Increased interstitial pressure improves nucleic acid delivery to skin enabling a comparative analysis of constitutive promoters

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Nucleic acid-based therapies hold great promise for treatment of skin disorders if delivery challenges can be overcome. To investigate one mechanism of nucleic acid delivery to keratinocytes, a fixed mass of expression plasmid was intradermally injected into mouse footpads in different volumes, and reporter expression was monitored by intravital imaging or skin sectioning. Reporter gene expression increased with higher delivery volumes, suggesting that pressure drives nucleic acid uptake into cells after intradermal injections similar to previously published studies for muscle and liver. For spatiotemporal analysis of reporter gene expression, a dual-axis confocal (DAC) fluorescence microscope was used for intravital imaging following intradermal injections. Individual keratinocytes expressing hMGFP were readily visualized in vivo and initially appeared to preferentially express in the stratum granulosum and subsequently migrate to the stratum corneum over time. Fluorescence microscopy of frozen skin sections confirmed the patterns observed by intravital imaging. Intravital imaging with the DAC microscope is a noninvasive method for probing spatiotemporal control of gene expression and should facilitate development and testing of new nucleic acid delivery technologies.

Introduction

Development of effective nucleic acid-based therapies is essential if we are to effectively use gene therapy approaches to treat difficult diseases. To date there have been over 1500 clinical trials reported (http://www.wiley.co.uk/genmed/clinical/). Although skin represents a promising target (due to large unmet clinical needs, easy access to tissue and relative ease of monitoring), only a few clinical trials have been carried out in the last decade, with the majority related to the treatment of melanoma. A large number of genetic skin disorders, that have few if any treatment options, are amenable to gene therapy but efforts to translate such approaches to the clinic are hampered by the difficulty of delivering nucleic acids across tissue and cellular barriers. Several methods have been used to deliver genes to the skin, with varying degrees of efficiency, including direct intradermal (i.d.) injection, gene gun, magnetofection, electroporation and topical formulations. However, only limited success with sustainable gene expression has been observed over wide areas of the skin. Although the mechanism of nucleic acid uptake in keratinocytes is poorly understood, it has been shown in other organ systems that high pressure facilitates delivery. For example, high pressure hydrodynamic intravascular injection produces moderate levels of gene expression in limb muscles. Intravenous (portal or tail vein) high volume/high pressure injections facilitate uptake and expression in the liver and, to a lesser extent, other organs such as spleen and kidney. 

Although i.d. injection of nucleic acid appears to be the most efficient delivery technique for gene transfer to skin (particularly when combined with other technologies such as electroporation), the localized and painful nature of hypodermic needle injection precludes its repeated use in treating wide areas of the skin. Therefore, development of novel, efficient and ‘patient-friendly’ delivery technologies is needed.

Intravital confocal microscopy can be used for spatiotemporal monitoring of reporter gene expression with cellular resolution and molecular specificity. A number of in vivo high resolution imaging technologies for skin have been reported, but have mainly focused on reflectance mode imaging to reveal tissue microanatomy or on en face features. We have developed a
dual-axis confocal (DAC) fluorescence microscope that has the capability of detecting fluorescent signals with high sensitivity to a depth of 150 μm with 488 nm excitation light. Like other confocal microscopes, the DAC system has the ability to optically, noninvasively, section tissues for 3-D reconstruction of images. We have recently reported that the DAC microscope can readily detect siRNA-mediated inhibition of green fluorescent protein (GFP) reporter expression in a transgenic GFP mouse model.

To investigate the utility of intravital imaging to monitor delivery of nucleic acids to skin, and to improve our understanding of the mechanisms, including high pressure, involved in nucleic acid uptake and functionality (that is, gene expression) in cells of the epidermis, we used fluorescence microscopy (including intravital DAC imaging) and in vivo bioluminescence imaging (BLI) to monitor the transfer of plasmid DNA and expression of encoded genes following i.d. injection.

