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Advances in sonoporation strategies for cancer

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1. ABSTRACT

We will focus on the therapeutic applications of ultrasound (US) for gene transfection or 'sonoporation'. Sonoporation therapy or 'sonotherapy' is an emerging physical method for delivering drugs and/or nucleic acids for treating cancer. Because of its non-invasive nature, sonotherapy has the potential to be competitive with other treatment delivery methods such as viruses or lipofection. For nucleic acid delivery, sonoporation in the presence of microbubbles (MB) significantly enhances transfection efficiency. Sonoporation is an ideal means of delivering pDNA, and it has a similar efficiency as electroporation or other physical gene therapy techniques, with potentially fewer side effects. Typically, nonphysical means of gene delivery have suffered from lower efficiencies compared to viral vectors, however, several studies suggest that sonoporation pDNA delivery could be a simple and inexpensive method that only requires a plasmid, MB, and a sonoporation device. Sonoporation could also be used to target MB to certain cells/tissues, delivering localized therapies. Using high-performance probes, more precise and safer sonoporation treatments will be developed, and newer therapeutic prospects will be realized.

2. INTRODUCTION

Therapeutic applications of ultrasound (US) predate its use in imaging. A range of biological effects can be induced by US, depending on the exposure levels used. At low levels, beneficial, reversible cellular effects may be produced, whereas at high intensities instantaneous cell death is sought. Therapeutic US can therefore be divided into "low" and "high" frequency applications. Low frequency applications include physiotherapy, fracture repair, sonophoresis, and sonoporation for drug or gene delivery, while the most common application of high frequency methods involves high intensity focused US. While useful therapeutic effects are now being demonstrated clinically, the mechanisms by which they occur are often not well understood (1). Therapeutic effects are obtained by both thermal and non-thermal interaction mechanisms. For example, at low power, acoustic streaming is likely to be significant, but at higher power, heating and acoustic cavitation mechanisms likely predominate such as shock waves (Figure 1A). Acoustic streaming is the movement of fluid due to an US wave. In clinical US, it is seen as movement of particulate matter within fluid during B-mode and Doppler examinations. The

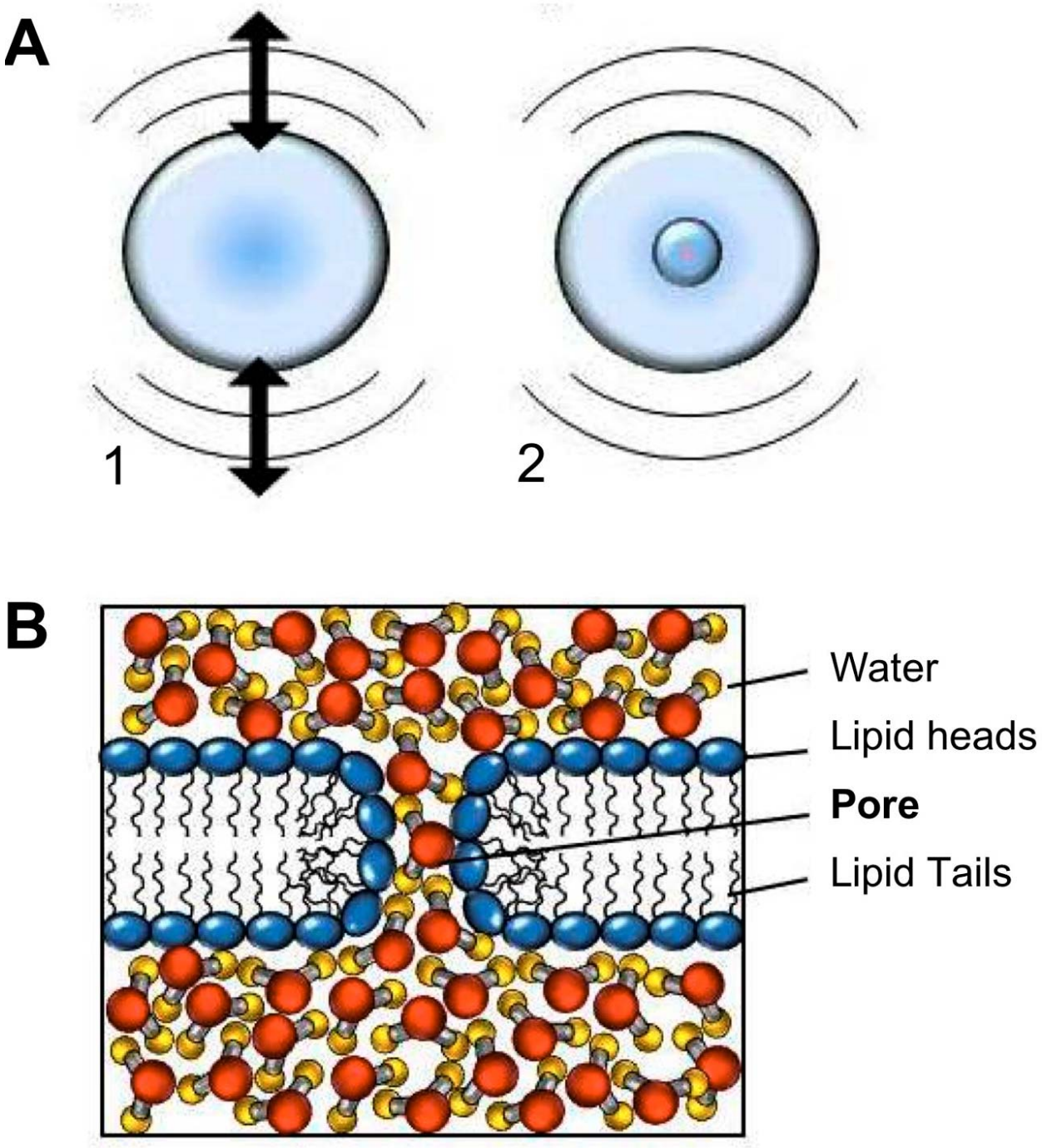

Figure 1. Sonoporation. a. Sonoporation mechanisms. (1), the acoustic streaming mechanism. MB can provoke cavitation by oscillating around their resonant size and generating velocities that induce shear stresses on cells and tissues. This mechanism is implicated in gene and drug delivery. (2), the shock waves mechanism. The sudden collapse of MB can lead to the formation of shock waves that are capable of causing tissue disruption and enhancing drug transport by sonoporation. b. Stable pore formation in lipid bilayer due to sonoporation. A diagram shows the formation of a stable pore structure in a phospholipids bilayer due to sonoporation. During sonoporation, the waves of US disturb the self-assembled phospholipids in a collective manner and provide sufficient energy for the surrounding water molecules to enter into the bilayer's hydrophobic core. The water molecules self-assemble into a cluster and push the phospholipids outwards and distort the bilayer structure. Depicted are phospholipids reorganizing around the cluster to mediate the opening of water-filled pores in the cell membrane.

Table 1. Commercially-available microbubbles for use in Sonoporation

	Mean diameter (μM)	Shell Composition	Surface Charge	Gas Type	References
BR14	2-6	Phospholipid	Neutral	C4F10	(9, 10, 31)
Definity	1-5	DPPC/DPPA/MPEG$_{5000}$/DPPE	Negative	C3F8	(46)
Optison	3-5	Human serum albumin	Negative	C3F8	(9, 10, 42, 63)
MB101	2-7	Stabilized lipid	Neutral	C4F10	(39)
Sonovue	2-5	DSPC/DPPG/PA	Negative	SF6	(10, 22, 25, 38, 40, 44, 45, 48)
Targeson	2-5	Surface modified with ligand	Variable	C4F10	(47)

Abbreviations: BR14, Bracco microbubble 14; C4F10, perfluuorobutane; DPPC, dipalmitoyl phosphatidylcholine, DPPA, diphenyl phosphoryl azide, MPEG, methoxypoly (ethylene glycol); DPPE, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, C3F8, perfluoropropane gas;, MB101, microbubbles 101; DSPC, 1,2-distearoyl-sn-glycero-phosphatidylcholine, Dipalmitoylphosphatidylglycerol, PA, palmitic acid; SF6, sulfur hexafluoride

Table 2. Other Agents (liposomes, lipoplexes) used with Sonoporation

	Shell Composition (gas)	References
Liposomes	DOTMA:Cholesterol	(32)
Lipoplexes	Man-PEG DSPE-PEG$_{2000}$-maleimide	(33) (55)
Other Microbubbles	DPPC:DSPE:DPPA (C3F8) PVA-MB (air) DSPC:DSPE-PEG$_{2000}$-OMe DSEPC:DSPC:PEG$_{40}$ DSPC:DSPE-PEG$_{2000}$:DSPE-PEG$_{2000}$-biotin	(35) (50) (29) (34) (49, 54)
Nanobubbles	DSPC:DSPE-PEG (C3F8)	(56)

Abbreviations: DOTMA, N- (1- (2,3-dioleyloxy)propyl)-N,N,N-tri- methylammonium chloride; Man-PEG, mannose-PEG2000; PEG, poly (ethyleneglycol);, DSPE, distearoyl phosphatidyl ethanolamine, DPPC, dipalmitoyl phosphatidylcholine, DPPA, diphenyl phosphoryl azide, C3F8, perfluoropropane gas;, PVA, polyvinyl alcohol, MB, microbubbles, DSPC, 1,2-distearoyl-sn-glycero-phosphatidylcholine, DSPE-PEG$_{2000}$-OMe, 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine s-methoxypolyethyleneglycol, DSEPC, 1,2-distearoyl-sn-glycero-3-ethylphosphocholine.

movement occurs in the direction of the beam away from the transducer and is due to energy transfer from the US wave to the fluid (2).

This review will focus on the therapeutic potential of low frequency US for gene transfection or 'sonoporation'. Sonoporation is an emerging and promising physical method for cancer gene therapy. Sonoporation typically operates in the range of 35 KHz- 1 MHz and has several advantages over other non-physical means of nucleic acid delivery, including the ability to also deliver drugs or small molecules. To further improve the efficiency of sonoporation, promising studies include complexing DNA, siRNA, small molecules, or viral vectors with lipid agents in different conformations. Lipid agents can be liposomes or, more commonly, gas-filled lipid molecules called microbubbles (MB). MB is gas–liquid emulsions consisting of a gaseous core surrounded by a shell and are usually 1 to 7 microns in size. In imaging applications, the MB shell prevents both gas leakage and aggregation of the MB. Different types of contrast MB have been synthesized by combining different shell compositions such as albumin, galactose, lipids, or polymers, with different gaseous cores such as air, or high molecular weight gases (perfluorocarbon, sulphur hexafluoride or nitrogen) and several types of MB are available commercially (Table 1). Heavy gases are less water-soluble and, thus, dissolve into the surrounding solution at a much slower rate, than for example, air. This prolongs the effective lifetime of MB. Other MB-like agents promising for gene and drug delivery also are reviewed herein and listed in Table 2.

