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Abstract. We investigate the feasibility of using nanosecond pulsed laser-induced stress waves (LISWs) for gene transfer into rat facial muscles. LISWs are generated by irradiating a black natural rubber disk placed on the target tissue with nanosecond pulsed laser light from the second harmonics (532 nm) of a Q-switched Nd:YAG laser, which is widely used in head and neck surgery and proven to be safe. After injection of plasmid deoxyribose nucleic acid (DNA) coding for Lac Z into rat facial muscles, pulsed laser is used to irradiate the laser target on the skin surface without incision or exposure of muscles. Lac Z expression is detected by X-gal staining of excised rat facial skin and muscles. Strong Lac Z expression is observed seven days after gene transfer, and sustained for up to 14 days. Gene transfer is achieved in facial muscles several millimeters deep from the surface. Gene expression is localized to the tissue exposed to LISWs. No tissue damage from LISWs is observed. LISW is a promising nonviral target gene transfer method because of its high spatial controllability, easy applicability, and minimal invasiveness. Gene transfer using LISW to produce therapeutic proteins such as growth factors could be used to treat nerve injury and paralysis. [DOI: 10.1117/1.3628313]

Keywords: gene transfer; optical transfection; lasers; facial muscles.

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1 Introduction

Regeneration in the peripheral nerve is poor after severe or chronic denervation. Promoting facial nerve regeneration is an important area of study because facial paralysis markedly decreases the quality of life. Previous reports demonstrated that topically applied growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF), enhance facial nerve regeneration.[1–4] Secretion of such proteins, upregulated by gene transfection, may be a promising treatment.

Viral and nonviral vectors are two major categories of gene delivery vehicles that are currently being applied for gene therapy. The viral delivery system has been shown to be efficient for gene transduction in muscle. Adeno-associated viral vectors, for example, can transduce muscle with high efficiency. However, therapeutic application in human gene therapy would require the resolution of serious problems such as mutagenesis and unexpected immune responses.[5]

For nonviral gene delivery, physical methods, such as electroporation[6,7] and ultrasound,[8] have been investigated. They are relatively safe and easy to apply, but have low transfer efficiencies and can cause tissue damage. Optical transfection, especially laser-mediated gene transfection has received much attention as a new method for target gene transfection because of the high spatial controllability of laser energy. Most reports on laser-mediated gene transfer have mainly dealt with single-cell-basis transfection by direct irradiation of the cell membrane with a focused laser beam; this approach cannot be applied to deep tissue because of limited optical penetration. However, we recently demonstrated that genes can be transferred both *in vitro* and *in vivo* by the use of laser-induced stress waves (LISWs) or photomechanical waves.[8–12]

Delivery of drug molecules by LISWs was developed by Lee and Doukas[13] and Doukas and Flotte.[14] We have extended the application of LISW to the delivery of genes. Using this method, a large number of cells can be transfected simultaneously. It also enables the treatment of deeper tissue than the methods based on direct laser irradiation, because stress waves can be propagated much more efficiently in tissue than can laser light. Using LISWs, we have previously transfected various types of cell lines with plasmid deoxyribose nucleic acid (DNA) for the expression vector of enhanced green fluorescent protein (EGFP).[11] We have also delivered luciferase, EGFP, and Lac-Z genes to rat skin,[12] and EGFP genes to the mouse central nerve system *in vivo*.[10]

Polyethylenimene (PEI) is a synthetic cationic vector and has been shown to be an efficient and versatile gene transfer tool. The plasmid DNA complexes formulated with 22 kDa PEI in 5%

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glucose are sufficiently small and stable in physiological fluid to provide high diffusibility and enhance gene transfection.[15,16]

In this study, we investigated the feasibility of gene transfer using LISWs and the DNA/PEI complex. We demonstrated that efficient, site-specific, and minimally invasive gene transfer can be achieved by applying this novel technique based on LISWs.

2 Materials and Methods

2.1 *Plasmid Deoxyribose Nucleic Acid–Polyethylenimene Complex*

Lac-Z plasmid encoding pSV-β-galactosidase was purchased from Promega (Madison, Wisconsin). The plasmid DNA was transformed into competent *Escherichia coli* cells using standard procedures and amplified and purified on a Qiagen column. The final concentration of the vector was 0.5 ug/uL. The plasmid DNA and DNA-PEI complex solution was prepared according to the manufacturer's instructions using 25 uL of DNA (2.0 ug/uL) and 8 uL of jetPEI (PolyPlus-transfection, Illkirch, France) in 10% glucose to obtain a PEI nitrogen/DNA phosphate (N/P) ratio of 8.

