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Microbubble-enhanced ultrasound for gene transfer into living skin equivalents

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Living skin equivalent

Summary

Background: Gene transfer to skin is an attractive therapeutic approach because of the accessibility of the skin and the high rate of cure for many cutaneous diseases. However, safety concerns over viral vectors and the low efficiency of most non-viral gene transfer techniques have encumbered their clinical application for gene transfer. By contrast, efficient gene transfers into various cell types using microbubble-enhanced ultrasound has been reported.
Objectives: The purpose of this study was to investigate whether ultrasound with microbubble enhancement allowed effective transfer of foreign genes into living skin equivalents (LSEs).
Methods: Microbubbles and plasmid DNA encoding green fluorescent protein (GFP) were added to the dermal—epidermal junctions of LSEs, which were then exposed to ultrasound. The LSEs were harvested at different time points to investigate transgene expression using confocal laser microscopy. Transfected LSEs were also transplanted onto nude mice, and the in vivo transgene expression was observed.
Results: From days 2 to 7 after transfection, most GFP-positive cells continued to migrate upward from the basal layer, while other GFP-positive cells lagged behind or remained in the basal layer on days 5 and 7. Transfection resulted in 20—30% GFP-

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positive cells. Multiple transfections further increased the percentage of transfected cells and resulted in multi-layer transgene expression. Grafts from the transfected LSEs survived on nude mice and continued to express GFP up to 2 weeks post-transplantation.
Conclusion: Gene transfer into LSE using ultrasound with microbubble enhancement is an effective alternative to viral and non-viral methods.
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1. Introduction

A human living skin equivalent (LSE) consists of keratinocytes seeded upon a fibroblast-populated type I collagen gel. LSEs are now readily cultured and applied in the treatment of burn wounds, ulcers, and other skin defects [1–3]. It has been suggested that this three-dimensional skin substitute could be genetically modified and transplanted in order to deliver therapeutic proteins locally and systemically for the treatment of cutaneous diseases and systemic disorders characterized by low or absent levels of circulating polypeptides [4,5]. LSE is an attractive ex vivo approach to gene delivery, as it allows confirmation of the efficiency of gene transfer and avoids the need to administer the vectors directly to patients, with the concomitant risk of systemic dissemination [6]. Virally transduced keratinocytes have been deposited onto dermal matrices to construct genetically engineered LSEs that expressed the transgene in vitro and on nude mice after transplantation [7,8] and that corrected the abnormal morphogenesis and malfunction of LSEs comprising cells expressing certain genodermatoses [9,10]. However, safety concerns regarding viral vectors, such as immunogenicity, insertional mutagenesis, and cytopathic effects, as well as the low efficiency of most non-viral gene transfers, have encumbered their use in clinical application [11–13].

Several non-viral approaches for gene transfer to skin have been introduced [14,15], among which, in vivo intradermal injection has successfully transduced naked DNA into keratinocytes [16] and the cytokine transgene expression has provided biological effects in the treated skin [17,18]. However, non-viral gene transfer into living skin equivalent has not been established. Recently, microbubble-enhanced ultrasound, as a new non-viral gene delivery approach, has efficiently transferred foreign genes into cardiac cells, muscle cells, and prostate cancer cells [19–22]. In the present study, we demonstrate that this approach is also effective in delivering genes into LSE.

2. Materials and methods

2.1. Monolayer cell culture

Normal human epidermal keratinocytes were cultured in MCDB 153 type II medium as previously described [23,24]. Keratinocytes in their fourth passage were used for LSE cultures.

Normal human dermal fibroblasts were isolated from normal human skin and cultured in DMEM (Gibco) supplemented with 10% FCS. Fourth- or fifth-passage cells were used in constructing the LSEs.

