

Lentiviral Transduction of Face and Limb Flaps: Implications for Immunomodulation of Vascularized Composite Allografts

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Background: Ex vivo introduction of an immunomodulatory transgene into a face or hand allograft may improve the risk-to-benefit ratio of vascularized composite allografts. Abrogation of the immunogenicity of the skin component of a face or hand allograft may decrease alloreactivity and permit the induction of immunologic tolerance. Proof-of-principle demonstrations of transduction of composite tissue have been established using adenoviral vectors, producing transient gene expression. The authors hypothesized that transduction, integration, and long-term expression of transgenes in a vascularized composite allograft could be achieved using lentiviral vectors.

Methods: Ex vivo transduction of heterogeneous primary rat cell lines representative of a composite tissue flap's cellular architecture was performed using a luc-enhanced green fluorescent protein (eGFP) human immunodeficiency virus-1–based lentiviral vector. Ex vivo injections of rat superficial inferior epigastric artery flaps with the viral vector were performed intraarterially, intramuscularly, and intradermally.

Results: Quantifiable reporter expression by flow cytometry (fluorescence-activated cell sorting) analysis and in vitro bioluminescence was observed. The luc-eGFP vector exhibited broad tropism and allowed transgene expression in relevant cell lines and throughout the flaps. Ex vivo intradermal transfection resulted in genomic integration and long-term constitutive gene expression (>150 days). Similarly, efficient intradermal transfection of face and hand flaps in a rat model corroborated this approach. Ex vivo intravascular perfusion of the vector proved inferior to intradermal injection.

Conclusions: Intradermal delivery of the transgenes proved superior to intravascular perfusion. Optimization of this gene-delivery approach may allow long-term, constitutive expression of immunomodulatory proteins in face and hand allografts. Future goals include replacement of the luciferase and eGFP reporter genes with key immunomodulatory proteins. (*Plast. Reconstr. Surg.* 129: 391, 2012.)

Increasing enthusiasm for vascular composite allografts has accompanied the recent successful hand and face transplants that have been

reported by a number of centers. As clinical experience in reconstructive transplantation accumulates and long-term successful outcomes become more commonplace, efforts to broaden applicability of this modality will increase. Most

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vascularized composite allograft surgeons would agree that progress with tolerance protocols and/or immunosuppression minimization is integral to the widespread application of vascularized composite allografts, and many centers are engaged in active research to this end.

Tolerance protocols involve the treatment of the allotransplant recipient such that the recipient will be immunologically tolerant of the allograft. Various approaches have been used and are reviewed elsewhere.

An alternative or perhaps adjunctive approach to tolerance induction or immunosuppression minimization in vascularized composite allografts involves immunologic manipulation of the allograft itself rather than of the recipient. This concept has evolved from the paradigm described by Gurtner and Levine that microvascular free flap tissue can be manipulated *ex vivo* by gene transduction, enabling the flap to generate proteins that treat infection, produce antitumor effects, or promote bone growth.¹

Most previous studies of *ex vivo* microvascular free flap transfection have used adenoviral vectors and produced only transient (≤ 3 weeks) gene expression and protein production—an attractive attribute in settings where short-term protein production would be desirable. Introducing one or more genes into a vascularized composite allograft to provide *long-term* expression of gene products using this *ex vivo* strategy represents an attractive opportunity for immunomodulation of a vascularized composite allograft recipient's immune response to the allograft. In this study, we investigated the *ex vivo* transduction of groin, face, and limb vascularized composite allografts using lentiviral vectors for genomic integration, efficient transduction, and long-term transgene expression.

MATERIALS AND METHODS

Production of Lentivirus

Plasmid pCCL-c-MNDU3c-LUC-muPGK-EGFP, referred to as self-inactivating human immunodeficiency virus in this publication, was provided as a gift by Donald Kohn, M.D. (University of California, Los Angeles, Los Angeles, Calif.), and encodes for a third-generation, self-inactivating lentiviral vector.² [See Document, Supplemental Digital Content 1, which describes the luciferase reporter gene, <http://links.lww.com/PRS/A450>.]

