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REVIEW

Gene therapy progress and prospects: Ultrasound for gene transfer

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Ultrasound exposure (USE) in the presence of microbubbles (MCB) (e.g. contrast agents used to enhance ultrasound imaging) increases plasmid transfection efficiency in vitro *by several orders of magnitude. Formation of short-lived pores in the plasma membrane ('sonoporation'), up to 100 nm in effective diameter lasting a few seconds, is implicated as the dominant mechanism, associated with acoustic cavitation. Ultrasound enhanced gene transfer (UEGT) has also been successfully achieved* in vivo, *with reports of spatially restricted and therapeutically relevant levels of transgene expression. Loading MCB with nucleic acids and/or disease-targeting ligands may further improve the efficiency and specificity of UEGT such that clinical testing becomes a realistic prospect.*
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In brief

Progress

- Technological developments have increased the efficiency of UEGT *in vitro* by up to 2 orders of magnitude over initial results.
- Research into the mechanisms underlying UEGT has increased our understanding of both the biophysical effects of ultrasound that are involved and the stages in the transfection pathway that are facilitated.
- The biophysical effect of ultrasound most clearly implicated in the mechanism of UEGT is acoustic cavitation.
- Mechanistic studies implicate plasma membrane 'sonoporation' (reversible pore formation, up to 100 nm in effective diameter with a half-life of a few seconds) as the dominant mechanism underlying UEGT.
- Studies of MCB oscillation and/or collapse during USE implicate both transient and stable cavitation in sonoporation.
- Multiple biophysical effects of ultrasound may contribute to sonoporation.
- The kinetics of sonoporation are complex and may involve active resealing of ultrasound-induced pores.
- Whether ultrasound affects later steps in the transfection process, particularly plasmid entry into the nucleus, remains unclear.
- Ultrasound-enhanced gene transfer *in vivo* has been successful, both with reporter and therapeutic gene constructs, showing both 'sufficiently efficient' gene transfer for biological effects and high spatial targeting of transgene expression.
- UEGT *in vivo* is relatively nontoxic.

Prospects

- Progress towards clinical application requires the development of standardized reporting of USE conditions to facilitate comparisons with diagnostic imaging and other UEGT publications.
- Defining the relative contribution and/or effectiveness of stable vs transient cavitation to sonoporation will facilitate the optimization of MCB and USE conditions for clinical application.
- The ultimate aim is to build upon preliminary studies that support the concept of producing disease-specific 'therapeutic' MCB comprising disease-targeting, nucleic acid-loaded MCB, specifically designed for UEGT, that can be injected intravenously, accumulate at the site of disease and respond optimally to the ultrasound beam focused on the diseased tissue.

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Technological developments have increased the efficiency of ultrasound enhanced gene transfer *in vitro* by up to 2 orders of magnitude over initial results

Ultrasound has many of the attributes of an ideal transfer system for gene therapy. It is non-invasive and well tolerated, with an extraordinary safety record over a wide range of frequency and intensity and high levels of public acceptability and understanding. Moreover, there are highly sophisticated, flexible, cost-effective and readily available diagnostic and therapeutic systems that can achieve site-specific transfer of ultrasound energy almost anywhere in the body, except perhaps the lung. The development of ultrasound enhanced gene transfer (UEGT) began when a number of research groups speculated that the physical perturbations induced by an ultrasound beam would encourage plasmid DNA uptake and transgene expression. Although the early results were at best modest, achieving 10- to 30-fold increases in transfection efficiency *in vitro*, technical refinements have meant that enhancements of up to several thousand fold *in vitro* are now being observed,[1–9] sufficient to encourage studies of UEGT *in vivo*.

Research into the mechanisms underlying UEGT has increased our understanding of both the biophysical effects of ultrasound that are involved and the stages in the transfection pathway that are facilitated

Understanding the mechanism(s) underlying UEGT is crucial to the further development of this technique towards clinical application. There are two interrelated aspects of this research. First, it is necessary to understand which of the many biophysical effects of ultrasound are crucial in the promotion of UEGT and whether these overlap with and/or are identical to any ultrasound bioeffects that may have toxic effects on the target cells and tissues. Such an understanding is crucial to maximizing the efficiency and safety/therapeutic index of UEGT. Second, it is essential to understand which of the many steps in the transfection pathway are facilitated by ultrasound exposure (USE), as this will help to identify any remaining rate-limiting steps that could, in theory, be overcome by combining ultrasound with other transfection methods. Many groups have contributed to our increased understanding of these issues, particularly through detailed optimization and analysis of UEGT *in vitro*.[1–30]

The biophysical effect of ultrasound most clearly implicated in the mechanism of UEGT is acoustic cavitation