2.2. Preparation of LSEs

1. Collagen gel was prepared as follows: six volumes of ice-cold porcine collagen type I solution (Nitta Gelatin Co., Osaka, Japan) were mixed with one volume of 8× DMEM (Gibco), 10 volumes of 1× DMEM supplemented with 20% FCS, and one volume of 0.1N NaOH. One millilitre of the resulting solution was added to each culture insert (Transwel, collagen membrane bottom, diameter: 24 mm, membrane pore size: 3 μm, Costar) in a six-well culture plate (Costar). After polymerization of the solution on the insert at 37 °C, 3.5 ml of fibroblast-containing collagen solution was applied onto each insert, followed again by polymerization at 37 °C. DMEM supplemented with 10% FCS and ascorbic acid (final concentration 50 ng/ml) was then added to the fibroblast-rich gel. After 5 days of culture, the fibroblasts had contracted the gel.
2. Five days after the dermal component was prepared, 6.0×10^5 keratinocytes in 60 μl MCDB 153 type II were seeded onto the concave surface of the contracted gel. The resulting LSE was maintained submerged in culture medium for 2 days. When the keratinocytes reached confluence, the LSE was raised to the air–liquid interface, and cornification medium (CM), composed of a 1:1 mixture of Ham's F-12 and DMEM supplemented with 2% FCS, 0.1 mM ethanola-

mine, 0.1 mM *o*-phosphoethanolamine, 0.4 μg hydrocortisone/ml, 5 μg insulin/ml, 12.2 μg adenine/ml, 5 μg transferrin/ml, 6.83 μg selenious acid/ml, 13.46 pg triiodothyronine/ml, 89.34 μg choline chloride/ml, 105.1 μg serine/ml, 20 μg linoleic acid/BSA/ml, and 50 μg ascorbic acid/ml was added. The medium was changed every other day.

2.3. Transfection of GFP plasmid DNA

A commercial reporter plasmid, pEGFP-C2 vector 6083-1 (Clontech, Japan), encoding green fluorescent protein (GFP), was used for transfection. Plasmid DNA was obtained from *Escherichia coli* JM 109 cultures and prepared with a kit (Qiagen Midi; Qiagen, Chatsworth) according to the manufacturer's instructions. Agarose gel electrophoresis was performed before and after restriction endonucleases digestion to verify the identity and purity of the plasmid DNA.

GFP plasmid DNA was transfected into the LSE on day 7 after air-lift. As stated in the protocol accompanying the MB-3 albumin-coated octafluoropropane-gas-filled microbubbles (purchased from Neppa Gene Co., Japan), GFP plasmid was mixed with the microbubbles (final DNA concentration, 200 μg/ml). The plasmid DNA and microbubbles (10 μl) were added to the dermal—epidermal junction of the LSEs, which were then immediately transferred to a six-well plate (Costar) and exposed to ultrasound using an ultrasound generator (Ultax Model No. UX-301, Celcom Inc., Japan), with the probe applied to the bottom of the plate. The optimal conditions for gene transfection were determined by testing various combinations of exposure parameters: frequency (from 1 to continuous), intensity (from 1 to 10), and exposure duration (from 1 to 60 s). Following the exposure to ultrasound, the transfected LSEs were kept in culture for 2 more days, after which the epidermis was peeled off and checked for GFP expression using confocal laser microscopy (LSM 510, Zeiss, Germany). The remaining layers of the LSEs were cultured continuously in air-lifted status and harvested on days 2, 5, and 7 after transfection.

Plasmid DNA re-transfection was also conducted. LSEs were divided into three groups. The first group was transfected on day 7 after air-lift; the second group was re-transfected on days 7 and 9, and the third group on days 7, 9, and 11. All the LSEs were harvested on day 13 after air-lift.

In the transfection and re-transfection trials, the epidermis was peeled off of the dermal matrix of the LSEs and cut into two pieces. One piece was mounted directly for observation of horizontal sections of the LSE; the other was snap frozen and sectioned vertically at 7 μm intervals. The sections were evaluated by confocal laser microscopy. Three independent experiments were performed.

