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Ultrasound-Mediated Gene Delivery

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1. Introduction

Human gene therapy holds great promise in treating not only hereditary genetic disorders, but also disease states such as cancer and viral infections, and contingencies such as stroke or myocardial infarctions. It can be achieved by delivery of a correct gene into target cells with genetic deficiency or mutations, or by transfer of a therapeutic agent such as agents targeting a cancer-causing oncogene, growth factor gene, antisense oligonucleotides (ODN), or small interfering RNA (siRNA) to correct the disease state using either viral or nonviral vectors. Viral gene therapy has succeeded in many animal disease models {Snyder 1999}, and has progressed to clinical trials {Hacein-Bey-Abina *et al.* 2002; Kay *et al.* 2000}. However, significant obstacles remain, including immune responses {Manno *et al.* 2006} or tumor genesis {Hacein-Bey-Abina *et al.* 2003}. A nonviral approach would provide a safer strategy. The potential for therapeutic ultrasound (US) to effect minimally invasive nonviral gene transfer has long been recognized, and a growing body of evidence indicates that significant enhancement of transgene expression can be achieved by using high frequency acoustic energy. In addition to its well-known role in providing inexpensive, real-time imaging capability, US has been used therapeutically for years {Herzog *et al.* 1999}. The most common therapeutic application involves low acoustic intensities and is intended to heat deep tissues; *e.g.*, as used in sports medicine. At the other 'end' of the acoustic intensity spectrum is HIFU (high intensity focused ultrasound), which can be used to ablate {Fischer *et al.* 2010} or to liquefy tissues {Hall *et al.* 2009}. US of intermediate intensities has been applied to many systems, together with exogenous microbubbles [MBs], to use the acoustically-forced behavior of the MBs to generate desired bioeffects. The latter usually involves changing the permeability of endogenous barriers to otherwise impermeable materials (*e.g.*, drugs or macromolecules). Many gene therapies have been attempted by direct intramuscular or intraparenchymal injection of gene vectors; these vectors gain immediate access to the interstitial space and must then traverse the plasma membrane of the targeted cells. US contrast agents are almost always administered intravascularly. When accompanied by a gene vector, the first barrier encountered is the vascular endothelium. The next are other vascular anatomical features (*e.g.*, the basement membrane, smooth muscle layer, *etc.*) and then the outer cell membrane of the cells one hopes to target. Finally, DNA needs to be transferred across the nuclear membrane to enter the nucleus for efficient gene expression.

This review will focus almost entirely on the use of ultrasound targeted microbubble destruction (UTMD) as a means by which to deliver foreign DNA (or drugs or photo

reactive nanoparticles, as other examples for which there are numerous publications) into targeted host tissues and cells. The literature on this topic is growing at an incredible rate, because almost immediately following the commercial availability of microbubble-based US contrast agents in the 1980s, it was recognized that acoustic cavitation could cause potentially undesirable bioeffects, desirable ones, or both.

Exploitation of acoustically-activated MBs for therapeutic effect remains an exciting topic. Indeed, harnessing the dynamical behavior of acoustic MBs is somewhat of the 'holy grail' of acoustically-targeted gene (or drug) delivery {Lindner 2009}. However, a healthy skepticism has been (see, *e.g.*, {Villanueva 2009} and should continue to be, applied to claims of great successes achieved using UTMD, as replication studies are few, and contradictory findings not unusual. Skepticism should obtain especially when considering 'black box' US studies.

A reasonably coherent picture of US-mediated gene therapy is emerging. With UTMD techniques, the issue ultimately reduces to the fact that US can force dynamic behaviors of MBs. Endogenous MBs are absent in most tissues of the body {Carstensen *et al.* 2000; Gross *et al.* 1985}. Exogenously-administered MBs are generally inert without acoustic exposure, but can be made to pulsate gently, or to undergo violent but highly localized dynamic behavior when driven by acoustic fields. Targeting can thus be achieved by methods as simple as co-administering MBs and gene vectors and exposing the targeted tissue to US, which will thus be the only site where bubble activation occurs. As we shall see, bioeffects typically arise when MBs are driven at pressure amplitudes sufficient to produce nonlinear bubble oscillations.

Here we will discuss the issues broadly, in the hope that the reader will gain a general understanding of the techniques, applications, apparent mechanisms, and some insights into what has been achieved. We focus most of our discussion on *in vivo* studies, as US-assisted gene therapy *in vivo* continues to be a more challenging problem than US-enhanced cell permeabilization *in vitro* or even in intact *ex vivo* tissues (see; *e.g.* {Kodama *et al.* 2005}). We have striven to be as jargon-free as possible, and have neglected mathematical treatments of the topics discussed here, as these can be found elsewhere. A few simple **abbreviations** are used: **US** (ultrasound); **MB** (microbubble); P_a or P_r (acoustic pressure amplitude or rarefaction pressure, respectively); and **pDNA** (plasmid DNA). Some words on US exposure metrics are also necessary. In some applications of therapeutic US (*e.g.*, tissue heating), the acoustic intensity in dimensions of W/cm^2 is the parameter of interest. In contrast, the occurrence and character of acoustic cavitation *in vivo* is largely determined by the presence or absence of exogenous MBs and by *peak acoustic intensity, or more properly, by the peak acoustic pressures*. These are expressed in units of mega- (MPa) or kiloPascals (kPa), where 1 MPa = 10 atm. Acoustic intensity scales as the square of the pressure amplitude. The quality of acoustic reporting in the US-mediated gene therapy literature varies widely. We will describe most acoustic exposures in terms of the pressure amplitude (P_a) or the peak rarefactional acoustic pressure (P_r), sometimes inferred by us. In any case, it is not our purpose here to be rigorously quantitative.

2. Ultrasound-targeted microbubble destruction: Physics & technology

There are many potential applications for UTMD gene delivery; a large body of work has been conducted using both *in vitro* and *in vivo* model systems to understand if, and how, UTMD 'works' to produce therapeutic effects. Most studies have as their ultimate goal the application of the technology to effect minimally-invasive treatment of disease or

contingency, although *in vitro* UTMD transfection techniques are being used for cell-based therapies {Otani *et al.* 2009}. Specific applications to various organs or for various clinical conditions will be discussed in **§5**. Here it is sufficient to note that in UTMD-based gene delivery studies, much of the work has focused on model or surrogate systems; *e.g.*, the delivery of reporter genes rather than therapeutic ones. However, therapeutic gene transfer effects such as tumor volume reduction have been reported in some model systems.

Therapeutic US has the potential for enhancing minimally invasive gene therapies. For gene therapies involving naked DNA vectors in particular, UTMD techniques have many desired characteristics. These include (1) low toxicity of all components of the treatment, (2) low immunogenicity of the vectors, (3) low invasiveness (*e.g.*, the vector and gas bodies can be administered intravascularly, and for sonographically-accessible organs, the therapeutic US can be applied through the skin), (4) there is good potential for repeated application, (5) organs can be targeted with high specificity, and (6) the technique has broad applicability (again related to sonographic accessibility). However, low efficiencies remain a problem.

2.1 What is ultrasound targeted microbubble destruction?

Here we hope to provide a sense of the types of MBs often used in US-mediated gene therapies, the ways in which US can cause these MBs to be destroyed (either gradually or abruptly) and/or otherwise activated, and the mechanisms by which UTMD-induced microvascular damage, extravasation, and target cell uptake of gene vectors may occur. A more comprehensive discussion of the physics can be found in {Wu & Nyborg 2008}.

In broad terms, the targeting 'part' of UTMD therapies is based *principally* on the fact that MBs present in tissues respond dynamically only if the area is exposed to US. Ligands incorporated into the MB shell may enhance accumulation of the MBs and any vector load they carry in a region of interest, but it is the selective acoustic exposure which 'activates' the MBs. As mentioned, site-specific ligands can be added to MB shells to improve retention in regions of interest. This field has received considerable attention {Ferrara *et al.* 2009}. Some degree of targeting can also be achieved by acoustically 'pushing' bubbles to a region of interest. A propagating acoustic field exerts a radiation force on MBs {Sarvazyan *et al.* 2010}, and this force can propel them in the direction of wave propagation. If the MB is undergoing radial oscillations {Emmer *et al.* 2007}, the bubble lurches forward, slowing as it expands and accelerating as it collapses. Radiation force can be used to concentrate MBs along a specific wall of an intracavitary space {Horie *et al.* 2010}, or blood vessel.

