# Targeting *KRAS* Mutation-bearing Lung Cancer *In Vivo* by Pulmonary Surfactant–Adenovirus-mediated Gene Transfer

TAKUYA FUKAZAWA[1], YUTAKA MAEDA[2], JUNJI MATSUOKA[3], TOSHIRO ONO[4], KATSUMI MOMINOKI[5], TOMOKI YAMATSUJI[1], KAORU SHIGEMITSU[1], ICHIRO MORITA[1], ICHIRO MURAKAMI[6], HIROTOSHI TANAKA[7], MARY L. DURBIN[8] and YOSHIO NAOMOTO[1]

*[1]Department of General Surgery, Kawasaki Medical School, Okayama, 700-8505, Japan;*
*[2]Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229-3039, U.S.A.;*
*Departments of [3]Gastroenterological Surgery, [4]Radiation Research,*
*Shikata Laboratory Advanced Science Research Center, and [5]Animal Resources Advanced Science Research Center,*
*Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, 700-8558, Japan;*
*[6]Division of Molecular Pathology, Faculty of Medicine, Tottori University, Tottori, 683-8503, Japan;*
*[7]Division of Clinical Immunology, Advanced Clinical Research Center,*
*Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan;*
*[8]Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525,U.S.A.*

**Abstract.** *Pulmonary surfactant has been used as a carrier to deliver a therapeutic virus to dysfunctional lung cells that reside within an intricate lung structure. To investigate whether pulmonary surfactant enhances the efficacy of intratracheal instillation of a therapeutic virus to target KRAS mutation-bearing lung cancer in vivo, we developed a recombinant adenovirus that induces cell death only in lung cancer cells and injected the adenovirus into a mouse model of KRAS mutation-positive lung cancer intratracheally with and without surfactant. A therapeutic adenovirus that induces cell death only in lung cancer cells was constructed by combining a cancer-specific human telomerase reverse transcriptase (hTERT) promoter fused to CCAAT/enhancer-binding protein alpha (CEBPα) with a modified lung-specific Clara cell-specific 10-kDa protein (CC10) promoter fused to cytotoxic adenovirus type 5 early region 1A (E1A). CEBPα is induced only in cancer cells and activates the CC10 promoter, which in turn induces cytotoxic E1A, and causes cell death only in lung cancer cells in vitro. This adenovirus was intratracheally administered to the model mice (CCSP-rtTA/Tet-op-K-Ras4bG12D bitransgenic mice) in the presence and absence of pulmonary surfactant. Intratracheally administered therapeutic adenovirus with pulmonary surfactant spread to airways, as well as to the alveolar region of the lung, and caused a reduction of lung tumors developed. The therapeutic adenovirus without pulmonary surfactant spread only to airways and was ten-fold less effective in tumor reduction. Here, we demonstrate that pulmonary surfactant is an efficient tool to intratracheally deliver a therapeutic virus to treat KRAS mutation-positive lung cancer in vivo.*

*Correspondence to:* Takuya Fukazawa, MD, Ph.D., Department of General Surgery, Kawasaki Medical School, Okayama 700-8505, Japan. Tel: +81 862252111, Fax: +81 862328343, e-mail: FukazawaT@aol.com

*Key Words:* Pulmonary surfactant, *KRAS* mutation, CC10, intratracheal instillation, lung cancer, gene transfer.

Delivery of therapeutic virus into the lung is an attractive approach for treating lung diseases including lung cancer, cystic fibrosis, α1-antitrypsin deficiency, and pulmonary hypertension (1-10). Intratracheal instillation of a therapeutic virus has also been validated as a safer approach than systemic injection of the virus (11, 12). One of the drawbacks of this approach is the lack of means to effectively deliver the therapeutic virus to target different kinds of cells that reside within an intricate lung structure. Pulmonary surfactant has been shown to enhance delivery of recombinant adenovirus throughout the lung structure in normal rabbit lungs (13). Pulmonary surfactant is a mixture of phospholipids, neutral lipids, and pulmonary surfactant protein which are secreted into the air spaces by alveolar type II cells and Clara cells of the distal pulmonary epithelium (14-16). Pulmonary surfactant has biophysical properties such as rapid surface adsorption, surface spreading and film formation that are critical for normal lung function. Exogenous pulmonary surfactant administration is a standard treatment for premature infants (17-19). Thus, pulmonary surfactant is a clinically validated safe intratracheal delivery carrier. However, it is not known whether pulmonary surfactant is applicable for delivering a therapeutic virus to

lung cancer cells *in vivo*. In order to test the efficacy of pulmonary surfactant for virus delivery, we treated the *KRAS* mutation-positive lung cancer mouse model (CCSP-rtTA/Tet-op-K-Ras4bG12D bitransgenic mice) using a conditionally replicative adenovirus (CRAd) that selectively eradicates lung cancer in the presence or absence of the pulmonary surfactant. This model is a well-established lung cancer mouse model that conditionally expresses oncogenic mutant KRAS.G12D in lung epithelium and develops lung adenomas/adenocarcinomas in 2-3 months (20). KRAS and epidermal growth factor receptor (*EGFR*) are the two most commonly mutated oncogenes in lung cancer. Currently, there is no effective therapy for treating *KRAS* mutation-driven lung cancer. A treatment for this form of cancer is thus critically needed (21).

## Materials and Methods

*Tissues and cell lines*. Human pulmonary adenocarcinoma H441 and H358 cells, human breast cancer MCF7 cells and human cervical cancer HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 (H358 cells) with high glucose Dulbecco's modified Eagle's medium (H441 cells and MCF7 cells) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human squamous cell lung carcinoma cells H226Br were obtained from Dr John D. Minna (University of Texas Southwestern Medical Center) and grown in RPMI-1640 supplemented with 10% heat inactivated FBS. Normal human lung fibroblasts (NHLF) obtained from Clonetics (San Diego, CA,USA) were grown in culture medium supplied by the manufacturer. All cell lines were cultured in 10% $CO_2$ at 37˚C. Lysates of human adult lung tissue were obtained from Novus Biologicals (Littleton, CO, USA).

*Plasmids*. The luciferase reporter construct pGL.CC10 -2300 was generated by excising the promoter region of CC10 -2300/+49 from pCC10-2300 CAT (kindly provided by Dr. Jeffrey A. Whitsett, Cincinnati Children's Hospital Medical Center, OH, USA) (22) with *HindIII* and ligating into the pGL3-Basic luciferase reporter construct (Promega Madison, WI, USA). pGL.CC10-175(-175 1×) was generated by subcloning -175/+49 amplified from pGL.CC10 -2300 using the primers (5'ttt*BglII*agatct$^{-175}$gtgagctcagtttcaatggga aaagaaactgggtt and 5'-ttt*HindIII*aagctt $^{+49}$gggctgtctgtagatgtgggctg atgttgtaatgt). The PCR-generated fragment was digested with *BglII* and *HindIII*, and subcloned directly into the pGL3-Basic. The CC10 region -175/-28 was generated from pGL.CC10 -1050 using the primers (5'ttt*NheI*gctagc$^{-175}$ gtgagctcagtttca atgggaaaagaaactgggtt and 5'-aaa*BglII*agatct$^{-28}$tgttcctggcagccca caagaggtaggggcagggat). The PCR generated fragment was digested with *BglII* and *HindIII*, and subcloned into pGL.CC10$^{-175}$ (-175 1×), termed pGL.CC10 -175 2×. The plasmid pGL.CC10 -175 3× was constructed in the same manner. The CC10 region -1050/-28 was generated from pGL.CC10 -1050 using the primers (5'-ttt*KpnI*ggtacc$^{-1050}$ ctcgaagcgcctcttca ggtcttccccgatgtccag, and 5'-aaa*NheI*gctagc$^{-28}$ tgttcctggcagcccac aagaggtaggggcagggat). The PCR-generated fragment was digested with *Kpn1* and *NheI*, and subcloned into pGL.CC10 -175 2×), termed pGL.CC10 -175 2× 1050. The cDNA of CCAAT/enhancer binding protein α (CEBPα) was generated from pcDNA3.1(–) CEBPα kindly provided from Dr Lung-Chih Yu (23) using specific primers (5'ttt*HindIII*aagctt*KOSAK*gccacc atggagtcggccgacttctacgaggcgga and 5'-ttt*XbaI*tctagactacgcgcagt tgcccatggccttgaccaaggagc, digested with *HindIII* and *XbaI*, and subcloned into pGL.hTERT (24) after excising the luciferase gene (pGL.hTERT/CEBPα). Bicistronic plasmid phTERT/CEBPα • CC10 -175 2× 1050/Luc was constructed by excising hTERT/CEBPα and SV40 late polyadenylic acid signal fragment from pGL.hTERT/CEBPα and ligating it into CC10 -175 2× 1050/Luc. All of the recombinant plasmids were sequenced to ensure insert identity and proper insert orientation.