2.4. Transplantation of GFP-transfected LSEs onto nude mice

On day 7 after air-lift, GFP-containing plasmid was transfected into the LSEs by microbubble-enhanced ultrasound as described above. On the 2nd day of transfection, the epidermal layers of the LSEs were peeled off and grafted onto full-thickness wounds on the backs of 8-week-old, female BALB/cAJc1-nu nude mice. The grafting protocol was approved by the Ethics Committee of Ehime University School of Medicine. Each mouse was anesthetized by an intraperitoneal injection of 0.3 ml avertin (1.25% tribromoethanol, 2.5% 2-methyl-2-butanol). An 8 mm skin biopsy punch was used to create a full-thickness wound deep to the fascia of the dorsal musculature,

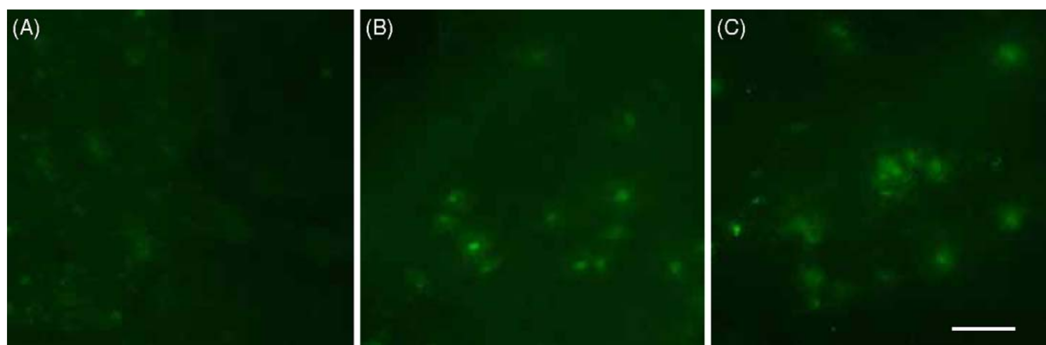

Fig. 1 The influence of the ultrasound exposure duration on gene transfer efficiency in LSEs. Microbubble-enhanced ultrasound gene transfer to living skin equivalents (LSEs) was performed at 2 Hz and an intensity of 8 for 10 s (A), 30 s (B), and 60 s (C). Green fluorescent protein (GFP) expression in the peeled epidermis is observed 2 days later using fluorescence microscopy. Scale bar, 50 μm.

and an LSE graft of corresponding size, removed by the same punch, was applied to the wound and covered with transparent film. On days 3, 5, 7, and 14 after transplantation, the nude mice were killed, the transplanted grafts were excised, and each graft was cut into two pieces.

One piece was fixed in 20% formalin, embedded in wax, and sectioned at 5 μm intervals for H&E staining. The other piece was frozen in OCT compound and sectioned at 7 μm intervals for observation using confocal laser microscopy. Three independent experiments were performed.

