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ORIGINAL ARTICLE

Amphiphilic triblock copolymer poly(p-dioxanone-co-L-lactide)-block-poly(ethylene glycol), enhancement of gene expression and inhibition of lung metastasis by aerosol delivery

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We describe the development of an aerosol system for topical gene delivery to the lungs of C57BL/6 mice. This system is based on the combination of the commercial cationic lipid Lipofectin with a novel amphiphilic triblock copolymer, poly(p-dioxanone-co-L-lactide)-block-poly(ethylene glycol) (PPDO/PLLA-b-PEG, and abbreviated in the text as polymeric micelles). After optimizing conditions for DNA delivery to the lungs of mice using the combination of polymeric micelles with Lipofectin and LacZ DNA, we used the Lipofectin/polymeric micelle system to deliver the tumor suppressor gene PTEN to the lungs of C57BL/6 mice bearing the B16-F10 melanoma. Lipofectin/PTEN/polymeric micelles significantly improved gene expression of PTEN in the lungs of mice with no evidence of cell toxicity or acute inflammation. Importantly, lung metastasis, as measured by lung weight, was significantly reduced ($P < 0.001$), as were total tumor foci in the lungs ($P < 0.001$) and size of individual tumor nodules in animals treated with Lipofectin/PTEN/polymeric micelles compared with control animals. Survival time was also extended. These results suggest that the Lipofectin/polymeric micelle system is appropriate for enhancing gene delivery in vivo *and that it can be applied as a non-invasive gene therapy for lung cancer.*

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Keywords: *aerosol; gene delivery; lung metastasis; polymeric micelles*

Introduction

Lung cancer is the single largest cause of cancer deaths, with approximately 175 000 deaths reported every year in the US alone. More than 80% of lung cancers do not respond favorably to irradiation or chemotherapy. A novel approach to suppress tumor growth and reverse molecular dysfunctions in cancer treatment is tumor suppressor gene replacement therapy. Among tumor suppressor genes, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10, also known as *MMAC-1* or *TEP-1*), a new tumor suppressor gene discovered in 1997, is now known to play major roles not only in suppressing cancer but also in embryonic development, cell migration and apoptosis. Mutation of *PTEN* is a common event in advanced and metastatic stages of diverse human malignancies, suggesting that *PTEN* plays an important role in the development and metastasis of cancers.

For the treatment of lung cancer, therapeutic genes have been delivered to tumors by intratumoral, intrabronchial and intrapleural injection.[1] These approaches have achieved limited success for several reasons. First, tumors are not always easily accessible to direct injection; second, intratumoral injections do not consistently deliver therapeutic genes to the entire tumor volume, leaving portions of the tumor untreated; and third, gene transfer to lung tumor cells is inhibited by soluble factors in malignant pleural effusions, thereby reducing the efficacy of treating tumor cells in this tissue compartment.[2] Therefore, aerosolized delivery is a potentially more efficient approach for gene delivery into the lung.[3]

Aerosol delivery systems represent a non-invasive alternative for delivery of therapeutic genes to the lungs.

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They do not carry the risks associated with intrathoracic injections and avoid the toxicities associated with systematic administration. Furthermore, aerosol delivery distributes the complexes uniformly. Despite these advantages, the efficacy of aerosolized gene transfer has been limited by the vector systems used. Aerosolized delivery of adenoviral and retroviral vectors has enabled gene transfer to lung cancers in animal models, but this approach is limited by variable viral receptor expression in lung cancer cells. Further, administration of viral vectors to the lung induces antiviral immune responses that reduce the efficiency of gene transfer.[4–6] Cationic lipids and polymers[7–16] encapsulating DNA have demonstrated high transfection efficiency in normal human bronchial epithelial cells[17] and lung tumor cells.[13,18,19] But they are associated with significant inflammatory reactions in the bronchial epithelium.[20,21] In addition to their limited transfection efficiencies, lipid and viral vectors are fragile and can be degraded during the jet nebulization process.[22] It is also well known that degradation of DNA can occur via numerous mechanisms: acid-catalyzed hydrolysis, enzymatic degradation by DNases, oxidation by molecular oxygen or free radicals and strand breaks due to hydrodynamic shear stress.[23–25]

