Chapter

Recent Advances about Local Gene Delivery by Ultrasound

Zhiyi Chen, Meng Du and Fei Yan

Abstract

Gene therapy has been widely explored as a pharmacological approach, with a great potential to treat various diseases. Generally, many diseases have definite lesion's site, especially for tumors. This feature results in a great demand on the delivery of therapeutic gene to the local lesion's site. Ultrasound combined with microbubbles provides a promising platform to deliver gene in a spatiotemporally controlled way. Ultrasound beam can be positioned and targeted onto the deep-seated lesion's site of diseases by an external mobile transducer. Microbubbles can serve as vehicles for carrying genetic cargo and can be destructed by ultrasound, resulting in the local release of genetic payload. Meanwhile, sonoporation effect will occur upon which the bubbles are exposed to the appropriate ultrasonic energy, producing the transient small holes on the adjacent cell membrane and thus increasing the vascular and cellular permeability. In this chapter, we will review the recent advances about local gene delivery by ultrasound.

Keywords: ultrasound, gene therapy, microbubbles, sonoporation

1. Introduction

Gene therapy, designed to deliver nucleic acid into cells to compensate for abnormal genes, is now considered a promising treatment option for some human diseases [1]. With the development of modern medicine and precise medicine, there is an increasing trend to change the traditional gene delivery mode into local gene delivery. At present, there are mainly two gene delivery approaches, virus-mediated transfection and nonvirus-mediated transfection [2]. The former method has high-transfection efficiency, but the preparation procedure of recombinant viruses is sophisticated, and their clinical application is restricted due to biosafety concerns [3]. Nonviral vector approaches, such as liposome-mediated methods and electroporation techniques, are relatively safe. However, poor targeting and low-transfection efficiencies limit their widespread use [4]. It is a current research hotspot to look for an effective and safe method to mediate gene delivery for biomedical application.

Ultrasound is a widely used diagnostic technique in clinic, which possesses the advantages of safety, real-time monitoring, and low cost. Recently, with the development of ultrasound contrast agents, ultrasound has evolved from a diagnostic tool to a treatment application for delivering locally therapeutic substances into the lesion's sites. Ultrasound-targeted microbubble destruction (UTMD) provides a promising platform to deliver genes in a spatiotemporally controlled way.

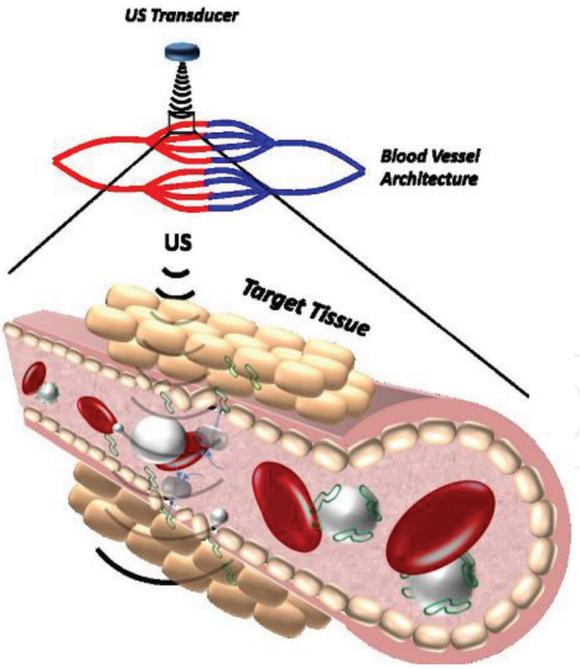

Figure 1.
Schematic model of ultrasound-mediated gene delivery. Bioeffect produced by ultrasound and microbubble interaction could enhance the permeability of vascular and promote the accumulation of gene (green) in tissue. (Quoted from: Sirsi and Borden [5]).

Microbubbles can serve as vehicles for carrying genetic cargo and can be destructed by ultrasound, resulting in the local release of genetic payload. Meanwhile, sonoporation effect will occur upon which the bubbles are exposed to the appropriate ultrasonic energy, producing the transient small holes on the adjacent cell membrane and thus increasing the vascular and cellular permeability **Figure 1**. In this chapter, we will briefly introduce the mechanism and review the recent advances about local gene delivery by ultrasound.

2. Mechanism of ultrasound-mediated gene transfection

2.1 Sonoporation

When ultrasound is irradiated locally with certain energy, the cavitation nuclei, such as ultrasound contrast agents and bubbles, could alternately occur expansion, contraction, splitting, fusion, and even rupture. This physical process is called cavitation effect. Accompanied by the cavitation effect, acoustic microstreaming, micro-jet, high temperature, and shockwave will occur in the medium, resulting in the formation of some temporary, reversible pores on the cell membrane, which is sonoporation [6, 7]. Generally, it is an accepted notion that the sonoporation from cavitation effect allows genes and drugs to enter cells [8].

There are a large number of studies, which have confirmed that sonoporation can increase the efficiency of gene delivery through enhancing the permeability of the cell membrane [9–12]. The number of pores, having a high impact on the gene delivery efficiency, can be affected by a lot of factors, such as acoustic pressure, irradiation duration time, and pulse repetition frequency [13–15]. Sonoporation pores trend to be larger along with the increase of acoustic pressure and irradiation time, which also enhance gene transfection efficiency [16]. However, excessive acoustic pressure or ultrasonic duration may reduce cell viability and even cause cell death, vascular rupture, and other side effects [17–19]. Therefore, to achieve a

high gene transfection efficiency and remain a cell viability as much as possible, it is important to optimize ultrasound irradiation parameters during gene transfection.

2.2 Endocytosis

In addition to sonoporation, cavitation effect can change the cell membrane structure through microstreaming and shear force. The mechanical force may cause cytoskeleton rearrangement and regulate various downstream cellular signaling pathways, helping the endocytosis of genetic cargo [20, 21]. Generally, there are three forms of endocytosis, including macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis [22]. After ultrasound irradiation, the reactive oxygen species are produced to stimulate the calcium influx and induce the occurrence of endocytosis [23]. In addition, cavitation effect and shear force induced by ultrasound can change cell structure and influence endocytosis through mechanosensors and signaling cascade [24]. Meijering et al. demonstrated that endocytosis was involved in the uptake of the macromolecular substances, while small molecules enter cells mainly through the pores of the membrane surface [25].

2.3 Sonoprinting

Recently, Cock et al. put forward a new viewpoint on the mechanism of ultrasound-mediated gene delivery [26]. By using the real-time scanning confocal microscopy, they found that nanoparticle-loaded microbubbles could deposit the nanoparticles in patches onto the cell membrane during ultrasound irradiation and promote the particles that enter cell through the fluidity of the membrane. In their opinion, this method, termed sonoprinting, is neither the traditional sonoporation nor the material swallowing. The underlying mechanisms still need to be explored.

3. Type of ultrasound contrast agents as gene vector

Genes administrated by the intravenous route are easily be degraded. Conventionally, genes such as plasmids, mRNA, siRNA, and miRNA need to be protected from degradation by extracellular and intracellular barriers **Figure 2**. The ideal gene vectors should have the following characteristics: (1) safe and nontoxic, long cycle time in vivo, protecting the nucleic acid molecules from being destroyed by extracellular nucleic acid enzymes; (2) possessing the characteristics of a targeting ability and delivering the gene to target tissue or target cells; (3) high gene-carrying capacity; (4) promoting the gene to enter cytoplasmic or nucleus and stable expressing; (5) ensuring the controllability of gene function; and (6) noninvasive evaluation of gene delivery effectiveness. In the field of ultrasound-mediated gene delivery, many ultrasound contrast agents, including microbubbles, nanobubbles, nanodroplets, and some nanoparticles, are being developed into gene vectors in gene delivery mediated by ultrasound.

The gene vector may help them to avoid degradation by extracellular and intracellular barriers, including serum endonucleases, immune detection, and endosome (Quoted from: Yin et al. [2]).

3.1 Microbubbles

Microbubbles are small, gas-filled microspheres with the particle size of 1–3 μm. As gene vectors, they not only can protect the genes from nucleic acid enzyme degradation and from reticuloendothelial system clearance but may also enhance