*Transient transfection reporter assays*. All transfections were carried out in six-well plates. Cells were seeded 24 hours prior to transfection at the following densities: $0.5\times10^6$/well for NHLF and $0.3\times10^6$/well for all other cells. Transfections were carried out with Lipofectin (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Transfected cells were harvested 24 hours post lipofection. The results of one representative experiment are presented as fold induction of relative light units normalized to β-galactosidase activity relative to that observed for the control vectors. Each experiment was repeated at least three times. Error bars indicate the standard deviation from the average of the triplicate samples in one experiment (24).

*Construction of the recombinant adenoviral vectors*. Bicistronic shuttle vector pShuttle hTERT/CEBPα • CC10 -175 2× 1050/Luc (pShuttle TCC/Luc) was constructed by excising hTERT/CEBPα with SV40 late polyadenylic acid signal fragment and CC10 -175 2× 1050/Luc with SV40 late polyadenylic acid signal fragment from pGL3. CC10 -175 2× 1050/Luc and ligating it into pShuttle. pShuttle TCC/E1A was constructed by ligating E1A into pShuttle TCC/Luc after excising the luciferase gene. Ad-TCC/E1A was generated by homologous recombination and plaque purified (25). The viral titer for each vector was determined by measuring absorbency at 260 nm and plaque assay. The optimal multiplicity of infection (MOI) was determined by infecting each cell line with Adenoviral vector expressing green fluorescent protein driven by cytomegalovirus promoter (Ad-CMV/GFP)(24) and assessing the expression of GFP by flow cytometric analysis. In *in vitro* experiments, all cells were infected with the recombinant adenoviral vectors at a multiphicity of infection (MOI) of 5000 viral particles per cell.

*Immunoblot analysis*. Cells were lysed in ice cold lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Cell lysates were clarified by centrifugation (10 min at 15,000 × *g* at 4˚C) and protein concentration was determined using the DC protein assay (BioRad, Hercules, CA, USA). Equal amounts of protein were separated on an sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was electrophoretically transferred to a Hybond PVDF transfer membrane (Amersham, Arlington Heights, IL, USA). The membrane was incubated with primary and secondary antibodies according to the Supersignal$^R$ West Pico chemiluminescence protocol (Pierce, Rockford, IL, USA) to detect secondary antibody binding. Antibody specific for TTF-1 was purchased from DAKO (Carpinteria, CA, USA). Anti-actin antibody was obtained from Sigma (St. Louis, MO, USA). Antibody specific for human CEBPα was obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody specific for Adenovirus type 5 E1A was obtained from Pharmingen, (San Diego, CA, USA). Secondary horseradish

peroxidase-conjugated goat anti-rabbit antibody and anti-mouse antibody were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

*Transgenic mice and doxycycline administration*. The tetracycline operator regulated K-Ras4bG12D (Tet-op-K-Ras4bG12D) responder mice were kindly provided by Dr William Pao (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The Clara cell secretory protein promoter-tet activator (CCSP-rtTA) mice were also kindly provided by Dr Jeffrey A. Whitsett (Cincinnati Children's Hospital Medical Center) (20). Tail DNA was isolated using Qiaprep Tail DNeasy isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Detection of the rtTA activator transgene and the K-Ras4bG12D transgene were performed using the following primers: rtTA: fwd, 5'-AAGGTTTAACAACCCG TAAACTCG-3 and rev, 5-GTGCATATAACGCGTTCTCTAGTG-3' (generates a 330-bp product); K-Ras4bG12D K-Ras fwd 5'-GGGA ATAAGTGTG ATTTGCCT-3' and rev 5' -GCCTGCGACG GCGGCATCTGC-3' (~300-bp product). Doxycycline (Sigma) was administered *via* the drinking water, freshly prepared twice a week, at a concentration of 500 mg/l.

*Pulmonary surfactant adenoviral suspensions and intratracheal administration*. Bovine lung source pulmonary surfactant, Surfacten (Mitsubishi Tanabe Pharma Corporation, Tokyo, Japan), was used that is approved for the treatment of respiratory distress syndrome in preterm infants. This pulmonary surfactant is made from organic solvent extracts of minced bovine lung and is supplemented with dipalmitoylphosphatidylcholine, tripalmitine, and palmitic acid and contains the hydrophobic pulmonary surfactant proteins SP-B and SP-C but not SP-A (26). Adenoviral vectors were diluted with sterile PBS or pulmonary surfactant to $1\times10^{10}$ or $1\times10^{11}$ viral particles per mouse and administered intratracheally in a total volume of 80 μl (27). The final concentration of pulmonary surfactant was adjusted to 10 mg/ml (13).

*Determination of LacZ gene expression*. Ten- to twelve-week-old mice of the ICR strain (Charles River Japan, Tokyo, Japan) were intratracheally administered with Ad-CMV/LacZ ($1\times10^{10}$ or $1\times10^{11}$ viral particles per mouse) in the presence or absence of pulmonary surfactant (N=8). Seventy-two hours after administration, mice were anesthetized *via* intraperitoneal injection of phenobarbital and sacrificed. For 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) staining, 5-μm frozen sections were fixed with 4% paraformaldehyde for 10 min before being stained with X-gal solution containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside at 37˚C for 8 hours. The sections were counterstained with Nuclear Fast Red (Sigma). For determination of *in situ* β-galactosidase expression, lungs were intratracheally instilled with X-gal solution described above. Lungs were incubated for 8 hours at 37˚C and then repeatedly washed with PBS and fixed with 2% buffered formalin. The quantitative expression of β-gal expression was evaluated by the β-Galactosidase Enzyme Assay System (Promega). The total protein content of the lung lysates was quantified using DC protein assay (BioRad) according to the manufacturer's protocol.

*A combination treatment of Ad-TCC/E1A with pulmonary surfactant in a KRAS-mutated lung cancer mouse model (CCSP-rtTA/Tet-op-K-Ras4bG12D bitransgenic)*. Ten weeks after doxycycline treatment, $1\times10^{11}$ viral particles of Ad-TCC/E1A, Ad-CMV/Luc or PBS with or without pulmonary surfactant was injected into CCSP-rtTA/Tet-op-K-Ras4bG12D bitransgenic mice (N=8) by an outer sheath of 21-gauge venous catheter (BD Biosciences, San Jose, CA, USA) using a mouse intubation system (Natsume Seisakusyo, Tokyo, Japan) during anesthesia with isoflurane. This treatment was performed three times every other week for CCSP-rtTA/Tet-op-K-Ras4bG12D bitransgenic mice along with six weeks for doxycycline administration. The mice were sacrificed 16 weeks after doxycycline administration.