We have introduced a non-ionic polymeric micellar system consisting of the novel amphiphilic triblock PPDO/PLLA-b-PEG copolymer, to improve the cationic lipid-mediated gene delivery system. This approach is based on our previous studies,[26–28] in which we defined the properties of Lipofectin associated with micelle formations of the PPDO/PLLA-b-PEG copolymer at low concentrations and on recent reports suggesting that these systems also enhance the transport of charged molecules across the cell membranes *in vitro*.[29]

First, we describe conditions for optimal enhancement of transgene expression in the lungs of C57BL/6 mice, utilizing nebulization (5% CO_2)[12,13] of formulations containing Lipofectin, polymeric micelles and *LacZ* as model DNA. Next, we demonstrate the therapeutic effect of the tumor suppressor *PTEN* gene, delivered by aerosol in a Lipofectin/*PTEN*/copolymer micelle complex, in the B16-F10 melanoma lung metastasis mouse model.

Results

Optimization of PPDO/PLLA-b-PEG copolymer formulation with Lipofectin/DNA

In order to optimize the formulation of the gene delivery complex, we varied the concentration of the PPDO/PLLA-b-PEG polymeric micelles, keeping Lipofectin/*LacZ* concentrations constant (4.5 mg plasmid DNA and 0.5 mg Lipofectin per 10 ml nebulized solution). Expression of the LacZ in the lung was greatest (~10-fold more than control, $P<0.001$) at 6 mg copolymer in 10 ml of the aerosol solution (Figure 1a). At concentrations of polymer greater than 6 mg, the aerosol solution became cloudy and thick. This likely accounts for the decrease in LacZ expression observed at 8–20 mg polymeric micelles ($P<0.05$).

Next, we determined the time course for transgene expression in the lungs of mice. Transgene expression was highest at 24 h ($P<0.001$) (Figure 1b). After 24 h, transgene expression in the lungs of mice decreased in a linear fashion; however, as statistically significant transgene expression persisted in the lungs of mice up to 120 h, our protocol provides for twice weekly treatments of the mice with Lipofectin/*PTEN* or *LacZ*/polymeric micelles aerosol complexes.

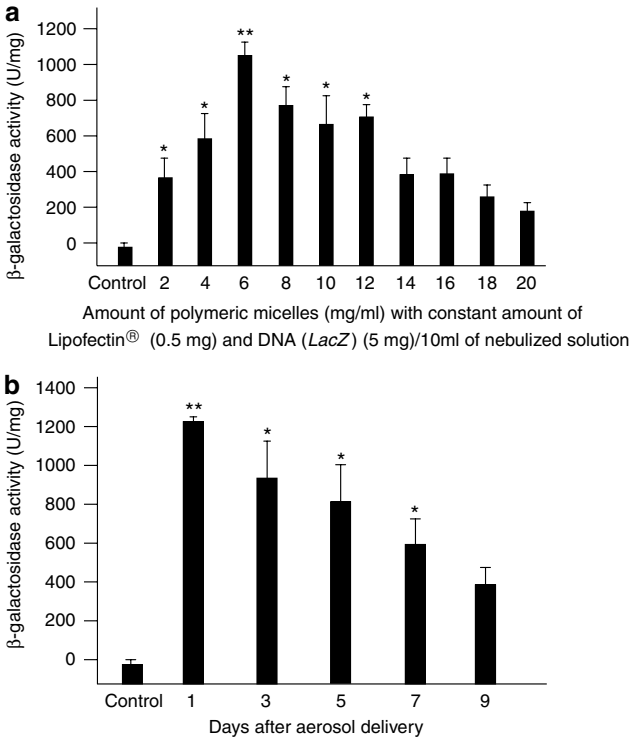

Figure 1 Dose dependence (**a**) and time course (**b**) of transgene expression. (**a**) β-Galactosidase activity in lung tissue 24 h after aerosol delivery of Lipofectin/*LacZ* and various concentrations of PPDO/PLLA-b-PEG polymer. (**b**) Time course of transgene expression in mice exposed to a single aerosol treatment of Lipofectin/*LacZ*/polymeric micelles at the optimum concentrations given in the text. Values are given as average means ± s.e. of triplicates or quadruples (U/mg total protein) ($n=6$). Statistical differences are denoted as $^{*}P<0.01$ and $^{**}P<0.001$.

