Published in final edited form as:

Nat Protoc. 2019 April ; 14(4): 1015–1026. doi:10.1038/s41596-019-0125-y.

Nonviral ultrasound-mediated gene delivery in small and large animal models

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Abstract

Ultrasound-mediated gene delivery (sonoporation) is a minimally invasive, nonviral and clinically translatable method of gene therapy. This method offers a favorable safety profile over that of viral vectors and is less invasive as compared with other physical gene delivery approaches (e.g., electroporation). We have previously used sonoporation to overexpress transgenes in different skeletal tissues in order to induce tissue regeneration. Here, we provide a protocol that could easily be adapted to address various other targets of tissue regeneration or additional applications, such as cancer and neurodegenerative diseases. This protocol describes how to prepare, conduct and optimize ultrasound-mediated gene delivery in both a murine and a porcine animal model. The protocol includes the preparation of a microbubble-DNA mix and in vivo sonoporation under ultrasound imaging. Ultrasound-mediated gene delivery can be accomplished within 10 min. After

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Author contributions

G.P., K.W.F. and D.G. designed the experiments. M.B., J.F. and G.S. performed the experiments. M.B. and G.S. analyzed the data. M.B., J.F. and G.S. created the figures. M.B., J.F. and G.S. wrote the manuscript. All authors approved the manuscript.

Competing interests

G.P. and D.G. are shareholders in GamlaStem Medical, which did not provide funds for this study. The other authors declare no competing interests.

Supplementary information is available for this paper at <https://doi.org/10.1038/s41596-019-0125-y>.

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Data availability

All materials are available from commercial sources or can be derived using methods described in this study. All relevant data are reported in the article.

DNA delivery, animals can be followed to monitor gene expression, protein secretion and other transgene-specific outcomes, including tissue regeneration. This procedure can be accomplished by a competent graduate student or technician with prior experience in ultrasound imaging or in performing in vivo procedures.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Introduction

Gene therapy holds immense potential to cure a multitude of diseases by replacing a malfunctioning gene or modulating gene expression. From classically targeted monogenic diseases to cancer therapy and regenerative medicine, gene therapy research has resulted in >2,400 clinical trials to date¹. Reported positive clinical outcomes include vision improvement in blind patients², correction of coagulopathies³ and eradication of resistant cancers⁴. Yet efficient and selective delivery has remained a major roadblock to translational success⁵. The delivery method must overcome complex cellular and tissue barriers to drive expression of a therapeutic gene without disrupting other cellular processes. Then large enough quantities of endogenous cells will need to be transfected to produce a therapeutic effect.

Comparison with other methods

Available delivery methods can be broadly divided into direct and cell-mediated methods⁶. The cell-mediated method primarily relies on administering cells that were transfected in vitro with a transgene and then implanted in vivo in order to deliver the therapeutic protein^{7,8}. Although this method may be more efficient and may enable better control of transfection in specific cells, it is substantially more complex and less cost-effective than in vivo delivery, mainly due to the prolonged time and resources required for cell harvest and expansion in vitro. The direct method uses viral and nonviral vectors to deliver the transgene to the target tissue or cells in vivo. Use of viral vectors is deemed more efficient but less safe than nonviral methods, mainly due to possible immune reactions to the viral proteins and the potential for cell transformation and vector genome mobilization^{9,10}. Hence, nonviral delivery appears to be a safer, simple and cost-effective method for in vivo delivery when short-term expression of a transgene is required, making it a leading candidate for clinical translation. However, nonviral delivery methods are mainly limited by their relatively low efficiency of transfection as compared with viral vectors⁷. Ultrasound-mediated gene delivery, i.e., sonoporation, is especially attractive because it induces transient pore formation in the cell membrane in a spatiotemporally controlled manner under direct visualization¹¹. Furthermore, ultrasound is already being extensively used in the clinic and is typically considered among the safest imaging modalities, as it is noninvasive and does not rely on radiation. Alternative physical methods for gene delivery include electroporation, which often uses needle electrodes to create electrical pulses in the target tissue with improved efficiency¹². The need for electrodes limits the use of electroporation to targets

that are close to the skin¹³. Alternatively, a surgical approach is needed to enable gene transfer to deeper tissues¹⁴. Therefore, sonoporation can be an attractive choice for clinical translation because of its noninvasiveness and ease of use.