The MB destruction 'part' of UTMD occurs in response to the acoustic exposure, and may result in release of lipid or aqueous-phase drugs {Smith *et al.* 2010} or produce small-scale damage to microvessels which allows normally impermeable materials (drugs, plasmids, even objects as large as cells or MB fragments) to escape the vascular lumen and enter the interstitial space. Cellular permeabilization is also associated with MB activation.

2.2 Ultrasound contrast agents and other stabilized gas bodies

Microbubble US contrast agent evolution from early, agitated saline or sugar solutions to protein-shelled agents containing air to lipid- or polymer-shelled agents using relatively insoluble gases was rapid. The evolution of these agents continues {Qin *et al.* 2009}, as do new applications for them {Cosgrove & Harvey 2009}. US contrast agents are typically micron-sized gas bodies which are stabilized against diffusion by a shell {Overvelde *et al.* 2010; Sarkar *et al.* 2009}; most contrast agent MBs have mean diameters of ~2 μm. New

agents currently under development for imaging or therapy are smaller still, and are considered to be nanobubbles {Krupka *et al.* 2009}. Multi-layered structures in which a gas body stabilized by a lipid monolayer is contained in an aqueous compartment bounded by a lipid bilayer (echogenic liposomes) are also under development. *When used for imaging, US contrast agents are unique amongst contrast agents in that they respond dynamically to the signal used to interrogate tissues for their presence.* It is this same property that is exploited for UTMD therapies or therapy models.

2.3 Microbubble destruction: Shell disruption and shell/gas body fragmentation

There are three principal mechanisms by which US or time can destroy a shelled MB {Chomas *et al.* 2001}. In increasing order of 'violence' to the MBs, these are: (1) static diffusion, in which gas dissolves into the surrounding host fluid. This may be rapid if the bubble is 'free' (without stabilizing shell) or very slow if a stabilizing shell is present; (2) acoustically-forced shell disruption, which leads to accelerated diffusive loss of gas relative to unperturbed shelled bubbles; and (3) shell fragmentation and rapid loss of gas.

A micron-sized free air bubble can be expected to dissolve in water in ~30 ms {Sarkar *et al.* 2009}, which is why relatively insoluble gases such as perfluorocarbons are now used in modern contrast agents, and a stabilizing shell is employed. The principal mechanism by which bubble shell materials stabilize MBs against diffusion is by reducing the surface tension at the bubble surface. Shell properties also affect the dynamic responses and stability of MBs at P_a below the shell fragmentation threshold {Emmer *et al.* 2007; Ferrara *et al.* 2009}.

Acoustically-forced MB dissolution can occur when the P_a is sufficient to drive MB oscillations which stretch and compress the shell sufficiently to produce relatively small defects in the shell, which may re-seal {Huang 2008}. Very high physical stresses develop in the shells as the MB oscillates, even when the driving pressures are only a few hundred kPa {Stride & Saffari 2003}. Small shell defects lead to more rapid dissolution of the gas body than would otherwise occur, but complete gas dissolution may require many acoustic cycles {Smith *et al.* 2010}. For examples, Optison MBs exposed to 3.5 MHz US of $P_a > 0.15$ MPa undergo accelerated loss of gas, indicating shell compromise {Porter *et al.* 2006}. Shell disruption P_a thresholds for albumin-stabilized Optison and surfactant stabilized Sonazoid are similar; at 1.1 MHz, these are 0.13 MPa or 0.15 MPa, respectively. At 3.5 MHz, these thresholds are somewhat higher (Optison: 0.48 MPa; Sonazoid: 0.58 MPa) {Chen *et al.* 2003}. Others have reported a somewhat lower threshold (0.15 MPa) for Optison shell disruption at 3.5 MHz {Porter *et al.* 2006}. Likewise, {Borden *et al.* 2005} studied the behavior of lipid monolayer-encapsulated MBs at 2.25 MHz using single-cycle pulses, and found that the P_r threshold for slow MB dissolution to be in the range of 0.4 – 0.6 MPa, and the P_r threshold for MB fragmentation to be 0.8 MPa. A P_a of around 0.3 MPa was also found to be the threshold for soft-shelled contrast agent MB disruption {de Jong *et al.* 2009}. Echogenic liposomes also appear to have two pressure thresholds; one associated with compromise of the shell of the interior gas body, and a higher one associated with disruption of the outer lipid bilayer {Smith *et al.* 2010}. Rapid fragmentation occurs on a time scale of microseconds when P_a is sufficient to buckle and completely rupture the shell {Marmottant *et al.* 2005}, creating free gas bodies which may then dissolve, grow by rectified diffusion, coalesce into larger bubbles, or dissolve, depending stochastically on exposure conditions.

2.4 Microbubble dynamics: An overview of how gas bodies respond to ultrasound

At very low P_a, MB volume oscillations are related linearly to P_a. At modest P_as of a few hundred kPa (or less), MB volume oscillations become non-linearly related to P_a, with bubble expansion being relatively slow and bubble collapse much faster, being governed by the inertia of the in-rushing surrounding fluid to a greater extent than by the compressive phase of the applied pressure field; hence the phenomenon of bubbles undergoing acoustically-driven expansions followed by rapid, inertially-dominated collapse is termed 'inertial cavitation'. This typically occurs when a MB has been driven by the rarefaction phase of the acoustic wave to a diameter roughly 2 – 3 times the initial diameter {Chomas *et al.* 2001}. At very modest P_as, inertial cavitation can be stable and sustained for an almost indefinite number of acoustic cycles. For example, {Church & Carstensen 2001} found that surfactant-coated Sonazoid MBs could undergo repetitive inertial collapses and rebounds when driven by 2.5 MHz US at acoustic pressures greater than about 0.3 – 0.4 MPa; for this agent, irreversible post-collapse fragmentation occurred at a P_a of ~ 1.5 MPa. Others have reported that a 3 μm bubble exposed to 2.25 MHz US can be expected to undergo expansion, inertial collapse, and fragmentation at a P_a just over 0.3 MPa {Chomas *et al.* 2001}. The albumin-shelled, first-generation US contrast agent Albunex began to emit acoustic signatures characteristic of nonlinear oscillations at acoustic pressures as low as 0.005 – 0.010 MPa {Krishna & Newhouse 1997}. At even modest P_a (*e.g.*, 0.6 MPa at 1 MHz), some contrast agent bubbles can expand to 10 times or larger than their equilibrium radius; under such conditions, inertial collapse is expected. Fragmentation of the inertially-collapsing bubble occurs at some instant near the time of minimum bubble radius {Postema & Schmitz 2007}. Inertial bubble collapse is so rapid and the gas so compressed that the maximum temperature inside the bubble can reach more than 5000 K {Apfel & Holland 1991}. Light emissions {Matula 2003} and reactive free radicals may be produced {Okada *et al.* 2009}. MB rebound and fragmentation can re-emit acoustic energy at many times the excitation pressures. These emissions also contain higher frequency spectral components which can produce local heating. In the present context, however, it appears that the most important determinant of UTMD gene delivery methods is that *bubble expansion and collapse can produce local tissue distortions or damage which are presumed to be the principal mechanism by which microvascular bioeffects become manifest, and target cell permeabilization occurs.*

Cavitation bubbles can interact with nearby boundaries; these interactions can produce high velocity fluid jets and induce bubble translation, with the directions of fluid jets and translation depending on the boundary's properties. For rigid boundaries, bubbles closer to the boundary than about twice the fully-expanded radius of the bubble {Kodama & Tomita 2000} may collapse asymmetrically, with a high velocity water jet 'punching through' the bubble and impinging on the rigid boundary. Near rigid, planar boundaries, an oscillating MB translates toward the boundary and if collapse jetting occurs, the jet is directed toward the rigid surface {Plesset & Chapman 1971} with sufficient water hammer pressure to damage the surface {Blake & Gibson 1987}. *In vivo*, however, this condition would seem rare.