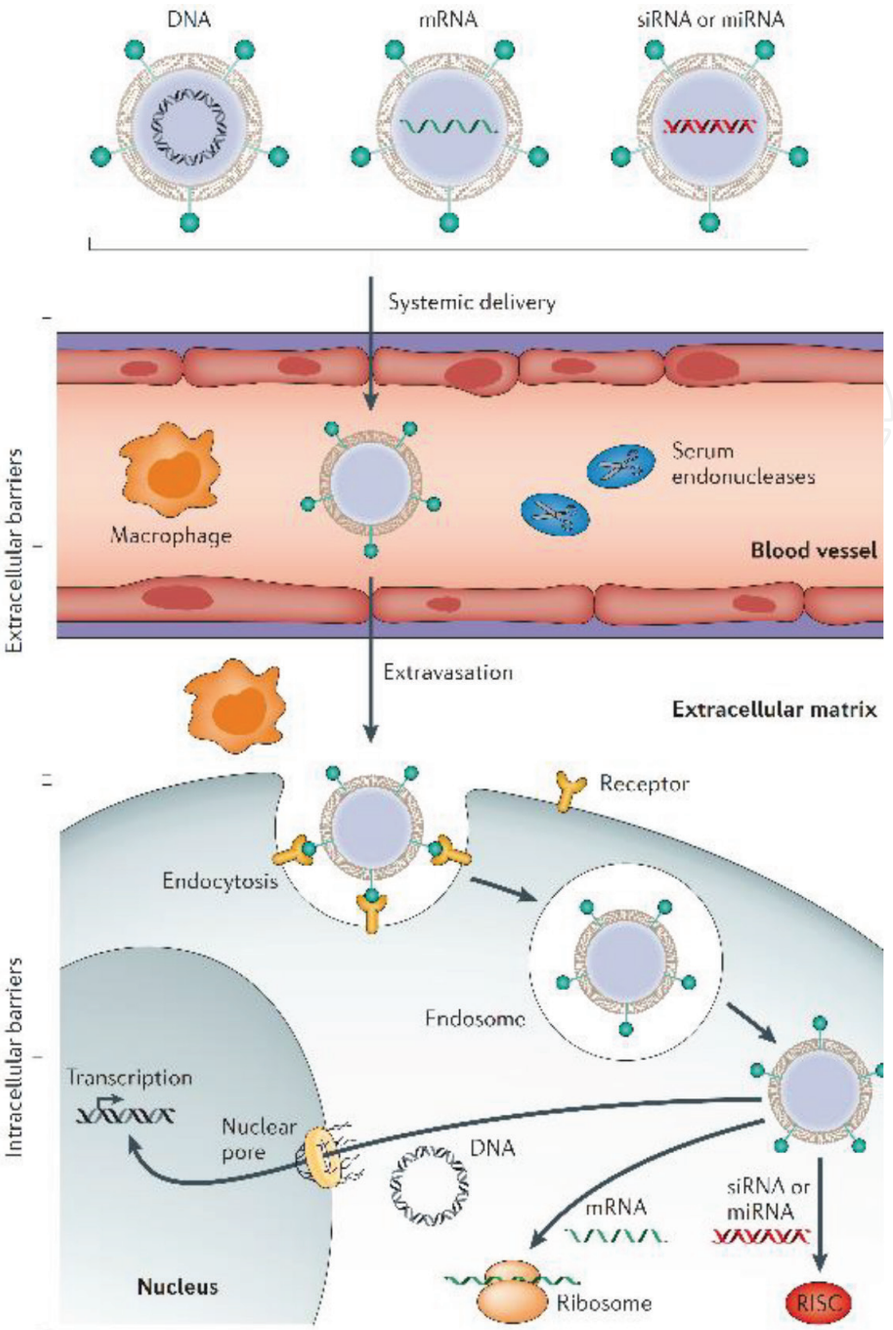

Figure 2.
Schematic model of transfection process of genes in carriers.

their local delivery through active and passive targeting. Traditional membrane materials consist of microbubbles, which include albumin, lipid, polymers, and surfactants. Different shell compositions have various characteristics. Albumin is commonly used in the preparation of commercial ultrasound contrast agents, but it is susceptible to degeneration due to temperature change. In addition, it is expensive and easy to cause immune response. The synthetic phospholipids are good and biocompatible, but their half life is short in vivo. Polymers are slightly inferior in biocompatibility, but it possesses better stability.

It has been proved that the application of ultrasound combined with commercial microbubbles and gene mixture could regulate gene expression and achieve therapeutic effect [27–30]. Wang et al. compared the effect of gene delivery by three kinds of typical commercial microbubbles—Optison, Sonovue, and Levovist. The mixture of microbubbles and plasmid DNA encoding green fluorescent protein was injected into tibialis anterior muscle of mice. After ultrasound irradiation,

the number of GFP-positive fibers was significantly increased in Optison- and Sonovue-treated groups, proving the efficiency of gene transfection by ultrasound combined with commercial microbubbles [31]. However, DNA is anionic molecules, and most microbubbles are negatively charged on the surface, which bring some difficulty for the formation of DNA/microbubble complexes. In order to address this issue, some cationic microbubbles are developed and applied as gene vector to enhance the gene-carrying capacity [32–37]. Wang et al. evaluated the difference of gene transfection rate between cationic microbubbles and neutral microbubbles in combination with ultrasound. Their results demonstrated that the expression of reporter gene in cationic microbubble group was 20-fold higher in vitro and 3-fold higher in tumor model than neutral bubbles [34]. Recently, Wei et al. applied Targesphere, a kind of commercial cationic microbubbles, as short hairpin (shRNA) vector for connective tissue growth factor (CTGF). It was showed that the expression of CTGF was decreased in renal fibrosis mouse model after ultrasound irradiation, which proved the great potential in gene delivery mediated by ultrasound combined with cationic microbubbles [38].

3.2 Nanoparticle, nanodroplet, and nanobubble

Nanoscale ultrasound contrast agents, with the particle size from 100 to 600 nm, are also developed in the recent years. Compared with traditional microbubbles, nanoscale contrast agents have smaller size and stronger penetrating ability. In addition, nanoscale contrast agents possess greater gene-carrying capacity due to their larger surface area. Common nanoscale ultrasound contrast agents include nanobubbles, solid nanoparticles, and liquid fluorocarbon nanoparticles. Most of the shell membrane of nanobubbles are lipid or polymer, and the core could be gas or liquid. Nanobubbles can cross through the blood vessels and aggregate in the tumors through the enhanced permeability and retention (EPR) effect [39]. It was proved that nanobubbles could achieve ideal gene transfection efficiency when combined with ultrasound [40, 41]. Horie et al. applied ultrasound combined with nanobubbles mediating tumor necrosis factor (TNF-α) DNA delivery to treat tumor-bearing mice and resulted in the decrease of the tumor vessel density and inhibition of tumor growth [42]. To enhance the gene-carrying capacity and local transfection efficiency, cationic nanobubble or targeted nanobubbles have been applied and showed excellent therapeutic effect in vitro and in vivo [43–45]. Yin et al. developed a new kind of siRNA-nanobubble, through a nanoparticle heteroassembly of siRNA-loaded polymeric micelles and liposomes, demonstrating their ideal therapeutic effect in cancer treatment [46]. Xie et al. used cell-permeable peptides (CPPs) to enhance the transferring rate of siRNA. They developed CPP-siRNA that targets oncogene c-myc and encapsulated it into nanobubbles. It was shown that the expression of c-myc mRNA was significantly decreased, and the growth of tumor was significantly inhibited after ultrasound irradiation [47].

Recently, liquid fluorocarbon nanodroplets have attracted wide attentions in the ultrasound-mediated gene delivery. These nanodroplets prepared from a lipid or a polymer shell can encapsulate liquid fluorocarbon emulsion (perfluoropentane, etc.). The liquid core would occur "acoustic droplet vaporization" (ADV) under ultrasound irradiation, which makes the nanodroplet transform into gas-containing microbubbles, greatly enhancing the cavitation effect of ultrasound **Figure 3**. Although nanodroplets have shown its therapeutic effect in high-intensity focused ultrasound (HIFU) and drug delivery, its application in gene delivery is still rare. Gao et al. synthesized a novel tumor-targeting cationic nanodroplet and applied it as gene vector to treat Her2-positive breast cancer. The results in their study

demonstrated that this nanodroplet could achieve better gene transfection efficiency, showing its potential in gene delivery by ultrasound [48, 49].

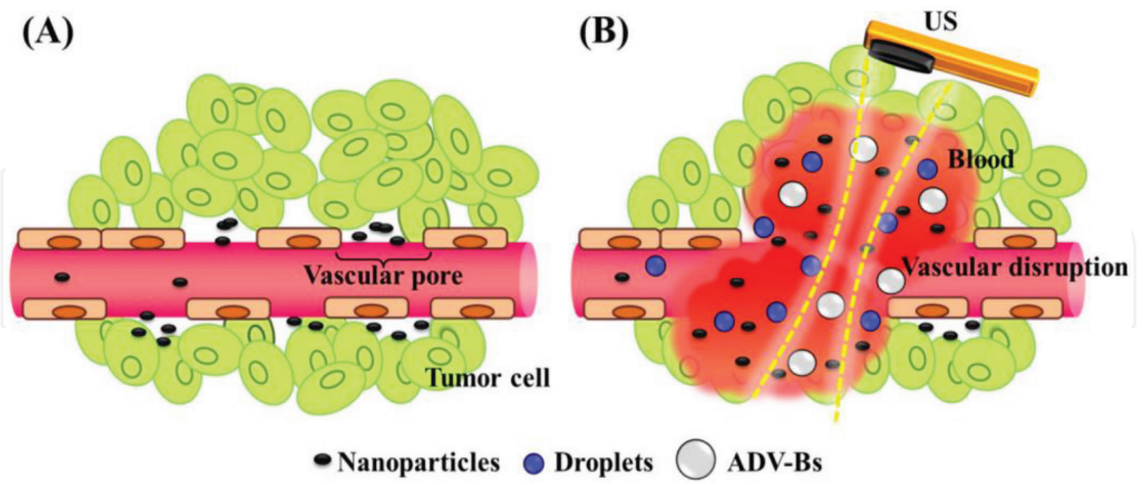

Figure 3.
Schematic model of acoustic droplet vaporization (ADV).

(A) Nanoparticles penetrate the tissue through the EPR effect; (B) droplets vaporize into microbubbles through ADV under certain acoustic pressure, which enhances the cavitation effect and changes the structure of tumor vessels. (Quoted from: Ho et al. [50]).

Nanoparticles commonly used in gene transfection include liposomes, polymer, and magnetic nanoparticles. Studies demonstrated that the cavitation effect produced by UTMD could increase the concentration of nanoparticles in targeted tissue and improved gene transfection efficiency. In the field of ultrasound-mediated gene delivery, liposome and polyethylenimine (PEI) are the most popular gene vectors.

