

# Therapeutic ultrasound facilitates antiangiogenic gene delivery and inhibits prostate tumor growth

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## Abstract

Gene therapy clinical trials are limited due to several hurdles concerning the type of vector used, particularly, the viral vectors, and transfection efficacy when non-viral vectors are used. Therapeutic ultrasound is a promising non-viral technology that can be used in the clinical setting. Here, for the first time, we show the efficacy of therapeutic ultrasound to deliver genes encoding for hemopexin-like domain fragment (PEX), an inhibitor of angiogenesis, to prostate tumors *in vivo*. Moreover, the addition of an ultrasound contrast agent (Optison) to the transfection process was evaluated. Prostate cancer cells and endothelial cells (EC) were transfected *in vitro* with cDNA-PEX using therapeutic ultrasound alone (TUS + pPEX) or with Optison (TUS + pPEX + Optison). The biological activity of the expressed PEX was assessed using proliferation, migration, and apoptosis assays done on EC and prostate cancer cells. TUS + pPEX + Optison led to the inhibition of EC and prostate cancer cell proliferation (<65%), migration (<50%), and an increase in apoptosis. *In vivo*, C57/black mice were inoculated s.c. with prostate cancer cells. The tumors were treated with TUS + pPEX and TUS + pPEX + Optison either once or repeatedly. Tumor growth was evaluated, after which histology and immunohistochemistry analyses were done. A single treatment of TUS + pPEX led to a 35% inhibition in tumor growth. Using TUS + PEX + Optison led to an inhibition of 50%. Repeated treatments of TUS + pPEX + Optison were found to significantly ( $P < 0.001$ ) inhibit prostate tumor growth by 80%, along with the angiogenic indices, with no toxicity to the surrounding tissues. These results depict the efficacy of therapeutic ultrasound as a non-viral technology to efficiently deliver genes

to tumors in general, and to deliver angiogenic inhibitors to prostate cancer in particular. [Mol Cancer Ther 2007;6(8):2371–82]

## Introduction

Antiangiogenic gene therapy is a promising approach to inhibit neovascularization via angiogenesis, the growth of new blood vessels from preexisting ones. Gene therapy vectors can localize the DNA at the site of interest, leading to the expression of the angiogenic inhibitor (1). Such an approach can overcome drawbacks associated with stability, quantity, and long-term administration, which involve antiangiogenic protein regimens. Nevertheless, clinical applications of gene therapy, in general, are still in the beginning stages due to several hurdles concerning transfection efficiency and the type of vectors used, particularly the viral ones, which are subject to safety issues (2, 3). On the other hand, non-viral vectors are emerging as a viable alternative, being safer in terms of mutagenesis and immunogenic response. Nevertheless, *in vivo* transfection levels and the efficiency of non-viral vectors are relatively lower when compared with the viral ones (4).

Ultrasound is a relatively new and promising non-viral approach that can deliver genes into cells and tissue in a noninvasive manner (5–7). Ultrasound waves can be directed to a specific tissue or organ, thereby localizing the transfection to a specific area and overcoming one of the major problems associated with other non-viral vectors (8, 9). Various ultrasound modalities were used for gene therapy applications. These include the diagnostic ultrasound, which operates at frequencies of 3 to 10 MHz and moderated intensities (9, 10), high-intensity (10–50 W/cm<sup>2</sup>; refs. 8, 11, 12) focused ultrasound and low-frequency (20–500 kHz) ultrasound (13). Therapeutic ultrasound, which is commonly used for physiotherapy, was recently used for gene delivery applications as well. Therapeutic ultrasound operates at moderate frequencies of 1 to 3 MHz and intensities of 0.5 to 3 W/cm<sup>2</sup> and is approved for clinical applications (14).

Using therapeutic ultrasound rather than low-frequency ultrasound or high-intensity focused ultrasound has several advantages. It has good penetration through soft tissues, does not damage cells and tissue, does not affect DNA integrity, and most importantly, is approved for clinical application. Nevertheless, unsolved issues remain regarding the efficiency and duration of gene expression using this technology. Recent studies have suggested using ultrasound contrast agents (USCA; gas-filled microbubbles) in the transfection process to overcome the low transfection efficiency associated with this modality (5, 6, 15–19). USCA are routinely used in diagnostics and are considered safe for therapeutic applications (5, 20).

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Nevertheless, the use of therapeutic ultrasound or therapeutic ultrasound in conjunction with USCA for cancer gene therapy in terms of efficacy outcome is barely, if at all, addressed.

This study shows, for the first time, the efficacy of therapeutic ultrasound to deliver naked cDNA encoding for PEX to prostate cancer. PEX is a COOH-terminally derived fragment of matrix metalloproteinase-2 (MMP2), which is known to inhibit angiogenesis *in vitro* and *in vivo* (21–23). The USCA Optison was used to increase transfection efficiency and thus prostate tumor inhibition. Repeated treatments of therapeutic ultrasound with PEX and Optison were found to significantly inhibit tumor growth and angiogenic indices with no toxicity observed in surrounding tissues. The results show the therapeutic efficacy of therapeutic ultrasound as a tool to deliver genes to tumors.

## Materials and Methods

### Plasmids

Expression plasmid DNA for human hemopexin-like domain, pTracer-PEX (pPEX), was constructed by subcloning the PEX-cDNA into pTracer plasmid (Invitrogen) using *EcoRI* and *NheI* restriction enzymes (Takara-Bio). The plasmid DNAs were amplified and purified using JET-Star kit (Genomed).

### Cell Culture

Human prostate cancer cell lines (LNCaP, American Type Culture Collection) were cultured in RPMI 1640 (Life Technologies, Invitrogen) supplemented with 10% FCS (Life Technologies) and 10 mmol/L HEPES buffer. Murine prostate cancer cells derived from transgenic adenocarcinoma of mouse prostate (TrampC2, PC2; received from Prof. R. Apte, Ben Gurion University of the Negev, Israel) were cultured in DMEM (Life Technologies) supplemented with 5% FCS and 5% Nu-Serum (BD Bioscience),  $10^{-8}$  mol/L of dihydrotestosterone (Sigma), and 5 µg/mL of insulin (Sigma). LNCaP and PC2 cells were used within 4 to 20 passages. Human umbilical vein endothelial cells (HUVEC) were isolated as described (24) and were cultured in M-199 medium (Biological Industries) supplemented with 20% FCS, vitamin solution (Biological Industries), and 2 ng/mL of basic fibroblast growth factor. Bovine capillary endothelia (BCE; received from Prof. J. Folkman, Harvard Medical School) were cultured in DMEM supplemented with 10% FCS. HUVEC and BCE cells were used within 4 to 10 passages. All media were supplemented with penicillin-streptomycin solutions and fungizone (Biological Industries) and the cultures were maintained in 37°C and 5% CO<sub>2</sub>.

### Ultrasound Apparatus and *In vitro* Gene Transfection

The ultrasound apparatus used for all experiments is a therapeutic ultrasound, which operates at a frequency of 1 MHz (UltraMax, XLTEK). The ultrasound setup and *in vitro* preparation of cells for therapeutic ultrasound application were as previously described (7, 25). The effect of USCA on transfection was evaluated using Optison

(Mallinckrodt). The plasmid DNA, with or without preincubation with Optison at 10% (v/v), was added to the cells, after which therapeutic ultrasound was applied at 30% duty cycle, 2 W/cm<sup>2</sup> for 30 min. Control cells received either pPEX only, pPEX + Optison without therapeutic ultrasound, or therapeutic ultrasound only. After 3 days, conditioned media from transfected cells was collected and transfection of pPEX was detected using Western blot analyses with primary antibodies against MMP2 (Oncogene Science) and secondary antibody peroxidase-conjugated (Sigma).