MBs driven to oscillate near pressure release boundaries (an air-water interface, for example), collapse jets which occur are directed away from the boundary, as is the direction of bubble translation {Chahine 1977; Robinson *et al.* 2001}.

Oscillating MBs collapsing near a 'soft', planar boundary (*e.g.*, gels or large vessel walls) also undergo translation and jetting, but the directions of jetting and translation can be either toward or away from the boundary, being determined by bubble size, bubble distance from

the boundary, and the mechanical properties of the boundary (see. *e.g.*, {Kodama & Tomita 2000; Shima *et al.* 1989}. Deformation of the elastic boundary stores energy which is 'returned' as the bubble collapses; this creates a hydrodynamic pressure gradient which in turn produces fluid flow away from the elastic boundary. If the pressure gradient is large enough, bubble collapse jets directed away from the boundary, and bubble translation away from the boundary, can result {Blake & Gibson 1987}. Liquid jets were observed in an early experimental study of acoustically forced MB behavior in a 200 μm diameter cellulose tube {Postema *et al.* 2004}. In vessel-simulating gel tunnels, at 1.7 MHz, the P_a at which Optison MBs began to emit broadband noise characteristic of inertial collapse and rebound was weakly dependent on tunnel diameter (~0.8 MPa in 90 μm diameter tunnels *vs*. ~0.6 MPa in 800 μm tunnels) {Sassaroli & Hynynen 2007}. Single-MB dynamics observations in actual microvessels were acquired using a rat cecum model; MBs translated toward the vessel walls and a toroidal bubble morphology consistent with the formation of a microjet was observed {Caskey *et al.* 2007}.

Where bioeffects attributable to inertial cavitation occur, cavitation jets directed toward cells or tissues have long been assumed to be causative. Vessel 'stretching' during bubble expansion has also been proposed as a mechanism of vessel damage {Miao *et al.* 2008}. Very high speed imaging of MBs driven to growth and inertial collapse in microvessels of rat mesentery {Chen *et al.* 2010; Chen *et al.* 2011} indicate that (1) MB collapse jets form frequently in the intravascular environment, (2) jets are typically directed *away* from the nearest blood vessel wall, (3) blood vessel expansion in response to bubble growth is minimal, and (4) tissues 'follow' the collapsing bubbles such that inward vessel distortions are much greater than outward distortions, with inward vessel wall motion having speeds of 5 – 10 m/s and the events occurring on microsecond time scales. This is perhaps one mechanism by which vessel permeabilization occurs. In any case, permeabilization of biological transport barriers is associated with some cell killing, both *in vitro* {Brayman *et al.* 1999} and *in vivo* {Ferrara 2008; Miller *et al.* 2011; Price *et al.* 1998; Skyba *et al.* 1998}

Finally, it is worth mentioning that MB-excitation techniques for cellular permeabilization need not use US to achieve MB activation or cell permeabilization; targeted laser illumination can also be used effectively. For a recent research paper on this topic, using a liposome MB contrast agent, see {Zhou *et al.* 2010}.

2.5 Effect of different shell compositions or agents on gene delivery

It is difficult to compare the efficacy of different contrast agents in US-mediated gene delivery between different studies, because MB type, concentration as injected, rate of injection, total dose, animal model, US exposure source, exposure conditions, and even the way the MBs are handled vary widely and can influence outcome. MB concentration is a determinant of UTMD-mediated pDNA expression in mouse liver {Miao *et al.* 2005; Shen *et al.* 2008}. Different agents have different 'native' concentrations, and agents with similar gas content but dissimilar shells can have different efficacies in enhancing gene delivery under otherwise comparable conditions (*cf.* effects of Optison and PESDA MBs; {Pislaru *et al.* 2003}). Relatively large differences between contrast agent types in UTMD gene delivery have been observed, but the reasons are not always clear. With equalized MB concentrations and either Optison, SonoVue or Sonazoid contrast agents, UTMD-mediated transfer of phosphorodiamidate morpholino oligomer to a dystrophin-deficient murine heart model induced dystrophin-positive cells in the hearts when harvested 7 days after treatment.

Optison and Sonazoid were equivalent in effectiveness, producing about four times greater expression than did SonoVue {Alter *et al.* 2009}.

2.6 Sensitivity of stabilized gas bodies to technique

Bubbles are buoyant, fragile, can coalesce into larger bubbles, or 'disappear' as gas is lost to diffusion. In many *in vivo* studies of UTMD, the MBs and gene vectors are infused slowly; this prolongs the time window in which acoustic treatments can occur, but aggravates the problem of time-dependent changes in MB distribution and delivery {Kaya *et al.* 2009}. It is worth noting that MBs can be destroyed by static pressure or tension; an over-pressure of only ~0.3 MPa can destroy them {Stringham *et al.* 2009}. MBs can also be destroyed by drawing up or injecting an MB suspension *too rapidly* when using a small gauge needle {Talu *et al.* 2008}; thus one can unwittingly alter experimental outcome by 'over-enthusiasm'.

3. Breaching the physical barriers to gene delivery using ultrasound

In order to achieve efficient gene expression following ultrasound-mediated gene delivery, multiple barriers need to be overcome to allow pDNA to enter into the nucleus of target cells including penetrating vascular and cellular membranes as well as trafficking through different intracellular compartments.

US contrast agents are intended to be intravascular agents. Whether the gene vector is administered as a simple mixture with the gas body suspension, or is in some way linked to the gas bodies, they are also intravascular agents in vascular UTMD methods. The first barrier encountered is the vascular endothelium. The next are other vascular anatomical features (*e.g.*, the basement membrane, smooth muscle layer, *etc.*) and then the outer cell membrane of the cells one hopes to target. Some intracellular membranous compartments must also be traversed. However, MBs have been used with intramuscular or intraparenchymal injections of vectors, and some successes reported. In UTMD-based gene transfer methods using vascular approaches, to mediate gene therapy *via* acoustic excitation, gas bodies must first exert their influence from within the vascular lumen.

Most of the available evidence from *in vivo* studies indicate that vessel permeabilization effects occur principally in the microcirculation; larger vessels are too robust to be penetrated by cavitation events, even if their vascular endothelium can be effectively destroyed by intraluminal inertial cavitation {Hwang *et al.* 2005}. Extravasation of dyes, nanoparticles or macromolecules through microvessels is almost always accompanied by extravasation of red cells (see below); since these have diameters on the order of 6 μm, breaches in the endothelial wall can be quite large. However, there is also some evidence that more subtle effects, such as partial opening of the tight junctions between endothelial cells, can also contribute. Assuming that the gene vector escapes the intravascular compartment and enters the interstitium, it must then enter the surrounding cells; thus the plasma membrane is the second major barrier encountered by the vectors. Lethal effects of cavitation occurring in the cardiac microcirculation can extend outward into the myocardium {Miller *et al.* 2011}; there is good reason to expect that sub lethal poration of cells located within a few cell diameters of the intravascular cavitation event(s) also occurs.

3.1 Extravasation of dyes, nanoparticles and cells

Evans blue [EB] is an azo dye which binds serum albumin with high affinity, and is normally unable to pass through the endothelium. Extravasation of EB through reversible or

irreversible capillary modification has been observed in small animal (rat) hearts exposed to low-MHz frequency US with the use of various contrast agent MBs, accompanied by premature ventricular contractions {Li *et al.* 2004}. The effect was sensitive to P_a (apparent threshold somewhat less than 1.6 MPa at 1.5 MHz) and to the concentration of the injected MBs {Miller et al. 2004}. The effect appears to be of mechanical origin {Miller *et al.* 2004} With injection of dilute Definity suspensions, extravasation of EB from canine hearts, and cell killing of cardiomycetes within the ultrasonically-interrogated area also occurs under diagnostically realistic exposure conditions {Miller *et al.* 2006}.