Biodistribution, toxicity and delivered genes expression in mouse lung following aerosol delivery of the Lipofectin/DNA/PPDO/PLLA-b-PEG copolymer

We next examined the biodistribution of delivered genes and determined whether toxicity resulted from aerosol delivery of Lipofectin/polymeric micelles or Lipofectin/*LacZ*/polymeric micelles. For these assays, different organs were collected from mice at various times after exposure to the micelle complexes. Aerosol delivery of Lipofectin/*LacZ*/polymeric micelles resulted in highly specific *LacZ* gene expression in lung tissue (Figure 2). Histological analysis of lung tissue from mice exposed to Lipofectin/*LacZ*/polymeric micelles also revealed the greatest localization of LacZ in bronchial epithelial cells with lesser localization (scattered pattern) in the lung parenchyma (data not shown). There was no evidence of any cellular inflammatory reaction, as judged by an absence of polymorphonuclear cell infiltration with hematoxylin/eosin staining, and no microscopic evidence of toxic and cytological disruptive effects caused by Lipofectin/*LacZ*/polymeric micelles in any of the tissues examined. We assessed neutrophil-related inflam-

Table 1 Evaluation of neutrophil infiltration into the lungs of mice after delivery of Lipofectin/polymeric micelles

Group	*Lung MPO activity*[a] *(U/g tissue)*
A	0.0398 ± 0.01
B	0.0404 ± 0.008

Abbreviation: MPO, myeloperoxidase.
[a]MPO activity was determined using *O*-diasinidine dihydrochloride and hydrogen peroxide, and values are averages of the lung MPO activity ± s.e. of quadruples (U/g tissue). A and B denote untreated mice (control) and mice treated with Lipofectin/polymeric micelles, respectively ($n = 6$).

Figure 2 Tissue distribution of transgene expression after a single aerosol delivery of Lipofectin/*LacZ*/polymeric micelles. Values are presented as the average means ± s.e. of triplicates (U/mg total protein) ($n = 6$).

mation in the lungs using the myeloperoxidase (MPO) assay and noted no significant difference between MPO levels in the control mouse lungs and the lungs of mice exposed to aerosol Lipofectin/polymeric micelles (Table 1). Moreover, there was neither difference in body weight nor abnormal behavior between the treated and untreated mice (data not shown).

Figure 3 demonstrates that the lungs of mice exposed to Lipofectin/*PTEN*/polymeric micelles express PTEN exclusively in alveolar epithelial cells (type II pneumocytes) and endothelial cells. Quantitative results from Western blot also revealed that Lipofectin/*PTEN*/polymeric micelle-treated mice showed about a 10-fold increase in the level of PTEN expression detected in lung tissue compared to control mice.

Inhibition of lung micrometastases and PTEN expression in tumor-bearing mice following aerosol delivery of the Lipofectin/PTEN/PPDO/PLLA-b-PEG copolymer

Suppression of tumor micrometastases in lung is of great concern in clinical settings. Whereas the lungs of untreated tumor-bearing mice had large numbers of tumor nodules (Figure 4a and b), tumor-bearing mice treated with Lipofectin/*PTEN*/polymeric micelles had very small and distinct tumor foci with no invasion into the chest wall. Further, the tumor-bearing mice treated with Lipofectin *PTEN*/polymeric micelles had a very low tumor index compared to all other control groups ($P < 0.001$). The lung weights of the *PTEN*-treated group also differed significantly ($P < 0.001$) from the control groups (Figure 4c). In addition, there were marked differences in body weight and behavior among the treated and untreated tumor-bearing mice (data not shown).

To test the relation of PTEN expression and development of tumor nodule in tumor-bearing mice, we examined PTEN expression in tumor foci with or without treatment of Lipofectin/*PTEN*/polymeric micelles by immunohistochemistry. Similar to what was observed for normal mice (Figure 3c), conducting airways, including the terminal airways and large airways, were intensely stained for expression of the transfected *PTEN* gene (Figure 5) in tumor-bearing mice exposed to Lipofectin/*PTEN*/polymeric micelles. There was also diffuse staining for PTEN in the alveolar lining cells and intense staining in some individual cells of the tumor-bearing lung. In contrast to the normal lung, there was slightly diffused staining for PTEN in the lungs of tumor-bearing control mice (Figure 5b and c), showing the endogenous PTEN activity. Following Lipofectin/*PTEN*/polymeric micelles treatment, the number of tumor foci was significantly reduced and expression of PTEN in tumor tissue appeared much more abundant (Figure 5d–f).

