

# Enhanced angiogenesis in grafted skins by laser-induced stress wave-assisted gene transfer of hepatocyte growth factor

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## 1 Introduction

Adhesion of grafted tissue largely depends on the provision of nutrients and oxygen by angiogenesis. In the case of skin grafting, vascular anastomosis is not employed, so the adhesion of grafts is determined by the spontaneous tissue healing

**Abstract.** Treatment to increase secretion of growth factors related to angiogenesis by gene transfection is a promising therapeutic solution for improving the outcome of tissue transplantation. We attempted to deliver a therapeutic vector construct carrying the human hepatocyte growth factor (hHGF) gene to skin grafts of rats using laser-induced stress waves (LISWs), with the objective of enhancing their adhesion. First we delivered the hHGF gene to rat native skin *in vivo* to determine the optimum gene transfer conditions. We then transferred the hHGF gene to excised rat skins, with which autografting was performed. We found that the density and uniformity of neovascularities were significantly enhanced in the grafted skins that were transfected using LISWs. These results suggest the efficacy of this method to improve the outcome of skin grafting. To our knowledge, this is the first experimental demonstration of a therapeutic efficacy based on LISW-mediated gene transfection. Since the present method can be applied not only to various types of tissues but also to bioengineered tissues, this technique has the potential to contribute to progress in transplantation medicine and future regenerative medicine. © 2007 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2745313]

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process. Thus, adhesion of grafts often requires a long time and sometimes even fails, resulting in a high risk of infection. Gene therapy has great potential as a strategy for improving the outcome of skin transplantation. Several types of growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF), are known to enhance angiogenesis and wound healing.<sup>1-3</sup> Thus, the secretion of such growth factors up-

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regulated by gene transfection can accelerate the adhesion of transplanted tissues.

Most of gene therapy trials for skin covering have been conducted using skin flaps with one edge remaining connected to the donor site, allowing blood to be supplied to the flap through the pedicle. In several studies, the gene coding for VEGF has been used to improve skin flap survival. Gurnunluoglu et al. reported that subdermal injections of an adenovirus vector coding for VEGF promoted the viability of epigastric skin flaps.<sup>4,5</sup> Taub et al. reported that survival of ischemic skin flaps can be enhanced by gene transfer of VEGF using an adeno-associated virus vector and liposomes.<sup>6</sup> It has also been reported that adenovirus-mediated gene transfer of transforming growth factor- $\beta$  ameliorated ischemic necrosis in skin flaps.<sup>7</sup> However, the use of viral vectors involves problems,<sup>8,9</sup> such as mutagenesis and enhanced innate immune responses through the activation of Toll-like receptor 9. Thus, a nonviral vector has also been used for gene transfection of skin flaps. Liu et al. used lipofectin for simultaneous transfer of three growth factor genes: VEGF, platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF-2), to enhance the survival of ischemic skin flaps.<sup>10</sup> However, skin flap surgery can be applied only to a limited area of the skin. There is a need for a method to accelerate the adhesion of separated skin grafts in transplantation medicine.

Laser-mediated gene transfection is a promising nonviral method for targeted gene transfection because of the high spatial controllability of laser energy.<sup>11-17</sup> In addition, since laser energy can be transmitted through an optical fiber, catheter-based gene transfer may come into practical use. For gene transfection, lasers have mainly been used for direct irradiation of cells or tissues to perforate cell membranes, but we recently demonstrated that genes can be transferred both *in vitro* and *in vivo* by the use of laser-induced stress waves (LISWs).<sup>18-21</sup> Delivery of drug molecules by LISWs was developed by Doukas et al. They have successfully introduced various types of exogenous molecules into targeted cells and tissues.<sup>22-24</sup> 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 methods based on direct laser irradiation, since stress waves can be propagated much more efficiently in tissue than can laser light. On the basis of LISW, various types of cell lines have been transfected with plasmid DNA for the expression vector of enhanced green fluorescent protein (EGFP) under a ubiquitous promoter. We have delivered luciferase, EGFP, and  $\beta$ -galactosidase genes to rat skin *in vivo*. We have also demonstrated that the mouse central nervous system can be transfected with plasmid DNA coding for EGFP.<sup>21</sup> Recently, Tang et al. have reported that small interfering RNA (siRNA) can be delivered to plant cells using LISW.<sup>25</sup> To our knowledge, however, no therapeutic effects have been demonstrated so far based on LISW-based gene delivery.

