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ORIGINAL ARTICLE

siRNA-mediated gene silencing in the salivary gland using *in vivo* microbubble-enhanced sonoporation

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OBJECTIVES: siRNA-induced gene silencing in the salivary gland using microbubble-enhanced sonoporation was used to develop an *in vivo* gene knockdown technique.

METHODS: siRNA targeting rat glyceraldehyde-3-phosphate dehydrogenas (GAPDH) was mixed with echo-enhanced microbubbles and reverse-injected into rat parotid glands using transdermal ultrasound. To compare direct and transdermal ultrasound efficiencies, an incision was made on the lateral neck to expose the parotid glands for direct application. The efficiency of gene suppression was determined using quantitative reverse transcription-polymerase chain reaction 24–72 h after siRNA delivery. Cytotoxicity was assessed using histological analysis.

RESULTS: Expression of rat GAPDH in the parotid glands was silenced 48 h after siRNA was delivered by ultrasound (frequency: 1 MHz; intensity: 2 W cm^{-2}; exposure time: 2 min). High-intensity ultrasound induced tissue damage and apoptotic change. Echo-enhanced microbubbles significantly improved siRNA-induced gene silencing by 10–50%. Compared with transdermal application, direct-exposure ultrasound was only slightly effective, and no significant difference in gene expression was observed.

CONCLUSION: The results indicate that microbubble-enhanced sonoporation can yield *in vivo* siRNA gene silencing in the rat parotid gland. This technique could be applied to provide gene knockdown organs for functional genomic analyses and to develop siRNA-based gene therapy.

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Keywords: sonoporation; siRNA; gene silencing; *in vivo*; salivary gland

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Introduction

The silencing of gene expression by siRNA is rapidly becoming a powerful tool for experimental analysis and disease treatment. However, the small siRNA constructs are not readily internalized by mammalian cells. Therefore, the problem of intracellular delivery must be solved before siRNAs can be used for specific gene silencing *in vivo*. High-pressure intravenous injection (hydrodynamic delivery) (Lewis *et al*, 2002; McCaffrey *et al*, 2002), electroporation (Kishida *et al*, 2004; Akaneya *et al*, 2005; Takabatake *et al*, 2005), and liposome- and atelocollagen-mediated delivery (Minakuchi *et al*, 2004; Yano *et al*, 2004) are methods that demonstrate the feasibility of using siRNA for gene silencing *in vivo*. Although gene silencing has been accomplished using these procedures, some have disadvantages for clinical applications. For example, the massive volume of aqueous fluid that is injected at once during high-pressure intravenous injection puts the recipient at risk for acute heart failure. Safety and tissue-specific delivery of siRNA present the greatest obstacles to the implementation of siRNA in a clinical setting. Viruses are efficient delivery systems for DNA and shRNA that silence genes using the same mechanisms as siRNA, but these vectors are associated with serious immunogenicity and cytotoxicity problems (Miller *et al*, 2002a). Viral vector-mediated procedures may induce complications associated with recombinant viruses, hindering clinical application of the systems to gene therapy.

A candidate for a more efficient, non-viral method of gene transfer is sonoporation of cells. In sonoporation, ultrasound is used to increase the porosity of the cell membrane, probably by the production of small and transient nonlethal pores (Ogawa *et al*, 2001). Ultrasound induces the formation of cavitation bubbles that, by mechanical action, cause enough damage to the cell membrane to allow entry into the cell, but not so much damage that the cells cannot reseal the membrane and survive (Miller *et al*, 2002b). Microbubble contrast agent-enhanced ultrasound technologies allow site-specific sonoporation and intracellular delivery of plasmid DNA (Nakashima *et al*, 2003; Tsunoda *et al*, 2005; Wang *et al*, 2005). Microbubbles, which are gas bubbles

of approximately 3 μm diameter, were developed primarily as contrast agents to improve ultrasonographic scans (Taniyama *et al*, 2002; Li *et al*, 2003; Lu *et al*, 2003). Microbubbles act as cavitation nuclei, effectively focusing ultrasonic energy, and can potentiate bioeffects (Ogawa *et al*, 2001), including sonoporation. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the target, we investigated the possibility of using microbubble-enhanced ultrasound to deliver chemically synthesized siRNA, reverse-injected via the excretional duct, to inhibit its expression in the salivary gland. The efficiency of gene suppression was determined with quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results presented in this study demonstrate that microbubble-enhanced ultrasound sonoporation remarkably enhances GAPDH gene suppression by reverse-injected siRNA.