The biophysical effects of ultrasound most relevant to UEGT include cavitation (the growth, oscillation and/or collapse of MCB within an acoustic field), radiation pressure (a net force in the direction of propagation of the acoustic field) and acoustic microstreaming (swirling shear forces in the vicinity of oscillating MCB).[31] Cavitation[32] has received the most attention, as many scientists have shown that the deliberate addition of echocontrast MCB[33,34] (used to enhance clinical ultrasound imaging) to the transfection medium before USE markedly increases subsequent transgene expression *in vitro*.[4,7,23,28,35–37] Cavitation may be subdivided into two categories (see Figure 1):

1. Stable cavitation occurs when MCB oscillate stably around a resonant diameter in a low-intensity acoustic field. This generates local shear forces and acoustic microstreaming.
2. At higher intensities, rapid bubble expansion during the rarefaction phase of the acoustic cycle is followed by forcible collapse because of the inertia of the fluid that rushes in during the compression phase; hence, the interchangeable terms inertial or transient cavitation. The dramatic compression forces within collapsing MCB generate highly localized extremes of temperature and pressure, which in turn are accompanied by secondary phenomena such as radial shock waves and free radical generation.

The likelihood of cavitation, which is increased by raising ultrasound intensity but is inversely related to frequency, is expressed as the mechanical index (MI) of an acoustic field. This figure is now displayed as a safety feature on the output displays of most clinical ultrasound machines. It is thought that cavitation is unlikely to occur at an MI of less than 0.7 (equivalent to pressure oscillations of seven atmospheres in positive and negative directions at a driving frequency of 1 MHz) although, importantly, the presence of echocontrast or other MCB in the acoustic field reduces this threshold by a rather unpredictable degree (see below).[38,39]

Mechanistic studies implicate plasma membrane 'sonoporation' (reversible pore formation, up to 100 nm in effective diameter with a half-life of a few seconds) as the dominant mechanism underlying UEGT

The prevailing dogma is that acoustic cavitation promotes UEGT principally through the generation of reversible pores in the plasma membrane ('sonoporation'), as evidenced by direct imaging and/or by analysis of increased plasma membrane permeability to marker compounds.[3,12,14,15,20,24,25,29,30,40–42] Recent studies have focused upon the role of transient cavitation in sonoporation. Sundaram *et al*.[12] examined the effect of low-frequency (20–93 kHz) ultrasound on uptake of the fluorescent dye calcein (molecular weight 623 Da, radius 0.6 nm) into mouse 3T3 fibroblasts in the absence of echocontrast agents but at MI levels (mostly above 1) sufficient to induce cavitation in non-degassed culture medium. There was a close and frequency-independent correlation between transient cavitation and calcein uptake. In contrast, there was no consistent relationship between calcein uptake and the extent of stable cavitation recorded during the same experiments. These data suggest that both stable and transient cavitation was occurring during insonation and that transient cavitation