**Results**

*Increased pressure during hydrodynamic i.d. injection of plasmid DNA improves reporter gene expression in keratinocytes*

Several laboratories have shown that keratinocytes efficiently take up and express naked plasmid DNA following i.d. administration possibly via a protein-mediated event. We have previously used BLI to quantitatively assess plasmid uptake and expression in skin following i.d. injection of a plasmid-encoding modified firefly luciferase as a reporter. As high pressure has been shown to facilitate gene transfer in several organs including liver, kidney and muscle, we tested the effects of pressure in gene transfer to skin. Equal amounts (10 μg) of the pGL3-CMV-Luc reporter plasmid were delivered by i.d. injection into mouse footpad skin (a single injection of the indicated volume was performed at the same pad location on each paw) in different volumes (10, 25, 50, 75 and 100 μl) of PBS vehicle (pressure-fecction). Mouse footpads were intradermally injected with a constant amount (10 μg) of pGL3-CMV-Luc expression plasmid over a range of volumes in PBS. 24 h later, luciferin was injected intraperitoneally into mice and luciferase activity measured by BLI (see Materials and methods). The effect of varying the volume was compared on each mouse by injecting a smaller volume into the left footpad whereas the right paw was injected with a larger volume: 10 vs 50 μl (a), 25 vs 75 μl (b) and 50 vs 100 μl (c). The ratio of the average radiance (photons/s/cm²/sr) was calculated by dividing the right (high volume) paw value with the left (low volume) paw value of each mouse. Each individual data point was plotted and the mean (horizontal line) and error (vertical line) calculated (d).

**Figure 1** Plasmid delivery and expression in mouse paws following i.d. injection is facilitated by high pressure (‘pressure-fecction’). Mouse footpads were intradermally injected with a constant amount (10 μg) of pGL3-CMV-Luc expression plasmid over a range of volumes in PBS. 24 h later, luciferin was injected intraperitoneally into mice and luciferase activity measured by BLI (see Materials and methods). The effect of varying the volume was compared on each mouse by injecting a smaller volume into the left footpad whereas the right paw was injected with a larger volume: 10 vs 50 μl (a), 25 vs 75 μl (b) and 50 vs 100 μl (c). The ratio of the average radiance (photons/s/cm²/sr) was calculated by dividing the right (high volume) paw value with the left (low volume) paw value of each mouse. Each individual data point was plotted and the mean (horizontal line) and error (vertical line) calculated (d).

**Gene transfer visualization of single keratinocytes in vivo using DAC microscopy**

Although gene transfer to skin has been previously reported by whole animal in vivo imaging using reporter genes that encode bioluminescent (for example, firefly luciferase), fluorescent (GFP) or chromogenic (LacZ) proteins, the localization and identification of individual reporter gene-expressing cells have been assessed only after killing of the mouse and removal of the skin for sectioning and microscopy. Using the DAC microscope, individual cells can be detected in vivo following i.d. injection of a GFP-encoding plasmid into mouse footpads (Figure 2). Within 15 h post-injection, humanized *Montastrea cavernosa* (‘monster’) green
fluorescence reporter protein (hMGFP) was detected at depths of 15–60\(\mu\)m (Figures 2a and b), consistent with expression in the stratum granulosum (verified by traditional skin cross sections obtained from similarly treated mice, see Figure 3). The signal accumulated in the cytoplasm (see arrows in Figure 2a as well as Supplementary Video 1). At 48 h post injection, the signal was mainly detected in the top layers of the epidermis from 0 to 30\(\mu\)m in depth (just a few cells were detected in lower layers, Figures 2c and d), and the cells showed the flat morphology and lack of nuclei typical of fully differentiated squames (see Supplementary video 2).

