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Inhibition of Tumor Angiogenesis and Growth by Nanoparticle-Mediated p53 Gene Therapy in Mice

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Abstract

Mutation of the p53 tumor suppressor gene, the most common genetic alteration in human cancers, results in more aggressive disease and increased resistance to conventional therapies. Aggressiveness may be related to the increased angiogenic activity of cancer cells containing mutant p53. To restore wild-type p53 function in cancer cells, we developed polymeric nanoparticles (NPs) for p53 gene delivery. Previous *in vitro* and *in vivo* studies demonstrated the ability of these NPs to provide sustained intracellular release of DNA, thus sustained gene transfection and decreased tumor cell proliferation. We investigated *in vivo* mechanisms involved in NP-mediated p53 tumor inhibition, with focus on angiogenesis. We hypothesize that sustained p53 gene delivery will help decrease tumor angiogenic activity and thus reduce tumor growth and improve animal survival. Xenografts of p53 mutant tumors were treated with a single intratumoral injection of p53NPs. We observed intratumoral p53 gene expression corresponding to tumor growth inhibition, over 5 weeks. Treated tumors showed upregulation of thrombospondin-1, a potent antiangiogenic factor, and a decrease in microvessel density vs. controls (saline, p53 DNA alone, and control NPs). Greater levels of apoptosis were also observed in p53NP-treated tumors. Overall, this led to significantly improved survival in p53NP-treated animals. NP-mediated p53 gene delivery slowed cancer progression and improved survival in an *in vivo* cancer model. One mechanism by which this is accomplished is disruption of tumor angiogenesis. We conclude that the NP-mediated sustained tumor p53 gene therapy can effectively be used for tumor growth inhibition.

Keywords

Neoplasms/*genetics/metabolism/*pathology; Cell Proliferation; Nanomedicine; Transfection

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Conflict of interest

The authors declare no conflict of interest.

Introduction

The p53 gene is the most widely studied tumor suppressor gene and one of the most important defenders of tumor development. It is best known for its role in governing cell-cycle progression, DNA repair, and apoptosis in response to cellular stress and damage, and therefore is often referred to as the “guardian of the genome.”¹ Mutations in p53 are one of the most frequent events in human cancer.² Mutant p53 proteins not only lose their tumor suppressive functions, but also gain new abilities to promote tumorigenesis.³ This change results in more aggressive disease, increased resistance to chemo- and radiation therapies, and poor clinical outcomes.^{4, 5} Consequently, significant research and development has been directed towards restoring functional p53 in tumors, either by rescuing the structure/function of the mutant protein or by introducing wild-type (wt) p53 in cancer cells.⁶ We are pursuing nonviral gene therapy as a strategy for delivering wt-p53 to solid tumors with the goal of stopping, slowing, and/or reversing disease progression.

In addition to maintaining the genomic stability of cells, p53 also plays a role in regulating the environmental conditions that impact tumor development. Many studies demonstrate a relationship between the p53 gene and angiogenesis,^{7, 8} which is essential for tumor growth and metastasis. Tumor cells with mutant p53 have a pro-angiogenic phenotype, marked by downregulation of angiogenic inhibitors and upregulation of pro-angiogenic factors.⁹ Intratumoral delivery of p53 can potentially affect tumor neovascularization,^{10, 11} in addition to directly inducing cell senescence and apoptosis of cancer cells. An understanding of the extent of the direct and “bystander” effects of p53 gene therapy is important in the development of this therapeutic approach.

We previously reported the effectiveness of nanoparticle (NP)-mediated p53 gene therapy for breast and prostate cancer *in vitro* and *in vivo*. Our NPs are engineered from biodegradable polymers that enable sustained release of p53 DNA within cells, resulting in less frequent dosing and prolonged gene expression compared to other nonviral gene delivery systems.^{12, 13} We administered p53-gene loaded NPs (p53NPs) intravenously in tumor-bearing mice and observed accumulation in tumor tissue without any systemic toxicity,¹³ bypassing several limitations associated with viral vectors which typically can only be administered by local injection due to toxicity/immunogenicity.¹⁴ Treatment with p53NPs both locally and systemically resulted in tumor inhibition and improved animal survival in a p53-null model of prostate cancer.¹³

The objective of this study was to evaluate the *in vivo* mechanisms of tumor inhibition by NP-mediated p53 gene therapy in a tumor model containing mutant p53, with focus on tumor angiogenesis. We selected a murine tumor model containing a commonly found missense mutation in p53 and treated the animals with one intratumoral injection of p53NPs. We hypothesized that sustained p53 gene expression in tumors would lead to sustained anti-angiogenic effects, thereby slowing disease progression.

Materials and Methods

Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA) (50:50, MW 143 kDa) was purchased from Durect Corp., Cupertino, CA. Acetylated bovine serum albumin (Ac-BSA) and polyvinyl alcohol (PVA, average MW 30–70 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS, heat inactivated), 1X trypsin-ethylenediaminetetraacetic acid (TE), Roswell Park Memorial Institute 1640 (RPMI 1640) medium, and penicillin-streptomycin were obtained from Gibco-BRL (Grand Island, NY). Human carcinoma (MDA-MB 435S) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). pCEP4 vector containing CMV-driven wild-type human p53 cDNA and the empty vector were provided by Dr Pi Wan Cheng (University of Nebraska Medical Center, Omaha, NE). Vectastain kitR and Avidin biotin blocking kit for immunohistochemistry were purchased from Vector Laboratories (Burlingame, CA). Primary antibody against TSP-1 was purchased from Abcam (Cambridge, MA). DeadEnd colorimetric TUNEL system was purchased from Promega (Madison, WI). QuantiTect SybrRGreen kit for Real time PCR was obtained from Qiagen (Valencia, CA).