We expected that gene transfer of HGF to transplanted tissue would accelerate angiogenesis in grafts, by which their adhesion can be enhanced. For this purpose, we planned to apply LISW-based gene transfer. HGF has pleiotropic effects such as morphogenic and mitogenic functions for various types of cells, including vascular endothelial cells.<sup>26,27</sup> It has been reported that HGF promotes angiogenesis in various or-

gans and tissues such as infarcted myocardium,<sup>28</sup> corneas,<sup>29</sup> and cutaneous wounds.<sup>30,31</sup> Nakamura et al. reported that the stimulatory effect of HGF on DNA synthesis in human endothelial cells is greater than the effects of bFGF and VEGF.<sup>32</sup> In the first part of this study, we delivered a therapeutic vector construct carrying the human HGF (hHGF) gene to rat native skin *in vivo* by the use of LISWs to determine the optimum gene transfer conditions. Next we attempted to deliver hHGF expression vector plasmid DNA to excised rat skins as grafts by the use of LISWs. Each graft was transplanted onto the donor site (autograft), and angiogenesis in the grafts was evaluated histologically. We demonstrated that angiogenesis can be accelerated significantly in grafted skins that have been transfected using LISWs, suggesting the efficacy of this method in enhancing the adhesion of transplanted grafts.

## 2 Materials and Methods

### 2.1 Plasmid DNA

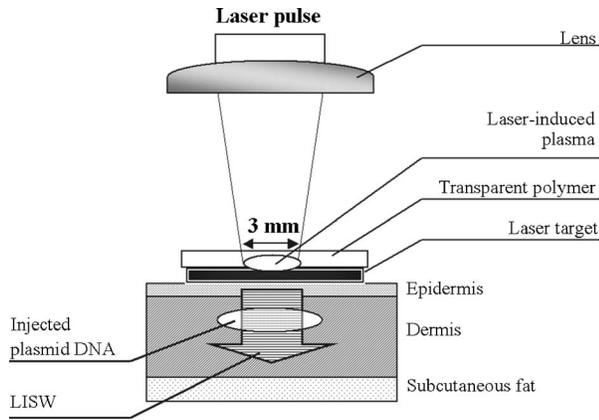
HGF expression vector plasmid DNA was constructed by insertion of hHGF cDNA into the *NotI* site of pcDNA3.1 (Invitrogen Corp., Carlsbad, California). A cDNA clone coding for hHGF was provided by Dr. T. Nakamura. The vector was driven by a cytomegalovirus promoter. *Escherichia coli* competent cells were transformed and amplified in media with ampicillin, and then plasmid DNA was purified on a Qiagen column (Qiagen, Inc., Hilden, Germany).

### 2.2 Laser-Induced Stress Waves

A black natural rubber disk 0.5-mm thick was used as the laser target. A transparent polyethylene terephthalate sheet 1-mm thick was bonded to the top surface of the target to confine the laser-induced plasma. Plasma confinement increases the peak pressure and pulse width of the generated stress waves.<sup>33</sup> LISWs were generated by irradiating the target with three laser pulses of 6-ns width (FWHM) from the second harmonic (532 nm) of a Q-switched Nd:YAG laser (Surelite I-10, Hoya Continuum). The pulsed light beam was focused with a plano-convex lens ( $f=170$  mm) on a spot 3 mm in diameter on the target.

### 2.3 In Vivo hHGF Gene Transfer into Rat Native Skin

We delivered hHGF expression vector plasmid DNA to rat skin *in vivo* to determine the optimum gene transfer conditions. Figure 1 shows the experimental configuration. We used Sprague-Dawley rats weighing 300 to 380 g. They were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg animal weight), and their dorsal hairs were clipped and depilated before plasmid DNA injection. Human HGF expression vector plasmid DNA (1.0  $\mu\text{g}/\mu\text{l}$ ) was intradermally injected using a syringe (80601, Hamilton Company, Reno, Nevada) with a 27-G needle (Terumo, Tokyo, Japan). A laser target was placed on the gene-injected skin (Fig. 1); silicone grease was used between the target bottom surface and the skin surface to ensure acoustic impedance matching. The target was irradiated with laser pulses to generate LISWs whose characteristics were the same as those that had been evaluated in our previous study.<sup>20</sup> Because the black natural rubber target does not transmit laser pulses, the laser beam



**Fig. 1** Configuration for HGF gene transfer into rat native skin by the use of LISWs.

and tissue do not interact with each other. Peak pressures of the LISWs were estimated to be 16, 26, 39, 45, 50, and 52 MPa at laser fluences of 0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 J/cm<sup>2</sup> per pulse, respectively. The pulse duration (FWHM) of LISW was approximately 1  $\mu$ s. Gene-injected skin was exposed to three pulses of LISW. Because the transparent sheet was detached from the black rubber by irradiation with a single laser pulse, the target was replaced for each pulse.

