Poly(β-amino ester) Nanoparticle Delivery of TP53 Has Activity against Small Cell Lung Cancer In Vitro and In Vivo

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Abstract
Small cell lung cancer (SCLC) is an aggressive disease with one of the highest case-fatality rates among cancer. The recommended therapy for SCLCs has not changed significantly over the past 30 years; new therapeutic approaches are a critical need. TP53 is mutated in the majority of SCLC cases and its loss is required in transgenic mouse models of the disease. We synthesized an array of biodegradable poly(β-amino ester) (PBAE) polymers that self-assemble with DNA and assayed for transfection efficiency in the p53-mutant H446 SCLC cell line using high-throughput methodologies. Two of the top candidates were selected for further characterization and TP53 delivery in vitro and in vivo. Nanoparticle delivery of TP53 resulted in expression of exogenous p53, induction of p21, induction of apoptosis, and accumulation of cells in sub-G1, consistent with functional p53 activity. Intratumoral injection of subcutaneous H446 xenografts with polymers carrying TP53 caused marked tumor growth inhibition. This is the first demonstration of TP53 gene therapy in SCLC using nonviral polymeric nanoparticles. This technology may have general applicability as a novel anticancer strategy based on restoration of tumor suppressor gene function. Mol Cancer Ther; 12(4); 1–11. ©2013 AACR.

Introduction
Small cell lung cancer (SCLC) represents 15% of all lung cancer cases and has one of the highest case-fatality rates of all cancers with nearly as many deaths as diagnoses per year. In 2011, more than 25,000 deaths were attributable to this disease in the United States alone (1). The median survival of patients diagnosed with SCLC is less than 1 year. This statistic has not changed significantly over the past 3 decades despite more than 52 randomized phase III clinical trials evaluating numerous cytotoxic chemotherapies (2). The combination of etoposide and cisplatin has been the standard first-line therapy for SCLC since the 1980s. In 2003, topotecan became the only drug approved for treatment of patients with relapsed SCLC. New therapeutic approaches are needed to improve long-term survival in this disease.

SCLC exhibits certain recurrent genetic alterations most notably inactivation of the tumor suppressor genes, TP53 and RB. TP53 encodes a transcription factor whose targets regulate cell-cycle progression, senescence, DNA repair, and apoptosis (3, 4). TP53 mutations are the most common genetic alteration in human cancer, occurring in more than 50% of cases (5, 6). Wild-type (WT) p53 activity can also be abrogated by endogenous MDM2 or viral proteins; the human papilloma virus E6 protein, SV-40 large T antigen, and adenovirus E1B 55-kDa proteins can bind and attenuate p53 activity resulting in cellular transformation (7–9). In transgenic mouse models, disruption of TP53 results in increased susceptibility to tumor development, most notably lymphomas and sarcomas. Restoration of p53 in these models results in potent antitumor activity in a cell-type–specific manner; TP53 reexpression induces apoptosis in autochthonous lymphomas but senescence in sarcoma and hepatocellular carcinoma models (10, 11).

In SCLC, TP53 alterations are prevalent; among 67 independent SCLC cell lines and 231 primary SCLC tumors, TP53 was mutated in 90% and 74% of cases, respectively (12). Support for the critical role of TP53 in SCLC pathogenesis also derives from transgenic mouse models in which Cre-mediated loss of TP53 and RB results in murine SCLC, which shares histopathologic features of human SCLC including neural cell adhesion molecule (NCAM; CD56) expression and elaboration of neuroendocrine (NE) markers such as synaptophysin and chromogranin (13). In this genetic background, AdenoCre placed under the control of the neuroendocrine cell–specific calcitonin/calcitonin-gene related peptide promoter but not a Clara cell–specific promoter, resulted in murine SCLC, implicating pulmonary neuroendocrine cells as the putative cell of origin for SCLC (14). In vitro, Adachi and
colleagues have shown that expression of wild-type (WT) p53 in a p53-null SCLC cell line results in apoptosis (15). As loss of TP53 appears to be critical in SCLC development, restoration of functional p53 may have therapeutic efficacy.

