

# Inhibition of Oncogenic K-ras Signaling by Aerosolized Gene Delivery in a Mouse Model of Human Lung Cancer<sup>1</sup>

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## ABSTRACT

**Purpose:** Transfer of growth-suppressive genes to lung tumors has therapeutic potential, but effective delivery techniques have not been developed. Here, we investigated gene delivery to lung tumors by aerosolization of adenoviral vectors incorporated into calcium phosphate precipitates.

**Experimental Design:** To investigate the efficacy of this delivery method in normal and neoplastic lung, an adenoviral vector expressing  $\beta$ -galactosidase was administered by jet nebulization to K-ras<sup>LA1</sup> mice, which develop lung adenocarcinomas through activation of a latent allele carrying mutant K-ras (G12D). Furthermore, we investigated whether aerosolized delivery of Ad-MKK4 (KR), an adenoviral vector expressing dominant-negative mutant mitogen-activated protein kinase kinase 4 (MKK4), can block Ras-dependent signaling in K-ras<sup>LA1</sup> mice.

**Results:** After a single administration,  $\beta$ -galactosidase was detected in lung tissue for up to 21 days, and expression was much greater in tumors than in normal lung tissue. MKK4 was activated in the lungs of K-ras<sup>LA1</sup> mice, and aerosolized treatment with Ad-MKK4 (KR) decreased c-Jun-NH<sub>2</sub>-terminal kinase but not extracellular signal-

regulated kinase activity, providing evidence that MKK4 was selectively inhibited.

**Conclusions:** These findings demonstrate a novel approach to targeting oncogenic pathways in lung tumors by aerosolized gene delivery.

## INTRODUCTION

Lung cancer remains the primary cause of cancer-related death in the United States, and its incidence is increasing in developing countries (1). Progress has been made in the treatment of other malignancies by targeting the protein products of mutated proto-oncogenes (2), and similar efforts have begun in the treatment of lung cancer patients. Interest has focused on the proto-oncogene K-ras, which is mutated in 30–50% of lung adenocarcinomas, the most common histological subtype of non-small cell lung cancer (3). K-ras has been inhibited through the development of small molecules that block posttranslational modifications required for K-ras activation and by gene therapeutic strategies that decrease its expression (4, 5).

Gene therapies have shown promise in the replacement of wild-type p53 in mouse lung tumors after intratracheal delivery of vector (6–9). Gene therapies for the treatment of lung cancer patients have been delivered through intratumoral, intrabronchial, and intrapleural injections (10). These approaches have achieved limited success for several reasons. First, tumors are not always easily accessible to direct injection, increasing the morbidity associated with this procedure. Second, intratumoral injections do not consistently deliver adenovirus to the entire tumor volume, leaving portions of the tumor untreated. Third, gene transfer to lung tumor cells is inhibited by soluble factors in malignant pleural effusions (11), reducing the efficacy of treating tumor cells in this tissue compartment.

Aerosolized delivery is a potentially more efficacious approach for gene delivery. It can reach a large surface area of the bronchial epithelium, does not carry the risks associated with intrathoracic injections, and avoids the toxicities associated with systemic administration. Despite these advantages, the efficacy of aerosolized gene transfer has been limited by the vector systems used. Cationic lipids and polymers encapsulating DNA have demonstrated high transfection efficiency in normal HBE<sup>3</sup> cells and lung tumor cells (6, 12), but they are associated with significant inflammatory reactions in the bronchial epithelium (13, 14). Aerosolized delivery of adenoviral and retroviral vectors has achieved gene transfer to lung cancers in animal models, but this approach is limited by variable viral receptor ex-

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<sup>3</sup> The abbreviations used are: HBE, human bronchial epithelial; ERK, extracellular signal-regulated kinase; JNK, c-Jun-NH<sub>2</sub>-terminal kinase; MKK4, mitogen-activated protein kinase kinase-4; X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; GST, glutathione S-transferase.

pression in lung cancer cells, and administration of viral vectors to the lung induces antiviral immune responses that reduce gene transfer (15–17). In addition to their limited transfection efficiencies, lipid and viral vectors are fragile and can be degraded during the jet nebulization process (18).