Materials and methods

In vivo *siRNA transfection by sonoporation in rat parotid glands*

Animals were treated according to procedures approved by the Animal Care and Use Committee of Tokyo Dental College. Male Wistar rats (7–9 weeks old, 200–250 g) were obtained from SLC, Inc. (Hamamatsu, Japan). The animals were raised with water and food *ad libitum* and kept on 12-h light/dark cycles. The rats were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital-Na at 50 mg kg^{-1} before the anterior and lateral areas of the neck were shaved with a razor. Atropine (2 mg kg^{-1}; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was administered i.p. 30 min prior to retroductal infusion of siRNA. A piece of modified Size 4 polyethylene tubing (Imamura Co., Ltd., Tokyo, Japan) was inserted into the parotid duct with the aid of a surgical microscope. Chemically synthesized siRNA (32 μg), a suppressor of rat (and human and mouse) glyceraldehyde-3-phospate dehydrogenase (Silencer GAPDH siRNA, Cat #4631; Ambion, Inc., Austin, TX, USA), or a negative control siRNA (Silencer Negative Control #1 siRNA, Cat #4611; Ambion, Inc.) was mixed with echo-enhanced microbubbles (Optison®; Amersham Health, Oslo, Norway) and saline (total = 60 μl). After 50-μl aliquot was reverse-injected into the parotid gland, the syringe was left in place for 10 min to prevent fluid backflow. Following siRNA delivery, a Sonoporation Gene Transfection System (Sonitoron 2000V; RICH-MAR, Inola, OK, USA) was used to deliver a transdermal application of ultrasound (probe: 12 mm; frequency: 1 MHz; intensity: 0.5–4.0 W cm^{-2}; exposure time: 2 min; duty cycle: 50%) to the parotid gland. An incision on the lateral neck exposed the parotid gland for a direct application of ultrasound irradiation (12 mm probe). The animals were euthanized with diethylether 24–72 h after sonoporation.

Analysis of gene silencing with quantitative RT-PCR (qRT-PCR)

Parotid gland tissues were removed, rinsed with ice-cold PBS (−), immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Total RNA was prepared from frozen tissues with the Nucleospin RNA II Total RNA Isolation Kit (MACHEREY-NAGEL GmbH & Co., KG, Düren, Germany) according to the manufacturer's instructions.

cDNA was synthesized using 4 μg of total RNA, which was denatured for 10 min at 65°C with 5 μM oligo-dT_{18} primer (Roche Diagnostics GmbH, Penzberg, Germany). Transcriptor Reverse Transcriptase (10 U per reaction; Roche Diagnostics), dNTP mix (1 mM of each dNTP), and Protector RNase Inhibitor (20 U per reaction; Roche Diagnostics) were added and the mixture was incubated for 30 min at 55°C. The reverse transcriptase was inactivated by incubating the reaction mixture at 85°C for 5 min, followed by cooling on ice.

The following primers for quantitative PCR of rat GAPDH and β-actin were designed and synthesized by Takara Bio, Inc., (Tokyo, Japan): GAPDH - 5′-TCC CTC AAG ATT GTC AGC AA-3′ (forward) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (reverse); β-actin - 5′-AGC CAT GTA CGT AGC CAT CC-3′ (forward) and 5′-CTC TCA GCT GTG GTG GTG AA-3′ (reverse). The PCR products were 309 bp (GAPDH) and 228 bp (β-actin) in size. The template cDNA was amplified using SYBR® Green Realtime PCR Master Mix (TOYOBO Co., LTD, Osaka, Japan) in a DNA Engine Opticon™ 2 System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The conditions for the PCR run were as follows: initial denaturation at 95°C for 15 min; 40 cycles of 94°C for 15 s, 59.2°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 15 min. The melting curves of the PCR products were observed from 72 to 95°C (with an incremental rate increase of 0.2°C s^{-1}). GAPDH data were normalized to β-actin data from the same template cDNA, and relative GAPDH mRNA expression was calculated using the comparative Ct method ($\Delta\Delta$ Ct).