Applications of the method

Various proteins and genes can be directly delivered using sonoporation. In addition, different lipids and polymers can be assembled into liposomes, micelles and other nanoparticles to entrap drugs and can be used to selectively deliver them to regions exposed to ultrasound¹⁵. Most sonoporation studies have combined plasmid DNA with microbubbles (low-diffusivity gas cores encapsulated by protein or lipid shells) to enhance membrane permeability in different tissues via the effects of microbubble volume fluctuations and/or collapse (Table 1)^{16–20}. The mechanisms for microbubble-mediated sonoporation depend on acoustic pressure^{21–23}. We were the first to show that osteogenic gene electroporation can be used to completely heal a critical size defect (a bone fracture that does not spontaneously heal)²⁴ and that sonoporation-based gene delivery leads to transient, well-localized gene expression²⁵ and bone formation in vivo¹². Sonoporation was found to be less efficient but also less invasive as compared with electroporation in these studies¹². We therefore optimized microbubble-enhanced ultrasound gene delivery in muscle tissue in mice²⁶ and showed that this method could be used to safely and efficiently regenerate a critical size bone defect²⁷ and enhance bone-ligament integration²⁸ in pigs' hindlimbs. We believe that sonoporation-based gene therapies have the potential to become a prominent clinical candidate for numerous medical conditions. They are minimally invasive have no systemic effects and could be adjusted to the individual patient depending on the target tissue area and the duration of gene expression that is needed. Further optimizations will be required to improve the efficacy of such approaches at different target sites.

Limitations

This protocol has been optimized for microbubble-enhanced DNA sonoporation in two animal models, mice and pigs. If the user is interested in changing the animal model or the molecule being delivered, it is conceivable that further optimization may be needed. For example, the amount of the molecule of interest, volume of injection and ultrasound settings may need to be adjusted. Furthermore, as ultrasound is strongly reflected at certain types of tissues where there is a substantial difference in impedance, it is imaginable that this method could not be implemented in tissues of hollow viscous organs (e.g., lungs, colon) or near any implanted materials with high reflectivity. The reflection of the acoustic waves may lead to different exposure levels and potential tissue damage. Another limitation of this protocol is that any nonviral gene delivery method (sonoporation included) leads to a transient gene expression profile, which may not be suitable for some gene therapies. A viral gene therapy approach should be considered in cases in which long-term expression is desired. Finally, the operation of ultrasound equipment is user dependent, and the initial results of sonoporation may vary for a new user. It may require several attempts and optimizations to achieve a consistent result. To assist in optimization of ultrasound delivery, the efficiency of this method can be validated through the use of a reporter gene. Direct visualization of microbubble oscillation by ultrasound generally implies that sonoporation exerts a positive effect on the target tissue. It should be noted that prolonged exposures to ultrasound using

the provided settings can lead to overheating and tissue damage; therefore, the therapy should not be repeated at short time intervals.

Overview of the procedure

First, the animal should be sedated and the target area prepared for injection and ultrasound application. A minimal distance of 3 cm must be created between the ultrasound probe and the targeted area. If the area is too superficial, a spacer must be made before the procedure itself (see Steps 1–5 and ‘Spacer adjustments’ below). Shortly before the injection, the microbubbles must be activated and mixed with the plasmid DNA (Steps 6–8). Next, the mixture must be injected under visual guidance of the ultrasound probe. Following successful injection into the area, a therapeutic array of ultrasound is applied to the injection site for ~2 min. Follow-up studies of transgene expression should be conducted at the desired time points, on the basis of transgene function (Step 10).

Experimental design

Animal models—This protocol has been optimized to use microbubble-enhanced sonoporation for gene delivery in murine (10-week-old FVB/N mice; gene delivered intramuscularly to the thigh muscle) and porcine (6- to 9-month-old Yucatan mini-pigs; gene delivered to a segmental fracture created in the tibia bone) animal models according to the ARRIVE guidelines²⁹. We have successfully used it on both male and female animals, and it has no age or animal strain limitation.

