

## ORIGINAL ARTICLE

# WT1 gene silencing by aerosol delivery of PEI–RNAi complexes inhibits B16-F10 lung metastases growth

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The Wilms' tumor gene 1 (WT1) is a universal tumor antigen and consequently a good therapeutic target for the development of gene therapy strategies. Earlier, we reported the *in vitro* efficacy of WT1 RNAi in the inhibition of B16F10 murine melanoma cell line growth. In this study, we used an aerosol system to deliver WT1 RNAi complexes, polyethyleneimine (PEI)–WT1-1 or PEI–WT1-2, to the lungs of mice with B16F10 lung metastasis. This treatment produced a statistically significant ( $P=0.020$ ) reduction in the number and size of lung tumor foci, resulting in decreased lung weight and tumor index in treated mice compared with controls. The WT1 RNAi treatment also reduced the number and size of tumor blood vessels, suggesting decreased angiogenesis. Furthermore, the treated lung tissue showed cells in the tumor infiltrations undergoing apoptosis and elevated expression of the proapoptotic genes Bcl-xS and Bax, suggesting an activation of the intrinsic apoptotic pathway. Overall, WT1-1 treatment prolonged the mean survival time of tumor-bearing mice in comparison with the control and WT1-2-treated mice. Our data show that WT1 gene silencing *in vivo* by aerosol delivery of PEI–WT1 RNAi complexes is an effective therapeutic strategy for the treatment of lung metastases.

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### Introduction

Gene therapy is an attractive strategy for the development of more effective anticancer therapies with reduced treatment-related toxicity.<sup>1</sup> There have been significant improvements in lung cancer treatment with gene therapy by targeting the protein products of mutated proto-oncogenes such as RAS,<sup>2</sup> tumor suppressor genes such as p53<sup>3</sup> or overexpressed genes associated with cancer progression and invasion, such as IGF-1R.<sup>4</sup> However, the efficacy of gene therapy is limited by the low efficiency of gene transfer and the technical difficulties of regimen delivery.

The first major limitation of gene therapy of the lung is the low efficiency of gene transfer. High gene transfection efficiency in the lung has been shown for DNA encapsulated by polymers and cationic lipids.<sup>5,6</sup> Recently, the cationic polymer polyethyleneimine (PEI) has been

shown to be an efficient vector for gene transfer *in vivo* and to have promising utility for aerosol-based gene delivery without the acute inflammatory cytokine responses caused by cationic liposomes and viral vectors.<sup>7–10</sup> The second major limitation of gene therapy is the route of delivery. Gene therapy regimens are typically delivered to the lung through intratumoral, intrabronchial or intrapleural injections.<sup>3,11,12</sup>

These types of direct intratracheal administration of therapeutic agents are technically cumbersome, uncomfortable and invasive. By contrast, aerosol delivery is more efficient and a simpler mode of gene therapy transport to the lungs. Aerosol delivery also has the advantages of uniform gene therapy agent distribution, lower risk of lung injury and access to a larger bronchial epithelium surface area.<sup>7,8</sup> Intranasal aerosol delivery of biological agents has been successfully shown for interleukin-12,<sup>13,14</sup> p53,<sup>3</sup> the therapeutic antibody cetuximab,<sup>15</sup> PTEN,<sup>16</sup> interleukin-2<sup>17</sup> and granulocyte macrophage colony-stimulating factor.<sup>18</sup> Several chemotherapeutic agents have also been tested for aerosol delivery to patients with lung carcinoma, including cisplatin and doxorubicin.<sup>19–22</sup> Thus, aerosol delivery cationic lipid-encapsulated DNA is a potentially more efficacious approach for the treatment of lung cancer with gene therapy regimens.

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The Wilms' tumor gene 1 (WT1) is a potential therapeutic target for the treatment of leukemia and other cancers.<sup>25-35</sup> WT1 encodes a transcription factor that is involved in gonadal development. Mutations in WT1 were initially found to cause urogenital disease and kidney tumors, suggesting that it is a tumor suppressor.<sup>36-39</sup> However, WT1 is considered to be an oncogene rather than a tumor suppressor gene because its increased expression is associated with the development and progression of diverse cancers including leukemia, mesothelioma, breast, gastric, colon, ovarian, melanoma and lung cancer. It also plays an important role in cell proliferation, differentiation of various tissue types, and apoptosis.<sup>40-42</sup> Increased expression of WT1 is associated with unfavorable prognosis of multiple malignancies and its downregulation by different gene therapy strategies leads to a down of cell proliferation and apoptosis.<sup>32-35</sup> Overexpression of WT1 mRNA has been detected in 54 of 56 (96%) *de novo* non-small cell lung cancers and protein overexpression was confirmed in 5 of 6 (83%) *de novo* small cell lung cancers by immunohistochemistry.<sup>43,44</sup> It is therefore a target for gene therapy with high potential for lung cancer treatment.