*Histology and immunohistochemistry (IHC)*. The lungs were excised, fixed with 10 ml of 4% paraformaldehyde overnight, then washed once for 5 min in PBS, placed into 70% ethanol, and embedded in paraffin (28). Tissue or tumor sectioning and staining were performed in the Histology Laboratory in the Department of Gastroenterological Surgery at Okayama University Graduate School of Medicine and Dentistry. For immunohistochemical analysis of the E1A protein, tumors were fixed in 20% formalin, embedded in paraffin, and then cut into 4 μm sections. To retrieve antigens, the sections were baked, deparaffinized, and heated in citrate buffer (10 mM citric acid, pH 6.0) in a steamer. After endogenous peroxidase was inactivated with 1.5% $H_2O_2$/methanol for 10 min, the sections were incubated with mouse anti-E1A polyclonal antibody (Pharmingen; 1:200 dilution) or rabbit anti-CEBPα polyclonal antibody (Cell Signaling Technology; 1:200 dilution) for 1 hour and then with biotinylated goat anti-mice or rabbit IgG antibody for 30 min. The specific binding was visualized with an avidin-biotin-peroxidase reagent and its substrate diaminobenzidine tetrachloride (DAKO), and subsequent counterstaining with Mayer's Hematoxylin.

*Analysis of serum alanine aminotransferase (AST) and aspartate transaminase (ALT)*. Blood was drawn from the tail vein of the mice seven days after the intraperitoneal injection of Ad-CMV/LacZ, Ad-TCC/E1A ($1\times10^{11}$ viral particles per mouse) or PBS with or without pulmonary surfactant. The levels of serum AST and ALT were measured by SRL Inc. (Tokyo, Japan).

*Statistical analysis*. Statistically significant differences between means and medians of studied groups were evaluated using Student's *t*-test and the non-parametric Mann-Whitney *U*-test, respectively. An analysis of variance (ANOVA) test, where appropriate, was used to identify statistical significance for multiple comparisons. Statistical significance was defined as $p<0.05$.

## Results

*Development of a lung cancer-specific promoter system*. In order to target lung cancer cells without harming normal cells, we developed a lung cancer-specific promoter system. We designed the system by combining two constructs: i) a construct of a cancer-specific hTERT (a component of the telomerase promoter)-driven C/EBPα, a lung transcription factor (the CCAAT/enhancer binding protein α) with ii) a construct of a lung specific *CC10* promoter-driven E1A, a therapeutic protein (a cytotoxic adenoviral protein that confers adenovirus replication only in lung cancer cells). In theory, the lung transcription factor CEBPα that is induced by the *hTERT* promoter only in cancer cells activates the

lung-specific *CC10* promoter through its CEBPα-binding element only in lung cancer cells. The activated *CC10* promoter drives the cytotoxic E1A and induces cell death only in lung cancer cells but not in other types of cells including normal cells such as fibroblasts. The lung-specific *CC10* promoter is activated by CEBPα through the 100-bp region flanking the 5' end of the *CC10* gene (22). CEBPα driven by the *hTERT* promoter activates the *CC10* promoter containing the region -175-bp to -28-bp only in lung cancer H441 and H226Br cells (Figure 1A and 1B). To further enhance specificity and promoter activity in lung cancer cells, we made constructs of tandem repeats of the *CC10* gene (Figure 1A). We obtained the highest and the most specific promoter activity in lung cancer H441, H226Br and H358 cells when the promoter region -175-bp to -28-bp was double tandemerized (pGL.CC10 -175 2× -1050) (Figure 1B). The promoter activity of pGL.CC10 -175 2× -1050 was low in MCF7 breast cancer cells, HeLa cervical cancer cells and NHLF (Figure 1B). Thus, we successfully developed a lung cancer-specific promoter system, in which CEBPα driven by the *hTERT* promoter in cancer cells activates the modified lung *CC10* promoter through its CEBPα-binding elements and in turn induces a therapeutic gene only in lung cancer cells. We termed the modified promoter TCC (hTERT promoter-driven CEBPα and *CC10* promoter) (Figure 1C).

*Adenovirus TCC/E1A vector targets and kills only lung cancer in vitro*. In order to kill lung cancer cells, we then fused the TCC promoter to adenoviral E1A cDNA and constructed a CRAd that selectively infects and lyzes cancer cells (Ad-TCC/E1A) (Figure 2A). Ad-TCC/E1A reduced the cell viability of H441 and H226Br lung cancer cells but not MCF7 breast cancer cells or NHLF lung fibroblasts (Figure 2B), demonstrating the specificity of the TCC construct. E1A protein was induced in Ad-TCC/E1A-infected H441 and H226Br lung cancer cells but not in MCF7 breast cancer cells and NHLF lung fibroblasts (Figure 2C). Ad-TCC/E1A induced cell death/lysis in H441 and H226Br lung cancer cells but not in MCF7 breast cancer cells and NHLF lung fibroblasts. The control vector (Ad-CMV/Luc) did not induce any cell death in these cells (Figure 2D). These results confirm that Ad-TCC/E1A induces cell death/lysis specifically in lung cancer cells.

*Pulmonary surfactant enhances adenovirus-mediated gene expression in mouse lungs*. Previously, pulmonary surfactant was demonstrated to enhance adenovirally mediated gene transfer and transgene expression in rabbit and rat lungs (13, 29, 30). Here we tested whether pulmonary surfactant would also facilitate adenovirus-mediated gene expression in mouse lungs. When adenovirus expressing LacZ under the control of the CMV promoter (Ad-CMV/LacZ) was intratracheally administered with pulmonary surfactant into mouse lungs, high levels of β-gal expression was observed in all lobes in both mouse lungs. On the other hand, when Ad-CMV/LacZ was intratracheally injected without pulmonary surfactant, β-gal expression was not widely observed (Figure 3A). We further assessed gene transfer using X-gal stained sections produced from Ad-CMV/LacZ-infected lungs with or without pulmonary surfactant. As shown in Figure 3B, β-gal expression was observed in distal airways and alveoli in lungs from mice infected with Ad-CMV/LacZ in the presence of pulmonary surfactant. In contrast, β-gal expression was limited to proximal airways in lungs from mice infected with Ad-CMV/LacZ in the absence of pulmonary surfactant. We also analyzed levels of β-gal expression quantitatively. We harvested Ad-CMV/LacZ-infected mouse lungs treated with or without pulmonary surfactant and measured β-gal activity using lung cell lysates. Significantly higher levels of β-gal activity were observed in lungs from mice infected with Ad-CMV/LacZ with pulmonary surfactant. Pulmonary surfactant increased the efficacy of gene transfer 8.34-fold after infection with $1\times10^{10}$ viral particles of Ad-CMV/LacZ and 10.2-fold more after infection with $1\times10^{11}$ viral particles infection (Figure 3C). These results suggest that pulmonary surfactant increases adenovirus vector-mediated gene expression and distribution in normal mouse lungs after intratracheal administration.