The *PTEN*-treated tumor-bearing mice also survived longer (mean = 78 ± 4 days) than the control mice (mean = 54 ± 2 days; $P < 0.001$) (Figure 6). No control mice survived beyond day 65, whereas almost 80% of *PTEN*-treated mice were alive on day 65. The reduction of tumor burden in the lungs of tumor-bearing mice exposed to Lipofectin/*PTEN*/polymeric micelles (Figure 4b) and their increased survival time correlates with the high expression of transfected PTEN in the lungs of these mice (Figure 5d–f).

Discussion

We have previously reported that Lipofectin/DNA and aqueous solution of the PPDO/PLLA-b-PEG polymeric micelles gives the highest level of reporter gene expression *in vitro*.[27] Our previous studies also suggested a therapeutic role for the *PTEN* gene in the context of tumorigenicity *in vitro* and *in vivo*, and in tumor cell metastasis.[30] In this study, we present evidence that optimization of the Lipofectin/DNA/polymeric micelle formulation for aerosol delivery of genes *in vivo* provides the basis for successful non-viral gene delivery for treatment of lung metastasis. Our further optimized results showed that Lipofectin/polymeric micelles are able to reduce the size of DNA (from 395 ± 3.8 to 130 ± 4.4 nm) with negative zeta potential (Supplementary Table 1).

In addition to efficiency of gene delivery, important factors in gene therapy are viability of the DNA, specificity of gene delivery and toxicity. An acknowledged problem in delivering naked DNA with either Lipofectin or polymeric micelles by aerosol is the significant degradation of the DNA (Supplementary Figure 1). The combination of DNA with Lipofectin and polymeric micelles significantly resolves this problem and subsequently protects the viability of DNA delivered to the lung.

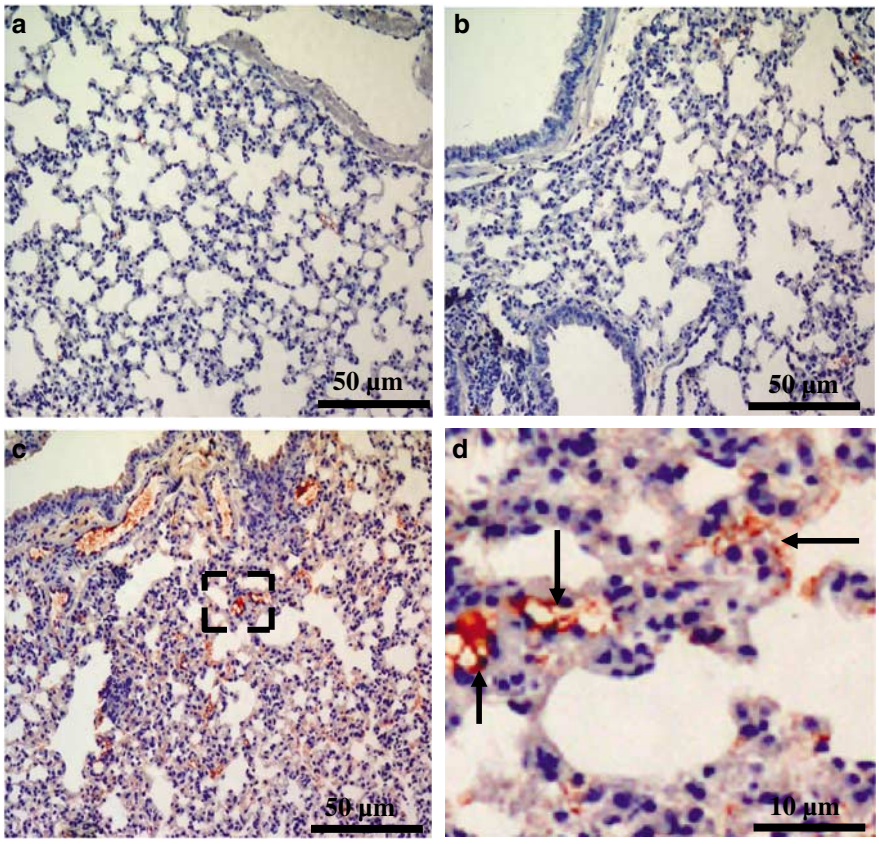

Figure 3 Immunohistochemistry: expression of transfected PTEN in normal lung tissue 48 h after aerosol treatment with Lipofectin/*PTEN*/polymeric micelles. (**a**) Untreated mice; (**b**) mice treated with Lipofectin/*LacZ*/polymeric micelles; (**c**) mice treated with Lipofectin/*PTEN*/polymeric micelles; (**d**) high magnification of (**c**) (bracketed area). Eleven sections (5 μm thickness) were examined from each lung harvested from normal and tumor-bearing mice. Histological analysis was performed as described in Materials and methods. Cells expressing PTEN are stained reddish-brown (arrows).