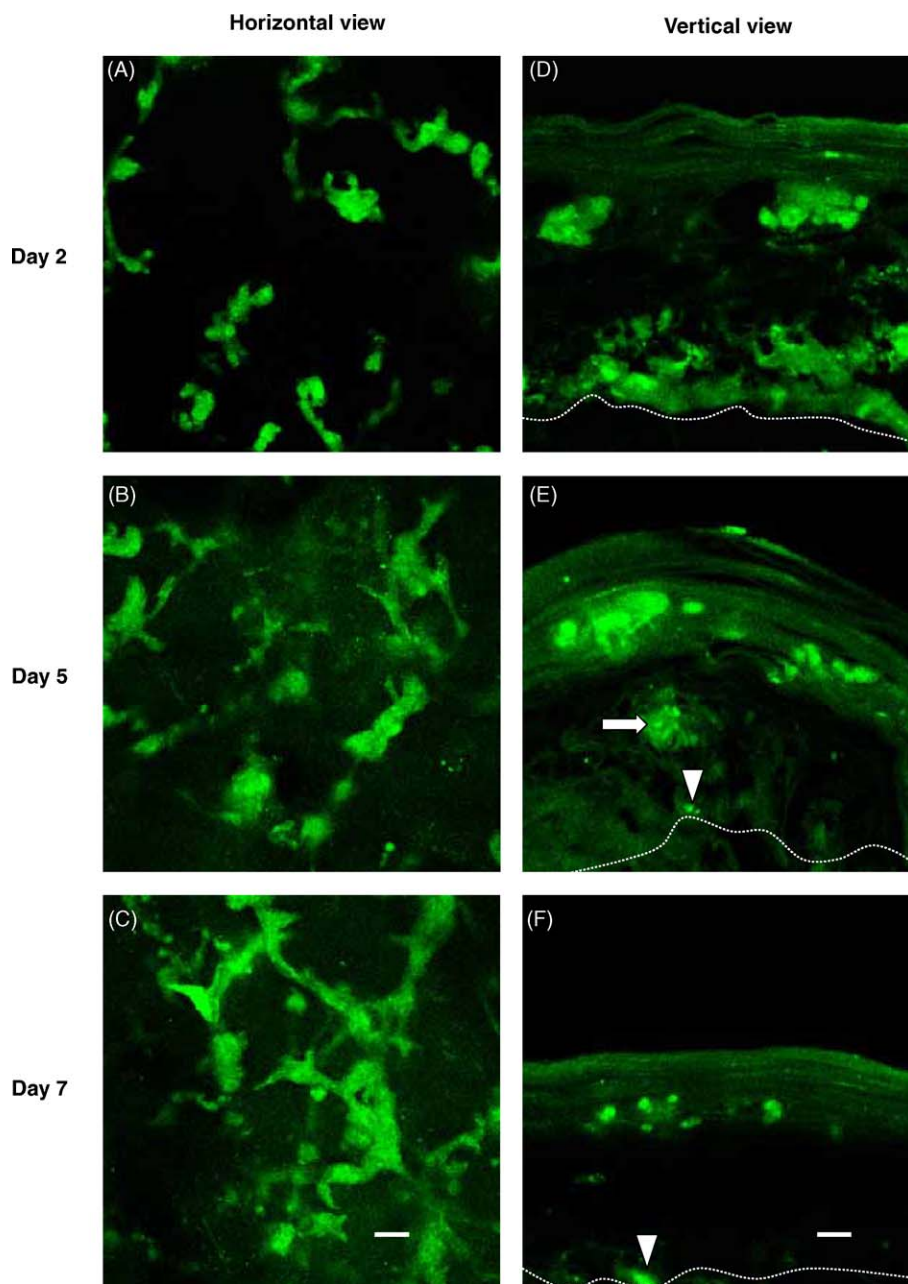

Fig. 2 The dynamics of GFP expression in LSEs after transfection. Samples were collected on day 2 (A, D), day 5 (B, E), and day 7 (C, F) after transfection. For each sample, horizontal (A—C) and vertical (D—F) microscopy sections were examined. The horizontal sections show that fluorescence-positive cells had spread and merged with each other from day 2 (A) to day 5 (B) to day 7 (C). The vertical sections show that most fluorescence-positive cells are located in the basal or suprabasal layer on day 2 (D) and move to the stratum corneum on day 5 (E) and day 7 (F), while some positive cells either lag behind in their upward migration (arrow in Fig. 2E) or remain in the basal layer on day 5 (arrow head in E) and day 7 (arrow head in F). The white-dotted line indicates the dermal—epidermal junction. Scale bar, 50 μm.

3. Results

3.1. Determination of optimal ultrasound exposure parameters

After testing various combinations of ultrasound frequency, intensity, and duration, an exposure of 2 Hz at an intensity of 8 for 60 s was found to most efficiently transfer the GFP plasmid into LSEs. Fig. 1 shows the influence of the ultrasound exposure duration on the GFP gene transfer efficiency. At a frequency of 2 Hz and an intensity of 8, more fluorescence-positive cells were found in the epidermis of the LSE exposed to ultrasound for 60 s (Fig. 1C) than for 10 s (Fig. 1A) or 30 s (Fig. 1B).