2.2 *Laser-Induced Stress Waves*

Figure 1(a) shows the experimental arrangement. A black natural rubber disk with a thickness of 0.5 mm and a diameter of 12 mm was used as the laser target. A 15-mm square transparent polyethylene terephthalate sheet with a thickness of 1 mm was bonded to the rubber disk surface to confine the laser-induced plasma. Plasma confinement increases the peak pressure and pulse width of the generated stress waves.[9,11,12] LISWs were generated by irradiating a target with 6-ns pulsed laser light from the second harmonics (532 nm) of a Q-switched Nd:YAG laser (Brilliant b; Japan Laser, Tokyo, Japan); the laser beam was focused with an uncoated planoconvex quartz lens ($f = 200$ mm). The spot diameter on the target can be changed but was kept constant at 6 mm in the present study [Fig. 1(b)].

2.3 *Gene Transfer*

We used male Sprague-Dawley rats (Japan SLC) weighing 300–400 g. The present study was carried out according to the institutional ethical guidelines for animal experiments proposed and approved by the Committee for Animal Research at the National Defense Medical College (Tokorozawa, Saitama, Japan). Animals were deeply anesthetized by intraperitoneal injection of ketamine chloride and medetomidine hydrochloride (75 and 0.5 mg/kg animal weight, respectively), and their facial hairs and vibrissae were clipped. The middle point between the unilateral nostril and corner of the mouth was targeted and marked with a permanent pen. Lac-Z expression DNA or the DNA-PEI complex was injected through a 27-gauge needle attached to a Hamilton syringe (80601, Hamilton Company, Reno, Nevada) from four points around the marked skin target into muscle layer under the center of the target. Total volume of plasmid DNA and DNA-PEI complex solution was 100 μl and injection speed was 2.5 μl/s in each experiment. A rubber target was placed on the facial skin. Ultrasound transmission mediums for diagnostic use (Echo Jelly, Hitachi-Aloka Medical, Tokyo, Japan) was used between the target and the skin for acoustic impedance matching.

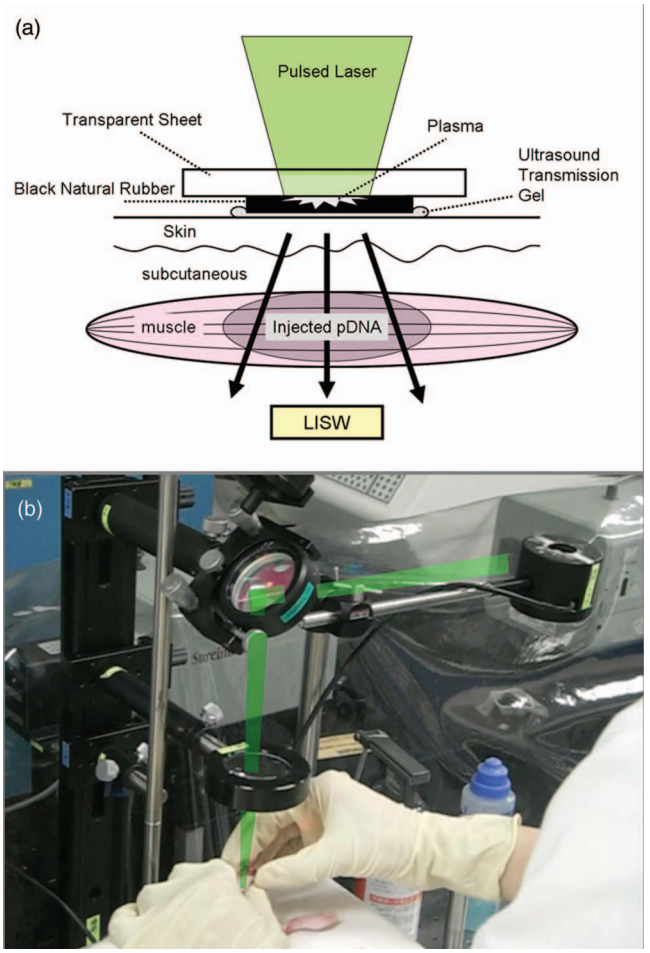

Fig. 1 Depictions of gene transfer using LISWs.