Our biodistribution studies indicate that *LacZ* delivered by aerosol in the Lipofectin/polymeric micelle complex is expressed almost exclusively in respiratory tissues (Figure 2). Histochemical staining of lung tissue revealed that LacZ expression was highest in the epithelial cells of conducting airways. Biochemical analysis and histological examination (Supplementary Figure 3) showed no evidence of toxicity or histological abnormality, indicating that the formulation is safe and highly specific for mouse lung.

We utilized these observations to test whether delivery of the *PTEN* tumor suppressor gene in the Lipofectin/polymeric micelle complex provided sufficient levels of PTEN expression to induce anti-tumor activity against B16-F10 melanoma metastasis in the mouse lung. The improved delivery system induced greater transfection efficiency of *PTEN* to the lung, with dramatic inhibition of tumor growth, exceeding expectations based on the level of PTEN expression. Importantly, PTEN expression was observed not only in peritumor area but also within tumor foci. This therapeutic response included prolongation of the survival time for tumor-bearing mice. Thus, aerosol delivery of *PTEN* via Lipofectin/polymeric micelles to the lung *in vivo* provides an attractive non-invasive alternative to viral or cationic lipid formulations for targeting therapeutic genes to the lungs.

There are several possible mechanisms by which PTEN may achieve anti-tumor effects. One possibility is that PTEN interferes with angiogenesis induced by and required for B16-F10 melanoma growth.[31,32] Another possibility, previously discussed,[30] is that suppression of tumor growth and metastasis by PTEN is a function of its ability to inhibit insulin-like growth factors and VEGF, growth factors correlated with high incidence of metastasis and poor prognosis in various cancers.

In conclusion, we have shown that Lipofectin/DNA complexed with polymeric micelles can increase the levels of gene expression when the gene is delivered by aerosol. Expression of the transfected gene occurs predominantly in the lungs and highly significant levels are detected 48 h after aerosol delivery. Lipofectin/polymeric micelles appear to be non-toxic at the optimal concentration for *in vivo* gene expression. Based on our results, we believe that aerosol delivery of Lipofectin/DNA complexes with the polymeric micelles can improve the efficiency of cationic lipid vector-mediated gene delivery and that it offers an inexpensive and safe alternative to viral delivery. Furthermore, we have shown that improved gene delivery of the *PTEN* tumor suppressor gene is sufficient to induce anti-tumor activity against B16-F10 melanoma lung metastasis in a therapeutic setting. Although the exact mechanism of