Methods

Plasmid DNA Preparation

The recombinant pCEP4 vector containing a cytomegalovirus-driven human wt-p53 cDNA was kindly provided by Dr. Pi-Wang Cheng (Biochemistry and Molecular Biology, University of Nebraska Medical Center).¹⁵ The vector was transformed and propagated in DH5-alpha *E. coli* (Invitrogen, Carlsbad, CA). Plasmid was isolated using an Endofree Plasmid Giga Kit (Qiagen, Valencia, CA) according to manufacturer's protocol.

Formulation of nanoparticles containing plasmid DNA

DNA-loaded NPs were formulated by a double emulsion-solvent evaporation technique.¹⁶ In brief, an aqueous solution containing 1 mg of p53 plasmid DNA and 2 mg of Ac-BSA in 200 μ L of TE buffer were emulsified into 1 mL of PLGA solution in chloroform (30 mg PLGA/mL) using a probe sonicator (XL 2015 Sonicator[®] ultrasonic processor, Misonix Inc., Farmingdale, NY) at 55 W of energy output for 1 min over an ice bath. The Ac-BSA is nuclease free and provides stability to the primary emulsion, resulting in better encapsulation of DNA in NPs.¹⁷ The primary emulsion was then emulsified into 6 ml of 2% w/v aqueous solution of PVA using sonication as above for 5 min to form a multiple (water-in-oil-in-water) emulsion. We have previously shown that sonication of the emulsion does not affect the integrity of the DNA or its transfection efficiency.^{18, 19} The emulsion was stirred overnight at room temperature to evaporate chloroform. NPs were recovered by ultracentrifugation at 30,000 rpm (Beckman L80, Beckman Instruments, Inc., Fullerton, CA), washed three times with TE buffer to remove PVA and untrapped DNA, resuspended in autoclaved water, and lyophilized. The washings following the recovery of NPs were saved to determine DNA encapsulation using an indirect method.¹⁶ In brief, the DNA concentration in the washings was determined by ultraviolet absorbance at 260 nm using a spectrophotometer. Washings from NPs without DNA were used to blank the

spectrophotometer. The amount of DNA encapsulated in the NPs was determined by subtracting the amount in the washings from the amount used in the preparation. DNA encapsulation efficiency was approximately 70%, resulting in DNA loading of 2% w/w. NP size was determined using quasi-elastic light scattering (PSS/NICOMP 380/ZLS Particle Sizing Systems, Santa Barbara, CA). The resulting NPs had an average hydrodynamic diameter of 280 nm (diameter by transmission electronmicroscopy was 100 nm).²⁰

Cell culture

MDA-MB-435 cells, which contain a missense mutation in the DNA binding region of p53,²¹ were obtained from American Type Culture Collection (ATCC, Rockville, MD). The origin of these cells, originally described as breast cancer cells, is not conclusive and we therefore refer to them as human cancer cells. Cells were grown in complete Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics. Prior to use, cells were detached using TE at 37 °C.

MDA-MB-435 carcinoma model

Female athymic nude mice (age 5–6 weeks, weight 20–30 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were allowed to acclimatize for 7 days before study initiation. Animals were housed in a pathogen-free environment in animal housing and were given water and chow *ad libitum*. Animals were handled according to the protocol approved by the Institutional Animal Care and Use Committee.

Approximately 1×10^6 MDA-MB 435 cells in 100 μ l of Hank's balanced salt solution (HBSS) were injected subcutaneously in the left abdomen region of female athymic nude mice. When the tumors reached 50–60 mm³, the animals were randomly divided into four groups of eight animals each. The animals were categorized into groups receiving either p53 gene-loaded NPs (p53NP) or NPs containing control (p53 negative) plasmid [p53(-)NP], p53 plasmid DNA alone (p53DNA), or saline. For the p53DNA group, 50 μ g of DNA was suspended in 50 μ l of HBSS and injected intratumorally. For the p53NP and p53(-)NP groups, NPs containing 50 μ g of either p53 or control DNA were suspended in 50 μ l of HBSS and sonicated for 15 min using a water bath sonicator (FS140, Fisher Scientific, Pittsburgh, PA) before injecting them into the tumors. Saline controls comprised 50 μ L of HBSS injected in each control mouse, designated as saline control. Treatments were given only once in the entire study. All the mice were continuously monitored and were sacrificed if any signs of distress, weight loss, or metastasis (swelling of area around liver and spleen) were observed.

Tumor size was measured twice weekly, and tumor volume was calculated using the formula

$$V = \frac{l \times x \times b^2}{2}$$

where l is the largest dimension, b is the perpendicular diameter of the largest dimension, and V is volume in cubic millimeters.

In a follow-up study to evaluate tumor characteristics, a similar treatment regimen was followed, except that three mice from each group were sacrificed at 1-, 3-, and 5-week intervals, and tumors were harvested for immunohistochemical and histopathological analysis as well as gene expression analysis. All tissue samples for histology were frozen and sectioned by embedding in the O.C.T.TM compound (Tissue-Tek[®], Sakura, Torrance, CA) and 8 μ m-thin sections were cut using a rotary microtome (AO 820, American Optical, Del Mar, CA). Sections were stored at -80 °C before further analysis. Tissue samples for gene expression analysis were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Gene expression analysis

Total RNA was isolated from tumor tissues 1, 3, and 5 weeks post treatment using the RNeasy Qiagen Kit (Qiagen) as per the manufacturer's instructions. RNA (500 ng) was used for RT-PCR. Reverse transcription was performed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. For PCR, the following primers were used: p53 forward 5'-TCCACCAGGTCATCTACC-3', p53 reverse 5'-CTCTGAGCCGTTTCATACACA-3'; GAPDH (internal control) forward: 5'-TGGGGAAGGTGAAGTTCGGA-3' and GAPDH reverse: 5'-GAAGGGGTCATTGATGGCAA-3'.