Just after gene injection, the target was irradiated with pulsed laser at a laser fluence of 1.0 J/cm^2 with a spot diameter of 6 mm on the target. Under this conditions, the rise time, peak pressure, and impulse of the photo mechanical wave (PMW) generated were 3.6 GPa/ns, 140 MPa, and 30 Pa s, respectively. Three laser pulses were applied for each gene transfer in all experiments. For multipulse irradiation, the target was changed pulse by pulse to ensure efficient plasma confinement. The conditions of the experiments were assigned to the following five groups: (a) control, use of neither LISWs nor DNA injection; (b) DNA injection only; (c) DNA-PEI complex injection; (d) use of LISWs following DNA injection; and (e) use of LISWs following DNA-PEI complex injection. In each condition, six rats were used. For quantification of gene expression, the number of Lac-Z-positive cells included in a randomly selected 200-μm^2 region was counted. Data are presented as mean (SE). Statistical analysis was performed on the basis of the Mann-Whitney U-test.

2.4 *Assay*

Animals were sacrificed on the third, seventh, and fourteenth days after gene transfection for evaluation of the time course of gene expression. Facial skin and muscles were dissected *en bloc* from facial bone. For analysis of Lac-Z expression, X-gal

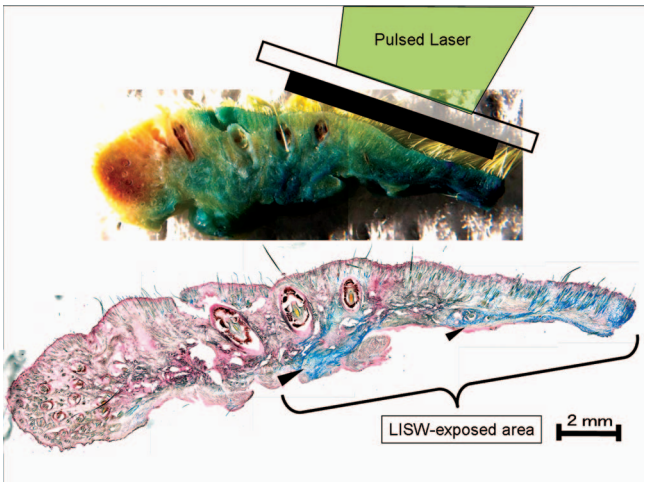

Fig. 2 Whole facial tissue obtained at seven days after gene transfer with LISWs. Scale bar: 2 mm.

staining was performed. Tissues were fixed for 3 h with 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed well in PBS, and stained with the X-gal solution at 38°C for 36 h. They were cryoprotected with 30% sucrose in PBS at 4°C overnight, then frozen in liquid nitrogen and sectioned with a cryostat microtome at a thickness of 20 μm. Eosin counterstain was performed, and then tissues were observed using optical microscopy (BX5; Olympus, Tokyo, Japan).

To evaluate tissue damage and inflammatory changes after use of LISWs, tissue exposed to LISWs without DNA injection was examined with hematoxylin-eosin stain seven days after irradiation.

3 Results

Figure 2 shows the sections of the whole facial tissue seven days after gene transfection using LISWs. Lac-Z expression was observed only in the tissue exposed to LISWs. The muscles existing several millimeters deep in the skin were especially well stained (arrowheads). Figure 3(a) shows the hematoxylin-eosin-stained tissue section taken from the tissue exposed to LISWs without DNA injection. Almost no detectable damage and inflammatory infiltrating cells were observed in the muscles seven days after application of LISWs. Figure 3(b) shows the facial muscles seven days after DNA transfection by LISWs. Intensive Lac-Z expression was observed in myofibers (arrowheads). No obvious damage was observed, but some myofibers seemed to be slightly atrophic.