### RNA Preparation and Reverse Transcription-PCR

Transcription of PEX from cells transfected with TUS + pPEX or TUS + pPEX + Optison was evaluated for a period of 21 days post-therapeutic ultrasound application using reverse transcription-PCR (RT-PCR). Total RNA was extracted using Tri-Reagent (Sigma), following standard protocols, and 250 ng from each sample were taken for synthesis of cDNA using random primers (AB-Gene). For the amplification of PEX, the following primers were used: 5'-TAATACGACTCACTATAGGG-3' (sense) which is the T7-promoter primer in pTracer-PEX and 5'-GCCTCGTATACCGCATCAAT-3' (antisense). The primer for T7 was used to distinguish the expression of PEX transcribed from the expression plasmid to endogenous MMP2 transcription by the cells. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as a control and was done using the primers: 5'-ACCCAGAAGACTGTGGATGG-3' (sense) and 5'-CTTGCTCAGTGTCTTGCTG-3' (antisense). PCR conditions were: 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. PCR products were analyzed by 1% agarose gel electrophoresis.

### Cell Proliferation Assays

Proliferation assays were done on HUVEC (20,000 cells/well) and BCE (15,000 cells/well) using a thymidine incorporation assay (26). Cells were cultured with conditioned media taken from prostate cancer cells transfected with TUS + pPEX or TUS + pPEX + Optison, 3 or 6 days after therapeutic ultrasound. For negative controls, conditioned media was taken from prostate cancer cells treated with therapeutic ultrasound only, pPEX only, pPEX + Optison only, or nontreated prostate cancer cells. After 48 h, [<sup>3</sup>H]thymidine (1 µCi/mL, Amersham Bioscience) was added to each well and cells were incubated for another 12 h. The cells were lysed with 0.2N NaOH and suspended in liquid scintillation and analyzed by β-counter for radioactivity. The percentage of proliferation is expressed as counts per minute (cpm) obtained from each sample divided by cpm obtained from the negative control [endothelial cells (EC) grown with conditioned media taken from nontreated prostate cancer cells].

The proliferation of EC and prostate cancer cells themselves were also evaluated after their transfection with TUS + pPEX or TUS + pPEX + Optison. After 3 days, [<sup>3</sup>H]thymidine was added and proliferation was measured as described. As a negative control, cells were treated with therapeutic ultrasound only, pPEX only, or pPEX + Optison only. As a positive control, different concentrations

(0.1–10  $\mu\text{g}/\text{mL}$ ) of purified PEX protein (purified in our laboratory, as described in refs. 21, 23) were added to prostate cancer cells. Cell proliferation was measured as described.

#### Cell Migration Assay

The migratory ability of EC grown with conditioned media taken from prostate cancer cells transfected with TUS + pPEX or TUS + pPEX + Optison was assayed as previously described (21). Briefly, one linear scar was drawn in the monolayer of confluent EC. The stripped area was marked and a set of photos were taken using Laboratory Universal Computer Image Analyses (LUCIA) software (Laboratory Imaging). The wells were washed, and conditioned media from prostate cancer cells transfected with TUS + pPEX or TUS + pPEX + Optison, was added to the wells. EC that were incubated with conditioned media taken from prostate cancer cells treated with therapeutic ultrasound only, with pPEX only, or from nontreated prostate cancer cells served as control. Photos were taken at different time intervals, superimposed and cells that migrated across the line drawn were counted. Means of all field areas from each group were calculated and presented as a percentage from the mean of the control.

#### Apoptosis Assays

Apoptosis assay was done on HUVEC incubated with conditioned media taken from prostate cancer cells 3 days after transfection with TUS + pPEX or TUS + pPEX + Optison. Apoptosis was measured using Annexin V-FITC (BD Bioscience) and propidium iodide (Sigma) by flow cytometry (FACSCalibur, BD Bioscience; ref. 27). As controls, cells were incubated with conditioned media taken from prostate cancer cells treated with (a) therapeutic ultrasound only, (b) pPEX only, or (c) pPEX + Optison only. Results are presented as the percentage of apoptotic cells measured in the various treatments.

#### *In vivo* Transfection Using Single Treatment of Therapeutic Ultrasound with pPEX or pPEX + Optison

Mice, C57/black male 4 to 5 weeks old, were inoculated s.c. in the flank with  $2 \times 10^6$  PC2 cells per mouse. When tumors reached  $\sim 100 \text{ mm}^3$ , animals were randomly divided into four groups of eight mice in each group. The treatment groups were: (a) control—no treatment, (b) mice treated with therapeutic ultrasound only, (c) mice injected intratumorally (i.t.) with 100  $\mu\text{g}$  of pPEX without therapeutic ultrasound application, and (d) mice injected i.t. with 100  $\mu\text{g}$  of pPEX and subjected to a single application of therapeutic ultrasound. To evaluate the effect of Optison, another set of experiments was done with the following groups: (a) control—no treatment, (b) mice treated with TUS + Optison only, (c) mice injected i.t. with 100  $\mu\text{g}$  of pPEX with Optison, without therapeutic ultrasound application, and (d) mice injected i.t. with 100  $\mu\text{g}$  of pPEX with Optison and subjected to a single application of therapeutic ultrasound. For therapeutic ultrasound applications, an ultrasound coupling gel was applied on the skin above the tumor and therapeutic ultrasound was applied at 30% duty cycle, 2  $\text{W}/\text{cm}^2$  for 20 min. Subcutaneous tumor growth was measured with a caliber every 2 days for 28 days, and

tumor volume was calculated (26). All mice were sacrificed after 28 days and tumor weights and volumes were measured. Immunohistochemistry and RT-PCR assays were done on the harvested tumors. All animal studies were approved by the Animal Ethics Committee at the Technion.

#### Duration of pPEX Transcription in Tumors Post-Therapeutic Ultrasound Application

Mice ( $n = 60$ ) were inoculated with PC2 cells and treated once with TUS + pPEX ( $n = 30$ ) or TUS + pPEX + Optison ( $n = 30$ ). At various points in time—1, 3, 7, 14, and 21 days—after therapeutic ultrasound application, six mice from each group were sacrificed. Tumors were taken for RNA analyses and RT-PCR was done using primers for pPEX and glyceraldehyde-3-phosphate dehydrogenase as described.

#### *In vivo* Transfection Using Repeated Treatments of Therapeutic Ultrasound with pPEX or pPEX + Optison

Based on the results obtained from the single treatment, additional *in vivo* experiments were done in which mice ( $n = 32$ ) inoculated with PC2 were subjected to repeated treatments of TUS + pPEX or TUS + pPEX + Optison. The treatment was carried out once a week for 4 weeks in the following groups of eight mice: (a) mice treated with TUS + Optison only, (b) mice injected i.t. with 100  $\mu\text{g}$  of pPEX + Optison without therapeutic ultrasound, (c) mice injected i.t. with 100  $\mu\text{g}$  of pPEX with Optison without therapeutic ultrasound, and (d) mice injected i.t. with 100  $\mu\text{g}$  of pPEX with Optison and subjected to therapeutic ultrasound application. Therapeutic ultrasound application and measurements of tumor volume and weight were done as previously described.