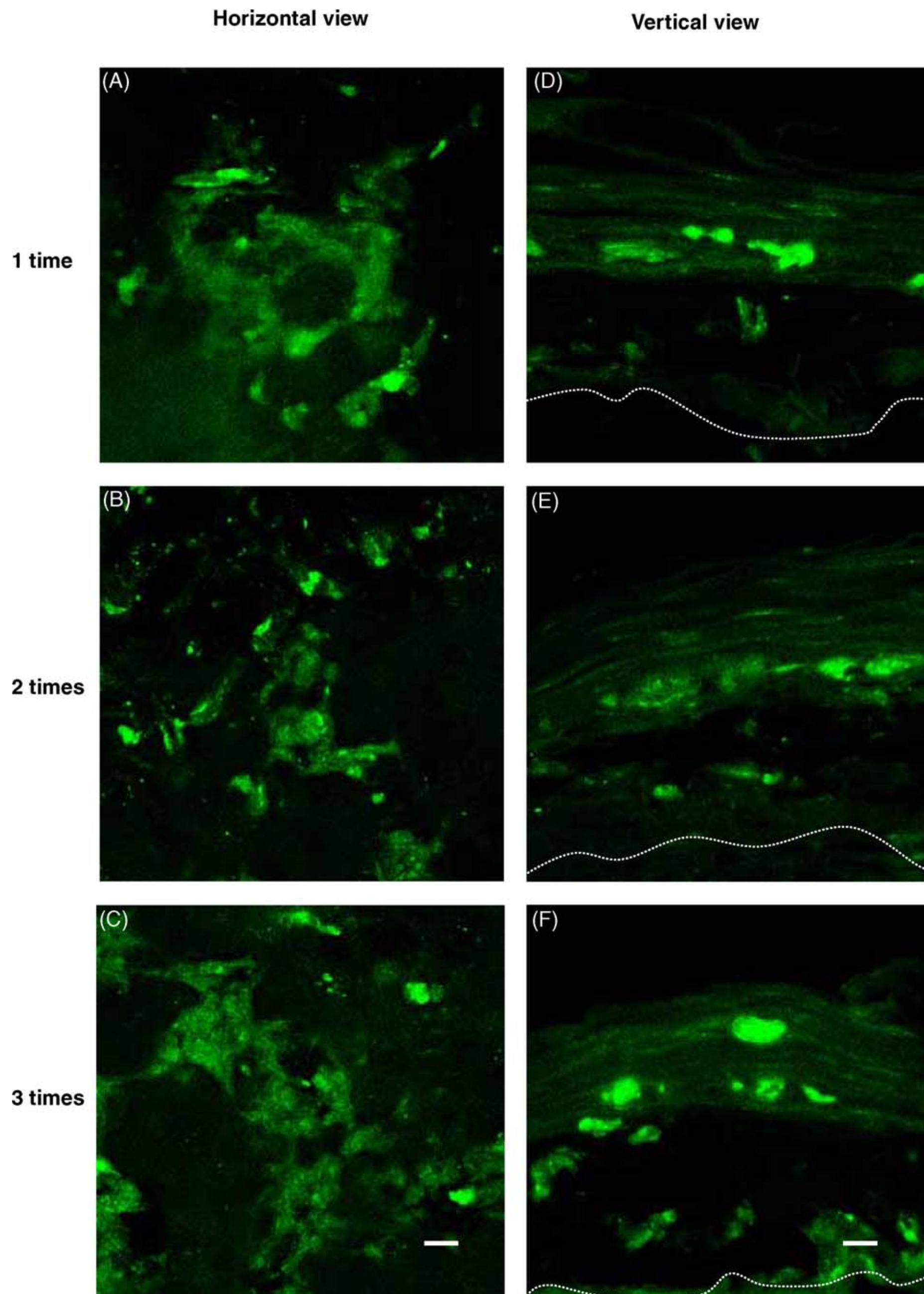

Fig. 3 GFP expression after multiple transfections. LSEs were transfected once (A, D), twice (B, E), or three times (C, F). The horizontal microscopy sections show that cells transfected three times (C) expressed more GFP than did those transfected once (A) or twice (B). The vertical sections show that the fluorescence-positive cells are located mainly in the stratum corneum in cells transfected once (D), in both the stratum corneum and the suprabasal layer in cells transfected twice (E), and in the stratum corneum, suprabasal, and basal layers in cells transfected three times (F). The white-dotted line indicates the dermal—epidermal junction. Scale bar, 50 μm.