MB have traditionally been developed as contrast agents used to enhance US imaging in the clinic (3), however, MB now play an increasingly significant role in both diagnostic and therapeutic applications of US. In drug delivery and gene therapy, MB are being developed as vehicles which can be loaded with the required therapeutic agent, traced to the target site using diagnostic US, and then dissociated with US of higher intensity to release the material locally, thus avoiding side effects associated with systemic administration. Additionally, it has been shown that the motion of MB disruption increases the permeability of cell membranes and the endothelium, thus enhancing therapeutic uptake, and can locally increase drug/nucleic acid transport across biologically inaccessible interfaces such as solid tumors (4). For nucleic acid delivery, sonoporation in the presence of MB has been shown to increase plasmid transfection efficiency *in vitro* by several orders of magnitude. Formation of short-lived pores in the plasma membrane, up to ~100 nm in diameter lasts a few seconds, and is implicated as the dominant mechanism associated with acoustic cavitation (5). Acoustic cavitation, enhanced by MB, perturbs cell membrane structures to cause sonoporation and increases the permeability to bioactive materials (Figure 1B). Drugs and nucleic acids can be incorporated into or on the surface of MB, and these complexes can circulate in the blood and retain their cargo until they are in the presence of a US stimulus. The effects of US+MB typically correlate to the cell location relative to the energy source (transducer) or their proximity to acoustically active MB. US-mediated gene delivery has been applied successfully to heart, blood vessels, lung, kidney, muscle, brain, and tumors with high gene transfection efficiency. However, transfection efficiency may depend on careful choice of ultrasonic parameters such as acoustic pressure, pulse length, duty cycle, repetition rate, and exposure duration, as well as MB

properties such as size, gas species, shell material, and surface rigidity (6). The drug delivery effect by US is highly related to the distance between the MB+drug/nucleic acid complex and the biological membrane. Kodama et al. (7) found that transient membrane permeability is induced within ~5 μm from the center of the MB, within which distance exogenous molecules may be delivered into the cytoplasm. It should be recognized also, however, that the transport of drugs/nucleic acids into the nucleus or other cellular compartments might depend on mechanisms independent of US effects. For example, addition of contrast agents increases pDNA uptake into the cytoplasm but not into the nucleus of cultured cells (8).

The type of MB or liposome chosen for sonoporation is highly relevant to obtaining efficient transfection. Several studies have compared different MB for their ability to deliver plasmid DNA (pDNA) or drugs. For example, three perfluorocarbon gas-containing MB, Optison, Sonovue, and BR14, were compared for their ability to deliver pCMV-luciferase (pLuc) to porcine heart cells in vitro (9). Interestingly, the lipid-stabilized microbubble, BR14 at 50%, gave >2.6x greater gene transfection than did Optison, perhaps due to size (since Optison MB can range up to 32m) or composition (since Optison is albumin-based). SonoVue and BR14 are examples of phospholipid-stabilized MB, which may be able to withstand higher or longer US pulses. For instance, when exposed to US pulses above the fragmentation threshold for Optison, lipid-shelled MB efficiently scatter incident pulses for >60s, while Optison is destroyed in ~5s (10). The mechanisms of ultrasonic destruction of MB are thought to occur via two mechanisms: acoustic dissolution at low acoustic pressure and fragmentation into two or more bubbles at high pressure (11). At ~1MHz US, the gas inside phospholipid shells steadily oscillates and lipid-shelled MB can expand from 2 μm to ~20-55 μm (12). When MB expand and collapse near a cell wall, for example, a fluid jet/shock wave is formed with an associated increase in vascular permeability (13). In this manner, drug or nucleic acid transport occurs by convection through a membrane pore (12).

In fact, the formation of pores on the cellular membrane following US exposure is thought to be the main mechanism for sonoporation-mediated gene or drug delivery. Many reports have supported this possibility and observed that cellular deformation correlates with the uptake of dyes, and membrane changes as assessed by scanning electron microscopy, membrane electrophysiology, and atomic force microscopy (8, 14, 15). Pores are thought to arise from shear stress placed on the membrane by 'acoustic streaming' or unidirectional flow currents within a fluid that are due to the presence of sound waves. Following pore formation, non-specific uptake of extracellular molecules can occur, the membrane is repaired, and molecules are therefore retained within cells. Mammalian cells have been shown to repair pores of up to ~1000 μm^2 within a short period (16), in a manner resembling the kinetics of membrane repair after mechanical wounding. During membrane repair, the pore size dictates the mechanism of resealing, and Ca^{2+} levels are thought to promote this response (17, 18). Small pores might be sealed passively, an inherent quality of lipid bilayers that is favored energetically, whereas the sealing of larger pores likely requires Ca^{2+} influx from the extracellular environment via a concentration gradient (19). Additionally, US-stimulated MB activity has been implicated as an initiator of the Ca^{2+} influx (19), followed by triggering of Ca^{2+}-responsive proteins that react with the cytoskeleton to promote depolymerization of filaments, allowing for lysosomes to approach the membrane pore. In fact, lysosomes can be stimulated by high Ca^{2+} concentrations to fuse with membranes (20) as well as with one another (21), to potentially patch the membrane pore. Supporting evidence for this mechanism is a recent report describing that upon exposure of Sonovue MB to US, a Ca^{2+} influx is elicited in rat cardiomyoblasts, which leads to activation of BK_{Ca} channels and a local hyperpolarization of the cell membrane. This localized membrane hyperpolarization facilitates the uptake of macromolecules likely through endocytosis and macropinocytosis (22). Additionally, there might be alternative mechanisms underlying the 'healing' of cell membranes following sonoporation, including the activation of cytoplasmic enzymes such as tissue transglutaminases, which are activated upon exposure to elevated Ca^{2+} concentrations. Tissue transglutaminases can crosslink extracellular matrix proteins, forming intracellular 'clots', a process thought to be implicated in wound healing (23). However, the relevance of such pathways in sonoporation gene or drug delivery has not been examined yet and will likely be the focus of future mechanistic investigations.

Therapeutic approaches using gene therapy are promising as an alternative or as an adjunct to conventional cancer treatment. Because of its non-invasive nature, sonoporation has the potential to be highly competitive with viral gene delivery and existing non-viral alternatives. Sonoporation is emerging as a successful technology for *in vivo* gene and drug delivery, with reports of spatially restricted and therapeutically relevant levels of transgene expression. In this review we will discuss the advances in sonoporation technology and discuss the future applications of this promising new method of therapeutic delivery. One important development in this field will be to enhance the current status of targeting nucleic acids or drugs in complexes with MB to specific cells or tissues in order to enhance specificity and efficiency of sonoporation technology.

3. ADVANCES IN MOLECULAR THERAPY USING SONOPORATION

3.1. Delivery of Small Molecules by Sonoporation

Sonoporation-induced destruction of drug-loaded MB has evolved as a promising strategy for non-invasive local drug delivery. Drugs can be delivered by sonoporation by either coupling the drug to the surface of the MB or by enclosing it within the particle. As the MB particle travels within the vasculature, an US stimulus can be applied locally to burst MB and release the drug, which is absorbed by the target tissue (Figure 2a). Recently, sonoporation has been employed in several molecular

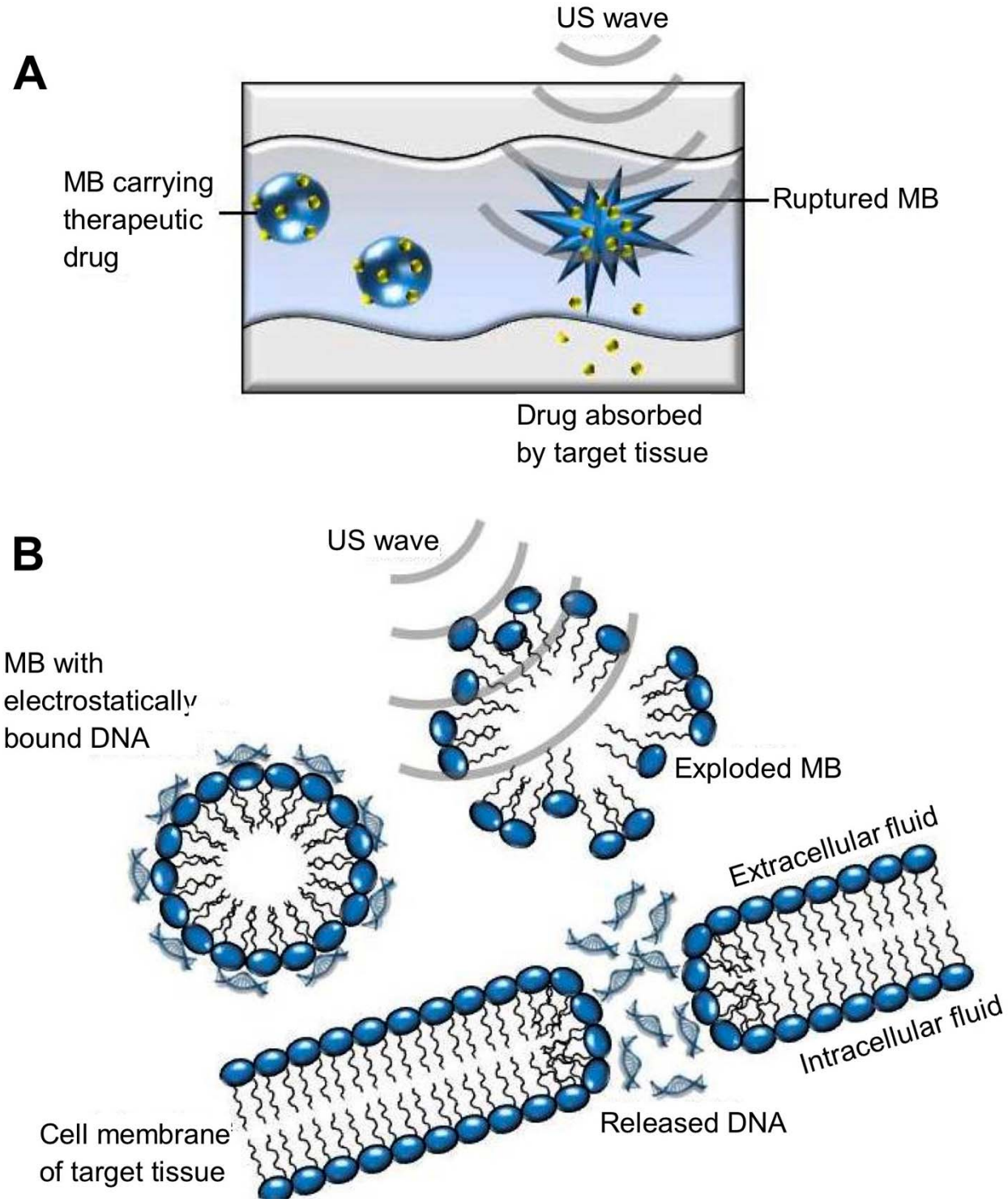

Figure 2. Sonoporation mechanisms for therapeutic delivery. a. Diagram of sonoporation for drug delivery. Drugs can be delivered by sonoporation. Microbubbles with drug attached to the surface or enclosed within the particle travel within capillaries. Upon exposure to an US stimulus or wave, MB rupture, releasing the drug contents. Drugs are absorbed by the target tissue. b. Schematic drawing of sonoporation for gene therapy. When plasmid DNA (pDNA)-containing MB are passed through blood vessels adjacent to tumor cells, insonated US waves rupture MB and release pDNA. Released pDNA penetrates into cells through their membranes by means of sonoporation.

therapy reports where it successfully can deliver drugs locally, reducing their systemic cytotoxicity. For example,

sonoporation delivery of Bleomycin managed to nearly eradicate Ca9-22 tumor xenografts. US delivery allowed for a lower dose of bleomycin to be used and augmented the apoptotic effect in tumors. Also, combination of Bleomycin plus a plasmid encoding for the cytolethal distending toxin B (cdtB), a periodontic bacterium gene product that induces cell cycle arrest and apoptosis, induced marked growth inhibition of Ca9-22 tumors also increased apoptosis. These findings suggest that local administration of cytotoxic agents by sonoporation is a useful method for molecular cancer therapy, and drugs and plasmid therapies can be combined for augmented effects (24).