The quantitative real-time PCR was performed on the 7500 Real Time PCR System (Applied Biosystems) using Power SYBR green PCR master mix (Applied Biosystems). PCR cycles were started with 50 oC for 2 min, 95 oC for 10 min, then 40 cycles of 30 s of denaturation at 94 oC, 40 s of annealing at 55 oC, and extension for 40 s at 72 oC, and were stopped/held at 4 oC. Melt curve analysis of PCR end products was performed to ensure there were no primer-dimer formations.

Data analysis was performed using 7500 System SDS Software, version 3.1 (Applied Biosystems). The comparative C_T method (C_T) method was used to calculate the relative expression. GAPDH was used an internal reference. Samples were normalized for GAPDH expression by the following expression:

$$\Delta C_T = C_T(p53) - C_T(GAPDH)$$

Saline control was used as a calibrator and the expression levels were calculated by the following equation:

$$\text{Relative expression level} = 2^{-\Delta\Delta C_T}$$

where $C_T = C_T(\text{Sample}) - C_T(\text{Saline})$. The log of the relative expression level was plotted over time for each group.

Immunohistochemical analysis for thrombospondin (TSP-1) in tumor cells

Frozen tissue sections were thawed to room temperature and fixed by incubating in 10 % buffered formalin fixative reagent for 15 min. The fixed tissue sections were then incubated with 0.3 % H₂O₂ at room temperature to block the endogenous peroxidase activity. The sections were then immunoassayed using a Vectastain kit (Vector Laboratories, Burlingame, CA). In brief, sections were incubated with 2.5 % serum for 30 min at room temperature to block nonspecific binding, followed by incubation with avidin and biotin blocking solutions for 15 min each. The sections were washed in between each step three times with 1X PBS to avoid carryovers. Sections were then incubated with primary mouse monoclonal antibody (anti-thrombospondin-1, 1:250 dilution, Abcam overnight at 4 °C. The following day, sections were washed thrice with PBS and incubated with secondary antibody provided with the kit for 30 min at room temperature. The sections were then incubated with biotin-horseradish peroxidase/Avidin (ABC reagent) for 45 min at room temperature. The antibody complex was visualized by staining with 3, 3' diaminobenzidine and counterstaining with hematoxylin-eosin. The sections were then washed in ethanol-glacial acetic acid (99.5% : 0.5%) solution, and slides were cleaned and mounted using PermountR. The sections were viewed under brightfield transmitted light using an Eclipse E600 microscope (Nikon, Melville, NY). The images were processed using Adobe Photoshop® 6.0 software (Adobe, San Jose, CA).

Evaluation of blood vessel density in tumors

Immunohistochemistry studies were performed in the Immunohistochemistry Core facility of Nebraska Health Systems using BMK iVIEW DAB procedure. In brief, frozen tissue sections were fixed in acetone. Samples were incubated with an I-VIEW inhibitor to block endogenous peroxidase activity and then incubated with purified anti-mouse CD31 (DAKO, Carpinteria, CA) at a dilution of 1:100. Sections were then incubated with I-VIEW biotin and I-VIEW streptavidine-horseradish peroxidase. Sections were visualized using DAB chromogen and were counterstained using I-VIEW copper.

Microvessel counting was performed as described by Weidner et al.²² Each stained section was screened at 10X (eyepiece) and 60X (objective) magnification to identify the areas of highest vascularization. Vessels were counted in 10 areas of highest vascular density. Branching areas were considered a single vessel. The microvessel count was expressed as the average number of vessels from 10 areas. For each treatment group, vessel counts are represented as the mean value obtained from tissues of three animals.

Detection of apoptosis by TUNEL assay

TUNEL assay was performed using DeadEnd colorimetric TUNEL system kit (Promega). In brief, formalin fixed tissue sections were incubated with biotinylated nucleotides in the presence of enzyme terminal deoxynucleotidyl transferase (TdT) for 1 hr at 37 °C after equilibration of tissues using equilibration buffer. Biotinylated nucleotides incorporated into the 3'-OH DNA using the TdT were visualized using DAB substrate after incubation with streptavidine-horseradish peroxidase for 30 min at room temperature. Sections were visualized under light microscope and pictures were taken as described in the immunohistochemical analysis section.

Statistical methods

All numerical data were expressed as the average of the values obtained, with error bars representing the standard error of mean unless otherwise noted. Tumor growth curves were analyzed by calculating the area under the curve from day 0 to the day on which animals began to die (day 45). The area under the curve provided estimates of percentage inhibition in tumor growth compared to control groups over the time course of the study, rather than at a single time point.²³ We performed Student's *t* test (without Bonferroni correction) to determine differences between treatment and control data sets. Analysis of the Kaplan-Meier survival curve was conducted by a log-rank statistical test. We considered $p = 0.05$ to be statistically significant.

Results

NP-mediated p53 gene delivery inhibits tumor growth and improves animal survival

A single intratumoral injection of p53NPs resulted in an overall reduction in tumor growth compared to controls (Figure 1). Tumor growth rates among control groups were similar; however, the p53NP group demonstrated a 43% reduction in tumor growth compared to saline control ($p = 0.012$). Complete tumor inhibition was observed in the p53NP group for 50 days post treatment, after which tumors started to grow again.

The overall reduction in tumor growth corresponded to improved animal survival in the p53NP group (Figure 2). Survival rates among the control groups, which included saline, p53 plasmid DNA alone (p53DNA), and NPs made with control plasmid (p53(-)NP), were similar, with median survivals of 138, 130, and 115 days, respectively. The survival of animals treated by p53NP was significantly greater ($p = 0.05$), with the majority of animals (6/8) surviving beyond 208 days.

NPs provide sustained transgene expression

Expression of the p53 gene was determined relative to saline controls (Figure 3). Since endogenous p53 expression in this cell line is possible, any increases in relative p53 gene expression were considered to be a result of treatment (i.e., transgene expression). In the p53NP group, an increase in intratumoral p53 expression was detected at all time points up to 5 weeks post treatment with p53NPs. This change corresponded to the time frame in which tumor growth inhibition was observed. Of the time points evaluated, the highest p53 expression levels – observed at 1 – week subsequently declined over 5 weeks. In contrast, no upregulation of p53 expression was observed in the p53DNA- and control NP (CNP)-treated tumors.