**Ex vivo validation of gene transfer to keratinocytes**

The intravital DAC imaging results were verified with conventional fluorescence microscopy of frozen skin sections of similarly treated skin. Examining vertical sections of footpad skin, localized regions of gene expression were detected, which were associated in some cases with needle tracks (data not shown). Fluorescence was predominantly localized in the upper layers of the epidermis, similar to what we previously reported in a hMGFP transgenic mouse model.\(^{35}\) The strong fluorescence signal of this reporter construct allowed clear visualization of single keratinocytes expressing hMGFP as early as 6 h (Figures 3a and b) after i.d. injection (in vivo detection of fluorescence using the DAC intravital imaging system was difficult due to low hMGFP signals at these early time points). Cells expressing hMGFP were mainly visualized in the granular layer, at 6–24 h after i.d. injection (Figures 3a–I). In contrast, only a few hMGFP-expressing cells were detected in the spinosum (Figures 3a and b) and labeled cells were rarely seen in the basal layer (data not shown). Finally, hMGFP expression began to accumulate in the lower part of the stratum corneum within 48–72 h post-injection (Figures 3g–j). At 144 h, hMGFP signal appeared mainly in the stratum corneum (Figures 3k and l).

**Figure 2.** Intravital imaging of hMGFP expression in mouse footpad epidermis using the dual-axis confocal (DAC) fluorescence microscope. The pCMV-hMGFP plasmid (total mass of 10 \(\mu\)g) was injected intradermally into mouse footpads and the paws were imaged using the DAC microscope at 15 and 48 h post-injection. The images show en face planes (left panels) and a vertical view (right panels) of a 3-D volume rendering of 145 \(\mu\)m \(\times\) 240 \(\mu\)m en face images through a depth of 90 \(\mu\)m. Individual cells expressing hMGFP were visualized throughout the granular layer (arrows in (a)) from 15 to 60 \(\mu\)m in depth 15 h after injection (b), where a single plane at 20 \(\mu\)m depth is shown in (a). At 48 h, most of the signal had accumulated at higher levels (that is, closer to the skin surface) consistent with fully differentiated squames (stratum corneum) from 0 to 30 \(\mu\)m in depth (d), where a single plane at 11 \(\mu\)m depth is shown in (c). Scale bar is 20 \(\mu\)m. The 2-D image stack videos of each data set are provided as Supplementary Materials.
Based on our data, differentiating keratinocytes transit approximately one cell layer every 20–24 h.

Comparative analysis of reporter gene expression expressed by different promoters in epidermal strata The localization of hMGFP expression within the granular layer and stratum corneum reported here and by others\textsuperscript{11} may be due to the effects of CMV enhancer elements (Soosan Ghazizadeh, personal communication). To investigate the role of these CMV promoter elements, we engineered plasmids expressing the dual function reporter under the control of promoters that do not contain CMV genetic elements. Plasmids expressing hMGFP/CBL from the Ubc, eIF4A1 or EF1a promoter were intradermally injected in mouse footpads and

Figure 3  Timecourse analysis of migration of hMGFP-transfected keratinocytes in murine epidermis during the differentiation process. The pCMV-hMGFP/CBL (10 μg in 50 μl PBS) expression constructs were intradermally injected into FVB mouse footpads as in Figure 1. Mice were killed at 6 (a, b), 15 (c, d), 24 (e, f), 48 (g, h), 72 (i, j) and 144 (k, l) h following i.d. injection, frozen in OCT, sectioned (10 μm) and reporter expression visualized by fluorescence microscopy as described in Materials and methods. Left panels show brightfield overlay. Scale bar is 10 μm. Nuclei are visualized with DAPI (blue).
expression analyzed following skin removal and sectioning. Each of these promoters resulted in more uniform expression as compared with plasmids containing the CMV promoter. Ubc and eIF4A1 promoters showed expression in the lower layers of the epidermis, mostly in the spinosum; however, a few cells were also detected in the basal layer (Figures 4a-d). The EF1a promoter showed preferential expression in the upper layers of the epidermis after i.d. injection (Figures 4e and f), although some GFP-expressing cells were also observed in lower compartments.