Adenovirus is one of the most widely studied gene therapy vectors; in non–small cell lung cancer (NSCLC), adenoviral-mediated TP53 (Ad.p53) delivery has been evaluated in several early-phase clinical trials (16, 17). Intratumoral (i.t.) delivery of Ad.p53 in combination with chemotherapy was found to be safe, and histologic examination of tumor tissue revealed apoptosis in Ad.p53-treated samples (16). A phase II study, however, failed to show increased response or local benefit of combined Ad.p53 and chemotherapy over chemotherapy alone (17). Adenoviral gene therapy has also been evaluated preclinically in SCLC models. Adenoviral delivery of an siRNA targeting the hepatocyte growth factor receptor, c-Met, in the H446 SCLC cell line resulted in decreased proliferation \textit{in vitro} and tumor growth inhibition \textit{in vivo} (18). Similarly, adenoviral delivery of fragile histidine triad complex, a putative tumor suppressor gene often mutated in SCLC, induced apoptosis in multiple SCLC cell lines (19).

The use of viral vectors has been limited by safety concerns including insertional mutagenesis and toxicity as well as limited cargo capacity and manufacturing challenges (20, 21). Many patients have preexisting humoral immunity to adenovirus, or rapidly develop neutralizing antibodies, limiting the potential of adenoviral therapies. Alternative approaches to gene delivery, using non-viral biomaterials such as inorganic nanoparticles, cationic lipids, liposomes, polymers, and peptides, have been limited by low efficiency, resulting in limited efficacy (22–24).

We have developed highly effective biomaterials for non-viral gene delivery to hard-to-transfect cells (23, 25–28). These poly(β-amino ester) (PBAE) polymers are biodegradable due to ester linkages throughout the polymer backbone, which allows for lower toxicity and release of DNA intracellularly. Through their secondary and tertiary amines, these polymers are also able to buffer the endosome, which facilitates endosomal escape (29, 30). In addition, subtle changes to PBAE structure can improve specificity of transfection, and these polymers have been adapted for gene delivery to various cell types including human umbilical vein endothelial cells (HUVEC), human retinal endothelial cells, and human mesenchymal stem cells as well as glioblastoma multiforme, ovarian, prostate, and pancreatic cancer cell lines (25, 27, 31–33).

In this study, we sought to develop non-viral nanoparticles that could deliver therapeutic genes with high efficiency to SCLC cells. We synthesized an array of PBAEs using combinatorial chemistry (34) and found several polymers with transfection efficiencies comparable to commercially available agents in SCLC cell lines. These polymers may be generally useful as efficient gene delivery vectors. As a proof-of-principle for this approach, we used 2 PBAE polymers to assess the activity of WT TP53 delivery to the p53-mutant H446 SCLC cell line \textit{in vitro} and \textit{in vivo}.

Materials and Methods

Plasmids, chemicals, and reagents

Unless otherwise stated, all reagents were purchased from Sigma Chemical Company or Fisher Scientific in the highest available purity. The CMV-GFP (EGFPN1) and CMV-LUC plasmids were purchased from Elim Biopharm. p53-CMV-GFP plasmid (Addgene) contained CMV promoter regulating the fusion of 2 gene segments: WT p53 and EGFPN1 backbone.

Monomers reagents

Monomers used for polymer synthesis were the following: from Acros Organics [1-(3-aminopropyl)pyrrolidine (E8)], Alfa Aesar [1,4-butanediol diacrylate (B4), 1-(3-aminopropyl)-4-methylpiperazine (E7)], Fluka [2-(3-aminopropylamino)ethanol (E6), 3-amino-1-propanol (S3), 4-amino-1-butanol (S4), 5-amino-1-pentanol (S5)], Mono-Polymer and Dajac Laboratories [1,3-propanediol diacrylate (B3), 1,5-pentanediol diacrylate (B5)], Sigma-Aldrich [1,3-butadiol diacrylate (B3m)], and TCI America [1,3-diaminopentane (E3), 2-methyl-1,5-diaminopentane (E4), (PEO)4-bis-amine (E5)] as previously described (34).

Preparation of Cy5-labeled dsRED DNA

pDsRed-Max-N1 DNA (Promega) was labeled with Cy5 fluorophore using the Label IT Cy 5 Tracker Kit (Mirus) as per manufacturer’s protocol and stored at –20°C in light protective conditions.

Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC) and grown in complete RPMI (RPMI-1640 (Quality Biologicals) supplemented with 10% FBS, l-glutamine, penicillin/streptomycin, sodium pyruvate, HEPES, and sodium bicarbonate) per ATCC recommendations. The H146, H187, and H446 cell lines were authenticated by short tandem repeat analysis by the Fragment Analysis Facility at Johns Hopkins University (Baltimore, MD). All cell lines were grown and multiple aliquots were cryopreserved after authentication. All cell lines were used within 6 months of resuscitation.

Synthesis of PBAEs

The PBAEs were synthesized in a 2-step reaction procedure (Fig. 1). In the first step, the base polymers were synthesized by mixing diacylates (B) with amino alcohols (S) at a molar ratio of 1:1:1 or 1:2:1. The reaction was conducted in glass scintillating vials with teflon stir bars at 90°C for 24 hours. The base polymer was then dissolved in anhydrous dimethyl sulfoxide (DMSO) to 167 mg/mL. In the second step of the reaction, 480 μL of the base polymer solution was mixed with 320 μL of 0.5 mol/L end-capping...
amine (E) in 1.5-mL Eppendorf tubes and allowed to react in a shaker for 24 hours. Polymers were then divided into smaller volumes and stored at \(-20^\circ C\) and with desiccant until needed. These polymers were characterized with gel permeation chromatography and proton nuclear magnetic resonance as previously described (34). Select polymers, including polymers used in vivo, were made in larger quantities and further purified. In this case, after the base polymer reaction step, the polymers are dissolved in tetrahydrofuran (THF), instead of DMSO, and then end-capped as above, but with end-capped monomers also diluted in THF. Once the reaction was complete, the polymer was mixed with 4× the volume of ethyl ether. These mixtures were vortexed vigorously and then centrifuged at 4,000 rpm for 5 minutes, after which the supernatant containing unreacted monomer and organic solvents was removed. This process was then repeated a second time and the purified polymers dried under vacuum for 2 days. The polymers were then dissolved in anhydrous DMSO and kept frozen with desiccant as described above.

**Nanoparticle preparation**

For each transfection, PBAE:DNA nanoparticles were prepared by separately dissolving PBAE polymer and DNA in 25 mmol/L sodium acetate (pH 5.0). The polymer and DNA solutions were mixed in equal volumes, gently pipetted, and incubated for 10 minutes at room temperature.
temperature. A range of w/w ratios were evaluated for the high-throughput screen and are described in the High-throughput screening of PBAE polymer library section found in the Supplementary Data. For all other experiments, PBAE:DNA ratios of 75 w/w and 30 w/w were used for in vitro and in vivo studies, respectively. Polymer and DNA stocks were stored at −20°C and thawed on ice before mixing and treating the cells. For Cy5-DsRed experiments, 0.1 µg of Cy5-labeled dsRED was included in the DNA mixtures and PBAE:DNA nanoparticles were prepared as described above.

**Nanoparticle characterization**

Plasmid DNA and PBAEs were separately diluted in 25 mmol/L sodium acetate buffer to 0.06 mg/mL and either 1.8, 3.6, or 4.5 mg/mL, respectively (corresponding to PBAE:DNA weight/weight, wt/wt, ratios of 30, 60, or 90). Equal volumes of the DNA and PBAE solutions were mixed and incubated for 10 minutes at room temperature. The resulting solution of nanoparticles was then used either for sizing or DNA complexation studies. In the case of sizing, the samples were further diluted in PBS and loaded into a Nanosight NS500 nanoparticle tracking analysis (NTA) instrument. The solutions were diluted anywhere from 50× to 200×, so that the nanoparticle concentrations were appropriate for NTA analysis (35). NTA videos were captured for 60 seconds and analyzed using the NTA software, version 2.1. To further examine the ability of the polymers to bind to the DNA, the nanoparticle solutions were run on a gel using electrophoresis and compared with naked DNA. A 1% agarose gel with 0.1 µL/mL ethidium bromide was made. The naked DNA solution was mixed with a 30% glycerol loading buffer with bromophenol blue dye, whereas the nanoparticle solutions were mixed with the loading buffer without the dye to prevent interference between the dye and PBAE–DNA interaction. The gel was run for 30 minutes at 100 V and imaged using the UVP BioDoc-It Imaging Center.