Ex Vivo Transduction of Heterogeneous Primary Cell Lines

Primary rat cell lines representative of a composite tissue flap cellular architecture were cultured according to vendor protocols (Cell Applications, Inc., San Diego, Calif.). Rat epidermal keratinocyte cells, rat epidermal keratinocyte precursor cells, Sprague-Dawley, rat dermal fibroblasts, rat aortic endothelial cells, rat mesenchymal stem cells, and human adipose-derived stem cells (no. R7788-110; Invitrogen, Carlsbad, Calif.) were transduced using the luc-enhanced green fluorescent protein (eGFP) human immunodeficiency virus-1–based lentiviral vector. Approximately 3 to 5×10^5 cells/well were plated 12 to 16 hours before transduction. The next day, cells were transduced at five different viral concentrations and incubated for 4 to 8 hours at 37°C and 5% carbon dioxide. After incubation, the medium containing the vector supernatant was removed and fresh medium was added. Luciferase expression was analyzed 48 hours after transduction.

Use of a Superficial Inferior Epigastric Artery Flap on the Rat Model

The study was approved by the Institutional Animal Care and Use Committee at the University of Southern California. Sprague-Dawley laboratory rats weighing approximately 300 to 500 g were used in our study. Food and water were provided to the animals *ad libitum*.

The superficial inferior epigastric artery flap, as described previously,³ was isolated and used as a transduction model for composite tissues. In brief, following intraperitoneal anesthesia, the flap (measuring 2.5×2.5 cm) was marked on the groin skin using a prefabricated template and elevated as an island flap based on the superficial inferior epigastric vessels. The flap was then treated according to the study group to which the animal had been assigned and the flap was sutured back in place using 4-0 silk sutures.

Intraarterial, Intramuscular, and Intradermal Transduction of Superficial Inferior Epigastric Artery Flaps with Self-Inactivating Human Immunodeficiency Virus-1–Based Lentiviral Vectors Expressing luc

Sprague-Dawley rats were used for our experiments ($n = 21$). Three experimental groups were generated to obtain flap transduction. In group 1 ($n = 7$), superficial inferior epigastric artery flaps were injected intraarterially; in

group 2 ($n = 2$), superficial inferior epigastric artery flaps were injected intramuscularly; in group 3 ($n = 12$), the superficial inferior epigastric artery flaps were injected intradermally. The intravascular lentiviral injection was performed as follows. After clamping of the superficial inferior epigastric vein, an arteriotomy and a venotomy were produced using microsurgical scissors. A 30-gauge tubing segment was inserted in the lumen of the superficial inferior epigastric artery and anchored to the vessel using 8-0 silk ties. Residual blood in the flap's vasculature was removed by flushing with heparinized solution. The vein was clamped proximally to the venotomy and the lentiviral vector was injected intraarterially and allowed to dwell for 1, 2, and 4 hours. Care was used to avoid lentiviral vector spill in the surrounding tissues during the procedure. At the end of the dwell time, the venotomy and arteriotomy were repaired using 8-0 Ethilon (Ethicon, Inc., Somerville, N.J.), the clamps were removed, and circulation to the flap was restored.

In group 2, the flaps were injected intramuscularly with the same lentiviral vector. The gracilis muscle was included in the flap design and the muscle was injected following flap dissection and vascular pedicle isolation. In group 3, transduction of the flaps was achieved by lentiviral vector injection intradermally. A negative control was included in each animal and consisted of contralateral phosphate-buffered saline injections through the same administration route used for the lentiviral injection.

Luciferase Expression Measurement In Vitro

The luciferase substrate (D-luciferin) was added to the cell culture media in the six-well plates and imaged at 1.5 minutes after injection. Scans were acquired using the Living Image 3.2 software (Caliper Life Sciences, Alameda, Calif.), and with the following settings: 60-second bioluminescent acquisition; Bin, medium; field of view, 26.1; f-stop, 1. All images were analyzed using the Living Image Software to measured bioluminescent signal in photons per second per square centimeter per steradian and fluorescent signal in photons per second.