NP-mediated p53 gene delivery reduces tumor angiogenesis

Tumors treated with p53NPs demonstrated greater staining for the anti-angiogenic protein thrombospondin (TSP-1) compared to saline, CNP, and p53DNA controls; this response was sustained over 5 weeks (Figure 4). The p53DNA group also showed staining for TSP-1 at higher levels than the saline and CNP group, indicating some activity of the plasmid alone, although this was not observed to the same extent as in the p53NP group.

CD31 staining for endothelial cells was notably lower in the p53NP group compared to controls (Figure 5), indicating less angiogenic activity, which is consistent with the presence of TSP-1. With time, there was an increase in CD31 expression in the control groups, especially at 5 weeks; however, the low level of CD31 staining in the p53NP group was sustained.

Quantification of microvessel density revealed a significant ($p < 0.05$) reduction in the number of blood vessels in the tumors treated with p53NPs compared to controls at all time points evaluated (Figure 6). Tumor vascularity increased over time in the saline and CNP groups, which is consistent with the increased CD31 staining. In the p53DNA group, tumor vascularity appeared relatively constant. Tumors treated with p53NPs demonstrated a 34, 76, and 84% reduction in blood vessels compared to the saline control group at 1, 3, and 5 weeks, respectively.

NP-mediated p53 gene delivery increases levels of apoptosis in tumors

Treatment with p53NPs resulted in sustained and greater levels of apoptosis (Figure 7) at all the time points studied, with greatest levels observed at 3 weeks post treatment, as determined by the TUNEL assay. The p53DNA group also demonstrated apoptosis at 3 weeks, but not at 1 or 5 weeks. In contrast, tumors treated with saline and CNP did not show a significant level of apoptosis.

Discussion

In this study, we demonstrated that NP-mediated p53 gene delivery provides sustained wt-p53 gene expression in tumors, which reduced tumor growth, decreased angiogenesis, and improved animal survival. After a single intratumoral injection of p53NPs, we observed p53 gene expression for as long as 35 days (5 weeks) and complete tumor inhibition for 45 days, after which point the tumors began to grow again, albeit at slower rates than in the control groups. With p53 DNA alone, p53 expression was not observed at any of the time points, although assays for TSP-1 and apoptosis indicate that treatment with p53DNA alone may have some effects. It is possible that p53 expression in the p53DNA group occurred but did not persist long enough to be detectable at 1 week post-treatment. In our previous study using a prostate cancer model, slight p53 expression after intratumoral injection of plasmid p53 DNA was observed after 3 days but diminished by 14 days.¹³ The NPs protect the p53 DNA from degradation and provide controlled intracellular release, which enhances the activity and efficacy of p53 DNA. NPs formulated with control plasmid did not demonstrate tumor inhibition, indicating that efficacy is due to the delivery of the p53 gene and not to any nonspecific effects of the NPs. The p53 gene expression with p53NP treatment showed a steady decline over the 5-week period evaluated, and it is likely that tumor growth after 45 days corresponded to insufficient p53 expression. This information provides a basis for optimizing dosing regimens to sustain p53 expression and consequently improve efficacy.

The ability to provide a sustained release profile is one of the key advantages of our PLGA DNA-loaded NPs. Therapy using the p53 gene has been studied extensively with both viral and nonviral gene systems.^{10, 24–26} Viral vectors are very efficient at cellular targeting and gene transfection; however, they are associated with serious safety issues, such as toxicity,

insertional mutagenesis, and immunogenicity (especially with multiple administrations).^{25, 27, 28} Nonviral vectors, such as liposomes, lipoplexes, and cationic polyplexes, also have cytotoxicity issues, since they gain entry into cells via fusion with the plasma membrane, which can lead to nonspecific cell lysis.¹⁴ In addition, the resulting gene expression profiles of such nonviral vectors are transient. As a result, they are often administered in low but frequent doses to achieve efficacy and limit nonspecific effects.¹⁵ Our ability to provide sustained gene transfection without vector-associated toxicity is a significant advantage over these gene vectors. This feature is especially important when considering systemic administration.¹³

Angiogenesis plays a key role in tumor growth and metastasis. Tumors must stimulate the growth of new capillary blood vessels to guarantee an adequate supply of oxygen and nutrients to their proliferating cells and for expansion of metastatic foci.⁹ Tumors with higher microvessel densities tend to demonstrate greater metastatic potential.⁸ An increase in microvessel density was observed in tumors treated with saline and control NPs, which corresponded to higher tumor growth rates and poorer animal survival compared to animals treated with p53NPs. Tumors treated with p53NPs demonstrated significantly lower microvessel density that did not increase over the 5-week period evaluated. The disruption of tumor angiogenesis likely contributed to the observed tumor inhibition and, we hypothesize, reduced metastasis, leading to improved animal survival.

One of the mechanisms by which disruption of angiogenesis occurred was upregulation of TSP-1. TSP-1 is a large, multifunctional matrix glycoprotein that serves as a potent inhibitor of neovascularization by inhibiting endothelial cell proliferation, migration, and angiogenesis in the presence of angiogenic stimuli.²⁹ TSP-1 expression in tumors has been inversely correlated with malignant progression in breast and lung carcinomas and melanomas.³⁰ Therapeutic strategies aimed at delivering the gene for TSP-1 and/or specific peptide sequences responsible for its anti-angiogenic effect have also demonstrated tumor inhibition and decreased cancer progression.^{31–33} TSP-1 is a transcriptional target of p53 (i.e., its promoter is regulated by p53).³⁴ In this study, we demonstrated upregulation of TSP-1 in response to p53 gene delivery. Interestingly, although p53 gene expression declined significantly after 1 week in the p53NP group, the levels of TSP-1 were sustained over 5 weeks. It may be that even low levels of p53 expression are sufficient to activate TSP-1 and/or that TSP-1 remains relatively stable.