#### Histology, Immunohistochemistry, and Immunofluorescence

Harvested tumors were embedded in optimal cutting temperature (Tissue-Tek, Sakura), frozen with liquid nitrogen and stored at  $-80^\circ\text{C}$ . Sections (7  $\mu\text{m}$ ) from each tumor were stained using H&E. Immunohistochemistry was carried out using Vectastain Elite ABC kit (Vector Laboratories). Primary antibodies include anti-CD31 (1:100; BD Bioscience) for microvessel staining, anti-Ki-67 nuclear antigen (1:100; LabVision) for proliferating cells, anti-cleaved caspase-3 (1:100; Cell Signaling) for the detection of apoptosis, anti-E-cadherin (1:100, Cell Signaling) for the detection of tumor invasiveness, and anti-human MMP2 (1:100; LabVision) for the detection of pPEX expression in the tumor. Detections were carried out using the 3,3'-diaminobenzidine chromogen (Vector Laboratories) and sections were counterstained with hematoxylin. Negative control slides were obtained by omitting the primary antibody. Microvessel density was assessed according to a method described elsewhere (28). The percentage of the area with microvessels and the area ratio of E-cadherin expression were determined by LUCIA image analysis software using 10 randomly chosen fields per section in at least five sections at  $\times 100$  magnification. The proliferation and apoptotic indices were defined as the percentage of positively stained cells of 100 nuclei from 10 randomly

chosen fields at  $\times 200$  magnification, as previously described (22). Dual-immunofluorescence for localization of apoptosis, proliferation, and pPEX expression in the tumor were done using secondary antibodies labeled with rhodamine (Jackson ImmunoResearch). Sections were counterstained with 4',6-diamidino-2-phenylindole for nuclei staining.

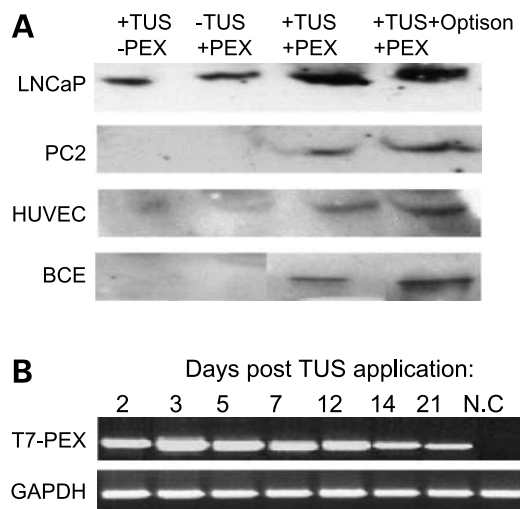
#### Statistical Analysis

All data are expressed as mean value  $\pm$  SE or expressed as a percentage relative to control  $\pm$  SE. Statistical differences between treatment groups were determined using Student's *t* test for independent samples. Statistical significance was defined as  $P < 0.05$ . All micrographs were representatives of at least 15 random micrographs taken from independent experiments.

## Results

### In vitro Transfection of Prostate Cancer Cells and EC Using Therapeutic Ultrasound

Western blot analyses were done on PC2, LNCaP, HUVEC, and BCE that were transfected with TUS + pPEX or TUS + pPEX + Optison. Medium taken from cells treated with therapeutic ultrasound only or with pPEX only served as controls. Results showed that all cells were successfully transfected with pPEX using therapeutic ultrasound with or without the addition of Optison (Fig. 1A). A low level of endogenous expression of PEX was observed only in LNCaP cells. Transfection of pPEX using therapeutic ultrasound was also evaluated using RT-PCR (Fig. 1B), showing that PEX was transcribed from PC2 cells 21 days after a single therapeutic ultrasound application.



**Figure 1.** Transfection of EC and prostate cancer cells using therapeutic ultrasound. Therapeutic ultrasound was applied at 1 MHz, 30% duty cycle, 2 W/cm<sup>2</sup> for 30 min. **A**, Western blot analyses of PEX expressed in conditioned media taken from LNCaP, PC2, HUVEC, and BCE cells transfected with pPEX using therapeutic ultrasound. **B**, RT-PCR analyses of pPEX transcribed from PC2 cells 21 d post-therapeutic ultrasound application. N.C., negative control, PC2 cells alone. Transcription of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is shown as control.

### Expression of pPEX from Prostate Cancer Cells Post-Therapeutic Ultrasound Transfection Inhibits EC Migration and Proliferation and Increases Apoptosis

The biological activity of PEX transcribed from prostate cancer cells post-therapeutic ultrasound transfection was assessed on EC migration, proliferation, and apoptosis. Conditioned media taken from PC2 and LNCaP cells 3 days after transfection with TUS + pPEX led to the inhibition of EC migration by 70% to 80% and 55% to 60%, respectively (Fig. 2A). The addition of Optison during therapeutic ultrasound transfection resulted in a slight increase in the inhibition of EC migration (not significant; Fig. 2A). In addition, PEX transcribed from prostate cancer cells after therapeutic ultrasound transfection induced apoptosis in HUVEC cells (Fig. 2B). Incubation of HUVEC with conditioned media taken from PC2 transfected with TUS + pPEX or with TUS + pPEX + Optison resulted in an increase in the percentage of apoptotic cells from  $7 \pm 2\%$  (control) to  $13 \pm 3\%$  ( $P < 0.05$ ) and  $17 \pm 4\%$  ( $P < 0.001$ ), respectively. Incubation of HUVEC with conditioned media taken from LNCaP transfected with TUS + pPEX or with TUS + pPEX + Optison resulted in  $15 \pm 3\%$  ( $P < 0.001$ ) and  $18 \pm 4\%$  ( $P < 0.001$ ) of apoptotic cells, respectively (Fig. 2B).

Proliferation assays revealed that EC cultured with conditioned media taken from prostate cancer cells not exposed to therapeutic ultrasound (negative control), treated with therapeutic ultrasound only, or pPEX only maintained their proliferation rate (Fig. 2C and D). However, when EC were cultured with conditioned media taken from PC2 or LNCaP 3 days posttransfection with TUS + pPEX, their proliferation was hindered by  $>55\%$ . When conditioned media was taken from prostate cancer cells 6 days posttransfection with TUS + pPEX, the proliferation of EC was inhibited by  $>45\%$ . The proliferation of EC cultured with conditioned media taken from prostate cancer cells transfected with TUS + pPEX + Optison was further inhibited, although not significantly. The highest inhibition was observed when HUVEC were grown with conditioned media taken from PC2 transfected with TUS + pPEX + Optison 3 days after therapeutic ultrasound reaching 28% relative to control (Fig. 2C).