Luciferase Expression Measurement In Vivo

Optical imaging was performed at the University of Southern California Molecular Imaging Center using the IVIS 200 (Xenogen Corp., Alameda, Calif.) in vivo imaging system. All animals were anesthetized using 2% isoflurane in oxygen

throughout the duration of the scan. Animals were placed into induction boxes with anesthetic gas before administration of the luciferase substrate, D-luciferin. The luciferase substrate was administered intravenously into rats at 50 mg/kg and imaged at 1.5 minutes after injection. Scans were acquired and analyzed using the Living Image 3.2 software, and bioluminescent signal was measured in photons per second per square centimeter per steradian.

In addition, pseudo-three-dimensional images were created using the diffuse light imaging tomography software from Caliper Life Sciences. Images were acquired using a sequence of six 1-minute scans during the plateau of signal intensity (12 to 18 minutes after injection).

Bioluminescence was measured at 48 hours after intraarterial, intramuscular, and intradermal injection of self-inactivating human immunodeficiency virus. Rats displaying signs of successful *luc* gene transduction were followed up on days 7, 15, 30 and monthly thereafter up to the study endpoint (day 150).

Isolation of DNA and Polymerase Chain Reaction Analysis

The *GFP* gene was detected by polymerase chain reaction. Rat genomic DNA was extracted from a full-thickness biopsy specimen of the skin flap at the specified time points using the Qiagen DNEasy Blood & Tissue extraction kit (Qiagen, Inc., Valencia, Calif.). Polymerase chain reaction primers were designed by importing the GFP coding sequence from GenBank into the primer design program Primer3.⁴ The 550-base pair GFP gene product was amplified by a standard polymerase chain reaction protocol. Briefly, the polymerase chain reaction mix was heated to 94°C for 2 minutes to denature the DNA; primers were annealed at 60°C and extended at 72°C for 30 seconds. This was repeated for 30 cycles. The products were analyzed by 1% agarose gel electrophoresis.

Immunohistochemistry

The GFP expression was detected by immunostaining. The rat skin tissue biopsy specimen was fixed in 4% paraformaldehyde overnight at 4°C. The biopsy specimen was then washed in phosphate-buffered saline and dehydrated in an ethanol gradient series. The biopsy specimen was then cleared in xylene and embedded in paraffin, and 7- μ m sections were cut. The paraffin sections were subjected to antigen retrieval in

10 mM citrate buffer for 30 minutes. Standard immunohistochemistry technique was used to detect GFP. Briefly, GFP primary antibody (ab290; Abcam, Cambridge, Mass.) was incubated overnight at 4°C. Biotin-linked antirabbit secondary antibody was incubated for 1 hour at room temperature. Streptavidin–horseradish peroxidase tertiary antibody was incubated for 30 minutes at room temperature. The AEC detection kit (Vector Laboratories, Burlingame, Calif.) was used to develop color that detected GFP expression patterns.

RESULTS

Lentiviral Vector Allows Expression of Luciferase and eGFP In Vitro

Heterogeneous primary rat cell lines, such as rat epidermal keratinocyte cells, rat epidermal keratinocyte precursor cells, Sprague-Dawley, rat dermal fibroblasts, rat aortic endothelial cells, and rat mesenchymal stem cells were successfully transduced using the luciferase-eGFP human immunodeficiency virus-1–based lentiviral vector. Stable transgene expression of all cell types normally present in the flap architecture was observed by bioluminescence following transduction with our lentiviral vector in vitro for the entire duration of the cell culture (approximately 2 weeks). High-level transduction of human adipose-derived mesenchymal stem cells was also obtained and represents important preliminary data supporting the future applications of this technique (Fig. 1).

