Insight Concerning the Mechanism of Therapeutic Ultrasound Facilitating Gene Delivery: Increasing Cell Membrane Permeability or Interfering with Intracellular Pathways?

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Abstract

Nonviral gene delivery methods encounter major barriers in plasmid DNA (pDNA) trafficking toward the nucleus. The present study aims to understand the role and contribution of therapeutic ultrasound (TUS), if any, in pDNA trafficking in primary cells such as fibroblasts and cell lines (e.g., baby hamster kidney [BHK]) during the transfection process. Using compounds that alter the endocytic pathways and the cytoskeletal network, we show that after TUS application, pDNA trafficking in the cytoplasm is not mediated by endocytosis or by the cytoskeletal network. Transfection studies and confocal analyses showed that the actin fibers impeded TUS-mediated transfection in BHK cells, but not in fibroblasts. Flow cytometric analyses indicated that pDNA uptake by cells occurs primarily when the pDNA is added before and not after TUS application. Taken together, these results suggest that TUS by itself operates as a mechanical force driving the pDNA through the cell membrane, traversing the cytoplasmic network and into the nucleus.

Introduction

A KEY FACTOR in gene therapy is the efficient delivery of DNA into a wide variety of cells and tissues. Ultrasound has been studied extensively as a nonviral physical method for gene delivery (Miller *et al.*, 1996; Huber *et al.*, 2003; Wei *et al.*, 2004). Among the various ultrasound modalities used for gene delivery, therapeutic ultrasound (TUS, 1–3 MHz; intensities, 0.5–2 W/cm², pulse mode) is considered safe in terms of tissue damage and is used for clinical applications. To enhance TUS transfection efficiency or to study the mechanism associated with this technology, various groups, including ours, have demonstrated that the use of ultrasound contrast agents (USCAs; gas-filled microbubbles) in the transfection process can increase transfection efficiency. However, USCAs were associated predominantly with cell membrane trafficking, rather than DNA intracellular trafficking, as they first come in contact with the cell membrane and do not penetrate the cytoplasmic compartment (Duvshani-Eshet *et al.*, 2006a; Fan *et al.*, 2013).

Nevertheless, the mechanism accounting for TUS gene delivery still remains a puzzle. Most studies addressing the underlying mechanism of action focus on the effect that TUS may have on cell membrane permeability, as this is the first

organelle that encounters the ultrasound energy (Brayman *et al.*, 1999; Taniyama *et al.*, 2002; Mehier-Humbert *et al.*, 2005). Once passing through the cell membrane barrier, the plasmid DNA (pDNA) still needs to bypass other major barriers. The pDNA needs to avoid endonucleases and lysosomal degradation, which face all nonviral gene delivery methods (Zabner *et al.*, 1995; Nishikawa and Huang, 2001; Munkonge *et al.*, 2003), and needs to be transported through the dense cytoskeletal network and into the nucleus.

It is possible that TUS, when delivering genes, affects the endocytic pathway leading to the internalization of DNA through plasma membrane invaginations, similar to the mechanism attributed to lipid-based and polymer-based gene delivery systems (Guy *et al.*, 1995; Zabner *et al.*, 1995; Nishikawa and Huang, 2001; Meijering *et al.*, 2009). However, as passive diffusion of large DNA molecules (>2 kb) into the cytoplasm is limited (Lukacs *et al.*, 2000), it is also likely that TUS plays a role in the active transport of molecules mediated by the dense cytoskeletal network, using microtubules or actin filaments (Wang and MacDonald, 2004; Vaughan and Dean, 2006). Therefore, whether TUS has a role in pDNA trafficking or only contributes to the internalization of pDNA into the cell cytoplasm still remains an unsolved question.

In the present study, we aim to address these hypotheses and further understand the role and contribution of TUS, if any, in pDNA trafficking in the cytoplasm. These questions were addressed by studying the effect of various factors associated with endocytic uptake and the cytoskeletal network on TUS-mediated transfection.