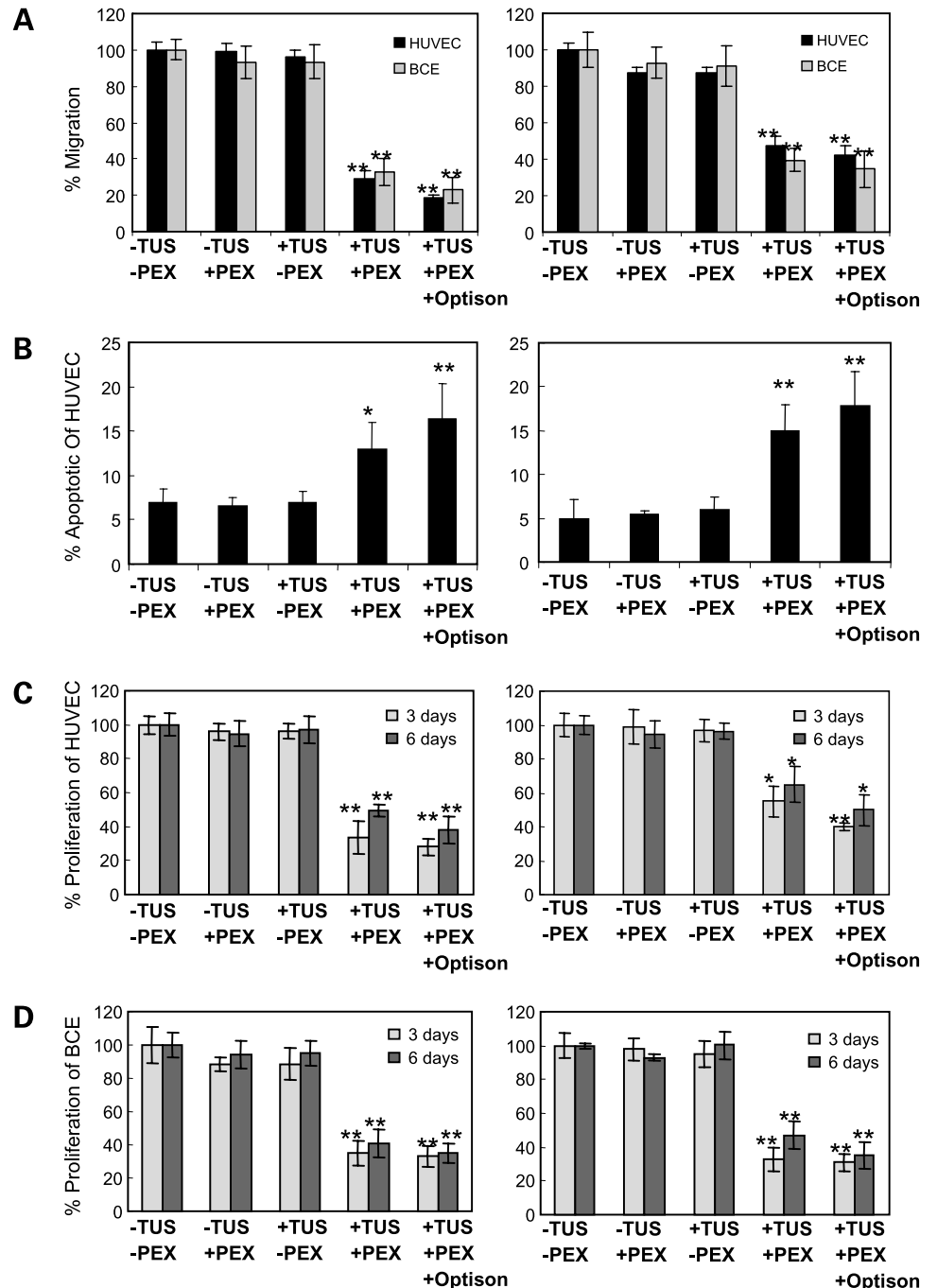
### Effect of the Factor PEX on Prostate Cancer Cell Proliferation

PEX is known to inhibit the proliferation of cancer cells as well as EC (21, 29). To evaluate the effect of PEX on the proliferation of prostate cancer cells, the cells were incubated with an increased concentration of purified PEX protein. For PC2 cells, 0.5  $\mu\text{g}/\text{mL}$  of PEX was sufficient to significantly inhibit their proliferation by  $47 \pm 7\%$  relative to the control ( $P < 0.05$ ). Incubation of LNCaP cells with 2.5  $\mu\text{g}/\text{mL}$  of PEX reduced their proliferation by  $52 \pm 12\%$  relative to the control ( $P < 0.05$ ).

### Effect of pPEX Expressed after Therapeutic Ultrasound on the Proliferation of Prostate Cancer Cells and EC

Prostate cancer cell proliferation was also evaluated after their transfection with TUS + pPEX or TUS + pPEX +

**Figure 2.** Effect of conditioned media taken from prostate cancer cells on EC proliferation, migration, and apoptosis. EC were cultured with conditioned media taken from PC2 (left) or LNCaP cells (right) transfected with TUS + pPEX or TUS + pPEX + Optison at 1 MHz, 30% duty cycle, 2 W/cm<sup>2</sup> for 30 min. **A**, migration of HUVEC and BCE, relative to control (-TUS, -PEX). **B**, percentage of HUVEC cells undergoing apoptosis. **C** and **D**, proliferation of HUVEC (**C**) or BCE (**D**) grown with conditioned media taken from LNCaP or PC2, 3 and 6 d posttransfection using therapeutic ultrasound. Proliferation is presented relative to control (-TUS, -PEX). X-axis, treatment of the prostate cancer cells; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  versus control.

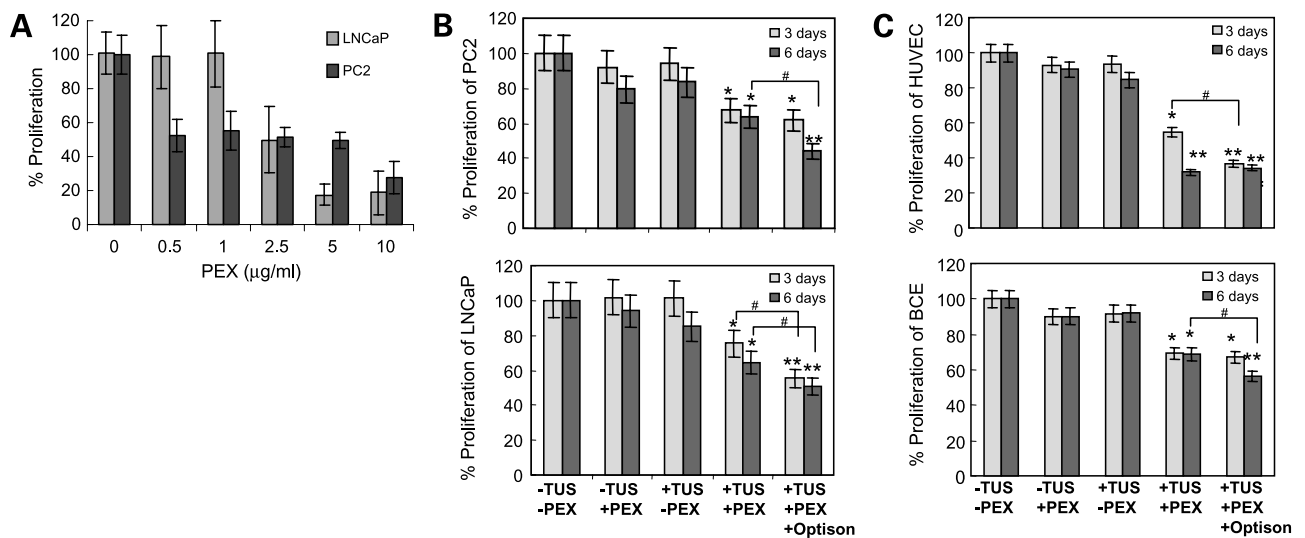


Optison. The proliferation of PC2 and LNCaP was inhibited by 30% and 25%, respectively, 3 days post-therapeutic ultrasound application (Fig. 3B). Therapeutic ultrasound application with pPEX + Optison resulted in additional inhibition of prostate cancer cell proliferation relative to control. Transfection of HUVEC using TUS + pPEX resulted in a 40% proliferation inhibition, whereas transfection of BCE with TUS + pPEX resulted in proliferation inhibition by >20% ( $P < 0.05$ ; Fig. 3C). The proliferation of HUVEC and BCE was further inhibited (60% and 40%,

respectively;  $P < 0.001$ ), when transfecting these cells with TUS + pPEX + Optison. Similar inhibitions were obtained 6 days posttransfection. No significant inhibition was observed in the proliferation of EC treated with therapeutic ultrasound only or with pPEX only (Fig. 3C).