In this study, we use a B16-F10 mouse melanoma lung metastasis model to show that aerosol delivery of PEI-RNAi can be used to silence the WT1 gene.

Silencing of WT1 reduced the number and growth of visible metastatic tumor foci in the lungs. It also reduced the number of blood vessels, induced apoptosis and increased the mean survival time of treated animals. These data suggest that the silencing of the WT1 gene with aerosol delivery of PEI-RNAi is an attractive strategy for the prevention and treatment of established tumor metastases in the lungs.

## Materials and methods

### Animals

Female C57Bl/6 mice (7–8 weeks old, 20–25 g) were obtained from Harlan Mexico (Mexico, DF). The animals were housed in a 12-h light/12-h dark cycle and were provided with an autoclaved rodent diet and water *ad libitum*. All experiments were carried out in accordance with prior approval from the local animal ethics committee.

### B16F10 murine melanoma cell line

The murine B16F10 melanoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultivated in Dulbecco's modified Eagle Medium (DMEMF-12; Life Technologies, Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin and 10% FBS. Cells were maintained in culture flasks at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. The B16F10 murine melanoma cell line produces lung metastases when administered intravenously.<sup>45</sup> The cells were used at passages 3–12. B16F10 cells (5 × 10<sup>5</sup>) in 150 µl of media

without serum were injected per mouse through the tail vein. Lung metastases are detected within 7 days after injection.

### Preparation of PEI-DNA complexes

A PEI (25 kDa branched form; Aldrich, Milwaukee, IL) stock solution was prepared at a concentration of 4.3 mg ml<sup>-1</sup> (0.1 M in nitrogen) in water. The DNA had 3 nmol of phosphate per microgram and 1 µl of the 0.1 M PEI solution had 100 nmol of amine nitrogen. The resulting charge ratio was expressed as the ratio of PEI nitrogen to DNA phosphorus (N/P). A 10:1 N/P was used for the experiments, which corresponds to a 1.29:1 PEI/DNA weight ratio.

### WT1 RNAi expression constructs

Two oligo pairs (sense and antisense) for WT1 RNAi were designed using the siRNA design guidelines online software (Ambion, Austin, TX), synthesized at a concentration of 0.05 µg (Ambion) and used to create two recombinant plasmids (termed WT1-1 and WT1-2). For plasmid WT1-1, the oligos were 5'-gatccggctgtccacttacag atggaagctgcatctgtaagtgggacagctttttggaag-3', 3'-gccgacag ggtgaatgtctactctcgaacgttagacattcacctgtcgaaaaaccttcgcc gg-5' and for plasmid WT1-2, the oligos were 5'-gatccggagg agacatacaggtgtgagaagctgtcacactgtatgtctcttttttgggaagc-3', 3'-gctcctctgtatgtccacactcttcgaacagtggtgacatgcagaggaaaaaaccttcgccgg-5'. The oligos were resuspended to a final concentration of 1 µg µl<sup>-1</sup>, annealed and ligated into pGSH1-GFP (Gene Therapy Systems, Inc., San Diego, CA).

### Delivery of PEI-DNA complexes by aerosol

For aerosol treatment, we used a nose exposure chamber system for four mice. The recombinant plasmids were aerosolized using a Pulmo-Aide compressor model 5650D, which includes a Micro-Mist Nebulizer (115v) (DeVilbiss Health Care Corp., Somerset, PA).<sup>46</sup> The mice were treated with 25 µg of PEI-DNA complexes (WT1-1 or WT1-2) per mouse in a final volume of 2 ml. The aerosol delivery of the 2 ml solution took about 10 min. The mice were treated twice a week for 4 weeks (eight treatments in total), starting 2 days after injection of the melanoma cell line. The mice of the untreated control group were aerosolized with PEI-pEGFP-N2 complex (Clontech, Palo Alto, CA).