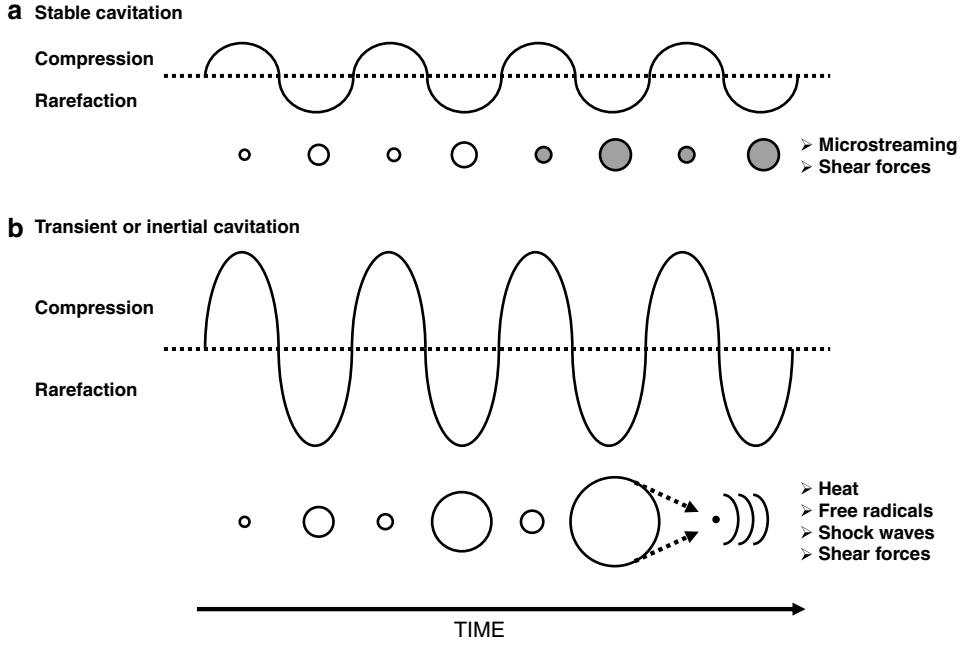

Figure 1 Schematic diagram illustrating the effects of acoustic fields of identical frequency but differing intensity on MCB behaviour. (**a**) Low-intensity ultrasound induces oscillation of pre-existing MCB, with a gradual increase in MCB diameter until a resonant diameter is reached, when stable oscillation occurs (filled circles). The term stable cavitation applies to the repeated oscillation of the MCB around this resonant diameter. (Note: MCB initially grow in size when insonified primarily because the surface area for dissolved gas to enter the MCB during the expansion (rarefaction) phase is greater than that available for gas to diffuse out of the MCB during the compression phase, a process known as rectified diffusion). (**b**) At higher intensities, the MCB grow rapidly for a few cycles. Very soon, however, the inertial energy of the fluid surrounding the MCB during the compression half cycle becomes so great that it cannot reverse direction when the next rarefaction half cycle arrives. It continues to rush in and forcibly collapses the MCB, generating highly localized extremes of temperature and pressure. This in turn generates shock waves, free radical production and local heat. This process is termed inertial or transient cavitation.

was the dominant influence on membrane permeabilization under these conditions. Very similar results were reported by Hallow *et al*.,[24] this time using 1–3 MHz ultrasound (MI 0.3–2) in the presence of the echocontrast agent Optison, which comprises an albumin shell encapsulating a perfluorocarbon gas. Once again, there was a very close correlation between calcein uptake and transient cavitation but not total acoustic energy transfer. These studies clearly demonstrate that transient cavitation is associated with membrane permeabilization in a 'dose'-dependent manner. Indeed, most studies of UEGT have employed USE conditions whereby MCB collapse is demonstrated directly or is likely to have occurred. The dose-dependent relationship between sonoporation and transient cavitation likely reflects the violence of the initial MCB collapse, and the effects of USE upon smaller gaseous bubbles released from the index MCB.

Multiple biophysical effects of ultrasound may contribute to sonoporation

Practical and theoretical experiments show that rapid bubble expansion, collapse and subsequent shock wave formation can generate shear forces that will disrupt cell membrane integrity and contribute to permeabilization.[12,16,43] Moreover, the geometry of MCB collapse is itself influenced by adjacent cell membranes, such that microjets of the surrounding fluid (which in transfection medium contains the exogenous nucleic acid of interest) may be 'injected' into the cell.[40,44,45]

More controversial is the potential role of extra and/or intracellular free radical production produced during transient cavitation.[25,46,47] We have reported that the presence of free radical scavengers in the transfection medium has no effect on transgene expression following UEGT, suggesting that free radicals are not required for sonoporation.[46] In contrast, Juffermans *et al*.[25] found that scavenging hydrogen peroxide (H_2O_2) with extracellular catalase largely prevented the increased calcium permeability induced by low MI (0.1–0.5) ultrasound (1–3.6 MHz) in H9c2 rat cardiomyoblast cells. However, these authors felt that only stable cavitation was generated in their system, despite the detection of increased intracellular H_2O_2 – somewhat surprisingly they did not report on extracellular levels.

Transient cavitation is a highly disruptive process that not only promotes UEGT but can also induce apoptosis, necrosis or cell disruption. Clarifying further whether free radicals are mediators of, or bystanders to, sonoporation is clearly crucial, not least because increased oxidative stress can itself have major deleterious effects in cells and intact tissues. In fact, it may not be an absolute requirement to induce transient cavitation to achieve sonoporation. Marmottant and Hilgenfeldt[11] showed that MCB are highly effective at 'concentrating' and focusing low-intensity long wavelength ultrasound so that it has profound effects at the micron or submicron level. Specifically, very low (<0.05) MI 180 kHz ultrasound (wavelength 8 mm) was shown to induce stable and gentle oscillations of MCB attached to a cuvette that, through the generation of microstreaming shear forces,

was able to induce disruption of lipid bilayers, an essential element of sonoporation. Such effects are also seen at higher MIs sufficient to produce stable oscillation without causing bubble collapse. For example, de Jong's group have used ultra high-speed (up to 10 MHz frame speed) real-time optical imaging to observe reversible cell deformation and membrane permeabilization in response to 0.4 MI 1 MHz ultrasound.[30] Both these and other studies indicate that close proximity between MCB and cell membranes facilitates sonoporation, suggesting that bringing MCB nearer to cells using acoustic radiation pressure and/or by ligand-targeting could improve the efficiency of UEGT and/or decrease the required acoustic power and dependence upon transient cavitation.