Histopathological analysis of tissue damage

Parotid glands were fixed in 10% neutral-buffered formalin/PBS, and 4-μm paraffin sections were stained with hematoxylin and eosin (H&E) for histopathologic examination. The sections were also used for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining to assess the apoptosis that is induced with tissue damage. TUNEL staining was performed using an *In situ* Apoptosis Detection Kit (Takara Bio, Inc., Shiga, Japan). A Zeiss Axiophot 2 microscope (Carl Zeiss Imaging Solutions, GmbH, Munich, Germany) was used for light microscopy (H&E staining) and immunofluorescence microscopy (TUNEL staining). Images were captured using a chilled three-chip color charge-coupled-device camera and digitized using image analysis software (AxioVision Rel.4.6.3.0; Carl Zeiss Imaging Solutions).

Results

Optimum ultrasound intensity and concentration of echo-enhanced microbubbles for siRNA sonoporation into rat parotid glands in vivo

siRNA against GAPDH was introduced into rat parotid glands *in vivo* by microbubble-enhanced sonoporation.

Optimal sonication parameters for siRNA-induced gene silencing were identified by evaluating the effect of ultrasound intensity. As intensity increased from 0 to 4 W cm^{-2} (frequency: 1 MHz; duty cycle: 50%; exposure time: 2 min), the level of GAPDH mRNA was significantly ($P < 0.05$) suppressed at 1 and 2 W cm^{-2} (Figure 1). The efficiency of siRNA-induced GAPDH gene silencing was saturated from 1 to 2 W cm^{-2}.

An examination of H&E stained sections showed that the parotid glands were not damaged by reverse-injection of siRNA plus Optison 48 h after treatment. Histologic images of tissues sonoporated by 1 or 2 W cm^{-2} of ultrasound also appeared normal (Figure 2, left row: 0/1/2 W cm^{-2}). In contrast, glands exposed to 4 W cm^{-2} showed tissue damage 48 h postapplication (Figure 2, lowest of left row). Many acinic cells were shrunken, and there was destruction of acini and inflammatory cell invasion. Also, interstitial acute inflammation (edema and inflammatory cell invasion) was observed in glands exposed to 4 W cm^{-2}. Apoptosis in ultrasound-exposed parotid glands was assayed using Hoechst 33258 and TUNEL staining and DNA laddering (Figure 2, right row). TUNEL staining, which is used to identify apoptotic cells, revealed an increase in the number of TUNEL-positive nuclei in the glands after 4 W cm^{-2} of ultrasound exposure *vs* glands exposed to lower intensity ultrasound.

Quantitation of mRNA levels by qRT-PCR revealed that Optison echo-enhanced microbubbles significantly improved the efficiency of siRNA-induced gene silencing at concentrations of 10–50% (Figure 3). Concentrations of 20–50% resulted in maximum gene silencing efficiency in rat parotid glands as assessed by GAPDH siRNA-induced suppression of mRNA levels.

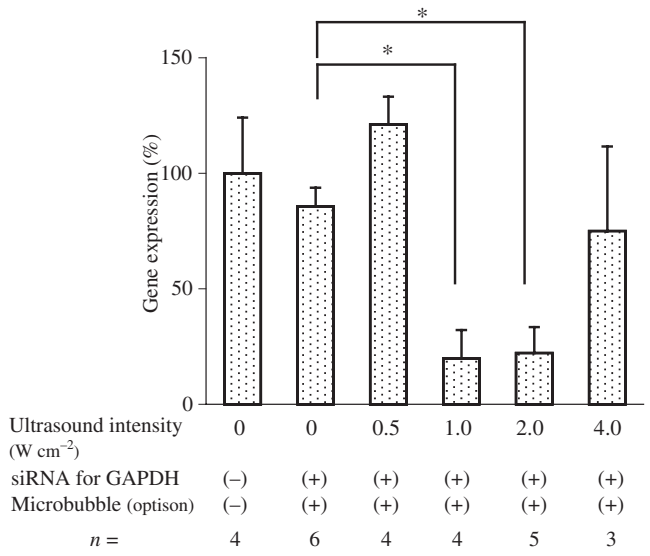

Figure 1 Effect of ultrasound intensity on microbubble-enhanced sonoporation. Fifty-microliter aliquots of siRNA containing 20% Optison were reverse-injected into rat parotid glands. The glands were then exposed to a transdermal application of ultrasound at various intensities (frequency: 1 MHz; intensity: 0, 0.5, 1.0, 2.0, or 4.0 W cm^{-2}; exposure time: 2 min; duty cycle: 50%). The rats were killed 48 h later and GAPDH mRNA expression in the parotids was measured by qRT-PCR. All data are presented as the means ± s.e.m. *$P < 0.05$ (*vs* the Ultrasound intensity 0 group)