Figure 4  Distribution of reporter gene expression when expressed by different promoters in footpad skin. Vertical footpad skin sections were prepared 24 h following i.d. injection of plasmids expressing hMGFP under the control of Ubc (a, b), eIF4A1 (c, d) or EF1a (e, f) promoters and visualized by fluorescence microscopy as described in Figure 3. Left panels show bright field overlay. Scale bar is 10 μm. Nuclei are visualized with DAPI (blue).

Discussion

The inability to effectively deliver nucleic acids to skin remains a major bottleneck in the development of nucleic acid-based therapeutics to treat dermatological diseases.12 Plasmids and a large number of nucleic acid-based inhibitors, including short interfering and short hairpin RNAs (siRNAs and shRNAs), antisense oligonucleotides, morpholinos, aptamers, peptide nucleic acids, and ribozymes, are promising candidates for treating skin diseases if delivery issues are overcome that allow efficient delivery in sufficient quantities to appropriate target skin cells.43–46 Here, we have used two intravital imaging technologies, BLI and DAC fluorescence microscopy to monitor plasmid expression to skin following i.d. injection. Properties of plasmids, including large size and a negatively charged phosphodiester backbone, limit efficient topical delivery across the stratum corneum barrier resulting in poor cellular uptake. Other nucleic acids, including siRNA, antisense oligonucleotides and ribozymes, may have similar delivery characteristics.47

Intradermal injection, with or without other enhancing methodologies such as electroporation, appears to be the most commonly used and most effective method identified to date to introduce nucleic acids into keratinocytes of the skin.6,9,15,19,20,31 Previous studies using i.d. injection suggested that delivery of plasmid or siRNA across the stratum corneum barrier to the vicinity of the keratinocyte allowed uptake by unknown mechanisms.37,38,48 Following these examples, we recently showed in vivo gene silencing following i.d. injection of unmodified and unformulated siRNA into the footpad of a transgenic skin reporter mouse.35 Furthermore, in the recently reported pachyonychia congenita phase 1b clinical trial, very large volumes (up to two ml) were intradermally injected and high pressure may have been responsible for the observed effect on keratin gene expression.7 Although i.d. injection of nucleic acids is currently the most efficient method for skin delivery, its appeal in the clinic is greatly limited due to: (1) the pain associated with hypodermic injection (for example, i.d. injections during the pachyonychia congenita clinical trial required oral pain medication and nerve blocks to make this treatment bearable); and (2) the localized nature of the technique, which limits its application to small regions of skin.9–38 Widespread acceptance of nucleic acid-based therapeutics will likely require the development of more patient-friendly delivery technologies rather than reliance on hypodermic needles.

The results presented here suggest that the presence of nucleic acid in the epidermis is not enough to allow keratinocyte uptake and that pressure or some other
A small survey of transgenic mouse models that express reporter genes in the epidermis showed that promoters lacking CMV promoter elements (for example, Rosa26,56 Ubc) show a more uniform distribution throughout the epidermis, consistent with the results obtained with i.d. injection of non-CMV expression plasmids (Supplementary Figures 1c, d and g). However, in some transgenic mouse models expressing EGFP or LacZ reporter genes under the control of the CAG promoter, reporter distribution was observed throughout the epidermis (Supplementary Figures 1e, f, see also57). In contrast, expression under the control of the CMV promoter was unevenly distributed within the epidermis, with the highest accumulation in the upper epidermal layers (particularly the stratum corneum). These observations suggest that CMV elements may be causing differential gene expression during epidermal differentiation; however, other unknown factors may also be involved in this process. For example, Ghazizadeh et al.11 used an organotypic model to investigate reporter gene expression in skin.rafting of human skin equivalents, using keratinocytes labeled with EGFP (produced by lentiviral transduction), on immunocompromised mice resulted in a pattern of EGFP reporter expression that was restricted to the upper epidermal layers. In this system, the researchers showed that inclusion of the woodchuck hepatitis virus element downstream of GFP sequence, which facilitates nuclear export of intron-less transcripts, resulted in enhanced transgene expression in basal keratinocytes,11 arguing for a role of additional non-CMV elements.9

An alternative explanation for the observed uneven reporter expression (that is, accumulation in the granular layer) is differential plasmid uptake and expression in differentiating keratinocytes. Sawamura et al.9 observed that reporter gene expression following i.d. injection was confined to upper layers of the epidermis, whereas keratinocytes in the lower epidermis were able to take up rhodamine-tagged pDNA but eliminated it from their cytoplasm in less than 8 h. Sawamura proposed that the lower efficiency observed in trafficking of these plasmids to the nucleus in basal keratinocytes could be an explanation for the lower transfection efficiency and reporter expression observed in these cells.