Erythrocyte extravasation has been observed in rat kidney glomerular capillaries exposed to diagnostic US with contrast agent MBs in the circulation {Miller et al. 2010a, Miller et al., 2010b}; such damage was associated with tissue inflammation at 1 week and fibrosis at 4 weeks {Miller *et al.* 2009}. A threshold P_a of ~0.8 MPa was indicated {Miller *et al.* 2007}. Glomerular capillary hemorrhage with contrast-enhanced diagnostic US also occurs in large animals (pigs) exposed to 1.5 MHz, 1.7 MPa US {Miller *et al.* 2010}. Petechial hemorrhage on intestinal blood vessels in an *in vivo* murine model was observed with the contrast agent Albunex, with apparent P_a thresholds of 0.4 MPa at 0.4 MHz, 0.8 MPa at 1.1 MHz, or 2.3 MPa at 2.3 MHz; the thresholds were lower with the agent Levovist than with Albunex {Miller & Gies 1998a, Miller & Gies 1998b}. At 2.25 MHz, a threshold P_a of between 0.85 and 1.0 MPa was indicated for vessel damage in exteriorized rat cremaster muscle containing Definity MBs {Samuel *et al.* 2009}. Similarly, intravital observation of red cell extravasation from rat cremaster muscle capillaries containing MB contrast agent was observed more than a decade ago {Price *et al.* 1998; Skyba *et al.* 1998}. Microscopic observations of US and MB-induced red cell extravasation have been reported in glass catfish {Maruvada & Hynynen 2004}. In exteriorized rat spinotrapezius muscle, 1 MHz US at a P_a of 0.75 MPa was shown to result in extravasation of 100 nm diameter microspheres co-injected with contrast agent MBs {Song *et al.* 2002}. In rats, *in vivo* exposure of the kidneys following injection of MBs resulted in red cell extravasation from the glomerular capillaries into Bowman's space with an apparent threshold P_a of ~ 0.73 MPa (at ~ 1 MHz), with nearly 40% of histological sections taken from the focal plane showing extravasation at a P_a of 1.8 MPa {Miller *et al.* 2007}. Intravital microscopy and concurrent cavitation detection was used to study the relationship between bubble dynamics and extravasation of red cells from rat cremaster muscle using 2.25 MHz US, and Definity contrast agent infused *via* the tail vein. Vascular damage and acoustic emissions from the MBs were correlated. The greatest amount of red cell extravasation and the greatest cumulated bubble acoustic emissions occurred at 10 Hz pulse repetition frequency **[PRF]**, indicating that the time for tissue refill with bubbles following each pulse was ~100 ms. In experiments in which pulses were applied at 100 Hz PRF and the P_a varied from 0 – 2.0 MPa, there was no vascular damage at $P_r \leq 0.85$ MPa, but unambiguous damage occurred at $P_r \geq 1.0$ MPa {Samuel *et al.* 2009}. Finally, there is evidence that low amplitude US induced MB oscillations (1 MHz, $P_r = 0.1$ MPa, SonoVue bubbles) can increase the permeability of primary endothelial monolayer cultures *in vitro*. Insonation produced immediate influx of Ca^{2+} ions into the cells, indicating poration of the endothelial plasma membranes. The effect was essentially abolished by application of catalase, strongly suggesting a role for extracellularly-produced H_2O_2 associated with nonlinear bubble oscillations. Moreover, histochemical staining for a protein associated with gap junctions showed an approximately 50% increase immediately after insonation, but returned to control levels within 30 minutes of insonation {Juffermans *et al.* 2009}.

3.2 Transient poration of the cell membrane

Two mechanisms by which US and MBs facilitate poration of cell membranes are prominent; these are: (1) cavitational; *e.g.*, the opening of transient holes in membranes in consequence to local shear forces exerted on membranes by fluid flow ('micro streaming') around oscillating bubbles, local shock waves (which produce large pressure gradients across a cell), or cavitation microjets, or (2) endocytosis {Doinikov & Bouakaz 2010; Walton & Shohet 2009}. Other mechanisms have been proposed, of course. However, it appears that shear stresses associated with bubble activation is probably the principal mechanism. A third mechanism by which normally 'tight' physiological barriers may be permeabilized is by sonochemical stimulation.

Transient pores of a few hundred nanometers in diameter and lifetimes of several seconds can be formed in cell membranes by acoustically-driven, single MB oscillations {Deng *et al.* 2004; Han *et al.* 2007; Zhou *et al.* 2008; Zhou *et al.* 2009}. Voltage clamp studies of *Xenopus* oocytes exposed to 1 MHz US showed that without MBs, there was no change in current at P_r as high as 1.2 MPa. With Optison MBs, opening and resealing of individual pores was observable even at P_r as low as 0.2 MPa. The transmembrane current was carried by influx of Ca^{2+} ions. The transmembrane current was greater at 0.4 MPa than at 0.2 MPa; in both cases, pore lifetime was ~2 s. More generally, the effect was P_a dependent, increasing slowly with increasing P_a over the range of 0.3 – 0.55 MPa, and then inflecting sharply upward at higher P_a {Deng *et al.* 2004}. Pore opening showed a high level of temporal correlation with inertial cavitation noise {Zhou *et al.* 2008}. Pore size was estimated as 220 ± 80 (mean ± SD) nanometers, and refined estimates of pore resealing times indicated closure in 3 – 5 s {Zhou *et al.* 2009}. *In vitro* studies of cells in contact with SonoVue MBs excited by 1 MHz US at P_a of 0.05 – 2.50 MPa have shown Ca^{2+} influx associated with poration {Juffermans *et al.* 2008}. Sonoporation to naked DNA *in vitro* is correlated with inertial cavitation activity {Qiu *et al.* 2010}, and depends in part on the fluidity of the membrane at the time of UTMD treatment {Zarnitsyn & Prausnitz 2004}, with higher reporter gene transfection rates at 37 ºC than at 21 ºC. This is consistent with the need for porated membranes to reseal rapidly in order to maintain viability. Still others have shown that 1 MHz US at P_a as low as 0.1 MPa can permeabilize cells *in vitro* to pDNA {Rahim *et al.* 2006}. In cell suspensions containing Definity MBs, the P_a thresholds for cell permeabilization to 70 kDa FITC-dextran, propidium iodide (indicating cell death) and MB destruction were 75 kPa at 0.5 MHz, 200 kPa at 2.5 MHz, and 600 kPa at 5 MHz {Karshafian *et al.* 2009}. However, the idea that cellular poration results from inertial cavitation has been challenged on the basis of observations of cavitation noise and permeabilization of *in vitro* cell monolayers to 500 kDa FITC dextran; the supporting data indicate that permeabilization precedes the occurrence of broadband noise associated with inertial collapse and rebound cavitation. The authors conclude that the forces associated with microstreaming around activated bubbles is the principal mechanism of sonoporation {Forbes *et al.* 2008}. More study is clearly needed.

US and MB-induced cell lysis and reversible poration in other cell types *in vitro* are strongly correlated with inertial cavitation {Chen *et al.* 2003a; Chen *et al.* 2003b; Lai *et al.* 2006}. Cell surface antigens may be stripped off the surface of viable cells during such events {Brayman *et al.* 1999}. While *in vitro* UTMD-based transfection with naked DNA is often associated with high cell mortality, this is not always the case {Wang *et al.* 2009}.