Liposome is used as a nanocarrier for gene transfection, with high gene-carrying capacity and transfection efficiency. Taking advantages of UTMD, researchers have demonstrated that the accumulation of gene-carrying liposomes can be improved in targeting cells or tissue [51, 52]. Yoon et al. proved that ultrasound combined with microbubbles and gene-carrying liposomes could be a superior gene transfection system [53]. Recently, Chertok et al. modified heparin on the surface of liposome to increase the accumulation of gene in tumor site and reduce the off-target effect. Compared with nonheparinized DNA-carrying liposomes, modified liposomes combined with UTMD could significantly enhance the gene transfection rate in tumor in vivo [54].

PEI is another commonly used gene vector with high-density positive charge. It can form stable complex with genes through electrostatic adsorption. Also, utilization of PEI can avoid DNA degradation by nucleic acid enzyme and improve the stability and integrity of genes in vivo. Meanwhile, PEI can assist gene delivery into nuclei through proton sponge mechanism and endosomal escape, which will enhance the expression of targeting gene [55] **Figure 4**. However, the cell toxicity is inevitable because of its strongly positive charge. UTMD may function as an effective method to balance the cytotoxicity and transfection efficiency of PEI. UTMD could not only temporarily mediate the opening of cell membrane and promote the PEI-DNA complex entering the cell but also improve the level of intracellular calcium and PKC protein expression, which can enhance the effect of endocytosis. It was confirmed that UTMD combined with PEI or chemical modified PEI could be an effective and safety gene transfection strategy in vitro or in vivo [56, 57]. Dang et al.

demonstrated that UTMD combined with PEI could achieve the same transfection efficiency as Lipofectamine 2000 and lower cytotoxicity [58]. Deshpande et al. found that ultrasound combined with PEI could enhance the DNA transfection rate up to 200-fold than naked DNA plasmids [59]. Park et al. applied UTMD combined with PEI mediating the adenine nucleotide translocase-2 (ANT2) shRNA to successfully increase the survival rate of xenograft mice and induce the tumor regression [60].

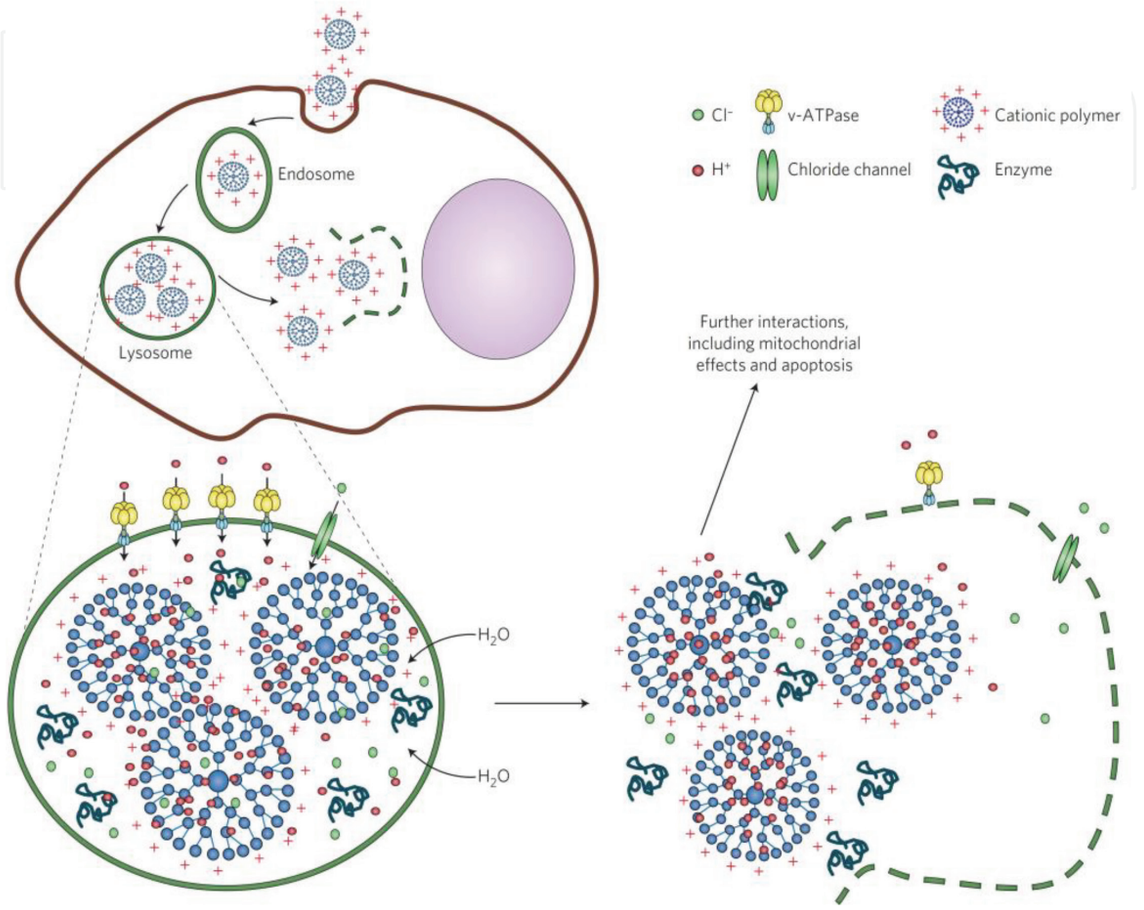

Figure 4.
Schematic model of the proton sponge effect by cationic nanoparticles.

PEI binds with cell membrane and is endocytosed. When they enter lysosome, the unsaturated amino groups are able to capture protons and cause the retention of Cl^- ion and water molecule, which will make lysosomal swelling and rupture, and then release the lysosomal content. (Quoted from: Nel et al. [55]).

4. Application of local gene delivery by ultrasound

4.1 Tumor

Tumor is a kind of genetically related disease. Its occurrence, development, and recurrence are closely related to the mutation and deletion of the gene. With the development of molecular biology, gene therapy has shown a great potential in cancer treatment. At present, the common strategy of gene therapy is to transfer tumor suppressor gene into tumor cells to restore normal phenotype of cells. Nande et al. applied UTMD to mediate tumor suppressor genes, including p53, Rb, p130, and significantly reduced tumor growth [61]. Chang et al. utilized the p53-loaded targeted microbubbles for ovarian cancer treatment and achieved higher transfection

efficiency than conventional nontargeted microbubbles [62]. Mishel et al. used ultrasound to mediate hSef-b delivery, another kind of human tumor suppressor gene, and demonstrated the efficacy of gene therapy mediated by ultrasound [63]. Recently, gene delivery by ultrasound was also applied in gene-directed enzyme prodrug therapy (GDEPT). The key process of GDEPT is effectively transferring gene encoding enzyme, which can convert a nontoxic prodrug into an activated cytotoxic agent [64]. Devulapally et al. used PEGylated-PLGA-PEI nanoparticles and mediated TK-NTR fusion gene delivery in tumor xenograft mice. Their results showed that the tumor size was reduced by 2.3-fold when compared with untreated mice [65].

In the field of tumor therapy, RNAi could selectively inhibit the expression of key genes in the development of cancer. It has been proved that co-delivery of siRNA and chemotherapy drugs by ultrasound could improve the therapeutic effect of tumor and reduce the dosage chemotherapy drugs [66–69]. Zhao et al. synthesized cationic porphyrin microbubbles for the delivery of FOXA1-siRNA, achieving an excellent therapeutic effect for breast cancer [70]. Cancer stem cells (CSCs), a group of tumor cells with self-renewal, multidirectional differentiation potential, are thought to be the key of tumor recurrence, metastasis, and drug resistance. Specific markers, such as CD133, are important targets for gene therapy. Liu et al. used UTMD to deliver shRNA-CD133 to liver CSCs and reversed the process of epithelial-mesenchymal transition [71].

4.2 Cardiovascular disease

Atherosclerosis is the main cause of coronary heart disease, cerebral infarction, and peripheral vascular disease. Studies demonstrated that ultrasound combined with microbubbles can deliver angiogenic genes to the ischemic region of the myocardium and enhance expression of angiogenesis-related factors and thus improve myocardial blood supply [72, 73]. Du et al. utilized UTMD and cationic microbubbles to mediate delivery of growth differentiation factor 11 (GDF11) plasmid to aged heart. Their results suggested that ultrasound could enhance GDF11 expression, increase the cardiac stem cell (CSC) proliferation, and rejuvenate the senescent heart from ischemic injury [74]. Castle et al. successfully enhanced the level of ApoA-I and high-density lipoprotein cholesterol (HDL-C) in vivo through delivering human apolipoprotein ApoA-I plasmids by ultrasound [75].

Heart failure, caused by various cardiac structures and functional disorders, will impair ventricular filling and ejection function and eventually cause cardiac output unable to meet body tissue metabolic needs. Lee et al. delivered survivin gene to cardiomyocyte by UTMD and observed its efficacy on cardiac function. The apoptosis rate of cadiomyocyte was significantly decreased, and the left ventricular systolic dysfunction was attenuated after 6 weeks, demonstrating that ultrasound-mediated gene delivery can be an effective treatment in heart failure [76].

4.3 Central nervous system diseases

Blood-brain barrier (BBB) is an important obstacle for central nervous system (CNS) diseases. BBB is mainly composed of cerebral capillary endothelial cells and their cells, matrix, astrocytes, and extracellular matrix [77]. To cross the BBB, researchers have tried various methods, including invasive surgery, hypertonic drugs, chemical modification of drugs to target delivery to brain, and micro-carriers [78–80]. Recently, ultrasound mediating BBB opening has attracted researchers' attention due to its characteristic of noninvasive, reversible, and targeted delivery. Hynymen et al. proved that microbubbles could be applied as cavitation nuclei to reduce the ultrasonic energy to open the BBB, reducing the risk of tissue damage and bleeding [81]. Based on this, numerous studies are exploring the therapeutic

effect of gene delivery mediated by ultrasound in CNS diseases [82, 83], such as glioma, Parkinson's disease, and Alzheimer's disease.