Comparison of transdermal and direct ultrasound exposure

The experiments mentioned earlier in this study utilized transdermal ultrasound irradiation. As ultrasound intensity can be weakened by skin tissues, transdermal ultrasound irradiation was compared with direct irradiation of surgically exposed parotid glands (direct sonication). Direct sonication was slightly more effective for siRNA-induced gene silencing, but no significant difference was observed (Figure 4).

Time course for siRNA gene silencing with siRNA against GAPDH or negative control siRNA

Based on the optimum conditions for intensity and microbubble concentration, the time course for specific gene silencing was compared with the nonspecific effect of siRNA. When ultrasound was used at a frequency of 1 MHz (duty cycle: 50%) with an intensity of 2 W cm^{-2} for 2 min, high-efficiency siRNA targeting GAPDH-induced rGAPDH gene suppression was observed 48 h after transfection (Figure 5). At 48 h after siRNA delivery, rat parotid glands exhibited an 80% decrease in GAPDH mRNA levels (*vs* naive glands). However, 72 h following siRNA delivery, mRNA levels had recovered to 83% of the naive level. In contrast, sonoporation of control siRNA led to a slight increase in GAPDH gene expression above naive levels observed 48 h after treatment.

Discussion

RNA interference (RNAi) using siRNA is a powerful tool for gene-expression silencing, with uses not only in basic biology but also in therapeutic applications (Bantounas *et al*, 2004; Uprichard, 2005). Currently, the major issue preventing more widespread use of siRNA is focused on the method of intracellular delivery, especially *in vivo*. Viral vector infection with siRNA is effective for RNAi *in vivo* (Xia *et al*, 2002). However, it is difficult to regulate the extent of the infected region when using viral vector, and there are severe limitations imposed by cytotoxicity and immunogenicity (Miller *et al*, 2002a). Although the efficiency of gene delivery is relatively low, the use of non-viral methods has many advantages for clinical applications of siRNA. Lipofection is an efficient delivery technique *in vitro*, but is less efficient *in vivo* and has shown serious cytotoxicity in some cases. Widely used methods for *in vivo* siRNA delivery are high-pressure intravenous (hydrodynamic) injection (Lewis *et al*, 2002; McCaffrey *et al*, 2002), electroporation (Kishida *et al*, 2004; Akaneya *et al*, 2005; Inoue *et al*, 2005), atelocollagen (Minakuchi *et al*, 2004; Takeshita *et al*, 2005), and the use of a naked plasmid vector encoding short hairpin RNA (shRNA) (Kong *et al*, 2004; Zhang *et al*, 2004). Hydrodynamic injection of siRNA puts the recipient at risk for acute heart failure, as a large volume of aqueous fluid must be injected into the circulation over a short