Other studies corroborate the anti-angiogenic effects of p53 gene delivery by various mechanisms. One study using cationic liposome:p53 DNA complexes demonstrated a reduction in the number of blood vessels within breast tumors, although upregulation of TSP-1 was not detected.¹⁰ Another study demonstrated that nonviral p53 delivery to lung tumors resulted in tumor inhibition via induction of TSP-1 expression.³⁵ Adenovirus-mediated p53 gene delivery was shown to inhibit angiogenesis in colon cancer by downregulating the pro-angiogenic factor, vascular endothelial growth factor.¹¹ It appears that restoration of wt-p53 in tumors may act as an antiangiogenic therapy via a number of possible pathways. Further studies are needed to understand the cellular targets for p53 gene therapy, and whether they are the cancer cells themselves or endothelial cells. Upregulation of p53 in endothelial cells has also been shown to arrest their cell cycle and growth.³⁶ In

addition, p53 gene delivery to peritumoral tissue could also influence the progression of tumor growth. Such mechanistic information would be useful in further engineering targeting strategies for NPs.

Cell-cycle arrest and apoptosis are critical functions of p53 in tumor suppression. Greater levels of apoptosis were observed in the p53NP-treated tumors compared to controls, with the greatest number of apoptotic cells observed at 3 weeks. However, tumors did not undergo regression but rather inhibition of growth. This observation is consistent with those of previous studies in which we evaluated p53NPs in p53-null models of prostate cancer. Growth rates of untransfected cells may have counterbalanced the rate of cell death. Alternatively, the majority of tumor cells may have undergone cell-cycle arrest rather than apoptosis, which would halt tumor growth but not necessarily lead to regression. The choice between growth arrest and apoptosis involves the complex interplay of numerous factors, including various post-translational modifications and protein-protein interactions.^{21, 37} It has been suggested that a threshold level of p53 activity is required to activate the low-affinity p53 binding sites on the promoters of apoptotic genes. In contrast, genes involved in growth arrest contain high-affinity p53 binding sites that will activate with relatively lower levels of p53.³⁸ The decreased blood supply may have also contributed indirectly to the levels of apoptosis. Further studies are needed to elucidate the transcriptional targets activated by NP-mediated p53 gene delivery related to cell-cycle arrest and apoptosis. This information could be useful in determining the extent of direct vs. bystander mechanisms of action and identifying synergies with other cancer treatment modalities. Such mechanisms may also be dose-dependent. Higher doses of p53NPs may not only lead to transfection of more cells but may result in greater activation of apoptosis vs. cell cycle arrest related genes. Our data suggest that a single dose of p53NPs provides tumor inhibition for 45 days. Higher and/or more frequent dosing may be required to achieve complete tumor regression.

It is interesting that at the 5-week time point, p53 expression was close to baseline and the number of apoptotic cells decreased; however, blood vessel density and TSP-1 expression were sustained at similar levels to the 1- and 3-week time points. During this period, tumor growth was inhibited. A single treatment of p53NPs provided a therapeutic effect that lasted 35–45 days, which may be attributed to the reduction in angiogenic activity even more so than to direct p53-mediated apoptosis. The success of nonviral gene delivery may depend not only on the engineered characteristics of the vehicle, but also on the selected transgene. The p53 gene is a potent inhibitor of tumor growth at low levels and exerts significant bystander effects, as demonstrated here by its impact on angiogenesis. Consequently, p53 may be well suited for nonviral approaches in which gene transfection efficiencies are typically lower compared to viral vectors. High levels of transfection may not be necessary to obtain tumor inhibition, since transfected cells have a significant effect the untransfected portions of the tumor. The wt-p53 protein is short-lived, and therefore a gene delivery vehicle that provides sustained release over time is beneficial for providing long-lasting therapeutic effects. Given the high prevalence of p53 mutations in many types of cancers, a safe and effective p53 gene delivery system could have a tremendous impact on the treatment of cancer.

In conclusion, we have demonstrated in a p53-mutant cancer model that NP-mediated p53 gene delivery resulted in sustained intratumoral p53 gene expression and upregulation of thrombospondin, which decreased angiogenic activity in tumors. This ultimately led to greater levels of apoptosis and fewer blood vessels in tumors, thereby inhibiting tumor growth and improving animal survival. While intratumoral injections are feasible in certain types and stages of cancer, future studies will be aimed at systemic delivery of p53NPs to treat or control the spread of metastatic tumors. The mechanistic understanding gained in the study provides a foundation for further optimization of a potentially valuable therapeutic tool against cancer. In addition to cancer, p53 plays a role in various conditions including metabolic disorders, ageing, and neurological disorders.¹ Hence, an effective delivery vector for p53 gene therapy could have significantly broader applications in treating various disease conditions.

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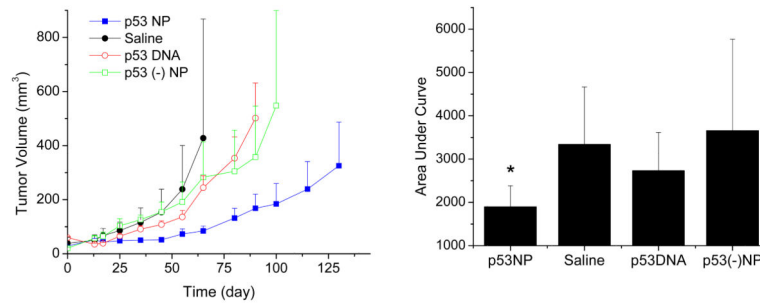


Figure 1. Tumor Growth Inhibition by p53NPs

(A) Tumor growth after a single intratumoral injection (day 0) of p53NP, p53DNA, p53(-)NP, or saline ($n = 8$ per group). (B) Area under the curve by day 45 (time of first animal death) demonstrates an overall reduction in tumor growth in the p53NP group compared to each control group. Statistical analysis was performed using Student's t test. * $p < 0.05$.