Glioma is the most common malignant tumor of the central nervous system. UTMD has a wide application prospect in the treatment of brain glioma. In 2016, Carpentier et al. developed an implantable ultrasonic irradiation system, named SonoCloud. They used this system to open the local area of BBB with microbubbles. In their study, 15 patients with recurrent brain glioma were selected to test the therapeutic effect of UTMD-mediated BBB opening. After intravenous administration of carboplatin and Sonovue combined with ultrasound treatment, it was proved that the BBB could be safely opened, and 9 of 15 patients showed no further tumor growth [84]. Fan et al. applied cationic microbubbles as therapeutic gene vectors and effectively mediated BBB opening for gene delivery in vitro and in vivo [85, 86]. Zhao et al. used targeted liposomes (NGR-liposomes) as vector for shRNA-Birc5 delivery and demonstrated the enhancement of local gene transfection and the inhibition of glioma progression [87].

Parkinson's disease (PD) is a common neurodegenerative disease of the nervous system due to the degeneration and death of dopaminergic neurons in substantia nigra and the significant decrease of dopamine content in striatum. Glial cell line-derived neurotrophic factor (GDNF) can protect the dopaminergic neurons and promote the regeneration of dopamine system in black striatum [88]. Fan et al. restored behavioral function in a PD animal model through delivering GDNF gene by transcranial focused ultrasound [89]. Lin et al. used the GDNF-loaded liposome-microbubble complexes and demonstrated the therapeutic effect of PD by using focused ultrasound-mediated BBB opening [90, 91].

In addition, ultrasound-mediated gene delivery was also applied in other CNS diseases. Song et al. developed PLGA nanobubbles for NGF delivery. NGF expression was significantly enhanced, and neuronal apoptosis in injured spinal cords was inhibited after ultrasound irradiation [92]. Wang et al. demonstrated that UTMD could successfully mediate VEGF gene delivery into brain and decreased infarct areas in a cerebral ischemic injury model [93].

4.4 Musculoskeletal disease

Arthritis is a common chronic inflammatory disease. Of these, the most common type is osteoarthritis and rheumatoid arthritis (RA). At present, the main treatment of arthritis is drug, including nonsteroidal anti-inflammatory drugs, cytotoxic drugs, and hormones. However, there are some drawbacks such as low local concentration and systemic side reaction. Ultrasound-mediated gene delivery has been proved to be effective in arthritis therapy. Xiang et al. applied UTMD-mediated enhanced green fluorescent protein (EGFP) gene delivery in antigen-induced arthritis rabbit model, and the significantly enhanced expression remained detectable for 40 days in the synovial pannus [94]. Tumor necrosis factor α (TNFα) secreted by synovial fibroblasts plays an important role in the progression of RA, which can cause bone destruction and joint dysfunction. Inue et al. transferred siRNA-TNFα to the articular synovial membrane of the rat through UTMD technique. They found that the expression of TNFα was inhibited, resulting in a significant remission of paw swallowing in comparison to control group [95].

In the field of fracture healing, bone morphogenetic protein-2 (BMP-2) is an ideal osteoinduction factor, which possesses the function of inducing cartilage and bone formation [96]. Some studies have confirmed that the transfection rate of BMP-2 gene in skeletal muscle cells and fibroblast cells could be enhanced by UTMD [97]. Osawa et al. delivered BMP-2 gene to the skeletal muscle in vivo, confirming the therapeutic effect of UTMD mediating gene transfection [98].

Tendon injury is a common disease in orthopedics with a significant impact on the quality of patient's life. Regulation of the expression of local cytokines in Achilles tendon by gene therapy is a potential therapeutic method to improve the prognosis of patient. Studies demonstrated that UTMD could increase the expression of genes in the Achilles tendon [99–101]. For example, Tang et al. transfected injured Achilles tendons of mice with insulin-like growth factor-1 (IGF-1) cDNA, showing that the maximum load, stiffness, and ultimate stress of treated Achilles tendons were higher than control group [102]. Bez et al. transferred BMP-6 encoding DNA by UTMD in Yucatan mini-pigs, showing the significantly enhanced osteointegration of all pigs after 8 weeks [103].

4.5 Ocular disease

For the treatment of ocular diseases, the most common method of drug delivery is surface administration or systemic administration. However, due to the unique structure of the eye, traditional drugs are difficult to enter the posterior eye segment, causing low bioavailability of drugs. As for ultrasound mediated gene delivery in ocular diseases, recent researches mainly focus on the cornea, retinoblastoma, and retinal neovascularization.

Cornea is a transparent tissue without blood vessels, which is an ideal target tissue for gene therapy because of its superficial position, transparent organization, and easy observation. Sonoda et al. confirmed that UTMD could mediate eGFP gene transfection to cornea epithelial cells of rabbit. In their study, they injected plasmid and microbubbles into the cornea of the rabbit and irradiated the eyes with ultrasound. They found that the corneal cells with GFP-positive expression were distributed around the injected region. No obvious tissue damage was observed in their study [104]. To optimize the gene transfection efficiency, Yamashita et al. developed a novel lipid microbubble, composed of polyethylene glycol (PEG) modified liposomes and perfluoropropane gas, and achieved a 27% gene transfection rate [105].

In the retina, there is a biological barrier similar to the blood-brain barrier named blood-retinal barrier (BRB), which is composed of tight connection between retinal endothelial cells and retinal pigment epithelial (RPE) cells. The presence of BRB prevents most systemically administered genes entering the retina, reducing the effectiveness of treatment. Park et al. demonstrated that UTMD could mediate BRB reversible opening without retinal damage [106]. Some studies have confirmed the effect of UTMD-mediated gene delivery into retinal in vitro and in vivo [107–110].

Retinoblastoma (RB) is a common ocular malignancy. Local treatment not only can retain part of the vision but also reduce the toxic side effects. Luo et al. applied wild-type 53 (wtp53) as a therapeutic gene. The in vitro experiment showed that the apoptosis rate of RB cells was higher (25.58%) than control group after ultrasound treatment [111]. To prove the therapeutic effect of gene delivery by ultrasound in vivo, Gao et al. transferred both wtp53 and Rb94 by UTMD to treat tumor-bearing mice. RB tumor growth was significantly inhibited, along with the decrease of the level of vascular endothelial factor and microvessel density [112].

Retinal neovascularization (RNV) is caused by hypoxic-ischemic ocular fundus diseases, characterized by retinal fibrous hyperplasia, retinal detachment, and even loss of vision. It has been reported that endostatin can be used for treating RNV because of its excellent antiangiogenic effect [113]. Xu et al. significantly enhanced the expression of endostatin by using cationic microbubbles to deliver endostatin gene under ultrasound irradiation. As a result, the growth of human retinal vascular endothelial cell was inhibited, suggesting that endostatin gene delivery mediated by UTMD may be a useful tool for RNV therapy [110].

4.6 Nephropathy

The blood flow of the normal kidney accounts for one-fourth to one-fifth of the total circulating blood volume. Therefore, a large number of microbubbles could enter the kidney blood vessels, which could be applied for ultrasound contrast imaging or targeted treatment. Based on this feature, some researchers applied UTMD to deliver genes to treat nephropathy, including diabetic nephropathy, hypertensive nephropathy, and renal fibrosis. Zhang et al. found that UTMD could increase the renal interstitial capillary permeability in diabetic nephropathy rat models [114]. Transforming growth factor β (TGF-β) is the key cytokine to promote the development of renal fibrosis. It can induce apoptosis of the podocytes on glomerular filtration membranes and promote the activation and proliferation of interstitial fibroblasts through TGF-β/SMAD signaling pathway. Lan et al. enhanced the expression of Smad7 in rat unilateral ureteral obstruction model by ultrasound as the gene delivery system, greatly attenuating tubulointerstitial fibrosis [115]. The therapeutic effect of UTMD-mediated Smad7 gene delivery in renal fibrosis was also proved in Smad7 gene knockout mice [116], diabetic nephropathy model mice [117], and angiotensin II-mediated hypertensive nephropathy [118].

In addition to the TGF-β/SMAD signal pathway, researchers have also explored the use of other signaling pathways in the treatment of renal fibrosis. RAP1 is a small molecule G protein that participates in the regulation of cell proliferation, differentiation, and intercellular adhesion [119]. Xiao et al. treated diabetic model rats with Rap1 gene delivery by ultrasound and microbubble (Optison). It was demonstrated that this treatment could protect the mitochondrial function of renal tubules and reduce the interstitial fibrosis [120]. In diabetic nephropathy, Yiu et al. confirmed the therapeutic effect of Kallistatin, which possesses the function of antioxidative and anti-inflammatory. The glomerulosclerosis and renal fibrosis were attenuated, and the renal function was improved after Kallistatin gene delivery by UTMD [121].

Recently, RNAi combined with UTMD therapy has been applied to the treatment of renal diseases. miR-29b is low expression in diabetes [122] and can function as a therapeutic targeting [123]. Chen et al. delivered miR-29b in diabetic mice by ultrasound combined with SonoVue. The results showed that this treatment could inhibit the inflammation induced by NF-κB/p63 and delay the progress of renal fibrosis [28]. Zhong et al. found that the level of miR-21 is highly associated with the development of renal fibrosis in diabetic mice and effectively improved renal fibrosis and inflammation by using UTMD-mediated miR-21 shRNA delivery [29]. Wei et al. applied UTMD combined with shRNA-CTGF to treat mouse models of renal fibrosis; the level of CTGF was significantly lower; and the renal fibrosis was attenuated, accompanied by the reduction of TGF-β and Type I collagen [38].