Materials and Methods

Plasmids

Reporter plasmid carrying the firefly luciferase gene (pGL3-Luc-control, 5.2 kb; Promega, Madison, WI) was used for the transfection measurement. For intracellular tracking studies, plasmids were labeled fluorescently with fluorescein or rhodamine, using a *Label IT* kit (Mirus Bio, Madison, WI).

Cell culture

Baby hamster kidney cells (BHK-21; American Type Culture Collection [ATCC], Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beit HaEmek, Israel), with 10% fetal calf serum (FCS). Primary fibroblasts were isolated from discarded human foreskins after circumcision. Both cultures were supplemented with 1% penicillin–streptomycin solutions (Biological Industries) and amphotericin B (GIBCO Fungizone; Life Technologies, Carlsbad, CA) and maintained at 37° C and 5% CO₂.

In vitro TUS gene transfection

TUS transfection was performed as previously described (Duvshani-Eshet and Machluf, 2005; Duvshani-Eshet *et al.*, 2006b). Briefly, cells were seeded in 6-well plates and administered pDNA (7.5 µg/ml). Cells were then exposed to 1-MHz TUS (Ultra-Max, XLTEK, Canada) at 30% duty cycle (DC), 2 W/cm² for a total exposure time of 30 min. Control cells received only pDNA, only TUS, or were transfected with jetPEI reagent (Polyplus-transfection, Illkirch, France), according to the recommended protocol. In all studies, cell viability was detected with methylthiazolyldiphenyl-tetrazolium bromide (MTT, 5 mg/ml; Sigma-Aldrich, St. Louis, MO).

To evaluate the effect of pDNA addition before or after TUS, pDNA was added at various times after TUS application and transfection was measured and compared with that of cells receiving pDNA before TUS application.

DNA uptake by cells

The effect of TUS on pDNA uptake by cells was evaluated with pDNA fluorescently labeled with rhodamine, which was added to the cells before TUS, or at various times after TUS application. To distinguish between live and dead cells, cells were cultured with calcein acetoxymethyl ester (calcein-AM; Sigma-Aldrich, Rehovot, Israel), which enters cells and is converted to the green fluorescent molecule calcein only in live cells. Cells were then washed twice with phosphate-buffered saline (PBS) and 20,000 cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

Effect of TUS on endocytic and cytoskeletal pathways

To study whether TUS mediates DNA delivery by affecting the endocytic or cytoskeletal pathway, cells were treated

with wortmannin, ammonium chloride (AC), cytochalasin B (CytoB), or nocodazole (Noc) (all from Sigma-Aldrich).

Wortmannin, which inhibits endocytosis via phosphatidylinositol 3-kinase inhibition, was added at 50 and 100 nM concentrations, 15 min before TUS transfection (Richards *et al.*, 2004). Ammonium chloride, which increases the pH in lysosomes, was added at 10 and 50 mM, 2 hr before TUS transfection. The effect of two cytoskeletal depolymerization agents, CytoB and Noc, was also evaluated. CytoB is an inhibitor of actin microfilament polymerization, and Noc binds microtubule subunits and prevents their polymerization. CytoB was added to the cells at 5 µg/ml, 2 hr before TUS transfection. Noc was added at 20 µM, 30 min before TUS transfection. Because both agents were dissolved in dimethyl sulfoxide (DMSO), controls (pDNA alone) also received the same concentration of DMSO.

After adding the factors, BHK cells and fibroblasts were transfected with pLuc, using TUS, and served as controls. The noncontrol cells were transfected with jetPEI reagent according to the recommended protocol. Medium was completely replaced 5 hr after transfection with pLuc or jetPEI.

Measurements of luciferase activity

Luciferase activity was determined 3 days posttransfection, as described previously (Duvshani-Eshet and Machluf, 2005). Luciferase activity is reported as the mean of relative light units (RLU) obtained from each sample divided by total protein weight measured for each sample, using bicinchoninic acid (BCA) protein assay reagents (Pierce Biotechnology, Rockford, IL). When the effect of various drugs was tested, results are presented as the fold increase in luciferase activity in the presence of the drug, compared with control.