### Evaluation of tumor burden

Mice were killed on day 27 after injection. The lungs were isolated, washed with phosphate-buffered saline (PBS), weighed and examined with an inverted microscope to count the visible foci. The growth of tumors contiguous to the lung was considered to be a part of the lung for the purpose of weighing the lungs. As lungs in the control groups had uncountable foci, we graded them on the basis of a scale of 1–5 as described earlier.<sup>47</sup> A value of 1 indicated that there were less than 10 tumor foci; 2 indicated that there were 10–100 tumor foci; 3 indicated that one lobe of the lung was filled with tumor; 4 indicated that both lobes were filled with tumor; and 5

indicated that the lungs were filled with tumor and the tumor was growing out of the lungs into the chest wall. The tumor index was calculated by the formula: tumor index = lung weights  $\times$  average grade for the group. Reported values are the mean  $\pm$  s.d. ( $n=4$  mice per group).

#### Quantitative real-time RT-PCR

Lungs were isolated and total RNA was extracted using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. RNA concentration and integrity were determined by measuring the absorbance at 260 nm and by electrophoresis on a 1% agarose gel. For reverse transcription (RT)-PCR, 5  $\mu$ g of total RNA was reverse transcribed using SUPERScript RT (Invitrogen) and an oligo (dT)<sub>12-18</sub> primer (Invitrogen). Real-time RT-PCR was performed in an iCycler detection system (Bio-Rad, Hercules, CA). The measurements of WT1 mRNA using the TaqMan probe were carried out in 20  $\mu$ l of reaction mixture containing 200 nM of forward (TCTGCGGAGCCCAATACAG) primer, reverse (CACATCCTGAATGCCTCTGAAGA) primer and 250 nM probe using the Universal PCR Master Mix (Applied Biosystems, Foster, CA). After an initial hold of 10 min at 94 °C, samples were cycled 40 times at 94 °C for 30 s and at 64 °C for 30 s. Expression of the mRNA was assessed by evaluating the threshold cycle values. The threshold cycle values were normalized to the expression level of mouse  $\beta$ -actin mRNA (Mouse ACTB, VIC-MGB; Applied Biosystems) and the relative amount of WT1 mRNA was calculated using the 'DCT' method and a reference gene.

#### Histology

The mouse lung tissue was fixed in 10% formalin and embedded in paraffin. Histological observations were made on 4- $\mu$ m-thick sections stained with hematoxylin and eosin (H&E). Three mice not injected with cancer cells were included in each of the treatment groups to detect signs of toxicity or any pulmonary inflammation owing to treatment, PEI or the aerosol delivery system.

#### Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

The *in Situ* Cell Death Kit, AP (Roche Diagnostics, Indianapolis, IN) was used to analyze apoptosis in lung tissue. Paraffin-embedded mice lung tissue was rehydrated according to standard protocols by heating at 60 °C, washing in xylene and rehydrating through a graded series of ethanol and double-distilled water. Tissue sections were incubated for 30 min at 37 °C with proteinase K working solution (20  $\mu$ g ml<sup>-1</sup>, 10 mM Tris/HCl, pH 7.4). The tissues were then washed with PBS, permeabilized for 8 min with 0.1% sodium citrate solution containing 0.1% Triton X-100, washed twice with PBS, stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture for 60 min at 37 °C, washed twice with PBS, incubated with Converter-AP solution for 30 min at 37 °C, washed three times with PBS and incubated with

substrate solution (NBT/BCIP solution) for 10 min at 25 °C in the dark. After three washes, tissue sections were stained with hematoxylin, and apoptotic cells were observed by microscopy (Olympus Optical Co. Ltd, Center Valley, PA).