*Intratracheal administration of adenovirus TCC/E1A vector with pulmonary surfactant reduced tumors in a KRAS mutation bearing lung cancer mouse model*. In order to test our Ad-TCC/E1A in an *in vivo* model, we intratracheally administered Ad-TCC/E1A with pulmonary surfactant into a KRAS mutation bearing mouse model mice in which K-Ras4b (G12D) is conditionally expressed in lung epithelium under the control of doxycycline. This mouse model develops pulmonary adenoma/adenocarcinoma 2-3 months after doxycycline administration (20). Ten weeks after doxycycline administration, Ad-TCC/E1A was intratracheally administered to the mice, with or without pulmonary surfactant every other week for 6 weeks (total of 3 injections). Sixteen weeks after doxycycline administration, multiple solid tumors were observed over all the lungs (Figure 4A, left panel). Whether used alone or in combination, pulmonary surfactant and control Ad-CMV/Luc adenovirus did not have any effect on the tumors. Ad-TCC/E1A without pulmonary surfactant reduced the number of tumors only slightly (40 tumors reduced to 30 tumors), while Ad-TCC/E1A with pulmonary surfactant significantly reduced the number of tumors (40 tumors reduced to 2) (Figure 4A, right panel).

In order to confirm tumor-specific replication of Ad-TCC/E1A in lung tumors, we intratracheally injected Ad-TCC/E1A into both non-doxycycline- (no tumor) and doxycycline (tumor)-treated *KRAS* mice. As shown in Figure

4B (left panels), human ectopic CEBPα and E1A were not expressed in non-doxycycline -treated mice infected with Ad-TCC/E1A. However, human ectopic CEBPα and E1A were highly expressed in doxycycline-treated mice infected with Ad-TCC/E1A (Figure 4B, right panels), indicating that Ad-TCC/E1A replicates only in lung tumor but not in normal lung cells.

*Minimal toxicity after intratracheal administration of Ad-TCC/E1A with pulmonary surfactant*. In order to confirm the non-toxicity of Ad-TCC/E1A administration with pulmonary surfactant, the liver function of the treated mice was examined by analyzing liver enzyme levels. Seven days after the last viral administration, blood was collected from the mice and serum levels of ALT and AST were determined. As shown in Figure 5, serum levels of ALT and AST were not altered in the treated mice compared to the control mice. These results suggest that intratracheal administration of Ad-TCC/E1A with pulmonary surfactant is a safe approach.

## Discussion

For almost 30 years, exogenous pulmonary surfactant has been clinically used to treat neonatal respiratory distress syndrome to compensate for the lack of endogenous surfactant and reduce surface tension in the lungs (17, 18). Pulmonary surfactant is a clinically validated safe material. In identifying further clinical utility of pulmonary surfactant, Jobe *et al*. found that pulmonary surfactant facilitated adenovirally mediated luciferase gene transfer and luciferase gene expression in normal rabbit lungs (13). Intratracheal instillation of a therapeutic virus to treat lung diseases has been considered an attractive approach for treatment, but there has been a limitation to the efficient delivery of the therapeutic virus throughout the lung. Intrigued by the finding of Jobe *et al*. (13), we attempted to treat lung cancer developed in the *KRAS* mutation-bearing lung cancer mouse model using our novel lung cancer-targeted therapeutic adenovirus in association with pulmonary surfactant in the present study. Our therapeutic adenovirus with surfactant was 10 times more effective in lung tumor reduction than the adenovirus without surfactant.

For non-small cell lung cancer treatment, intratumoral injection of a therapeutic adenovirus carrying tumor suppressor p53 using bronchoscopy has been clinically performed (11, 12). A drawback of the trial was that intratumoral injection of a therapeutic virus using bronchoscopy was limited to relatively large tumors in bronchia and was not able to target lung cancer cells that are not detectable by computed tomography. In the present study, we were able to target peripherally spreading lung tumors and cells by combining our therapeutic adenovirus with pulmonary surfactant. The mechanism by which pulmonary surfactant functions is derived from its surface-active properties, helping the therapeutic virus to spread rapidly and effectively into distal lung tissue (13). Pulmonary surfactant also facilitates free airflow through narrow tubes, alters mucus properties and facilitates airway transport (31, 32). The therapeutic adenovirus without pulmonary surfactant might be rapidly absorbed in the airway mucosa, which probably limits its spreading to the distal airways and air spaces. In contrast, the therapeutic adenovirus with pulmonary surfactant is rapidly dispersed and deposited in the alveolar spaces. The pulmonary surfactant also has an anti-microbial effect that is also potentially beneficial for obstructive pneumonia caused by lung cancer. At the same time, pulmonary surfactant does not inactivate the infectivity of the therapeutic adenovirus (13, 33-35). Thus, pulmonary surfactant is an ideal carrier to deliver a therapeutic adenovirus to distally spreading lung tumors.

One concern regarding the use of adenovirus for gene therapy is that host immunity may remove the adenovirus. In our present study, intratracheal administration of Ad-CMV/LacZ with pulmonary surfactant resulted in significant expression of β-gal in immunocompetent control mice (Figure 3). Furthermore, repeated treatment with Ad-TCC/E1A therapeutic adenovirus significantly suppressed tumor growth in this *KRAS* mutation-bearing lung cancer model (Figure 4). These results indicate that intratracheal administration of the adenoviral vector with pulmonary surfactant is able to induce target gene expression and inhibit tumor growth in the lungs of mice with host immunity. However, lung diseases such as lung cancer often relapse. In the event that lung cancer relapses and the adenovirus-mediated gene therapy succumbs to host defense removal, a lentiviral vector that avoids host defense would be the next line of treatment (36). It is not known whether pulmonary surfactant enhances lentivirally mediated gene transfer and gene expression, and this needs to be investigated.

Another established approach for delivering a therapeutic virus to the lung is aerosol delivery. Aerosol delivery is noninvasive to lungs, and was expected to be an ideal approach to treat lung diseases including lung cancer (37-39). However, the distribution of aerosol virus did not favor transgene expression in the lung parenchyma relative to intratracheal instillation (35). Considering the pathogenesis of lung cancer, it is essential that the cancer is completely eradicated. Intratracheal instillation of a therapeutic virus in association with pulmonary surfactant is a more effective approach for delivering a therapeutic virus throughout the lung in order to treat lung diseases, especially lung cancer, than in aerosol delivery.

In summary, we used pulmonary surfactant as a carrier to deliver a therapeutic virus to lung tumors developed in a *KRAS* mutation-bearing lung cancer model. The therapeutic