#### 2.4 Measurement of hHGF Protein Expressed in Rat Skin

Punch biopsy 3 mm in diameter was performed for gene-transferred skins with subcutaneous tissues. Tissue samples were homogenized in a homogenizer (24,000 rpm) in an hHGF extract solution containing 1 mM/L phenylmethylsulfonyl fluoride (Institute of Immunology, Tokyo, Japan) for 1 min. The homogenate was centrifuged at 15,000  $\times$  g for 30 min at 4 °C. The concentration of hHGF protein expressed in the tissue sample was measured, based on enzyme-linked immunosorbent assay (ELISA) using an anti-human-HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The experiments were performed 8 to 11 times for each condition to evaluate reproducibility.

#### 2.5 Gene Transfer of hHGF into Skin Grafts

We attempted to deliver the hHGF gene to rat skin grafts using LISWs with the objective of enhancing their adhesion by autografting. Autografts are most desirable for achieving efficient adhesion because of their lack of rejection. This experiment was performed on the basis of the optimum experimental conditions for gene transfection of native skins described earlier. Dorsal skin of a rat, measuring 20 mm  $\times$  20 mm, was excised for use as a graft, and its subcutaneous fat was removed. Human HGF expression vector plasmid DNA (10  $\mu$ l, 1.0  $\mu$ g/ $\mu$ l) was injected into the graft from the reverse side using a syringe (80601, Hamilton Company, Reno, Nevada) with a 27-G needle (Terumo, Tokyo, Japan). The graft was placed on a plastic plate 3-mm thick. LISWs were generated by irradiating the laser target that was placed on the dermal side of the graft with laser pulses of 6-ns width (FWHM) at a laser fluence on the target of 1.2 J/cm<sup>2</sup>. Gene-

injected skin was subjected to three pulses of LISW. After application of LISWs, autografting was performed, i.e., the graft was transplanted onto the donor site of the rat. Grafts to which a control vector, pcDNA3.1 (Invitrogen Corp., Carlsbad, California), was injected were also used for comparison.

#### 2.6 Assessment of Angiogenesis

Grafted skin was excised from the rat, and angiogenesis in the graft was assessed based on immunohistochemical staining using anti-rat CD31/PECAM-1 (platelet endothelial cell adhesion molecule-1) antibody. CD31/PECAM-1 is an integral membrane glycoprotein that is expressed on endothelial cells. Endothelial cells of blood vessels are thus stained with the antibody.<sup>34</sup> A deparaffinized section of the graft was incubated overnight with anti-rat CD31 (BD Biosciences, Franklin Lakes, New Jersey) at a dilution of 1:5 and then incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) at a dilution of 1:200. For digital histological images, CD31-positive pixels were discriminated on the basis of RGB values. For quantitative evaluation of angiogenesis, the total number of CD31-positive pixels at each depth was counted; the depth profile of CD31-positive pixels, i.e., the depth profile of the density of neovasculatures, was obtained. The experiments were performed using five rats in each experimental condition ( $n=5$ ).

#### 2.7 Statistical Analysis

Statistical analysis was performed based on the nonparametric Mann-Whitney test. A value of  $p < 0.05$  was considered statistically significant. All values are expressed as means  $\pm$  SEM.

### 3 Results

#### 3.1 In Vivo hHGF Gene Transfer into Rat Native Skin

Figure 2 shows the dependence of hHGF concentration expressed in rat skins on the injected volume of plasmid DNA. The laser fluence was held constant at 1.2 J/cm<sup>2</sup> in this experiment. For comparison, the results for skins to which no LISWs were applied (naked plasmid DNA injection) are also shown. The skins to which LISWs were applied showed higher mean hHGF concentrations at all injected plasmid DNA volumes. At 10  $\mu$ l of plasmid DNA, a significant difference was found between hHGF concentration in skins to which LISWs had been applied and that in skins without LISW application; this volume (10  $\mu$ l) was used in the following experiments.