Choice of microbubbles

Both protocols include preparation of a microbubble-DNA mix and delivery under ultrasound guidance in vivo. In this protocol, we opted to use FDA-approved Definity microbubbles, which are lipid-encapsulated. Other possible microbubble suspensions that can be used for sonoporation include the albumin-coated Optison product³⁰. Both Definity and Optison have a neutral surface charge, and the latter requires minimal activation before use. Newer experimental cationic microbubble solutions are available that allow DNA-microbubble coupling, which can potentially increase the efficacy of sonoporation³¹.

Choice of ultrasound probe

The choice of the ultrasound probe design may also affect the results of sonoporation. This protocol uses imaging array probes to induce sonoporation. With single-element probes (which can have a flat surface to emit plane waves, or a concave surface to emit focused waves at a given depth), the transducer must be positioned precisely to sonicate the area to be treated. Flat elements can sonicate a large volume but cannot generate high pressure, whereas focused ones can generate high pressure in a small volume. With an array (i.e., the use of multiple elements in a single probe), different delays can be applied to each of the array elements in order to focus the pressure wave at a particular point in space, which can be observed on the monitor. The delaying is an automatic process done by choosing a focus plane on the monitor, and it offers flexibility because the focus can be displaced simply by changing the delays applied to the elements.

Spacer adjustments

To allow later use in human gene therapy trials, clinical-grade ultrasound systems are used in this protocol and are not adjusted for small animal models. To compensate for the small size of murine animal models, a spacer made of agarose is mounted over the ultrasound probe to create distance between the surface of the probe and the animal's tissues. This allows proper focusing of the ultrasound beam at the targeted site, which is not possible if the small animal tissues are placed directly in contact with the probe. For other animal models in which the target site is >3 cm away from the probe surface, the use of a spacer might not be necessary. Arrays allow changing of the focal distance, but doing so can cause a substantial change in the applied acoustic pressure and may require prior calibration to ensure that the intended pressure is applied at the targeted site.

Ultrasound settings

Based on the acoustic pressure values used in this protocol (which are considered low), any commercially available ultrasound system coupled with a standard imaging array probe within the bandwidth of 1–3 MHz can generate the required pressure without any software modifications. In the described protocol, we opted to use a Philips S3 probe with a bandwidth of 1–3 MHz. Other suitable alternatives to this probe include the Philips/ATL P4–1 array (phased array; 1- to 4-MHz bandwidth), GE M5Sc (phased array; 1.5–4.6 MHz), GE C1–6 (curved linear array; 1–6 MHz) and Siemens/Acuson PA230 (phased array; 1–4 MHz), among others that work in the same bandwidth. We have also provided more advanced parameters (excitation frequency, pulse repetition frequency (PRF) and pulse duration) that are machine dependent and usually cannot be changed on commercial systems. A programmable ultrasound system, such as the Verasonics Vantage, can easily replicate the provided sequence for optimal results.

Follow-up studies

After DNA delivery, animals can be followed to study gene expression, protein secretion and other transgene-specific outcomes. In our studies, we monitored bone regeneration outcomes through osteogenic differentiation marker expression, bone structural imaging and biomechanical analysis. Performing power calculations to estimate the number of animals needed is recommended when planning the experiment. In our studies, groups of five mice (transgene expression), three pigs (protein or gene expression) and five pigs (bone formation) were sufficient to achieve statistical significance. In addition to the experimental group treated with a microbubble-DNA mix and ultrasound, the following controls are suggested: no treatment ($n = 3$), ultrasound only ($n = 3$), DNA and microbubble injection without ultrasound ($n = 3$), DNA injection with ultrasound ($n = 3$) and microbubble injection with ultrasound ($n = 3$). Taken together with the experimental group, the users can deduce the relative contribution of each component of the combined therapy, namely microbubbles, DNA and ultrasound, to the overall outcome. A positive control group for local gene delivery, such as electroporation, can also be used.

Materials

Biological materials

- Mice. FVB/N mice (Charles River, strain no. 207) were used for this study. **!**
CAUTION Appropriate institutional regulatory board permission for animal experiments must be obtained. All animal work in this study was performed in accordance with the institutional animal care and use guidelines of the Cedars-Sinai Medical Center.
- Mini-pigs. Yucatán mini-pigs (S&S Farms) were used for this study. **!**
CAUTION Appropriate institutional regulatory board permission for animal experiments must be obtained. All animal work in this study was performed in accordance with the institutional animal care and use guidelines of the Cedars-Sinai Medical Center.

