

Sustained *in Vivo* Gene Delivery from Agarose Hydrogel Prolongs Nonviral Gene Expression in Skin

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ABSTRACT

Prolonging gene expression in skin using safe, nonviral gene delivery techniques could impact skin regeneration and wound healing, decrease infection, and potentially improve the success of tissue-engineered skin. To this end, an injectable, agarose-based delivery system was tested and shown to prolong nonviral gene expression in the skin. DNA was compacted with polylysine to improve DNA stability in the presence of nucleases. Up to 25 μg of compacted luciferase plasmid with or without agarose hydrogel was injected intradermally in rodents. Bioluminescence imaging was used for longitudinal, noninvasive monitoring of gene expression *in vivo* for 35 days. Injections of DNA in solution produced gene expression for only 5–7 days, whereas the sustained release of compacted DNA from the agarose system prolonged expression, with more than 500 pg (20% of day 1 levels) of luciferase per site for at least 35 days. Southern blotting confirmed that the agarose system extended DNA retention, with significant plasmid present through day 7, as compared with DNA in solution, which had detectable DNA only on day 1. Histology revealed that agarose invoked a wound-healing response through day 14. Tissue-engineering and wound-healing applications may benefit from the agarose gene delivery system.

INTRODUCTION

THE USE OF DRUG DELIVERY TECHNIQUES to influence the phenotypic expression of cells and promote tissue regeneration and healing has been the mainstay of tissue engineering. Using gene therapy techniques, to influence genotypic capabilities further expands the repertoire of tissue engineers in promoting tissue regeneration and healing, including skin regeneration and wound healing.

Some tissue-engineering approaches have combined gene therapy with scaffold materials, such as poly(lactic-co-glycolic acid) (PLGA) and collagen, to transfect cells *in vivo* and promote granulation tissue formation and neovascularization,¹ wound healing,² and bone regeneration.^{3–5} A system for the slow release of nonviral DNA may extend the duration of gene expression, particularly in applications in which gene expression is short-lived.

In the skin, DNA encoding growth factors could be used to aid in regeneration and wound healing.^{1,2} How-

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ever, existing nonviral gene delivery methods in the skin result in gene expression peaking within 24 h and falling below detection levels within 7 days.^{6–10} This time course of expression may not be clinically useful for tissue regeneration efforts.

Our laboratory and others have used agarose, a biocompatible hydrogel derived from red algae, for sustained release of proteins *in vivo*^{11,12} and have shown the sustained release of nonviral DNA *in vitro*.¹³ Agarose provided the sustained release of compacted, functional DNA for more than 50 days at 37°C.¹³ DNA compacted with a polylysine–polyethylene glycol (PEG)-based conjugate has been shown to be effective in transfecting cells *in vivo*,¹⁴ and compaction increases the resistance to nuclease degradation as compared with uncompact DNA.¹⁵ Thus, DNA compaction should help improve nuclease resistance during a prolonged period *in vivo*. The agarose system is advantageous because it does not require organic solvents or harsh physical forces that may alter or degrade the DNA during the process of incorporating the DNA into the hydrogel.^{16,17}

To evaluate the gene delivery system *in vivo*, the firefly (*Photinus pyralis*) luciferase reporter gene was utilized, because detection techniques are highly sensitive.¹⁸ Traditionally, luciferase activity is detected in tissue lysates with a luminometer. Bioluminescence imaging (BLI) is a technique used to monitor luciferase activity *in vivo* without killing animals at each time point.^{19,20} BLI utilizes a highly sensitive cooled charge-coupled device (CCD) camera to detect light production. BLI has been used successfully to monitor gene expression *in vivo*.^{21–25}

Our feasibility study described here demonstrates the ability of the agarose hydrogel delivery system to prolong gene expression *in vivo*, using compacted luciferase plasmid DNA and a rodent model of intradermal gene therapy. BLI was used to noninvasively monitor the time course of gene expression in a quantitative fashion. The agarose system resulted in prolonged gene expression with significant expression lasting at least 35 days, whereas DNA without the delivery system produced significant gene expression for only 7 days.