The use of the collapse jets of MBs generated by laser pulses to selectively and directionally sonoporate individual cells has reached a high level of sophistication, producing pore sizes of ~ 200 nm {Sankin *et al.* 2010}, which may someday prove useful for cell therapies. This is very

similar to pore size determined by sonoporation of *Xenopus* oocytes using 1.07 MHz US of 0.3 MPa P_a with Definity MBs; *viz.*, a mean pore size of 220 ± 80 nm diameter {Zhou Y *et al.* 2009}. Lipoplexes, which are normally taken up by cells *via* endocytosis, are taken up poorly when 'PEGylated' (coated with polyethylene glycol). However, when PEGylated pDNA/lipolexes were attached to acoustically activated MBs, large increases in PEGylated lipoplex transfection were achieved in an *in vitro* model system, relative to ether 'free' PEGylated lipolexes, or lipolexes + MB simple (un-linked) mixtures {Lentacker *et al.* 2009}. The investigators also used a number of endocytosis inhibitors; their results indicate that endocytosis was not the primary mechanism involved. They postulate that lipoplex-loaded MBs collapsing near cell monolayers in culture plates released the lipolexes, resulting in high local lipoplex concentrations, that some of the released lipolexes became entrained in MB collapse jets and were 'injected' into the cells with the fluid jet (see also {Miller 2000}). However, Meijering and colleagues, using fluorescently labeled dextrans of ~4 – 500 kDa, present data which indicate that the principal mechanism by which UTMD treatment (1 MHz US, 0.2 MPa P_r, SonoVue MBs) facilitates macromolecule delivery across the plasma membrane is *via* induction of endocytosis. Sonoporation was also observed, but did not seem to be the mechanism for macromolecular uptake {Meijering *et al.* 2009}. It is clear that the our understanding of the mechanism(s) of UTMD-enhanced uptake of macromolecules remains incomplete.

3.3 Entry of pDNA into the nucleus

Following delivery of pDNA across the plasma membrane of cells into cytoplasm, pDNA may travel through multiple cellular compartments and finally enter the nucleus *via* diffusion {Liang *et al.* 2004} or other assisted mechanism to produce efficient transgene expression. UTMD may facilitate overcoming some of these barriers most likely *via* cavitation bioeffects. It was reported that long-term exposure to therapeutic ultrasound (1 MHz, 2 W/cm², 30% duty cycle for 30 mins) can overcome the rate-limiting step of driving DNA into the cell nucleus {Duvshani-Eshet & Machluf 2005}. One thousand fold higher gene expression levels of luciferase was achieved with minimal loss in cell viability (<20%) in three different cell types (BHK, LNCaP, and BCE). These data implied that therapeutic US is the main driving force delivering pDNA not only to the cell cytoplasm but also to the nucleus. However, in this case, no MB was used. The same group {Duvshani-Eshet *et al.* 2006} showed that adding Optison further increased transfection levels. Confocal and atomic force microscopy studies indicated that long-term therapeutic US application localizes the pDNA in cell and nucleus regardless of Optison addition. In addition, the use Optison did not affect the kinetics of protein expression, indicating Optison did not affect DNA trafficking to the nucleus. They hypothesized that US application by itself plays a major role in delivering DNA to the nucleus.

An interesting recent report used Doxorubicin (DOX) as a molecular nanotheranostic agent to study UTMD-mediated intracellular delivery and nuclear trafficking {Mohan & Rapoport 2010}. DOX is a popular research tool due to its inherent fluorescence and was encapsulated in poly(ethylene glycol)-co-polycaprolactone (PEG-PCL) micelles or PEG-PCL stabilized perfluorocarbon nanodroplets in this study. US triggered DOX trafficking into cell nuclei; the trafficking was further enhanced in the presence of phase-transition nanodroplets which become gas MBs upon US exposure of sufficient P_r. This was believed to be due to cavitation induced transient permeabilization of both plasma and nuclear membranes, thus

allowing DOX penetration into the cell nuclei. Whether MBs and/or nanodroplet emulsions can significantly augment DNA delivery to the nucleus needs further investigation.

Once pDNA enters the nucleus, some DNA can be condensed by histones and form persistent nucleosome-like structures. Persistent gene expression from these stable episomal pDNA genomes requires the introduction of specific cis-acting elements in the gene transfer constructs. We have demonstrated that incorporation of locus control region and intron elements into specific gene transfer constructs can achieve persistent expression of therapeutic-levels of coagulation proteins in the liver following nonviral gene therapy {Miao *et al.* 2001; Miao *et al.* 2003; Ye *et al.* 2003}. The locus control region {Yant *et al.* 2003} can contribute to the open chromatin structure of the pDNA genome and avoid silencing of the transgene expression cassette {Miao *et al.* 2005}. Furthermore, addition of an intron element significantly enhanced the transcription efficiency of stable mRNA {Miao *et al.* 2005}. Thus, gene transfer of episomal pDNA into slow-dividing or terminally differentiated cells facilitated by UTMD has high potential to achieve a therapeutic effect to treat specific diseases. For gene transfer into dividing cells where integration of target genes are required, incorporation of other systems such as sleeping beauty transposons {Aronovich *et al.* 2011; Yant *et al.* 2007} or PhiC31 integrase {Keravala *et al.* 2011} with UTMD-mediated nonviral gene transfer methodology can lead to long-term transgene expression.

4. The vectors: Packaging desired genes for ultrasound-mediated delivery

4.1 Naked DNA: Plasmids

Naked plasmid DNA provides many advantages as a nonviral gene transfer vector, including: (1) ease of preparation, (2) cost-effectiveness, (3) minimum toxicity, and (4) it is least immunogenic of the vectors. Indeed, the immunogenic CpG moiety can be modified easily if needed. Most importantly, quality control is quite easy compared to other pDNA complexes containing synthetic vehicles. However, it has been a very challenging problem to deliver naked pDNA into specific cells due to its large size and negative charge. *In vivo* delivery of naked pDNA is especially difficult with additional impediments of instability of pDNA in blood serum as well as in cellular sub-compartments such as cytosol, endosome, and nucleus after cell entry. Recently a hydrodynamic approach has been developed to drive efficient gene delivery into liver {Liu *et al.* 1999} and muscle {Danko *et al.* 1997} resulting in therapeutic levels of transgene expression in animal disease models, including hemophilia B {Miao *et al.* 2003} and others {Zhang *et al.* 2000}. This method in its current form is not suitable for clinical use; however, notable recent advances have been made in large animal models (*vide, e.g.*, {Fabre *et al.* 2008; Kamimura *et al.* 2009; Suda *et al.* 2008}. Alternatively, innovative US and MB technology to facilitate delivery of naked pDNA is a potential clinically feasible nonviral gene therapy approach {Miao *et al.* 2005; Shen *et al.* 2008; Song *et al.* 2011}.

The potential for sonoporation to increase pDNA loading of cells was recognized years ago {Fechheimer *et al.* 1987}. Sonoporation-enhanced transport of nanoparticles into cells is dependent on molecular size; uptake of particles ≤ 37 nm diameter was enhanced by sonoporation without gross damage, but particle uptake generally declined as particle size increased {Mehier-Humbert *et al.* 2005a}. pDNA gene expression is faster with sonoporation than with liposome-based methods which depend on endocytosis {Mehier-Humbert *et al.* 2005}, indicating that pDNA enters cells through transient pores. Moreover, due to the risk of pDNA degradation by serum nucleases and removal by phagocytes {Niidome & Huang

2002}, injection of a nonviral gene transfer vector long before US treatment occurs is unlikely to be effective. With simultaneous delivery of pDNA and US exposure, UTMD can significantly facilitate the transfer of naked pDNA up to several hundred fold *in vitro* {Miller *et al.* 2002; Newman & Bettinger 2007} and up to several thousand fold *in vivo*, depending on different US and transfection systems {Chen *et al.* 2010; Miao *et al.* 2005; Shen *et al.* 2008; Song *et al.* 2011}. Furthermore, with introduction of specific cis-acting elements in the gene transfer constructs, persistent expression of near-therapeutic levels of proteins can be achieved from episomal plasmids following UTMD-mediated gene therapy {Miao *et al.* 2005}. These results demonstrate that development of UTMD has high potential to achieve a therapeutic effect for treating specific diseases.

4.2 Encapsulated or compacted DNA

Polyanionic solutes can be complexed to phospholipid polar head groups *via* Ca^{2+} bridges {Huster *et al.* 1999}. Thus, anionic DNA molecules can be compacted onto cationic MB lipid shells. Polylysine has also been used to link naked DNA to phospholipid MB shells {Wang *et al.* 2009}. It appears to be widely believed that enhanced efficiency of gene delivery can be obtained with pDNA in close proximity to MBs and any cell membrane 'defects' they may create, thereby increasing the probability that pDNA will be available to enter through these pores prior to their closure. Thus far, however, there has been inconsistent support for this idea. For example, pDNA coupled electrostatically with cationic MBs were used for local delivery of DNA to vascular muscle cells. One percent of cells were transduced with 40% of the cells remaining viable {Phillips *et al.* 2010}. However, reporter plasmid bound to the cationic MB preparation MRX-225 was used to transfect canine myocardium which was exposed to diagnostic US. Reporter gene activity was only observed in the myocardium of those animals that received MB-linked DNA and were exposed to US but not in control untreated animals {Vannan *et al.* 2002}. It is not clear if the pDNA-MB linkage influenced the experimental outcome.