Confocal studies

Cells seeded on coverslipped cell chamber slides (Lab-Tek; Electron Microscopy Sciences, Hatfield, PA) were supplemented with fluorescently labeled pDNA and transfected by TUS. For the endocytosis studies, cells were also transfected with jetPEI. At various times points after TUS application (immediately and 2, 5, and 24 hr afterward) cells were washed in PBS and fixed in 4% paraformaldehyde. Cells with pDNA alone served as controls. Nuclei were stained with DRAQ5 (0.5 µl/ml; Biostatus, Shephed, Leicestershire, UK). Endosomes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-early endosomal antigen-1 (EEA1; BD Biosciences). Lysosomes were stained with rhodamine-LysoTracker (Molecular Probes/Life Technologies) at a final concentration of 100 nM. Actin filaments were stained with phalloidin-FITC (Sigma-Aldrich) at a final concentration of 0.01 mg/ml. Microtubules were stained with anti-β-tubulin (BD Biosciences) and with a cyanine-3 (Cy3)-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA). After staining, all cells were mounted with Fluoromount-G (Electron Microscopy Sciences). Confocal analyses were performed with a confocal microscope (MRC-1024; Bio-Rad, Hercules, CA) and micrographs were taken across 8 µm of the Z-range at intervals of 0.5 µm to determine the location of pDNA in the cell.

Colocalization analyses were performed with LSM software (Carl Zeiss, Jena, Germany). Colocalization coefficient

values were calculated using Pearson's correlation coefficient and Manders's overlap coefficient values (Manders *et al.*, 1993).

Statistical analysis

All data are presented as means $\pm$ SEM or as a percentage of the mean from control $\pm$ SEM. Statistical differences were determined by analysis of variance (ANOVA) or Student *t* test for independent samples and statistical significance was defined as $p < 0.05$. Transfection conditions were performed in four repeats and each experiment was repeated on three separate occasions. Confocal micrographs are representative of three different experiments and 10 random fields.

Results

Effect of TUS on endocytic pathways

The effect of TUS on pDNA intracellular pathways was investigated using inhibitors or accelerators for the endocytic pathways followed by transfection measurements. As seen in Fig. 1A, the addition of ammonium chloride did not significantly affect TUS transfection of BHK cells and fibroblasts. When using jetPEI, the addition of ammonium chloride increased transfection in BHK cells and fibroblasts in a dose-dependent manner. The increase in transfection was significantly higher than that obtained in control cells receiving the higher ammonium chloride concentration (50 mM). Adding wortmannin did not affect significantly TUS or PEI transfection of BHK cells and fibroblasts (Fig. 1B).

Localization of pDNA in endocytic organelles posttransfection

BHK cells and fibroblasts were transfected with fluorescently labeled pDNA, and endosomes and lysosomes were also fluorescently stained (Fig. 2).

As seen in Fig. 2A and B, most of the pDNA did not colocalize with the endosomes or lysosomes immediately, 2 hr, or 5 hr after TUS transfection into BHK cells. Quantification of the percentage colocalization coefficient value of the pDNA channel with the endosome or lysosome channel revealed that when using TUS, less than 15% of the pDNA was colocalized with endosomes or lysosomes (Fig. 2E). However, when using jetPEI a vast amount of the pDNA was colocalized with endosomes (Fig. 2A) and with lysosomes (Fig. 2B), as indicated by the yellow-orange color in the images. Quantification analyses showed that when using jetPEI, $40 \pm 10\%$ of the pDNA had colocalized with the endosomes or lysosomes by 5 hr posttransfection (Fig. 2E). When fibroblasts were transfected by TUS and immediately imaged, most of the pDNA was not colocalized with endosomes (Fig. 2C). At 2 and 5 hr after TUS, a small amount of pDNA was detected in endosomes (10–15%; Fig. 2F). In contrast, when using jetPEI, a higher amount of pDNA was detected in endosomes (Fig. 2C), reaching $35 \pm 5\%$ and $50 \pm 15\%$ at 2 and 5 hr posttransfection, respectively. Moreover, when using TUS, a small amount of pDNA appeared to be in the lysosomes, mainly at 2 and 5 hr posttransfection, reaching $30 \pm 5\%$. However, when using jetPEI, $50 \pm 10\%$ of the pDNA was located in the lysosomes, 5 hr posttransfection (Fig. 2D and F).