#### RT-PCR

Lung tissue was harvested and the total RNA was extracted using 1 ml of Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and integrity were determined by measuring the absorbance at 260 nm and by electrophoresis on 1% agarose gel. For RT-PCR, 5  $\mu$ g of total RNA was reverse transcribed using SUPERScript RT (Invitrogen) and oligo (dT)<sub>12-18</sub> primers (Invitrogen). The cDNA obtained was amplified using Taq DNA polymerase (Invitrogen) and the following primers: 5'-TTGGACAATGGACTGGTTGA-3' and 5'-GTAGAGTGGATGGTCAGTG-3', producing a 591 base-pair product (BclXS) or a 780 bp product (BclXL). For bax amplification, 5'-GGCCACCAGCTCTGAGCAGA-3' and 5'-GCCACGTGGCGTCCCAAAGT-3' primers were used to generate a 479 bp product. Amplifications for Bax and BclX were performed with 35 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s).<sup>48</sup> The primer sequences designed for amplification of a  $\beta$ -actin cDNA fragment (541 bp) were 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-GTCCCTAATGTCACGCACGATTTC-3'. Amplification was performed according to Laux *et al.*<sup>49</sup> PCR products were analyzed by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide (Anco and Rhenium Industries Ltd, Jerusalem, Israel).

#### Survival evaluation

To evaluate the length of survival after the aerosol delivery of PEI-WT1 RNAi constructs, a parallel experiment was carried out with eight tumor-bearing mice per group exposed to PEI-pEGFP-N2 (control), WT1-1 or WT1-2 RNAi. The mice were treated for 4 weeks, twice a week, beginning 2 days after tumor cell injection and then monitored over time for their survival.

#### Statistics

All experiments were carried out in triplicate, and statistical analysis was performed with the Mann-Whitney test using the SPSS 12.0 program (SPSS Inc.) with a significance of  $P < 0.05$ .

## Results

#### Suppression of B16F10 lung metastases by aerosol delivery of PEI-WT1 RNAi

To assess the effect of PEI-WT1-1 and PEI-WT1-2 RNAi on development of lung tumor metastases, the lung weight was measured and the numbers of metastatic lung surface nodules were observed for treated and untreated control animals. In comparison with the untreated control group (PEI-pEGFP-N2), the RNAi-treated groups showed

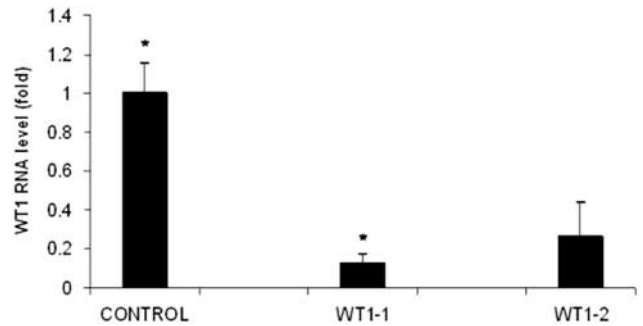
reduced numbers of tumor foci and statistically significant reductions in mean lung weights and tumor index (Figures 1a and b). The average lung weight of the treated mice was 0.20 g for WT1-1 and 0.22 g for WT1-2. These values were significantly reduced ( $P=0.020$ ) in comparison with those the untreated control group (0.69 g; Figure 1c).

The tumor index represents the tumor burden as the mean lung weight relative to the mean tumor grade of the group. The treated groups had a mean grade of two (10–100 tumor foci) and the untreated group had a mean grade of five (lungs covered by tumor with metastasis into the chest cavity). The mice treated with WT1-1 had a mean tumor index of 0.375 as compared with those treated with WT1-2 having a mean tumor index of 0.415 ( $P=0.020$ , control group tumor index = 3.265; Figure 1d). These results show the efficacy of aerosol delivery of WT1 RNAi against lung cancer.