MATERIALS AND METHODS

DNA compaction

The plasmid pKCRlucSV5.9 (gift of Copernicus Therapeutics, Cleveland, OH), containing the luciferase gene under the control of the elongation factor 1 α promoter and the cytomegalovirus enhancer, was selected as the reporter plasmid. The compaction peptide (gift of Copernicus Therapeutics) consisted of an N-terminal cysteine residue and 30 lysine residues (CK₃₀) coupled to a

10-kDa PEG chain, resulting in CK₃₀PEG10k. Acetate was used as the negative counterion. DNA was compacted as previously described^{13,15} to a concentration greater than 4 mg/mL in 150 mM NaCl.

Agarose delivery system

SeaPlaque agarose hydrogel (Cambrex, East Rutherford, NJ), which melts at 65°C and gels at 26–30°C, was prepared at a concentration of 2% (w/v) in 150 mM NaCl and sterilized by passage through a 0.45- μ m pore size syringe filter. After cooling the solution to 37°C, 12.5 μ L of 2% SeaPlaque was mixed with 25 μ g of compacted DNA in 37.5 μ L of 150 mM NaCl. The mixture was gelled at room temperature in a 0.3-mL syringe.

Rodent model of skin gene therapy

Adult male Harlan Sprague Dawley rats (HSD, 7–8 weeks old; Harlan, Indianapolis, IN) were injected intradermally with compacted luciferase plasmid: 2.5 μ g in 150 mM NaCl, 25 μ g in 150 mM NaCl, and 25 μ g in agarose. Negative control injections included 150 mM NaCl and 0.5% SeaPlaque agarose without DNA. The animals were anesthetized with a rodent anesthesia cocktail (0.5–0.7 mL/kg body weight: ketamine-HCl [42.9 mg/mL], xylazine-HCl [8.57 mg/mL], and acepromazine [1.43 mg/mL]). After clipping the hair and cleansing the skin, the sample (50 μ L in a 0.3-mL syringe) was injected with a 28.5-gauge needle. Each of eight animals received up to eight randomized injections at separate, randomized locations on the dorsal skin at least 2 cm apart. Each injection type had a sample group size of at least 11. After the injections, the animals were returned to their cages and monitored until they awoke. All procedures were approved by and performed in compliance with the Institutional Animal Care and Use Committee at Case Western Reserve University (Cleveland, OH).

In vivo imaging

On days 1, 3, 5, 7, 14, 28, and 35 postinjection, each animal was imaged in the BLI system. The system (Roper Scientific, Trenton, NJ) consisted of a light-tight sample chamber with an adjustable stage, a liquid nitrogen-cooled CCD camera, a controller, and data acquisition software. The camera was equipped with a 50-mm f/0.7 lens (Nikon, Tokyo, Japan), and the camera temperature was maintained below –115°C to minimize electronic read (background) noise. The data acquisition software, Winview (Roper Scientific), allowed hardware control of the camera systems via the controller.

Each rodent was anesthetized with rodent anesthesia cocktail, shaved, and administered an intraperitoneal injection of D-luciferin (126 mg/kg body weight; Promega, Madison, WI).²⁶ The luciferin was prepared at a concentration of 50 mg/mL in phosphate-buffered saline

(PBS; GIBCO-BRL Invitrogen Life Technologies, Carlsbad, CA) and sterilized by passage through a 0.22- μm pore size syringe filter. Approximately 6 min after the luciferin injection, the animal was placed in the sample chamber. To further prevent light contamination, the chamber was covered externally with a photographer's dark cloth. A dim light source located within the chamber was utilized for photographic images. The stage height was adjusted to focus on the dorsal skin, and a photographic image was taken to locate the injection sites. The light was then switched off and a series of images was collected for 40–60 min using a 6-min integration time and 2×2 binning. The animal was removed from the chamber, monitored until it awoke, and returned to the animal facilities to be imaged at later time points.

After imaging on day 35, the animals were euthanized with carbon dioxide gas. A skin biopsy (8-mm punch) was removed from each injection site and fixed in formalin (10% phosphate buffered, Fisher Scientific, Pittsburgh, PA) for paraffin sectioning and histological analysis.