Due to large size of DNA molecules and the concerns of enzymatic degradation of the injected pDNA as well as the low pDNA concentration in the vicinity of sonoporated cell membranes, polymer-coated MBs that can bind and protect the pDNA have been developed for UTMD-mediated gene delivery. Coating albumin-shelled MBs with poly(allylamine hydrochloride) (PAH) makes the surface charge of the MBs positive, but did not affect the size distribution of the MBs. The cationic coating allowed the MBs to bind to 100 fg of pDNA per MB and protected the bound DNA against nucleases. The PAH coating also significantly increased the lifetime of MBs (half-life ~7 h), making them more convenient for *in vivo* applications {Lentacker *et al.* 2006}.

Another approach to compacting DNA onto MBs is to first incubate the pDNA with a cationic lipid such as GL67 or a cationic polymer such as polyethylenimine (PEI), followed by mixing with MBs. PEI/DNA mixed with SonoVue MBs were injected intravenously in tumor–bearing mice. Following US exposure, reporter gene expression in tumor xenografts was significantly enhanced without causing any apparently adverse effect. Furthermore, with UTMD and PEI complex, vectors carrying a short hairpin RNA (shRNA) targeting human survivin were efficiently delivered into the tumor site, leading to inhibition of surviving gene expression and apoptosis of the tumor cells {Chen *et al.* 2010}.

DNA loading of MB shells can be impressive, but may also be limiting to high yield gene therapies using UTMD. By first forming pDNA-Lipofectamine 2000 complexes, mixing

these with home-made phospholipid mixtures, agitating under a perflutren atmosphere to generate MBs, and then repeatedly washing the bubbles to remove unassociated DNA, a per bubble loading of 50 x 10^{-15} gram/MB was achieved {Chen *et al.* 2006}. Similar binding rates (approximately 100 fg/MB) were reported by others {Carson *et al.* 2011}. Using a layer-by-layer approach, DNA loading of pre-existing cationic MBs was increased 10-fold by first bringing anionic DNA to the surface of the cationic bubble, followed by binding a coating of poly-cationic polylysine to the DNA, followed by more DNA, *etc.* Loading as high as ~2500 fg DNA/MB was achieved, without apparent impact on the bubbles' dynamical response to acoustic excitation {Borden *et al.* 2007}. However, others have found that *in vitro* transfection rates are not enhanced by conjugation of reporter pDNA to MB shells {Tlaxca *et al.* 2010}, as found in the *in vivo* canine myocardium example discussed earlier in this section.

4.3 Viral vectors

There have been many reports that US treatment alone, or in combination with MBs, can increase transgene uptake by cells {Miller *et al.* 2002; Newman & Bettinger 2007}. Even with the difficulty of translating UTMD-mediated results obtained under tissue culture conditions (*e.g.*, infinite media for suspended cells *vs.* nearby noncompliant boundaries for monolayers) to the fully 3-D, viscoelastic intravascular environment *in vivo*, *in vitro* experiments often have the advantage of being better controlled and the results therefore more easily understood. This is not always the case, however. Zheng and colleagues exposed two different endothelial pigment cell lines to adenoviral gene vectors in combination with US or US and MBs (1 – 3 W/cm^2; P_r appears to have been ~0.17 – 0.30 MPa at 1 MHz; SonoVue MBs were used when bubbles were employed). They found that treatment with US and MBs increased adenoviral gene transfer in human retinal pigment epithelium cells, but was without effect on rat retinal pigment epithelial cells under otherwise identical conditions {Zheng *et al.* 2009}. The differences were speculated to arise in consequence to differing ability of the two cell lines to phagocytize the SonoVue MBs, to which the adenoviral vectors were believed to be attached.

It is difficult to limit the specificity of delivery of viral vectors, which are usually delivered systemically. By using retrovirus-loaded MBs, UTMD facilitated the delivery of viral vectors in a restricted area of cells exposed to P_r of 0.4 MPa or greater, despite uniform dispersion of the vector {Taylor *et al.* 2007}. An envelope-deficient retroviral vector was combined with cationic MBs and added to target cells. Transduction efficiencies and sites can thus be controlled by means of US exposure. These results emphasize that UTMD can not only facilitate the delivery of nonviral vectors, but also has the potential to enhance efficiencies and restrict targeted sites of viral gene transfer.

4.4 Small RNAs and oligonucleotides

Small RNAs and oligonucleotides (ODN) have recently been developed as promising therapeutics to treat diseases like viral infections, cancer, and several genetic disorders. Among these, small inhibitory RNA (RNAi or siRNA) based therapeutics have been investigated for treating a number of different diseases, including viral infections (*e.g.*, hepatitis, HIV, influenza), cancer, Huntington's disease, and others. Other therapeutic agents including microRNA (miRNA), transfer RNA (tRNA), and antisense ODN are also being developed to regulate gene or cell functions as treatment regimens. Since these agents are small, it is expected that they will be good candidates for augmented delivery by UTMD.

The US contrast agent pioneer Thomas Porter recognized early that UTMD delivery of oligonucleotides had the potential to influence vascular tissue remodeling after injury. In a 2001 study, an oligonucleotide which inhibits vascular smooth muscle cell proliferation was bound to albumin-shelled MBs and UTMD effected by transcutaneous application of 20 kHz US to porcine carotid artery walls following balloon catheter injury. Thirty days after treatment, the percent area stenosis in UTMD-treated animals was half that in controls {Porter *et al.* 2001}. The uptake of ODNs into intact *ex vivo* human saphenous veins and isolated smooth muscle cells from the veins was also potentiated by US {Kodama *et al.* 2005}. In addition, UTMD facilitated the delivery of antisense ODN targeting the human androgen receptor (AR) in prostate tumor cells, resulting in 49% transfected cells, associated with a decrease in AR expression compared to untreated controls {Haag *et al.* 2006}.

UTMD-mediated sonoporation (frequency: 1 MHz; intensity: 2 W/cm^2; exposure time: 2 min) was capable of enhancing *in vivo* siRNA delivery into salivary gland of rats, leading to significant GAPDH gene silencing by 10-50% for 48 hours {Sakai *et al.* 2009}. No gene silencing was observed with exposure to US only in the absence of Optison MBs. Intraventricular co-injection of siRNA-GFP and MB BR14 with concomitant ultrasonic exposure resulted in a substantial reduction in EGFP expression in the coronary artery in EGFP transgenic mice {Tsunoda *et al.* 2005}. Liposomal MBs combined with US can efficiently delivery siRNA with only 10s of US exposure *in vitro*. siRNA was also efficiently delivered into the tibialis muscles using the same system and the gene-silencing effect could be sustained for more than 3 weeks {Negishi *et al.* 2008}. These results demonstrate that UTMD-mediated delivery of siRNA can serve as a very useful tool for loss-of-function genetic engineering both *in vitro* and *in vivo*.

4.5 Transduced cell therapy

Cell therapy is a promising strategy for many applications, including genetic diseases, cancer, regenerative medicine, and others. However, it is very difficult to transfect certain cell types and maintain their viability following transfection, including hematopoietic and mesenchymal stem cells, T cells, and others which are important targets for cell therapy using the transfection methods currently available. UTMD has been demonstrated to facilitate the delivery of siRNA into mesenchymal stem cells (MSCs) {Otani *et al.* 2009}, which knocked down mRNA expression of specific genes, leading to the improvement of cellular function and viability. The application of UTMD has high potential to facilitate the delivery of genetic materials into target cells and can be expanded for use in a variety of cell therapy protocols.