#### A Single Treatment with TUS + pPEX or pPEX + Optison Leads to Tumor Inhibition

Tumor growth was significantly inhibited 28 days after a single treatment of TUS + pPEX resulting in tumor volume of  $1,300 \pm 250$  compared with  $2,000 \pm 300$  mm<sup>3</sup> in the



**Figure 3.** Proliferation of prostate cancer cells and EC. **A**, proliferation of prostate cancer cells grown with different concentration of purified PEX protein, presented as a percentage relative to control. **B** and **C**, proliferation of prostate cancer cells (**B**) and EC (**C**), 3 and 6 d after their own transfection with TUS + pPEX or TUS + pPEX + Optison. Therapeutic ultrasound was applied at 1 MHz, 30% duty cycle, 2 W/cm<sup>2</sup> for 30 min. Proliferation is presented as a percentage relative to control (-TUS, -PEX). X-axis, treatment of the cells; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  versus control; #,  $P < 0.05$  TUS + pPEX versus TUS + pPEX + Optison.

control ( $P < 0.05$ ; Fig. 4A). Tumor weight was also significantly lower ( $0.65 \pm 0.15$  g) compared with control ( $1.05 \pm 0.25$  g,  $P < 0.05$ ; Fig. 4A). Additional inhibition of tumor growth was obtained when tumors were transfected with TUS + pPEX + Optison using a single treatment of therapeutic ultrasound (Fig. 4B). In these mice, tumor volume ( $1,000 \pm 250$  mm<sup>3</sup>) and tumor weight ( $0.52 \pm 0.12$  g) were also significantly lower compared with the control group ( $P < 0.01$ ; Fig. 4B) and compared with the group receiving TUS + pPEX ( $P < 0.05$ ). These results indicate that PEX transcribed in tumors post-therapeutic ultrasound transfection was biologically active and retained its efficacy.

#### Duration of pPEX Transcription in Tumors Post-Therapeutic Ultrasound Application

RT-PCR done on tumors receiving a single treatment of therapeutic ultrasound with pPEX revealed that the highest expression of pPEX was achieved on the 3rd day after therapeutic ultrasound application (Fig. 4C). A reduction in the transcription of pPEX was observed both 7 and 14 days after therapeutic ultrasound application. At 21 days, almost no transcription of pPEX was detected. Similar results were obtained when tumors were treated with therapeutic ultrasound and pPEX + Optison (Fig. 4C).

#### Repeated Treatments of Therapeutic Ultrasound with pPEX and pPEX + Optison in Tumors

To extend pPEX expression by the transfected tumor, mice were injected i.t. once a week for 4 weeks with TUS + pPEX or TUS + pPEX + Optison (Fig. 4D). The results clearly show that tumor growth was markedly inhibited compared with the control groups ( $P < 0.001$ ) as well as when compared with tumors treated once with TUS + pPEX ( $P < 0.05$ ; Fig. 4A) or TUS + pPEX + Optison

( $P < 0.05$ ; Fig. 4B). Repeated treatments of tumors with TUS + pPEX led to tumor volumes of  $750 \pm 250$  mm<sup>3</sup>, whereas repeated treatments with TUS + pPEX + Optison led to tumor volumes of  $410 \pm 230$  mm. Tumor weights were also significantly lower,  $0.24 \pm 0.06$  g for tumors treated with TUS + pPEX and  $0.17 \pm 0.07$  g for tumors treated with TUS + pPEX + Optison, compared with the control groups or the groups which received a single treatment of therapeutic ultrasound ( $P < 0.001$ ; Fig. 4D). Maximum inhibition in tumor growth (volume and weight) was observed after repeated treatments of TUS + pPEX + Optison, with significantly lower rates than in tumors receiving repeated treatments of TUS + pPEX only ( $P < 0.05$ ; Fig. 4D).

#### Expression of pPEX in Tumors Posttransfection Using Therapeutic Ultrasound

The expression of pPEX in tumors was evaluated using immunohistochemistry and immunofluorescence with anti human MMP2. This antibody recognizes human PEX only and does not recognize the endogenous mice PEX. As seen, PEX expression in tumors transfected with a single treatment of TUS + pPEX + Optison or repeated treatments of TUS + pPEX or TUS + pPEX + Optison was higher than in the control (treated with pPEX + Optison without therapeutic ultrasound; Fig. 5A). Expression of pPEX in the tumors was detected as clusters. Quantification of pPEX expression revealed that in tumors treated repeatedly with TUS + pPEX + Optison, pPEX expression was significantly higher than in nontreated tumors ( $16 \pm 5$  compared with  $4 \pm 3$ ,  $P < 0.001$ ; Fig. 5B), and compared with tumors treated repeatedly without the addition of Optison ( $11 \pm 3$ ,  $P < 0.05$ ; Fig. 5B).

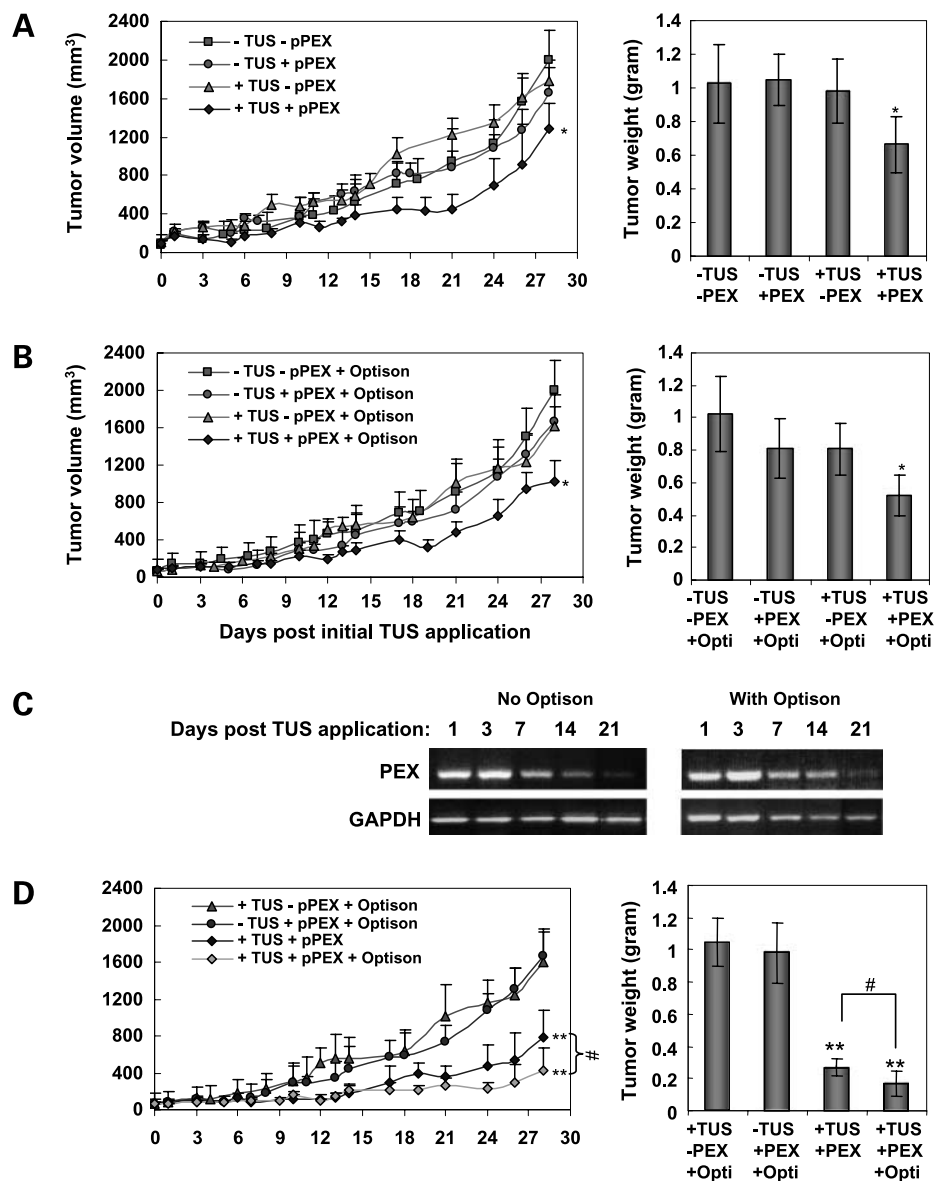
The localization of pPEX expression in the tumor relative to the microvasculature was evaluated using dual