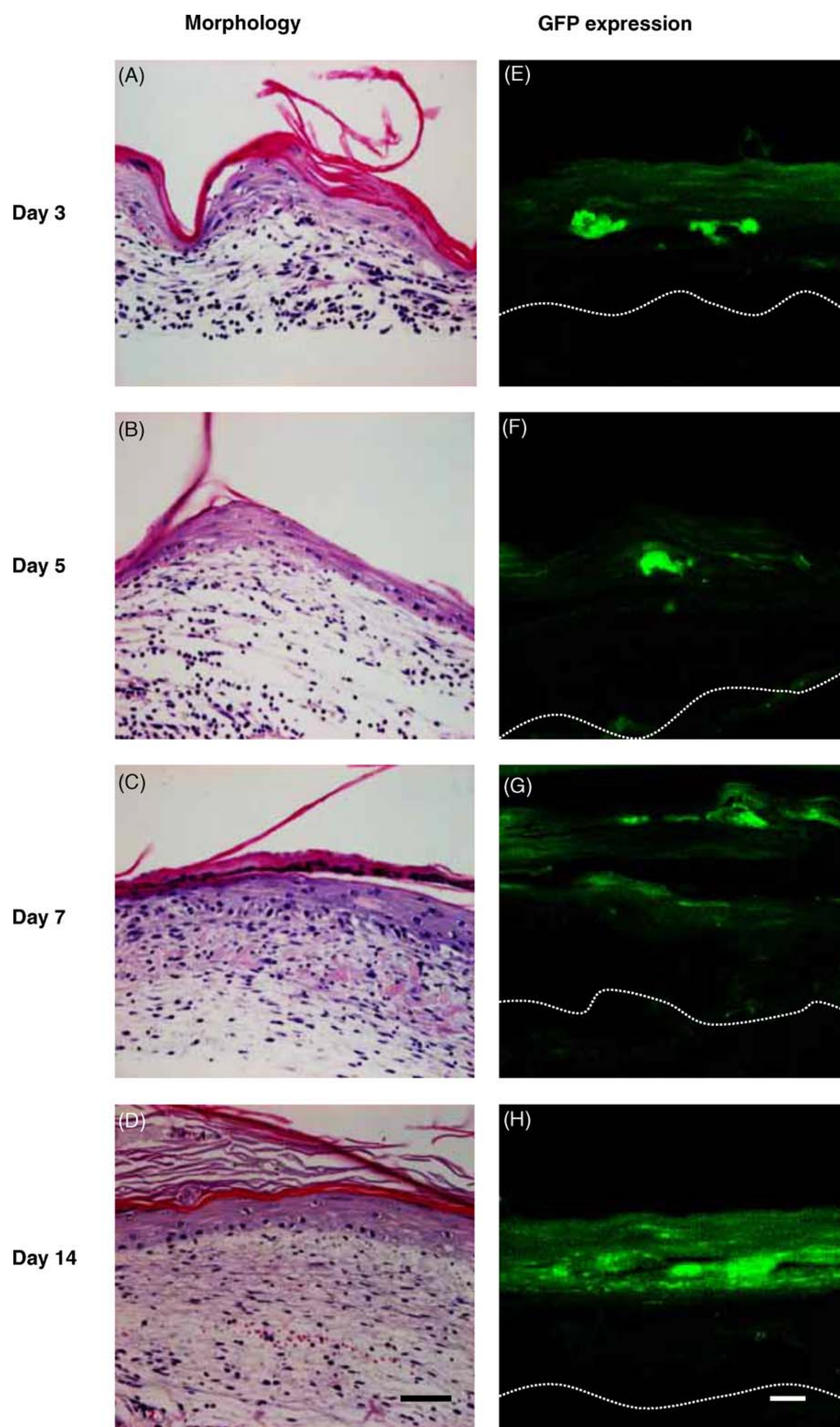

Fig. 4 Morphology of the epidermis and GFP expression in transfected LSE grafts in vivo. Transfected LSE grafts were collected on day 3 (A, E), day 5 (B, F), day 7 (C, G), and day 14 (D, H) after transplantation. H&E staining (A—D) shows that the transfected LSE grafts survive on nude mice and that the epidermis is well stratified and differentiated. Frozen sections (E—H) viewed by confocal laser microscopy show that the fluorescence-positive cells stayed in the suprabasal layer on day 3 (E) and moved to the stratum corneum on day 5 (F) and day 7 (G). By day 14, all of the cells were restricted to the stratum corneum (H). The white-dotted line indicates the dermal—epidermal junction. Scale bar, 50 μm.