Reagents

- Plasmid DNA, e.g., pUb-Luciferase2 (Sheyn et al.³²), pcDNA3-CMV-rhBMP-6 (Zachos et al.³³)
- Microbubble suspension (Definity; Lantheus Medical Imaging, cat. no. DE4) **▲**
CRITICAL A new batch of microbubbles should be made up fresh before each sonoporation procedure, as they lose their potency within 1 h after preparation. Microbubble dilution should be performed immediately before injection, if possible.
- Agarose powder (Avocado Research Chemicals, cat. no. A10752)
- PBS (Thermo Fisher Scientific, cat. no. 10010023)
- Purified water (Thermo Fisher Scientific, cat. no. 15230162)
- Ultrasound gel (Aquasonic, cat. no. 01–34)
- Acepromazine (Vetone, cat. no. 501075)
- Ketamine (Vetone, cat. no. 501090)
- Atropine (Vetone, cat. no. 510221)
- Propofol (Fresenius Kabi, cat. no. 4463021)
- Isoflurane (Piramal, cat. no. 66794–017)
- 70% (vol/vol) Ethanol (Decon Labs, cat. no. 2716)
- Luciferin (Promega, cat. no. E1601)
- 0.1% (wt/vol) Collagenase (type 1A; Sigma-Aldrich, cat. no. C0130)
- Medical-grade oxygen
- RNeasy isolation kit (Qiagen, cat. no. 74104)
- Reverse transcription kit (Promega, cat. no. A3500)

Equipment

- VialMix shaker (Lantheus Medical, cat. no. VMIX)
- Siemens Antares system with a colinear phased array probe (Siemens Health Care, Ultrasound Division, cat. no. PX4–1)
- Sonos 5500 with a phased array S3 probe (Philips Healthcare, cat. no. 21311a)
- Fluoroscanner mini C-arm imaging system (Hologic, cat. no. INSIGHT2)
- IVIS Spectrum system (PerkinElmer, cat. no. 124262)
- Intubation equipment and ventilation system (Highland Medical Equipment, cat. no. 16–2080)
- Isoflurane anesthesia machine (Highland Medical Equipment, cat. no. 16–7001)
- 1-ml Syringe (Becton Dickinson, cat. no. Z192147)
- 18-gauge Needle (Becton Dickinson, cat. no. Z192554)
- Particle counter (Beckman Coulter, cat. no. 6605698)
- Microwave (Samsung, model ME18H704SFG)
- Shaver (miniARCO Clipper Kit; Kent Scientific, cat. no. CL8787-KIT)
- ABI 7500 Prism system (Applied Biosystems, cat. no. 4351106)

Procedure

Spacer production ● Timing 3.5 h

▲ **CRITICAL** A spacer should be produced if the distance between the ultrasound probe and the targeted area is <3 cm (see ‘Spacer adjustments’ in ‘Experimental design’).

1. Combine 4 g of agarose and 200 ml of purified water in a flask to obtain a 2% (wt/vol) suspension (Fig. 1a).
2. Mix and microwave the suspension at 90 °C until completely dissolved (Fig. 1b).
3. Place the flask in a vacuum for 10 min for degassing (Fig. 1c).
4. Pour the dissolved agarose into a cubic mold larger than the surface area of the ultrasound probe and with a minimal depth of 3 cm; allow the agarose spacer to solidify for at least 3 h at room temperature (23 °C; Fig. 1d).
5. Cut the spacer down to fit the surface area of the ultrasound probe. The final thickness of the spacer should be 3 cm (Fig. 1e).

■ **PAUSE POINT** Spacers can be prepared in advance and stored overnight at 4 °C.

DNA-microbubble preparation ● Timing 5 min

6. Activate the microbubble suspension by shaking the original vial for 45 s using a VialMix shaker.

7. Roughly estimate the volume of the targeted area for sonoporation. This can be achieved by obtaining prior radiographic or sonographic images of the target area.
8. First, use a particle-counting device (e.g., Coulter particle counter) to estimate the number and the average size of microbubbles. In brief, withdraw 20 μl of the activated suspension from the vial and place it in a separate vial. Run the vial in the counting device to obtain the concentration and the size of the microbubbles. The expected diameter range of Definity microbubbles is 1.1–3.3 μm . Next, mix $3.2 \times 10^6/\text{cm}^3$ microbubbles and $320 \mu\text{g}/\text{cm}^3$ plasmid DNA based on the volume of the targeted area and transfer the mixture to a 1-ml syringe. For our pig study, we mixed 10^7 microbubbles and 1 mg of plasmid DNA diluted with PBS to a final volume of 500 μl (calculated for a target area volume of 3.14 cm^3). For our murine model, we mixed 5×10^5 microbubbles and 50 μg of plasmid DNA to a final volume of 20 μl (calculated for a target area volume of 0.155 cm^3).