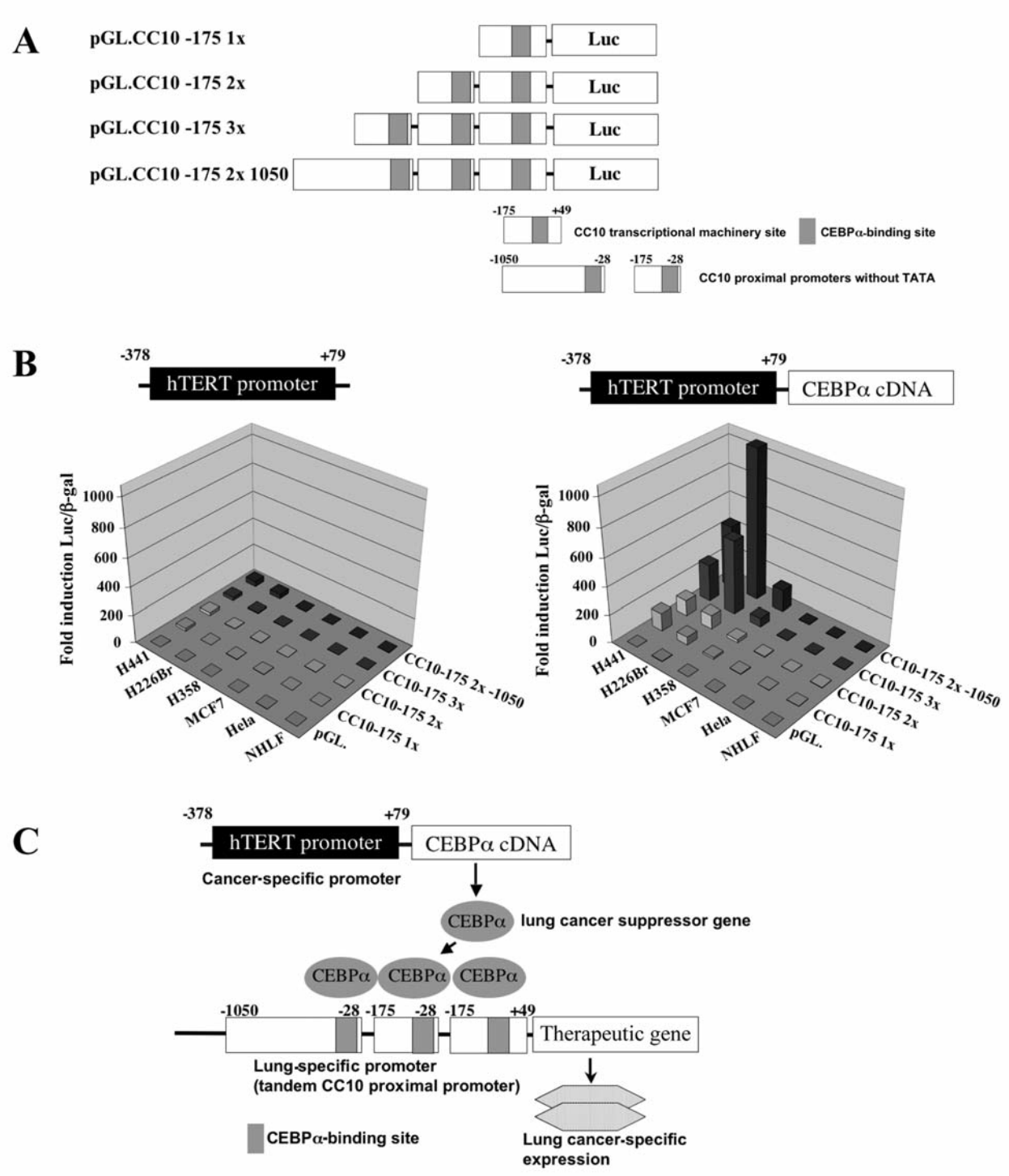



Figure 1. *Development of lung cancer-specific promoter TCC system. A: Schematic representation of CC10 tandem copies reporter constructs. One or two tandem copies of the CC10 promoter region from -175 to -28 were subcloned into pGL.CC10 containing promoter fragment -175 to +49 (CC10 -175 1×, CC10 -175 2× and CC10 -175 3×). The CC10 promoter region from -1050 to -28 was subcloned into pGL.CC10 -175 2× (pGL.CC10 -175 2× 1050). B: Transient transfection reporter assays in H441 and H358 pulmonary adenocarcinoma cells, H226Br lung squamous cell lung carcinoma cells, MCF7 breast cancer cells, HeLa cervical cancer cells, and normal human lung fibroblast NHLF with indicated CC10 tandem luciferase reporter constructs (2 μg) and pCMV.β-gal (2 μg) co-transfected with hTERT promoter only (left panel) or hTERT promoter driven CEBPα. Results are presented as fold induction of relative light units normalized to β-galactosidase activity relative to that observed for the control constructs. C: Schematic of lung cancer-specific TCC promoter system (TCC: hTERT promoter driven CEBPα combined with CC10 promoter).*

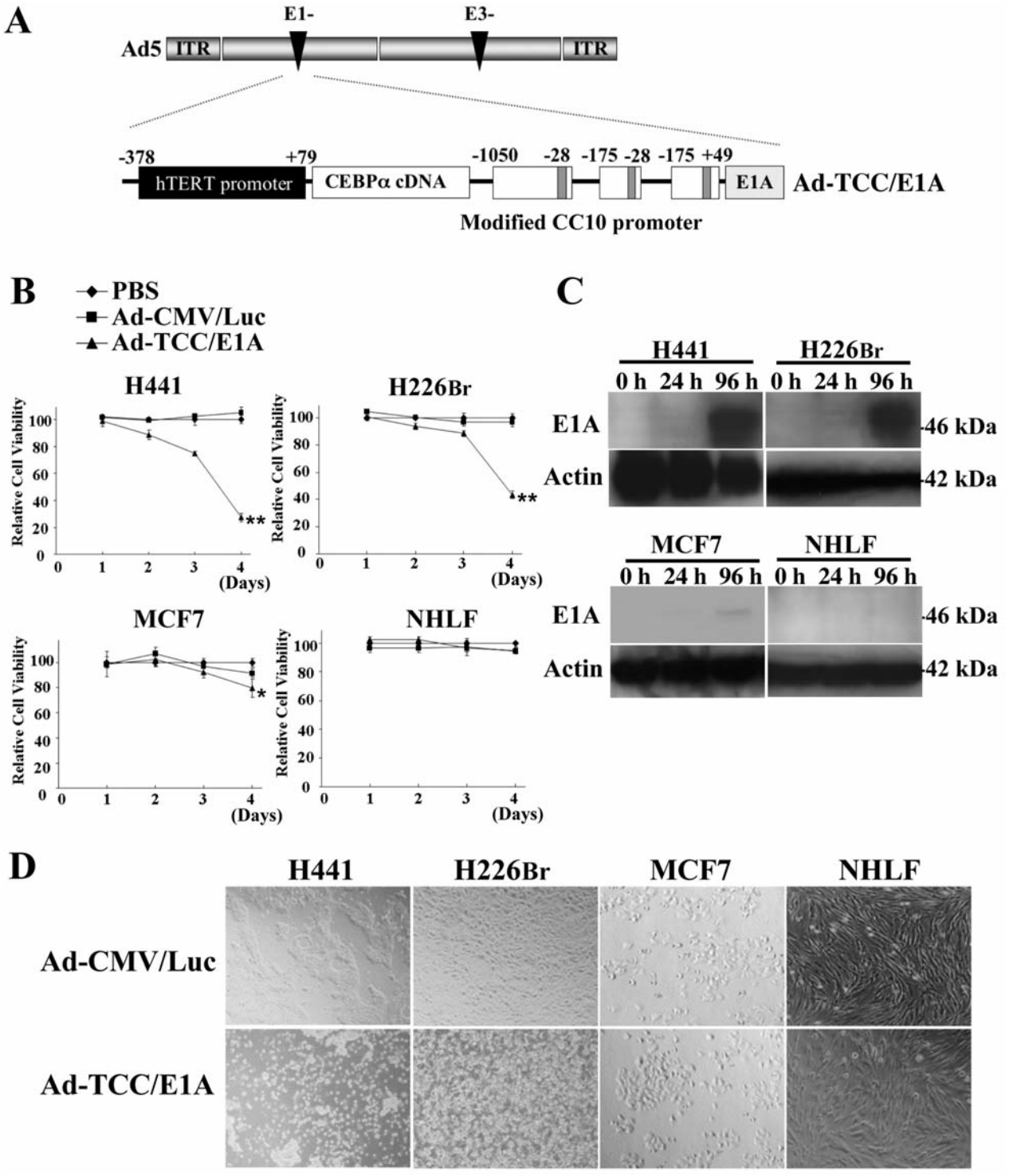



Figure 2. *Induction of lung cancer-specific cell death induced by TCC promoter system combined with adenoviral protein E1A. A: Schematic of Ad-TCC/E1A. B: Effect on cell viability induced by Ad-TCC/E1A infection in vitro. Cells were plated in 96-well plates at a density of $2\times10^3$ cells/well 24 hours prior to infection and treated with Ad-CMV/Luc or Ad-TCC/E1A at an MOI of 5000 vp. Cell viability was evaluated at 0, 1, 2, 3, and 4 days following adenoviral infection by MTS assay (CellTiter 96; Promega) according to the manufacturer's protocol. Statistically significant differences are shown as *p<0.05 and **p<0.01. C: Immunoblot analysis of adenoviral E1A protein expression at 0, 24, and 96 hours after infection of Ad-TCC/E1A in indicated cells. Actin is shown as a control. D: Phase-contrast photomicrographs of H441 pulmonary adenocarcinoma cells, H226Br squamous cell lung carcinoma cells, MCF7 breast cancer cells, and NHLF infected with Ad-CMV/Luc or Ad-TCC/E1A. Cell morphology was evaluated 4 days after infection. Photomicrographs were taken at a magnification of ×100.*