The kinetics of sonoporation are complex and may involve active resealing of ultrasound-induced pores

It has long been assumed that sonoporation is a transient phenomenon, but only recently have the kinetics of the process been studied in detail using fluorescent molecules or by measuring changes in ionic conductivity.[3,14,20,29,30,41] Estimates of the membrane recovery time range from a few seconds to at most a few minutes, with differing kinetics for small and larger molecules, and some evidence of separate pore populations that close at fundamentally different rates. The degree of sonoporation can be varied through changes in USE conditions, particularly the pulse repetition frequency and ultrasound intensity. Estimates of pore size based on the physical diameter of marker compounds are most commonly in the range of 30–100 nm. Microscopy studies, however, suggest that USE may induce up to micron diameter disruptions in the plasma membrane, but that these pores have a sieve-like character that only allows passage of molecules considerably smaller than the diameter of the defect.[29] From a UEGT perspective, we have found that USE can promote the transfer of plasmids carrying genomic DNA inserts, albeit at a much lower efficiency than standard reporter gene constructs (unpublished data). Recent data suggest that resealing of ultrasound-induced pores is an energy-dependent process that exhibits morphological features consistent with an active and vesicle-based wound-healing response by the cell.[29]

Whether ultrasound affects later steps in the transfection process, particularly plasmid entry into the nucleus, remains unclear

An important rate-limiting step for non-viral gene transfer is plasmid entry into the nucleus. Many groups have noted that sonoporation delivers compounds to the cytoplasm,[3,15,41,46] with nuclear exclusion of any but the smallest molecules (which can gain access through the nuclear pore complex). Moreover, the increase in transgene expression following UEGT is much less than might be expected from the increment in plasmid uptake.[15] These findings are intuitively correct, as the wavelength of kilo- and low megahertz ultrasound (0.5–50 mm) is much longer than cells and is unlikely to cause direct perturbations in the nuclear membrane. Moreover, any physical effects of extracellular MCB oscillation and/or collapse will be damped by the viscosity of the cytoplasm. Recently, however, Machluf's group have reported that exposing baby hamster kidney cells to 0.16 MI 1 MHz ultrasound for 30 min in the presence of Optison MCB yielded more rhodamine-labelled plasmid in the nucleus compared with a 10 min exposure.[22,23] Whether this truly reflects a direct effect of prolonged USE upon nuclear uptake is difficult to assess as there was also more plasmid in the cytoplasm after USE for 30 min, which clearly altered the plasmid concentration gradient from cytoplasm to nucleus compared with a 10 min USE. Of note was the observation that some Optison MCB remained intact after 30 min, again consistent with, but not proof of, a role for stable cavitation in promoting UEGT.

Ultrasound-enhanced gene transfer *in vivo* has been successful, both with reporter and therapeutic gene constructs, showing both 'sufficiently efficient' gene transfer for biological effects and high spatial targeting of transgene expression

The last few years have seen the establishment of UEGT as a viable technique for *in vivo* gene transfer. Most *in vivo* studies have concentrated on organs and tissues that are readily imaged by diagnostic ultrasound machines, including skeletal muscle, heart and kidneys.[35,36,48–62] Table 1 summarizes the results of some recent studies in these tissues using reporter genes,[35,36,48–54,58,60,61] and broadly similar data have begun to emerge from studies in the vascular,[4,63,64] neurology[65–67] and oncology[18,37,68] fields, the pancreas[69] and in hematology.[70] On first inspection the relatively modest enhancements in plasmid-mediated transfection after USE *in vivo* may appear disappointing compared with the results obtained *in vitro*. Indeed, we found that *ex vivo* transfection of saphenous vein segments using ultrasound yielded only 50-fold enhancements compared with the 3000-fold effects of ultrasound upon vascular smooth muscle cell transfection *in vitro*.[4] However, *in vivo* transfection is always less efficient than *in vitro* and several of the studies in Table 1 also report that the maximum efficiency of UEGT was similar to or better than viral or liposome-mediated transfection. Transgene expression post UEGT is transient, certainly when using standard promoters such as cytomegalovirus, generally peaking at 4–7 days and with rare exceptions is essentially gone by 21 days. Whether transgene expression could be prolonged using strategies to, for example, avoid promoter silencing remains to be determined. Perhaps most impressive is the consistent observation that reporter gene expression was highly restricted to ultrasound-exposed tissues, without any significant expression in the liver or other remote organs, in stark contrast to the situation with 'standard' adenoviral vector transfer. Table 1 also exposes one of the current problems with the field of UEGT, namely the inconsistent manner in