In this study, we investigated a novel aerosolized treatment strategy. We delivered recombinant adenoviral vectors incorporated into calcium phosphate precipitates, a modification that enhances adenoviral gene delivery in airway epithelia, which lack the receptor activity to bind adenovirus fiber protein (19, 20). Gene transfer was achieved in lung tumors that arise in K-ras<sup>LA1</sup> mice, which express mutant K-ras through stochastic activation of a latent allele. Aerosolized delivery of an adenovirus expressing dominant-negative mutant *MKK4* inhibited Ras-dependent signaling in the lungs of K-ras<sup>LA1</sup> mice. These findings provide evidence that aerosolized delivery of calcium phosphate-precipitated adenoviral vectors is an effective means of gene transfer to lung tumors that should be considered for therapeutic trials in lung cancer patients.

## MATERIALS AND METHODS

**Animals, Cells, and Materials.** We conducted studies on K-ras<sup>LA1</sup> mice, which carry a latent K-ras allele with two copies of exon 1, one wild type and the other mutant (G12D; Ref. 21). The latent allele is activated stochastically in cells through homologous recombination, which results in the deletion of the wild-type copy of exon 1 and expression of an oncogenic form of the K-ras gene. Multifocal lung adenocarcinomas develop spontaneously in 100% of the mice. Mutant K-ras mRNA and protein are detectable in tumors but not in normal lung tissue. Tumors are first detectable at 2–3 weeks, grow to essentially fill the thoracic cavity, and cause pulmonary insufficiency to which the mice succumb at 8–10 months of age (21). The spontaneously immortalized HBE cell line HB56B (22) was grown in keratinocyte serum-free medium (Life Technologies, Inc., Gaithersburg, MD) on standard plasticware (Falcon; Becton-Dickinson, Bedford, MA) at 37°C with a pCO<sub>2</sub> of 5%. We purchased rabbit polyclonal antibody against human ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal antibody against human JNK1 (Santa Cruz Biotechnology), and rabbit polyclonal antibody against human phospho-MKK4 (Thr261; New England Biolabs, Beverly, MA).