Image analysis

Bioluminescence images were analyzed with public domain ImageJ software (NIH, Bethesda, MD). Because the detected light peaked during the imaging session, the image with the maximum values was used for analysis. A region of interest (ROI) was traced around each site, and the area and mean intensity value were calculated. Four additional areas were selected on noninjected dorsal skin of each animal to serve as negative controls. To account for electronic readout noise from the camera, a large ROI outside the area occupied by the animal was used for background subtraction. Background subtraction was performed by taking the mean intensity value of the large background ROI and subtracting it from the mean intensity value of each injection and negative control site. After background subtraction, the corrected intensity values were multiplied by their respective areas to obtain the integrated (total) intensity, with units of gray levels, of each site. Each injection type group was compared with the negative control group from the same day to determine whether expression was significant.

Pseudocolor images were developed in MATLAB (The Mathworks, Natick, MA), and an overlay program was written to superimpose the pseudocolor bioluminescence images and the gray-scale photographic images. The pseudocolor relative intensity ranged from dark blue to red, with red being the most intense.

Data were normalized by two methods. First, the integrated intensity from each site was normalized to its day 1 value by expressing the data as a percentage of the day 1 levels. The data were then expressed as the percentage of sites with an integrated intensity significantly above that of the negative controls at each time point.

To determine whether gene expression at a particular site and time point was significant, the integrated intensity from that site was compared with the integrated intensity from negative control sites from the same animal and time point. This was done in two comparisons. First, because a series of images was collected at each time point, the series of integrated intensities from the site of interest was compared with the series of integrated intensities from the negative controls on the same animal, using the Student *t* test. A *p* value less than 0.05 indicated significant luciferase activity at the injection site. In addition, significant light activity resulted in a single peak in light output during the imaging session. Therefore, the second indicator of significant expression was a single peak in integrated intensity at an injection site. These two measures were used to determine whether the light produced at an individual injection site was significant.

Conversion of integrated intensity to protein mass

To convert the integrated intensity from the imaging system into protein mass, two curves were developed and combined. For the correlation between integrated intensity and luminometer relative light units (RLU), three rats were injected with 0, 1, 5, 10, 25, and 50 μg of compacted DNA in 150 mM NaCl. After 24 h, the rats were imaged on the BLI system, and biopsies were taken and frozen immediately in liquid nitrogen. The tissue samples were minced into small pieces with a cold razor blade and then crushed into a fine powder, using a liquid nitrogen-cooled mortar and pestle. The tissue powder was mixed with 800 μL of $1 \times$ cell culture lysis reagent (CCLR; Promega) and incubated on ice for 60 min with occasional vortexing. After two freeze–thaw cycles, the lysates were centrifuged ($12,000 \times g$, 5 min). The lysate supernatant was transferred to a new tube, and the final volume was measured. To measure the luciferase activity, 20 μL of lysate supernatant were mixed with 100 μL of luciferase assay reagent (LAR; Promega), and the resulting light was quantified on the luminometer (Tropix; Applied Biosystems, Foster City, CA) for 10 s. Total protein levels were measured using the DC protein assay (Bio-Rad, Hercules, CA). Total luminometer RLU produced at each site was calculated by multiplying the RLU (for 1 μL of tissue lysate) and the total volume of lysate collected.

To convert luminometer RLU to picograms of luciferase, recombinant luciferase (Promega) was used to develop a standard curve on the luminometer. Luciferase solutions (0.005–0.5 ng/mL) were made in $1 \times$ CCLR containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO). To measure the luciferase activity, 20 μL of each dilution was mixed with 100 μL of LAR, and light output was measured on the luminometer for 10 s.

Nine separate dilution sets were measured in triplicate and averaged. These two curves were combined to determine the relationship between the integrated intensity from BLI and the mass of luciferase produced.

Injections for blotting and histology

Nine HSD rats were injected intradermally with compacted DNA (2.5 μg , 25 μg , and 25 μg in agarose), 150 mM NaCl, or 0.5% SeaPlaque agarose. To locate the injection sites at later time points, 1% (v/v) sterile Pelikan Fount India ink was added to the samples before injection.²⁷ Each animal received at least one of each injection type. On days 1, 7, and 14 postinjection, three animals were euthanized. Half of the sites were removed as 8-mm punch biopsies and frozen in liquid nitrogen for Southern blotting. The remaining sites were removed and fixed in formalin for histological analysis.