5. Selected applications of ultrasound-mediated gene delivery

5.1 Cardiovascular

Recent reviews of cardiac applications of UTMD are provided elsewhere {Laing & McPherson 2009; Porter 2009}. Desired endpoints may be improved vascular function (*e.g.*, following ischemia) or inhibition of vascularization to 'starve' tumors. The following discussion is intended only to provide an indication of some of the exciting avenues of research in the area.

Cardiovascular graft remodeling: Tissue remodeling after transplantation surgery is required for long term transplant success. It has been shown that US-mediated gene therapy can improve transplanted vessel patency after surgery. Carotid interposition saphenous vein

grafts in pigs were treated *ex vivo* prior to transplantation with 1 MHz US at ~1.8 MPa P_a with both a MB contrast agent and a plasmid encoding for metalloproteinase 3 (TIMP-3; the enzyme inhibits post-graft vessel restriction) present during US exposure. At 4 weeks, luminal diameters in animals receiving the transfected grafts were significantly greater than in controls {Akowuah *et al.* 2005}. Similarly, US treatment enhanced the delivery of an adenoviral vector to the aortic root, yielding a 2.5-fold enhancement in gene delivery {Beeri *et al.* 2002}. A technical issue of note is that a balloon catheter was used to briefly occlude the aortic root above the sinuses to increase the dwell time of the injected MBs and adenoviral vectors.

Reperfusion therapies: US-mediated gene therapy to improve myocardial reperfusion following induced myocardial infarcts in mice was studied using Definity MBs, and UTMD achieved using high frequency (8 MHz), relatively high P_a (estimated ~4.5 MPa) US from a diagnostic US machine. Plasmids were either empty (controls) or encoded for Stem Cell Factor (SCF; expected to enhance reperfusion by recruitment of cells during tissue remodeling) or VEGF (expected to stimulate angiogenesis). At 21 days, UTMD with either VEGF or SCF-bearing pDNA increased the microvessel density and blood flow relative to controls {Fujii *et al.* 2009}. Similarly, reperfusion of ischemic rat hind limbs was improved by US-mediated gene therapy. Cationic lipid-shelled MBs and a pDNA encoding for VEGF-165 were used; US frequency and amplitude were 1.3 MHz and ~2 MPa, respectively {Kobulnik *et al.* 2009}. A murine cardiac infarct model treated with UTMD with pDNA encoding for either VEGF or stem cell factor (SCF) has been reported to increase reperfusion; the observation that SCF-encoding pDNA increased perfusion was interpreted as evidence for the recruitment of reparative cells into the area of infarction {Fujii *et al.* 2009}.

Expression of a reporter plasmid gene delivered to the myocardium by UTMD methods was relatively brief (4 d), but was improved by retreatment {Bekeredjian *et al.* 2003}. Under similar exposure conditions, damage to the heart was negligible {Bekeredjian *et al.* 2004}. However, UTMD-enhanced gene delivery to the heart is often attended by at least minimal damage, which can include extravasation of large molecules and red cells {Hernot *et al.* 2010}. It is noteworthy that under diagnostic US exposure conditions, premature ventricular contractions can occur with the use of US contrast agents (see, *e.g.* {Miller *et al.* 2005}), and in contrast with the results of {Hernot *et al.* 2010} these have been unambiguously correlated with cell killing of cardiomycetes. In rats, using 1.7 MHz ultrasound, premature complexes and cardiomycete death were observable at P_as of 2 MPa or greater {Miller *et al.* 2011}. Premature contraction complexes appear to be related directly to extravascular cell killing, so even absent gross side effects, some side effects can be expected in gene therapies involving UTMD. This has important implications not only for safety, but also efficacy; *i.e.*, one hopes to transfect the target cells, not kill them. It seems unlikely that transfection to meaningful extents can be achieved without some cell killing, so attempts to optimize UTMD treatments must strive to achieve an acceptable balance between desired effect (transfection) and undesired effect (killing of the target cells).

When treating the heart using reporter genes, MBs and a diagnostic scanner as the acoustic source, superior results were obtained by moving the scan head about to 'paint' a larger volume of tissue {Geis *et al.* 2009}. Another noteworthy finding was that moving the beam relative to the heart did not increase Evans blue dye extravasation, which suggests less microcirculation damage per unit transgene expression when the insonifying beam is moved relative to the target.

Inhibition of neovascularization: A substantial literature on therapies to inhibit neoangiogenesis exists. Here we mention one recent example: MBs and pDNA encoding for pigment epithelium derived factor, which inhibits neovascularization in the retina, were injected into the vitreous humor of rats having laser-induced choroidal injury, which leads to neovascularization. The eyes were treated immediately with 0.3 MHz US of P_a estimated to be in the range of 0.1 – 0.3 MPa. At 28 days post treatment, choroidal neovascularization was inhibited in the UTMD group relative to untreated controls {Zhou XY *et al.* 2009}. Similarly, pDNA carrying a silencing sequence for the gene coding for survivin were introduced into implanted murine tumors using UTMD methods. Treated tumors were sonicated with 3 MHz US at an intensity of 2 W/cm² (estimated P_r: 0.2 - 0.5 MPa). Transgene expression was significantly increased in tumors treated with UTMB. It was proposed that the technique could be applied therapeutically to tumors to increase in tumor cell apoptosis *via* the silencing effect on survivin expression in transfected cells {Chen *et al.* 2010}.

5.2 Skeletal muscle

Among the applications for gene therapy in skeletal muscle is the treatment of muscular dystrophy (see, e.g., {Alter *et al.* 2009} and references therein). As a specific recent example, Kodama and colleagues used various means to attempt delivery of PGL3 luciferase pDNA into murine skeletal muscle. Optison, human albumin MBs or acoustic liposomes (all formulated with C_3F_8 gas) were compared, using a 1 MHz US source; P_a was 0.2 MPa. Treatment with plasmid, US and either Optison or liposomes increased luciferase expression relative to controls. The ~200 nm diameter acoustic liposomes produced the highest expression rates, presumably because of their very high concentration {Kodama *et al.* 2010}.

5.3 Pancreas

Pancreatic gene therapies have focused principally on treatments for diabetes types I and II. The feasibility of using pDNA and UTMD methods to deliver transgenes to the pancreas has been described by Grayburn and colleagues. Following injection of reporter gene pDNA attached to the MB phospholipid shells, the reporter gene was expressed with fairly high spatial specificity, declining after 4 days, but with measurable expression persisting for as long as 21 days ({Chen *et al.* 2006}; see internal citations for a discussion of viral approaches). US of ~3 MHz was used, with an apparent P_a of ~1 MPa at the pancreas. When plasmid vectors carrying human insulin and hexokinase I gene were delivered using UTMD, transgene expression was obtained in islets and decreased circulating glucose levels were observed in treated rats. These data indicate that UTMD allows relatively noninvasive delivery of genes to pancreatic islets to modulate beta cell function. More recently, the same group reported that delivery of NeuroD1 gene driven by a rat insulin promoter (RAP3.1) into rats by UTMD-mediated gene delivery targeting pancreas *in vivo* promoted islet regeneration from surviving beta-cells, with normalization of glucose, insulin and C-peptide levels at 30 days {Chen *et al.* 2010}.

5.4 Liver

Liver is a major organ for protein synthesis, and therefore represents an important target for gene therapy. We will discuss here UTMD-mediated gene therapy targeting liver for treating non-tumoral diseases.

Our group has extensively studied gene delivery of reporter and therapeutic genes into the liver. We have demonstrated that UTMD (1 MHz US) can significantly enhance gene transfer of naked pDNA into the mouse liver in the presence of either Optison {Miao 2005} or Definity MBs {Shen *et al.* 2008}. Transgene expression was dependent exponentially on P_r, with an inflection point usually between 1 and 2.5 MPa followed by a plateau above 3 MPa {Song *et al.* 2011a}, consistent with an inertial cavitation mechanism. More than thousand-fold enhancement of gene transfer efficiencies was obtained compared to control experiments in the absence of UTMD. Recently we have gained preliminary success in scaling up pDNA delivery in larger animal models, including rats {Song *et al.* 2011b } and dogs {Noble *et al.* 2011}. Previously we have shown that near therapeutic levels of factor IX were achieved by UTMD-mediated gene delivery in mice. Technical improvements to further enhance gene transfer of factor VIII for treatment of hemophilia A and factor IX for treatment of hemophilia B are currently being pursued in small and large animal models.