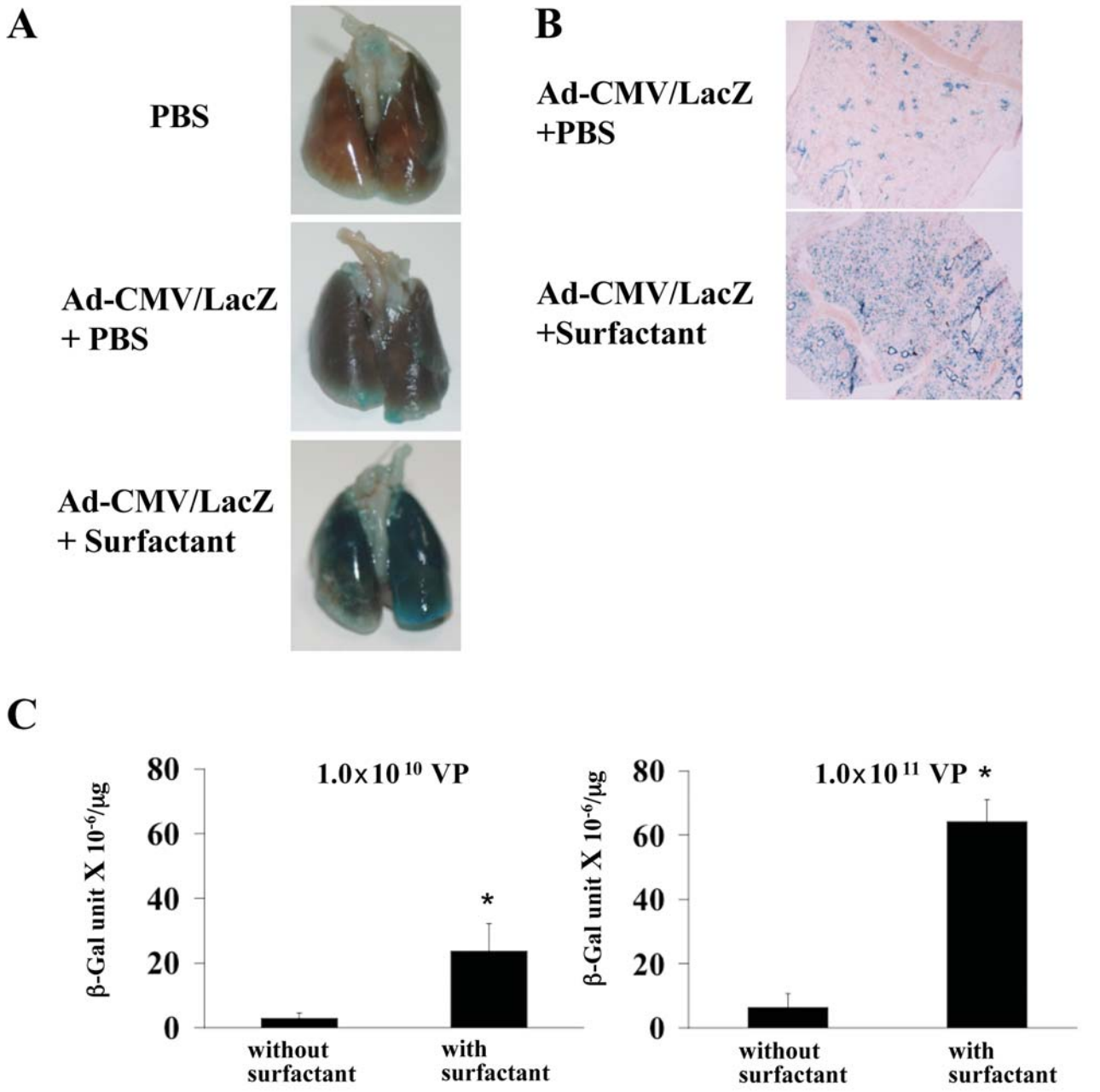
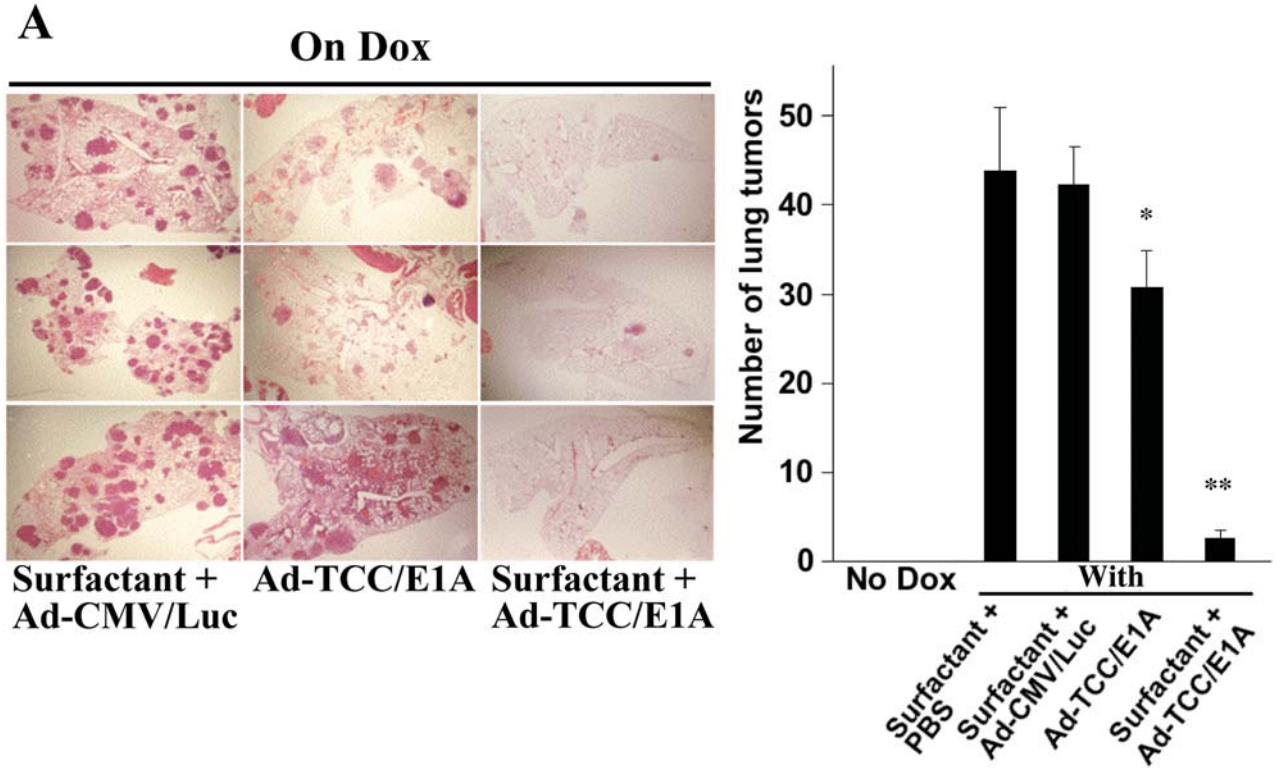