Another example of how sonoporation can enhance the uptake of chemotherapeutics in malignant cells used HT1080 fibrosarcoma cells treated with a combination of Sonovue MB and taurolidine (TRD) plus tumor necrosis factor-related apoptosis inducing ligand (TRAIL). Apoptosis was enhanced by 25% following sonoporation delivery of TRD and TRAIL to human fibrosarcoma cells. Changes in proapoptotic genes correlated with US+MB chemotherapy delivery (e.g. BIRC3, NFKBIA and TNFAIP3). Some of these genes had been shown previously to play a role in human fibrosarcoma apoptosis (HSPA1A/HSPA1B, APAF1, PAWR, SOCS2), and interestingly, one gene had previously been associated with sonication-induced apoptosis (CD44) (25).

Sonoporation can be an asset for local delivery of otherwise systemically toxic chemotherapeutics such as Doxorubicin (DOX), a potent drug that displays severe adverse effects such as cardiac toxicity and myelosupression. Drug targeting thus is highly desirable for DOX delivery, aiming at increasing its local drug concentration while reducing its systemic side effects. A recently developed novel DOX-loaded microbubble (DOX-MB) formulation has been tested *in vitro,* showing optimal DOX loading capacity, ideal physical characteristics and retention of its antiproliferative efficacy. In a rat model, DOX-MB were not found to be toxic systemically. In a s.c. pancreatic carcinoma rat model, DOX-MB plus US (1.3 MHz; mechanical index 1.6) led to a 12-fold higher concentration of DOX in tumor tissue and significantly reduced tumor growth in sonoporated compared to contralateral control tumors. All rats survived the DOX-MB administration without any adverse effects. This study was promising since it highlighted the potential systemic safety and efficacy of MB to deliver a chemotherapeutic drug by sonoporation *in vivo* (26).

4. ADVANCES IN SONOPORATION FOR CANCER GENE THERAPY

4.1. Delivery of pDNA

Sonoporation therapy or 'sonotherapy' is emerging as an effective biotechnology tool especially for gene therapy applications and the number of scientific reports in this field is expanding rapidly. When plasmid DNA (pDNA)-containing MB pass through blood vessels adjacent to tumor cells and are exposed to a US wave stimulus, they rupture and release pDNA or other nucleic acids. Released nucleic acids penetrate into cells through the cellular membranes by means of sonoporation (Figure 2b). Therefore, on principle, sonoporation delivery may be particularly well suited for superficial tissues such as the skin. And indeed, sonoporation is efficient in delivering pDNA for healing skin wounds in diabetic mice. A Minicircle pDNA expressing the human vascular endothelial growth factor $(VEGF)_{165}$ cDNA (minicircle-VEGF) promoted accelerated wound closure and enhanced skin blood perfusion following sonoporation. Minicircle-treated wounds were fully restored to normal skin architecture compared with the untreated diabetic controls, which retained marked edema and abnormal skin morphology (27). This approach may therefore be highly promising for treating skin following surgical removal of lesions, for example in the context of skin malignancies. However, sonoporation application is not restricted to the skin. Sonoporation can be applied to treat tissues or tumors more deeply within the body and in a localized manner. Several classes of nucleic acid-based therapies can be delivered to tumors by sonoporation, including pDNA (immunogenes, cytotoxic genes), pDNA+polymers, siRNA, and even viruses. We will discuss each of these modalities in detail as follows.

4.1.1. Immunogene pDNA sonotherapy

A highly effective form of sonotherapy includes the delivery of immune stimulatory gene for treating cancer or other inherited or acquired diseases. This approach has great potential for treating multiple cancer types. Sonoporation is an ideal means of delivering immunogene pDNA, as it a similar efficiency as electroporation or other physical gene therapy techniques, with potentially fewer side effects such as injury at the site of delivery. For example, a recent report compared efficacy and safety of electroporation and sonoporation for delivering pDNA encoding for granulocyte-macrophage colony-stimulating factor (GM-CSF) and the T cell B7-1 costimulatory molecule. GM-CSF has been shown to enhance primary immune responses attributable to enhanced antigen-presenting cell (APC) efficiency since it stimulates the growth of APCs such as dendritic cells (DC) and macrophages. The costimulatory factor B7-1 acts by binding to CD28 on T cells, stimulating the production of multiple cytokines by CD4+ and CD8+ T cells. This pGM-CSF+B7-1 sonoporation strategy used US parameters of 1.0 W/cm^2 at 20% duty cycle for 5 min. *In vivo* sonoporation delivery resulted in a 55% cure rate in tumor-bearing animals. The immunological response invoked was cell-mediated, conferring resistance against re-challenge and against tumor challenge following transfer of splenocytes to naïve animals. Sonoporation did not cause any injury to treated tissue, and importantly, therapeutic efficacy was comparable to the electroporation-based approach (28). Another immunetherapy approach to successfully use sonoporation delivered interleukin-12 (IL-12) pDNA. IL-12 has a variety of immunomodulatory anti-tumor effects including induction of interferon- (IFN-) secretion by stimulation of T cells and natural killer cells, and promotion of cytotoxic T lymphocyte maturation. In addition, IL-12 induces antiangiogenic effects, which render IL-12 a very effective anti-tumor agent. In this

report, MB were used containing the US imaging gas perfluoropropane and liposomes were composed of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine s-methoxypolyethyleneglycol (DSPE-PEG_{2000}-OMe). Sonoporation delivery of pIL-12 using these MB dramatically suppressed tumor growth. The therapeutic effect was CD8+ T-cell dependent, as confirmed by an *in vivo* depletion assay. Sonoporation delivery is therefore an excellent non-viral system to achieve robust antitumor IL-12 gene therapy (29).

Another immunestimulatory approach tested the efficacy of sonoporation delivery of interferon-beta pDNA (pIFN) gene therapy for treating murine colon cancer liver metastases by using cationic liposomes instead of MB. The combination of liposome+pIFN+US induced higher IFN expression than pIFN+US or liposome+pIFN alone. This sonoporation strategy showed an antitumor effect *in vitro*. Moreover, when the pIFN sonotherapy was combined with the anticancer drug cis-diaminedichloroplatinum, a synergistic effect could be observed with increased and reduced tumor growth (30). A study delivering pIFN sonotherapy using BR14 MB sought to treat hepatocellular carcinoma (HCC). Human hepatic cancer cells (SK-Hep1) and plasmid cDNAs expressing pIFN and green fluorescent protein (pGFP). *In vivo*, pGFP and pIFN pDNA were mixed with BR14 MB and directly injected into subcutaneous SK-Hep1 tumors, followed by transcutaneous tumor sonoporation. Transfection efficiency *in vivo* was roughly 50% of cells as assessed by GFP fluorescence microscopy. *In vitro*, transfection efficiency was highest following sonoporation of SK-Hep1 cells in the presence of BR14 (a 6-fold increase) and up to 24% of cells were GFP+ as assessed by flow cytometry. Sonoporation of IFN pDNA *in vivo* reduced tumor size significantly, suggesting IFN sonotherapy could be a promising new therapy for HCC or other cancers (31).

Localized IL-12 sonotherapy was examined in SCCVII murine tumors using systemically administered of DOTMA:cholesterol liposome+pIL-12 (liposome+pIL-12) complexes followed by localized US application to achieve sonoporation delivery restricted to tumors. Systemic administration of liposome+pIL-12 to tumor-bearing mice resulted in IL-12 expression in the tumor but also in other tissues, primarily lung. Interestingly, if US was applied either before or after the liposome+IL-12 injection, a significant increase in gene transfer to tumors was observed (but in not other organs), with gene expression upregulation of up to 270-fold as compared to 'no US' control. A combination of pDNA quantitation and fluorescence microscopy showed that US increased pIL-12 tumor uptake through the tumor vasculature. IL-12 sonotherapy was sufficient to inhibit tumor growth compared to controls, demonstrating that sonoporation may be an effective method for delivering gene therapy to tumor endothelium and achieving significant systemic antitumor effects (32).

DNA vaccination has attracted much attention for preventing metastasis of malignant tumors. One example of a sonoporation strategy for anti-melanoma DNA vaccination employed the antigen presenting cell (APC)-selective gene carrier Mannose- polyethylene glycol ($PEG)_{2000}$ lipoplexes (Man-PEG). Immunization was induced by sonoporating a pDNA expressing ubiquitylated melanoma-specific antigens gp100 and TRP-2 (pUb-M) with Man-PEG. Following immunization, the secretion of Th1 cytokines and the activities of cytotoxic T lymphocytes were enhanced and there were potent and sustained DNA vaccine effects specifically against solid and metastatic B16 murine melanomas. These findings suggested that sonoporation using Man-PEG lipoplexes could be efficient agents for DNA vaccination aimed at the prevention of metastatic cancers (33). In combination, these approaches for sonoporation delivery of immunogenes highlight the potential for sonotherapy to become a major factor in the development of nonviral gene delivery approaches, especially ones that can deliver potent immunestimulatory molecules that even transiently could provide a significant antitumor and thus therapeutic effect.

4.1.2. Cytotoxic and proapoptotic sonotherapy

Another therapeutic gene class delivered by sonoporation to treat cancer includes cytotoxic genes. For example, the herpes simplex virus thymidine kinase (HSV-TK) cell killing gene delivers a robust cytotoxic therapy that has long been utilized for gene therapy. Only cells transfected with the HSV-TK gene convert GCV to its toxic phosphorylated form, resulting in tumor cell death. Sonoporation is well suited to deliver HSV-TK gene therapy, as evidenced recently in a mouse model of squamous cell carcinoma (SCCVII). Sonoporation was first used to deliver pDNA harboring reporter genes (luciferase (pLuc) or green fluorescent protein (pGFP)) both to tumor cells and the perivascular areas within tumors. Lipoplexes were prepared from a mixture of 1,2-distearoyl-sn-glycero-3-ethylphosphocholine (DSEPC), distearoylphosphatidyl-choline (DSPC) and PEG_{40}. HSV-TK sonotherapy reduced the tumor doubling time and enhanced apoptosis, as compared to GFP-treated tumors (34). Another recent study examined the antitumor effect of HSV-TK sonotherapy on mouse hepatoma. MB were a mixture of dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidyl ethanolamine (DSPE), diphenyl phosphoryl azide (DPPA) and contained perfluoropropane gas (C3F8). HSV-TK sonotherapy induced higher transgene expression and tumor inhibition than controls, with a significant improvement in the survival time (35). These results suggest HSV-TK sonotherapy could be effective against multiple tumor types, representing a promising new strategy for cancer treatment. One issue, however, is the safety and toxicity of this cytotoxic approach since it might elicit bystander effects on normal cells or other organs and it might be best suited for localized sonotherapy. Safety and toxicity of HSV-TK sonotherapy will have to be examined in larger and more detailed animal studies prior to translation.