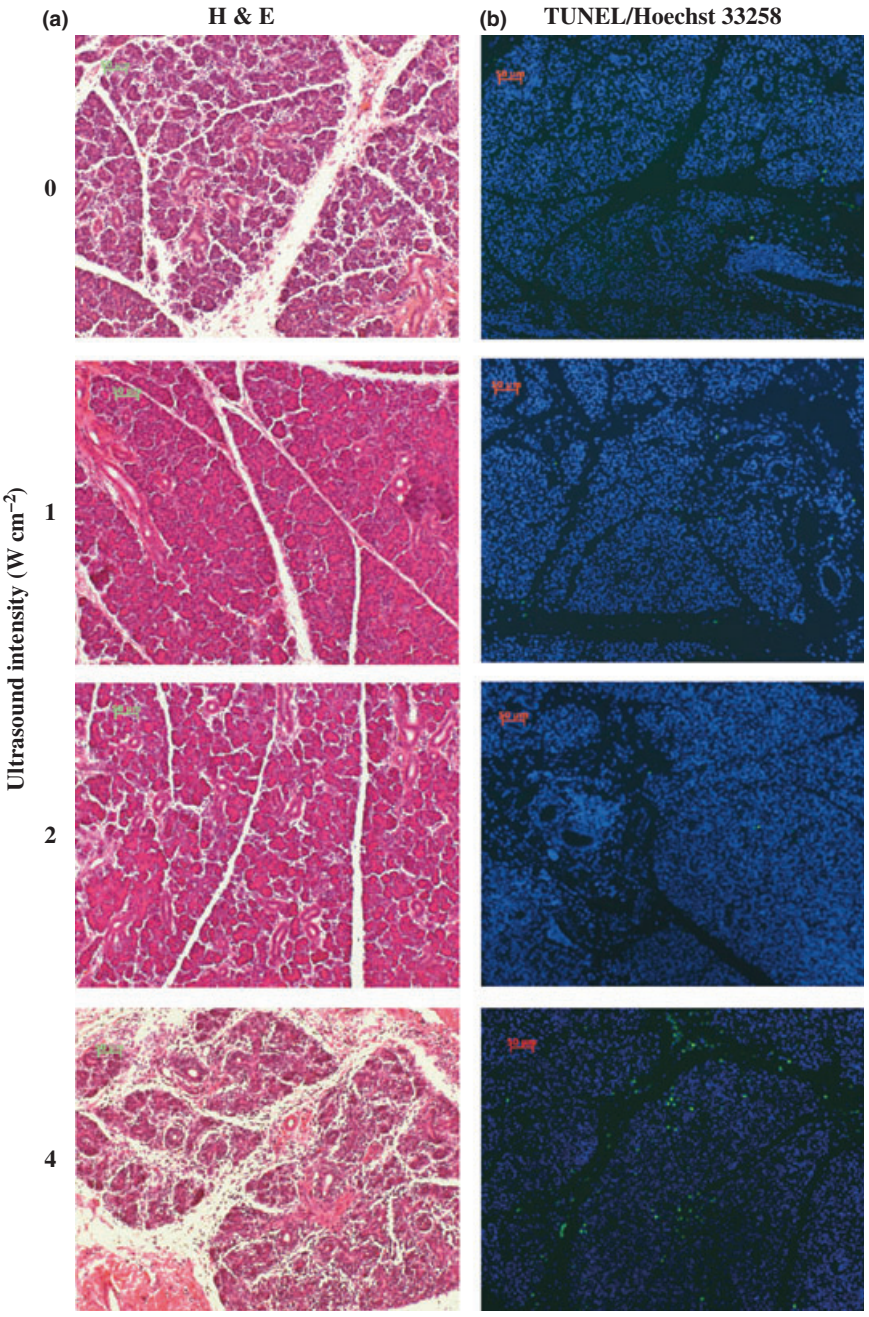

Figure 2 Histologic demonstration of tissue damage 48 h after siRNA sonoporation. Rat parotid glands were treated with 50-μl aliquots of siRNA containing 20% Optison. The glands were then exposed to the same range of ultrasound intensities. Shown are representative microscopic images of parotid glands on day 2 (48 h) of H&E staining (row **a**) or FITC-labeled (green) immunofluorescent TUNEL staining (row **b**). In TUNEL staining, nuclei are stained with Hoechst 33258 (blue)

period of time. In addition, as some of these methods deliver siRNA throughout the body, undesirable suppression of other genes may be induced in untargeted organs. Although some reports have shown that electroporation is useful for the intracellular delivery of siRNA to specific organs (Kishida *et al*, 2004; Akaneya *et al*, 2005; Inoue *et al*, 2005), this method requires the insertion of electrodes into the target area.

Advances in ultrasound and microbubble technologies have enhanced the ultrasound-echo contrast technique for nucleic acids, including siRNA, and raised the possibility of using ultrasound for gene-transfer (Miller *et al*, 2002b). Sonoporation transiently alters cell membrane permeability, an effect that is enhanced by the addition of microbubble ultrasound contrast agents that act to produce ultrasound-enhanced cavitation energy (Ogawa *et al*, 2001; Miller *et al*, 2002b). This technique enables site-specific delivery of naked plasmid DNA (Taniyama *et al*, 2002; Li *et al*, 2003; Nakashima *et al*, 2003) and siRNA (Kinoshita and Hynynen, 2005; Tsunoda *et al*, 2005), both *in vitro* and *in vivo*.