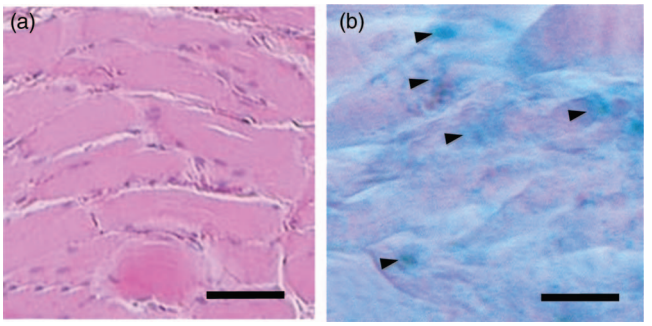

Fig. 3 (a) Hematoxylin-eosin staining of muscle fibers at seven days after exposure of LISWs without injection of DNA. Scale bar: 10 μm. (b) X-gal staining of muscle fibers at seven days after gene transfection using LISWs. Scale bar: 10 μm.

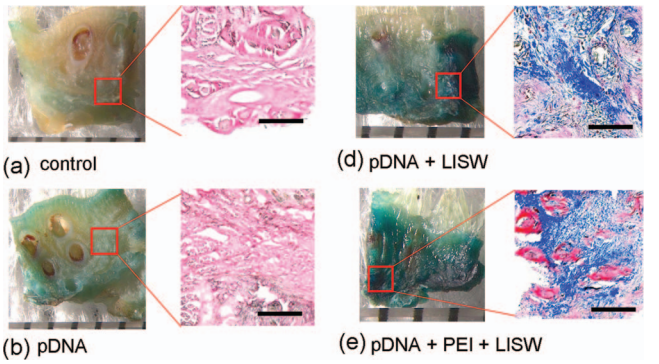

Fig. 4 Comparison of the intensities of Lac-Z expression under the four conditions at seven days after gene transfer. Scale bar: 200 μm.

Figure 4 shows the difference in intensity of Lac-Z expression at seven days after gene transfer under four experimental conditions. Detectable expression of Lac-Z was observed neither in the muscle layer of control tissue (group a) nor in the muscle layer only with pDNA injection (group b). On the other hand, apparent gene expression was observed in both cases of LISW application (groups d and e). In high-power fields, strong Lac-Z expression was observed in the deep muscle layer exposed to LISWs. When the DNA-PEI complex was used (if both LISWs and DNA-PEI complex were used), gene expression seemed to be slightly stronger than that with pDNA alone. Figure 5 shows the count of Lac-Z-positive cells in the tissue of groups b–e at seven days after gene transfection. A highly significant increase in the number of Lac-Z-positive cells was observed in both cases of LISW application (groups d and e) (both $p < 0.01$). A significant increase was also observed in

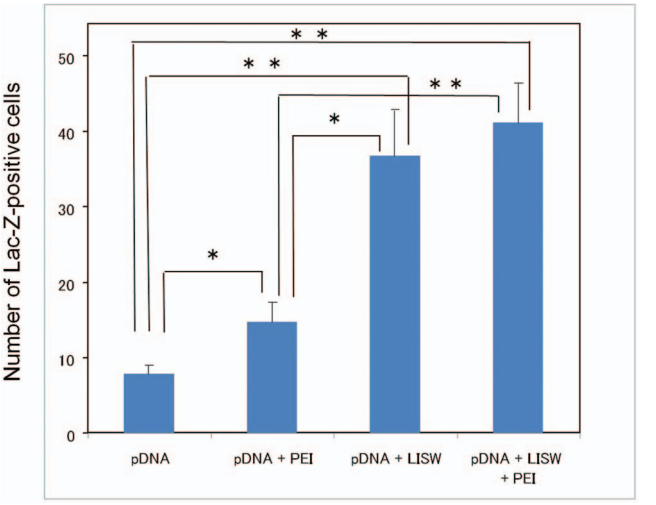

Fig. 5 Comparison of the transfection efficiencies evaluated by counting of Lac-Z-positive cells at seven days after gene transfer. Statistical analysis was performed using the Mann-Whitney *U*-test. The error bars represent standard error (*$p < 0.05$, **$p < 0.01$).