Figure 3 shows the dependence of hHGF concentration in rat skins on laser fluence at 24 h after gene transfection. Without application of LISWs, hHGF concentration was 0.8 pg/mg tissue. A fourfold increase in hHGF concentration was obtained at a laser fluence of 1.2 J/cm<sup>2</sup> when compared with that in skins without LISW application ( $p < 0.05$ ). No further increase in hHGF concentration was observed at the fluences higher than 1.2 J/cm<sup>2</sup>. The time-courses of hHGF concentration after gene transfection are shown in Fig. 4. Human HGF concentration decreased with elapse of time at all laser fluences: 0 J/cm<sup>2</sup> (no LISW), 1.2 J/cm<sup>2</sup>, and 1.8 J/cm<sup>2</sup>. The skins to which LISWs had been applied showed higher expression levels than those in skins without

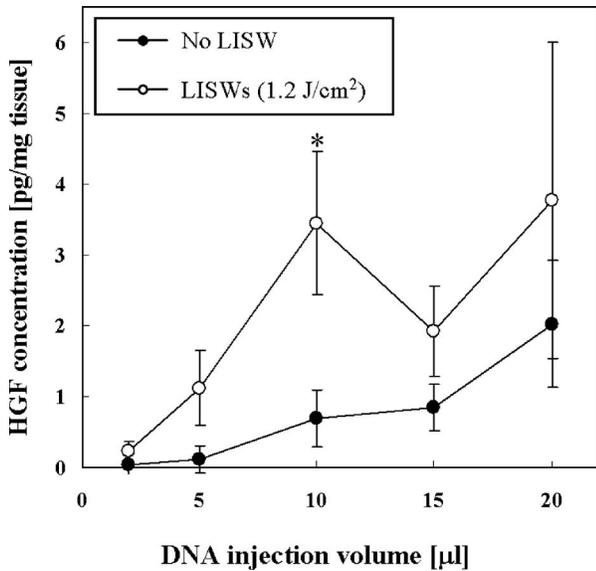


Fig. 2 Effect of DNA injection volume on hHGF concentration expressed in rat skins at 24 h after LISW application.  $N=8$  to 11. Values are expressed as means±standard error ( $*p<0.05$ , LISWs versus no LISW).

LISW application, even 5 days after gene transfection. In skins with LISW application at a laser fluence of 1.8 J/cm<sup>2</sup>, hHGF concentration at 5 days after gene transfection was comparable to that at 24 h after gene transfection without LISW application (naked plasmid DNA injection).

### 3.2 Human HGF Gene Transfer into Skin Autografts

Angiogenesis in the transplanted grafts was observed to a limited extent within 2 days of gene transfection (data not shown). Figure 5 shows typical cross-sectional images of the grafted skins 3 days after grafting. Red-brown staining indicates CD31-positive blood vessels. We found that most of the

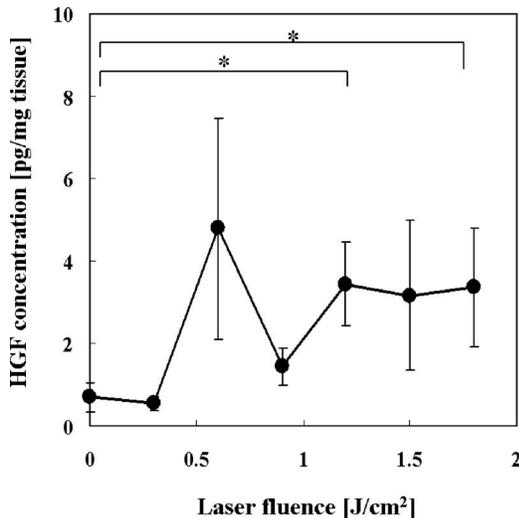


Fig. 3 Effect of laser fluence on hHGF concentration expressed in rat skins at 24 h after LISW application.  $N=11$ . Values are expressed as means±standard error. ( $*p<0.05$ ).