Also promising is proapoptosis sonotherapy, which has included strategies reexpressing the tumor suppressor gene p53 in tumor cells. Delivery of a plasmid expressing p53 (pP53) into Y79 cells led to the observation that MB alone might have a transfecting effect. MB

composition was not described in this report but they contained a C3F8 core. Both the pDNA+MB+US and the pDNA+MB group displayed enhanced cell apoptosis as assessed by flow cytometry, but not the pDNA+US (naked DNA) or the untreated groups. p53-mediated apoptosis was highest in the pDNA+MB+US group (26%), followed by pDNA+MB (19%), with the other two groups having a low apoptosis rate (< 10%) (36).

4.1.3. Optimizing conditions for pDNA sonoporation *in vivo*

Prior to starting sonotherapy, a transducer system has to be chosen and conditions optimized *in vivo* using the plasmid DNA and MB of choice. An example of using a reporter gene pDNA for optimizing sonoporation from our laboratory can be found in Figure 3. We have utilized sonoporation at 1MHz to deliver reporter gene pDNA as well as therapeutic plasmids *in vivo*. In this example, a humanized renilla luciferase-expressing pDNA (phRLuc) was constructed harboring the reporter gene under the control of the hEF-1/HTLV promoter. phRluc mixed either in 0.9% sterile saline alone (naked) or in 30% sonovue MB in saline. pDNA solutions were injected into RM1 prostate tumors growing subcutaneously in C57/BL6 male mice. Tumors were then immediately exposed to the US stimulus (+US) or left unirradiated (-US control). 24-48h following US exposure, reporter gene expression was assessed in tumors *ex vivo* by addition of coelenterazine (hRLuc substrate) and images of light output captures for 3-5min using a Xenogen IVIS50 CCD detection instrument, as previously described (37). Several variables can interfere with successful sonoporation. For example, the selection of a larger probe (transducer) can yield a loss in gene expression, as seen in Figure 3a (left panel). A 20mm probe appears to deliver more diffuse US stimulus and reporter gene expression decreases when tumors are irradiated with US. Also, too long of an US exposure time also can result in decreased reporter gene expression, as seen in Figure 3a (right panel). In this example, a 6mm probe was used for the extended period of time of 10min and likely also provided too high of an energy input and a decrease in gene expression was observed. DNA concentration also has to be optimized, and we found that ~45-50µg of pDNA in 30% sonovue represent an optimal dose for prostate tumor sonotherapy. Doubling the DNA amount does not necessarily enhance transfection efficiency by US, for example. When 90µg phRLuc (in 30% Sonovue) was administered, reporter gene expression was not significantly enhanced (Figure 3b) compared to optimal conditions using 45µg (Figure 3c). The most efficient MB tested by us was Sonovue and it was most efficient at 30% with 1 MHz frequency and 50% duty cycle at 1 W/cm^2. The best probe was found by us to be of 6mm for a 2min exposure using a KTAC4000 sonoporator. With these conditions, we typically observed up to ~100-300x gene expression increases in several sonoporated murine or human prostate tumors. And finally, delivery of naked pDNA also could be augmented by US irradiation (Figure 3d), although at typically ~10-100x lower levels as with pDNA+sonovue. We utilized these defined experimental conditions to deliver therapeutic genes using pDNA+MB+US (sonoporation) or viral vector delivery (Adenovirus) (Figure 3e). Shown are the differences in relative tumor size, where the group with the highest antitumor effect was the sonoporated therapeutic pDNA. Therapeutic Ad delivery did not result in an efficient antitumor effect, suggesting sonoporation under certain conditions might be able to surpass the efficiency of some viral vectors, which may be promising for therapeutic applications. US has a well-established record in a clinical setting, and thus sonoporation has the potential to deliver pDNA efficiently and more safely relative to viral vectors, which typically trigger strong immune reactions and rapid viral clearance following 1-2 administrations.

Another set of experimental conditions by Tsai *et al.* revealed that different tissues have distinct gene expression patterns following sonoporation. Sonoporation of reporter gene plasmids (pLuc, pGFP) directly to liver tumors or muscle indicated that the best expression levels occur in muscle, followed by subcutaneous liver tumors, and orthotopic liver tumors (38). Optimal conditions were 50% sonovue for tumors and 30% sonovue for muscle, with 10 min of US exposure (20% duty cycle and 0.4 W/cm^2 intensity). Therefore, sonoporation using sonovue can be efficient to deliver pDNA directly (intratumorally) or indirectly (intramuscularly). Intratumoral delivery likely is more transient than intramuscular, which typically can be used for producing large amounts of gene products from muscle for the purpose of gene therapy. Suitable genes for this purpose are immunogenes.

An interesting variation in pDNA sonoporation strategy from another group involved delivering an episomal pDNA (which remains in the cytoplasm following cell division) for prolonging reporter gene expression over time. A MB101 MB-assisted gene transfection procedure was used with an episomal pLuc using 1 MHz at a duty cycle of 25% at intensities ranging from 1-4 W/ cm^2. Under these conditions, expression of luciferase was detectable for a period of >80 days in the mouse hind leg muscle and also in tumors *in vivo*. Therefore, if prolonged gene expression is desired for therapeutic purposes, the use of an episomal vector could be warranted and could further the impact of sonotherapy strategies (39).

4.2. Other nucleic acid sonoporation gene therapy

4.2.1. Sonotherapy with pDNA and polymer complexes

Polymers such as PEI (positively-charged) are typically used in nonviral gene delivery strategies to condense pDNA (negatively-charged). Combination with PEI has recently been tested for enhancing the efficiency of sonoporation *in vitro*. A cationic branched polyethylenimine (BPEI) polymer was used to condense pDNA containing the cDNA expressing human $VEGF_{165}$ (pVEGF) (40). pVEGF+BPEI complexes were transfected into CHO, HEK293, or NIH3T3 cells using Sonovue MB-based sonoporation. At the intensity of 1 MHz, the transfection efficiency of sonoporation was enhanced when MB alone were used (MB+pDNA+US) with no detrimental effects. In contrast, the addition of BPEI reduced cell viability, despite its effect in enhancing transgene expression. These results suggest that sonoporation gene therapy might be the safest technique to be used in clinical

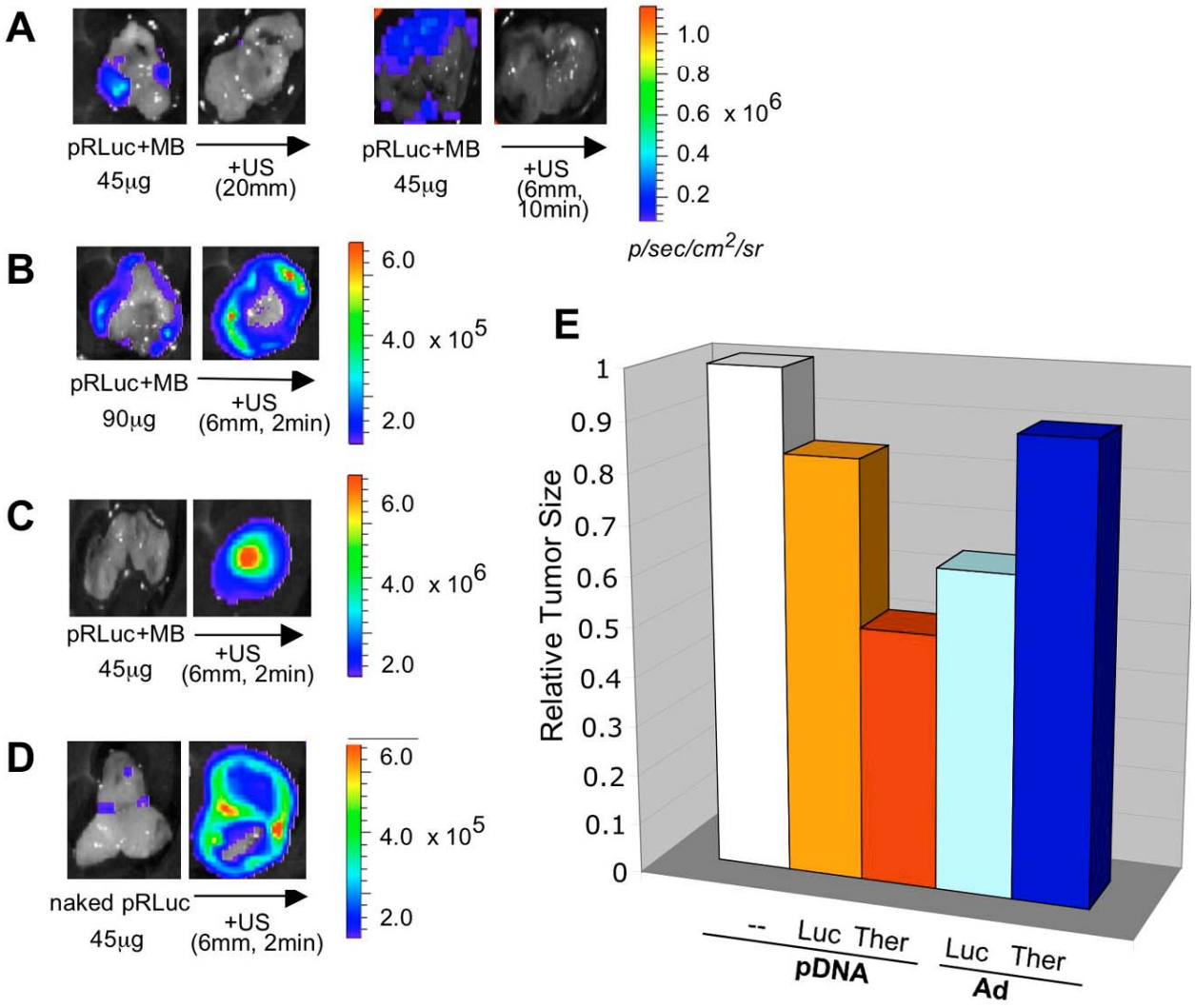

Figure 3. Defining the conditions for optimal Sonoporation of tumors *in vivo*. a. Probe selection: a 20mm probe delivers more diffuse US wave energy and no gene expression is seen with US (Left); a 6mm probe for 10min also provides too high of an energy input and does not achieve efficient transfection (Right). 45μg phRLuc pDNA plus 30% Sonovue MB were used for each experimental condition. b. DNA concentration effects: doubling the DNA amount does not necessarily enhance transfection efficiency by US. In this example, 90μg phRLuc plus 30% Sonovue MB is shown. c. Optimal concentration and conditions for sonoporation of plasmids into RM1 prostate tumors. The best conditions for sonoporation were 45-50μg DNA and 30% Sonovue with 1MHz frequency and 50%duty cycle, and 2Hz burst rate. The best probe was found to be 6mm for 2min exposure using a KTAC4000 sonoporator. d. Naked pDNA: US of naked pLuc also can augment its transfection. e. Application to a therapeutic model. Sonoporation treatment with 4 doses of 45μg therapeutic pDNA vector complexed with 30% sonovue plus US surpasses efficacy of therapeutic viral (Ad) vectors in an immunecompetent mouse model of prostate cancer. Shown are the relative TRAMP tumor sizes on day 44.

practice, and combination with BPEI should be attempted with caution *in vivo* systems and its toxicity potential well-characterized prior to translational applications.