▲ **CRITICAL STEP** Rotate the syringe periodically and immediately before injection to avoid microbubble aggregation and adhesion to syringe walls.

▲ **CRITICAL STEP** Following microbubble and DNA mixture, the solution should be injected as soon as possible. Longer time periods will cause microbubble aggregation and loss of their potency.

? TROUBLESHOOTING

Gene delivery ● Timing 10 min

9. Two different approaches to gene delivery can be used, depending on the animal model you choose. Use option A for small animal models (e.g., rodents) or in the case that the targeted area for gene delivery is superficial (<3 cm from the skin). This option requires the use of the Siemens Antares ultrasound system with a colinear array transducer or an equivalent multifrequency array and system, as well as a spacer (refer to Steps 1–5 for instructions on how to prepare the spacer)^{34–36}. Use option B for large animal models (e.g., canine, feline, equine and bovine). This option requires the use of Philips Sonos 5500 ultrasound model with an S3 probe, or an equivalent low-frequency array and system, and does not require a spacer.

(A) Murine model

! **CAUTION** Appropriate institutional regulatory board permission for animal experiments must be obtained. All animal work in this study was performed in accordance with the institutional animal care and use guidelines of the Cedars-Sinai Medical Center.

- (i) Set the ultrasound imaging mode to 'Cadence Contrast Agent Imaging mode' at 5.33 MHz.
- (ii) Set the transducer to face upward and use ultrasound gel to couple the spacer to the transducer (Fig. 2a).

- (iii) Apply ultrasound gel on top of the spacer to couple the target area to the spacer.
- (iv) Anesthetize the animal by continuous administration of 1–3% (vol/vol) isoflurane mixed with 100% medical-grade oxygen through a nose cone.

? TROUBLESHOOTING

- (v) Remove all fur from the target area (thigh) by shaving it.
- (vi) Swab the target area with 70% (vol/vol) ethanol and place it on the spacer (Fig. 2b).
- (vii) Cover the area with ultrasound gel; the target area should be completely immersed in the gel to prevent reflections.

▲ **CRITICAL STEP** Having a sufficient amount of gel surrounding the target area is needed to prevent any pockets of air between the animal and the gel. Any tissue/air interface will strongly reflect the ultrasound and may create higher-than-desired pressure values in the targeted area.

- (viii) Using ultrasound guidance, locate the target area and inject the plasmid DNA and microbubble mix into the ROI (Fig. 2c,d). The injected microbubbles should be observed by the ultrasound.

▲ **CRITICAL STEP** Do not apply excessive pressure while injecting the mixture. Overpressure in the syringe can cause microbubble destruction.

? TROUBLESHOOTING

- (ix) To begin sonoporation, use ultrasound to locate the injected mixture and apply an ultrasonic pulse using Cadence Contrast Agent Imaging mode to allow better visualization of the microbubbles at a transmission frequency of 1.4 MHz and a mechanical index (MI) of 0.2. For research-grade ultrasound equipment that allows programmable settings, the following parameters can be used: acoustic pressure of 240 kPa, 100-cycle pulse length and 540-Hz pulse repetition frequency (PRF), with the contrast pulse sequence imaging frame acquired every second for ~2 min until microbubble oscillation is no longer visualized by the ultrasound (Fig. 2e,f).

?TROUBLESHOOTING

- (x) After the animal has recovered from anesthesia, return it to its cage.

TROUBLESHOOTING

(B) Large animal model (pig)

! CAUTION Appropriate institutional regulatory board permission for animal experiments must be obtained. All animal work in this study was performed in accordance with the institutional animal care and use guidelines of the Cedars-Sinai Medical Center.

