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Ultrasound/Microbubble Enhances Foreign Gene Expression in ECV304 Cells and Murine Myocardium

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Abstract Although viral vectors are efficient systems to transfer foreign genes into cells or target tissues, safety issues remain in relation to human gene therapy. Microbubbles currently used as ultrasound contrast agents have been applied in transfection of genes. This study was designed to test the transfection efficiency and the expression of exogenous gene mediated by ultrasound irradiation enhanced air filled albumin microbubbles in ECV304 cell line *in vitro* and the heart of the mouse *in vivo*. Air filled microbubbles (2.0–4.0 μm in diameter) were created by sonicating the mixture of human albumin, glucose, mannitol and special additive that was designed for stabilization. Plasmid DNA loading the reporter genes was gently mixed with microbubbles. The mixture of plasmid DNA and microbubbles was administrated to cultured ECV304 cells and BALB/c mice (tail vein injection) under different ultrasound/microbubble conditions, and then the transfection and expression efficiency were examined. The results both *in vivo* and *in vitro* demonstrated that microbubble with ultrasound irradiation could significantly elevate the exogenous gene expression as compared with microbubble or ultrasound only. Overall, the present study showed that the ultrasound-target microbubble destruction method enhanced the exogenous gene expression *in vivo* and *in vitro*, and provided a gene therapy way not only efficient but also easy to be manipulated and carried out in clinical.

Key words microbubble; ultrasound; transfection; gene therapy

The success of gene therapy is largely dependent on the development of vectors or vehicles that can selectively and efficiently deliver a therapeutic gene to cells or target tissues with minimal toxicity. Viruses are efficient transducing vectors. However, the safety concerns regarding the use of virus vector in human make nonviral delivery system an attractive focus. Nonviral vectors are particularly suitable with respect to the simplicity of use, possibility of large-scale production and lack of specific immune responses [1]. The targeted diseases ranged from single gene deficiency diseases to more complex diseases such as cancers, inflammatory diseases and some cardiovascular diseases [2].

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Ultrasound exposure has been proved to permeabilize the plasma membrane [3–6] and reduces the thickness of the unstirred layer of the cell surface [7,8], which would encourage the DNA entry into cells. Many research reports provided evidences that ultrasound could increase the gene expression when being applied to the naked DNA. Microbubble as diagnostic contrast agent has been found for decades. While the use of it for therapy is in the infancy. It has been found that microbubble can be used as cavitation nuclei for drug and gene delivery. As the microbubbles reach the region of insonation, they will cavitate and locally release the drug or gene that has been pre-incorporated into the microbubbles [9]. The threshold of microbubble cavitation can be lowered by ultrasound energy. At the present time, drug and gene delivery with microbubble is not only a laboratory research tool, but also a new field in clinical [1]. However, the delivery strategies currently used are invasive (either catheter insertion

or vascular cutting down). Therefore, further efforts are still necessary to explore much safer and non-invasive methods.

In this study, we tested the transfection efficiency and the expression of reporter genes in endothelial cell line *in vitro* and the myocardium of the BALB/c mice *in vivo* mediated by air filled albumin microbubbles enhanced by different intensities of ultrasound irradiation, to explore the possibility for transfer of therapeutic genes into targeted cells.

Materials and Methods

Plasmids, cells and reagents

pGL3-control vector and pSV-β-Galactosidase control vector were from Promega Company. Plasmid pEGFP-N_1 encoding enhanced green fluorescent protein (EGFP) was purchased from Clontech Company. Plasmid maxi kit was purchased from Qiagen Company. Luciferase reporter assay kit was purchased from Promega Company. β-Galactosidase enzyme assay system was also from Promega Company. Human umbilical vein endothelial cell line (ECV304) was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Plasmid preparation

For the transfection of cells, plasmid DNA was prepared with Qiagen maxi kit following the protocol provided by the manufacturer. Briefly, DH5α transformants of enough high density that could express the target plasmid were made and lysed by the lysing solution in the kit. The lysate was passed through the Qiagen column. The plasmid DNA was isolated from the genomic DNA by the DNA-specific resin in the column, and was collected. Agarose gel electrophoresis was performed before and after restriction endonuclease digestion to verify the identity and purity of the plasmid DNA.