5. Conclusion and prospect

With the development of ultrasound contrast agents and the understanding of the biological effects of ultrasound, ultrasound-mediated gene delivery has been proven the great potential in the treatment of various diseases. Ultrasound contrast agents, including microbubbles, nanoparticles, and nanobubbles, can be used as gene vectors through intravenous or local injection into lesion site. With ultrasound irradiation at a certain level of acoustic intensity, the cavitation effect, sonoporation, and thermal effects occur, which can enhance the permeability of local tissue and promote the gene delivery into the pathological tissue.

Although ultrasound-mediated gene delivery has a broad application in animal study, there is still a long way for its application in human body. The main

problems, which need to be solved, may include the following aspects. First, many cationic materials are applied for the preparation of the ultrasound contrast agents. They have high gene-carrying capacity, but their biocompatibility is still doubtful. Second, ultrasound security is also an important concern. Unlike diagnostic ultrasound energy, the intensity applying in gene delivery is greater. Studies have shown that severe cavitation effects can lead to membrane rupture, DNA rupture, nuclear fragmentation, endothelial cell damage, microvascular leakage, hemolysis, myocardial injury, and even left ventricular function [19, 124]. More investigations need to be made to optimize the ultrasonic parameters so as to maximize the gene transfection efficiency and reduce the adverse side effects on the normal tissues and organs. In addition, the different types of ultrasound equipment used in various laboratories also bring some difficulties for the repeatability, which hinder the progress of ultrasound-mediated gene transfection technology to some degree. At the same time, it is believed that ultrasound will make more progress in gene delivery and bring about greater medical revolution in the future.

Acknowledgements

This work is supported by National Natural Science Foundation of China (Nos. 81671707, 81571701, and 81530056), Natural Science Foundation of Guangdong Province (Nos. 2017A030313651 and 2016A030311054), Research Projects of Guangzhou Science Technology and Innovation Commission (No. 201607010201), and Research Fund for Lin He's Academician Workstation of New Medicine and Clinical Translation.

Conflict of interest

The authors declare no competing financial interest.

Author details

Zhiyi Chen[1], Meng Du[1] and Fei Yan[2*]

1 Department of Ultrasound Medicine, Laboratory of Ultrasound Molecular Imaging, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, P. R. China

2 Paul C. Lauterbur Research Center for Biomedical Imaging, Institute of Biomedical and Health Engineering, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, P. R. China

*Address all correspondence to: fei.yan@siat.ac.cn

References

[1] Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018;**359**. DOI: 10.1126/science.aan4672

[2] Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. Nature Reviews. Genetics. 2014;**15**:541-555. DOI: 10.1038/nrg3763

[3] Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nature Reviews. Genetics. 2003;**4**:346-358. DOI: 10.1038/nrg1066

[4] Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. Nature Reviews. Genetics. 2005;**6**:299-310. DOI: 10.1038/nrg1577

[5] Sirsi SR, Borden MA. Advances in ultrasound mediated gene therapy using microbubble contrast agents. Theranostics. 2012;**2**:1208-1222. DOI: 10.7150/thno.4306

[6] Deng CX, Sieling F, Pan H, Cui J. Ultrasound-induced cell membrane porosity. Ultrasound in Medicine & Biology. 2004;**30**:519-526. DOI: 10.1016/j.ultrasmedbio.2004.01.005

[7] Lentacker I, De Cock I, Deckers R, De Smedt SC, Moonen CT. Understanding ultrasound induced sonoporation: Definitions and underlying mechanisms. Advanced Drug Delivery Reviews. 2014;**72**:49-64. DOI: 10.1016/j.addr.2013.11.008

[8] Yu H, Xu L. Cell experimental studies on sonoporation: State of the art and remaining problems. Journal of Controlled Release. 2014;**174**:151-160. DOI: 10.1016/j.jconrel.2013.11.010

[9] Park J, Fan Z, Deng CX. Effects of shear stress cultivation on cell membrane disruption and intracellular calcium concentration in sonoporation of endothelial cells. Journal of Biomechanics. 2011;**44**:164-169. DOI: 10.1016/j.jbiomech.2010.09.003

[10] Karshafian R, Bevan PD, Williams R, Samac S, Burns PN. Sonoporation by ultrasound-activated microbubble contrast agents: Effect of acoustic exposure parameters on cell membrane permeability and cell viability. Ultrasound in Medicine & Biology. 2009;**35**:847-860. DOI: 10.1016/j.ultrasmedbio.2008.10.013

[11] Mayer CR, Geis NA, Katus HA, Bekeredjian R. Ultrasound targeted microbubble destruction for drug and gene delivery. Expert Opinion on Drug Delivery. 2008;**5**:1121-1138. DOI: 10.1517/17425247.5.10.1121

[12] Suzuki R, Oda Y, Utoguchi N, Maruyama K. Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles. Journal of Controlled Release. 2011;**149**:36-41. DOI: 10.1016/j.jconrel.2010.05.009

[13] Fan Z, Liu H, Mayer M, Deng CX. Spatiotemporally controlled single cell sonoporation. Proceedings of the National Academy of Sciences of the United States of America. 2012;**109**:16486-16491. DOI: 10.1073/pnas.1208198109

[14] Qin P, Xu L, Han T, Du L, Yu AC. Effect of non-acoustic parameters on heterogeneous sonoporation mediated by single-pulse ultrasound and microbubbles. Ultrasonics Sonochemistry. 2016;**31**:107-115. DOI: 10.1016/j.ultsonch.2015.12.001

[15] De Cock I, Zagato E, Braeckmans K, Luan Y, de Jong N, et al. Ultrasound and microbubble mediated drug delivery: Acoustic pressure as determinant

for uptake via membrane pores or endocytosis. Journal of Controlled Release. 2015;**197**:20-28. DOI: 10.1016/j.jconrel.2014.10.031

[16] Qiu Y, Zhang C, Tu J, Zhang D. Microbubble-induced sonoporation involved in ultrasound-mediated DNA transfection in vitro at low acoustic pressures. Journal of Biomechanics. 2012;**45**:1339-1345. DOI: 10.1016/j.jbiomech.2012.03.011

[17] Miller DL, Song J. Tumor growth reduction and DNA transfer by cavitation-enhanced high-intensity focused ultrasound in vivo. Ultrasound in Medicine & Biology. 2003;**29**:887-893

[18] Hwang JH, Brayman AA, Reidy MA, Matula TJ, Kimmey MB, Crum LA. Vascular effects induced by combined 1-MHz ultrasound and microbubble contrast agent treatments in vivo. Ultrasound in Medicine & Biology. 2005;**31**:553-564. DOI: 10.1016/j.ultrasmedbio.2004.12.014

[19] Vancraeynest D, Havaux X, Pasquet A, Gerber B, Beauloye C, et al. Myocardial injury induced by ultrasound-targeted microbubble destruction: Evidence for the contribution of myocardial ischemia. Ultrasound in Medicine & Biology. 2009;**35**:672-679. DOI: 10.1016/j.ultrasmedbio.2008.10.005

[20] Davies PF. Flow-mediated endothelial mechanotransduction. Physiological Reviews. 1995;**75**:519-560. DOI: 10.1152/physrev.1995.75.3.519

[21] Mizrahi N, Zhou EH, Lenormand G, Krishnan R, Weihs D, et al. Low intensity ultrasound perturbs cytoskeleton dynamics. Soft Matter. 2012;**8**:2438-2443. DOI: 10.1039/C2SM07246G

[22] Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, et al. The use of inhibitors to study endocytic pathways of gene carriers: Optimization and pitfalls. Molecular Therapy. 2010;**18**:561-569. DOI: 10.1038/mt.2009.281

[23] Lariccia V, Fine M, Magi S, Lin MJ, Yaradanakul A, et al. Massive calcium-activated endocytosis without involvement of classical endocytic proteins. The Journal of General Physiology. 2011;**137**:111-132. DOI: 10.1085/jgp.201010468

[24] Lionetti V, Fittipaldi A, Agostini S, Giacca M, Recchia FA, Picano E. Enhanced caveolae-mediated endocytosis by diagnostic ultrasound in vitro. Ultrasound in Medicine & Biology. 2009;**35**:136-143. DOI: 10.1016/j.ultrasmedbio.2008.07.011

[25] Meijering BD, Juffermans LJ, van Wamel A, Henning RH, Zuhorn IS, et al. Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation. Circulation Research. 2009;**104**:679-687. DOI: 10.1161/CIRCRESAHA.108.183806

[26] De Cock I, Lajoinie G, Versluis M, De Smedt SC, Lentacker I. Sonoprinting and the importance of microbubble loading for the ultrasound mediated cellular delivery of nanoparticles. Biomaterials. 2016;**83**:294-307. DOI: 10.1016/j.biomaterials.2016.01.022

[27] Zolochevska O, Xia X, Williams BJ, Ramsay A, Li S, Figueiredo ML. Sonoporation delivery of interleukin-27 gene therapy efficiently reduces prostate tumor cell growth in vivo. Human Gene Therapy. 2011;**22**:1537-1550. DOI: 10.1089/hum.2011.076

[28] Chen HY, Zhong X, Huang XR, Meng XM, You Y, et al. MicroRNA-29b inhibits diabetic nephropathy in db/db mice. Molecular Therapy. 2014;**22**:842-853. DOI: 10.1038/mt.2013.235