Table 1 Summary of recent studies investigating UEGT *in vivo* using marker gene expression

	Model system (all normal tissues)	*Transgene/nucleic acid*	*Microbubbles used/delivery method*	*Nucleic acid delivery method*	*Ultrasound conditions*	*Time course of expression/ delivery*	*Optimal expression/ delivery after USE c.f. other methods*	*Site specificity*	*Toxicity*	*Additional comments*
(a) Normal skeletal muscle										
Pislaru *et al*.[35]	Rat triceps brachii/ gastrocnemius	Luciferase	PESDA i.m. or i.a.	20–200 μg plasmid i.m. or 400 μg plasmid i.a. into iliac/ femoral artery	(1) 1.7 MHz; PW; MI set at 0.9–1.7; −0.32 to 0.56 Mpa (hydrophone); 3 min (2) 1 MHz; CW; 0.32–0.41 MI, −0.32 to 0.41 MPa, 0.5–0.75 W/cm^2; 3 min	Luciferase activity measured at day 3	10 × vs plasmid alone (i.m.) 5 × vs Lipofectamine (i.m.) 0.5 × vs Adenovirus (6x10^9 p.f.u. i.m.) i.a. results equivalent to i.m.	Negligible activity in remote organs and untreated muscles except ++ liver expression with i.m. adenovirus	Minor inflammation along needle track – no different to controls	PESDA alone without USE had no effect on transfection
Christiansen *et al*.[51]	Rat hindlimb skeletal muscle	Luciferase	Bespoke cationic MCB carrying plasmid i.v. or i.a.	40 μg MCB-coupled plasmid into jugular vein or 4 μg via femoral artery	1.75 MHz; PW; MI set at 1.9; −1.14 Mpa (hydrophone); pulses every 7 s for 15 min	Luciferase activity measured at day 4	Transfection after i.a. equivalent to 20 μg plasmid i.m. without MCB and 200 × greater than after i.v. injection	Nil detected in the lung or liver (i.v. injection)	Occasional microhaemorrhages	Surprisingly high transfection after direct i.m. injection of plasmid alone
Li *et al*.[53]	Mouse quadriceps	GFP	Albunex Levovist Optison i.m.	25 μg plasmid i.m.	1 MHz; PW; 50% DC; 2 W/cm^2: 2 min	Number of GFP fibres measured at day 7	10 × vs plasmid alone using Optison No effect with Levovist or Albunex	Not described	No evidence of inflammation or necrosis	Similar (10 ×) enhancement in CHO cells *in vitro* at 2% (v/v) Optison
Lu *et al*.[54]	Mouse (4 weeks and 6 months old) tibialis anterior	GFP	Optison i.m.	10 μg plasmid i.m.	1 MHz; PW; 20% DC; 3 W/cm^2 spatial peak power; 60 s	Number of GFP fibres measured at day 7	30 × vs plasmid alone in older mice	Not described	Inflammation reduced by Optison ±USE	Optison without USE effective in young mice (10–12 ×)
Wang *et al*.[61]	Mouse (4- to 5-week old) tibialis anterior	GFP	Optison Levovist SonoVue i.m.	10 μg plasmid i.m.	1 MHz; PW; 20% DC; 2 W/cm^2 spatial peak power; 30 s	Number of GFP fibres measured at day 7	3 × vs plasmid alone using SonoVue in these young mice	Not described	No increased toxicity except with Levovist	Optison effective without USE (4 ×) Levovist reduced efficiency
(b) Normal cardiac muscle										
Chen *et al*.[50]	Rat myocardium	Luciferase	Bespoke liposome-MCB containing plasmid i.v.	Up to 600 μg of MCB-associated plasmid via jugular vein over 20 min	1.3 MHz; PW; MI set at 1.6–2.0; ECG triggered; 20 min	Luciferase activity measured at day 4	Luciferase expression with plasmid MCB equivalent to adenovirus-MCB (0.25×10^{12} p.f.u.)	Highly restricted expression; anterior > posterior LV. Liver expression ++ with adenoviral MCB only	No arrhythmias or negative inotropic effects	USE enhanced adenoviral delivery to heart
Bekeredjian *et al*.[49] (same group as Chen *et al*.[50])	Rat myocardium	Luciferase	Bespoke albumin and lipid MCB containing plasmid i.v.	Approx. 350 μg of MCB-associated plasmid over 20 min i.v.	1.3 MHz; PW; MI set at 1.5; ECG triggered; 20 min	Luciferase activity measured up to 28 days post delivery and after repeated administration	Luciferase mRNA and enzyme activity peaked at 2–4 days; minimal residual activity at day 28 Retransfection successfully increased activity	Minor activity in the liver and/or pancreas ($\ll$1% c.f. myocardium)	Not reported	