**Transfection protocol**

**Adherent cells.** H446 cells were seeded at low density and allowed to adhere overnight. PBAE:DNA nanoparticles were added to cells followed by gentle shaking. After 4 hours, the media were replaced with fresh media, and the cells were incubated at 37°C for 48 hours.

**Suspension cells (H146, H187).** Cells were seeded in complete RPMI overnight then incubated with PBAE:DNA nanoparticles for 4 hours with gentle shaking. After 4 hours, cells were centrifuged at low speed for 2 minutes, fresh media were added and the cells further incubated at 37°C for next 48 hours.

**Fluorescence microscopy**

GFP-positive cells were analyzed using a Motic AE31 inverted microscope using a fluorescein isothiocyanate (FITC) 480 nm filter. Cy5-labeled cells were visualized and analyzed using a Zeiss Axio Observer microscope fitted with a Cy5-specific filter (emission wavelength of 690 nm).

**Flow cytometry**

GFP fluorescence was used to indicate successful transfection, propidium iodide (PI) was used for dead cell discrimination, and Cy5 fluorescence to indicate successful nanoparticle association using a FACSCalibur flow cytometer (BD Biosciences). Briefly, adherent cells were trypsinized, centrifuged, and resuspended in 500 µL of fresh media and analyzed by flow cytometry. A total of 10,000 cells were acquired per analysis. All analyses were run with triplicate samples. Data analysis was conducted using BD CellQuest Pro software (BD Biosciences).

**Western blot analysis**

Whole-cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors and clarified by low-speed centrifugation. The protein extracts were quantitated by BCA assay, resolved by SDS-PAGE on 4%–12% Bis–Tris gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blotted with a 1:500 dilution of primary antibodies to p53 (Santa Cruz Biotechnologies), p21 (Calbiochem), anti-GFP (Abcam), and actin (Santa Cruz) and detected using horseradish peroxidase-conjugated secondary antibodies and visualized by ECL per protocol (Fierce).

**Cell-cycle analysis**

Cells were prepared at 48 and 72 hours posttreatment for cell-cycle analysis following a standard PI staining protocol and analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software. Each assay was run in triplicate, and all data are presented as the mean ± SEM.

**Annexin V staining**

To determine early apoptotic events, cells were assessed for Annexin V positivity. Cells were plated and transfected as described above. At the indicated time after transfection, cells were detached from the tissue culture plates using Accutase (Sigma), stained with Annexin V Cy5 (BD Biosciences) per manufacturer’s directions, and analyzed on a FACSCalibur flow cytometer. Cisplatin (10 µmol/L)-treated cells were used as a positive control. Each assay was run in triplicate and all data is represented as the mean ± SEM.

**Fluorescence-activated cell sorting**

Cells were sorted using a using a FACS Aria II (BD Biosciences) into GFP-positive and -negative populations 18 hours posttransfection. Untreated cells passed through the sorting machine were used as an additional control. Post-sort analysis was completed within 2 hours of collection, cells were replated in complete RPMI and assessed at 48 and 72 hours posttransfection.
In vivo experiments  
Three to 4-week-old athymic female nude mice were injected subcutaneously with $1.5 \times 10^6$ H446 cells suspended in PBS and mixed 1:1 in Matrigel (BD Biosciences). Once tumor volumes reached 200 mm$^3$, the mice were randomized ($n = 3–4$) into 3 groups: (i) untreated, (ii) 457: CMV-LUC, and (iii) 457:CMV-p53-LUC. The PBAE:DNA nanoparticles were prepared at 30 w/w; 50 μg of DNA was used per i.t. injection and the mice were treated twice a week for 3 weeks. Tumor measurements, using a digital caliper, and weights were collected twice a week. Tumor volume was calculated as follows: tumor volume (mm$^3$) = length (mm) × width (mm)$^2$/2. Toxicity was monitored by weight loss and animal activity following ACUC protocols. Tumor growth curves are presented as mean ± SEM. The area under curve (AUC) was calculated using GraphPad Prism estimating total AUC from days 1 to 18 for respective treatment groups. The percentage inhibition in tumor growth was compared among all the treatment groups over the total time course of the study. Statistical significance was then determined by 2-way ANOVA followed by Bonferroni test. ($^* P \leq 0.001$; CMV-p53-LUC vs. CMV-LUC; $^\# P \leq 0.01$; CMV-p53-LUC vs. untreated). All animal experiments were carried out following JHU Animal Care and Use Committee regulations.