5.5 Kidney

A number of kidney diseases could be potentially treated with gene therapies. Hydrodynamic approaches have met with some success. Xing *et al.* attempted to improve on these results by combining hydrodynamic and UTMD approaches to naked DNA reporter gene delivery to surgically-exposed rat kidneys. Combined US (unspecified frequency) from a Sonotron 2000 hand-held diagnostic US machine and hydrodynamic therapy together yielded better reporter gene transfection than hydrodynamic therapy alone, producing an approximately 4-fold increase in reported gene expression when the estimated P_a was in the range of 0.3 – 0.8 MPa and no MBs were used. The effect was intensity-dependent. When Optison MBs were injected with the naked DNA during hydrodynamic therapy and US exposure, the same effect (4-fold increase in gene expression) was observed at an intensity of only 1 Watt/cm^2 (estimated 0.2 – 0.5 MPa P_a) {Xing *et al.* 2009}.

5.6 Skin (DNA vaccine)

The failure of wounds to heal in diabetic patients is a significant clinical problem. Gene therapies which promote angiogenesis represent a promising approach to this problem. VEGF-encoding gene vectors (either minicircle naked DNA; a supercoiled form with a molecular weight estimated as 331 g/mol, or naked DNA borne on the gene carrier branched polyethylinimine) were tested for efficacy in inducing circulating VEGF expression and accelerating wound healing in an induced diabetic mouse model. Wounds were treated by peripheral injection of gene vectors with or without exposure to US (1 MHz, 2 W/cm^2, 20% duty cycle; estimated 0.25 - 0.5 MPa). In some treatments, SonoVue MBs were injected with the microcircle DNA prior to sonication. Markedly greater levels of circulating VEGF were observed in mice treated with [VEGF-encoding minicircle DNA + US + MBs] relative to controls, but not as high as those obtained using the polyethylinimine gene carrier. Nonetheless, the [minicircle DNA + US + MBs] treatment produced a significant improvement in healing rates of the treated skin wounds {Yoon *et al.* 2009}.

5.7 Other solid organs

Brain: Much work has been done on 'opening' (*i.e.*, making more permeable) the blood-brain barrier, which so tightly regulates traffic between the vascular space and the brain that chemotherapeutic agents often cannot cross the barrier {Meairs & Alonso 2007}. Much

success with tracer molecules (*e.g.*, Evans blue dye, gadolinium MRI contrast agents, *etc.*) has been achieved using UTMD methods, principally in small animal models. However, low energy US applied through the temporal bone of swine produced short-term permeabilization of the blood-brain barrier with exogenous MBs (see {Xie *et al.* 2008} and citations within). However, we have found no reports of UTMD gene therapies attempted in the brain.

5.8 Endothelium

There are many papers which indicate that endothelial cells *in vitro* can express transgenes delivered by UTMD methods (see {Su *et al.* 2010} and internal references for recent examples). Su and colleagues found that treatment with 1 MHz US of 1 MPa P_r together with pGL3 pDNA and MBs yielded a 100-fold increase in luciferase expression relative to pDNA treatment alone. It remains to be determined if endothelial cells *in vivo* can be 'recruited' as effective synthesizers and secretors of therapeutic gene expression products.

5.9 Cell therapies: *Ex vivo* gene therapy

In this section, we discuss several examples of US-mediated gene delivery using cell cultures *in vitro*; these may have some mechanistic relevance to the *in vivo* condition. However, some of the results obtained using *in vitro* cells may have direct bearing on cell therapies.

Shock waves and MBs have been used *in vitro* to increase pDNA uptake in cultured HEK cells; the technique worked, but was associated with substantial cell killing {Bekeredjian *et al.* 2007} as might be expected (see, *e.g.*, {Brayman *et al.* 1999; Hwang *et al.* 2006; Hwang *et al.* 2003}). As a general rule, only a small fraction (~5 – 10%) of cells insonated with MBs *in vitro* undergo transient poration; these yields are often associated with cell killing rates of ~50% (see, *e.g.* {AIUM 2000; AIUM 2008} and references therein). If those cells can be sorted (*e.g.*, by flow cytometry) and cultured to increase cell numbers, this may be acceptable for cell therapies – if the gene vector is integrated into the host genome. An obvious problem for cell therapies based on this approach with naked DNA, however, is that the pDNA does not replicate with the host cell, and so there would be little to be gained by increasing cell numbers of the transfected subpopulation of cells. However, using suspended KHT-C cells, and 3 V% Definity, high levels of *in vitro* permeabilization to 70 kDa fluorescein isothiocyanate (FITC) - dextran were achieved using 0.5 MHz US at a P_a of 0.57 MPa, 16 cycle pulses repeated at 3 kHz PRF. About 30% of the cells were permeabilized, with >95% retention of viability {Karshafian *et al.* 2009}. If similar results can be obtained with naked DNA, the prospects for cell therapies using cells transfected using *in vitro* UTMD seem much brighter.

5.10 Gene therapies for tumoricidal activity

Strategies currently under investigation for treatment of cancers include anti-angiogenic therapies, the introduction of 'suicide genes' which either induce apoptosis or sensitize cells to subsequent treatment with drugs {Aoi *et al.* 2008; Azuma *et al.* 2008; Daigeler *et al.* 2010; Kirn *et al.* 2002; Zhou *et al.* 2010}, or down-regulate oncogenes {Wang *et al.* 2009}. Naked DNA bearing the Herpes-derived 'suicide gene' for thymidine kinase has been delivered to murine squamous cell carcinomas by UTMD methods (1.3 MHz, estimated P_r ~1.8 MPa). The DNA was bound to lipid shelled MBs at a rate of about 100 fg DNA/MB. With daily ganciclovir treatments beginning 3 d after UTMD and pDNA treatment, tumor doubling times were significantly reduced (by ~17%) in the UTMD-treated tumors {Carson *et al.* 2011}.

In tumors as in other systems, extravasation of gene vectors seems to be a constraint for delivery of antitumoral therapeutics which are delivered intravascularly. Here, too, UTMD appears to aid in increasing vascular permeability. For example, in implanted subcutaneous hepatomas, extravasation of Evans blue dye was 5-fold higher in the UTMD and plasmid-treated tumors than in untreated control tumors; there was no increase in Evans blue extravasation when US was applied without MBs. In this case, however, there was no significant transfection by the pDNA {Bekeredjian *et al.* 2007}. UTMD techniques have been used successfully to introduce the gene for tumor suppression protein p53 into murine retinoblastoma xenografts; when insonated in the presence of MBs or liposomes, significant expression of p53 resulted; there was no expression in the plasmid-only or plasmid + US groups {Luo *et al.* 2010}.

6. Concluding remarks

Much exciting work on ultrasound-mediated gene delivery has already been done, but the field remains young and even preclinical applications involving therapeutic genes are relatively few. A few generalities may be put forth: In most (but not all) cases, ultrasound-mediated gene delivery is dependent on, or greatly facilitated by, exogenous microbubbles. Where this is the case, the acoustic exposures are usually such that the bubbles can be expected to behave in a nonlinear way. Given the intense effort being devoted to the topic, we can anticipate with some confidence that successful gene therapies in large animals and early human trials will be achieved in the near future.

7. Acknowledgements

8. References

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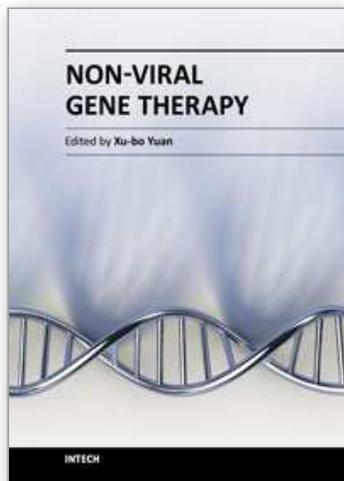
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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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