Table 1 Continued

	Model system (all normal tissues)	*Transgene/ nucleic acid*	*Microbubbles used/delivery method*	*Nucleic acid delivery method*	*Ultrasound conditions*	*Time course of expression/ delivery*	*Optimal expression/ delivery after USE c.f. other methods*	*Site specificity*	*Toxicity*	*Additional comments*
Guo *et al*.[36]	Mouse myocardium	Luciferase and β-Gal	Bespoke air-containing albumin MCB	Up to 60 μg plasmid mixed with MCB and injected via tail vein (TV)	1.3 MHz; PW; MI set at 1.5; 2 min	Transgene activity measured up to 24 h post USE	8 × c.f. plasmid alone	Not reported	Transient elevation in cardiac enzymes	Comparable *in vivo* and *in vitro* enhancements with USE
Tsunoda *et al*.[60]	Mouse myocardium	Luciferase, β-Gal and GFP	BR14	500 μg plasmid mixed with BR14 injected directly into LV cavity or TV over 10 s	1 MHz; PW; 10–50% DC; 1.0–2.0 W/cm^2; 60 s	Transgene expression measured at days 2–7	Negligible transfection without USE; LV injection 60 × better than TV Peak expression at 4 days in subendocardium nearest transducer	No transgene detected in remote organs	Temporary AV block but no effect on contractility	High MCB concentration needed for optimal transfection
(c) Normal kidney										
Azuma *et al*.[48]	Mouse kidney	Luciferase	Optison	50 μg plasmid mixed with MCB and infused into renal artery *in situ* with renal vein clamped USE performed *ex vivo* and kidney retransplanted as autograft	2 MHz; 2.5 W/ cm^2; 30 s–8 min	Luciferase activity measured at day 4 post autograft	25 × c.f. plasmid alone using 1 min USE and 25% (v/v) Optison	Not reported	Not reported	Levovist ineffective
Lan *et al*.[52]	Rat kidney	FITC-labelled oligonucleotides	Optison	5 μmol oligo injected into surgically exposed renal artery with renal artery and vein clipped USE performed *in situ*	1 MHz; CW; '5% power output'; 2 min	Fluorescence microscopy 45 min after injection and USE	Up to 95% glomerular and 70–80% tubular cells positive for FITC-ODN – 1000 × c.f. without USE	Not reported	No histological or functional abnormalities	
Koike *et al*.[58]	Rat kidney	Luciferase	Optison	50 μg plasmid injected as in Lan *et al*.[52]	1 MHz; CW; '5% power output'; 60 s	Luciferase activity measured days 4–21 post transfection	USE with 25% (v/v) Optison up to 100 × c.f. USE without MCB and 6 × c.f. HVJ liposomes	Peak expression at day 7, still detectable at day 21	Histological damage due to high Optison concentrations (>25% (v/v)), not USE	

Abbreviations: AV, atrioventricular; CW, continuous wave; DC, duty cycle; GFP, green fluorescent protein; LV, left ventricle; MCB, microbubble; MI, mechanical index; MPa, MegaPascals; PW, pulsed wave; USE, ultrasound exposure.

which the USE conditions used are reported. This hampers our ability to compare the results of different studies and makes it difficult to draw generalizable conclusions about which aspects of the ultrasound beam are most crucial in optimizing the efficiency of UEGT (see below).

Encouraging results using reporter genes in healthy tissues have led several groups to investigate whether UEGT is 'sufficiently efficient' to achieve 'therapeutic' non-viral gene transfer. Table 2 summarizes some recent examples using animal models and therapeutic transgenes that have potential clinical relevance.[4,37,55,56,69,70] Although these and other *in vivo* studies provide proof-of-concept that UEGT can indeed achieve therapeutically effective gene transfer, the delivery approaches used (e.g. direct injection of plasmid/MCB into the liver or left ventricular cavity, UEGT requiring temporary cessation of blood flow in target organs) are in many cases unattractive for clinical use, certainly if repeat administrations for chronic conditions are contemplated.