Southern blotting

Frozen skin biopsies from days 1, 7, and 14 were ground into a fine powder as described earlier. Cellular DNA was isolated with a QIAamp DNA minikit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The DNA (2.5 μg of total DNA from each site) was cut with *Hind*III (Promega) at 37°C to linearize the plasmid and digest the genomic DNA. Negative controls contained 2.5 μg of genomic DNA from skin that was not injected with luciferase DNA. Positive controls contained plasmid DNA (0.5, 0.05, and 0.005 ng) with 2.5 μg of negative control genomic DNA.

Digested samples were electrophoresed, transferred to nylon membranes (Nytran SuPerCharge; Schleicher & Schnell BioScience, Keene, NH), and probed according to standard molecular biology protocols. The probe was a 1587-bp fragment cut from the luciferase plasmid with *Pvu*II and *Xba*I (Promega) and labeled with peroxidase, using an ECL direct labeling kit (Amersham Biosciences, Piscataway, NJ). The membranes were developed with chemiluminescent SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Rockford, IL). Images were captured on a Fluor-S MultiImager system (Bio-Rad).

Histology

To examine the inflammatory response to the injections, tissue samples from days 1, 7, and 14 were removed and fixed in formalin. Day 35 samples were taken from the imaged animals and fixed in formalin. Paraffin sections were cut and stained with hematoxylin and eosin (H&E) according to standard histological protocols. Using light microscopy, the extent of inflammation was evaluated on a scale of 0 to 5, with 5 indicating the most severe inflammation.

Statistical analysis

Before statistical analysis, the data were converted to log-scale values (log base 10) for a normal distribution to allow the use of parametric statistical tests. The time course of gene expression was analyzed by Student *t* test, with a *p* < 0.05 indicating a significant difference.

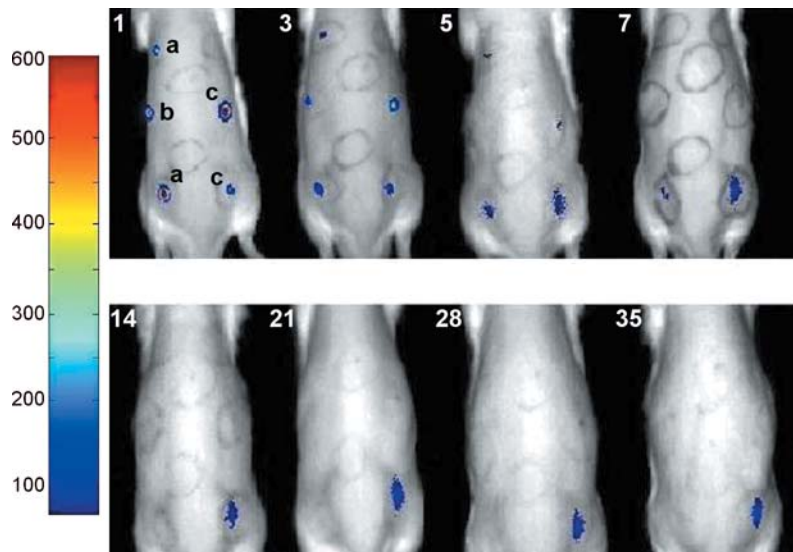


FIG. 1. Gene expression measured by BLI: Overlay of pseudocolor luminescence images and gray-scale photographic images showing the time course of gene expression in rodent skin (days 1, 3, 5, 7, 14, 21, 28, and 35). Injected samples included 2.5 μg of compacted DNA (a), 25 μg of compacted DNA (b), and 25 μg of compacted DNA in agarose (c). Images were integrated for 6 min, starting 6 min postinjection.

RESULTS

Duration of gene expression

The time course of gene expression was longitudinally monitored by BLI. Figure 1 shows a sample time course of gene expression in a typical rat. A total of eight animals were injected and imaged.