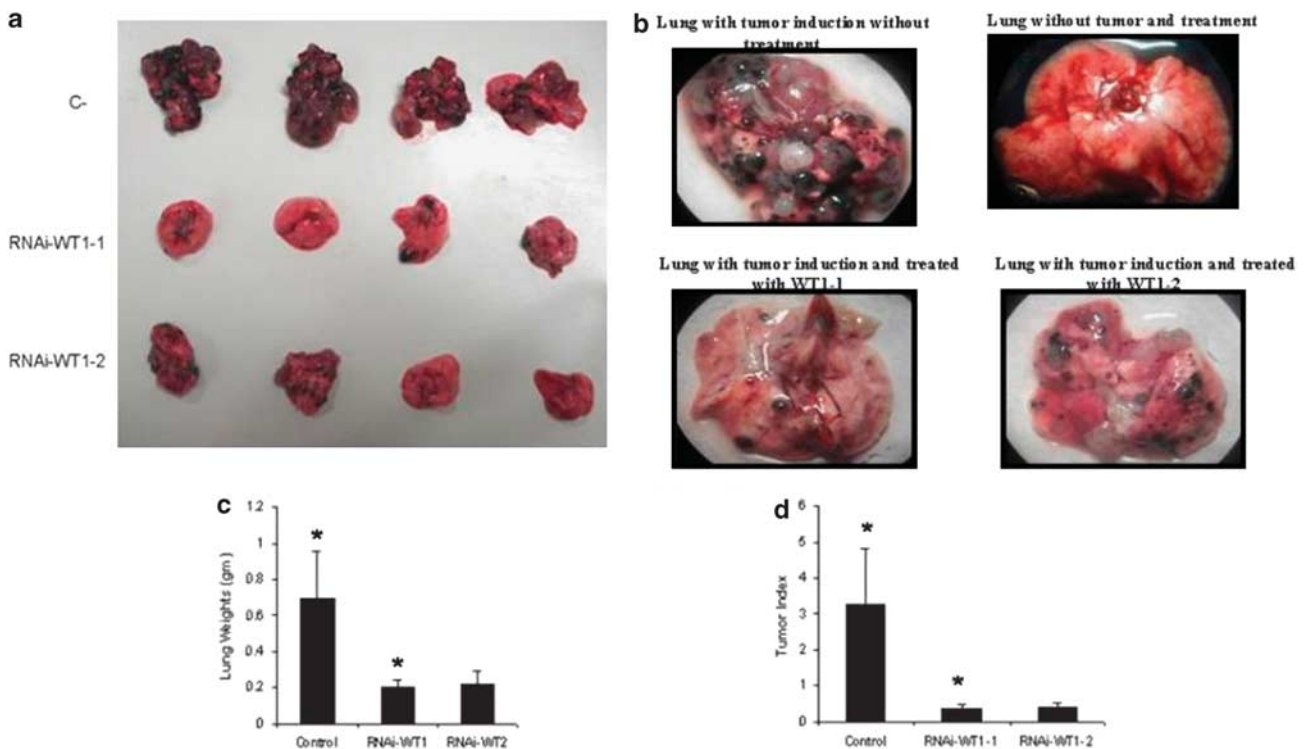
*Aerosol delivery of PEI-RNAi WT1 complexes downregulates WT1 mRNA*

To determinate whether the reduction in the number of metastatic lung tumors correlated with WT1 gene silencing, we evaluated WT1 mRNA levels in the lungs of mice after the treatment strategy using real-time RT-PCR. Quantitative analysis with real-time RT-PCR showed that aerosol delivery of WT1 RNAi significantly

reduced ( $P=0.020$ ) WT1 mRNA levels by 87% with WT1-1 and by 73% with WT1-2 in lung tissue in comparison with the untreated control group (Figure 2). These data suggest that the reduced tumor burden after RNAi treatments was due to silencing of WT1 expression in the lungs of treated mice.



**Figure 2** The Wilms' tumor gene 1 (WT1) mRNA expression in the lungs of mice after aerosol treatment with WT1-1 or WT1-2. The mice were treated with 25 μg of PEI-DNA complexes (WT1-1 or WT1-2) twice a week for 4 weeks. The control mice were exposed to PEI-pEGFP-N2. The mice were killed on day 27, lungs were harvested and tissue was assayed for WT1 mRNA expression by quantitative real-time RT-PCR. \*show that the greatest statistical difference was presented between control and the group treated with WT1-1. This significance was performed by Mann-Whitney test using the SPSS 12.0 program (SPSS Inc.) with a significance of  $P<0.05$ .



**Figure 1** Effects of the Wilms' tumor gene 1 (WT1) gene silencing by aerosol treatment with WT1-1 or WT1-2 on the growth of B16F10 experimental lung metastases. (a and b) Representative lungs from PEI-pEGFP-N2 control group and WT1-1 and WT1-2-treated mice. (c) Lung weights of PEI-pEGFP-N2 control group and WT1-1 and WT1-2-treated groups. (d) Tumor index was calculated by the formula: tumor index = lung weights × average grade for the group. Data are representative of at least two experiments. \*show that the greatest statistical difference was presented between control and the group treated with WT1-1. This significance was performed by Mann-Whitney test using the SPSS 12.0 program (SPSS Inc.) with a significance of  $P<0.05$ .