immunofluorescence staining with antibodies against CD31 and human MMP2 (Fig. 5C). The results show that pPEX is expressed by both tumor cells and EC, and its expression is in the tumor cell mass rather than in the blood vessels lining the tumor. Detection of pPEX expression in the tumors was also evaluated by RT-PCR at 28 days after initial treatment. The results revealed that pPEX expression was detected in all the mice that received repeated treatments (Fig. 5D).

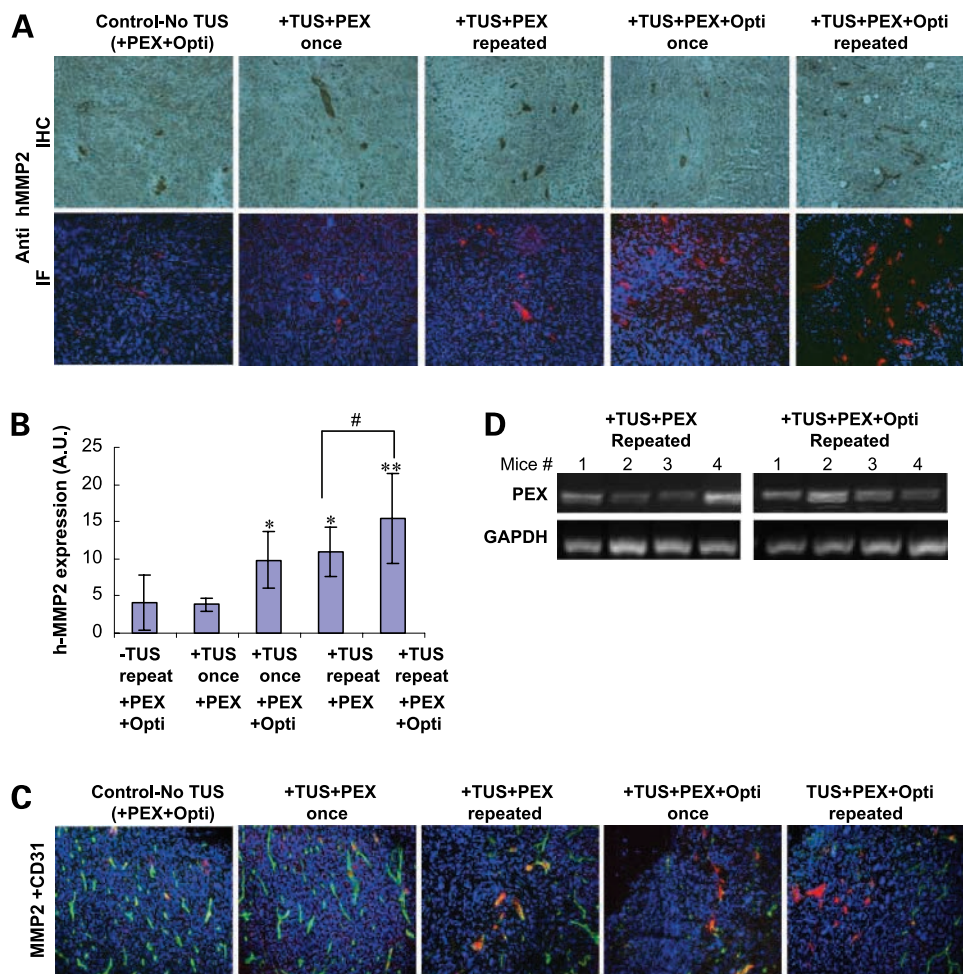
#### Histology and Immunohistochemistry of Tumors Posttransfection with TUS + pPEX

Immunohistochemistry of tumor sections after a single treatment with TUS + pPEX revealed that there was no significant change in the vascularization, proliferation, and apoptosis indices compared with the control groups (Fig. 6A and B). However, apoptosis was significantly

higher ( $127 \pm 22\%$ ,  $P < 0.05$ ) and proliferation was significantly lower ( $73 \pm 11\%$ ,  $P < 0.05$ ) in tumors receiving a single application of TUS + pPEX + Optison compared with the control groups (100%; Fig. 6B). When mice received repeated treatments of therapeutic ultrasound with pPEX, the vascularization ( $64 \pm 8\%$ ) and proliferation ( $79 \pm 12\%$ ) indices were significantly lower ( $P < 0.05$ ) than the control group (100%). In these tumors, E-cadherin expression was significantly higher ( $3.6 \pm 0.7$ ,  $P < 0.01$ ) compared with the control groups ( $1.6 \pm 0.4$ ). The most significant changes in the angiogenic indices were achieved when mice received repeated treatments of TUS + pPEX + Optison. In these tumors, apoptosis ( $166 \pm 15\%$ ) and E-cadherin ( $3.8 \pm 0.5$ ) expression were significantly higher ( $P < 0.001$ ), proliferation ( $52 \pm 10\%$ ) and vascularization ( $34 \pm 12\%$ ) were significantly lower ( $P < 0.001$ ) when



**Figure 4.** *In vivo* therapeutic ultrasound transfection of pPEX in C57/Bl mice inoculated with prostate cancer tumors. Prostate cancer tumors were inoculated s.c. in C57/Bl mice. Tumors were injected i.t. with pPEX or pPEX + Optison and therapeutic ultrasound was applied to tumors at 1 MHz, 30% duty cycle, 2 W/cm<sup>2</sup> for 20 min. Controls received only plasmid DNA (pPEX or pPEX + Optison), only therapeutic ultrasound, or no treatment at all. **A** and **B**, tumor volume during 28 d post-initial treatment and tumor weight at the end of the experiment after treatment with TUS + pPEX (**A**) or TUS + pPEX + Optison (**B**). **C**, RT-PCR for transcription of pPEX in tumors at 1, 3, 7, 14, and 21 d post-single transfection of TUS + pPEX or TUS + pPEX + Optison. Transcription of glyceraldehyde-3-phosphate dehydrogenase is shown as control. **D**, tumor volume during 28 d post-initial treatment and tumor weight at the end of the experiment after repeated treatments. Mice were transfected with TUS + pPEX or TUS + pPEX + Optison once a week for four times. *Opti*, Optison. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  versus control (-TUS, -PEX); #,  $P < 0.05$  TUS + pPEX versus TUS + pPEX + Optison.