UEGT *in vivo* is relatively non-toxic

UEGT can, like other transfection techniques, be associated with intrinsic toxicity that offsets the beneficial effects of therapeutic gene transfer. For example, we have reported that *ex vivo* transfection of porcine saphenous vein segments with human tissue inhibitor of metalloproteinase 3 plasmid reduces the deleterious vascular remodelling that occurs after interposition grafting into the arterial circulation.[4] However, the net beneficial effect was reduced by the fact that USE alone increased neointima formation, possibly through a free radical-dependent mechanism. USE can also induce microcapillary rupture and extravasation of erythrocytes and marker compounds into the tissue interstitium. This response is dependent upon the ultrasound and MCB 'dose' and different tissues exhibit varying susceptibility to this phenomenon.[71] Whether increased microcapillary permeability is an important mechanism underlying UEGT *in vivo*, or is an unwanted/unnecessary and potentially deleterious side effect remains an unanswered question. Conversely, ultrasound-induced microcapillary rupture (UIMR) may have therapeutic potential as there are reports that UIMR induces angiogenesis and encourage stem/progenitor cell recruitment to ischaemic skeletal muscle and myocardium.[72,73] This could be combined with UEGT-mediated proangiogenic gene transfer to further encourage revascularization of ischaemic tissues. Overall, there seems to be a reasonable therapeutic index for UEGT, as very little toxicity has been observed in a wide variety of tissues. Whether this will still pertain if and when the efficiency of UEGT is increased further by continued refinement of USE parameters remains to be determined. What is clear is that UEGT exhibits quite remarkable tissue specificity, with transgene expression highly restricted to insonified tissues even after systemic injection of plasmid DNA. In some cases, indicated in Tables 1 and 2, the nucleic acid was physically combined with the MCB, which may explain some of this spatial restriction in transgene expression. This spatial targeting has been observed using standard imaging transducers as well as high intensity focused ultrasound equipment.[68,74] The fact that UEGT can be so precisely targeted *in vivo* is a major potential safety advantage over many viral vector systems. With regard to translation to studies in man, it is often quoted that UEGT can be effectively achieved using 'diagnostic ultrasound', with the implication that there should be a simple and safe transition to studies of UEGT in man.

Although many investigators do indeed use diagnostic ultrasound machines to achieve UEGT, the total energy transfer is often much greater than is employed during diagnostic examinations. Moreover, the MCB concentrations used in most animal studies are much higher than those licensed for echocontrast imaging in patients, which is important as the presence of MCB dose dependently reduces the threshold for ultrasound-induced cavitation and associated bioeffects.[31,32,75–77] This again emphasizes the need for caution and for a standardized format for reporting of USE conditions in the UEGT literature, so that comparison with the instantaneous USE parameters and total 'dose' pertaining to clinical examinations and other individual studies of UEGT can be made. The need for such a standardized reporting format has been recognized by the International Society for Therapeutic Ultrasound, and a consensus statement on this topic is under active development.

Prospects

Recent developments in the biotechnology of echocontrast agents have permitted ligand modification of the MCB surface to facilitate targeting and accumulation at disease sites that express one or more cognate ligands.[33] This approach has been used extensively for the molecular imaging of inflammation and angiogenesis and to promote thrombus selectivity and MCB retention for ultrasound-enhanced thrombolysis. Whether using ligand-modified MCB will significantly improve the efficiency and spatial targeting of therapeutic UEGT *in vivo* remains to be determined. It is also possible to attach anionic plasmid DNA and other nucleic acids to MCB, either through charge interactions with cationic MCB or by mixing the DNA with the MCB components during their preparation. As mentioned above, there are theoretical reasons to believe that plasmid-bearing MCB will promote UEGT at the cellular level. The concept of injecting a single 'therapeutic' MCB that carries the plasmid payload is also attractive from a practical perspective and the results using plasmid-loaded MCB in animal studies are encouraging. The ultimate 'therapeutic' MCB for gene therapy, particularly when repeated administration is contemplated, would be one that carries the therapeutic plasmid, is ligand targeted to specific disease sites and is protected against plasmid degradation by circulating nucleases. Dual loading of MCB with plasmid and targeting ligands appears technically feasible, but once again the added value of ligand targeting (which may reduce the plasmid carrying capacity) remains unclear as ultrasound delivery itself can be highly targeted to the disease site(s). Protection against nucleases may be achieved through coating with novel polymers that do not affect the acoustic characteristics of the MCB.[78] It may also be possible to design 'bespoke' MCB that are specifically

Table 2 Summary of recent studies investigating UEGT *in vivo* using 'therapeutic' genes in normal and diseased tissues