- (i) Sedate the animal using intramuscular acepromazine (0.25 mg/kg), ketamine (20 mg/kg) and atropine (0.02–0.05 mg/kg).
- (ii) Administer propofol (2 mg/kg) intravenously and intubate endotracheally.
- (iii) Maintain anesthesia using 1–3.5% (vol/vol) inhaled isoflurane for the duration of the procedure.
- (iv) Locate the target area for sonoporation (bone defect located in the tibia) using a Fluoroscanner mini C-arm imaging system (continuous X-ray imaging device) and insert an 18-gauge needle into the center of the area (Fig. 3a).
- (v) Inject the DNA-microbubble mix under visualization, using a Sonos 5500 unit equipped with an S3 probe set to B mode with the focus matching the location of the target area (Fig. 3b).

TROUBLESHOOTING

- (vi) Apply an ultrasonic pulse using Cadence Contrast Agent Imaging mode at a transmission frequency of 1.3 MHz, MI of 0.6 and a depth of 4 cm for ~2 min until microbubble oscillation is no longer observed (Fig. 3c).

TROUBLESHOOTING

Evaluation of gene expression

10. The decision about the preferred method of evaluation largely depends on the properties of the expressed protein. In our murine model, we delivered a gene encoding luciferase and monitored its expression in vivo using bioluminescence imaging. Tissues that express luciferase will emit a bioluminescent signal following the administration of luciferin (the substrate of luciferase) to the animal. We demonstrate how to perform option A only in our murine model, but it is feasible to use this method with any type of animal model. In our porcine model (option B), we delivered the gene encoding BMP-6 and monitored the expression of its RNA by RT-qPCR. This approach for evaluating gene expression is also applicable to both models.

(A) In vivo luciferase expression ● Timing 15 min

- (i) 10 min before bioluminescence imaging, administer intraperitoneally (or by any other method of systemic administration) luciferin diluted with PBS (wt/vol; 126 mg/kg body weight).
- (ii) Anesthetize the mouse using continuous administration of 1–3% (vol/vol) isoflurane mixed with oxygen.
- (iii) Evaluate bioluminescent light emission using an IVIS Spectrum system (bioluminescence imaging device), with the exposure time set to automatic. This procedure can be repeated on the same animals at different time points. Appropriate time points to check the duration of expression following gene delivery are on days 2, 4, 7 and 14.

? TROUBLESHOOTING

(B) Ex vivo transgene expression analysis ● Timing 2 h

- (i) Euthanize the animals according to the institutional animal regulatory board guidelines at the desired time point for evaluation of transgene expression in target cells.
- (ii) Post mortem, extract the tissues subjected to sonoporation in their entirety, wash with PBS, cut into pieces of 1-cm diameter and digest for 1 h in enough volume of 0.1% (wt/vol) collagenase to immerse the dissected tissues.
- (iii) Use a 70-µm cell strainer to filter the solution, and centrifuge at 180g at 4 °C for 7 min and collect the cell pellet.
- (iv) Following cell isolation, extract the RNA using an RNeasy extraction kit according to the manufacturer's instructions. Then, reverse-transcribe the RNA, using random primers and reverse transcriptase. After obtaining cDNA, analyze gene expression using qRT-PCR. In our pig model, we delivered a gene encoding for BMP-6. We analyzed its expression using an ABI 7500 Prism system with Hs00233470_m1 primer. 18S RNA was used as a housekeeping gene control.

? TROUBLESHOOTING

Troubleshooting—Troubleshooting advice can be found in Table 2.

Timing

Steps 1–5, spacer production: 3.5 h

Steps 6–8, DNA-microbubble preparation: 5 min

Step 9, gene delivery: 10 min

Step 10A, in vivo evaluation of luciferase expression: 15 min

Step 10B, ex vivo transgene expression analysis: 2 h

Anticipated results

Using this protocol, one can non-virally introduce any gene of interest in a localized manner. The ease of application, high spatiotemporal control over the introduced gene and a short-term localized gene expression make sonoporation a safe and attractive approach for future gene therapies designed to treat humans. Spatial control can be obtained by directly visualizing the injected site with ultrasound and using a focused ultrasonic pulse to create a localized effect. This protocol has been successfully used to overexpress several genes, in both small and large animal models.