One report has characterized in more detail the conditions needed for optimal sonoporation with pDNA plus polymer microparticles using *in vivo*. These studies examined the feasibility of sonoporation gene delivery in CC531 rat liver tumors using a pDNA expressing the *E. coli* lacZ gene (pLacZ). pLacZ was encapsulated in controlled release formulations loaded in poly (D,L-lactide-co-glycolide) microparticles (PLGA-MP) as a complex with poly (L-lysine). A single injection of pLacZ+PLGA-MP was administered either intraarterially or intravenously with simultaneous US exposure (color Doppler mode, maximum mechanical index) as the microparticles passed through pancreatic tumor capillaries to assess efficiency of pLacZ transfection. The tumors with the highest expression of B-gal reporter gene were ones treated with a combination of pLacZ+PLGA-MP and US. Subsequently,

the therapeutic effectiveness of this delivery system was tested in nude mice bearing Capan-1 human pancreatic adenocarcinoma by using a plasmid containing the tumor suppressor gene p16 (pP16). The tumor suppressor gene p16 is deleted in Capan-1 cells, so the rationale of this therapy strategy is one of gene replacement to induce cell cycle arrest and apoptosis. Mice were treated with intravenous infusion of pP16+PLGA-MP or control substances with or without US exposure. This strategy differs from typical single intratumoral injections, and infusion was followed by a relatively short US treatment. In this example, the pP16+PLGA-MP solution is infused intravenously and tumors receive a relatively long US stimulus (12 min). The goal of this modified controlled release procedure is to maximize pDNA release at the target site to achieve longer lasting gene expression. This US-controlled release of pP16 from microparticles led to significant inhibition of tumor growth. This suggests that a combination of pDNA and novel microparticles for sonoporating pancreatic cancer might comprise an effective approach for treatment of this deadly disease (41).

4.2.2. siRNA sonotherapy

The field of sonoporation gene therapy will likely also continue to expand towards delivering siRNA molecules for tumor therapy. Sonoporation already has been used to successfully deliver siRNA to induce gene silencing in tumors and other tissues, including the salivary gland. One example is a study where a siRNA targeting rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was delivered in a complex with 20% Optison MB by reverse-injection into rat parotid glands using transdermal US. Expression of GAPDH was silenced 48h after low-intensity US siRNA delivery (at 1 MHz, 0.5-2 W/cm^2, 2 min), while high-intensity US (4 W/cm^2) induced tissue damage and apoptosis instead. Addition of MB significantly improved siRNA-induced gene silencing by up to 50%. Interestingly, if parotid glands received US via direct exposure (surgery), transduction efficacy was not significantly different than when receiving noninvasive US (transdermal). This report indicated that MB-enhanced sonoporation can yield effective siRNA gene silencing *in vivo* just as efficiently from a noninvasive as an invasive US approach, and this finding may have important implications for siRNA sonotherapy targeting distant cancer metastases (42).

In cancer cells, short hairpin RNA (shRNA) interference therapy targeting the human survivin gene has been compared with PEI transfection. First, the transfection efficiency of the combination sonoporation+PEI system was assessed using a reporter gene plasmid (pRFP, red fluorescent protein-expressing). Transfection efficiency could be enhanced by US when in the presence of an undisclosed MB formulation and it was similar to that of PEI in several cancer cell lines (HeLa, HepG2, Ishikawa, MCF-7, and B16-F10). The pRFP+PEI+MB+US group had significantly higher gene expression (~35% transfection efficiency) as compared with pRFP alone, pRFP+US, or pRFP+MB groups (all <5% transfection efficiency). The cell viability was relatively unaltered (~85%) by using PEI in combination with sonoporation. This transfection system was used to deliver shRNA therapy targeting Survivin, a member of the mammalian inhibitors of the apoptosis protein (IAP) family, which is upregulated in various malignancies to protect cells from apoptosis. A modest improvement in the gene expression knockdown was observed with shRNA+US+MB (85%), compared to shRNA+PEI (80%). Nevertheless, this modified PEI:shRNA sonotherapy could be valuable in downregulating survivin or other growth-promoting genes to induce tumor cell apoptosis (43).

In vivo, a study examined the effectiveness of another shRNA targeting survivin formulated in PEI and combined with 50% Sonovue MB for sonotherapy of HeLa tumor xenografts (44). Complexes were prepared and injected intravenously (tail vein) into mice harboring HeLa subcutaneous xenografts. US exposure significantly increased tissue transfection of a red fluorescing protein (dsRed) expressing plasmid (pSIREN-DNR-DsRed or pdsRed) or a pLuc as compared to naked pDNA, and sonoporation in the presence of PEI-condensed pDNAs enhanced gene expression further (Figure 4). Regardless of US irradiation, there was no obvious dsRed expression in groups A and B (~1-5%, Figure 4a). Without US, there were only a few cells expressing RFP in pdsRed+SonoVue group and red fluorescent signals were weak in the majority of samples (~5-10%, Figure 4a). However, dsRed expression was enhanced in the pdsRed+MB+US group (~80%) but the intensity still was weaker than with pdsRed+MB+PEI (~80-95%, Figure 4a). When US was added (pdsRed+MB+US+PEI), dsRed expression was increased significantly, with apparently both a wider transfected area and stronger intensity signals per cell (~100%, Figure 4a). Similar observations were made when the pLuc reporter plasmid was used in the same conditions. Luc expression was unaltered when US was used in the presence of naked pLuc. Also, in the absence of US exposure, Sonovue MB did not significantly enhance luc expression in tumors. However, when US was combined with MB, transfection efficiency was augmented by about 14-fold (Figure 4b). The transfection efficiency was the highest when US+MB were combined with PEI. The luc activity of tumors exposed to US was increased further by about 10-fold as compared to 'no US' tumors. And compared to 'no PEI' groups, luc expression was increased by >100-fold in the presence of PEI (Figure 4b). This interesting study demonstrated that the combination of sonoporation and PEI significantly augmented the transfection efficiency. Also, when a shRNA targeting the surviving gene was transfected, apoptosis was observed correlating with downregulation of survival genes survivin and bcl-2, and upregulation of proapoptotic genes bax and caspase-3. Importantly, no detrimental effects were seen on organs adjacent to the tumor receiving the US stimulus, even at 3Mhz intensity as used in this report, and adjacent organs did not have significant gene transfection (Figure 4c).

4.2.3. Enhancing viral delivery by sonoporation

The efficiency of sonoporation has been compared to that of adenovirus (Ad) in some studies. For instance, one study addressed the ability of these two methods of gene delivery to deliver gene therapy to the