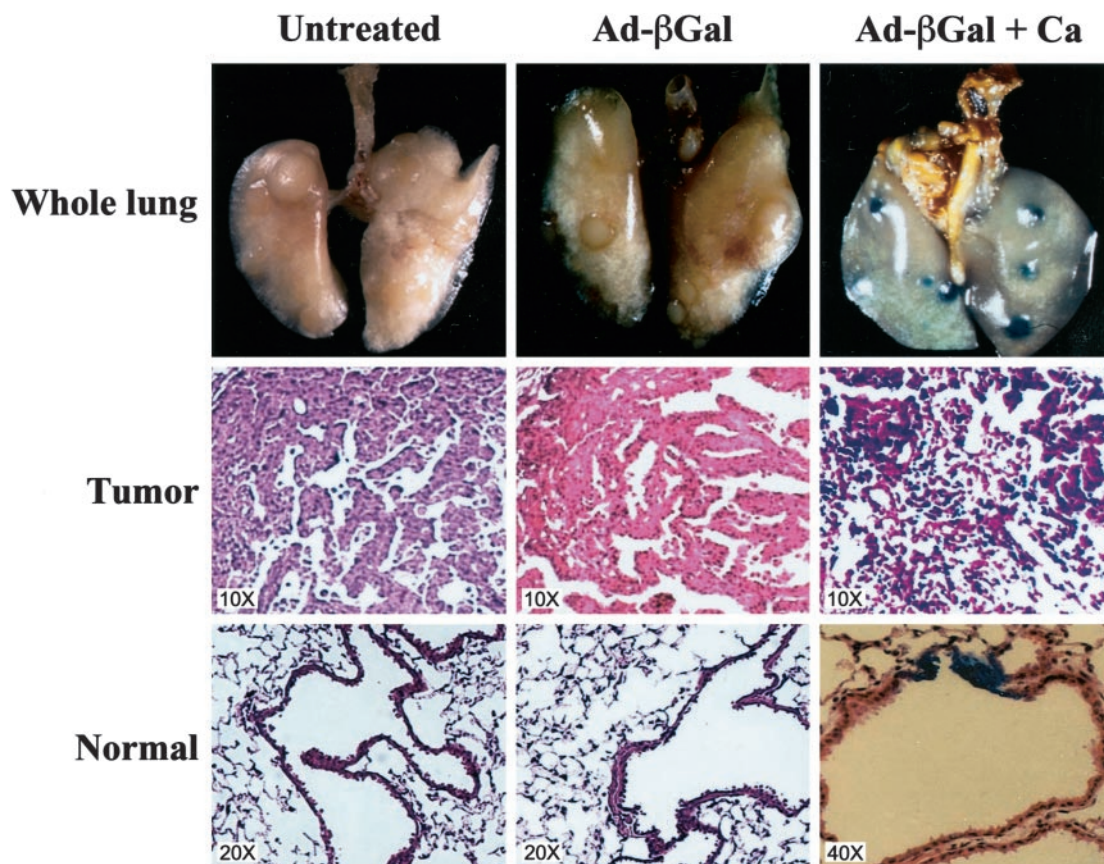
**Preparation and Administration of Calcium Phosphate-precipitated Adenovirus.** Particles ( $5 \times 10^9$ ) of adenovirus expressing  $\beta$ -galactosidase (Ad- $\beta$ Gal), dominant-negative mutant *MKK4* (Ad-MKK4-KR; Ref. 23), or parental adenovirus (Ad5CMV) were incubated in Eagle's Minimal Essential Medium (Sigma Chemical Co., St. Louis, MO) containing 5.8 mM Ca<sup>2+</sup> and 0.86 mM P<sub>i</sub> for 30 min. Adenoviruses were administered unmodified or as calcium phosphate precipitates by continuous aerosolization using an Aerotech II nebulizer (CIS-USA, Inc., Bedford, MA) flowing at 10 liters of dry, compressed air/min over a 30-min period into an airtight box housing K-ras<sup>LA1</sup> mice. Three days after a single administration, the mice were sacrificed. The lungs were perfused with 1× PBS, fixed with 10% formaldehyde, and stained with X-Gal as described previously (19). Lungs were then paraffin-embedded, cut into 5- $\mu$ m sections, and stained with H&E.

**Western Analysis.** Lung tissues were collected, frozen in dry ice, and kept at –70°C until use. Lung tissues were ground into powder in liquid nitrogen and suspended in 500  $\mu$ l of 1× PBS containing 1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged at 15,000 × *g* for 20 min. The pellet was suspended in 400–800  $\mu$ l of cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2 mM EGTA, 1% NP40, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 5 mM sodium orthovanadate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mg/ml pepstatin, and 1 mM benzamide] and left on wet ice for 20 min. Samples were vortexed vigorously for 15 s and clarified by centrifugation at 15,000 × *g* for 20 min. Whole cell lysates of H441 cells were isolated by incubation in lysis buffer for 20 min on ice followed by centrifugation at 15,000 × *g* for 20 min. Protein concentration was determined with the Protein Assay Kit (Bio-Rad, Hercules, CA). Protein was separated by electrophoresis on a SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA), and immunoblotted overnight at 4°C with primary antibodies, and antibody binding was detected using the enhanced chemiluminescence kit (Amersham, Inc., Arlington Heights, IL) according to the manufacturer's directions.