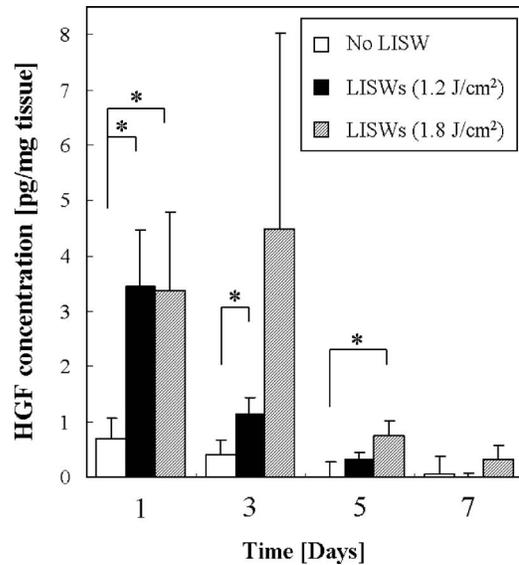
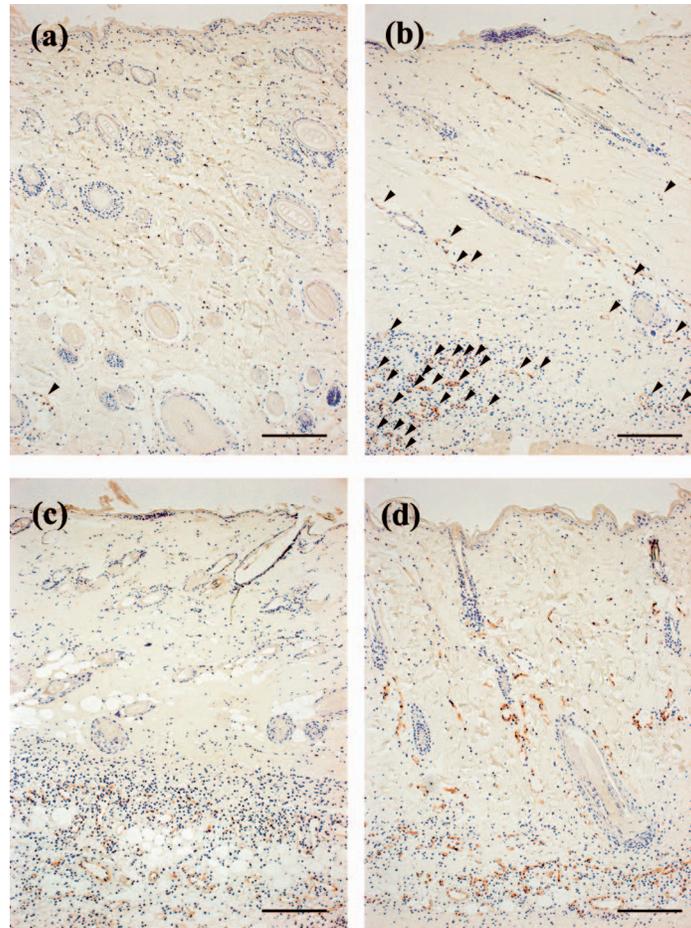


Fig. 4 Time-courses of hHGF concentration expressed in rat skins after gene transfection.  $N=8$  to 11. Values are expressed as means±standard error. ( $*p<0.05$ ).

CD31-positive blood vessels were characterized by neovascularity-specific size and conformation. The images of the grafted skins with hHGF-expression vector injection without LISWs (hHGF alone) (b) and control vector injection with LISWs (c) showed more CD31-positive vessels than did the images of normal grafting in which neither hHGF-expression vector nor LISW were applied (a). However, the distributions of stained blood vessels were inhomogeneous in these cases. Much more heavily stained vessels, the sites of angiogenesis, were observed in the image of the graft both with the hHGF-expression vector injection and LISWs (d) when compared with others. The stained blood vessels ranged from 5 to 20 μm in diameter. Although the number of lymphocytes increased in the perivascular tissues in (b), (c), and (d), no notable tissue damage can be observed.

Figure 6 shows the depth profiles of averaged CD31-positive pixel numbers for five rats in each condition ( $n=5$ ). In the normally grafted skins, the number of CD31-positive pixels was small over the whole depth range of the skins. Angiogenesis was enhanced in the skins injected with the hHGF gene alone and in the skins injected with the control vector plus LISW application. In skins injected with hHGF gene and subjected to LISW, a relatively uniform distribution of a large number of CD31-positive pixels was seen in the depth range of 0.7 to 1.5 mm in the skin. Figure 7 shows a comparison of the total numbers of CD31-positive pixels for all the sets of conditions. The number of pixels indicating angiogenesis in the skins injected with hHGF gene with LISW application was significantly larger than the numbers of pixels in skins treated under the three other conditions. The skins injected with the hHGF gene alone and those injected with the control vector with LISW application also showed higher mean values than those normally grafted, but no significant differences were obtained.