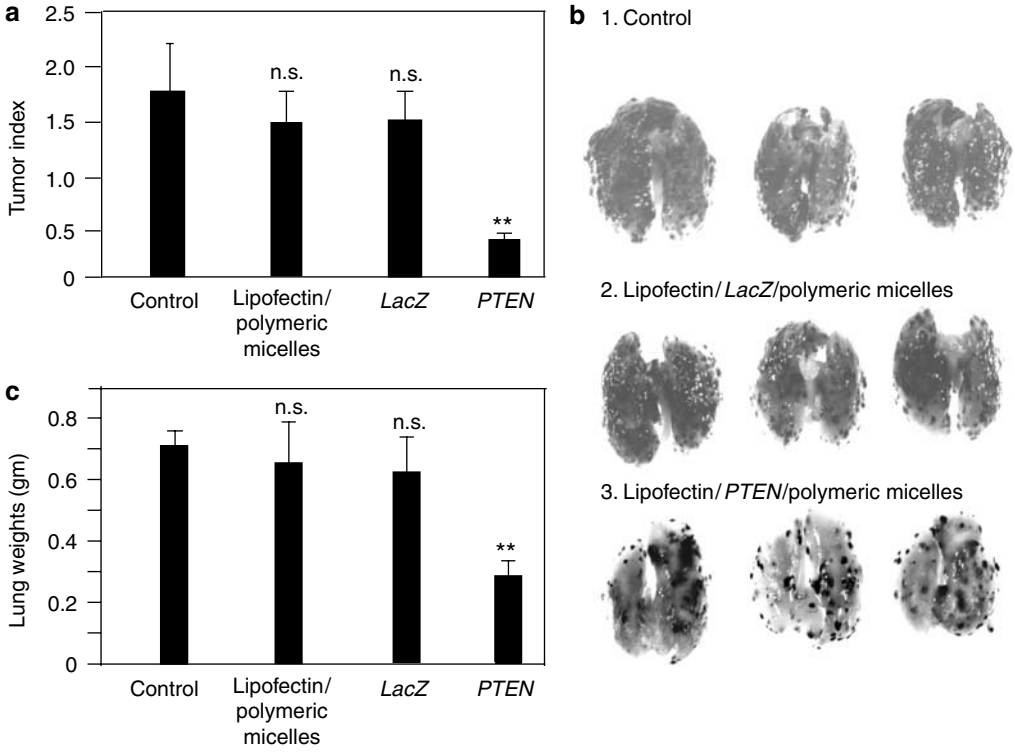

Figure 4 Inhibition of B16-F10 lung metastasis by *PTEN* delivered to mouse lung by aerosol with Lipofectin/*PTEN*/polymeric micelles. (**a**) Tumor burden as tumor index (lung weight × number of foci × size of foci); (**b**) representative lungs; (**c**) lung weights. Lungs from mice treated with Lipofectin/polymeric micelles alone were similar in shape, size and number of tumor foci to those shown in control (data not shown in (**b**)). Values are average means ± s.e. of triplicates or quadruples ($n = 7$). $^{**}P < 0.001$; n.s. = no significant difference.