FIG. 1. Effect of endocytic drugs on transfection using therapeutic ultrasound (TUS) and jetPEI. Baby hamster kidney (BHK) cells and fibroblasts were transfected by TUS (30% duty cycle [DC], 2 W/cm^2 , 30 min) and by jetPEI with pLuc, without any inhibitor (control) or with two concentrations of (A) ammonium chloride (AC) or (B) wortmannin (Wort). Results are presented as fold increase in luciferase activity compared with the control group. Cell viability was measured with methylthiazolyldiphenyl-tetrazolium bromide (MTT). $n = 16$; $*p < 0.05$.

FIG. 2. Localization of DNA in BHK cells or fibroblasts relative to endosomes or lysosomes after TUS or jetPEI transfection. BHK cells (A and B) and fibroblasts (C and D) were transfected by TUS (30% DC, 2 W/cm², 30 min) or jetPEI with fluorescently labeled plasmid, and fixed immediately after TUS or 2 and 5 hr after TUS or jetPEI. Cells without treatment served as controls. (A and C) Endosomes stained with FITC-conjugated anti-EEA1 (green) and pDNA stained with rhodamine (red). (B and D) Lysosomes stained with rhodamine-LysoTracker (red) and pDNA stained with fluorescein (green). Images are representatives of 10 micrographs based on confocal analyses. Scale bars: For BHK cells, 10 μ m; for fibroblasts, 20 μ m. (E and F) Quantification of colocalization coefficient of pDNA with endosomes or lysosomes in BHK cells (E) and fibroblasts (F). $n = 10$; * $p < 0.05$.

TUS effect on cytoskeletal network

Involvement of the cytoskeletal network in the trafficking of pDNA after TUS was evaluated using factors that affect the structure of this network. BHK cells and fibroblasts were treated with CytoB, an inhibitor of actin microfilament polymerization and Noc, which binds microtubule subunits and prevents their polymerization. When using CytoB, luciferase activity was increased by 2-fold ($p < 0.05$) in BHK cells (Fig. 3A) but was not significantly affected in fibroblasts (Fig. 3B). Addition of Noc decreased the luciferase activity by 1.3-fold (not significant) in BHK cells (Fig. 3A) and by 1.8-fold ($p < 0.05$) in fibroblasts (Fig. 3B). Both cell types were compared with cells transfected with TUS-pLuc alone. In addition, in both types of cells, using both factors, luciferase activity after TUS application was significantly higher ($p < 0.001$) than in cells treated with the factors, without TUS. Cell viability was more than 80% with the addition of CytoB or Noc, even after TUS application.

Interaction of pDNA with cytoskeleton after TUS transfection

Confocal analyses were performed to determine whether the TUS transfection mechanism is associated with the actin fibers and/or microtubules, and whether TUS affects these cytoskeletal components (Fig. 4). As seen in Fig. 4A, BHK cell actin fibers did not constitute a major component of the cytoplasm and were oriented mostly at the cell's boundaries. Applying TUS did not significantly affect these fibers or their location. After TUS application, pDNA was detected in the cell cytoplasm and nucleus, but not necessarily in association with actin. Addition of CytoB resulted in depolymerization of these fibers, leading to their unorganized accumulation in the cytoplasm. TUS-pDNA application on these cells did not lead to the association of pDNA with these fibers. More pDNA, however, seemed to be located near and inside the nucleus. A similar phenomenon was observed when using fibroblasts (Fig. 4B). TUS did not significantly affect the

FIG. 3. Effect of cytoskeletal depolymerization factors on transfection by TUS. **(A)** BHK cells and **(B)** fibroblasts were transfected with pLuc, using TUS at 30% DC, 2 W/cm² for 30 min, with and without the addition of cytochalasin B (CytoB) and nocodazole (Noc). Luciferase activity is presented as relative light units (RLU) per milligram of protein and as fold increase compared with control cells (cells treated with TUS plus pLuc without any factor). $n=12$; * $p<0.05$.

fibroblast actin structures, which are a major component of the fibroblast cytoplasm. They cross the whole cell and form a tight network of fibers throughout the cytoplasm (Fig. 4B). In contrast to the BHK cell actin fiber experiments, pDNA appeared to be associated with these fibers (Fig. 4B). Cells were also treated with CytoB before TUS application. Applying TUS to these cells did not change the location of pDNA in the cells when compared with control cells, and the pDNA did not seem to be linked to the actin fibers.