**Immune Complex Kinase Assay.** Immune complex kinase assays were performed as described previously (23) on lysates from mouse lung tissues and HB56B cells. HB56B cells were transiently transfected for 1 h with expression vectors (5  $\mu$ g) containing K-ras (G12D) or empty vector using LipofectAMINE (Life Technologies, Inc.) followed by infection for 48 h with calcium phosphate-precipitated Ad-MKK4 (KR) (24) or Ad5CMV ( $10^4$  particles). Cell lysates were prepared and subjected to immunoprecipitation (100  $\mu$ g/sample) using antibodies (1  $\mu$ g) that recognize JNK1 or ERK1/2 (Santa Cruz Biotechnology) by rotation at 4°C for at least 2 h. Protein A/G-agarose beads (20  $\mu$ l; Santa Cruz Biotechnology) were added and incubated at 4°C for 1–2 h. The beads were washed three times with lysis buffer and once with kinase buffer [20 mM HEPES (pH 7.5), 20 mM  $\beta$ -glycerol phosphate, 10 mM p-nitrophenyl phosphate, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 50 mM sodium vanadate]. Kinase assays were performed by incubating the beads with 30  $\mu$ l of kinase buffer containing 20  $\mu$ M cold ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (2000 cpm/pmol), and 1  $\mu$ g of GST-cJun or myelin basic protein as a substrate for JNK and ERK, respectively. The kinase reaction was performed at 30°C for 20 min. The samples were suspended in Laemmli buffer, boiled for 5 min, and analyzed by SDS-PAGE. The gel was dried and autoradiographed.

## RESULTS

**Calcium Phosphate Enhanced Aerosolized Adenovirus Delivery to K-ras<sup>LA1</sup> Mice.** K-ras<sup>LA1</sup> mice were treated with unmodified Ad- $\beta$ Gal or Ad- $\beta$ Gal incorporated into calcium phosphate precipitates (Ad- $\beta$ Gal + Ca) by continuous aerosolization into an airtight box. Three days after a single administration, the mice were sacrificed, and the lungs were fixed in formalin and stained with X-Gal. Whole lung preparations from mice treated with Ad- $\beta$ Gal + Ca demonstrated intense staining in tumors and less prominent staining in normal lung paren-



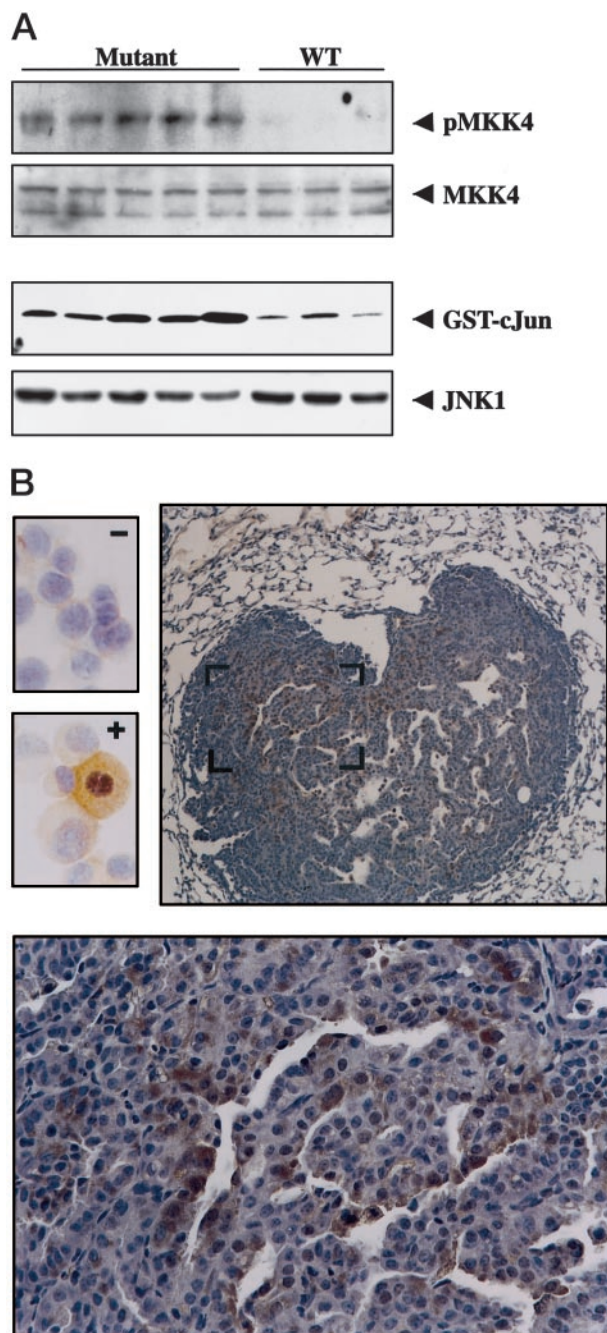
**Fig. 1** Aerosol delivery of Ad- $\beta$ Gal to the lungs of K-ras<sup>LA1</sup> mice. K-ras<sup>LA1</sup> mice (14 weeks old) were subjected to no treatment (*Untreated*) or to aerosolized treatment with unmodified Ad- $\beta$ Gal (*Ad- $\beta$ Gal*) or calcium phosphate-precipitated Ad- $\beta$ Gal (*Ad- $\beta$ Gal + Ca*). Three days after treatment, the mice were sacrificed, and the lungs were perfused with 1 $\times$  PBS, fixed with 10% formalin, and stained with X-Gal overnight at 37°C. Whole lung preparations are illustrated. These lungs were serially sectioned, counterstained with H&E, and subjected to histological analysis. Representative sections of tumor and normal bronchiole are illustrated. Magnifications are indicated in the *bottom left corner* of each image.