During each imaging session, luciferase activity peaked 15–30 min after the luciferin injection. The image with the peak values was used to quantify the integrated intensity from each site. The time course of gene expression for each injection type was plotted as the integrated BLI signal after background removal (Fig. 2).

Gene expression peaked on day 1, with similar luciferase activity levels for all three injection types on days 1 and 3. After day 5, DNA in agarose continued to produce sustained luciferase levels with significant expression lasting at least 35 days. Without agarose, gene expression from compacted DNA was transient, with significant expression lasting only 5 and 7 days for 25 and 2.5 μg of DNA, respectively.

When each site was normalized to its day 1 integrated intensity, the average activity from DNA in agarose remained over 20% of its day 1 value through day 35 (Fig. 3A). DNA in solution maintained levels of 2 and 0.5% of the day 1 values for 2.5 and 25 μg of DNA in solution, respectively. In the group treated with 25 μg of DNA in agarose, almost 60% of the injection sites retained significant gene expression for 35 days, whereas in the groups treated with 2.5 and 25 μg of DNA in solution, this percentage was much lower, especially after day 14 (Fig. 3B).

Luciferase standard curve

To convert the integrated intensity from the BLI system into units of protein, a calibration curve was devel-

oped to calculate the mass of luciferase produced at each site (Fig. 4). Using this relationship (Fig. 4A), the mass of luciferase produced at each site was estimated (Fig. 4B). On day 1, approximately 3500, 6600, and 7500 pg of luciferase were produced per site from 2.5 μg of DNA, 25 μg of DNA, and 25 μg of DNA in agarose, respectively. DNA in solution produced no significant protein during days 7–35. However, DNA in agarose maintained luciferase levels above 500 pg per site through day 35.

Southern blotting

Southern blotting was performed on tissue biopsies from days 1, 7, and 14 (Fig. 5). On day 1, significant plasmid levels were seen (lanes 5–7 in Fig. 5A) consisting of a band of intact, linearized plasmid at 5900 bp and smearing present throughout the lanes. Sites receiving 25 μg of DNA and 25 μg of DNA in agarose both had significantly greater amounts of plasmid than the 2.5- μg site. On day 7, only sites receiving 25 μg of DNA in agarose retained significant levels of intact plasmid along with low molecular weight fragments (lane 7, Fig. 5B). Sites injected with DNA in solution showed insignificant levels of DNA on day 7. On day 14, there were no differences among the sites, with no significant DNA present (Fig. 5C).

Inflammatory response

Evaluation of H&E-stained tissue sections revealed low levels of acute inflammation on day 1 for all injection types except saline solution without DNA. Sites injected with agarose, either with or without DNA, showed inflammation that persisted through day 14 but disappeared by day 35. This response was characterized by fibroblast infiltration and collagen synthesis typically found in wound healing. Table 1 provides the level of inflammation at each site.

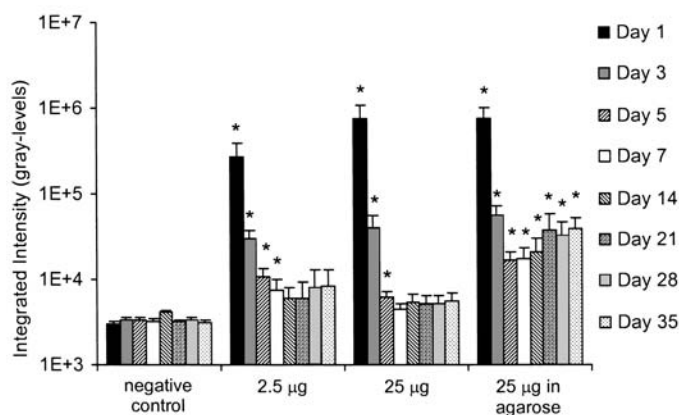


FIG. 2. Time course of luciferase activity for each injection type. Negative control represents rat skin that was not injected. Columns and error bars represent the average \pm SEM, with $n > 11$. * $p < 0.05$ compared with the negative control for the same day.