Results  
We generated a polymer array of 30 structurally distinct PBAEs and more than 120 nanoparticle formulations (Fig. 1). This array contained base polymers composed of 4 backbone monomers that differed by single carbons between the acrylate groups (B3, B3m, B4, and B5 corresponded to 3 carbons, 3 carbons + 1 methyl group, 4 carbons, and 5 carbons, respectively) and 3 side chain monomers (S3, S4, and S5 that differed by having 3, 4, and 5 carbons, respectively, between amine and alcohol groups). These base polymers were terminated with one of 6 amine-containing small-molecule end groups. Structures were validated by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR; ref. 34). We conducted a high-throughput luciferase-based screen of the PBAE array to identify leading polymers optimized for efficient transfection of SCLC cells. Gene delivery of exogenous luciferase plasmid resulted in luminescence that was quantified and normalized to untreated cells (Supplementary Fig. S1). The leading candidate polymers, such as 456 (Fig. 1), were able to transfect H446 cells several orders of magnitude better than other nanoparticles formulations and resulted in transfection efficiencies as high or higher than both positive controls, FuGENE HD and Lipofectamine 2000. The size of the leading PBAE: DNA nanoparticles was examined using nanoparticle tracking analysis (35) and quantified by nanoparticle number average size (Fig. 2A). DNA encapsulation and binding by the leading polymers was determined by gel electrophoresis, showing that at the optimized PBAE:DNA weight:weight ratios used in our study, the DNA is completely complexed by the polymers (Fig. 2B). In general, the nanoparticle diameters range from 100 to 200 nm, with the range for the leading polymers, a more narrow 100 to 150 nm. It has been previously reported that within this range, the size of the particle does not correlate strongly with transfection efficacy (29).

The extensive luciferase-based screen identified PBAEs that could induce high levels of expression, but to identify those PBAEs, which could transfect the highest percentage of cells, we used a GFP-based analysis. We selected 32 of the top candidates from the initial screen and quantitated the transfection efficiency by fluorescence microscopy and flow cytometry (Fig. 3A and B). Two of the top polymers, 456 and 457, exhibited transfection efficiencies of approximately 40%, comparable to the commercially available transfection agent, FuGENE HD (Fig. 3C, gray bars). The majority of the polymers also induced higher level GFP expression, quantitated by geometric mean fluorescence, compared with FuGENE HD (Fig. 3C, black bars). We adapted our transfection protocol for

![Figure 2](image-url)
suspension cells and found that 456 polymers could transfect H146 and H187 SCLC cell lines with efficiencies of 33% and 22%, respectively (Fig. 3D–F). As suspension cells are notoriously difficult to transfect, we found these results encouraging. We further assessed the ability of 456 polymers to transfect nontransformed cell lines. The transfection efficiency of our nanoparticles in the WI-38 and IMR-90 human lung fibroblast lines was 13% and 11%, respectively; therefore, the 456 polymers appeared to be fairly selective for SCLC over nontransformed cells (Supplementary Fig. S2). To determine whether the transfection efficiency was limited by poor nanoparticle association and uptake by SCLC cells, we transfected H446 cells with PBAEs complexed with a Cy5-labeled plasmid. We observed that more than 95% of cells were Cy5-positive (Supplementary Fig. S3), suggesting that events downstream of nanoparticle uptake such as intracellular trafficking, endosomal release, or nuclear uptake and processing may be additional determinants of PBAE transfection efficiency.