chyma (Fig. 1). Lungs from mice treated with unmodified Ad- $\beta$ Gal demonstrated no visible staining (Fig. 1). X-Gal staining was detected in lung tumors of mice sacrificed up to 21 days after treatment (data not shown). We performed histological analysis to investigate X-Gal expression in tumor and normal lung tissues. X-Gal staining was detected in lung tumor cells and detected less frequently in normal bronchial epithelial cells of mice treated with Ad- $\beta$ Gal + Ca but was not detected in lung tissues from mice treated with unmodified Ad- $\beta$ Gal (Fig. 1). These findings indicate that calcium phosphate precipitation was required for adenoviral gene expression in tumors.

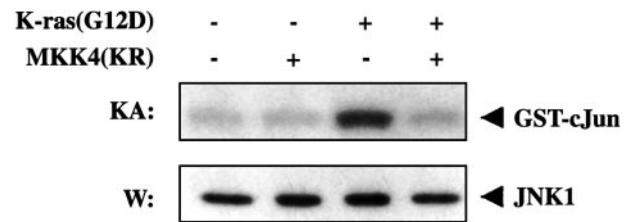
**MKK4-dependent Signaling Is Activated in the Lungs of K-ras<sup>LA1</sup> Mice.** Ras activates MKK4 through the GTPase Rac1, which plays a key role in Ras-induced cellular transformation (25). We examined MKK4 activity in the lungs of wild-type mice and K-ras<sup>LA1</sup> mice by Western and immunohistochemical analysis using antibodies that recognize phospho-MKK4 (Thr261) and used *in vitro* kinase assays of whole lung extracts to indirectly measure the activity of JNK, a substrate of MKK4. We detected phospho-MKK4 in whole lung extracts of K-ras<sup>LA1</sup> mice, but not wild-type mice (Fig. 2A). Immunohistochemical staining revealed phospho-MKK4 specifically in

lung tumors (Fig. 2B). JNK activity was higher in the lungs of K-ras<sup>LA1</sup> mice than in those of wild-type mice (Fig. 2A). However, JNK activity was detectable in lung extracts of wild-type mice, demonstrating basal JNK activation in the absence of mutant K-ras, which could occur through Ras-dependent and/or -independent pathways. Our finding that wild-type mice had detectable JNK activity but not phospho-MKK4 probably reflects the higher sensitivity of *in vitro* kinase assays compared with that of Western blotting. Moreover, these findings provide evidence that MKK4 is activated in the lungs of K-ras<sup>LA1</sup> mice.