The ability to image individual cells expressing reporter in intact skin of living animals allowed visualization of hMGFP reporter expression over time. As the transfected keratinocytes terminally differentiate, they were observed to move from the granular layer through the stratum corneum. Reporter expression in granular cells could be detected as early as 6 h following injection by fluorescence microscopy of skin sections. This early detection agrees well with published results by Hengge et al.,36 who showed expression of a reporter transgene in keratinocytes in the granulosum layer as early as 4 h following i.d. injection. Over time, the fluorescent cells could be seen in our study in the stratum corneum, suggesting migration of labeled cells. The transit time presented here is of approximately one cell layer per ~20–24 h. This roughly corresponds to previously published mouse footpad data showing that it takes approximately 6–7 days for a keratinocyte to transit from the basal layer to the stratum corneum,56 as well as the time required (~10 days) for a basal keratinocyte to terminally differentiate and be ‘sloughed off’.59
The observation that pressure facilitates uptake and expression of unmodified and unformulated nucleic acids suggests new delivery approaches are needed. For example, much emphasis has been placed on using methods that allow penetration through the stratum corneum barrier such as microneedles, topical formulations, iontophoresis and sonophoresis. These methodologies are mainly designed to allow placement of the nucleic acids within reach of keratinocytes and may be of little or no help in facilitating cellular uptake. Thus, additional consideration needs to be taken to find ways to enhance nucleic acid uptake by keratinocytes. The use of intravital imaging including the DAC microscope should facilitate the study of nucleic acid uptake and expression by allowing spatiotemporal analysis of a given set of cells and tissues in the same mouse over time. Therefore, the efficiency of different delivery methodologies (for example, electroporation, sonoporation, topical formulations alone or in combination), as well as nucleic acid conjugates (including peptide transduction domains or other enhancers that facilitate cellular uptake) may be more effectively screened using these intravital technologies.

Materials and methods

Animals

6–8 week old female FVB and CD1 (Charles River, Wilmington, MA, USA) mice were used according to the guidelines for Animal Care of both NIH and Stanford University. C57BL/6-Tg(CAG–EGFP)1Osb/J mice were purchased from Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME, USA). C57BL/6-Tg(UBC–GFP)30Sc/a/J and B6;129S-Gt(Rosa26) Sor/J transgenic animals (Jackson Laboratories) were kindly provided by Cory Nicholas, Stanford University.

Plasmids

The pGL3-CMV-Luc (Promega Corp, Madison, WI, USA) plasmid expresses the firefly luciferase under the control of the CMV promoter. The pCMV-hMGFP/CBL plasmid produces a fusion protein consisting of humanized Montastrea cavernosa (‘monster’) green fluorescence reporter protein followed by click beetle luciferase (CBL), under the control of the CMV promoter. The CMV promoter in pCMV-hMGFP/CBL was replaced with the following promoters: elf4A1, Ubc and EFIa (TD153, TD154 and TD151, respectively). All enzymes were purchased from New England Biolabs (Ipswich, MA, USA). The elf4A1 promoter (0.5 kb) from BamHI/ HindIII-digested elf4A1-blunt43 (provided by Jonathan Hardy and Michael Bachmann, Stanford University) was cloned into BgIII/HindIII-digested pCMV-hMGFP/CBL resulting in pTD153. The Ubc promoter was prepared by digestion of pTJ1-80 (provided by Thomas Kledal and Michael Bachmann) with Nhel followed by Klenow treatment to generate `blunt ends.' The linearized plasmid was then digested with HindIII generating a 1.6 kb fragment containing the Ubc promoter which was ligated into pCMV-hMGFP/CBL (previously digested with BgIII, and treated with DNA polymerase (Klenow) to generate `blunt ends,' and finally digested with HindIII) to create the final pTD154 construct. The EFIa promoter was PCR amplified from pHREFI1–GFP–WPRESIN18 (provided by Soosan Ghazizadeh 62,63) with forward primer-233 5'-GGATCCCTCGAGGGCTTCGCTGGCCCTGTAAGGCT-3' and reverse primer-232, 5'-AAGCTTTCACGACACCTGGAAG-3', introducing a 5'-BamHI and a 3' HindIII restriction site (underlined). The resulting 1.2 kb PCR fragment was cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA, USA), which was digested with BamHI and HindIII to release the 1.2 kb fragment containing the EFIa promoter. This fragment was ligated into BgIII/HindIII-digested pCMV-hMGFP/CBL resulting in pTD151.