Figure 3. *Pulmonary surfactant enhances LacZ gene delivery and expression mediated by Ad-CMV/LacZ. A: Representative mouse lung infected with Ad-CMV/LacZ using PBS or pulmonary surfactant. B: Photomicrograph of sections (5 μM) of lungs infected with Ad-CMV/LacZ using PBS or pulmonary surfactant. Representative photomicrographs from one of three experiments were shown. Photomicrographs were taken at magnification of X 20. C: Quantative β-galactosidase activity in homogenates produced from lungs infected with Ad-CMV/LacZ at an MOI of $1\times10^{10}$ or $1\times10^{11}$ viral particles per mouse with or without pulmonary surfactant. Eight mouse lungs were used for each experimental condition. Statistically significant differences are shown as *p<0.05 and **p<0.01.*

effect on the mice treated with the therapeutic virus in the presence of pulmonary surfactant was ten times better in tumor reduction than those treated in the absence of pulmonary surfactant. This approach, in which pulmonary surfactant is used as a delivery carrier for a therapeutic virus, can be applied to target other lung diseases including other types of lung cancer, cystic fibrosis, α1-antitrypsin deficiency and pulmonary hypertension.

## Conflict of interest

None declared.



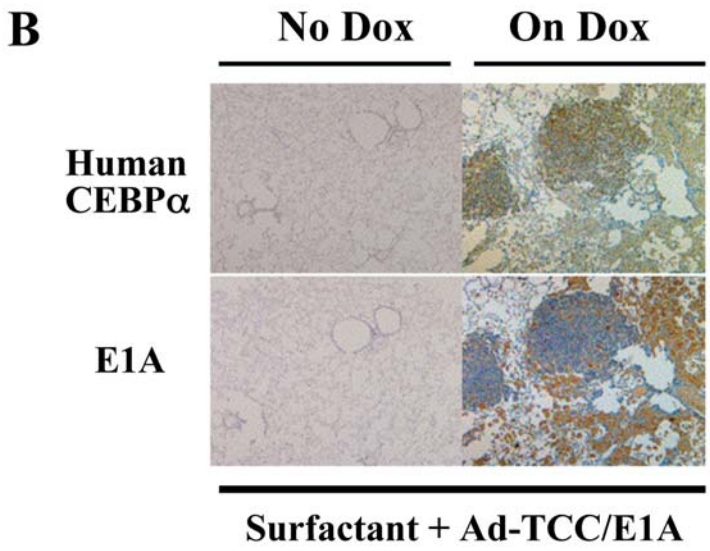




Figure 4. *Reduction of lung tumors in KRAS mutation-bearing lung cancer mouse model by Ad-TCC/E1A with pulmonary surfactant. A: Left panel: H&E stained sections from lungs of mice treated with Ad-CMV/Luc with pulmonary surfactant or Ad-TTC/E1A in the presence or absence of pulmonary surfactant. Tumor growth was significantly reduced by the combined treatment of Ad-TTC/E1A with pulmonary surfactant compared to lungs treated using Ad-CMV/Luc with pulmonary surfactant or a single treatment of Ad-TTC/E1A. Photomicrographs were taken at a magnification of ×20. Right panel: the numbers of left lung tumors with a long axis exceeding 1 mm in lungs from mice after intratracheal administration of pulmonary surfactant, Ad-CMV/Luc with pulmonary surfactant or Ad-TCC/E1A in the presence or absence of pulmonary surfactant. Statistically significant differences are shown as *p<0.05 and **p<0.01. B: Induced expression of CEBPα and E1A in lungs from mice after intratracheal administration of Ad-TCC/E1A with pulmonary surfactant. Left panels: Control lung. Right panels: KRAS mutation-bearing lung cancer. Photomicrographs were taken at a magnification of ×100.*

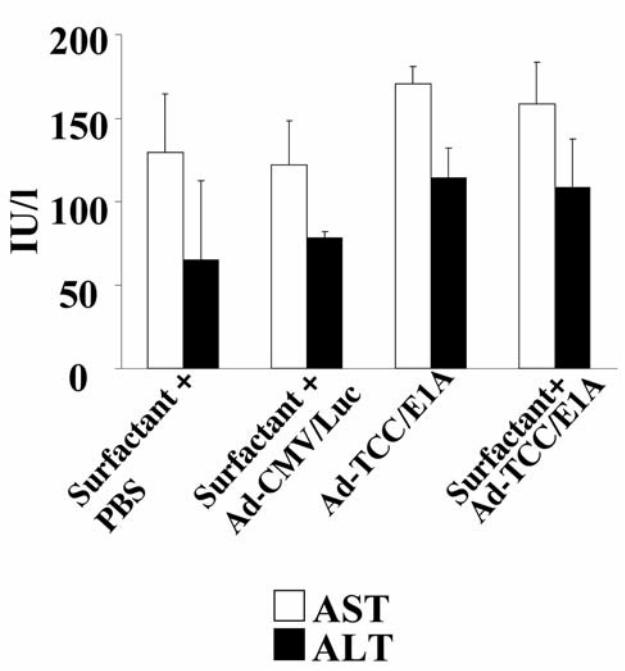



Figure 5. *No liver toxicity by Ad-TCC/E1A with pulmonary surfactant. Serum levels of AST and ALT seven days after treatment as indicated. Values (IU/l) are the means of three animals per group. Bars indicate SD; *p<0.05 and **p<0.01.*

## Acknowledgements

We thank T. Nakai, T. Yamanishi, M. Arakawa M. Fujii and N. Yata for technical assistance, M. Ikegami for technical advice, and J. Whitsett and H. Miyoshi for critical reading of the manuscript.

## References

1 Flotte TR and Laube BL: Gene therapy in cystic fibrosis. Chest *120*: 124S-131S, 2001.

2 Griesenbach U, Ferrari S, Geddes DM and Alton EW: Gene therapy progress and prospects: cystic fibrosis. Gene Ther *9*: 1344-1350, 2002.

3 Bou-Gharios G, Wells DJ, Lu QL, Morgan JE and Partridge T: Differential expression and secretion of alpha 1 anti-trypsin between direct DNA injection and implantation of transfected myoblast. Gene Ther *6*: 1021-1029, 1999.

4 De B, Heguy A, Leopold PL, Wasif N, Korst RJ, Hackett NR and Crystal RG: Intrapleural administration of a serotype 5 adeno-associated virus coding for alpha1-antitrypsin mediates persistent, high lung and serum levels of alpha1-antitrypsin. Mol Ther *10*: 1003-1010, 2004.

5 Behera AK, Kumar M, Lockey RF and Mohapatra SS: Adenovirus-mediated interferon gamma gene therapy for allergic asthma: involvement of interleukin 12 and STAT4 signaling. Hum Gene Ther *13*: 1697-1709, 2002.

6 Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O and Fujino M: Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci USA *98*: 5175-5180, 2001.

7 Champion HC, Bivalacqua TJ, Toyoda K, Heistad DD, Hyman AL and Kadowitz PJ: *In vivo* gene transfer of pre-procalcitonin gene-related peptide to the lung attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. Circulation *101*: 923-930, 2000.

8 Weill D, Mack M, Roth J, Swisher S, Proksch S, Merritt J and Nemunaitis J: Adenoviral-mediated *p53* gene transfer to non-small cell lung cancer through endobronchial injection. Chest *118*: 966-970, 2000.

9 Merritt JA, Roth JA and Logothetis CJ: Clinical evaluation of adenoviral-mediated *p53* gene transfer: review of INGN 201 studies. Semin Oncol *28*: 105-114, 2001.