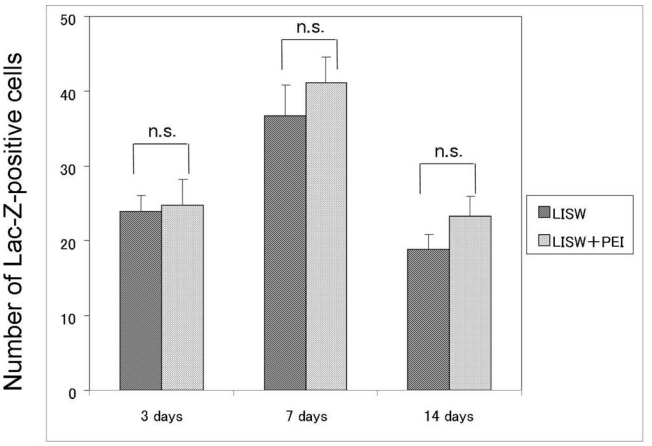

Fig. 6 Time course of the Lac-Z-positive cells in two conditions, using LISWs and a combination of LISWs and the DNA/PEI complex. Statistical analysis was performed using the Mann-Whitney *U*-test. The error bars represent standard error. (n.s. $p > 0.05$).

case of DNA-PEI complex alone (group c) ($P < 0.05$). There was significant difference between group c and groups d and e ($p < 0.05$, $P < 0.01$, respectively), but there was no significant difference between groups d and e, regardless of use of Jet-PEI.

Figure 6 shows the time course of expression in cases of using LISWs (groups d and e). The peak expression was observed at seven days after gene transfer, and thereafter, expression tended to decrease. However, gene expression was sustained for at least 14 days after gene transfer.

4 Discussion

We previously showed that plasmid DNA could be transfected into skin and central nervous system (CNS) cells *in vivo* by use of LISWs.[10,12] Moreover, we delivered a therapeutic vector construct carrying the *hHGF* gene into a rat skin graft for autografting and observed enhanced angiogenesis.[8] We believe this to be the first experimental demonstration of LISW-mediated gene transfection showing therapeutic efficacy.

In the present study, we demonstrated the applicability of LISW-mediated gene transfer into rat facial muscles as a basic experiment to test facial nerve regeneration. Muscle is an ideal target for gene therapy because it is easily accessible and able to induce the local and systemic secretion of therapeutic proteins.[5–7] We proved that LISWs enabled efficient DNA delivery into facial muscles existing several millimeters deep from the face surface under the laser spot on the target. No skin incision or exposure of muscles was required. Moreover, only limited damage and inflammatory change were caused by LISW application in the target muscle and its surrounding tissue. To be precise, some muscle fibers showed slight atrophic change. Several studies have suggested that toxicity could be caused by the intracellular presence and expression of DNA themselves.[6,17,18] Thus, some tissue alteration might be inevitable in gene transfer using plasmid DNA.

In the condition using LISWs with the DNA-PEI complex, the intensity of Lac-Z expression was slightly stronger than in experiments using only LISWs. The results showed some transduction efficiency of PEI, but a significantly small number of βGal positive cells are observed compared to that of LISWs. PEI could possibly have an additional effect on gene transfer using LISWs, but sufficient gene expression can be achieved by using LISWs alone. The most obvious Lac-Z expression was observed at seven days after gene transfection, and the expression tended to decrease with elapsed time. This time-dependent decrease in gene expression indicates that the transgene was hardly inserted into chromosomal DNA, suggesting a lower risk of adverse effects such as tumorigenesis due to insertion mutagenesis or chromosomal aberrations compared to the viral methods. These results suggest that this method might be suitable to prevent muscle atrophy and to promote nerve regeneration during at least the initial two weeks.

According to a previous report on gene transfer by electroporation, transient gene expression is observed mainly by transfection of mature muscle fibers. When satellite cells that are involved in the growth and regeneration of muscle are activated, and DNA is taken into these cells, stable gene expression was shown to last for several months up to a year.[8] If we apply LISWs to a degeneration muscle model, DNA will also be transfected into proliferated satellite cells and longer-term gene expression will be observed in regenerated myofibers.

Topically applied growth factors are known to enhance peripheral nerve regeneration. Watabe *et al*. demonstrated that adenoviral gene transfer of GDNF, BDNF, TGFβ2, and HGF into the facial canal prevented death of injured motoneurons after facial nerve avulsion.[2] Kohmura *et al*. showed that locally applied BDNF aterocollagen minipellets accelerated facial nerve regeneration in the rat model of facial nerve transection and immediate repair with suture.[1] Araki *et al*. proved that in a rat crush model for the recurrent nerve, a cranial motor-nerve-like facial nerve, treatment with topical injection of an adenovirus vector of GDNF led to significantly improved motor nerve conduction velocity and vocal fold movement at two and fout weeks after treatment and a significantly larger axonal diameter and improved remyelination compared to controls.[19] By use of LISWs, therapeutic genes can be more safely transfected into muscles and can impart neurotrophic effects on cases of acute nerve degeneration such as those that occur in facial injury and paralysis.