In this report, we investigated the possibility of using microbubble-enhanced sonoporation for siRNA delivery to rat parotid glands *in vivo*. We found that 2 min of microbubble-enhanced sonoporation (frequency: 1 MHz; intensity: 2 W cm^{-2}; duty cycle: 50%; microbubble concentration: 20%) could be used to deliver siRNA to the parotid gland. Gene silencing was

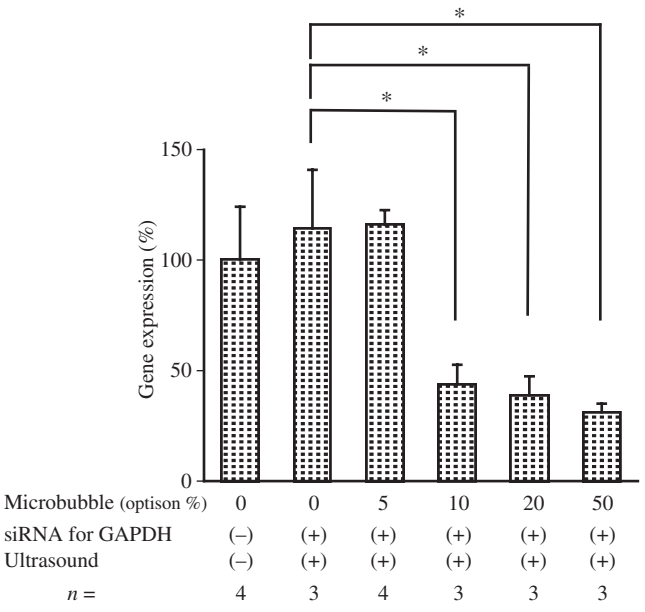

Figure 3 Effect of microbubble (Optison) concentration on bubble-enhanced sonoporation. Fifty-microliter siRNA aliquots containing various concentrations of Optison were reverse-injected into rat parotid glands. The glands were then exposed to a transdermal application of ultrasound (frequency: 1 MHz; intensity: 2.0 W cm^{-2}; exposure time: 2 min; duty cycle: 50%). The rats were killed 48 h later and GAPDH mRNA expression in the parotid glands was measured by qRT-PCR. All data are presented as the means ± s.e.m. $*P < 0.01$ (*vs* Optison 0% group)

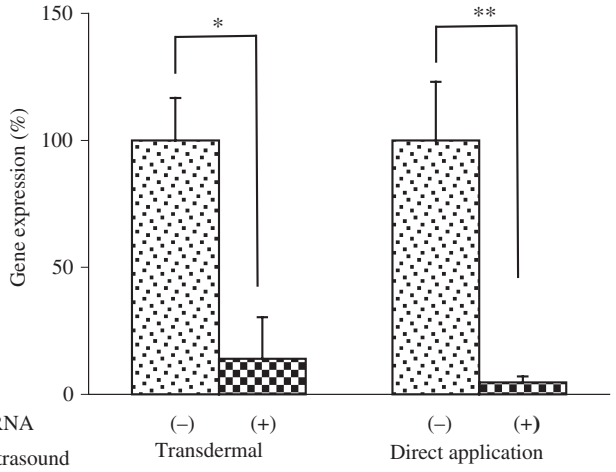

Figure 4 Comparison of transdermal and direct sonoporation for siRNA-induced gene silencing. Fifty-microliter siRNA aliquots containing 20% Optison were reverse-injected into rat parotid glands. For direct (open) ultrasound irradiation, an incision was made on the lateral neck to expose the parotid glands and transdermal or direct ultrasound (frequency: 1 MHz; intensity: 2.0 W cm^{-2}; exposure time: 2 min; duty cycle: 50%) was applied. The animals were killed 48 h later and GAPDH mRNA expression in the parotid glands was measured by qRT-PCR. All data are presented as the means ± s.e.m. of three different experiments. $*P < 0.05$, $**P < 0.01$ (*vs* without siRNA group)

confirmed by qRT-PCR analysis of parotid gland tissue. The transfection of GAPDH siRNA transiently suppressed GAPDH mRNA expression at 24–48 h