**Fig. 5** Cross-sectional images of rat skin grafts with immunohistochemical staining using anti-rat CD31/PECAM-1 antibody 3 days after gene transfection: (a) neither hHGF gene nor LISW was applied (normal grafting); (b) hHGF gene was injected without LISW application (hHGF alone); (c) control vector plasmid DNA was injected with LISW application (control vector with LISWs); (d) LISWs were applied after hHGF gene injection (hHGF with LISWs). Arrowheads indicate pronounced blood vessels indicated by immunohistochemistry [(a) and (b)]. No arrowheads are shown in (c) and (d) because there are too many nanovasculatures. Scale bars indicate 200  $\mu\text{m}$ .

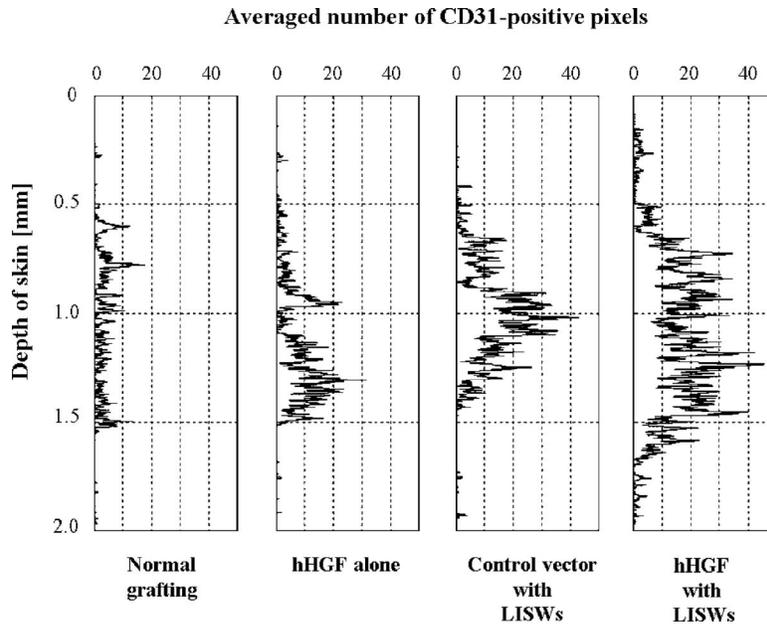
#### 4 Discussion

In this study, we delivered a therapeutic vector construct carrying the hHGF gene to skin grafts of rats by applying LISWs and used the grafts for autografting. Enhanced angiogenesis was observed in the grafted skin, demonstrating the validity of our gene transfection technique in improving the outcome of skin grafting. To our knowledge, this is the first experimental demonstration of LISW-mediated gene transfection showing therapeutic efficacy.

The hHGF expression level in skin decreased with elapse of time in skins with and without LISW application (Fig. 4). However, in the skins subjected to LISW at a laser fluence of 1.8 J/cm<sup>2</sup>, hHGF concentration at 5 days after gene transfection was comparable to that at 24 h after gene transfection in skins without LISW application. The time-course of hHGF concentration observed in the present study was similar to that observed in our previous study using a luciferase expression vector.<sup>20</sup> The time-dependent decrease in the protein concentration indicates that the transgene was not inserted into genomic DNA, suggesting a low risk of adverse effects such as tumorigenesis due to insertion mutagenesis or chromosomal

aberrations. A permanently high expression level of HGF is not necessary to accelerate graft adhesion.

HGF contains four kringle domains located on the  $\alpha$ -subunits,<sup>26</sup> many proteins with kringle domains have roles in vascular endothelial activities. HGF is known to be a mitogen for endothelial cells, based on which it has been reported that angiogenesis can be enhanced in various types of tissues.<sup>27–30</sup> In the skins injected with hHGF-expression vector without LISW application, the distribution of CD31-positive cells was nonhomogenous. The density of CD31-immunoreactivity was highest at a depth of 1.3 mm (Fig. 6), indicating that angiogenesis occurred near the graft beds; the thicknesses of skin grafts ranged from 1.3 to 1.7 mm. Unexpectedly, it was found that the skin grafts with control vector injection plus LISW application showed a significantly larger number of CD31-positive cells than those seen in the normal grafts. The reason for this is not clear, but one possible explanation is the effect of LISWs of stimulating biological activity in the tissue. Further study is needed to elucidate this phenomenon. The skins with hHGF gene injection plus LISW application showed significantly enhanced angiogenesis com-