Using a reporter gene (*pUb-Luc2*), we were able to determine the kinetics of gene expression in mice³⁷. Mice were injected once with 50 µg of plasmid DNA premixed with 5×10^5 microbubbles and then were treated with an acoustic pressure of 200 kPa for 2 min (DNA + MBs + US group). This group was compared with mice that were injected once with 50 µg of plasmid DNA premixed with 5×10^5 microbubbles (DNA + MBs group). The bioluminescent signal measured for mice in the group treated with ultrasound indicates that these sonoporation conditions result in efficient gene delivery and prolonged gene expression (up to 21 d) (Fig. 4a,b)³⁷. In addition, we introduced a therapeutic gene (encoding BMP-6) for a pig study of bone regeneration in a tibia nonunion fracture model. A mixture of 1 mg of plasmid DNA premixed with 10^7 microbubbles was injected directly into fracture sites of the experimental pigs. Then the injected fracture sites were exposed to ultrasound with a frequency of 1.3 MHz and a MI of 0.6 for 2 min. The control group consisted of pigs injected with the plasmid DNA and microbubble mixture but not exposed to ultrasound. Equal numbers of animals from the control and experimental groups were randomly selected to be sacrificed 2, 5 or 10 d after gene delivery. Tissues were collected post mortem to characterize *BMP-6* gene and protein expression. Quantitative RT-PCR was done to estimate *BMP-6* gene expression following ultrasound gene delivery, and ELISA was used to estimate BMP-6 protein expression in the defect over time. Using this protocol, we achieved transient and localized therapeutic gene expression (up to 10 d) in a clinically relevant animal model (Fig. 4c)²⁷. Reporter gene expression in the murine model was prolonged as compared with that in the pig model. We believe that the sonoporation efficacy should remain the same among different animal models; the differences could be attributed to the nature of the target tissue and the effect of the expressed protein, as proteins that lead to increased cell turnover may affect the duration of their expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge funding support from the California Institute for Regenerative Medicine (CIRM; (grant TR4-06713 to D.G.) and NIH grants R01EB026094 (to D.G.) and R01CA112356 (to K.W.F.).

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Fig. 1 |. Spacer production.

a, Agarose and purified water mixture before heating. **b**, Completely dissolved mixture following microwave heating. **c**, Mixture during the degassing process. **d**, Degassed mixture is poured into a cubic mold. **e**, Solidified mixture is shaped to match the surface area of the ultrasound probe.

Fig. 2 |. Sonoporation in mice.

a,b, Ultrasound gel is used to couple the targeted area (red arrow) to a spacer on the ultrasound transducer. The targeted area is completely immersed in ultrasound gel. **c,d,** With the aid of ultrasound guidance (**c**, the thigh muscle (marked by a red dashed circle) can be seen on top of the spacer (marked by a green dashed line)), we inject the muscle (**d**, needle in the target area (marked by blue arrowheads) with microbubbles (marked by a red arrow)) with the plasmid DNA-microbubble mix. **e,f,** During treatment (**e**, red arrow marks microbubbles) and immediately afterward (**f**), the injection site was visualized using a contrast-enhanced mode. Permission was obtained from the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center for all procedures performed within this protocol. Adapted with permission from Shapiro et al.²⁶, Elsevier.

Fig. 3 |. Sonoporation in pigs.

a, Injection of DNA-microbubble mixture into the tibia bone fracture site. The ultrasound probe is placed adjacent to the needle for optimal visualization of the injected mixture. **b**, Ultrasound B-mode visualization of the target site (marked by a red dashed square). **c**, Ultrasound contrast-agent imaging of the target site (red dashed square) during DNA and microbubble injection. Permission was obtained from the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center for all procedures performed within this protocol. Adapted with permission from Bez et al.²⁷, American Association for the Advancement of Science.