	Clinical disease target	*Animal model*	*Therapeutic transgene/ constructs*	*Microbubbles used/ delivery*	*Nucleic acid delivery method*	*Ultrasound conditions*	*Effectiveness of UEGD*	*Toxicity*
Kondo *et al*.[55]	Myocardial remodelling post-acute myocardial infarction (MI)	Rat; acute left coronary artery ligation; UEGT immediately after ligation	Plasmid encoding human hepatocyte growth factor; CMV promoter	20% (v/v) Optison infused at 0.2 ml/min for 5 min i.v.	1500 μg plasmid via LV catheter in 1.5 ml over 1.5 min, beginning 1 min after onset of MCB infustion	1.3 MHz; PW; end systole; −2.1 kPa, 2–4 min	Up to 4 × higher HGF expression of plasmid alone with more homogeneous distribution 20% reduction in LV mass at 3 weeks with improved function Myocardial scar reduced by >100% Improved angiogenesis	No difference in mortality of no ultrasound controls
Zhigang *et al*.[56]	Therapeutic myocardial angiogenesis post MI	Rat; left anterior descending coronary artery ligation; UEGT 3-day post ligation	Plasmid encoding $VEGF_{121}$	Quanfuxian (albumin/ perfluorocarbon MCB) i.v.	2000 μg MCB-associated plasmid i.v.	1.8 MHz PW; 'maximum MI'; ECG triggered	Significant enhancement in VEGF expression and 80% increase in capillary density in ischaemic myocardium	Not reported
Sakakima *et al*.[37]	Hepatocellular carcinoma (HCC)	Nude mice; subcutaneous HCC	Plasmid encoding human interferon-β (IFN-β); CMV promoter	BR14 injected into tumour	50 μg plasmid injected into tumour	1 MHz; 50% DC; 2 W/cm^2; 10 min	Relative tumour growth reduced by 70% over 6 weeks and 60% of nodules completely resolved in UEGT group c.f. none in controls without USE	No toxic effects c.f. controls
Akowuah *et al*.[4]	Prevention of saphenous vein graft failure	Porcine saphenous vein interposition grafts into carotid artery	Plasmid encoding human tissue inhibitor of metalloproteinase 3 (TIMP-3); CMV promoter	BR14 (50% v/v)	Segments of vein instilled and bathed in plasmid at 33 μg/ml	1 MHz; PW; 6% DC; 1.8 MI; 2 min total USE performed *ex vivo*	>50% increase in lumen area in TIMP-3/USE group c.f. controls	USE alone associated with increased neointima formation
Miao *et al*.[70]	Hemophilia	Wild-type mice; hepatic transfection	Plasmid encoding human factor IX; liver-specific promoter	Bespoke MCB	50 μg plasmid mixed with MCB injected directly into liver	1 MHz; PW; −4 MPa	Factor IX levels of up to 63 ng/ml, near therapeutic, 66 × c.f. plasmid alone	Transient mild liver inflammation
Chen *et al*.[69]	Type I diabetes mellitus	Rat; normal pancreatic islets	Plasmids encoding (1) human insulin and (2) hexokinase driven by rat insulin 1 promoter	Bespoke phospholipid MCB carrying plasmids i.v.	250 μg MCB-associated plasmid over 20 min i.v.	1.3 MHz; PW; ECG triggered; MI 1.2–1.4; 20 min; focused on pancreas	(1) Up to 20 × serum insulin/C-peptide for 5–10 days, and 20% reduction in serum glucose (2) 4 × increase in serum insulin and 30% reduction in serum glucose	No histological evidence of pancreatic inflammation, no change in serum amylase or lipase

designed to promote sonoporation and UEGT, and indeed recent studies indicate that hard shell (polymer-based) echocontrast MCB may be more effective for UEGT than earlier generation 'soft shell' albumin or phospholipid formulations (T Bettinger, unpublished data). Combining UEGT and/or MCB with liposomes,[79] detargeted viruses or viral components[35,80] or other DNA-condensing transfection reagents[5] and/or modifying plasmid promoters and constructs also offers the possibility to improve expression levels and tissue selectivity. Finally, the ease with which UEGT may be performed safely in a 'no-touch' sterile environment *ex vivo* has great attraction in the context of modifying stem cells harvested from patients before reinjection, either to enhance homing to disease sites and/or to induce therapeutic transgene expression.

Summary

UEGT has in recent years 'come of age' as a potential delivery system for gene transfer *in vivo*. The promise of low toxicity and high targetability has in large measure been borne out by recent experimental studies, and the efficiency of UEGT appears adequate to achieve biological effects in a variety of normal and diseased tissues in animals. The clinical application of UEGT in man will depend on:

- the identification of optimal disease targets for this approach,
- further refinements to minimize the dose of MCB required while increasing efficiency,
- the production of bespoke MCB for targeted gene transfer and
- further optimization of USE parameters to bring them as close as possible to the exposure conditions used in clinical imaging, preferably concentrating on inducing stable rather than transient cavitation, as this gives the greatest likelihood that UEGT will prove safe in humans.

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