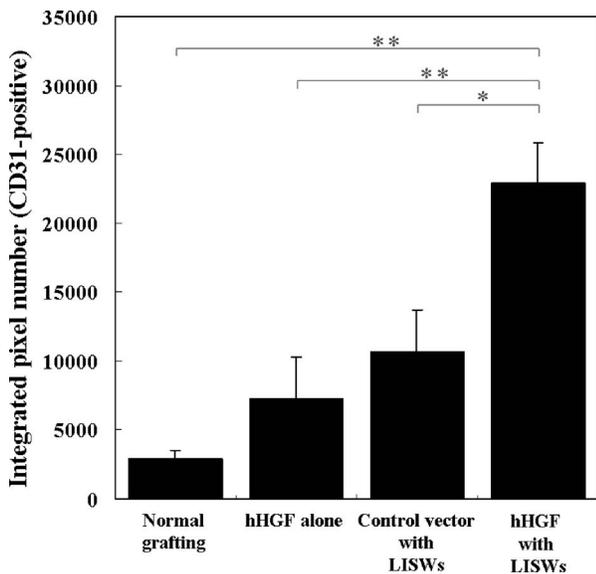
**Fig. 6** Depth profiles of the number of CD31-positive pixels for grafted skins treated under four conditions: (a) neither injection of hHGF gene nor application of LISWs (normal grafting); (b) injection of hHGF gene without LISW application (hHGF alone); (c) injection of control vector plasmid DNA with LISW application (control vector with LISWs); (d) application of LISWs after hHGF gene injection (hHGF with LISWs). Transverse axes show average number of CD31-positive pixels from five rats in each condition.

pared with skins treated under the other conditions (Fig. 7). In this case, neovascularities were distributed uniformly in the depth range of 0.7 to 1.5 mm in the skins, suggesting the efficient provision of nutrients and oxygen to the major part of the graft. This is likely to be an important factor in accelerating the adhesion of grafted skins.

Our method is expected to be effective not only for autografts but also for allografts and for cultured skin substitutes. Autografts are most desirable for achieving efficient ad-

hesion because of lack of rejection. However, skin for autografting is often limited in cases of extended full-thickness skin injuries such as burns. Thus, allografts and cultured skin substitutes are used for immediate coverage of wounds to improve healing, to control pain, and to prevent loss of fluids.<sup>35,36</sup>

Rapid closure of the wound is also important to reduce the risk of infection. Cultured skin substitutes have been developed since the introduction of cultured human epidermal cells by Green et al. in 1979.<sup>37</sup> However, cultured skin substitutes lack a vascular plexus, which often leads to grafting failure, resulting in an increased risk of infection due to ischemia and nutrient deprivation. While thicker skin substitutes contain more skin components, their survival rates are generally low due to poor blood circulation after grafting. Thus, recently, bioengineered skin substitutes prepared by genetic modification as a means of overcoming such problems have received much attention. It has been reported that up-regulation of growth factors by gene transduction accelerates the proliferation of epidermis and angiogenesis in grafts. In most cases, gene transduction of keratinocytes has been performed using a retrovirus vector;<sup>38-40</sup> the use of nonviral vectors has been limited to cultured skin substitutes. Rio et al. reported that the angiogenic response in host stroma was enhanced by lipofectamine-mediated gene transfection of pig primary keratinocytes.<sup>41</sup> However, this method requires culturing transfected keratinocytes on a fibrin matrix before grafting, which is a time-consuming process. On the basis of our method, it might be possible to transfer a gene into skin substitutes immediately before grafting, which would be a promising approach to developing high-performance bioengineered skins.



**Fig. 7** Comparison of total numbers of CD31-positive pixels for grafted skins treated under the four conditions, derived from Fig. 6. ( $p < 0.05$ ,  $**p < 0.01$ ).

With other physical gene transfer methods, such as electroporation and ultrasound, electrodes or a transducer need to be moved point-to-point to treat large-area grafts. With the LISWs-based gene transfer, large-area treatment is readily achieved by scanning a laser beam and can easily be automated, realizing high-speed processing. Furthermore, because the present method can also be applied to transplantation of various types of tissues and cells, the introduction of automated gene transfer systems of this type to skin banks and tissue engineering facilities will greatly contribute to progress in transplantation medicine and future regenerative medicine.

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