We next evaluated the ability of the 456 polymer to deliver functional p53. Transfection of H446 cells with 456:CMV-p53-GFP, but not 456:CMV-GFP, induced morphologic changes and punctate GFP localization (Fig. 4A). At 48 hours posttransfection, 22% of cells transfected with 456:CMV-p53-GFP were strongly GFP-positive, compared with 41% of those transfected with 456:CMV-GFP (Fig. 4B and C). p53 GFP expression was seen as early as 2 hours and peaked at 18 to 24 hours posttransfection (Fig. 4D). Consistent with functional p53 activity, we observed p21 induction at 18 to 24 hours, a significant increase of Annexin V–positive cells at 48 hours and cellular

Figure 3. A GFP-based secondary screen identifies several PBAE polymers that can deliver genes to adherent and suspension SCLC cell lines at efficiencies comparable to commercially available reagents. A and B, H446 cells were transfected with PBAEs complexed with CMV-GFP DNA and then analyzed by microscopy and flow cytometry. C, the transfection efficiency and geometric mean fluorescence of 15 PBAE polymers are shown; the PBAE polymers are indicated across the x-axis. Percentage of transfection is presented as the mean ± SEM of triplicate runs, and the geometric mean fluorescence is the geometric mean FL1 signal ± SEM of triplicate runs. FuGENE HD was used as a control. Statistical significance of geometric mean fluorescence was determined by one-way ANOVA comparing each group with FuGENE HD (\(, P < 0.01\)). D–F, two SCLC suspension cell lines, H146 and H187 cells, were transfected with 456:CMV-GFP and analyzed by fluorescence microscopy and flow cytometry.
accumulation in sub-G₁ at 72 hours posttransfection with 456:CMV-p53-GFP (Fig. 4D–F). To ascertain the effect of WT p53 restoration in more homogeneous populations, we sorted H446 cells transfected with either 456:CMV-GFP or 456:CMV-p53-GFP into GFP-positive and -negative populations (Fig. 5A). Postsort (20 hours posttransfection) flow cytometry confirmed that the sorted cells were relatively homogeneous (>80%) and GFP expression was maintained over 72 hours (Fig. 5B). At 48 and 72 hours, we observed robust p53 GFP expression and p21 induction in GFP-positive cells transfected with 456:CMV-p53-GFP (population 4) but in none of the other sorted populations (Fig. 5C). Cell-cycle analysis also revealed more than 40% of the population 4 cells had accumulated in sub-G₁ at 48 and 72 hours, consistent with functional p53 activity (Fig. 5D and E).

Successful nanoparticles delivery of WT p53 would be expected to result in cell-cycle arrest, induction of apoptosis, and inhibition of tumor progression in vivo. To determine whether PBAE-mediated p53 delivery had antitumor activity in vivo, we administered nanoparticles carrying CMV-p53-LUC intratumorally into subcutaneous H446 xenografts. For this proof-of-principle analysis, we selected another leading PBAE polymer from our in vitro studies, 457, as this polymer appeared to better target subcutaneous tumors in pilot in vivo experiments (data not shown). Nude mice bearing H446 xenografts received twice weekly i.t. injections of 457:CMV-p53-LUC, 457:CMV-LUC, or neither nanoparticle formulation, and the tumors were serially measured (Fig. 6). We observed more than 50% tumor growth inhibition with i.t. injection of 457:CMV-p53-LUC relative to either 457:CMV-LUC or no treatment.
treatment control \((P \leq 0.01\) for comparison to either control). Thus, when effectively delivered to tumors, PBAE-mediated nanoparticle delivery and exogenous expression of WT p53 can inhibit tumor growth of human SCLC xenografts.

**Discussion**

Here, we report on a nonviral, biodegradable PBAE nanoparticle that self-assembles with DNA and can deliver WT TP53 to SCLC cells. Using a luciferase-based high-throughput screening approach, we were able to identify PBAE polymers that could transfect SCLC lines at efficiencies comparable to commercially available transfection reagents. We showed that the 456 polymer could deliver functional p53 to SCLC cell lines resulting in p21 induction, induction of apoptosis, and accumulation in sub-G1. Finally, we showed that i.t. delivery of 457 CMV-p53-LUC significantly suppressed tumor growth, whereas injection of the polymer with CMV-LUC had no effect. Thus, when effectively delivered, exogenously expressed WT p53 suppresses tumor growth of H446 xenografts.