### Histological analysis of lung tissue from aerosol WT1 RNAi-treated mice

Observation of H&E-stained lung sections from untreated and treated tumor-bearing mice showed the presence of tumor infiltrations and the architecture of thickened alveolar septum in both groups. A higher number of blood vessels was observed in the tumors of the untreated control group (10–20 blood vessels per field; Figure 3a) in comparison with the WT1-1 and WT1-2-treated groups (3–7 blood vessels by field; Figure 3b). Furthermore, the RNAi-treated samples contained cells with cystic cytoplasm and fragmented nuclei, typical signs of cells undergoing apoptosis (Figure 3). We did not observe damage associated with acute inflammatory responses in lung tissues independently of neutrophil infiltration in any of the samples.

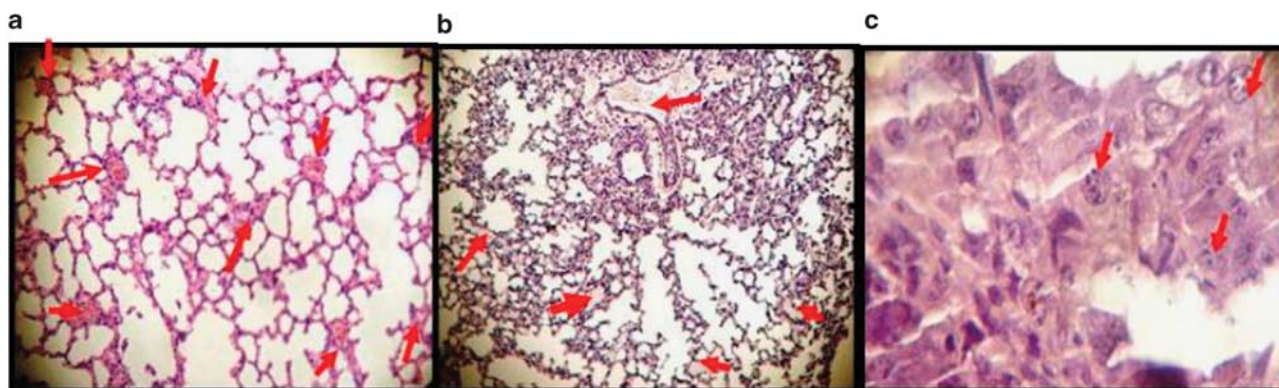
### Apoptosis in lung tumor foci of treated mice detected by TUNEL

We performed TUNEL assays to determine whether the cells with cystic cytoplasm and fragmented nuclei in the

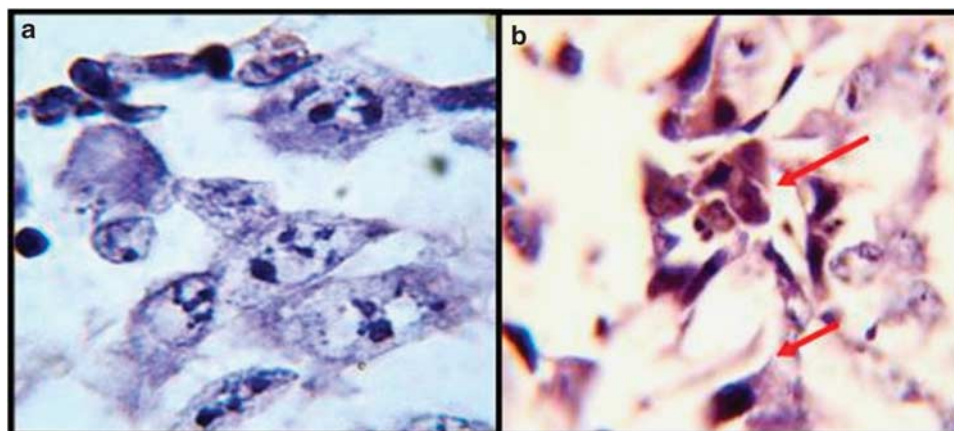
WT1-1- and WT1-2-treated groups were undergoing apoptosis. TUNEL-positive nuclei were detected in the WT1 RNAi-treated samples, but not in the PEI mock-treated samples (Figure 4). The apoptotic cells were present in the periphery of the tumor foci colocalized with tumor infiltrations. These results indicate that treatment with WT1 RNAi induces apoptosis in the lungs of B16F10 tumor-bearing mice.