Fig. 4 |. Anticipated results.

a, Representative image obtained using bioluminescence imaging (IVIS Spectrum) to visualize the measured luciferase signal 4 d after sonoporation of pUb-Luc2 into the thigh muscle in mice. The injected thigh muscle is indicated by a red oval. **b**, Luciferase expression profile in mice injected with plasmid DNA premixed with microbubbles and treated with sonoporation (DNA + MBs + US group; $n = 6$) compared to control group injected with plasmid DNA premixed with microbubbles (DNA + MBs group; $n = 6$). The treatment effect was monitored using bioluminescent imaging for 21 d after treatment (*** $P = 0.0003$); P values were determined by two-way ANOVA with multiple comparisons. **c**, BMP-6 expression and secretion monitored using RT-qPCR (gene expression measured as relative quantification (RQ), left-side y axis label) and ELISA (protein secretion measured in picograms (pg), right-side y axis label), respectively, at days 2, 5 and 10 following sonoporation into a tibia bone fracture model in pigs. Data are means $\pm$ s.e.m. BMP, bone morphogenetic protein. Institutional regulatory board permission was obtained from the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center for all procedures performed within this protocol. **a,b**, adapted with permission from Shapiro et al.²⁶, Elsevier. **c** adapted with permission from Bez et al.²⁷, American Association for the Advancement of Science.

Table 1 |

In vivo sonoporation studies on various tissues or organs

	Gene name	Target tissue and species	Sonoporation parameters
Fujii et al. ³⁸	Stem cell factor (SCF), stromal cell-derived factor (SDF)-1a	Cardiac tissue, rat	7 MHz, MI: 1.6 for 20 min
Leong-Poi et al. ³⁹	Vascular endothelial growth factor (VEGF)	Hind-limb ischemia, rat	1.3 MHz, transmit power of 0.9 W for 10 min
Zolochavska et al. ⁴⁰	IL-27	Prostate cancer, rat	1 MHz, 50% duty cycle, acoustic pressure of 0.12 MPa for 2 min
Hauff et al. ⁴¹	p16	Pancreatic cancer, rat	5 MHz, MI: 1.0
Shimamura et al. ⁴²	Luciferase	Brain, rat	1 MHz, 26% duty cycle, acoustic pressure of 0.55 MPa
Takeuchi et al. ⁴³	Hepatocyte growth factor (HGF)	Brain, mouse	1 MHz, 2.0 W/cm ²
Nakashima et al. ⁴⁴	Growth/differentiation factor (GDF) 11	Dental pulp, canine	1 MHz, 0.5 W/cm ² for 30 s
Anderson et al. ⁴⁵	Factor IX	Liver, mouse	1 MHz, peak negative pressure 1.6–1.7 MPa for 2 min
Chen et al. ⁴⁶	Rat insulin 1 promoter (RIP)-luciferase	Pancreatic islets, rat	1.3 MHz, MI: 1.2–1.4

Table 2 |

Troubleshooting table

Step	Problem	Possible reason	Solution
8	Microbubbles have aggregated to form large bubbles (can be identified by phase separation within the syringe, the mixture changing color to become less milky-white)	The microbubbles were activated too far ahead of injection	Prepare a fresh batch of microbubbles
9A(iv)	Animal gasping under anesthesia	Excessive isoflurane delivery	Reduce the concentration of isoflurane delivered to the animal
9A(viii) and 9B(v)	Resistance to injection of DNA-microbubble mixture	The needle is too close to bone structures	Take the needle back a few millimeters
	Microbubbles are not visualized on the ultrasound image after injection	Microbubbles were not injected at the target site or microbubbles were destroyed during injection (overpressure in the syringe)	Move the array probe to see if microbubbles are present in a different imaging plane. If no microbubbles are present, repeat the injection
9A(ix) and 9B(vi)	Strong reflections on ultrasound imaging	Not enough ultrasound gel or presence of air bubbles in the gel	Add fresh ultrasound gel under the application site while ensuring that no air pockets or bubbles are present
9A(x)	The animal is slow to recover from anesthesia	The animal is hypothermic	Use a heating pad for the animals to recover upon or add any other source of heating (e.g., heating lamp)
10A(iii) and 10B(iv)	The duration of gene expression is too short	(i) Not enough plasmid delivered (ii) Type of vector not compatible with target tissue (e.g., promoter silencing) (iii) Low expression capability of the target cells	(i) Optimize the DNA-microbubble mix by adding more DNA or microbubbles, or increase the acoustic pressure applied by the ultrasound. Alternatively, the treatment can be repeated at a later time point (ii) Design the vector to suit the expression profile of the target tissue. Use an alternative gene delivery method (e.g., electroporation) to validate proper gene expression (iii) Low tissue turnover and a high fraction of senescent cells can limit the expression of the vector. Consider targeting a different area with different cell populations for improved gene expression