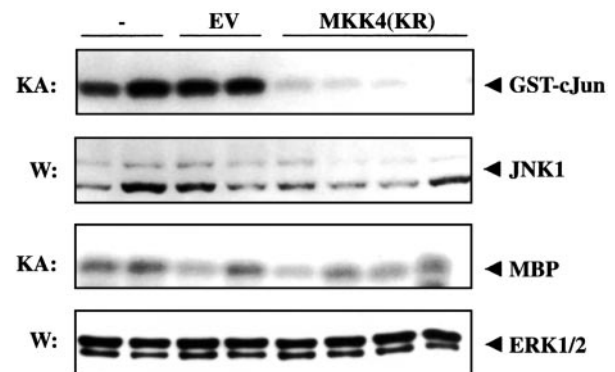
**Aerosolized Ad-MKK4 (KR) Blocks Ras-dependent Signaling in K-ras<sup>LA1</sup> Mice.** We sought a genetic approach to inhibit Ras-dependent pathways in the lungs of K-ras<sup>LA1</sup> mice. For this purpose, we investigated Ad-MKK4 (KR), an adenoviral vector expressing a dominant-negative mutant MKK4 that contains a lysine 129 (KR) mutation in the ATP binding region (24). We examined the ability of Ad-MKK4 (KR) to inhibit MKK4 activation by K-ras (G12D) in HB56B immortalized HBE cells, which contain no K-ras gene mutations at codons 12, 13, or 61 (22). HB56B cells were transiently transfected with K-ras (G12D) and infected with Ad-MKK4 (KR). The cells were then subjected to *in vitro* kinase assays of



**Fig. 2** MKK4 was activated in lungs of K-ras<sup>LA1</sup> mice. **A**, top panels, Western analysis was performed on whole lung extracts prepared from K-ras<sup>LA1</sup> mice (*Mutant*) and wild-type (*WT*) mice, using antibodies that recognize phospho-MKK4 (Thr261) (*pMKK4*) and pan-MKK4 (*MKK4*) to control for relative MKK4 levels. **A**, bottom panels, *in vitro* kinase assays of JNK activity were performed on whole lung extracts using GST-cJun as substrate. JNK1 Western analysis was performed on these extracts to control for relative JNK1 levels. **B**, top right panel, immunohistochemical analysis of MKK4 expression was performed on lung tissue derived from K-ras<sup>LA1</sup> mice using antibodies against phospho-MKK4 (Thr261). Illustrated is MKK4 staining of a lung tumor ( $\times 10$ ). **B**, bottom panel, high magnification ( $\times 40$ ) of the bracketed area in the top right panel. Cells staining positively are brown. **B**, top left panels, specificity of phospho-MKK4 antibody for activated MKK4 was examined in H441 non-small cell lung cancer cells that were serum-starved



**Fig. 3** MKK4 (KR) blocked JNK activation by mutant K-ras. HB56B cells were transiently transfected with a plasmid containing K-ras (G12D) or empty vector (-) and infected for 48 h with Ad-MKK4 (KR) or empty virus (-). Cell extracts were prepared and subjected to *in vitro* kinase assays (KA) using GST-cJun as substrate and to Western analysis (W) using anti-JNK1 antibodies to control for relative JNK1 levels.



**Fig. 4** Aerosolized delivery of Ad-MKK4 (KR) selectively inhibited JNK activity in the lungs of K-ras<sup>LA1</sup> mice. K-ras<sup>LA1</sup> mice were subjected to a single aerosolized treatment with calcium phosphate-precipitated Ad-MKK4 (KR), empty virus (EV), or no treatment (-). Three days later, the mice were sacrificed, and whole lung extracts were subjected to *in vitro* kinase assays (KA) using GST-cJun and myelin basic protein (MBP) as substrates for JNK and ERK, respectively. The extracts were also subjected to Western analysis (W) of JNK and ERK using anti-JNK1 and ERK1/2 antibodies to control for relative JNK and ERK levels.