Cell culture

Human umbilical vein endothelial ECV304 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS) and 50 μg/ml Gentamycin (Sigma) at 37 °C in 5% CO_2. The day before ultrasound exposure, cells were trypsinized, counted under the microscope, seeded in 6-well plate at 0.5–1.5×10^6 cells per well, and the expected 70%–80% confluent of the cells was reached on the day of ultrasound exposure.

Preparation of DNA-loading microbubbles

After the human albumin (Bayer), glucose (Sigma), mannitol (Sigma) and a special additive were fully mixed, the mixture was sonicated for 20 s [10] and incubated for 5 min at room temperature. The lower liquid phase containing microbubbles was ready for use. The density of the microbubbles was higher than 1.5×10^{11} microbubbles per liter, with diameter being 2.0–4.0 μm, and the half-life was about 120 min. Thereafter, the quantified plasmid pEGFP-N_1 was added and mixed with the microbubbles gently.

Microbubble disruption

After washing with serum-free DMEM medium, cells were cultured in medium without serum 880 μl per well and incubated at 37 °C for 5 min. Then 120 μl DNA-loading microbubbles were added into each well. The final concentration of added plasmid DNA and microbubbles was 10 μg/ml and 10%, respectively, in a total volume of 1 ml. Thereafter, ultrasound exposure was performed for 30 s with a custom-built, 20-mm-diameter and 0.8 MHz piezoelectric transducer placed in the transfection medium 2 mm above the cell monolayer. During the ultrasound exposure, the 6-well plate was suspended in a sponge water bath at 37 °C to minimize acoustic reflections and consequent standing wave formation. The transducer was calibrated to produce pulse-wave 0.8 MHz ultrasound at a spatial average temporal, average intensity of 1 W/cm^2 and a duty cycle of 10%. Five minutes after the first time exposure, another 30 s ultrasound exposure was performed under the same conditions. Ultrasound exposure caused only minor acute damage to the cell monolayer and had no effect on plasmid DNA integrity as assessed by agarose gel electrophoresis. The transfection lasted for 6 h at 37 °C in 6-well plate and stopped by dilution with 1 ml of fresh standard culture medium. Then, cells were incubated for additional 48 h.

Evaluation of GFP expression

After 48 h culture, the GFP positive cells were detected under the laser confocal microscope (Axiovert, Zeiss) at the excitation wavelength of 488 nm and photographed. Simultaneously, the cells were harvested, washed once in phosphate buffered saline (PBS) and centrifuged at 800 rpm for 3 min. Afterwards, the supernatant was decanted and the cells were resuspended in 500 μl PBS at a concentration of 5×10^8 cells/L. FACS analysis was performed on an FACS flow cytometer (Becton Dickinson, Germany).

5×10^4 cells were collected for each sample and the gene expression levels were analyzed. Cell viability was assayed by Trypan blue exclusion. Ten mililiter of cell suspension were mixed with the same volume of Trypan blue dye (Sigma). Blue (dead) and white (living) cells were counted in a hemocytometer under the microscope (IX-50, Olympus).

Animals

BALB/c mice (male, body weight 18–22 g, 8–11 weeks old) were obtained from Experimental Animal Centre in Nanjing Medical University, and were treated and housed according to approved guidelines (Guidelines for the Care and Use of Laboratory Animals).

Preparation of plasmid DNA/microbubble mixture

Plasmid pGL3-control vector and pSV-β-Galactosidase control vector containing CMV-driver firefly luciferase gene and LacZ protein cDNA were purified with Qiagen maxi kit following the protocol as mentioned above, redissolved in sterile 147 mM NaCl, 4 mM KCl and 1.13 mM $CaCl_2$ (Ringer solution), and stored at –20 °C. Microbubbles were prepared as the procedure mentioned above and mixed gently with the plasmid DNA prepared previously.