The mechanism of gene transfer using LISWs is not clear, but there are two possibilities: acceleration of exogenous genes and/or transient disruption of the plasma membrane by sheer force. We consider that exogenous genes are accelerated by large impulse pressure and enter the cells through lipid bilayers. Sheer force induced by LISWs may also cause reversible disruption of the cell membrane, through which exogenous genes are diffused into the cells due to the concentration gradient.[9,11] The mechanisms of LISWs appear to be different from those of conventional ultrasound; waveforms of LISWs were dominated by positive pressure components, unlike those of ultrasound. Increased permeabilization of the plasma membrane due to interaction with ultrasound is mediated mostly by cavitation. A contrast agent and a large number of shots are required to achieve efficient gene transfection based on the cavitation effect.[18,20] With the present method, on the other hand, efficient gene transfer can be achieved with only a few pulses, without the need for any contrast medium or long time of operation.

The most notable advantage of our method compared to other physical methods is its high spatial controllability. Genes can be delivered to a pinpointed target tissue by applying finely focused

laser light, while large-area treatment is also possible by using expanded or scanned laser light. Additionally, as laser light can be delivered through fiber optics, catheter-based gene transfer into deep and inaccessible target organs may be possible. With electroporation, it would be difficult to realize such highly controllable targeted gene delivery because it is difficult to control the path of electric current in tissue. Furthermore, electroporation easily causes tissue damage, making it unsuitable for large-area treatment.

Another advantage of LISW-based gene transfer is its minimal invasiveness and easy applicability. Q-switched Nd:YAG lasers are widely used in head and neck surgery and proven to be safe. They are commercially available, cost effective, and very easy to operate and maintain. Recently, gene transfer by the use of a titunium-sapphire femtosecond laser has been reported.[21] DNA-injected muscle in mice was directly irradiated with focused laser pulses *in vivo*, and efficient gene expression was observed in the irradiated tissue. However, Q-switched Nd:YAG lasers are cheaper, more compact, and easier to control when compared to femtosecond lasers. Therefore, this LISW-mediated method seems to have fewer problems that need to be solved before clinical use than other nonviral gene delivery method.

5 Conclusions

In this study, we successfully demonstrated gene transfer into the facial muscle by applying LISWs. This technique enables site-specific, safe, and convenient gene transfer. We plan to study the therapeutic effect of facial nerve regeneration based on this technique.