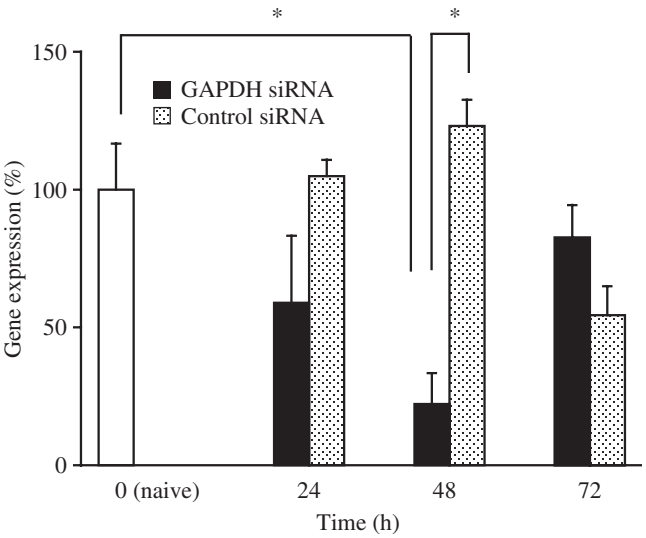

Figure 5 Microbubble-enhanced sonoporation of siRNA transiently silenced GAPDH mRNA expression in rat parotid glands *in vivo*. Thirty-two micrograms of chemically synthesized siRNA (black bars) targeting rat glyceraldehyde-3-phospate or *negative control siRNA* (gray bars) was mixed with 50 μl of saline containing 20% echo-enhanced microbubbles (Optison). Aliquots were reverse-injected into rat parotid glands. Following siRNA delivery, transdermal ultrasound was applied to the parotid glands (frequency: 1 MHz; intensity: 2.0 W cm^{-2}; exposure time: 2 min; duty cycle: 50%) with a 12-mm probe. Rats were killed 0 (naive), 24, 48, or 72 h later, and GAPDH mRNA expression was measured by qRT-PCR. All data are presented as the mean ± s.e.m. of four different experiments. $*P < 0.01$

(Figure 5). Such transient silencing by siRNA has also been reported for other methods (Filleur *et al*, 2003; Kishida *et al*, 2004). Because mRNA was significantly inhibited for only a short period of time, the suppression of a protein that is slowly degraded and/or is present in great quantity may prevent detection of a major decrease in protein content. In fact, immunoblot analysis did not detect a significant decrease in GAPDH protein content (data not shown). The shortened period of siRNA-induced gene silencing may be due to degradation of siRNA by endogenous RNase, not only in sonoporation but also in other non-viral gene silencing techniques such as chemically synthesized 21-mer siRNA *in vivo*. This transient detection prevents practical use of *in vivo* non-viral siRNA-induced gene silencing to create a new niche for experimental gene analysis and clinical therapeutics. Obtaining a longer period of siRNA silencing would require the use of RNase inhibitors or siRNA modifications, such as 2'-O-methyl-, 4'- O- alkyl-, 4'-thio-, and/or phosphorothioate (Choung *et al*, 2006; Dande *et al*, 2006; Zhang *et al*, 2006), to prevent siRNA degeneration. In addition, high-purity and relatively long (25–29 bp) double-stranded RNA (Kim *et al*, 2005; Siolas *et al*, 2005) might increase the specificity and efficiency of gene silencing *in vivo*. In fact, a preliminary study using chemically modified 25-mer siRNA extended mRNA suppression and produced a significant decrease of protein levels in immunoblot assays (data not shown).