[29] Zhong X, Chung AC, Chen HY, Dong Y, Meng XM, et al. miR-21 is a key therapeutic target for renal injury in a mouse model of type 2 diabetes. Diabetologia. 2013;**56**:663-674. DOI: 10.1007/s00125-012-2804-x

[30] Duvshani-Eshet M, Adam D, Machluf M. The effects of albumin-coated microbubbles in DNA delivery mediated by therapeutic ultrasound. Journal of Controlled Release. 2006;**112**:156-166. DOI: 10.1016/j.jconrel.2006.02.013

[31] Wang X, Liang HD, Dong B, Lu QL, Blomley MJ. Gene transfer with microbubble ultrasound and plasmid DNA into skeletal muscle of mice: Comparison between commercially available microbubble contrast agents. Radiology. 2005;**237**:224-229. DOI: 10.1148/radiol.2371040805

[32] Tlaxca JL, Anderson CR, Klibanov AL, Lowrey B, Hossack JA, et al. Analysis of in vitro transfection by sonoporation using cationic and neutral microbubbles. Ultrasound in Medicine & Biology. 2010;**36**:1907-1918. DOI: 10.1016/j.ultrasmedbio.2010.05.014

[33] Sun L, Huang CW, Wu J, Chen KJ, Li SH, et al. The use of cationic microbubbles to improve ultrasound-targeted gene delivery to the ischemic myocardium. Biomaterials. 2013;**34**:2107-2116. DOI: 10.1016/j.biomaterials.2012.11.041

[34] Wang DS, Panje C, Pysz MA, Paulmurugan R, Rosenberg J, et al. Cationic versus neutral microbubbles for ultrasound-mediated gene delivery in cancer. Radiology. 2012;**264**:721-732. DOI: 10.1148/radiol.12112368

[35] Panje CM, Wang DS, Pysz MA, Paulmurugan R, Ren Y, et al. Ultrasound-mediated gene delivery with cationic versus neutral microbubbles: Effect of DNA and microbubble dose on in vivo transfection efficiency. Theranostics. 2012;**2**:1078-1091. DOI: 10.7150/thno.4240

[36] Negishi Y, Endo-Takahashi Y, Matsuki Y, Kato Y, Takagi N, et al. Systemic delivery systems of angiogenic gene by novel bubble liposomes containing cationic lipid and ultrasound exposure. Molecular Pharmaceutics. 2012;**9**:1834-1840. DOI: 10.1021/mp200554c

[37] Zhang L, Sun Z, Ren P, You M, Zhang J, et al. Localized delivery of shRNA against PHD2 protects the heart from acute myocardial infarction through ultrasound-targeted cationic microbubble destruction. Theranostics. 2017;**7**:51-66. DOI: 10.7150/thno.16074

[38] Wei S, Xu C, Rychak JJ, Luong A, Sun Y, et al. Short hairpin RNA knockdown of connective tissue growth factor by ultrasound-targeted microbubble destruction improves renal fibrosis. Ultrasound in Medicine & Biology. 2016;**42**:2926-2937. DOI: 10.1016/j.ultrasmedbio.2016.07.022

[39] Guvener N, Appold L, de Lorenzi F, Golombek SK, Rizzo LY, et al. Recent advances in ultrasound-based diagnosis and therapy with micro- and nanometer-sized formulations. Methods. 2017;**130**:4-13. DOI: 10.1016/j.ymeth.2017.05.018

[40] Cavalli R, Bisazza A, Trotta M, Argenziano M, Civra A, et al. New chitosan nanobubbles for ultrasound-mediated gene delivery: Preparation and in vitro characterization. International Journal of Nanomedicine. 2012;**7**:3309-3318. DOI: 10.2147/IJN.S30912

[41] Zhang B, Chen M, Zhang Y, Chen W, Zhang L, Chen L. An ultrasonic nanobubble-mediated PNP/fludarabine suicide gene system: A new approach for the treatment of hepatocellular carcinoma. PLoS One. 2018;**13**:e196686. DOI: 10.1371/journal.pone.0196686

[42] Horie S, Watanabe Y, Ono M, Mori S, Kodama T. Evaluation of antitumor effects following tumor necrosis factor-alpha gene delivery using nanobubbles and ultrasound. Cancer Science. 2011;**102**:2082-2089. DOI: 10.1111/j.1349-7006.2011.02056.x

[43] Wu M, Zhao H, Guo L, Wang Y, Song J, et al. Ultrasound-mediated nanobubble destruction (UMND) facilitates the delivery of A10-3.2 aptamer targeted and siRNA-loaded cationic nanobubbles for therapy of prostate cancer. Drug Delivery. 2018;**25**:226-240. DOI: 10.1080/10717544.2017.1422300

[44] Song Z, Ye Y, Zhang Z, Shen J, Hu Z, et al. Noninvasive, targeted gene therapy for acute spinal cord injury using LIFU-mediated BDNF-loaded cationic nanobubble destruction. Biochemical and Biophysical Research Communications. 2018;**496**:911-920. DOI: 10.1016/j.bbrc.2018.01.123

[45] Xie X, Lin W, Li M, Yang Y, Deng J, et al. Efficient siRNA delivery using novel cell-penetrating peptide-siRNA conjugate-loaded nanobubbles and ultrasound. Ultrasound in Medicine & Biology. 2016;**42**:1362-1374. DOI: 10.1016/j.ultrasmedbio.2016.01.017

[46] Yin T, Wang P, Li J, Zheng R, Zheng B, et al. Ultrasound-sensitive siRNA-loaded nanobubbles formed by hetero-assembly of polymeric micelles and liposomes and their therapeutic effect in gliomas. Biomaterials. 2013;**34**:4532-4543. DOI: 10.1016/j.biomaterials.2013.02.067

[47] Xie X, Yang Y, Lin W, Liu H, Liu H, et al. Cell-penetrating peptide-siRNA conjugate loaded YSA-modified nanobubbles for ultrasound triggered siRNA delivery. Colloids and Surfaces. B, Biointerfaces. 2015;**136**:641-650. DOI: 10.1016/j.colsurfb.2015.10.004

[48] Gao D, Gao J, Xu M, Cao Z, Zhou L, et al. Targeted ultrasound-triggered phase transition nanodroplets for Her2-overexpressing breast cancer diagnosis and gene transfection. Molecular Pharmaceutics. 2017;**14**:984-998. DOI: 10.1021/acs.molpharmaceut.6b00761

[49] Gao D, Xu M, Cao Z, Gao J, Chen Y, et al. Ultrasound-triggered phase-transition cationic nanodroplets for enhanced gene delivery. ACS Applied Materials & Interfaces. 2015;**7**:13524-13537. DOI: 10.1021/acsami.5b02832

[50] Ho YJ, Chang YC, Yeh CK. Improving nanoparticle penetration in tumors by vascular disruption with acoustic droplet vaporization. Theranostics. 2016;**6**:392-403. DOI: 10.7150/thno.13727

[51] Bai WK, Zhang W, Hu B, Ying T. Liposome-mediated transfection of wild-type P53 DNA into human prostate cancer cells is improved by low-frequency ultrasound combined with microbubbles. Oncology Letters. 2016;**11**:3829-3834. DOI: 10.3892/ol.2016.4477

[52] Sun QF, Du F, Xu Y, Yang PS. Ultrasound-mediated microbubble destruction enhances bone morphogenetic protein-2 gene expression in mouse skeletal muscles. Hua Xi Kou Qiang Yi Xue Za Zhi. 2009;**27**:84-87

[53] Yoon YI, Kwon YS, Cho HS, Heo SH, Park KS, et al. Ultrasound-mediated gene and drug delivery using a microbubble-liposome particle system. Theranostics. 2014;**4**:1133-1144. DOI: 10.7150/thno.9945

[54] Chertok B, Langer R, Anderson DG. Spatial control of gene expression by nanocarriers using heparin masking and ultrasound-targeted microbubble destruction. ACS Nano. 2016;**10**:7267-7278. DOI: 10.1021/acsnano.6b01199

[55] Nel AE, Madler L, Velegol D, Xia T, Hoek EM, et al. Understanding biophysicochemical interactions at the nano-bio interface. Nature Materials. 2009;**8**:543-557. DOI: 10.1038/nmat2442

[56] Wang YJ, Zhou Q, Cao S, Hu B, Deng Q, et al. Efficient gene therapy with a combination of ultrasoundtargeted microbubble destruction and PEI/DNA/NLS complexes. Molecular Medicine Reports. 2017;**16**:7685-7691. DOI: 10.3892/mmr.2017.7510

[57] Arami S, Mahdavi M, Rashidi MR, Yekta R, Rahnamay M, et al. Apoptosis induction activity and molecular docking studies of survivin siRNA carried by Fe_3O_4-PEG-LAC-chitosan-PEI nanoparticles in MCF-7 human breast cancer cells. Journal of Pharmaceutical and Biomedical Analysis. 2017;**142**:145-154. DOI: 10.1016/j.jpba.2017.04.025

[58] Dang SP, Wang RX, Qin MD, Zhang Y, Gu YZ, et al. A novel transfection method for eukaryotic cells using polyethylenimine coated albumin microbubbles. Plasmid. 2011;**66**:19-25. DOI: 10.1016/j.plasmid.2011.03.003

[59] Deshpande MC, Prausnitz MR. Synergistic effect of ultrasound and PEI on DNA transfection in vitro. Journal of Controlled Release. 2007;**118**:126-135. DOI: 10.1016/j.jconrel.2006.12.010

[60] Park DH, Jung BK, Lee YS, Jang JY, Kim MK, et al. Evaluation of in vivo antitumor effects of ANT2 shRNA delivered using PEI and ultrasound with microbubbles. Gene Therapy. 2015;**22**:325-332. DOI: 10.1038/gt.2014.120