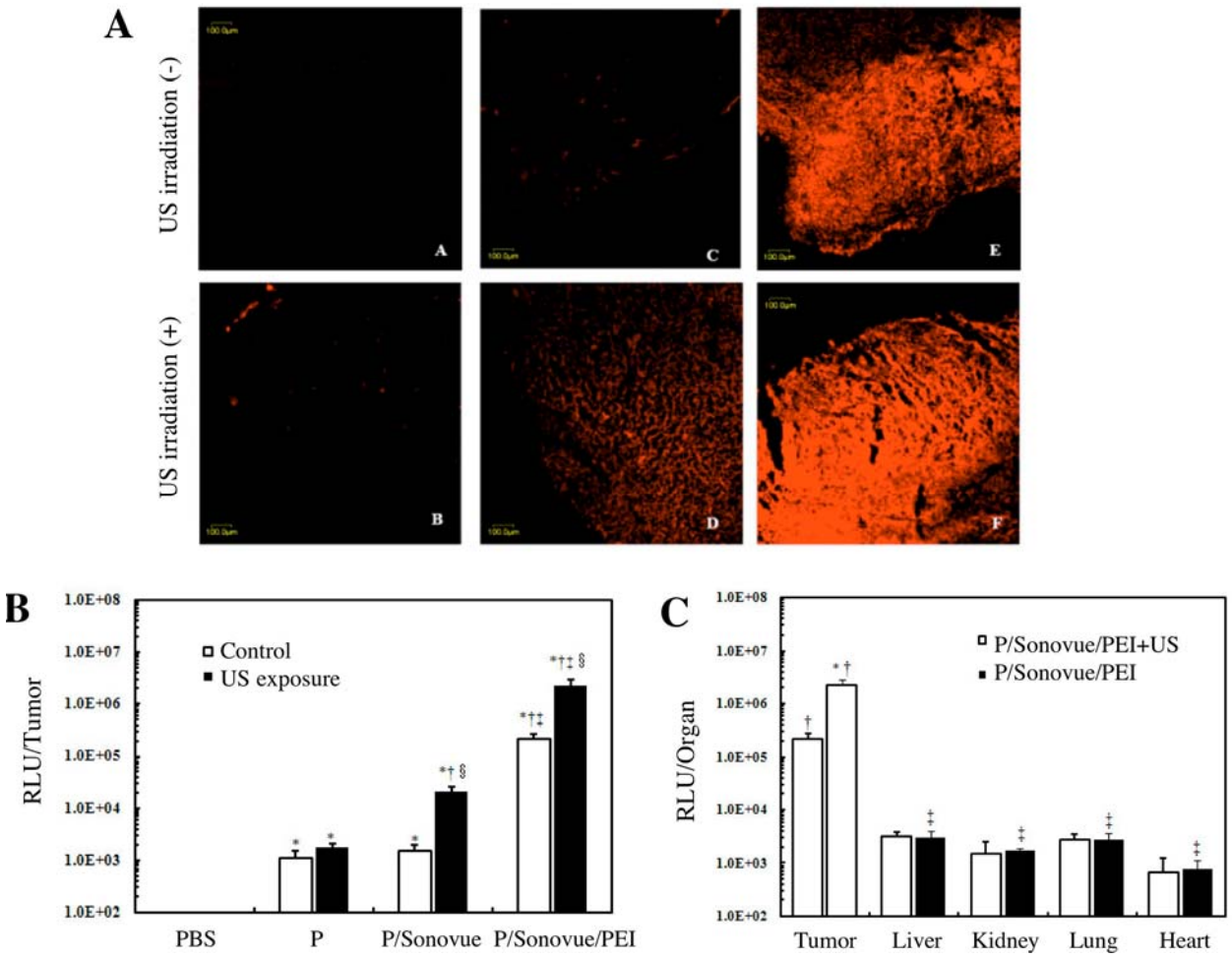

Figure 4. Efficiency of Sonoporation as assessed by reporter gene transfection in vivo. Red fluorescence microphotographs of HeLa tumor xenografts after intravenous injection of ~30 µg naked pSIREN-DNR-DsRed-Express (expresses a red-fluorescing reporter protein). (A, B), pSIREN-C plus ~50% Sonovue complex (C, D), and pSIREN-C+Sonovue+PEI complex (E, F) in 200 µl 0.9% saline with or without US irradiation. US parameters were time=2 min, intensity, 2 W/cm2, frequency= 3 MHz, and duty cytle= 20%. UTMD= Ultrasound targeted microbubble destruction; PEI= polyethylenimine; bar= 100 µm. Luciferase expression in HeLa tumor xenografts following US exposure and PEI. Control: no US exposure; P: pCMV-LUC; in the same condition (control or ultrasound exposure), as compared with PBS group, *P < 0.01; as compared with P group, †P < 0.01; as compared with P/SonoVue group, ‡P < 0.01; as compared with control group, §P < 0.01. Luciferase expression in non-target organs following US exposure and PEI of HeLa tumors in vivo. Ultrasonic energy at 3Mhz was more focused, and had no significant impacts on other adjacent irradiated organs. P: pCMV-LUC; as compared with non-irradiated tumors, *P < 0.01; as compared with other organs, †P < 0.01; as compared with P/SonoVue/PEI complexes injection alone, ‡P > 0.05. (From reference (44); this is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited).

walls of arteries for preventing atherosclerosis. A plasmid expressing TFPI-2 (pTFPI-2), an inhibitor of blood coagulation, regulates remodeling of the extracellular matrix and can potentially prevent atherosclerosis. Sonotherapy using pTFPI-2 and Sonovue MB successfully inhibited the proliferation of vascular endothelial cells *in vivo*. And although TFPI-2 sonotherapy showed similar gene transfection efficiency as an Ad, sonoporation delivery of TFPI-2 more strongly inhibited thrombosis and arterial re-stenosis. Moreover, sonoporation was considered a less damaging approach when transfecting genes into the arterial wall. These data indicate that sonoporation might have advantages as a transfection method over viral vectors (45), and perhaps these findings may extend to tumor tissues as well.

In the mouse salivary gland, sonoporation delivered pLuc via retroductal cannulation efficiently, as monitored by *in vivo* imaging. pLuc was delivered to the salivary gland complexed with Definity MB in two different concentrations (100% and 15% volume/volume). An Ad vector with the same CMV-Luc expression cassette

was used as a positive control at two different dosages. Four 30 s US bursts were applied at the following parameters: 1 MHz, 50% duty cycle and 2 W/cm^2, with 10 s in between pulses. Whereas sonoporation using 100% MB was weak and rapidly extinguished, sonoporation using the 15% microbubble solution was robust and stable for up to 28 days. Sonoporation therefore appears to be a promising method for non-viral gene delivery to the salivary glands (46), and application may be extended to tumor therapy also.

For tumor therapy, Ad have been considered a viable approach for late-stage cancers. In fact, Ad serotype 5 (Ad5) is the most commonly used gene delivery vector for cancer therapy, but progress using this vector has been hampered by concerns over the safety and practicality of using Ad for intravenous delivery in the clinic. Major challenges for effective therapy using Ad are the limited infectivity and the inability to specifically deliver the therapeutic directly into diseased tissue without trapping of the vector in the liver or its elimination by the immune system. The shortcoming in using Ad is mostly attributed to a reduction in expression of the Ad entry receptor, coxsackie-adenovirus receptor, on the surface of cancer cells. Interestingly, it has been proposed that systemic Ad gene delivery could be improved by using sonoporation delivery. For instance, sonoporation has been tested as a method to improve Ad delivery directly into prostate tumor tissues. The rationale has been that MB could help shield the Ad from the immune response while in circulation and also could help with targeting of Ad to a particular tumor. Typically, intratumoral injections of an Ad expressing the secreted proapoptosis cytokine IL-24 or melanoma differentiation-associated gene-7 (Ad.mda-7), inhibits prostate cancer growth. However, Ad.mda-7 is ineffective in tumors that overexpress antiapoptotic proteins such as Bcl-2 or Bcl-x_L. When Ad.mda-7 were complexed with Targeson MB (the specific type was undisclosed), tumor burden was significantly reduced not only in DU145, but also in DU145/Bcl-x_L tumors, a type of tumor resistance to mda7 therapies (47). A very promising result that is relevant to treating metastases was the finding that opposite flank (untreated) tumors also received a therapeutic benefit from Ad.mda-7, presumably through enhanced cytokine levels in circulation. This sonoporation strategy also could deliver a conditionally replicating Ad (CRAd) containing mda-7 following direct intratumoral injections (47). These findings highlight potential therapeutic applications of this sonotherapy strategy for either guiding tumor injection of Ad+MB complexes or to improve local delivery of Ad vectors to tumors. Also, the Ad/MB strategy might enable also therapy of advanced prostate cancer patients with distant metastatic disease. This unique strategy could provide a path to translation from the laboratory into the clinic for developing more effective gene therapies for prostate cancer. Still, expression of gene therapies from Ad or pDNA are transient transfection methods and multiple applications may be necessary to achieve complete tumor or metastases elimination. Incorporation of functional imaging methods will likely help refine and improve the choice of the safest and most effective mode of gene therapy delivery to each type of tumor in the clinic.

5. SONOPORATION AND FUTURE DIRECTIONS

5.1. Targeting of Microbubbles

The addition of ligands or antibodies to the surface of MB will likely enhance the specificity of sonoporation as a drug delivery system for cancer therapy. MB have been successfully targeted towards cancer cells or blood vessels (Figure 5). Gas-filled MB can be designed so that the interior is loaded with drugs and gas. Alternatively, drugs can be loaded on the surface of MB as well by charge associations. Drugs may be incorporated directly within the MB or if insoluble in water, in an oil layer. MB may be targeted to a specific cell or tissue via incorporation of molecules on the bubbles' surface, for example, targeting ligands or antibodies (Figure 5a). It is critical to employ targeting strategies in order to improve efficiency of targeting tumors. MB can be modified to allow non-covalent binding to tumor cells, or can harbor specific antibodies or ligands on the MB surface to help direct the particles specifically to tumor antigens. Also, drugs can be incorporated within the targeted MB particle to allow specific 'homing' towards a target site (i.e. PSMA inhibitors for prostate cancer homing) (Figure 5b).

For cancer cells, a specific drug delivery system for squamous cell carcinoma (SCC) was developed that utilizes an antibody for epidermal growth factor receptor (EGFR) for *in vitro* sonoporation. Sonoporation delivery of a low dose of bleomycin (BLM) using Sonovue MB combined with the F_{ab} fragment of an anti-EGFR antibody produced a marked growth inhibition of human ginvival SCC Ca9-22 cells (48). These findings indicated that sonoporation in the presence of the anti-EGFR antibody might allow more efficient and specific drug uptake, suggesting a novel application for chemotherapy and gene therapy treatments for oral squamous cell carcinoma. One limitation of this study, however, was a lack of direct coupling of the antibody to the MB, therefore the specific targeting mechanisms at play resulting in the tumor cell growth inhibition remain to be fully characterized.

Other studies of targeting in cancer cells have explored the use of a drug-loaded MB with a surface-bound peptide. In one example, MB were coated with a breast tumor-homing peptide (LyP-1) on the surface of paclitaxel-loaded MB (PTX-MB) through a biotin-avidin linkage. MB were composed of DSPC: DSPE-PEG_{2000}: DSPE-PEG_{2000}-biotin and a perfluoropropane (C3F8) gas core. The targeted PTX-MB encapsulated PTX efficiently (~40-60%) for delivering formulations from 0.015 mg-0.11mg PTX/10^8 MB. About 95% of MB were successfully coated as assessed by using fluorescent LyP-1 peptides, suggesting a high effectiveness of the biotin-avidin bridge system. PTX-MBs showed an excellent acoustic destructibility, with the lowest half-life of about 43s. The attachment of targeted MBs to human MDA-MB-231 breast cancer cells was highly efficient and stable even in the presence of US. The cellular uptake of targeted MBs was ~4- to 8-fold higher than that of non-targeted MB, suggesting that these LyP-1-coated PTX-MB are promising as a potential targeted chemotherapy delivery for breast cancer (49).