In previous reports, ultrasound intensity and microbubble concentration affected the efficiency and

cytotoxicity of gene delivery by sonoporation (Li *et al*, 2003; Nakashima *et al*, 2003; Kinoshita and Hynynen, 2005). Our investigation of sonication parameters for siRNA-induced gene silencing showed that maximum efficiency was obtained at an intensity of 1 and 2 W cm^{-2} (Figure 1). As high-intensity ultrasound is cytotoxic (Lu *et al*, 2003; Feng *et al*, 2008; Ide *et al*, 2008; Miller and Dou, 2009), we examined intensity-dependent cytotoxicity in rat parotid glands. Histologic examination of parotid glands exposed to 0, 1, 2, and 4 W cm^{-2} of ultrasound (Figure 2) showed no significant damage up to 2 W cm^{-2}. Toxic changes (atrophy of acinic cells, destruction of acini, and inflammatory cell invasion) in H&E staining (Figure 2, row A) and an increase in TUNEL-positive cells were seen at 4 W cm^{-2} (Figure 2, row B). As the choice of ultrasound intensity is dependent on the depth and thickness of tissues surrounding the target tissue, it may be necessary to regulate ultrasound intensity based on hypodermic structural information provided by imaging such as ultrasound echo.

As shown in Figure 3, the optimal concentration of echo-enhanced microbubbles (Optison) for siRNA delivery was 20–50%. These results were similar to the optimal intensity and microbubble concentration for plasmid DNA delivery to the mouse heart (Tsunoda *et al*, 2005). Higher intensity and microbubble concentration showed high efficiency of mRNA suppression after siRNA delivery.

The key mechanism of microbubble-enhanced siRNA delivery is thought to be the cavitation energy created by collapse of the microbubbles (Guzman *et al*, 2003). Optison is an ultrasound contrast agent consisting of albumin microbubbles filled with octafluoropropane (OFP) (Podell *et al*, 1999). These microbubbles are elastic and compressible, have lower density than water, and create an acoustic impedance mismatch from biological tissues and fluids (Forsberg *et al*, 1997). Furthermore, microbubbles are efficient reflectors of ultrasound and lower the threshold of energy for cavitation (Apfel and Holland, 1991). It has been shown that ultrasound frequency and bubble concentration may play a role in the amount of cavitation by collapsing the microbubbles (Unger *et al*, 2001; Ng and Liu, 2002). The transient perforation of the cellular membrane produced by the collapsing microbubbles might cause the increase in gene transfer efficiency. Indeed, ultrasound-enhanced microbubbles have been shown to improve gene transfer efficiency *in vitro* (Kinoshita and Hynynen, 2005) and *in vivo* (Li *et al*, 2003; Lu *et al*, 2003; Nakashima *et al*, 2003) significantly. The finding in this study that Optison (microbubbles) significantly improves siRNA-induced gene silencing (Figure 3) is consistent with the previous reports. However, it has been reported that increasing the microbubble concentration and ultrasound acoustic pressure enhances cell killing (Li *et al*, 2003; Nakashima *et al*, 2003). Therefore, optimization of microbubble concentration and ultrasound intensity might be necessary.

In all of previous reports that used microbubble-enhanced sonoporation *in vivo*, the technique was performed by direct exposure of ultrasound to tissues (Taniyama *et al*, 2002; Lu *et al*, 2003; Nakashima *et al*, 2003). Typically, tissue was surgically exposed and ultrasound was irradiated via conductivity gel. However, the clinical application of this technique with minimum invasion, transdermal irradiation, would be more beneficial. We compared transdermal ultrasound irradiation with direct irradiation of surgically exposed rat parotids (direct sonication) (Figure 4). Direct sonication was slightly more effective for siRNA-induced gene silencing but no significant difference was seen, showing that sonoporation by transdermal irradiation is effective for gene delivery to shallow tissue(s) such as the parotid gland. Consequently, transdermal sonoporation may prove advantageous for the clinical application of siRNA therapies.

In conclusion, sonoporation enables *in vivo* GAPDH gene silencing by chemically synthesized siRNA in the rat parotid gland. Albumin-based microbubbles (Optison) remarkably enhanced the efficiency of gene silencing. This technique may be applied to provide siRNA-induced site-specific gene silencing *in vivo* for functional genomic analyses without gene-knockout animals, as well as to search for candidate molecules for gene therapy. The expanded therapeutic window, optimized parameters for siRNA cellular delivery, and potential for tissue damage by microbubble-enhanced sonoporation must be assessed for future use of this technique.

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Author contributions

T Sakai primarily designed the study, did the experiment, analyzed the data and wrote the paper. M Kawaguchi helped Sakai with animal management and edited the manuscript. Y Kosuge did the histological evaluations.

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