Tail vein injection and ultrasound radiation

Before the injection procedure, animals were anaesthetized by ether and kept at a high ambient temperature to dilate the tail veins. A Sonos 5500 machine (Agilent Technologies) with an S3 transducer was put on the thorax of the mouse and connected to an echocardiographic scanner. The heart was continuously imaged at a low mechanical index (0.5) to observe the microbubbles. DNA/microbubble mixture was immediately administered to the animals by injecting 50 μg/ml of pGL3-control vector and pSV-β-Galactosidase control vector respectively in Ringer solution at the total volume of 1.0–1.2 ml via tail vein and the final concentration of the microbubbles was 15%. When the asystole occurred and the myocardium was filled with microbubbles, the ultrasound mechanical index was raised to 1.5 to disrupt the microbubbles, which was maintained for about 2 min until the microbubble depletion was noted, and then the probe was removed. Animals were sacrificed 8 hours, 16 hours and 24 hours after the injection and radiation.

Extraction of luciferase and β-Galactosidase from the mouse heart samples

The entire heart samples (100 mg wet weight) were excised and frozen in liquid nitrogen immediately after the animals were sacrificed. Each frozen sample was individually pulverized into fine powder by hand grinding with a liquid nitrogen-chilled porcelain mortar and pestle, and the powder was stored in 1.5 ml Eppendorf tube at –78 °C until for extraction. Frozen powder of each sample was thawed and 400 μl of "lysis buffer" (Promega) was added per tube. The mixture was vortexed for 15 min, frozen and thawed three times using alternating liquid nitrogen and 37 °C water bath, and centrifuged for 3 min at 10,000 *g*, 4 °C. The supernatant was transferred to another 1.5-ml tube and stored at –78 °C until for luciferase and β-Galactosidase activity assay [11].

Luciferase and β-Galactosidase activity assay

Luciferase activity was measured with a luminometer (TD-20/20 luminometer, Turner Designs Inc., Sunnyvale, CA) using 100 μl of sample extract and the same volume of luciferase assay reagent (Promega Corp., Madison, WI, USA), and the light units (LU) were recorded several times within 5 s after assay reagent was added in. An aliquot of the same cell lysate for each sample was used in measuring the β-Galactosidase activity to normalize the luciferase activity. β-Galactosidase activity was measured by using the Spectronic genesys spectrophotometer (Milton Roy Company, USA). 100 μl of sample extract was diluted with 1×"lysis buffer" (Promega) to a total volume of 150 μl, and 150 μl 2×assay buffer was added and mixed well. Incubate the reaction mixture at 37 °C for 30 min, stop the reaction by adding 500 μl of 1 M Tris base. The absorbance of each reaction mixture at 420 nm was recorded.

Measurement of clinical biochemistry parameters

0.8 ml whole blood withdrawn from retroorbital vein of each animal was centifuged for 5 min at 8000 *g*, 4 °C, and 0.5 ml serum was seperated. Serum concentration of alkaline phosphatase (ALP), creatine kinase (CK), creatine-kinase muscle-brain (CK-MB), lactate dehydrogenase (LDH), Na^+, K^+ and Cl^- were measured by automated Hitachi Clinical Analyzer 7600 in the clinical chemistry laboratory at the Nanjing General Hospital.

Statistical analysis

Data were expressed as mean ± SD. Comparison between groups was performed with SPSS 10.0, and difference was considered significant at $P<0.01$.

Results

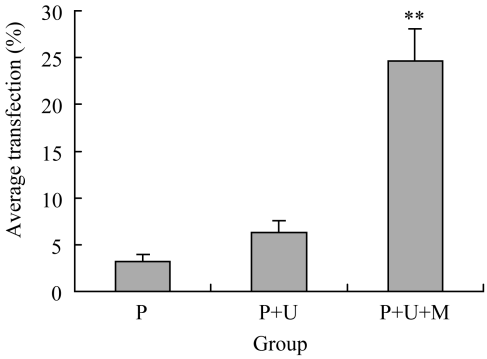

Fig. 1 Transfection effiency in human umbilical vein endothelial ECV304 cells with different treatments

Group P, plasmid DNA without microbubbles and ultrasound; group P+U, plasmid DNA with ultrasound; group P+U+M, plasmid DNA with microbubbles and ultrasound. Values are expressed as the average percentage of transfection, n=6 from 3 independent experiments. **P<0.01 vs. group P.