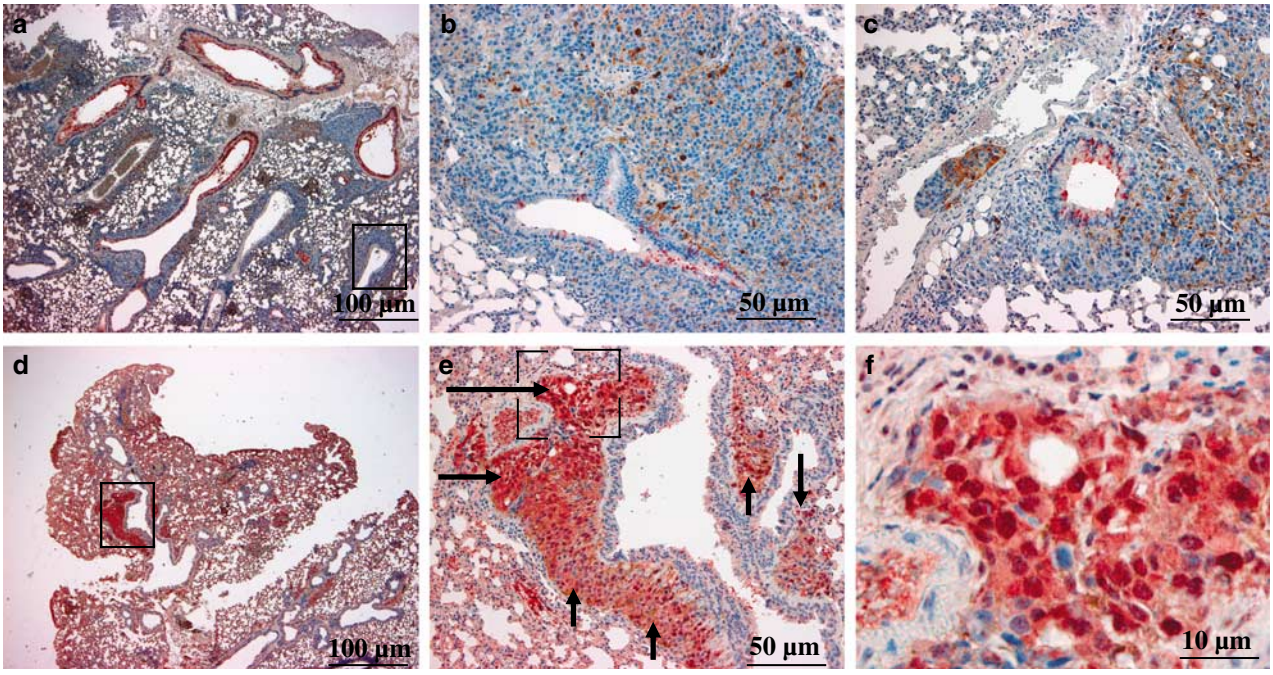

Figure 5 Histological analysis of PTEN expression in the lung tissue of B16-F10 tumor-bearing mice. (**a**) Untreated mice; (**b**) mice treated with Lipofectin/*LacZ*/polymeric micelles; (**c**) higher magnification of bracketed portion of (**b**); (**d**) mice treated with Lipofectin/*PTEN*/polymeric micelles; (**e**) a higher magnification of the bracketed area of (**d**); (**f**) a higher magnification of the bracketed area of (**e**). Note that the lowest magnifications (**a** and **d**) allow estimation of tumor loads (dark-brown cells). Eleven thin sections (5 μm thickness) were examined from each harvested lung and histological analysis was performed as described in Materials and methods. Arrows indicate cells expressing the PTEN.

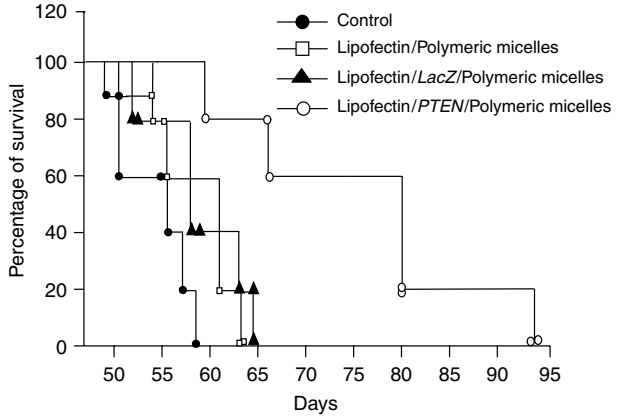

Figure 6 Prolonged survival of tumor-bearing mice treated with Lipofectin/*PTEN*/polymeric micelles. Animal survival was estimated by using the Kaplan–Meier and Wilcoxon signed-rank tests. Survival in *PTEN*-treated mice was significantly longer ($P<0.001$) than in control groups ($n=7$).

tumor suppression by PTEN remains to be elucidated, our data strongly support the potential use of aerosol-delivered Lipofectin/*PTEN*/PPDO/PLLA-b-PEG polymeric micelles for inhibition of lung metastasis and as a therapy complementary to surgery, chemotherapy and radiation.

Materials and methods

Materials, animals and cell culture

The synthesis[33,34] and molecular characterization[26,27,33] of the PPDO/PLLA-b-PEG copolymer has been described previously. *PTEN* cDNA was obtained by reverse transcriptase-PCR using primers 5′-CCAGACATGA CAGCCATCA-3′ and 5′-AATTCAGACTTTTGTAAT-3′ from MCF-7 cells (human breast adenocarcinoma, ATCC HTB22) and subcloned into the pcDNA3 expression vector[30] (Invitrogen, Groningen, The Netherlands).

Female C57BL/6 mice were purchased from the Korean Research Institute of Chemical Technology (Daejeon, Chuungnam, Korea), and were housed in an environment-controlled rearing system. The mice were maintained in animal facilities at the Chonbuk National University and used in accordance with the guidelines of the University. All mice used in the experiment were 7–8 weeks of age.

B16-F10 cells (murine melanoma) were cultured in MEM (Life Technologies, Grand Island, NY, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate at 37°C in a 5% CO_2 incubator. Tumor cells from a mid-log phase culture were harvested by brief exposure to 0.05% trypsin–0.02% ethylenediaminetetraacetic acid solution, washed twice and resuspended in culture media at the concentration indicated for injection.[30]

Preparation of complexes for aerosol delivery

Lipofectin/DNA complexes were prepared by mixing 0.5 mg Lipofectin and 4.5 mg pcDNA3.1/*LacZ* or pcDNA3.1/*PTEN*[30] with the various concentrations of copolymer in distilled water to a total volume of 10 ml in polypropylene tubes. The complexes were incubated for 30 min at room temperature before transferring to the nebulizer. Mice were placed in plastic cages sealed with tape before aerosol delivery.[35] The copolymer/DNA complexes were aerosolized using an Aerotech-2222 nebulizer (AT-2222) (CIS-US Inc., Bedford, MA, USA) at 10 l/min flow rate using air or air containing 5% CO_2.[36,37] Approximately 7 h of aerosol exposure was required to finish the required volume.