**Figure 5.** Expression of pPEX in tumors treated with TUS + pPEX or TUS + pPEX + Optison. **A**, representative micrographs of immunohistochemistry (brown) and immunofluorescence (red) staining of pPEX in tumors (magnification,  $\times 200$ ). The antibody against human MMP2 was used to distinguish pPEX expression from endogenous MMP2 expression. **B**, quantification of pPEX expression in tumors at 28 d post-initial treatment. Quantification was done using LUCIA image analysis software, taken from a total of 25 micrographs of five different mice. A.U., arbitrary units. Opti, Optison; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  versus control; #,  $P < 0.05$  TUS + pPEX versus TUS + pPEX + Optison. **C**, representative micrographs (magnification,  $\times 200$ ) of dual immunofluorescence of pPEX expression (red) and microvessel density (CD31 staining, green) in sections from tumors. **D**, RT-PCR analyses of pPEX expression taken from four tumors receiving repeated treatments of TUS + pPEX or TUS + pPEX + Optison at the end of the experiment. Transcription of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as control.

compared with the control groups (Fig. 6A and B). Vascularization and proliferation were also significantly lower, and apoptosis was significantly higher in this group, compared with tumors receiving repeated treatments with TUS + pPEX without Optison ( $P < 0.05$ ; Fig. 6B).

To locate the areas in the tumor that undergoes apoptosis, tumor sections were stained for cleaved caspase-3 and imaged at low magnification ( $\times 40$ , inset, Fig. 6A). These micrographs clearly show that in the treatment groups, the apoptotic areas were inside the tumor cell mass near the injection site. In the control group, the apoptotic areas were smaller and were sporadically distributed in the tumor. In the tumors receiving repeated treatments, the apoptotic area was much larger.

Coimmunofluorescence staining for CD31 with Ki-67, and CD31 with cleaved caspase-3, indicated that proliferating cells were sporadically spread throughout the tumor, whereas the apoptotic areas were not localized near the vasculature (Fig. 6C).

## Discussion

Recent studies have shown the potential of different ultrasound modalities for cancer gene therapy applications.

These studies have mainly used marker genes such as green fluorescent protein (GFP),  $\beta$ -Gal, and luciferase to show transfection rather than efficacy (8, 11, 12, 30, 31). Moreover, these studies have used different ultrasound modalities such as high-intensity focused ultrasound (8, 11, 30) or high-intensity ultrasound modalities (12, 31) for the transfection process, which are considered destructive to various tissues. In contrast, therapeutic ultrasound, which is considered safe for clinical applications, was applied *in vivo* mainly for the delivery of cDNA to muscles (15, 16), liver (17), kidney (18), and the vasculature (5, 6, 19). We have recently shown that therapeutic ultrasound application *in vitro* can lead to a transfection efficiency of 50%, which is similar to the transfection obtained with other non-viral vectors tested (7, 25, 32). The application of therapeutic ultrasound also enabled the delivery of cDNA, not only to the cell cytoplasm, but more importantly to the nucleus, resulting in rapid expression of the DNA (25).

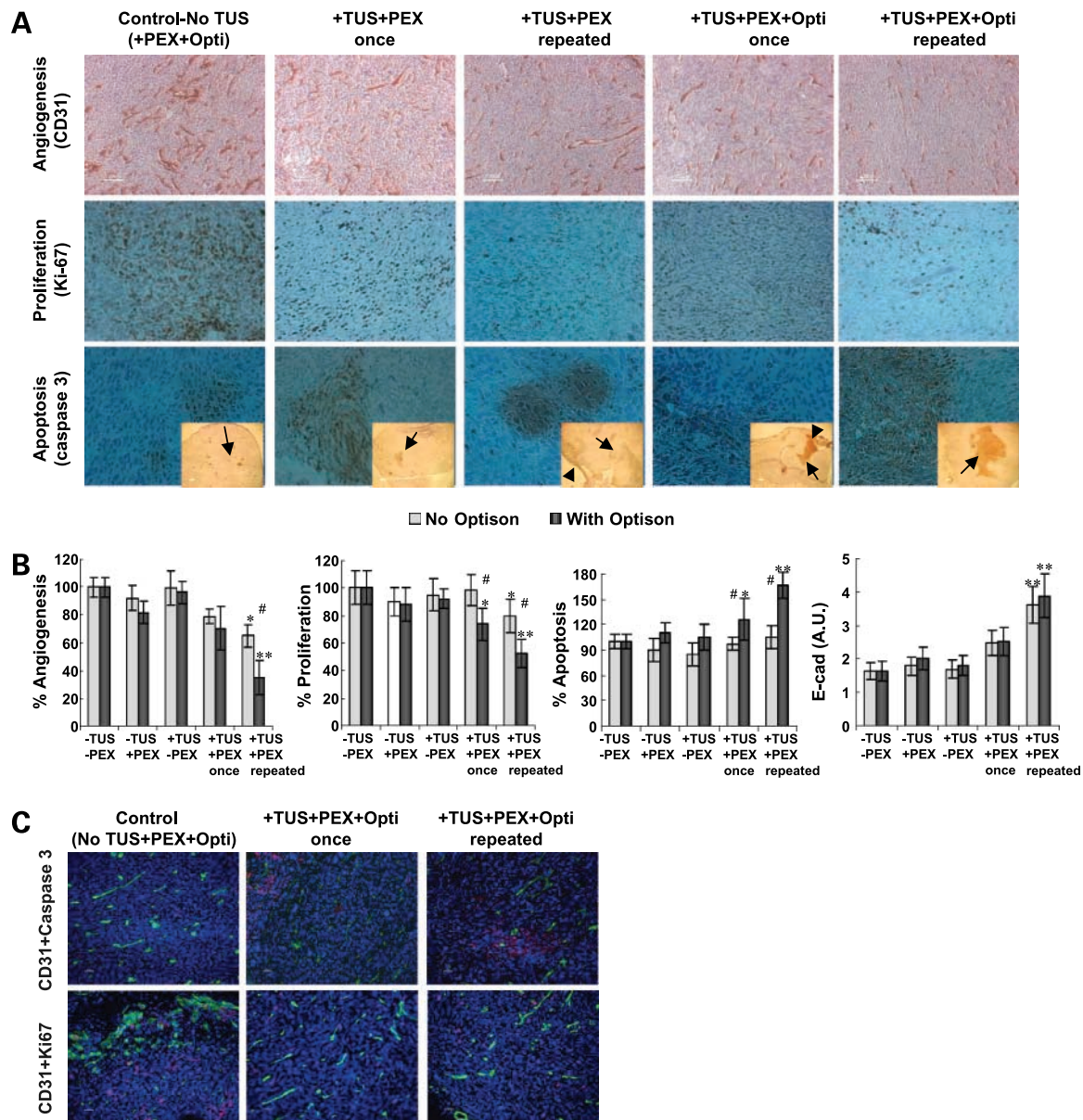
Here, we report on the use of long-term therapeutic ultrasound (20 min) for the transfection of prostate cancer, a highly vascularized tumor, which depends on angiogenesis and on MMPs for its growth (33–35). For our efficacy studies, we used a cDNA that encodes for PEX, an inhibitor



of angiogenesis. PEX is a unique factor which possesses multiple effects on cancer cells as well as EC. PEX interacts with integrins  $\alpha_v\beta_3$  and inhibits MMP2 (21, 29). Several studies showed the *in vivo* efficacy of PEX to inhibit tumors, mainly glioma (21–23) and melanoma (29, 36). PEX was also detected in the medium of prostate cancer cells and was shown to inhibit the proliferation of prostate

cancer cells *in vitro* (21). The *in vivo* effect of PEX on prostate cancer tumors has not yet been evaluated.