EGFP protein transfection in ECV304 cell line

Flow cytometer was used to examine the GFP positive cells that would emit green fluorescence, which was counted into the events of transfection efficiency. The examination was performed according to three different groups: P, plasmid DNA; P+U, plasmid DNA+ultrasound; P+U+M, plasmid DNA+ultrasound+microbubble.

As shown in Fig. 1, group P exhibited low transfection efficiency (3.22 ± 0.58)%; the transfection efficiency of group P+U remained low although showed a slight elevation (8.62 ± 1.40)%; while group P+U+M had significantly higher percentage of transfection efficiency (24.67 ± 3.29)% compared with group P and P+U (P<0.01). Fig. 2 illustrated the GFP positive cells photographed under the laser confocal microscope. It was observed that there were

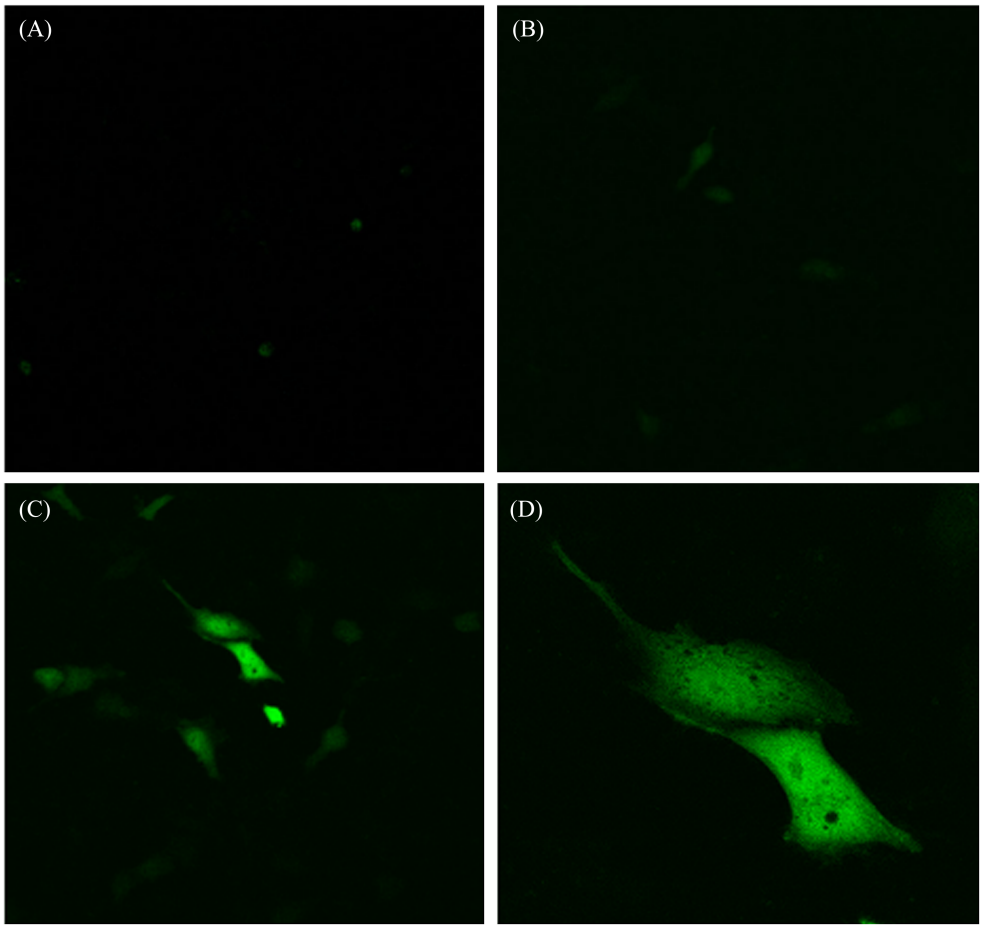

Fig. 2 Laser confocal microscope views of human umbilical vein endothelial cells (ECV304) transfected with pEGFP-N_1 under different ultrasound/microbubbles conditions