In addition to p53 deletion or inactivation, some p53 mutations result in dominant-negative (DN) or gain-of-function activities. The ability of WT p53 to overcome a gain-of-function p53 mutation is unclear. In spontaneous p53-mutant lymphoma and sarcoma models, WT p53 restoration resulted in tumor growth arrest but not tumor regression (39). In these experiments, p53 was restored to
endogenous levels, and the authors suggested that the ability of p53 to overcome a DN p53 mutant may be dose-dependent. As an alternative approach, small molecules that can restore mutant p53 to its WT conformation have been explored. One such molecule, PRIMA-1 (p53-dependent reactivation of massive apoptosis), is able to restore the transcriptional activity of mutant p53 and has efficacy in several preclinical cancer models including SCLC (40).

A primary limitation is the requirement for mutant p53; PRIMA-1 would not be effective in tumors harboring a null mutant or that are driven by overexpression of p53-binding proteins such as MDM2. The H446 SCLC cell line harbors a G154V alteration in the DNA-binding domain of TP53 and expresses high basal levels of the mutant protein. We showed that CMV-driven expression of p53 was effective in this p53-mutant cell line; our cell sorting experiments show that H446 cells that expressed high-level exogenous WT p53 had robust p21 induction and accumulated in sub-G1.

This study shows that PBAE-mediated gene transfection is feasible both in vitro and, importantly, in tumor-bearing animals. Furthermore, this work shows that this approach can be used to restore activity of a silenced or absent tumor suppressor, resulting in specific inhibition of tumor growth. This same approach could be used to restore multiple silenced genes or to deliver other therapeutic agents. These polymers are amenable to a variety of modifications that may improve their efficiency in targeted delivery of an anticancer payload.

A better understanding of the limitations on DNA delivery is required to improve the use of this non-viral gene transfer system. While nearly 100% of the cells take up the particles, about 40% of the cells successfully expressed the target protein in our initial experiments. This indicates that events downstream may be limiting transfection efficiency and PBAEs are able to successfully escape endosomes (41), thus cytosolic transport and nuclear import are possible barriers and are the subject of active investigation (23). Delivery of therapeutic agents that are not reliant on 100% transfection that can induce a bystander effect may be well suited for this system. For example, nanoparticle delivery of the TRAIL induces apoptosis and tumor regression in the A549 NSCLC cell line (42). TRAIL-induced death, however, is reliant on host factors, including the presence of TRAIL receptors, DR4 or DR5, and caspase-8. Agents such as mitomycin C, doxorubicin, etoposide, and epigenetic modulators are able to sensitize cells to TRAIL by upregulating of DR4/5 or caspase-8 (43–45). Another approach, gene-directed enzyme prodrug therapy, is based on cell-specific delivery of an enzyme that can convert a systemically administered prodrug into a toxin. While the direct effects are cell-specific, this system could be adapted to induce a bystander effect by selecting a drug whose metabolite can traverse gap junctions.

Our in vivo data show that if effectively delivered to tumors, PBAE nanoparticles can successfully transfect SCLC xenografts and inhibit tumor growth. Our work is further supported by Huang and colleagues showing that PBAE nanoparticles can deliver diphtheria toxin and induce tumor regression when delivered locally in an ovarian cancer xenograft model (46). Systemic delivery and in vivo stability are major challenges in the clinical development of these nanoparticles. PBAE particles are readily amenable to approaches to add electrostatic coatings to improve these properties (42, 47, 48). In SCLC, cell surface expression of CD56 is nearly universal and can be targeted effectively by antibodies. A mantaysinoid-conjugated anti-CD56 antibody is being evaluated clinically in SCLCs and other tumors (49, 50). Coatings that incorporate NCAM-binding moieties may enhance SCLC specificity and improve in vivo stability of PBAE particles.

Therapeutic gene delivery has held great promise for decades but has been limited by the lack of stable, safe
delivery vehicles. Here, we report the first demonstration to our knowledge of effective non-viral polymeric delivery of therapeutic genes in SCLCs in vivo. The ability to deliver high-level gene expression using a nontoxic biodegradable nanoparticle has multiple advantages over viral-based delivery systems, including flexibility in cargo capacity, ability to deliver mixed nucleic acids targets, lack of insertional mutagenesis, and minimal immunogenicity. PBAEs fulfill all of these criteria and, with optimization of DNA delivery and systemic stability, may ultimately provide a powerful platform for therapeutic restoration of gene expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C.D. Kamat, J.J. Green, C.L. Hann
Development of methodology: C.D. Kamat, J.J. Green, C.L. Hann
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