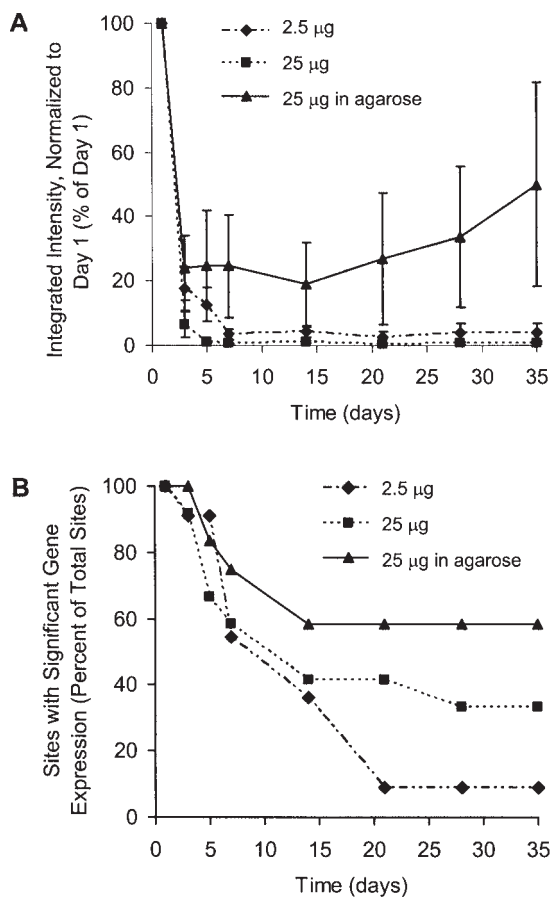


FIG. 3. Normalized gene expression. (A) Time course of gene expression with each site expressed as a percentage of its day 1 value. Data points and error bars represent the average \pm SEM. (B) Percentage of injection sites with significant luciferase activity.

DISCUSSION

An injectable agarose delivery system, compacted DNA, and BLI were combined and used to confirm the hypothesis that a sustained gene delivery system can prolong intradermal gene expression *in vivo*. Compacted plasmid DNA was used because of its enhanced nuclease resistance and its ability to transfect postmitotic cells,¹⁵ and BLI quantitatively demonstrated that gene expression was prolonged when using the agarose hydrogel compared with similar injections of DNA in solution without a delivery system. Southern blotting confirmed that retention of plasmid DNA at the injection site was increased by using agarose.

The model of skin gene therapy using a luciferase plasmid and BLI to monitor gene expression was ideal for testing the agarose delivery system *in vivo* and is appropriate for testing other delivery techniques. Luciferase produced from the transgene remained localized at each

injection site, so numerous samples could be tested on one animal without cross-contamination, reducing the number of animals needed. The light-emitting properties of the luciferase–luciferin reaction combined with the semitransparent properties of mammalian tissue allowed for the use of BLI as a noninvasive means of detecting gene expression *in vivo*. Because the injections were intradermal, signal reduction due to light absorption, which increases as a function of depth, was minimal, and scattering was the significant light interaction, allowing good measurement sensitivity.²⁸ Using BLI further reduced the number of animals necessary for this study by 87.5%, because each animal provided data for all eight time points. Overall, the combination of skin gene therapy, the luciferase gene, and BLI is advantageous for evaluating new gene delivery systems.

Using this model, the duration of gene expression from compacted DNA in solution and in agarose was evaluated. Gene expression extended beyond day 7 only with DNA in agarose. Although gene expression peaked on day 1, no significant changes were observed from day 5 to 35 for the agarose group, indicating a sustained, significant level of gene expression. DNA in solution, on the other hand, produced no significant expression after day 7.

Our data allow for the possibility that a small percentage of the compacted DNA injected intradermally resulted in persistent gene expression. Increasing the mass of DNA injected should increase the level of persistent expression, explaining only one persistent site for the low dose of DNA in solution and four persistent sites for the high dose of DNA in solution. Furthermore, the sustained release of DNA from agarose provided the cells with DNA for at least 1 week, according to the Southern blotting data. This continual exposure to DNA would provide additional opportunities for uptake and sustained expression, thereby explaining the results that seven sites injected with DNA in agarose had prolonged expression through day 35. Others have also found that nonviral transfection techniques can result in the stable transfection of cells, including keratinocytes.²⁹

Using the calibration curve, the mass of protein produced at each injection site was determined. DNA in agarose produced more than 500 pg of luciferase at each time point tested. This conversion to protein mass is useful when optimizing the delivery system for a particular application.