(A) Group P with plasmid DNA only, magnification 200×. (B) Group P+U with plasmid DNA and ultrasound, magnification 200×. (C) Group P+U+M with plasmid DNA, ultrasound and microbubbles, magnification 200×. (D) Group P+U+M with plasmid DNA, ultrasound and microbubbles, magnification 400×.

much fewer GFP-positive cells in group P or P+U than that in group P+U+M. For ECV304 cells which were transfected with pEGFP-N_1, the green fluorescence distributed around the whole intracellular area [Fig. 2(C,D)].

The cell viability of ECV304 cells for group P, P+U, and P+U+M was (84.1 ± 4.6)%, (80.4 ± 3.7)% and (81.3 ± 3.3)%, respectively, which suggested that ultrasound and albumin microbubbles had no obvious effect on cell viability (P>0.05).

Myocardial perfusion imaging

As it is shown in Fig. 3, when a diagnostic ultrasound transducer is placed on the thorax of mouse, the microbubbles as ultrasound contrast agents bearing plasmid DNA have been administered intravenously. As the microbubbles enter the region of insonation, they distribute within the chamber and myocardial tissue of the mouse via the vascular bed. This phenomenon suggested that the microbubbles might cavitate the endothelium of the myocardial tissue capillaries and release the genetic material into the capillary wall.

Luciferase activity assay

To test the transfection efficiency under ultrasound/microbubble, we also studied the relative activity of the luciferase versus β-Galactosidase. As shown in Fig. 4, the application of ultrasound with microbubble (group P+U+M) had induced a high luciferase activity (315.37 ± 58.46) compared with those of two other groups (P< 0.01). Consistent with the study *in vitro*, the application of plasmid DNA (group P) alone failed to achieve high luciferase activity (36.65 ± 8.89), and in the other control group administrated with ultrasound (group P+U), the luciferase activity only show a slight elevation (160.65 ± 33.24).

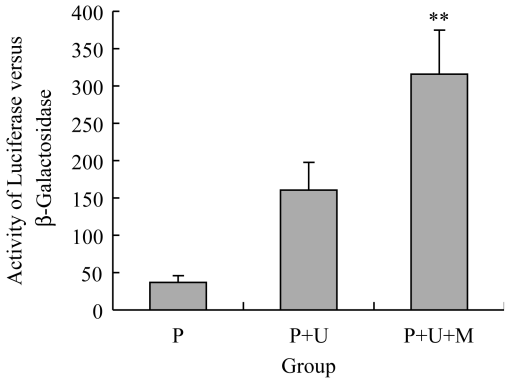

Fig. 4 Comparison of average activity of luciferase in myocardial tissue of the mouse with plasmid DNA by different means

Group P, plasmid DNA without microbubbles and ultrasound; group P+U, plasmid DNA induced by ultrasound; group P+U+M, plasmid DNA and microbubbles with the use of ultrasound. Values were expressed as the average activity of luciferase, n=6 from 3 independent experiments. **P<0.01 vs. group P with only plasmid DNA.