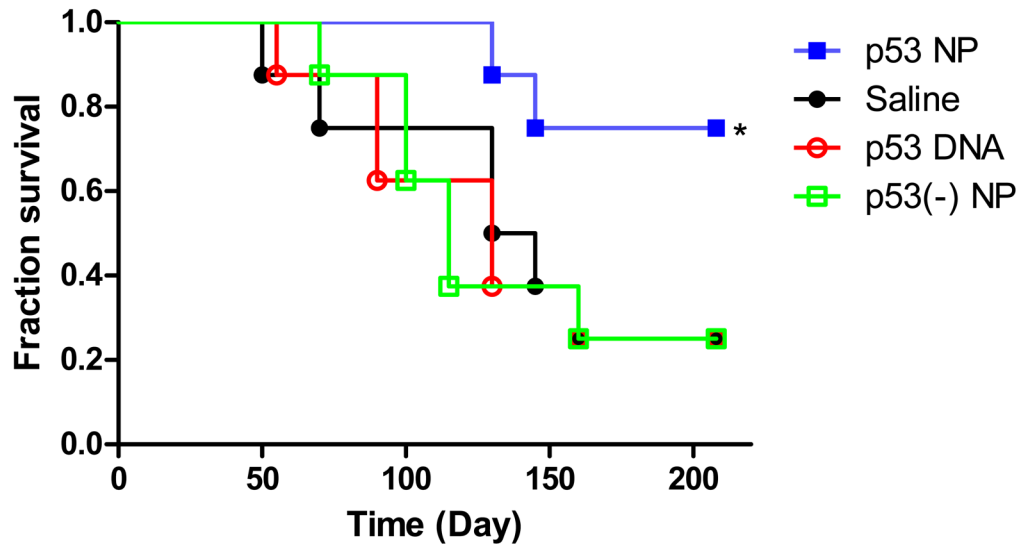


Figure 2. Improved Animal Survival with p53NP

Kaplan-Meier plot shows improvement in animal survival after a single intratumoral injection with p53NPs compared to controls. Log-rank test of p53NP and each control group yields $p < 0.05$ (*).

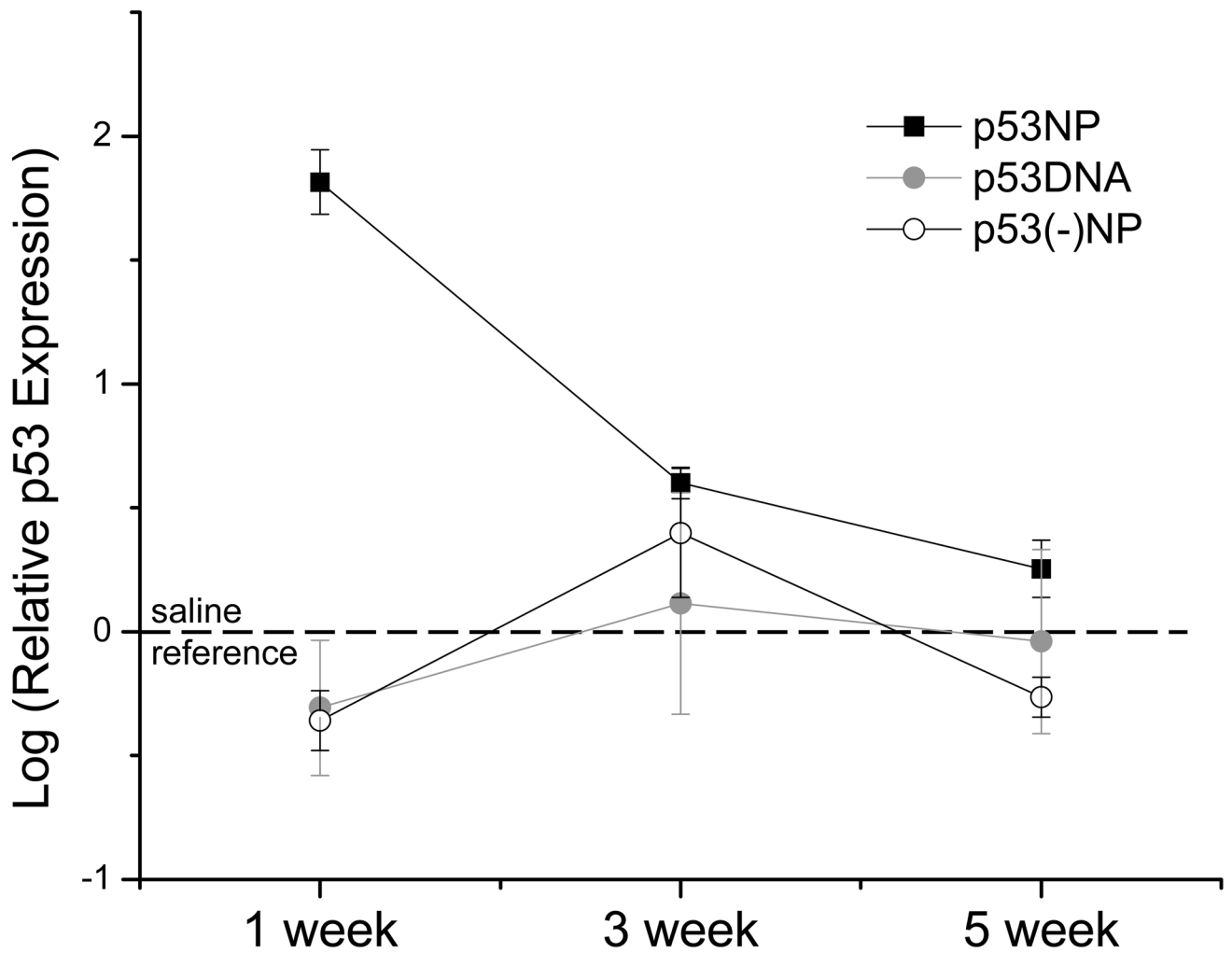


Figure 3. Intratumoral p53 Gene Expression

Expression of p53 relative to saline controls for p53NP, p53DNA, and p53(-)NP groups at 1, 3, and 5 weeks post treatment as determined by real-time RT-PCR. Data represented as mean \pm standard deviation ($n = 1-4$ tumors, triplicate samples). Tumors treated with p53NP demonstrated higher levels of p53 expression, which was sustained over 5 weeks.