References

1. E. Kohmura, T. Yuguchi, T. Yoshimine, T. Fujinaka, N. Koseki, A. Sano, A. Kishino, C. Nakayama, T. Sakaki, M. Nonaka, O. Takemoto, and T. Hayakawa, "BDNF atelocollagen mini-pellet accelerates facial nerve regeneration," *Brain Res.* **849**(1–2), 235–238 (1999).
2. K. Watabe, T. Sakamoto, T. Ohashi, Y. Kawazoe, K. Oyanagi, T. Takeshima, K. Inoue, Y. Eto, and S. U. Kim, "Adenoviral gene transfer of glial cell line-derived neurotrophic factor to injured adult motoneurons," *Hum. Cell.* **14**(1), 7–15 (2001).
3. T. Sakamoto, K. Watabe, T. Ohashi, Y. Kawazoe, K. Oyanagi, K. Inoue, and Y. Eto, "Adenoviral vector-mediated GDNF gene transfer prevents death of adult facial motoneurons," *Neuroreport* **11**(9), 1857–1860 (2000).
4. T. Sakamoto, Y. Kawazoe, J. S. Shen, Y. Takeda, Y. Arakawa, J. Ogawa, K. Oyanagi, T. Ohashi, K. Watanabe, K. Inoue, Y. Eto, and K. Watabe, "Adenoviral gene transfer of GDNF, BDNF and TGF beta 2, but not CNTF, cardiotrophin-1 or IGF1, protects injured adult motoneurons after facial nerve avulsion," *J. Neurosci. Res.* **72**(1), 54–64 (2003).
5. D. J. Wells, "Viral and non-viral methods for gene transfer into skeletal muscle," *Curr. Opin. Drug Discov. Devel.* **9**(2), 163–168 (2006).
6. C. Trollet, C. Bloquel, D. Scherman, and P. Bigey, "Electrotransfer into skeletal muscle for protein expression," *Curr. Gene Ther.* **6**(5), 561–578 (2006).
7. S. Miyazaki and J. Miyazaki, "*In vivo* DNA electrotransfer into muscle," *Dev. Growth Differ.* **50**(6), 479 –483 (2008).
8. M. Terakawa, S. Sato, D. Saitoh, H. Tsuda, H. Ashida, H. Okano, and M. Obara, "Enhanced angiogenesis in grafted skins by laser-induced stress wave-assisted gene transfer of hepatocyte growth factor," *J. Biomed. Opt.* **12**(3), 034031 (2007).
9. M. Terakawa, S. Sato, H. Ashida, K. Aizawa, M. Uenoyama, Y. Masaki, and M. Obara, "*In vitro* gene transfer to mammalian cells by the use of laser-induced stress waves: effects of stress wave parameters, ambient temperature, and cell type," *J. Biomed. Opt.* **11**(1), 014026 (2006).
10. Y. Satoh, Y. Kanda, M. Terakawa, M. Obara, K. Mizuno, Y. Watanabe, S. Endo, H. Ooigawa, H. Nawashiro, S. Sato, and K. Takishima, "Targeted DNA transfection into the mouse central nervous system using laser-induced stress waves," *J. Biomed. Opt.* **10**(6), 060501 (2005).
11. M. Terakawa, M. Ogura, S. Sato, H. Wakisaka, H. Ashida, M. Uenoyama, Y. Masaki, and M. Obara, "Gene transfer into mammalian cells by use of a nanosecond pulsed laser-induced stress wave," *Opt. Lett.* **29**(11), 1227–1229 (2004); Erratum: *Opt. Lett.* **29**(18), 2190 (2004).
12. M. Ogura, S. Sato, K. Nakanishi, M. Uenoyama, T. Kiyozumi, D. Saitoh, T. Ikeda, H. Ashida, and M. Obara, "*In vivo* targeted gene transfer in skin by the use of laser-induced stress waves," *Lasers Surg. Med.* **34**(3), 242–248 (2004).
13. S. Lee and A. G. Doukas, "Laser-generated stress waves and their effects on the cell membrane," *IEEE J. Sel. Top. Quantum Electron.* **5**(4), 997–1003 (1999).
14. A. G. Doukas and T. J. Flotte, "Physical characteristics and biological effects of laser-induced stress waves," *Ultrasound Med. Biol.* **22**(2), 151–164 (1996).
15. D. Goula, J. S. Remy, P. Erbacher, M. Wasowicz, G. Levi, B. Abdallah, and B. A. Demeneix, "Size, diffusibility and transfection performance of linear PEI/DNA complexes in the mouse central nervous system," *Gene Ther.* **5**(5), 712–717 (1998).
16. S. Wang, N. Ma, S. J. Gao, H. Yu, K. W. Leong, "Transgene expression in the brain stem effected by intramuscular injection of polyethylenimine/DNA complexes," *Mol Ther.* **3**(5 Pt 1), 658–664 (2001).
17. A. C. Durieux, R. Bonnefoy, T. Busso, and D. Freyssenet, "*In vivo* gene electrotransfer into skeletal muscle: effects of plasmid DNA on the occurrence and extent of muscle damage," *J. Gene Med.* **6**(7), 809–816 (2004).
18. Q. L. Lu, H. D. Liang, T. Partridge, and M. J. Blomley, "Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle *in vivo* with reduced tissue damage," *Gene Ther.* **10**(5), 396–405 (2003).
19. K. Araki, A. Shiotani, K. Watabe, K. Saito, K. Moro, and K. Ogawa, "Adenoviral GDNF gene transfer enhances neurofunctional recovery after recurrent laryngeal nerve injury," *Gene Ther.* **13**(4), 296–303 (2006).
20. Y. Taniyama, K. Tachibana, K. Hiraoka, M. Aoki, S. Yamamoto, K. Matsumoto, T. Nakamura, T. Ogihara, Y. Kaneda, and R. Morishita, "Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle," *Gene Ther.* **9**(6), 372–380 (2002).
21. U. K. Tirlapur and K. König, "Targeted transfection by femtosecond laser," *Nature* **418**(6895), 290–291 (2002).