[61] Nande R, Greco A, Gossman MS, Lopez JP, Claudio L, et al. Microbubble-assisted p53, RB, and p130 gene transfer in combination with radiation therapy in prostate cancer. Current Gene Therapy. 2013;**13**:163-174

[62] Chang S, Guo J, Sun J, Zhu S, Yan Y, et al. Targeted microbubbles for ultrasound mediated gene transfection and apoptosis induction in ovarian cancer cells. Ultrasonics Sonochemistry. 2013;**20**:171-179. DOI: 10.1016/j.ultsonch.2012.06.015

[63] Mishel S, Shneyer B, Korsensky L, Goldshmidt-Tran O, Haber T, et al. Delivery of the gene encoding the tumor suppressor Sef into prostate tumors by therapeutic-ultrasound inhibits both tumor angiogenesis and growth. Scientific Reports. 2017;**7**:15060. DOI: 10.1038/s41598-017-12408-1

[64] Zhang J, Kale V, Chen M. Gene-directed enzyme prodrug therapy. The AAPS Journal. 2015;**17**:102-110. DOI: 10.1208/s12248-014-9675-7

[65] Devulapally R, Lee T, Barghava-Shah A, Sekar TV, Foygel K, et al. Ultrasound-guided delivery of thymidine kinase-nitroreductase dual therapeutic genes by PEGylated-PLGA/PIE nanoparticles for enhanced triple negative breast cancer therapy. Nanomedicine (London, England). 2018;**13**:1051-1066. DOI: 10.2217/nnm-2017-0328

[66] Bae YJ, Yoon YI, Yoon TJ, Lee HJ. Ultrasound-guided delivery of siRNA and a chemotherapeutic drug by using microbubble complexes: In vitro and in vivo evaluations in a prostate cancer model. Korean Journal of Radiology. 2016;**17**:497-508. DOI: 10.3348/kjr.2016.17.4.497

[67] Yin T, Wang P, Li J, Wang Y, Zheng B, et al. Tumor-penetrating codelivery of siRNA and paclitaxel with ultrasound-responsive nanobubbles hetero-assembled from polymeric micelles and liposomes. Biomaterials. 2014;**35**:5932-5943. DOI: 10.1016/j.biomaterials.2014.03.072

[68] Wang TY, Choe JW, Pu K, Devulapally R, Bachawal S, et al.

Ultrasound-guided delivery of microRNA loaded nanoparticles into cancer. Journal of Controlled Release. 2015;**203**:99-108. DOI: 10.1016/j.jconrel.2015.02.018

[69] Florinas S, Kim J, Nam K, Janat-Amsbury MM, Kim SW. Ultrasound-assisted siRNA delivery via arginine-grafted bioreducible polymer and microbubbles targeting VEGF for ovarian cancer treatment. Journal of Controlled Release. 2014;**183**:1-8. DOI: 10.1016/j.jconrel.2014.03.025

[70] Zhao R, Liang X, Zhao B, Chen M, Liu R, et al. Ultrasound assisted gene and photodynamic synergistic therapy with multifunctional FOXA1-siRNA loaded porphyrin microbubbles for enhancing therapeutic efficacy for breast cancer. Biomaterials. 2018;**173**:58-70. DOI: 10.1016/j.biomaterials.2018.04.054

[71] Liu YM, Li XF, Liu H, Wu XL. Ultrasound-targeted microbubble destruction-mediated downregulation of CD133 inhibits epithelial-mesenchymal transition, stemness and migratory ability of liver cancer stem cells. Oncology Reports. 2015;**34**: 2977-2986. DOI: 10.3892/or.2015.4270

[72] Zhang M, Yu WZ, Shen XT, Xiang Q, Xu J, et al. Advanced interfere treatment of diabetic cardiomyopathy rats by aFGF-loaded heparin-modified microbubbles and UTMD technique. Cardiovascular Drugs and Therapy. 2016;**30**:247-261. DOI: 10.1007/s10557-016-6639-4

[73] Zhao YZ, Tian XQ, Zhang M, Cai L, Ru A, et al. Functional and pathological improvements of the hearts in diabetes model by the combined therapy of bFGF-loaded nanoparticles with ultrasound-targeted microbubble destruction. Journal of Controlled Release. 2014;**186**:22-31. DOI: 10.1016/j.jconrel.2014.04.054

[74] Du GQ, Shao ZB, Wu J, Yin WJ, Li SH, et al. Targeted myocardial delivery of GDF11 gene rejuvenates the aged mouse heart and enhances myocardial regeneration after ischemia-reperfusion injury. Basic Research in Cardiology. 2017;**112**:7. DOI: 10.1007/s00395-016-0593-y

[75] Castle JW, Kent KP, Fan Y, Wallace KD, Davis CE, et al. Therapeutic ultrasound: Increased HDL-cholesterol following infusions of acoustic microspheres and apolipoprotein A-I plasmids. Atherosclerosis. 2015;**241**:92-99. DOI: 10.1016/j.atherosclerosis.2015.04.817

[76] Lee PJ, Rudenko D, Kuliszewski MA, Liao C, Kabir MG, et al. Survivin gene therapy attenuates left ventricular systolic dysfunction in doxorubicin cardiomyopathy by reducing apoptosis and fibrosis. Cardiovascular Research. 2014;**101**:423-433. DOI: 10.1093/cvr/cvu001

[77] Patel MM, Patel BM. Crossing the blood-brain barrier: Recent advances in drug delivery to the brain. CNS Drugs. 2017;**31**:109-133. DOI: 10.1007/s40263-016-0405-9

[78] Timbie KF, Mead BP, Price RJ. Drug and gene delivery across the blood-brain barrier with focused ultrasound. Journal of Controlled Release. 2015;**219**:61-75. DOI: 10.1016/j.jconrel.2015.08.059

[79] Ikeda M, Bhattacharjee AK, Kondoh T, Nagashima T, Tamaki N. Synergistic effect of cold mannitol and Na(+)/Ca(2+) exchange blocker on blood-brain barrier opening. Biochemical and Biophysical Research Communications. 2002;**291**:669-674. DOI: 10.1006/bbrc.2002.6495

[80] Lee HJ, Zhang Y, Pardridge WM. Blood-brain barrier disruption following the internal carotid arterial perfusion of alkyl glycerols. Journal of

Drug Targeting. 2002;**10**:463-467. DOI: 10.1080/1061186021000038337

[81] Hynynen K, McDannold N, Vykhodtseva N, Jolesz FA. Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits. Radiology. 2001;**220**:640-646. DOI: 10.1148/radiol.2202001804

[82] Huang Q, Deng J, Wang F, Chen S, Liu Y, et al. Targeted gene delivery to the mouse brain by MRI-guided focused ultrasound-induced blood-brain barrier disruption. Experimental Neurology. 2012;**233**:350-356. DOI: 10.1016/j.expneurol.2011.10.027

[83] Negishi Y, Yamane M, Kurihara N, Endo-Takahashi Y, Sashida S, et al. Enhancement of blood-brain barrier permeability and delivery of antisense oligonucleotides or plasmid DNA to the brain by the combination of bubble liposomes and high-intensity focused ultrasound. Pharmaceutics. 2015;**7**:344-362. DOI: 10.3390/pharmaceutics7030344

[84] Carpentier A, Canney M, Vignot A, Reina V, Beccaria K, et al. Clinical trial of blood-brain barrier disruption by pulsed ultrasound. Science Translational Medicine. 2016;**8**:342r-343r. DOI: 10.1126/scitranslmed.aaf6086

[85] Fan CH, Chang EL, Ting CY, Lin YC, Liao EC, et al. Folate-conjugated gene-carrying microbubbles with focused ultrasound for concurrent blood-brain barrier opening and local gene delivery. Biomaterials. 2016;**106**:46-57. DOI: 10.1016/j.biomaterials.2016.08.017

[86] Chang EL, Ting CY, Hsu PH, Lin YC, Liao EC, et al. Angiogenesis-targeting microbubbles combined with ultrasound-mediated gene therapy in brain tumors. Journal of Controlled Release. 2017;**255**:164-175. DOI: 10.1016/j.jconrel.2017.04.010

[87] Zhao G, Huang Q, Wang F, Zhang X, Hu J, et al. Targeted shRNA-loaded liposome complex combined with focused ultrasound for blood brain barrier disruption and suppressing glioma growth. Cancer Letters. 2018;**418**:147-158. DOI: 10.1016/j.canlet.2018.01.035

[88] Fan CH, Lin CY, Liu HL, Yeh CK. Ultrasound targeted CNS gene delivery for Parkinson's disease treatment. Journal of Controlled Release. 2017;**261**:246-262. DOI: 10.1016/j.jconrel.2017.07.004

[89] Fan CH, Ting CY, Lin CY, Chan HL, Chang YC, et al. Noninvasive, targeted, and non-viral ultrasound-mediated GDNF-plasmid delivery for treatment of Parkinson's disease. Scientific Reports. 2016;**6**:19579. DOI: 10.1038/srep19579

[90] Lin CY, Hsieh HY, Chen CM, Wu SR, Tsai CH, et al. Non-invasive, neuron-specific gene therapy by focused ultrasound-induced blood-brain barrier opening in Parkinson's disease mouse model. Journal of Controlled Release. 2016;**235**:72-81. DOI: 10.1016/j.jconrel.2016.05.052

[91] Lin CY, Hsieh HY, Pitt WG, Huang CY, Tseng IC, et al. Focused ultrasound-induced blood-brain barrier opening for non-viral, non-invasive, and targeted gene delivery. Journal of Controlled Release. 2015;**212**:1-9. DOI: 10.1016/j.jconrel.2015.06.010