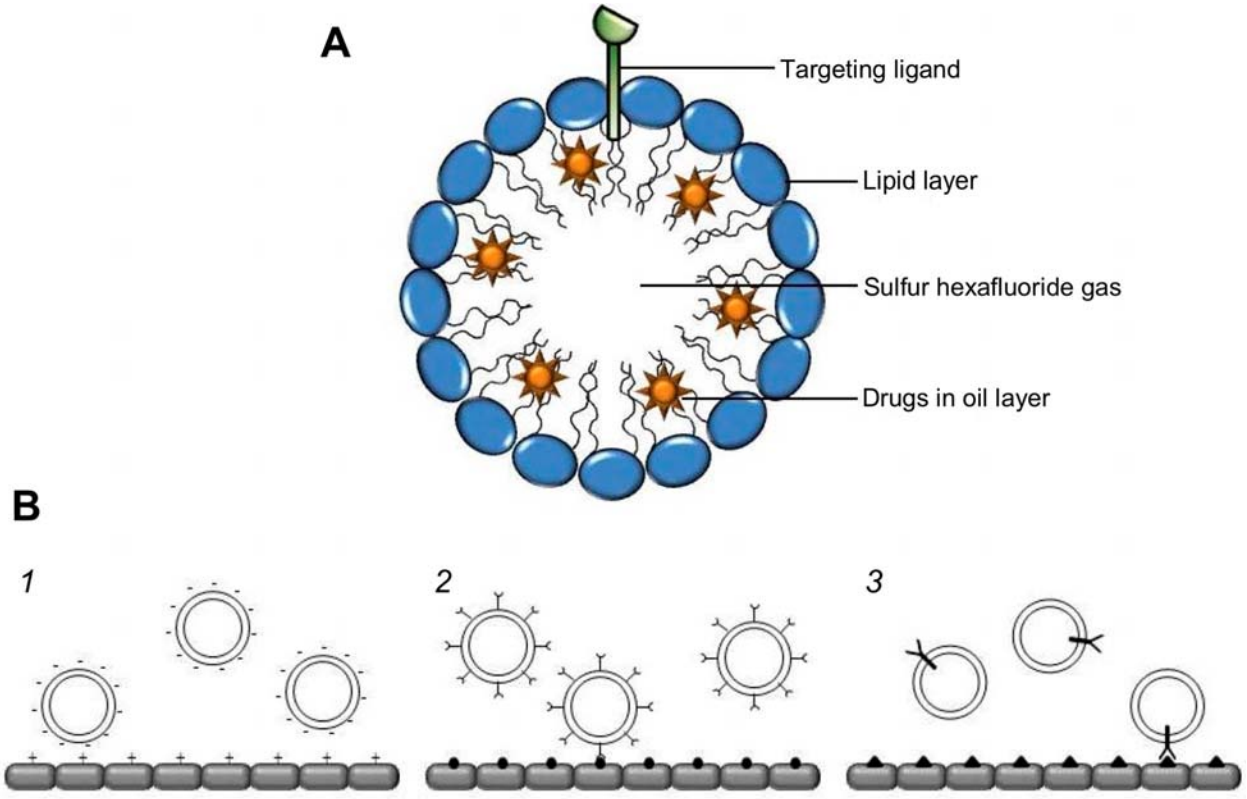

Figure 5. The future of sonoporation: targeted MB. (a) A diagram depicting a microbubble constructed for gene delivery. Gas-filled MB can be designed so that the interior is loaded with drugs and gas. Here, a lipid is depicted as the stabilizing material and it encases the perfluorocarbon gas to form MB. Drugs may be incorporated by themselves or, if insoluble in water, in an oil layer. MB may be targeted to a specific cell or tissue via incorporation of molecules on the bubbles' surface, for example, targeting ligands or antibodies. (b) Targeting strategies for delivering MB to tumor tissues. (1) Inherent chemical or electrostatic properties of microbubble (MB) shells allow non-covalent binding to tumor cells, (2) Attachment of specific antibodies or ligands on the MB surface help direct MB specifically to tumor antigens, and (3) incorporation of therapeutics into MB allows specific 'homing' capacity towards a target site.

An example of controlled drug delivery includes a system made of MB containing a synthetic biocompatible polyvinyl alcohol polymer shell and an air-filled core (PVA-MB) (50). The chemical versatility of the polymer surface offers a variety of coupling modalities useful for coating and specific targeting. The conjugation strategy on PVA-MB enables modifications of the polymer surface with oxidized hyaluronic acid (HAox) to localize drug delivery to tumor cells expressing cellular receptors recognized by HA. HA recognizes CD44 (Cluster Determinant 44), RHAMM (Receptor for HA Mediated Motility), and ICAM-1 (Intercellular Adhesion Molecule-1) receptors. CD44 and ICAM-1 are cell adhesion molecules and CD44 is a marker of several cancer types (prostate, ovarian, breast, and colon) (51). In this respect, HA is an excellent candidate to target PVA-MB to tumor cells. HAox-coated MB were used to release doxorubicin (DOX) in HT-29 tumor cells. HAox coating of MB enabled them to be more efficient in delivering DOX to cells, a prerequisite for a 'theranostic', i.e. a diagnostic and therapeutic, use of polymer-based targeted MB (52). Future developments will likely include characterization of these systems *in vivo*, using improved designs of MB surface modifications including the covalent linkage of DOX or other drugs to MB in a manner that could allow for hydrolysis by proteolytic enzymes present in the cell cytoplasm and subsequent drug release. This will allow for a more localized and controlled release of drugs.

Diagnostic applications of US already document the successful contrast enhancement possible with the use of MB, which are typically trapped in vasculature due to size constraints. For furthering 'theranostic' delivery, MB can be used to specifically bind blood vessels via modifications targeted towards selectins, $_{v3}$ or $_{51}$ integrins, glycoprotein IIb/IIIa, intracellular adhesion molecule (ICAM-1), or vascular endothelial growth factor receptor (VEGFR-2) (53). Targeted MB have been increasingly used *in vivo*, as illustrated by a recent study where a cyclic arginine-glycine-aspartic acid (cRGD) peptide for targeting integrins was conjugated to the surface of MB harboring the heterobifunctional agent pyridyldithio-propionate (PDP). MB were an aqueous dispersion consisting of disteroylphosphatidylcholine, distearoyl phosphatidylethanolamine-PEG_{2000}-pyridyl dithiopropionate (DSPE-PEG-PDP), and PEG_{40} stearate

sparged with decafluorobutane. As negative controls, MB were left either without a ligand or a scrambled sequence ligand (cRAD) was added to the PDP group. To enable characterization of peptides bound to MB surfaces, the cRGD peptide was labeled with FITC and a 5-fold enhancement in targeted adhesion of cRGD-MB was demonstrated in a flow adhesion assay against recombinant murine $_{v3}$ integrin protein and integrin-expressing endothelial cells (EC). The specificity of cRGD-MB for integrin was demonstrated by treating EC with a blocking antibody. A murine model of mammary carcinoma was used to assess targeted adhesion and ultrasound efficacy by molecular imaging *in vivo*. The cRGD ligand concentration on the MB surface was 8.2 x 10^6 molecules/MB. cRGD-MB, but not nontargeted MB or cRAD-MB, showed significantly enhanced contrast signals with a high tumor-to-background ratio. The results demonstrate the functionality of a novel MB contrast agent covalently coupled to an RGD peptide for ultrasound molecular imaging of integrin and integrin-expressing EC and the feasibility of quantitative molecular ultrasound imaging and therapy delivery *in vivo* (54). Another novel MB strategy for targeting blood vessels utilized MB coupled to a recombinant single-chain vascular endothelial growth factor construct (scVEGF) for ultrasound imaging of tumor angiogenesis. MB were a complex of phosphatidylcholine, distearoyl phosphatidylethanolamine-PEG 2000-maleimide (DSPE-PEG-maleimide), and PEG-40 stearate with decafluorobutane. MB reacted with scVEGF and led to the conjugation of 1.2x10^5 molecules scVEGF/MB. scVEGF-MB exhibited 5-fold higher adhesion to both recombinant VEGFR-2 substrates and VEGFR-2-expressing EC compared with nontargeted control MB. Importantly, in an *in vivo* model of tumor angiogenesis, scVEGF MB showed ~8-fold ultrasound contrast signal enhancement in tumors compared with nontargeted control MB. These results demonstrate that a novel scVEGF-bearing MB contrast agent is functional and could be useful for molecular imaging of VEGFR-2 or targeting of MB towards VEGFR2 expressing cells (55).

5.2. Developing superior localized sonotherapy

The major obstacle in the use of gene therapy is the lack of safe and efficient methods for localized gene delivery to targeted sites within tumors or to metastatic lesions. Sonoporation can be developed as a localized technique for these purposes. Several new methods are in development to improve localized sonoporation delivery. A new system recently reported the utilization of dual-intensity US with nanobubbles (NB) instead of MB, enabling improved localized sonotherapy. This system consists of low- and high-US intensities and so far only has been tested in the mouse bladder *in vivo*. The bladder was chosen as a prototype-testing organ since it is balloon-shaped and a closed organ in which the behavior of NB can be controlled spatially and temporally by US exposure. This method hypothesizes that when dual-intensity US is used in the presence of NB, the low-intensity US stimulus would serve to direct NB to targeted cells, while the high-intensity US stimulus would disrupt the NB, promoting cell membrane permeability and sonotherapy delivery. The NB used in this study were acoustic liposomes composed of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxy-polyethyleneglycol (DSPE-PEG), sonicated in the presence of C3F8 gas and were ~200 nm in diameter. NB were exposed to 0.05 W/cm^2 US for 10 s to induce NB movement to designated areas, and NB were exposed to 37 W/cm^2 for 0.5-1.0 s for NB destruction (both at 2.2 MHz) (56). Using high-frequency US imaging, this study characterized the movement and fragmentation patterns of NB in the bladder. Confocal microscopy revealed that TOTO-3 fluorescent molecules were delivered locally to the bladder wall, and histochemical examination indicated that an impressive degree of localization and transfection efficiency was observed in the urothelium (Figure 6).

A bioluminescence imaging system showed also that a pLuc was delivered to the bladder wall in a highly localized manner, suggesting that this approach could facilitate localized sonotherapy and render this approach feasible for the management of bladder cancer. There are some limitations to this method, however. First, noninvasive, low-intensity US can accumulate NB only in a limited area. This is not optimal since it would be desirable for NB to be able to reach several bladder or tumor areas since several separate lesions may form simultaneously within a given affected organ. A transducer-tipped US catheter such as those already developed for treating vascular thrombosis (57) could potentially be used to better manipulate NB localization. Another limitation is the nonspecificity of this method toward cancer cells, which can be overcome by using targeted NB. With future progress in liposome technology, NB will likely be modifiable to harbor targeted ligands or encapsulate therapeutic agents to be released specifically at the site of bubble collapse, and such developments may greatly enhance the translational applications of this precision controlled sonoporation method.

5.3. Extending sonotherapy to treat the tumor microenvironment: cancer-associated bone lesions

One important aspect of therapies that aim to erradiacate cancer metastases is to be able to also treat the surrounding bone, which is typically affected by tumor-induced osteoblastic and osteolytic bone lesions. Bone lesions are typically tumor promoting and aid tumor cells in seeding and expanding within the permissive bone microenvironment. These lesions result in many cases in fracture and bone loss, with osteoblastic lesions resulting also in the formation of immature of woven bone, which lacks several properties of normal bone (58). Therefore, for tumor sonotherapy of metastases to ultimately be successful, the bone microenvironment must also be restored to a normal state. Sonotherapy can be used for this dual purpose, i.e., treating both the tumor and restoring bone health. For example, the protein bone morphogenetic protein-2 (BMP-2) is able to promote bone healing and regeneration (59). However, previous studies using BMP-2 in clinical applications have been hampered due to the lack of an efficient, safe and simple delivery system, and rather require expensive recombinant protein production and/or the use of biological/engineered matrices. Gene therapy using sonoporation is a promising option for delivering

