cell cycle regulation in the lungs of 9-week-old K-ras^{LA1} mice (Figures 4A and 4B). Similar patterns of change in Akt and cell cycle-related signaling pathway were observed in the lungs of CTMP-delivered normal mice (Figure E2).

The Akt pathway has been reported to regulate activity of apoptosis and Bcl-2 family members (34). We demonstrated that the inhibition of akt1 activity may be sufficient to facilitate apoptosis and thus to inhibit angiogenesis. Our result showed that repeated aerosol delivery of lentivirus-CTMP significantly increased the expression levels of proapoptotic proteins, such as Bad and Bax, whereas a decrease in expression of antiapoptotic proteins, such as Bcl-2, was observed in the lungs of 13-week-old K-ras^{LA1} mice (Figure 5A). An increase in the Bax stimulates the release of cytochrome c from mitochondria into the cytosol. The cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 (35). Here, we report that repeated aerosol delivery of lentivirus-CTMP increased cytochrome c and apaf-1 protein levels and activated caspase-3 activity and apoptosis in the lungs of 13-week-old K-ras^{LA1} mice (Figure 5B). These results suggest that aerosol-delivered CTMP could facilitate apoptosis in the lung adenocarcinoma. Tumor angiogenesis is an important prognostic marker for many solid

tumors, including lung cancer, that independently predicts pathologic stages of tumor progression and tumor growth rate (36). VEGF is a multifunctional cytokine that increases microvascular permeability and directly stimulates endothelial cell growth and angiogenesis (22). Furthermore, degradation of the extracellular matrix by MMPs is a critical process in progression of malignant tumors, including NSCLC (37). Also, platelet endothelial cell adhesion molecule (PECAM-1/CD31), a member of the immunoglobulin superfamily expressed at high levels on endothelial cells, has been recently implicated in angiogenesis (38). In this study, we observed that repeated aerosol delivery of CTMP could inhibit VEGF, MMP2, and CD31 expression levels in the lungs of 13-week-old K-ras^{LA1} mice and therefore overexpression of CTMP may lead to a reduction of metastasis (Figure 6).

Our results demonstrated that lentivirus-mediated CTMP overexpression suppressed Akt activity and inhibited tumor progression. We have shown that tumor progression (from adenomas to adenocarcinomas) in the lungs of 9-week-old mice was inhibited by aerosol delivery of CTMP with a corresponding decrease in mTOR/p70SK signaling pathway and regulation of cell cycle, whereas CTMP aerosol delivery suppressed adenocarcinoma tumor growth in the lungs of 13-week-old mice and inhibited angiogenesis and induction of apoptosis. These results suggest that this *in vivo* approach has the potential for the elucidation of the relative roles of other specific signaling pathways in lung cancer development, which in turn may provide clues for clinically relevant targets for gene delivery in lung cancer patients.

In conclusion, repeated aerosol gene delivery may provide an effective noninvasive model of gene delivery and understanding the role of CTMP in the multistage lung tumorigenesis may be essential to developing effective therapeutics for lung cancer.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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