Serum biochemistry test

The potential toxic effects of our procedure on animals were assessed by serum biochemistry test. These tests include the determination of the major ion concentration (Na^+, K^+ and Cl^-), the concentration of heart specific

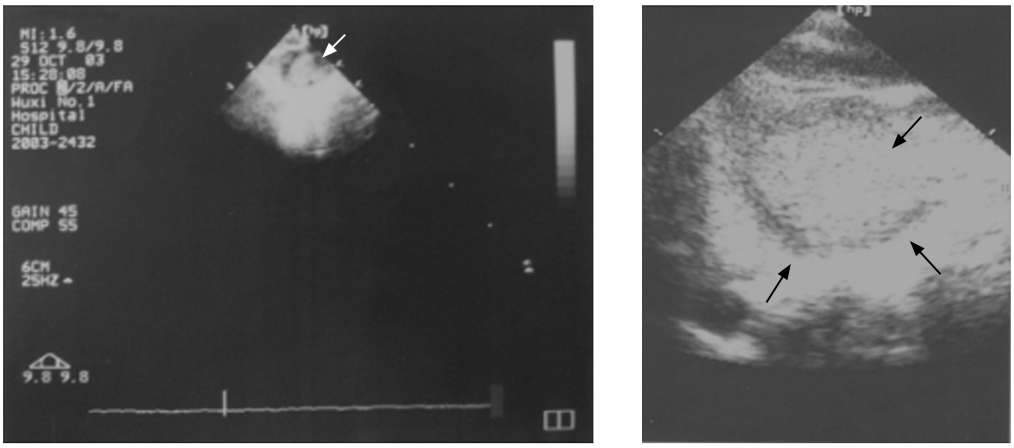

Fig. 3 Two-dimensional echocardiograms of the chamber of the mouse view after microbubble/plasmid injection to tail vein

The arrows indicate the microbubbles perfusion in chamber of the heart (left) and the myocardium (right).

Table 1 Effect of ultrasound-targeted microbubble destruction on serum biochemistry

Time after injection (h)	Serum concentration						
	Na^+ (mM)	Cl^- (mM)	ALP (mU/ml)	LDH (mU/ml)	K^+ (mM)	CK (mU/ml)	CK-MB^{-1} (mU/ml)
8	146 ± 9	102 ± 2	487 ± 15	1872 ± 25	6.5 ± 0.2	2120 ± 72	306.4 ± 9.2
16	143 ± 6	102 ± 3	314 ± 26	1572 ± 69	7.6 ± 0.6	1132 ± 40	254.7 ± 11.0
24	148 ± 1	106 ± 2	292 ± 10	1298 ± 16	7.6 ± 0.4	1113 ± 63	168.8 ± 11.3
Normal mice	146 ± 7	106 ± 6	237 ± 15	1162 ± 17	7.8 ± 0.8	384 ± 32	60.0 ± 6.7

ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase; CK-MB, creatine-kinase muscle-brain. Data represented mean ± SD from six animals.

enzymes including ALP, CK, CK-MB, and LDH. Both the short-term (8 h) and long-term (24 h) effects of applied procedure on these parameters were evaluated. Data presented in Table 1 showed that the ion biochemical parameters evaluated were all in the normal range as compared with those of normal animals, while the heart specific enzymes increased 8 h after the injection and then gradually descended to almost a normal range (ALP and LDH) or remained at a mild higher level (CK and CK-MB).

Discussion

The application of viral vectors in gene therapy is hampered by the fact that viral proteins elicit an immune response within the targeted host/tissue [12]. Recently, gene transfer into the target cells using naked DNA has been proved to be a simple and safe approach and improved by the combining with physical techniques, for example, electroporation, gene gun, ultrasound and hydrodynamic pressure [13]. Some methods even can reach an efficiency close to that achieved with viral vectors [14].

Plasmid DNA, although as a complete molecule itself, is substantially easier in large-scale producing and quality controlling than viral vectors. Besides, the safety of naked DNA has been demonstrated by numerous experiments including clinical trials [15,16]. These issues, together with the low immunogenicity and lack of tendency of integrating into chromosome DNA of target cells, make it a highly attractive reagent for human gene therapy. The only challenge for practical use might be improving the efficiency when being transfected into the target cells.

In this study, by using room air-filled human albumin-coated microbubbles, we demonstrated that recently emerged ultrasound-mediated destruction of microbubbles loaded with plasmid DNA was a feasible and efficient technique for therapeutic gene delivery *in vitro* and *in vivo*.