[92] Song Z, Wang Z, Shen J, Xu S, Hu Z. Nerve growth factor delivery by ultrasound-mediated nanobubble destruction as a treatment for acute spinal cord injury in rats. International Journal of Nanomedicine. 2017;**12**:1717-1729. DOI: 10.2147/IJN.S128848

[93] Wang HB, Yang L, Wu J, Sun L, Wu J, et al. Reduced ischemic injury

after stroke in mice by angiogenic gene delivery via ultrasound-targeted microbubble destruction. Journal of Neuropathology and Experimental Neurology. 2014;**73**:548-558. DOI: 10.1097/NEN.0000000000000077

[94] Xiang X, Tang Y, Leng Q, Zhang L, Qiu L. Targeted gene delivery to the synovial pannus in antigen-induced arthritis by ultrasound-targeted microbubble destruction in vivo. Ultrasonics. 2016;**65**:304-314. DOI: 10.1016/j.ultras.2015.09.011

[95] Inoue H, Arai Y, Kishida T, Shin-Ya M, Terauchi R, et al. Sonoporation-mediated transduction of siRNA ameliorated experimental arthritis using 3 MHz pulsed ultrasound. Ultrasonics. 2014;**54**:874-881. DOI: 10.1016/j.ultras.2013.10.021

[96] King GN, Hughes FJ. Bone morphogenetic protein-2 stimulates cell recruitment and cementogenesis during early wound healing. Journal of Clinical Periodontology. 2001;**28**:465-475

[97] Sun QF, Liu Y, Yang PS, Du F. Ultrasound-mediated microbubble destruction enhances exogenous gene expression in NIH3T3 cells in vitro. Hua Xi Kou Qiang Yi Xue Za Zhi. 2008;**26**:198-200, 205

[98] Osawa K, Okubo Y, Nakao K, Koyama N, Bessho K. Osteoinduction by microbubble-enhanced transcutaneous sonoporation of human bone morphogenetic protein-2. The Journal of Gene Medicine. 2009;**11**:633-641. DOI: 10.1002/jgm.1331

[99] Qiu L, Zhang L, Wang L, Jiang Y, Luo Y, et al. Ultrasound-targeted microbubble destruction enhances naked plasmid DNA transfection in rabbit Achilles tendons in vivo. Gene Therapy. 2012;**19**:703-710. DOI: 10.1038/gt.2011.165

[100] Delalande A, Bureau MF, Midoux P, Bouakaz A, Pichon C. Ultrasound-assisted microbubbles gene transfer in tendons for gene therapy. Ultrasonics. 2010;**50**:269-272. DOI: 10.1016/j.ultras.2009.09.035

[101] Delalande A, Bouakaz A, Renault G, Tabareau F, Kotopoulis S, et al. Ultrasound and microbubble-assisted gene delivery in Achilles tendons: Long lasting gene expression and restoration of fibromodulin KO phenotype. Journal of Controlled Release. 2011;**156**:223-230. DOI: 10.1016/j.jconrel.2011.08.020

[102] Tang Y, Leng Q, Xiang X, Zhang L, Yang Y, Qiu L. Use of ultrasound-targeted microbubble destruction to transfect IGF-1 cDNA to enhance the regeneration of rat wounded Achilles tendon in vivo. Gene Therapy. 2015;**22**:610-618. DOI: 10.1038/gt.2015.32

[103] Bez M, Kremen TJ, Tawackoli W, Avalos P, Sheyn D, et al. Ultrasound-mediated gene delivery enhances tendon allograft integration in mini-pig ligament reconstruction. Molecular Therapy. 2018;**26**:1746-1755. DOI: 10.1016/j.ymthe.2018.04.020

[104] Sonoda S, Tachibana K, Uchino E, Okubo A, Yamamoto M, et al. Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. Investigative Ophthalmology & Visual Science. 2006;**47**:558-564. DOI: 10.1167/iovs.05-0889

[105] Yamashita T, Sonoda S, Suzuki R, Arimura N, Tachibana K, et al. A novel bubble liposome and ultrasound-mediated gene transfer to ocular surface: RC-1 cells in vitro and conjunctiva in vivo. Experimental Eye Research. 2007;**85**:741-748. DOI: 10.1016/j.exer.2007.08.006

[106] Park J, Zhang Y, Vykhodtseva N, Akula JD, McDannold NJ. Targeted and reversible blood-retinal barrier disruption via focused ultrasound and microbubbles. PLoS One. 2012;**7**: e42754. DOI: 10.1371/journal.pone.0042754

[107] Li HL, Zheng XZ, Wang HP, Li F, Wu Y, Du LF. Ultrasound-targeted microbubble destruction enhances AAV-mediated gene transfection in human RPE cells in vitro and rat retina in vivo. Gene Therapy. 2009;**16**:1146-1153. DOI: 10.1038/gt.2009.84

[108] Wan C, Qian J, Li F, Li H. Ultrasound-targeted microbubble destruction enhances polyethylenimine-mediated gene transfection in vitro in human retinal pigment epithelial cells and in vivo in rat retina. Molecular Medicine Reports. 2015;**12**:2835-2841. DOI: 10.3892/mmr.2015.3703

[109] Li H, Qian J, Yao C, Wan C, Li F. Combined ultrasound-targeted microbubble destruction and polyethylenimine-mediated plasmid DNA delivery to the rat retina: Enhanced efficiency and accelerated expression. The Journal of Gene Medicine. 2016;**18**:47-56. DOI: 10.1002/jgm.2875

[110] Xu Y, Xie Z, Zhou Y, Zhou X, Li P, et al. Experimental endostatin-GFP gene transfection into human retinal vascular endothelial cells using ultrasound-targeted cationic microbubble destruction. Molecular Vision. 2015;**21**:930-938

[111] Luo J, Zhou X, Diao L, Wang Z. Experimental research on wild-type p53 plasmid transfected into retinoblastoma cells and tissues using an ultrasound microbubble intensifier. The Journal of International Medical Research. 2010;**38**:1005-1015. DOI: 10.1177/147323001003800327

[112] Gao R, Zhou X, Yang Y, Wang Z. Transfection of wtp53 and Rb94 genes into retinoblastomas of nude mice by ultrasound-targeted microbubble destruction. Ultrasound in Medicine & Biology. 2014;**40**:2662-2670. DOI: 10.1016/j.ultrasmedbio.2014.05.012

[113] Bai YJ, Huang LZ, Zhou AY, Zhao M, Yu WZ, Li XX. Antiangiogenesis effects of endostatin in retinal neovascularization. Journal of Ocular Pharmacology and Therapeutics. 2013;**29**:619-626. DOI: 10.1089/jop.2012.0225

[114] Zhang Y, Ye C, Xu Y, Dong X, Li J, et al. Ultrasound-mediated microbubble destruction increases renal interstitial capillary permeability in early diabetic nephropathy rats. Ultrasound in Medicine & Biology. 2014;**40**:1273-1281. DOI: 10.1016/j.ultrasmedbio.2013.12.006

[115] Lan HY, Mu W, Tomita N, Huang XR, Li JH, et al. Inhibition of renal fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model. Journal of the American Society of Nephrology. 2003;**14**:1535-1548

[116] Chen HY, Huang XR, Wang W, Li JH, Heuchel RL, et al. The protective role of Smad7 in diabetic kidney disease: Mechanism and therapeutic potential. Diabetes. 2011;**60**:590-601. DOI: 10.2337/db10-0403

[117] Ka SM, Yeh YC, Huang XR, Chao TK, Hung YJ, et al. Kidney-targeting Smad7 gene transfer inhibits renal TGF-beta/MAD homologue (SMAD) and nuclear factor kappaB (NF-kappaB) signalling pathways, and improves diabetic nephropathy in mice. Diabetologia. 2012;**55**:509-519. DOI: 10.1007/s00125-011-2364-5

[118] Liu GX, Li YQ, Huang XR, Wei LH, Zhang Y, et al. Smad7 inhibits

AngII-mediated hypertensive nephropathy in a mouse model of hypertension. Clinical Science (London, England). 2014;**127**:195-208. DOI: 10.1042/CS20130706

[119] Dube N, Kooistra MR, Pannekoek WJ, Vliem MJ, Oorschot V, et al. The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions. Cellular Signalling. 2008;**20**:1608-1615. DOI: 10.1016/j.cellsig.2008.05.006

[120] Xiao L, Zhu X, Yang S, Liu F, Zhou Z, et al. Rap1 ameliorates renal tubular injury in diabetic nephropathy. Diabetes. 2014;**63**:1366-1380. DOI: 10.2337/db13-1412

[121] Yiu WH, Wong DW, Wu HJ, Li RX, Yam I, Chan LY, Leung JC, Lan HY, Lai KN, Tang SC. Kallistatin protects against diabetic nephropathy in db/db mice by suppressing AGE-RAGE-induced oxidative stress. Kidney International. 2016;**89**:386-398. DOI: 10.1038/ki.2015.331

[122] Du B, Ma LM, Huang MB, Zhou H, Huang HL, et al. High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells. FEBS Letters. 2010;**584**:811-816. DOI: 10.1016/j.febslet.2009.12.053

[123] Roderburg C, Urban GW, Bettermann K, Vucur M, Zimmermann H, et al. Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. Hepatology. 2011;**53**: 209-218. DOI: 10.1002/hep.23922

[124] Miller DL. Overview of experimental studies of biological effects of medical ultrasound caused by gas body activation and inertial cavitation. Progress in Biophysics and Molecular Biology. 2007;**93**:314-330. DOI: 10.1016/j.pbiomolbio.2006.07.027