The calibration curve, which showed a high correlation between the BLI measurements and the mass of luciferase per site (Fig. 4A), was produced under several assumptions. First, the entire area of injected skin was assumed to be removed in each 8-mm biopsy. This was based on evaluating sites injected with India ink, in which the ink spread less than 8 mm in diameter. Because the ink particles are much smaller than compacted DNA, the

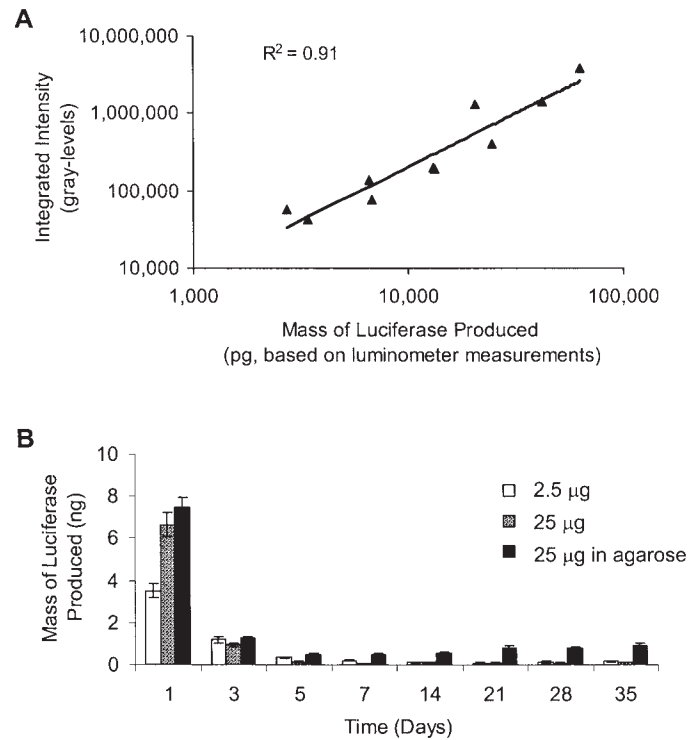


FIG. 4. Conversion of integrated intensity to mass of luciferase. **(A)** Relationship between integrated intensity from BLI and picograms of luciferase. The data points represent the experimental values, and the solid line is the best fit curve ($R^2 = 0.91$). **(B)** Mass of luciferase produced at each injection site. These values represent the conversion of the integrated intensity in Fig. 2 to an estimated mass of luciferase.

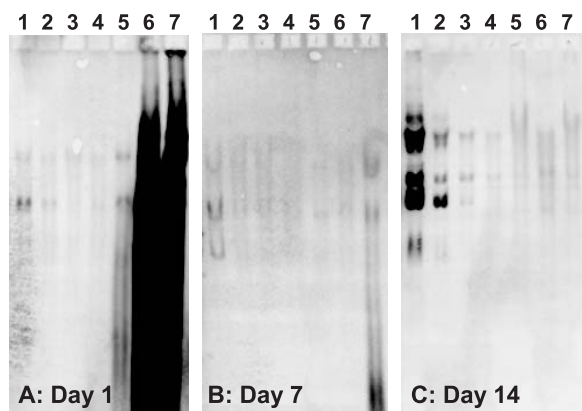


FIG. 5. Southern blotting of genomic DNA for days 1 **(A)**, 7 **(B)**, and 14 **(C)**. Positive controls contained 0.5 ng (lane 1), 0.05 ng (lane 2), and 0.005 ng (lane 3) of luciferase plasmid along with 2.5 µg of genomic DNA from noninjected skin. The negative control (lane 4) contained genomic DNA from noninjected skin without any plasmid. Genomic DNA samples (2.5 µg per lane) in the remaining lanes were from sites injected with the following: lane 5, 2.5 µg of compacted DNA; lane 6, 25 µg of compacted DNA; lane 7, 25 µg of compacted DNA in agarose.

ink should diffuse farther than the DNA. (None of the imaged animals were injected with India ink.)