Unlike actin fibers, microtubules form an extended network throughout the cell, in both BHK cells and fibroblasts (Fig. 4C and D). This network was not affected by TUS application, but was markedly affected by the addition of Noc. TUS application on BHK cells and particularly on fibroblasts led to the association of pDNA with these microtubules. When treating the cells with Noc and then applying TUS, less pDNA was detected adjacent to the nucleus compared with cells not treated with Noc (Fig. 4D), but again this effect was more notable in fibroblasts than in BHK cells.

Effect of TUS on pDNA delivery into cells

The effect of TUS on pDNA delivery into cells was evaluated by adding pDNA at various time points after TUS application and comparing the achieved transfection with that achieved in cells to which pDNA was added before TUS application. From Fig. 5A it is evident that when adding pGL-Luc to the cells immediately after TUS, the achieved transfection was lower by three orders of magnitude than that achieved when the pDNA was added before TUS application. This transfection was further decreased when pDNA was added 15 or 30 min, and longer (up to 24 hr), after TUS application. The transfection was about four orders of magnitude less than when pDNA was added before TUS (Fig. 5A).

To further evaluate the effect of TUS on pDNA uptake by cells, rhodamine-labeled pDNA (rpDNA) was added to BHK cells before or at various time points after TUS application. In order to analyze only the pDNA uptake only by viable cells,

CAM was added to the cells and the cells were analyzed by flow cytometry (fluorescence-activated cell sorting, FACS). The FACS analyses demonstrated that without TUS application there is a basal percentage of viable cells (0–1%) with rpDNA (Fig. 5B). When rpDNA was added before TUS, rpDNA was detected in 76% of the viable cells. However, when rpDNA was added to the cells immediately or 5 hr after TUS application, only 28 and 4% of the viable cells had rpDNA, respectively. The cells were analyzed 24 hr post-treatment (Fig. 5B).

Discussion

TUS is a promising technology by which to deliver genes into cells and tissues. TUS has been extensively demonstrated, using various marker and therapeutic genes, to affect cell and tissue transfection (Kim *et al.*, 1996; Tata *et al.*, 1997; Lawrie *et al.*, 1999; Duvshani-Eshet and Machluf, 2005; Duvshani-Eshet *et al.*, 2006b; Newman and Bettinger, 2007). Nevertheless, few studies have attempted to understand the mechanism underlying this technology, which may lead to better control of this approach and possibly improve the transfection process and efficiency. It is possible that TUS affects the cell membrane by altering it and creating holes in it (Brayman *et al.*, 1999; Taniyama *et al.*, 2002; Mehier-Humbert *et al.*, 2005; Newman and Bettinger, 2007) or by other processes associated with the cell membrane, such as inducing endocytosis and membrane invaginations. Moreover, once bypassing the cytoplasmic membrane barrier, the pDNA still needs to reach the nucleus by trafficking through the cytoskeletal network and avoiding lysosomal degradation. It was also suggested that in addition to its effect on the cell membrane TUS may lead to acceleration of DNA escape from endosomes, alter intracellular trafficking, induce upregulation of genes and protein translation, and more (Lawrie *et al.*, 1999).

Previously we demonstrated that TUS induces local shear forces and/or acoustic microstreaming on the cells during the transfection process (Duvshani-Eshet *et al.*, 2006b). In the

FIG. 4. Localization of pDNA in relation to the cytoskeletal network after TUS. TUS was applied at 30% DC, 2 W/cm^2 for 30 min. **(A and B)** Effect of CytoB on DNA (red) and actin (green) in **(A)** BHK cells and **(B)** fibroblasts. **(C and D)** Effect of Noc on DNA (green) and microtubules (red) in **(C)** BHK cells and **(D)** fibroblasts. Scale bars: For BHK cells, $10\text{ }\mu\text{m}$; for fibroblasts, $20\text{ }\mu\text{m}$. Images are representative micrographs of confocal images.