Our *in vitro* experiments show that therapeutic ultrasound can effectively deliver cDNA encoding for PEX to various prostate cancer cell lines and to primary EC, whereas retaining biological activity. This was shown by the significant inhibition of proliferation, migration, and



**Figure 6.** Immunohistochemistry of angiogenic indices in tumors transfected with pPEX or pPEX + Optison using therapeutic ultrasound post-initial treatment. Tumors were harvested 28 d post-initial treatments and sections were stained with antibodies against CD31, Ki-67, cleaved caspase-3, and E-cadherin. **A**, representative micrographs of sections stained with CD31 (magnification,  $\times 100$ ), Ki-67 (magnification,  $\times 200$ ), and cleaved caspase-3 (magnification,  $\times 200$ ) staining. *Insets* (arrows), areas in the tumor which underwent apoptosis (magnification,  $\times 40$ ). **B**, quantification of the percentage of microvessels in a field (% angiogenesis stained with CD31), the percentage of Ki-67 – positive cells (% proliferation), the percentage of cleaved caspase-3 (% apoptosis), and E-cadherin expression (A.U., arbitrary units). All quantifications were done on a total of 25 micrographs from five different mice using LUCIA image analysis. *Opti*, Optison; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  versus control; #,  $P < 0.05$  TUS + pPEX versus TUS + pPEX + Optison. **C**, representative micrographs of dual immunofluorescence staining of CD31 (green) and caspase-3 (red, top), and CD31 (green) and Ki-67 (red, bottom); magnification,  $\times 200$ .

increase in the number of apoptotic EC, when cultured with conditioned media taken from prostate cancer–transfected cells. Moreover, significant inhibition of EC and prostate cancer cell proliferation was achieved after their own transfection with TUS + pPEX. Preincubation of pPEX with Optison, an USCA, prior to therapeutic ultrasound transfection resulted in further inhibition of cell proliferation, migration, and increased apoptosis. These data support our previous findings, and others, using marker genes such as GFP and  $\beta$ -Gal in combination with USCA (5, 6, 18, 25, 37).

Recently, we have shown that i.t. administration of pGFP, together with Optison, and subsequent therapeutic ultrasound application led to an increase in the number of cells expressing GFP compared with tumors transfected with therapeutic ultrasound-pGFP alone (32). Analyses of the tumor bed revealed that GFP was distributed all over the tumor tissue. Most importantly, therapeutic ultrasound (2 W/cm<sup>2</sup>, 30% duty cycle for 20 min) led to a 12% transfection efficiency in the tumor tissue and to a 25% transfection efficiency when Optison was used (32). Nevertheless, expression of marker genes does not necessarily assure therapeutic efficacy. Therefore, in the current study, we have used the same therapeutic ultrasound variables to deliver a gene encoding for a therapeutic protein and assessed its efficacy on a prostate cancer tumor. Using therapeutic ultrasound enables the localization of the cDNA at the tumor bed, thus targeting the therapeutic effect and significantly reducing the amount of therapeutics needed to inhibit tumor growth, as opposed to systemic protein therapy (23, 38). Most importantly, the duration of gene expression and thus the therapeutic efficacy can be monitored. From the *in vivo* studies, it is evident that a single transfection with TUS + pPEX led to a significant inhibition in tumor growth. In addition, preincubation of pPEX with Optison and subsequent single therapeutic ultrasound application led to a more significant inhibition in tumor growth, suggesting that the addition of USCA in therapeutic ultrasound transfection not only increases transfection efficiency, but also increases the therapeutic effects. Tumor RT-PCR studies showed that the transcription of pPEX declined with time, indicating a transient transfection, which is typical for non-viral vectors as opposed to some viral vectors. These results are consistent with previous studies using luciferase and GFP (18, 32, 39). Because therapeutic ultrasound application is a noninvasive process and is safe in terms of tissue integrity, we hypothesized that repeated treatments may increase the duration of transfection thus achieving a more significant tumor inhibition. In contrast to viral vectors, repeated treatments of therapeutic ultrasound should not pose major limitations or side effects. Moreover, repeated treatments may be beneficial when there is a need to control the duration and amount of protein expression. Therefore, to achieve longer expressions of pPEX, and thus, a more significant inhibition of the tumors, an additional set of experiments was done in which mice received repeated treatments (once a week for 4 weeks) of pPEX or pPEX +

Optison with therapeutic ultrasound application. This therapeutic regimen led to a significant inhibition (80%) in tumor volume and weight when compared with the control group ( $P < 0.001$ ) and to the group that received a single treatment ( $P < 0.05$ ). Moreover, immunohistochemistry and immunofluorescence have shown that in tumors receiving repeated treatments of TUS + pPEX + Optison, the angiogenic indices were markedly decreased. Microvessels and proliferation rate were significantly lower, whereas apoptosis and E-cadherin expression were significantly higher, indicating a lower grade of the prostate cancer tumor (35, 40) and the efficacy of this technique. Repeated treatments with the addition of Optison (TUS + pPEX + Optison), were also significantly ( $P < 0.05$ ) therapeutically effective compared with repeated treatments with TUS + pPEX only. Several studies have shown that antiangiogenic gene therapy for prostate cancer is an efficient strategy leading to the inhibition of tumor growth (41–43). These studies have used viral vectors (Adenovirus and Adeno-associated virus) and obtained tumor growth inhibitions of 70% to 85%, respectively, which is similar to the tumor inhibition achieved using our repeated treatment (41–43).

Using such a regimen enabled us to overcome the transient gene expression associated with a single treatment of therapeutic ultrasound. Expression of pPEX was found in both EC and prostate cancer cells and it was sufficient to mediate apoptosis in EC and also in prostate cancer cells as indicated by dual immunofluorescence staining. Recently, Sakakima et al. (44) administered IFN- $\beta$  gene in combination with USCA (BR14) into hepatocellular carcinoma inoculated s.c. in mice and applied ultrasound at 2 W/cm<sup>2</sup> 50% duty cycle for 10 min. Single treatment with ultrasound led to a reduction in tumor size, but the effect of repeated treatments were not evaluated. It was also reported that BR14 was rapidly destroyed by ultrasound application, whereas we have previously shown that Optison is a stable microbubble, resulting in increased transfection when using long-term therapeutic ultrasound application *in vitro* (37) and *in vivo* (32). It is possible that the high-energy input used is due to the high duty cycle, leading to the destruction of BR14.

In summary, the present study shows, for the first time, the efficacy of therapeutic ultrasound as a non-viral gene delivery approach for the treatment of prostate cancer tumors. The combination of approved ultrasound modality with approved USCA and DNA encoding for angiogenic inhibitor can overcome the difficulties associated with the vector itself and the angiogenic inhibitor factors. The use of therapeutic ultrasound offers a way to target and localize the transfection without systemic side effects. Most importantly, this approach enables repeated treatments without any side effects as opposed to viral DNA delivery, allowing control over the expression. Although the therapeutic ultrasound used in this study is an unfocused one, and that tumors were inoculated s.c., the results from this study may set the stage for clinical application in orthotopic prostate cancer models. For

clinical application, the therapeutic ultrasound transducer can be easily focused, like optical waves, to a specific organ in the body, including orthotopic prostate tumor (8, 12). The focusing process of therapeutic ultrasound results in a very tight focus area that affects only the tissue that lie within the focal volume, whereas all other tissue in the ultrasound beam path are unaffected (45). In the case of gene delivery application, the energy in the focal plane will be the same as used for the s.c. model, i.e.,  $2 \text{ W/cm}^2$ , and the only required adjustment of the device will be the focus of the wave. Moreover, a therapeutic ultrasound transducer can be designed as a catheter as the one used in endovascular applications, with a narrow size and a central infusion lumen of  $180 \mu\text{m}$  (46). Taking this into account, it is apparent that therapeutic ultrasound can be focused and used for orthotopic prostate cancer, thus bringing this technology into clinical application. Further studies will be required to demonstrate this approach, as well as investigating the effects of combining this approach with other therapies, such as radiation and chemotherapy.

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