JNK activity. K-ras (G12D) increased JNK activity, and MKK4 (KR) inhibited JNK activation by K-ras (G12D) (Fig. 3), indicating that MKK4 (KR) effectively blocked K-ras (G12D)-dependent signaling in HBE cells.

We subjected K-ras<sup>LA1</sup> mice to aerosolized treatment with calcium phosphate-precipitated Ad-MKK4 (KR) or empty virus (Ad5CMV). The mice were sacrificed 3 days later, and whole lung extracts were subjected to *in vitro* kinase assays of JNK and ERK activity. ERK is not a substrate of MKK4 and was investigated as a negative control in this experiment. Ad-MKK4 (KR) inhibited the activity of JNK but not ERK (Fig. 4), providing evidence that aerosolized administration of Ad-MKK4 (KR) selectively inhibited MKK4 activation by mutant K-ras.

(-) or serum-stimulated (+), trypsinized, cytospun onto slides, and stained with phospho-MKK4 (Thr261) antibody (illustrated at  $\times 40$  magnification).

## DISCUSSION

Calcium phosphate precipitation was originally investigated as a way to increase aerosolized adenovirus gene delivery to normal airway epithelia in cystic fibrosis models (19). Here we present the first evidence that calcium phosphate precipitation enhances adenoviral gene transfer to lung tumors *in vivo*. Calcium phosphate crystals and other encapsulating materials such as cationic liposomes enhance gene delivery, in part, by acting as a physical barrier, reducing trauma to the viral capsid during the nebulization process (18). In addition to its properties as a physical barrier, calcium phosphate increases virus binding to normal lung cells through mechanisms independent of adenoviral fiber receptor (19).

Previous studies have shown that MKK4 expression and activity are altered in human tumor cells and support a role for MKK4 as both a promoter and a suppressor of human tumorigenesis. The *MKK4* gene is deleted or mutated in a subgroup of pancreatic, biliary, and breast carcinomas, and reintroduction of *MKK4* inhibits the metastatic ability of certain tumor cells, demonstrating that *MKK4* has tumor suppressor activity (26–28). Potentially mediating this effect, Ras pathway activation induces the expression of p53 and p16<sup>INK4a</sup>, which induce premature cellular senescence, and inactivation of p53 or p16 prevents Ras-induced growth arrest (29). In contrast to these studies, MKK4 is known to be a downstream mediator of Rac1, and Rac1 activation contributes to Ras-induced cellular transformation (25), indicating that MKK4 plays a role in cellular transformation. Supporting the latter hypothesis, we found that MKK4 activity was increased in the lungs of K-ras<sup>LA1</sup> mice, and phospho-MKK4 was detected mostly in lung tumor cells. This supports *in vitro* studies on lung cancer cells demonstrating that MKK4-dependent pathways play a dominant role in mutant Ras-induced colony formation (30, 31). Thus, MKK4 and its downstream mediators play apparently contradictory roles in the regulation of cellular growth and transformation that may depend on the presence of cell type-specific factors or the activity of tumor suppressor pathways that inhibit the mitogenic and transforming effects of MKK4.

We found that aerosolized delivery of calcium phosphate-precipitated Ad-MKK4 (KR) demonstrated selectivity at the molecular level, inhibiting the activity of JNK but not ERK in the lungs of K-ras<sup>LA1</sup> mice. Based on our findings, this technique has several potentially useful applications. First, it can be used in animal models of human lung cancer to investigate, in an *in vivo* setting, the role of specific intracellular pathways in lung tumorigenesis. Second, it can be implemented in the clinical setting for the delivery of genes with anticancer activity to lung cancer patients. Similar to previous reports (19), short-term treatments of K-ras<sup>LA1</sup> mice caused no histological evidence of inflammation to the airway epithelium (data not shown), suggesting that calcium phosphate may be less toxic to normal tissues than other materials used for adenoviral encapsulation (13, 14). In future studies, it will be important to investigate the antitumor efficacy of this approach and issues related to chronic adenoviral administration, such as toxicities to normal bronchial tissues and the development of antiadenoviral immune responses that reduce gene transfer efficiency.

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