### Expression of *Bcl-xL*, *Bcl-xS* and *Bax* in lung tissues of RNAi-treated mice

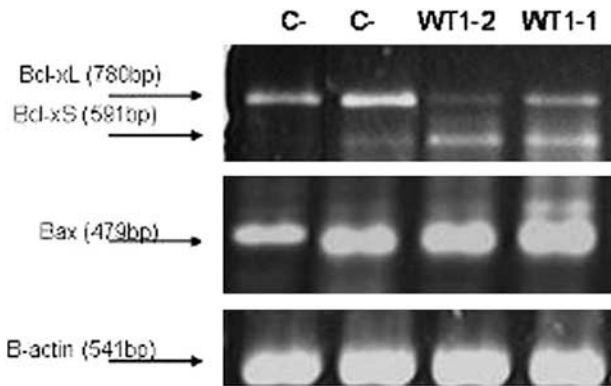
We analyzed the expression of apoptosis-related genes in the lung tissues of untreated or WT1-1 and WT1-2 RNAi-treated tumor-bearing mice. The expression of proapoptotic *BclxS* was preferentially expressed in the treated samples. By contrast, antiapoptotic *Bcl-xL* was preferentially expressed in the untreated samples. In addition, the *Bax* gene was also preferentially expressed in treated samples in comparison with the untreated samples (Figure 5). These results suggest that the WT1 RNAi



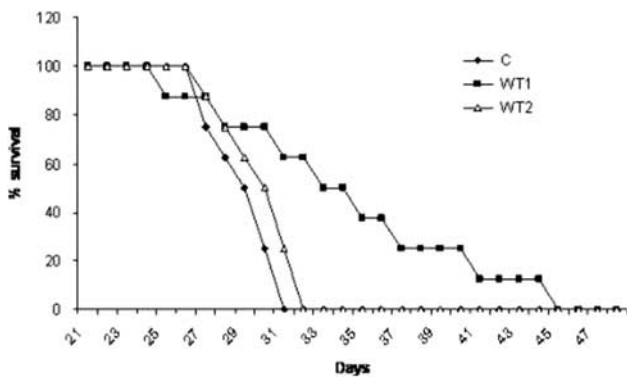
**Figure 3** Histological analysis of lung sections. Mice were injected i.v. with  $5 \times 10^5$  B16F10 cells. After eight aerosol treatments with WT1-1 recombinant plasmid (twice a week), lungs were fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin (H&E) staining. (a) Control group (PEI-pEGFP-N2) had 10–20 blood vessels per field. (b) The WT1-1-treated mice had 3–7 blood vessels per field. Arrows indicate blood vessels. (c) The WT1-1 cells had cystic cytoplasm and fragmented nuclei in the tumor infiltrations.



**Figure 4** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis of apoptosis in lung tumor foci of the Wilms' tumor gene 1 (WT1)-treated mice. After the WT1 treatment of the tumor-bearing mice, lungs were isolated, fixed in 10% formalin, processed for TUNEL and stained with hematoxylin. (a) PEI-pEGFP-N2 control tumor cell infiltrations were negative for TUNEL staining. (b) WT1-1-treated samples had TUNEL-positive staining in the tumor cell infiltrations.



**Figure 5** Expression of Bcl-xL, Bcl-xS and Bax genes by reverse transcription (RT)-PCR in lung tissues of control and RNAi-treated mice. After the eight treatments by aerosol with WT1-1 and WT1-2, lungs were harvested and apoptotic gene expression assessed by RT-PCR.



**Figure 6** Survival time of mice treated with WT1-1 and WT1-2 recombinant plasmids. Mice were injected i.v. with  $5 \times 10^5$  B16F10 cells and treated biweekly with PEI-pEGFP-N2, WT1-1 or WT1-2 for 4 weeks starting 2 days after tumor cell injection. Mice were monitored over time for their survival.

treatment initiates an intrinsic apoptotic pathway involving the expression of the Bcl-xS and Bax genes.