FIG. 5. Effect of DNA addition after TUS on transfection and pDNA uptake by cells. **(A)** TUS was applied to BHK cells at 30% DC, 2 W/cm² for 30 min, and pLuc was added before TUS or at various times after TUS application. Luciferase activity was measured for 3 days after TUS. $n=12$. **(B)** BHK cells were exposed to TUS at 30% DC, 2 W/cm² for 30 min. Rhodamine-labeled plasmid DNA (rpDNA) was added to the cells before TUS, immediately after TUS, or 5 hr after TUS. Cells were incubated for another 24 hr, calcein acetoxymethyl ester (CAM) was added to the cells, and cells were analyzed by fluorescence-activated cell sorting for their relative fluorescence and percentage of pDNA uptake by cells. BHK cells without any treatment, and BHK cells to which CAM and rpDNA were added without TUS, served as controls. $**p<0.001$.

present study we have shed light on other possible mechanisms of TUS gene delivery. We addressed the possible effects of TUS on various membrane pathways, in parallel to possible alteration of the cytoskeletal network. Using compounds that affect the endosome-lysosome pathway, and by comparing transfection using TUS to that of jetPEI (Mislick and Baldeschwieler, 1996), our results show that TUS-pDNA delivery is not mediated by endocytosis. This was also confirmed by adding wortmannin, and by using ammonium chloride. If TUS promotes pDNA escape from the lysosomes, the addition of ammonium chloride should have increased transfection as compared with TUS alone or as was observed with jetPEI. However, the addition of ammonium chloride to cells did not result in higher TUS transfection levels. This observation was supported by confocal studies in which the pDNA, endosomes, and lysosomes were fluorescently labeled. In BHK cells, less than 15% of the pDNA was colocalized with endosomes or lysosomes, 2 and 5 hr after TUS transfection. In contrast, when using jetPEI, about 30–50% of the pDNA was colocalized in endosomes and lysosomes at 5 hr posttransfection. In fibroblasts, which are morphologically much larger than BHK cells, less than 10% of the pDNA was localized in endosomes and lysosomes immediately after TUS, but 2 and 5 hr after TUS, a higher percentage of pDNA (15–35%) was localized in endosomes and lysosomes. As with BHK cells, this localization was still significantly lower ($p<0.05$) when compared with the transfection by jetPEI at the same time posttransfection. The different observations may be due to the difference in size of these two

types of cells. It is possible that, due to the large surface area of the fibroblast membrane, some pDNA is absorbed by the endosome-lysosome pathway (Meijering *et al.*, 2009). Lentacker and colleagues (2009) also demonstrated that PEGylated lipoplexes, released from the microbubbles on ultrasound application, are not taken up by endocytosis, the most common route for nanoparticles to enter cells. The authors suggested that the PEGylated lipoplexes are most probably able to passively diffuse through the cell membrane pores or become injected into the cytoplasm of the target cells, in contrast to free lipoplexes.

We have also addressed the possibility that TUS energy may affect pDNA trafficking to the nucleus by altering the cytoskeletal network. This hypothesis was based on studies showing that plasmids can use the cell's own machinery and be transported through cell cytoplasm, using the cytoskeletal network (Wang and MacDonald, 2004; Vaughan and Dean, 2006). Studies have shown that TUS exposure can alter the cytoskeleton's actin filaments, leading to changes in cell morphology (Adler *et al.*, 1993; Hrazdira *et al.*, 1998; Raz *et al.*, 2005). Furthermore, TUS can initiate mechanotransduction signals through activation of stress fibers, leading to upregulation of gene expression (Kano *et al.*, 2000; Saito *et al.*, 2004; Zhou *et al.*, 2004). Using confocal microscopy, we did not observe any significant changes in the actin filaments or microtubules of both cell types after TUS application. This could be explained by the low TUS intensity applied in our experiments. Still, the possibility that TUS mediates pDNA transport via other alterations of the