3.2. GFP expression following plasmid DNA transfection

To investigate the transgene expression and distribution, the transfected epidermis was harvested at different time points. Horizontal and vertical sections of the epidermis were viewed under a confocal laser microscope to investigate the three-dimensional, dynamic distribution of positively transfected cells. The horizontal sections revealed that a fraction of keratinocytes were transfected and expressed GFP. The fluorescent areas increased from days 2 to 7 after transfection and appeared to merge with each other by days 5—7 (Fig. 2A—C). The vertical sections showed that most of the fluorescence-positive cells migrated from the basal layer to the upper layer of the LSE between days 2 and 7 after transfection (Fig. 2D—F), while, on day 5, other cells still lagged behind (Fig. 2E), and a few persisted in the basal layer on days 5 and 7 (Fig. 2F).

3.3. Multiple GFP plasmid DNA transfections increased transgene expression in LSE

To increase the number of positively transfected cells, re-transfection was carried out. The results showed that the number of positively transfected cells increased with increasing frequency of transfection (single, double, or triple transfection), as revealed by analyzing horizontal and vertical sections of the epidermis of the transfected LSEs. Between 20 and 30% of the epidermal cells were GFP-positive (Fig. 3A—F). As seen in the vertical sections, GFP-positive cells were located mainly in the upper layer of the epidermis after single transfection and in the middle and upper layers after double transfection. Following triple transfection, GFP expression was distributed not only in the stratum corneum and suprabasal layer but also in the basal layer of the epidermis. With histogram of Zeiss LSM Image Examiner, we measured the unit fluorescence intensity on horizontal sections of three samples transfected 1, 2 or 3 times, respectively. The total intensity for each sample was counted, and the mean values of three samples were calculated. The relative fluorescence intensity of 2-time and 3-time transduction was 1.3 ($p < 0.05$) or 1.8 ($p < 0.05$) compared to 1-time transduction, respectively.

3.4. Transgene expression in transfected LSE grafts in vivo

To investigate the transgene expression in vivo, the LSEs were transplanted onto full-thickness wounds on the backs of nude mice, and samples were harvested on days 3, 5, 7, and 14 after transplantation. H&E staining of sectioned LSE grafts showed that, from day 3 up to day 14, the grafts survived well and the epidermis was well differentiated and stratified without any signs of toxicity (Fig. 4A—D). The frozen sections of the samples showed that the fluorescence-positive cells kept moving upward and were almost all restricted in stratum corneum at day 14, rarely seen at day 21 (data not shown), probably lost during skin scaling. On day 3, the GFP-positive cells stayed in suprabasal layer (Fig. 4E); on day 5 (Fig. 4F) and day 7 (Fig. 4G), positive cells were located mainly in the stratum corneum. By day 14, GFP-positive cells had spread and merged with each other in the stratum corneum such that it was difficult to identify individual positive cells (Fig. 4H).

4. Discussion

This study was designed to investigate whether ultrasound with microbubble enhancement could efficiently transfer foreign genes into human LSE. We have shown that GFP plasmid DNA was efficiently delivered into the epidermis of the LSE. The transfected LSE graft survived on full-thickness wounds of nude mice and expressed the transgene for up to 2 weeks. Physical parameters are known to influence the efficiency of ultrasound-mediated gene transfection and the viability of the target cells [21]. Optimal conditions vary according to cell type and concentration, the size and composition of the microbubbles, and whether transfection is conducted in vivo or in vitro [19—21,25]. In the ex vivo LSE transfection experiments described here, a mixture of albumin-coated, octafluoropropane-gas-filled microbubbles (2.0—4.5 μm) and plasmid encoding GFP were applied to the dermal—epidermal junction of the LSE, which was then exposed to ultrasound under a variety of conditions. After testing several combinations of ultrasound parameters, i.e., frequency, intensity, and exposure duration, we found that an exposure of 2 Hz for 60 s at an intensity of 8 resulted in the efficient transfer of GFP plasmid DNA into the LSE epidermis. In another trial, keratinocytes (at different concentrations) in culture medium were mixed with the microbubbles and GFP plasmid DNA and then exposed to ultrasound. Contrary to our expectations, very few GFP-positive cells were obtained using either the optimal conditions for LSE described above or any other combination of exposure parameters (data not shown).