MPO assay

Twenty-four hours after aerosol exposure to the Lipofectin/DNA/polymeric micelles, mice ($n=6$) were anesthetized with isoflurane and killed by exsanguination via the abdominal aorta. The lungs were harvested after perfusion through the heart with saline. The tissue was homogenized in hexadecyltrimethylammonium bromide (0.5% HTAB in 50 mM phosphate buffer, pH 6.0; 5 ml HTAB/g of tissue).[38] After centrifugation, the MPO activity in the supernatant was determined using *O*-diasinidine dihydrochloride (0.167 mg/ml) and 0.0005% H_2O_2. The absorbance (A) was measured at 460 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).[38] Naïve mice were used as controls. MPO activity is defined as follows: MPO activity = U/g lung weight or lung tissue (g).

β-Galactosidase reporter gene assay

A galacto-Star Kit (Tropix, Bedford, MA, USA) was used to measure *in vivo* reporter gene expression. Briefly, at defined times after aerosol delivery, mice were killed, the lung and other organ tissues harvested and homogenized for 20 s with 1 ml lysis buffer containing protease inhibitors cocktail (Boehringer Mannhein, Mannheim, Germany) and centrifuged at 12 500 *g* for 10 min at 4°C. The supernatant fluid was heated at 48°C for 60 min to inactivate endogenous β-galactosidase activity.[39] The sample was centrifuged again and total protein concentration was measured. Two hundred micrograms of protein from each sample was mixed with 70 μl reaction buffer in Monolight Luminometer cuvettes (Pharmingen, San Diego, CA, USA) and incubated at room temperature for 60 min. The β-galactosidase activity is expressed as relative light units per milligram protein (U/mg).

Lung micrometastasis studies

The animal model for B16-F10 melanoma lung micrometastasis has been described extensively.[30] For tumor suppression studies, C57BL/6 mice were injected via tail vein with 2.5×10^4 B16-F10 cells on day 0. Micrometastases were allowed to develop for 14 days[30] before initiating aerosol treatment. The mice were treated with Lipofectin/*PTEN*/polymeric micelle in aerosol (4.5 mg *PTEN* plasmid/10 ml of aerosolized solution) twice weekly for 5 weeks (10 treatments) starting on day 14 after inoculation of the cancer cells. The amount of DNA delivered per mouse is estimated to be 4–5 μg in normal air. In the presence of 5% CO_2, higher amounts of DNA are believed to be delivered owing to the increase in tidal and minute volumes.[12] For tumor studies, control groups included untreated mice and mice treated with Lipofectin/polymeric micelles or Lipofectin/*LacZ*/polymeric micelles. The mice were monitored for survival times. The control animals started dying at ~day 50 after tumor cell inoculation.

Evaluation of tumor burden

One week after the last treatment, mice from all groups were killed. The lungs were resected, weighed, fixed in Fekets solution (90% ethanol, 5% acetic acid and 5% formaldehyde) and examined under a dissection microscope to count the visible foci.[30] Tumors contiguous to the lung were included. The tumor burden (tumor index) was calculated using the following formula: tumor index = lung weight × number of foci × size of foci.

Histological analysis of tissue sections

The mice were killed and lungs were isolated, cannulated and fixed by inflation with Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) in saline. Cryosections were cut at 5 μm, mounted on slides, air-dried, incubated with 3% H_2O_2 in methanol to inactivate endogenous peroxidase activity and washed in phosphate-buffered saline at pH 7.4. After preincubation with blocking serum (Vector Laboratories, Burlingame, CA, USA), sections were incubated with anti-PTEN monoclonal antibody (Oncogen, Boston, MA, USA) at a 1:200 dilution in blocking serum for 30 min. The secondary antibody, rabbit anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) conjugated to biotin, was used in a 1:600 dilution for 30 min. Antibody binding was visualized using a streptavidin–peroxidase complex followed by addition of the 3,3′-diaminobenzidine peroxidase substrate (Sigma, St Louis, MO, USA) for 5 min.

Statistical analysis

Results are expressed as means ± standard errors (s.e.). *P*-values of <0.05 were considered statistically significant. Statistical significance of the experimental results was calculated using unpaired Student's *t*-test. All statistical analyses were performed using the program StatView 5.0.

Acknowledgements

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Supplementary information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)