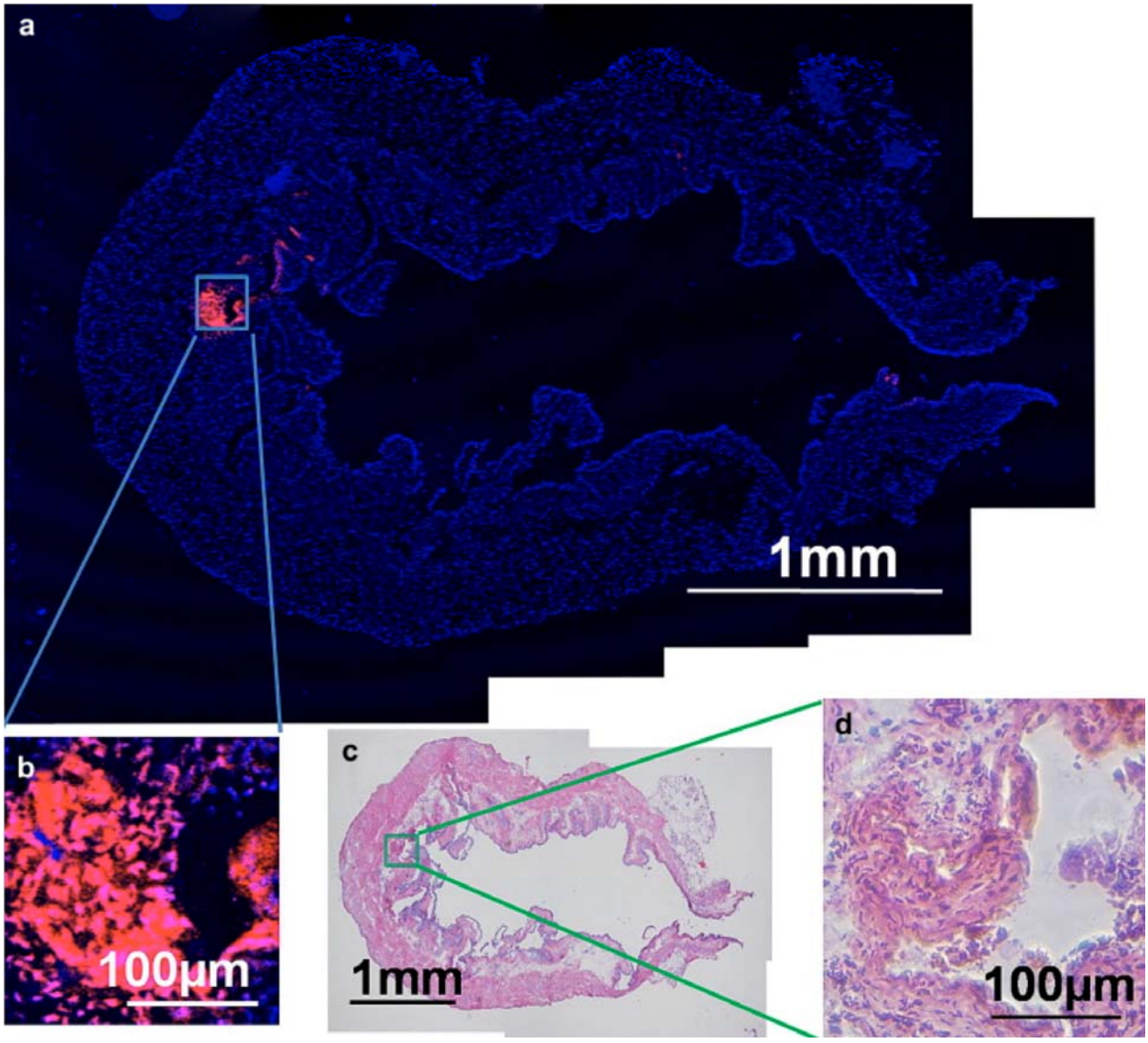

Figure 6. Therapeutic potential of localized sonotherapy. a. A representative confocal image (cross-section) of a bladder following red fluorescence molecules delivery by nanobubbles (NB) and US. For this sample, three sets of low-intensity US (0.05 W/cm^2 for 10 s) and high-intensity US (37 W/cm^2 for 1.0 s) sequences were used. DAPI stain (blue) shows the location of nuclei in bladder cells. TOTO-3 (red) fluorescent molecules emit signals at the localized area where the molecules were delivered. b. Confocal image in higher magnification of the bladder shown in (a). c. A serial HE-stained section adjacent to the confocal image shown in (a). d. Higher magnification of the sonoporated area of the bladder shown in (c). This image shows that the tissue sonoporated was bladder urothelium. Reprinted with permission from (56).

BMP-2 to bone. In a recent study, the BMP-2 gene was transferred into the skeletal muscle of mice by transcutaneous sonoporation using pDNA encoding for BMP-2 (pBMP-2). Following sonoporation, both human BMP-2 and bone formation and differentiation marker genes were expressed (alkaline phosphatase and osteocalcin). Moreover, mature bone formed within pBMP-2 sonoporated mouse muscle, confirming that transcutaneous sonoporation can promote osteoinduction *in vivo*. Skeletal muscle was used since direct sonoporation of bone is attenuated by the presence of calcified matrix components. These results suggest the possibility of the effective clinical use of BMP-2 sonotherapy using transcutaneous sonoporation (60).

Another study to successfully achieve US-based osteogenic gene delivery delivered naked pDNA encoding for the osteogenic gene human bone morphogenetic protein-9 (pBMP-9). The pBMP-9 plasmid was sonoporated into the thigh muscles of transgenic mice expressing the Luc gene under the control of a human osteocalcin promoter. Following pBMP-9 sonoporation, osteocalcin-dependent Luc expression lasted for 24 days and peaked on day 10, suggesting new bone formation

occurred. Bone tissue was formed at the site of pBMP-9 delivery, as shown by both micro-computerized tomography and histology. The sonoporation method was promising for gene delivery but found to be less efficient than electroporation, at least for delivering naked pBMP-9 (61). This study did not examine the effect of adding MB on enhancing delivery of pBMP-9 by sonoporation and this strategy would likely augment gene expression to levels higher that that observed for naked pBMP-9 sonoporation.

Interestingly, US exposure alone might have a positive impact on bone regeneration as well. There has been evidence that while high intensities of US can damage bone or delay healing, low intensities can enhance repair rates and reduce healing times. Experiments in a rat fibula fracture model show that US exposures during the inflammatory and early proliferative phase of bone repair promote accelerated healing with direct ossification. However, if US exposure is delayed until the late proliferative phase, cartilage growth is stimulated instead. For bone regeneration, US at 1.5 MHz appears to be more effective than at 3 MHz (0.5 W/cm^2, pulsed 2 ms: 8 ms for 5 min), suggesting a non-thermal mechanism (62). In a rabbit osteotomy model, fibulae strength returned to that of the uninjured bone within 17 days following US exposure at 1.5 MHz (0.03 W/cm^2, pulsed 200μs: 800μs for 20 min daily), compared to 28 days for untreated controls. The way in which bone repair may be accelerated by ultrasound is still not well understood. *In vitro*, 1.5 MHz and 0.1/ W/cm^2 US exposure stimulates collagen and non-collagenous protein synthesis (62). Mechanisms may include changes in signal transduction, enhanced gene expression, blood flow changes, tissue remodeling or micro-mechanical stresses, although these mechanisms warrant further study.

5.4. Microdevices

Adaptation of sonotherapy using microdevices and/or miniature probes is likely to be the new frontier for translational applications in the field of gene delivery. A miniature plate device has already been developed and tested for delivering antisense oligonucleotides. This plate consisted of a piezoelectric lead zirconate titanate (PZT) (0.5 cm^2 x 0.75 mm) element, which was used to transfect human umbilical vein endothelial cells (HUVEC) and human prostate cancer cells (PC3) using sonoporation. Following sonoporation, the transfection rate for HUVEC increased by 96% compared to controls, while for PC3, transfection rate increased by 31% compared to controls. This plate also showed peak intensity at 25 W/cm^2 and efficiently delivered pGFP to Chinese hamster ovary (CHO) cells when Optison MB were used (63). This research could potentially be applied in developing a microelectromechanical system (MEMS)-based device for future sonotherapy delivery for cancer treatment.

Importantly, a miniature probe must be developed for translational applications since it is difficult to transmit US from an external transducer to points deep within the human body at adequate intensity and position due to the attenuation of ultrasound by air, bone and tissue, and the deflection of US. A probe that contains a more focused ultrasonic transducer is in development for sonoporation translational applications (64). This probe might be able to deliver drugs, pDNA, and US transmission gel via a hole of ~500 mm in diameter and could be introduced at locations deep in the body via a catheter or an endoscope. The field of bioengineering will undoubtedly continue to advance to produce microdevices and/or miniature probes that will be used for enhancing specificity and precision of gene delivery by sonoporation.

6. SUMMARY AND PERSPECTIVE

Nonviral means of gene delivery have suffered from lower efficiencies compared to viral vectors currently used in the clinics. Also, novel means to delivering drugs *in vivo* are needed for reducing toxicity of chemotherapeutics such as Bleomycin or Doxorubicin. The novel drug and gene therapy delivery strategy of Sonoporation is emerging as a relatively effective, safe, and inexpensive method that only requires a plasmid, MB, and an ultrasound source plus transducer. Sonoporation brings to mind the science fiction movie ‘‘Fantastic Voyage’’ (1966), in which a diplomat is nearly assassinated and in order to save him, a submarine is shrunken to microscopic size and injected into his blood stream to deliver a small crew of miniaturized individuals. Although such a fantastic scenario is unlikely, sonoporation can become the tool by which microbubbles can serve as a shuttle to target specific cells or tissues. The efficiency of sonoporation strategies will likely continue to evolve, delivering increasingly precise therapies by using miniaturized medical endoscopes, catheters, or probes. Using higher-performance equipment, with targeted microbubbles, and with the guidance of diagnostic imaging, more precise and safe treatment will be possible using sonoporation, and newer therapeutic prospects will be realized.

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Abbreviations: US, ultrasound; MB, microbubbles; pDNA, plasmid DNA; Luc, luciferase; hRLuc, humanized renilla luciferase; TRD, taurolidine; TRAIL, tumor necrosis-factor related apoptosis ligand; DOX, doxorubicin; VEGF, vascular endothelial growth factor; GM-CSF, granulocyte macrophage colony stimulating factor; APC, antigen presenting cell; DC, dendritic cell; IL-12, interleukin 12; IFN-γ, interferon gamma; DSPC, 1,2-distearoyl-sn-glycero-phosphatidylcholine; DSPE-PEG_{2000}-OMe, 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine s-methoxypolyethyleneglycol; IFNβ, interferon beta; GFP, green fluorescent protein; DOTMA, N- (1- (2,3-dioleyloxy)propyl)-N,N,N-tri-methylammonium chloride; SCC, squamous cell carcinoma; PEG, poly (ethyleneglycol); Man-PEG, mannose-PEG2000; DSEPC, 1,2-distearoyl-sn-glycero-3-ethylphosphocholine, DPPC, dipalmitoyl phosphatidylcholine ; DSPE, distearoyl phosphatidyl ethanolamine, DPPA, diphenyl phosphoryl azide; C3F8, perfluoropropane gas; HSV-TK, herpes simplex virus thymidine kinase; CCD, charged-coupled device; BPEI, branched polyethylenimine; PLGA-MP, poly (D,L-lactide-co-glycolide) microparticles; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; shRNA, short hairpin RNA; RFP, red fluorescent protein; PEI, polyethylenimine; Ad, adenovirus serotype 5; PTX, paclitaxel; EGFR, epidermal growth factor receptor; PVA, polyvinyl alcohol; HAox, oxidized hyaluronic acid; CD44, cluster determinant 44, RHAMM, receptor for HA mediated motility; ICAM-1, intercellular adhesion molecule 1; VEGFR2, VEGF receptor 2; PDP, pyridyldithio-propionate; scVEGF, single chain VEGF; EC, endothelial cells; NB, nanobubbles; BMP-2, bone morphogenetic protein 2; BMP-9, bone morphogenetic protein 9; HUVEC, human umbilical vein endothelial cells; MEMS, microelectromechanical system.

Key Words: Sonoporation, Gene Therapy, Cancer, Sonotherapy, Gene Delivery, Microbubbles, Ultrasound, Review

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