It was assumed that injecting luciferin and imaging the animal 2–3 h before taking tissue samples for analysis in the luminometer would not significantly affect the luminometer RLU. This was tested and found to be a reasonable assumption (data not shown). Another assumption was that the injections were made at similar depths. All injections were performed by one person to minimize variability. Significant changes in injection depth would affect the BLI system measurements because of increased light scattering but should not affect the luminometer measurements. The high correlation between the two systems (Fig. 4A) indicated that the injection depths were not different enough to significantly affect the integrated intensity measurements.

Because each animal received up to eight injections, it was assumed that each injection site could be studied as a separate sample. To verify this, tissue samples were taken from noninjected skin found between and around the injection sites and tested for luciferase activity, using a luminometer. No significant luciferase activity was detected (data not shown), confirming that neither the in-

TABLE 1. EVALUATION OF INFLAMMATORY RESPONSE^a

<i>Injection</i>	<i>Day 1</i>	<i>Day 7</i>	<i>Day 14</i>	<i>Day 35</i>
Saline solution	0	+/-	0	0
Agarose	1	3	2	0
Compacted DNA				
2.5 μ g	1	0	1	0
25 μ g	1	2	0	0
Compacted DNA, 25 μ g in agarose	2	1	2	0

^aOn a scale of 0–5, with 5 being the most severe response.

jected DNA nor the resultant luciferase protein diffused through the skin and affected luciferase activity at other sites.

The final assumption was that the only significant light contributing to the bioluminescence measurements was from the luciferase–luciferin reaction. This was confirmed when no significant light was detected at sites injected with saline solution or with plain agarose without any DNA (data not shown). To rule out the presence of intrinsic bioluminescence, some animals were imaged before the luciferin injection and found to have no significant light production. Thus, all assumptions were validated.

Southern blotting confirmed that the gene delivery system prolonged the residency time of the DNA. Only agarose showed significant DNA present on day 7, although some of the DNA was degraded (Fig. 5B). It is likely that DNA retained within the agarose delivery system to be released at a later time point was degraded by nucleases present *in vivo*. Although agarose itself does not protect DNA from degradation *in vitro* (data not shown), compacting the DNA provides increased protection from nuclease degradation.^{13,15} Because the DNA was slowly released from the agarose, compaction should aid in stabilizing the DNA during its prolonged exposure to nucleases *in vivo*. To further enhance DNA stability and potentially extend the release of intact DNA, protective chemicals, such as EDTA,³⁰ could be incorporated into the delivery system.

Evaluation of the inflammatory response revealed a mild to moderate acute inflammatory response on day 1 to all injections. All injections evaluated for inflammation contained India ink, which may have contributed to mild inflammation. DNA in solution did not invoke any persistent reactions. However, agarose produced a wound-healing response through day 14. Agarose is known to be biocompatible, so the injection of agarose, in producing a physical separation within the dermis, may have wounded the tissue. If agarose were injected in another location, such as the subcutaneous space, wound healing might not be evident because the tissue disruption would be reduced.

The sustained levels of luciferase (>500 pg) resulting from injections of DNA in agarose should be sufficient for tissue-engineering approaches in the skin. Intradermal injections have been shown to transfect primarily keratinocytes in the epidermis,^{8,31–33} so monogenic skin diseases, such as junctional epidermolysis bullosa and lamellar ichthyosis, could be treated with the *LAMC2*³⁴ and *TGMI*³⁵ genes, respectively. The agarose system is versatile, and if protein production was insufficient, additional injections could be made or the mass of DNA injected could be increased.

Additional cells, including those within the dermis¹⁰ and the underlying smooth muscle cells,^{36,37} have also been transfected by intradermal injection. By targeting specific cells in the skin using chemical targeting ligands coupled to the DNA complex or a specific promoter, the gene expression profile could be further customized for a specific application.

Many tissue regeneration applications may benefit from prolonged gene expression, and the success of this delivery system in the skin may enable it to be used in other locations. Because the delivery system is injectable and nonrigid, it could be used by itself or included in a scaffold and injected into a variety of locations *in vivo* to promote the local production of a therapeutic protein.

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