Analysis of reporter expression

In vivo bioluminescence imaging. Fixed amounts (10 µg) of pGL3-CMV-Luc expression vector were intradermally injected into mouse footpads in increasing volumes (10, 25, 50, 75 and 100 µl) of PBS as previously described. At 24 and 48 h following i.d. injection, luciferin (100 µl of 30 mg/ml; ~150 mg/kg body weight) was injected into the peritoneal cavity of the mice and live, anesthetized mice were imaged after 10 min in the IVIS Spectrum Imaging System (Xenogen product from Caliper LifeSciences, Alameda, CA, USA) as previously described. The ratio (higher volume/lower volume) was calculated for each mouse and divided in three cohorts: 7 mice for the 50 µl/10 µl group, 12 mice for 75 µl/25 µl and 9 mice for 100 µl/50 µl. These data were analyzed statistically using a 2-tailed, paired t-test.

Skin sectioning. pCMV-hMGFP/CBL, pTD151, pTD153 and pTD154 (10 µg in 50 µl PBS) were intradermally injected into mouse footpads as described above. Mice were killed at various time points (6, 15, 24, 48, 72 and 144 h for analysis of pCMV-hMGFP/CBL expression) and at 24 h (for pTD plasmids) after i.d. injection. Skin tissues were removed from the footpad, embedded in OCT compound (Tissue-Tek, Torrance, CA, USA) and frozen in dry ice. Vertical cross sections (10 µm) were cut and mounted with Hydromount (National Diagnostic, Highland Park, NJ, USA) containing DAPI (1 µg/ml, Sigma, St Louis, MO, USA) for nuclear staining. Tissue sections were imaged as previously described.

Intravital DAC fluorescence imaging. Plasmids were injected intradermally at a total mass of 10 µg of pCMV-hMGFP/CBL in 50 µl PBS and imaged with a DAC microscope in living mice at the indicated times following injection. Intravital imaging was performed with the mouse under isoflurane anesthesia. Optical gel (NyoGel OC-431A-LVP, Nye Lubricants Inc., Fairhaven, MA, USA, index of refraction = 1.46) was used as a coupling agent between the footpad skin and the microscope. The footpads were scanned for hMGFP signal, and image stacks were acquired at sites expressing hMGFP. 3-D volumes were later reconstructed using Amira software (Visage Imaging, Carlsbad, CA, USA).
Conflict of interest
Roger Kaspar, Robyn Hickerson and Ryan Spitter are employees of TransDerm Inc. and Christopher Contag is a founder of Xenogen Corp. now part of Caliper LifeSciences.

Abbreviations
DAC, dual-axis confocal; CBL, click beetle luciferase; hMGFP, humanized monster green fluorescent protein; siRNA, small-interfering RNA; RNAi, RNA interference; Ubc, ubiquitin C; eIF4A1, eukaryotic translation initiation factor 4A, isoform 1; EF1a, elongation factor 1a; BLI, bioluminescence imaging; i.d., intradermal

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