Microbubbles used in present experiment were prepared in our lab and had overcome the defect of the first generation microbubble which can not survive for a long time to remain intact in blood. Considering the source limitation and potential toxicity of high molecular weight gas, the microbubbles here were filled with room air and coated with human albumin, glucose, mannitol and a special additive that was designed to stabilize the microbubble. The diameter of produced microbubbles was 2.0–4.0 μm. Our previous study showed these microbubbles could pass through the pulmonary circulation for the left ventricular imaging in a dog [10]. Its firmness was enough to avoid destroy by blood pressure. The half-life of the microbubbles is about 2 hours *in vitro* and half an hour *in vivo*, which is longer than that of the microbubbles reported earlier, so the microbubbles are more stable. The present experiment further demonstrated that it was effective in carrying plasmid DNA without distinct harmful effects.

By varying the power level and duration of ultrasound irradiation in our experiment, we found that the application of 0.8 MHz ultrasound for 30 s at an intensity of 1.0 W/cm^2 for twice and 10% duty cycle would be more optimal to get higher efficiency of transfecting and lower cell death rate. However, the optimal condition should be carefully tested for different cell lines.

Plasmid pEGFP-N_1 contained the reporter gene encoding EGFP, which can be expressed in the target cells after transfection and emits green fluorescence when excited by suitable wavelength laser. As exhibited in Fig. 2, ECV304 cells transfected with pEGFP-N_1 by ultrasound/microbubble showed that the green fluorescence was distributed around the whole cell. Moreover, the transfection efficiency examined by flow cytometer was higher than that obtained without using microbubble.

The study *in vivo* showed the similar results to that *in vitro* as mentioned above. The transfection efficiency

by means of ultrasound and microbubbles was elevated as represented by measuring the activity of luciferase, and the control group applied with plasmid only or combined with ultrasound did not elevated the expression of luciferase so high. We administrated the low-level ultrasound at the initial of injection and the high-intensity focused ultrasound was used when the microbubbles appeared on the echocardiographic scanner, which resulted in well imaging of the chamber and even the myocardium when fully filled with microbubbles. It has been suggested that acoustic cavitation can disrupt microbubbles and produced transient membrane permeabilization, thus enhancing the delivery of plasmid to the cytoplasm. Our results may serve as evidences for supporting such hypothesis. In addition, some other work [17] shows that transgene expression by ultrasound-targeted microbubble destruction is related to the mechanical index of the ultrasound and inversely related to ultrasound frequency. These findings suggest that disruption energy of microbubbles plays an important role in plasmid transfer. Further effort will be necessary to understand the precise mechanism(s) of this method.

The toxicity tests showed that all animal used in our experiments appeared to recover well from the procedure. The serum ion concentrations remained in the normal level duing the whole period while a transient increase of serum concentration of heart-specific enzymes was observed and showed a minor acute damage of the myocardium. Importantly, these animals appear to recover from such damage quickly. Twenty-four hours after the injection, the value of the enzymes fell, and some of them even reached the normal level.

Recently, some other studies have reported that ultrasound with microbubbles agent can enhance the gene expression in various tissues such as cardiac muscle [18], skeletal muscle [19], and carotid artery [20]. Compared with these invasive methods (either catheter insertion or vascular cutting down), we employ the simple and noninvasive method of tail vein injection to minimize the influence of physical manipulation on the animal and it is also a method easy to carry out in the clinical utility of a therapeutic strategy.

In summary, ultrasound-targeted albumin microbubble destruction in this study is a simple, convenient and efficient method to introduce and express exogenous genes in cultured cells *in vitro* and animals *in vivo*. The greatest advantages of this technique, compared with other vector-mediated gene deliver systems such as viral and nonviral systems, are based on its safety and high efficiency without complex techniques and procedures. We believed that it should be expected to create new therapeutic options by this method *in vivo* in some diseases such as cancer, inflammatory diseases, and some cardiovascular diseases, and provide a useful new tool for manipulating gene expression in the living animals.

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