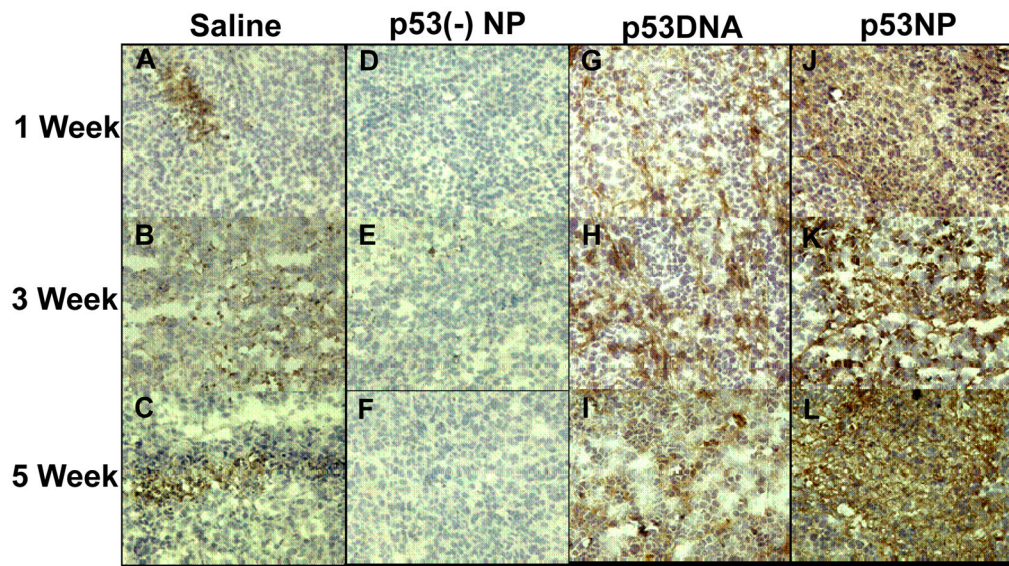


Figure 4. Intratumoral thrombospondin (TSP-1) protein expression
Immunohistochemical analysis of TSP-1 in tumors 1, 3, and 5 weeks post treatment with saline (A-C), p53(-)NP (D-F), p53DNA (G-I), and p53NP (J-L).

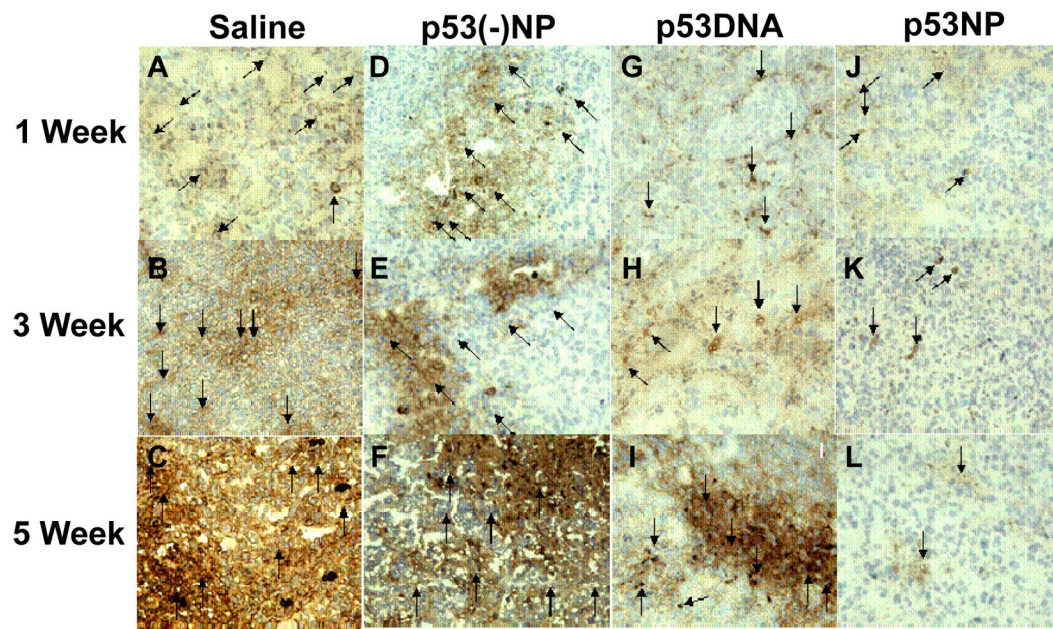


Figure 5. Intratumoral expression of angiogenesis marker CD31

Immunohistochemical analysis for CD31 (marker of angiogenesis) after 1, 3, and 5 weeks post treatment with saline (A-C), p53(-)NP (D-F), p53DNA (G-I), and p53NP (J-L).

Arrows indicate blood vessels (positive stain for CD31).