cytoskeletal network cannot be ruled out. The fibers most studied for their effect on pDNA trafficking are microtubules, and their involvement in gene delivery was seen when using liposomes (Hasegawa *et al.*, 2001; Wang and MacDonald, 2004; Afadzi *et al.*, 2013), polymers (polyethylenimine, PEI) (Tait *et al.*, 2004), and electroporation and/or direct pDNA injection (Vaughan and Dean, 2006). Our confocal studies showed that in BHK cells and particularly in fibroblasts, some pDNA seemed to be associated with the microtubules.

Moreover, increased amounts of pDNA were located near and inside the nucleus after TUS application, in cells treated without nocodazole when compared with cells treated with nocodazole, in a time-dependent manner, suggesting that microtubules may have a role in pDNA trafficking to the nucleus. Thus, it can be hypothesized that in large cells, such as fibroblasts, where the pDNA needs to traverse longer distances toward the nucleus, the effect of microtubules is more prominent. Vaughan and Dean observed that the addition of nocodazole to electroporation-transfected cells or direct injection of pDNA into the cytoplasm led to a 4-fold decrease in transfection (Vaughan and Dean, 2006). Although we observed a decrease in TUS transfection with nocodazole, this effect was minor (1.3-fold for BHK cells and 1.8-fold for fibroblasts; $p=0.05$), which may indicate that pDNA trafficking to the nucleus is not mediated purely by microtubules. Consequently, we addressed the possible role that actin filaments may have in pDNA trafficking after TUS application. Actin filaments were demonstrated to be the principal barrier of size-dependent DNA mobility in the cytoplasm (Dauty and Verkman, 2005). It was also suggested that actin filaments are required for receptor-mediated endocytosis (Lamaze *et al.*, 1997).

From our studies it is evident that CytoB affected the TUS transfection level of BHK cells when compared with that of fibroblasts. This may be due to the different distribution and deposition of actin filaments in these cells. In BHK cells the actin fibers do not constitute a major component of the cytoplasm and are oriented mostly at the cell's boundaries.

In fibroblasts, the actin structure seems to be a major component of the cytoplasm, crossing the whole cell and forming a tight network of fibers. Such dense structure occupying all the cytoplasm means that there is a greater barrier in fibroblasts than in BHK cells. However, CytoB did not increase the transfection level in fibroblasts when TUS was applied, implying that it is not a limiting factor for the TUS transfection process. Moreover, using TUS with or without CytoB led to the same transfection level, indicating that the actin filaments in fibroblasts do not constitute a barrier for TUS transfection.

Taking together, our data suggest that pDNA trafficking in the cytoplasm after TUS is not mediated by the endocytic pathways and is not restricted to interaction with the cytoskeletal network. These data further support our hypothesis that the mechanical pressure applied by TUS is probably the main driving force mediating pDNA passage through the cytoplasm and toward the nucleus.

To strengthen our theory, we studied the kinetics of pDNA entry into cells and its expression after TUS application. The results showed that when pDNA was added at various time points after TUS, transfection was negligible and significantly lower compared with cells transfected by TUS when pDNA was added before TUS ($p<0.001$) or when

pDNA was added immediately after TUS ($p<0.05$). FACS analyses further demonstrated that the percentage of cells containing pDNA was reduced significantly ($p<0.01$) when it was added immediately after TUS (30%), and negligible when it was added 5 hr after TUS (5%), compared with cells in which pDNA was added before TUS application (75%).

In conclusion, these data, along with previous studies, reinforce our hypothesis that TUS operates as the mechanical force delivering pDNA to the cell through the cytoplasmic network and into the nucleus. Most importantly, these data suggest that by changing TUS parameters, pDNA delivery and trafficking in the cytoplasm can be better controlled. Nonetheless, further studies are needed to investigate the role of other factors in the cytoplasm that may affect the transfection mechanism and address the effect that TUS may have on the transport of pDNA through the cell nuclear membrane.

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Author Disclosure Statement

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