#### Survival time of mice treated with WT1-1 and WT1-2 complexes

The mice treated with the WT1-1 recombinant RNAi plasmid had prolonged survival ( $P=0.003$ , mean =  $36.5 \pm 9.5$  days) in comparison with the untreated control group ( $29.5 \pm 1.5$  days). Almost 80% of the mice treated with WT1-1 were alive on day 31, by which time all the untreated mice were deceased. By contrast, WT1-2 treatment did not produce a statistically different change in the mean survival time of the control tumor-bearing mice ( $30 \pm 2$  days; Figure 6).

#### Discussion

WT1 has been considered to be an excellent potential target for the development of gene-therapy strategies to

treat cancer because it is overexpressed in many types of neoplasias.<sup>40-42</sup> Different strategies have been used to decrease the WT1 production and, thereby, inhibit cancer cell proliferation, induce tumor apoptosis and sensitize tumor cells to chemotherapy and radiotherapy.<sup>23-31</sup> In previous studies, we showed that WT1 is expressed in B16F10 murine melanoma cell line and that *in vitro* RNAi silencing of WT1 expression results in the inhibition of cell proliferation, induction of apoptosis, caspase-3 activation and poly-(ADP-ribose)polymerase cleavage *in vitro*.<sup>30</sup> In this study, we extended this analysis to show that *in vivo* aerosol delivery of PEI-WT1-1 RNAi or PEI-WT1-2 RNAi complexes effectively reduces the metastatic tumor burden in the lungs of B16F10 mice. The PEI-WT1-1 RNAi or PEI-WT1-2 RNAi complexes significantly ( $P=0.020$ ) reduced WT1 RNA expression in lung tissue and this correlated with reduction in the number and size of lung tumor foci.

Histological analysis of lung tissue revealed two major differences between the treated and untreated groups. First, we observed a reduction in the number and size of blood vessels in tumor samples from treated mice. The reduced number of blood vessels in the treated samples suggests that silencing of WT1 inhibits angiogenesis. This is consistent with the observation that WT1 activates the ETS-1 gene, which is important for tumor vascularization through regulation of endothelial cell proliferation and migration,<sup>50</sup> and with studies about WT1 transcriptionally regulating vascular endothelial growth factor expression. These molecular mechanisms may be responsible for the role of WT1 in angiogenesis and cancer progression.<sup>51</sup>

The second major histological and molecular observation was the presence of cells in apoptosis induced by WT1 RNAi. These apoptotic cells were observed in the infiltrated peripheral areas of the tumor, but were absent from the tumor foci. These results are consistent with the results of Gautam *et al.*,<sup>52</sup> which showed that the peripheral areas of the tumor are most easily accessible to gene therapy. WT1 has also been shown to be required for the inhibition of apoptosis *in vitro* and *in vivo*. Several bcl-2 family members are either direct or indirect WT1 target genes, including the antiapoptotic bcl-2 and the proapoptotic family members Bak and Bax.<sup>53-55</sup> Thus, silencing of WT1 would be expected to influence cell viability by favoring the expression of proapoptotic bcl-2 family member genes. Our results suggest that the silencing of WT1 in the tumor-bearing mice activates an apoptotic pathway, and for this reason we analyzed some of the Bcl-2 family related genes involved in the intrinsic apoptotic pathway. We observed an overexpression of the proapoptotic genes Bcl-xS and Bax in the treated mice in comparison with the controls. These results show the activation of the intrinsic apoptotic pathway by WT1 gene silencing and corroborate, in part, the report that shRNA silencing of WT1 induces apoptosis of fibrosarcoma cells.<sup>56</sup>

WT1-1 RNAi treatment significantly increased ( $P=0.003$ ) the mean survival time of tumor-bearing mice in comparison with the mice treated with WT1-2 RNAi or the untreated mice. Studies are ongoing to assess the

potential of using 5% CO<sub>2</sub> during nebulization to increase the efficacy of aerosol delivery because it has been reported that this addition enhances transgene expression in the lung for PEI-DNA vectors.<sup>7</sup> In conclusion, *in vivo* WT1 gene silencing by aerosol delivery of PEI-WT1 RNAi complexes effectively decreases B16F10 lung metastases by reducing the number and size of tumor foci, activates the expression of genes that initiate the intrinsic apoptotic pathway and increases the mean length of survival of tumor-bearing mice. Aerosol delivery of RNAi targeting WT1 is therefore an attractive strategy for the treatment of lung cancer and may complement conventional anticancer therapies.

### Conflict of interest

The authors declare no conflict of interest.

### Acknowledgements

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