The combined use of microbubbles and ultrasound can greatly increase gene transfer efficiency [26], and the number of positively transduced cells

is further increased by repeated exposure to microbubble-mediated ultrasound [27]. In this study, multiple transfections were carried out, resulting in both multi-layer GFP expression and an increase in the number of epidermal cells expressing the transferred gene. These results indicate that re-transfection is another approach to increase transfection efficiency in LSEs.

It was observed that, in transfected LSEs, most of the GFP-positive cells migrated upward from the basal layer, both in culture and after transplantation. In the transfected LSEs transplanted onto nude mice, the positively transfected cells persisted, moving from the basal layer to the stratum corneum, until day 14 in stratum corneum and by day 21 almost all fluorescence was lost. This process coincided with one turnover time of the epidermis. In epidermis, most keratinocytes continue to differentiate and eventually slough off the skin surface, so that a gene transfected into these keratinocytes might eventually be lost. Nonetheless, transient transgene expression may play a role in supplementing growth factors and cytokines that are lacking in abnormalities linked to inadequate wound healing. For example, it has been reported that the transduction of hepatocyte growth factor into keratinocytes caused transient epidermal hyperproliferation in an LSE [28]. The mixture of GFP plasmid DNA and mibrobubble was administered at the dermal-epidermal junction of the LSEs, and then ultrasound was applied. We investigated the GFP expression in the peeled epidermis and the frozen sectioned collagen gel, respectively. By contrast to the epidermis, we rarely observed fluorescence-positive cells in the collagen gel (data not shown). Similar phenomenon was reported in in vivo or ex vivo gene delivery by intradermal injection, which was supposed due to the relatively fewer fibroblasts in the dermis or the inefficiency of the dermal cells to take-up and express the injected naked DNA [16].

To obtain sustained gene expression, gene transfer should be targeted to stem cells that possess unlimited growth potential and self-renewal capacity. Recent findings have suggested that integrin β1, integrin α6, CD 71, CD 34, keratin 15, keratin 19, and p63 can serve as epidermal stem cell markers [29—37], although none of these is sufficient to unambiguously identify stem cells in vivo. Until now, there have been few reports confirming the existence of stem cells in LSE constructed by seeding keratinocytes onto fibroblast-populated collagen gels. However, it is highly likely that stem cells are present in LSE as an Apligraf skin equivalent continued to survive on nude mice for over 1 year after transplantation [38] and transgene expression in keratinocytes transfected by lentiviral or retroviral vectors in skin equivalents was observed up to 13 weeks (4—6 epidermal turnovers) post-grafting [8].

Ultrasound with microbubble enhancement has proved to be effective as a non-viral gene transfer technique in many cell types in vivo and in vitro [19—22,39]. In addition, the mechanism of this technique in gene transfer [40,41] does not imply limitations in transferring foreign genes into either dividing or non-dividing cells. In this study, we found that some fluorescence-positive cells lagged behind others in their upward migration and that some remained in the basal layer as late as days 5—7 after gene transduction. These lagging or remaining cells might be transit-amplifying cells or stem cells. Among the 20—30 frozen slices prepared from samples taken on day 5 or 7 after transduction, only a few contained GFP-positive cells in the basal layer of the epidermis. Stem cells represent only a small portion of the basal cells in the interfollicular epidermis [42] and may be even less frequent in the LSE, as there is no stem cell influx from the multipotent stem cells lodged in the bulge areas of the hair follicles. However, keratinocyte progenitor cells or stem cells could be transfected using the ultrasound technique, implying that the number of stem cells thus transfected could be increased under more appropriate conditions and that sustained transgene expression could be expected.

In the ex vivo experiment described here, keratinocytes of LSEs were efficiently transfected with microbubble-enhanced ultrasound. The transfected LSEs that were subsequently transplanted onto nude mice survived well and continued to express the transgene. This proves not only that efficient gene transfer into human LSE can be obtained by this technique but also that the use of genetically modified LSE is a practical strategy in the treatment of cutaneous disorders.

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