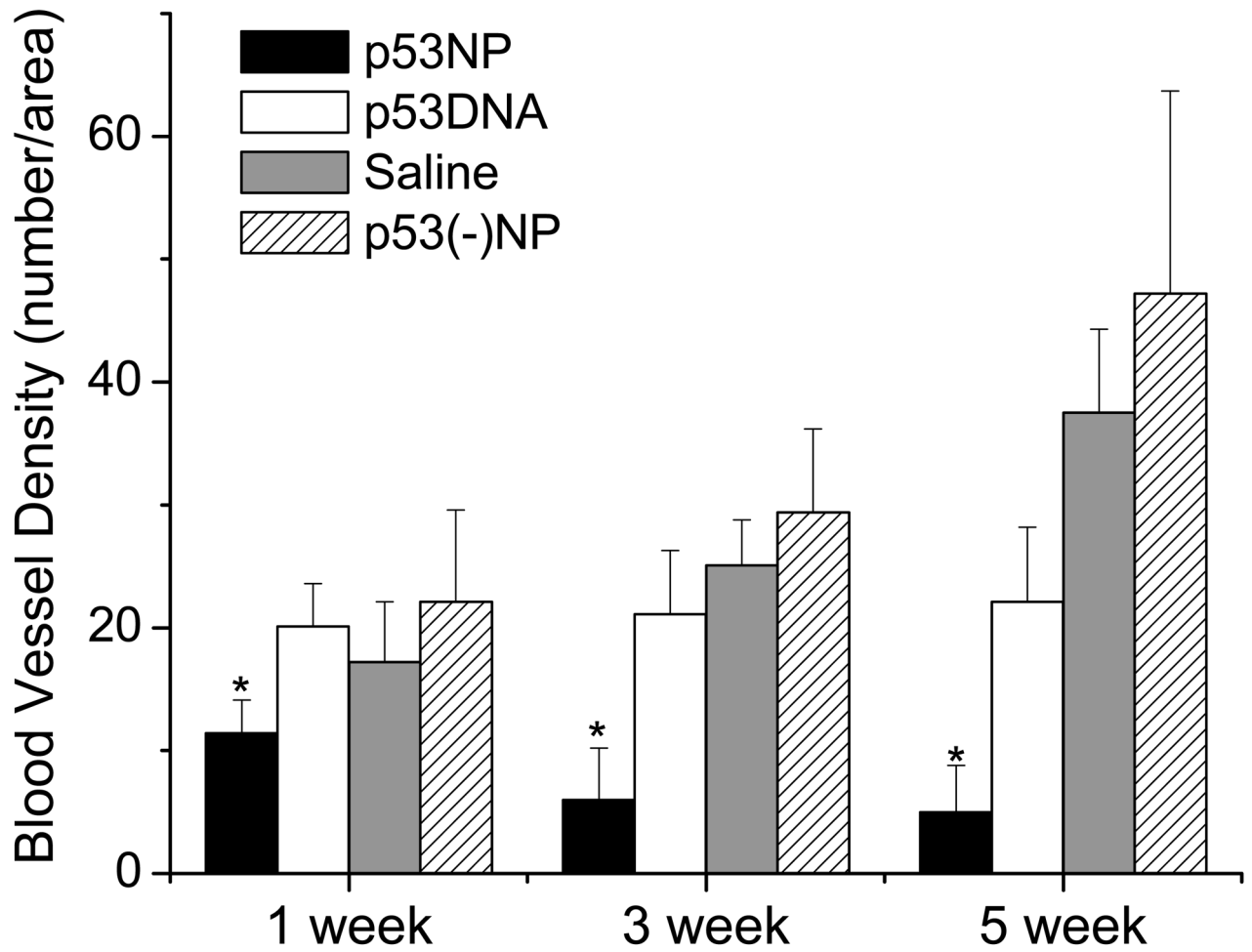


Figure 6. Intratumoral microvessel density

Quantification of microvessels (represented as number of blood vessels per field) in tumors after 1, 3, and 5 weeks post treatment with p53NP, saline, p53DNA, p53(-)NP. Tumors treated with p53NPs were less vascular compared to controls at all time points (* $p < 0.05$).

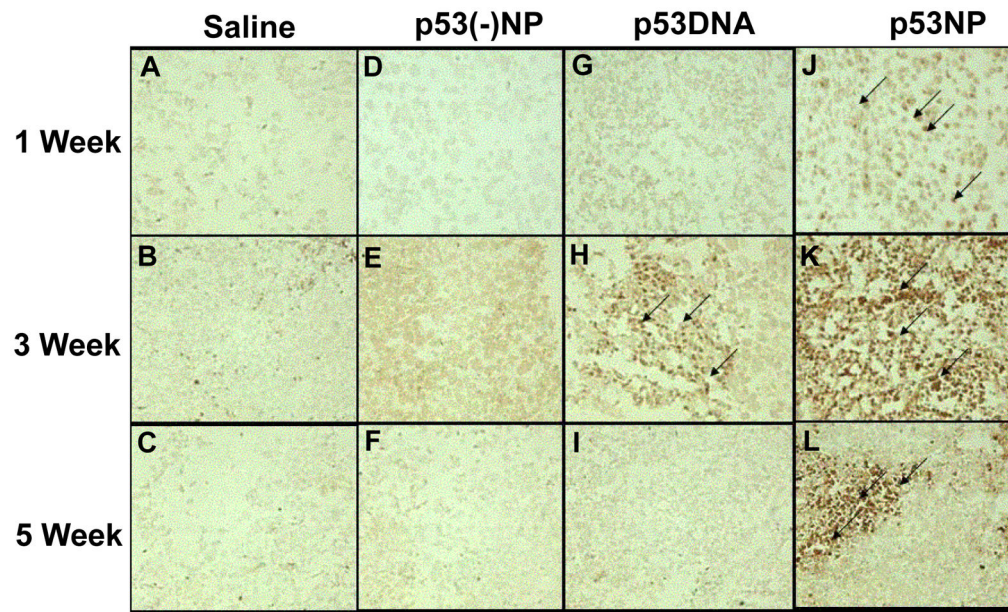


Figure 7. Apoptosis in tumors
TUNEL staining for apoptotic cells (arrows) 1, 3, and 5 weeks post treatment with saline (A-C), p53(-)NP (D-F), p53DNA (G-I), and p53NP (J-L).