10 Sinn PL, Burnight ER and McCray PB Jr.: Progress and prospects: prospects of repeated pulmonary administration of viral vectors. Gene Ther *16*: 1059-1065, 2009.

11 Swisher SG, Roth JA, Nemunaitis J, Lawrence DD, Kemp BL, Carrasco CH, Connors DG, El-Naggar AK, Fossella F, Glisson BS, Hong WK, Khuri FR, Kurie JM, Lee JJ, Lee JS, Mack M, Merritt JA, Nguyen DM, Nesbitt JC, Perez-Soler R, Pisters KM, Putnam JB Jr, Richli WR, Savin M, Schrump DS, Shin DM, Shulkin A, Walsh GL, Wait J, Weill D and Waugh MK: Adenovirus-mediated *p53* gene transfer in advanced non-small cell lung cancer. J Natl Cancer Inst *91*: 763-771, 1999.

12 Nemunaitis J, Swisher SG, Timmons T Connors D, Mack M, Doerksen L, Weill D, Wait J, Lawrence DD, Kemp BL, Fossella F, Glisson BS, Hong WK, Khuri FR, Kurie JM, Lee JJ, Lee JS, Nguyen DM, Nesbitt JC, Perez-Soler R, Pisters KM, Putnam JB, Richli WR, Shin DM, Walsh GL, Merritt J and Roth J: Adenovirus-mediated *p53* gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. J Clin Oncol *18*: 609-622, 2000.

13 Jobe AH, Ueda T, Whitsett JA, Trapnell BC and Ikegami M: Surfactant enhances adenovirus-mediated gene expression in rabbit lungs. Gene Ther *3*: 775-779, 1996.

14 Korfhagen TR, Bruno MD, Ross GF, Huelsman KM, Ikegami M, Jobe AH, Wert SE, Stripp BR, Morris RE, Glasser SW, Bachurski CJ, Iwamoto HS and Whitsett JA: Altered surfactant function and structure in SP-A gene targeted mice. Proc Natl Acad Sci USA *93*: 9594-9599, 1996.

15 Buckingham S and Avery ME: Time of appearance of lung surfactant in the foetal mouse. Nature *193*: 688-689, 1962.

16 Klaus M, Reiss OK, To Oley WH, Piel C and Clements JA: Alveolar epithelial cell mitochondria as source of the surface-active lung lining. Science *137*: 750-751, 1962.

17 Fujiwara T, Maeta H, Chida S, Morita T, Watabe Y and Abe T: Artificial surfactant therapy in hyaline-membrane disease. Lancet *1*: 55-59, 1980.

18 Clements JA and Avery ME: Lung surfactant and neonatal respiratory distress syndrome. Am J Respir Crit Care Med *157*: S59-66, 1998.

19 Whitsett JA and Weaver TE: Hydrophobic surfactant proteins in lung function and disease. N Engl J Med *347*: 2141-2148, 2002.

20 Fisher GH, Wellen SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, Whitsett JA, Koretsky A and Varmus HE: Induction and apoptotic regression of lung adenocarcinomas by regulation of a *K-Ras* transgene in the presence and absence of tumor suppressor genes. Genes Dev *15*: 3249-3262, 2001.

21 Riely GJ, Marks J and Pao W: *KRAS* mutations in non-small cell lung cancer. Proc Am Thorac Soc *6*: 201-205, 2009.
22 Stripp BR, Sawaya PL, Luse DS, Wikenheiser KA, Wert SE, Huffman JA, Lattier DL, Singh G, Katyal SL and Whitsett JA: *cis*-acting elements that confer lung epithelial cell expression of the *CC10* gene. J Biol Chem *267*: 14703-14712, 1992.
23 Twu YC, Chen CP, Hsieh CY, Tzeng CH, Sun CF, Wang SH, Chang MS and Yu LC: I branching formation in erythroid differentiation is regulated by transcription factor C/EBPalpha. Blood *110*: 4526-4534, 2007.
24 Fukazawa T, Maeda Y, Sladek FM and Owen-Schaub LB: Development of a cancer-targeted tissue-specific promoter system. Cancer Res *64*: 363-369, 2004.
25 He TC, Zhou S, da Costa LT, Yu J, Kinzler KW and Vogelstein B: A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA *95*: 2509-2514, 1998.
26 Takahashi A, Nemoto T and Fujiwara T: Biophysical properties of protein-free, totally synthetic pulmonary surfactants, ALEC and Exosurf, in comparison with surfactant TA. Acta Paediatr Jpn *36*: 613-618, 1994.
27 Epaud R, Ikegami M, Whitsett JA, Jobe AH, Weaver TE and Akinbi HT: Surfactant protein B inhibits endotoxin-induced lung inflammation. Am J Respir Cell Mol Biol *28*: 373-378, 2003.
28 Wert SE, Dey CR, Blair PA, Kimura S and Whitsett JA: Increased expression of thyroid transcription factor-1 (TTF-1) in respiratory epithelial cells inhibits alveolarization and causes pulmonary inflammation. Dev Biol *242*: 75-87, 2002.
29 Weiss DJ, Mutlu GM, Bonneau L, Mendez M, Wang Y, Dumasius V and Factor P: Comparison of surfactant and perfluorochemical liquid enhanced adenovirus-mediated gene transfer in normal rat lung. Mol Ther *6*: 43-49, 2002.
30 Katkin JP, Husser RC, Langston C and Welty SE: Exogenous surfactant enhances the delivery of recombinant adenoviral vectors to the lung. Hum Gene Ther *8*: 171-176, 1997.
31 Liu MY, Wang LM, Li E and Enhorning G: Pulmonary surfactant will secure free airflow through a narrow tube. J Appl Physiol *71*: 742-748, 1991.
32 Rubin BK, Ramirez O and King M: Mucus rheology and transport in neonatal respiratory distress syndrome and the effect of surfactant therapy. Chest *101*: 1080-1085, 1992.
33 Wu H, Kuzmenko A, Wan S, Schaffer L, Weiss A, Fisher JH, Kim KS and McCormack FX: Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. J Clin Invest *111*: 1589-1602, 2003.
34 Casals C: Role of surfactant protein A (SP-A)/lipid interactions for SP-A functions in the lung. Pediatr Pathol Mol Med *20*: 249-268, 2001.
35 Jobe AH, Ikegami M, Yei S, Whitsett JA and Trapnell B: Surfactant effects on aerosolized and instilled adenoviral-mediated gene transfer. Hum Gene Ther *7*: 697-704, 1996.
36 Kobinger GP, Weiner DJ, Yu QC and Wilson JM: Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia *in vivo*. Nat Biotechnol *19*: 225-230, 2001.
37 Flotte TR, Schwiebert EM, Zeitlin PL, Carter BJ and Guggino WB: Correlation between DNA transfer and cystic fibrosis airway epithelial cell correction after recombinant adeno-associated virus serotype 2 gene therapy. Hum Gene Ther *16*: 921-928, 2005.
38 Lee HY, Suh YA, Lee JI, Hassan KA, Mao L, Force T, Gilbert BE, Jacks T and Kurie JM.: Inhibition of oncogenic K-ras signaling by aerosolized gene delivery in a mouse model of human lung cancer. Clin Cancer Res *8*: 2970-2975, 2002.
39 Hwang SK, Lim HT, Minai-Tehrani A, Lee ES, Park J, Park SB, Beck GR Jr. and Cho MH: Repeated aerosol delivery of carboxyl-terminal modulator protein suppresses tumor in the lungs of K-rasLA1 mice. Am J Respir Crit Care Med *179*: 1131-1140, 2009.

*Received September 6, 2010*
*Revised November 1, 2010*
*Accepted November 2, 2010*