Long-Term Expression of Transgenes In Vivo When Lentiviral Vector Is Injected Intradermally

Animals in group 1 (intraarterial administration of lentiviral vectors) and group 2 (intramuscular administration of lentiviral vector) did not display eGFP or luciferase expression at any time point regardless of the location of the area injected. Furthermore, optical imaging for luc assessed at 48 hours, 72 hours, and 7 days did not show an increase in gene expression. We hypothesize that the lack of signal depends on the tropism of the pseudotyped vector for endothelial cells, which would limit transduction of extravascular tissues in the flap. This phenomenon would explain the finding in histologic sections of eGFP-positive vasculature in the flaps that were injected intravascularly. Conversely, animals in group 3 (intradermal administration of lentiviral vector) expressed the transgene products on the superficial inferior epigastric artery flaps for over 150 days after a single intradermal injection with luc-eGFP-lentiviral vector (Table 1). No attenuation of the photon emission was observed in any experimental animals, and in two animals some increase in gene expression intensity was noted. The contralateral internal control flaps injected with phosphate-buffered saline showed no signal throughout the entire duration of the study in all animals. Furthermore, standard immunohistochemistry techniques showed the presence of eGFP throughout the flap when the lentiviral vector was injected intradermally (Fig. 2) but not when injected in-

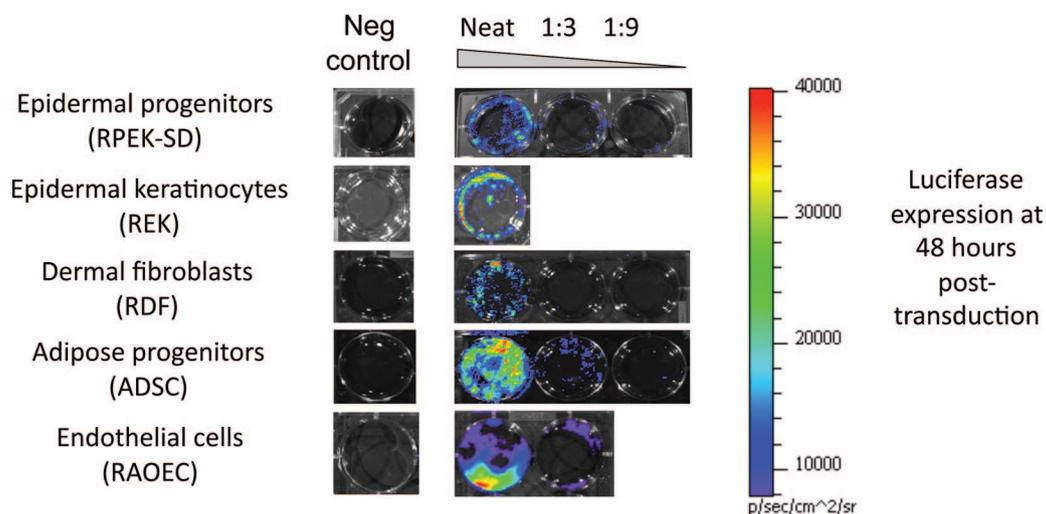


Fig. 1. Primary rat cell lines representative of a composite tissue flap cellular architecture were transduced at different vector concentrations. The adipose progenitors displayed in this figure are human adipose-derived stem cells. Luciferase expression was analyzed 48 hours after transduction and was present in all cell types.

Table 1. Experimental Groups

Group	No.	Self-Inactivating HIV Administration Route	Flap Used	Luc Expression (days)
1	7	Intraarterial	a) SIEA flap b) Hemiface c) Lower limb	a) No b) No c) No
2	2	Intramuscular	SIEA island flap (gracilis flap)	No
3	12	Intradermal	a) SIEA island flap b) Hemiface c) Lower limb	a) 150 ($n = 4$), 120 ($n = 2$), 60 ($n = 2$) b) 60 ($n = 2$) c) 60 ($n = 2$)

HIV, human immunodeficiency virus; SIEA, superficial inferior epigastric artery.

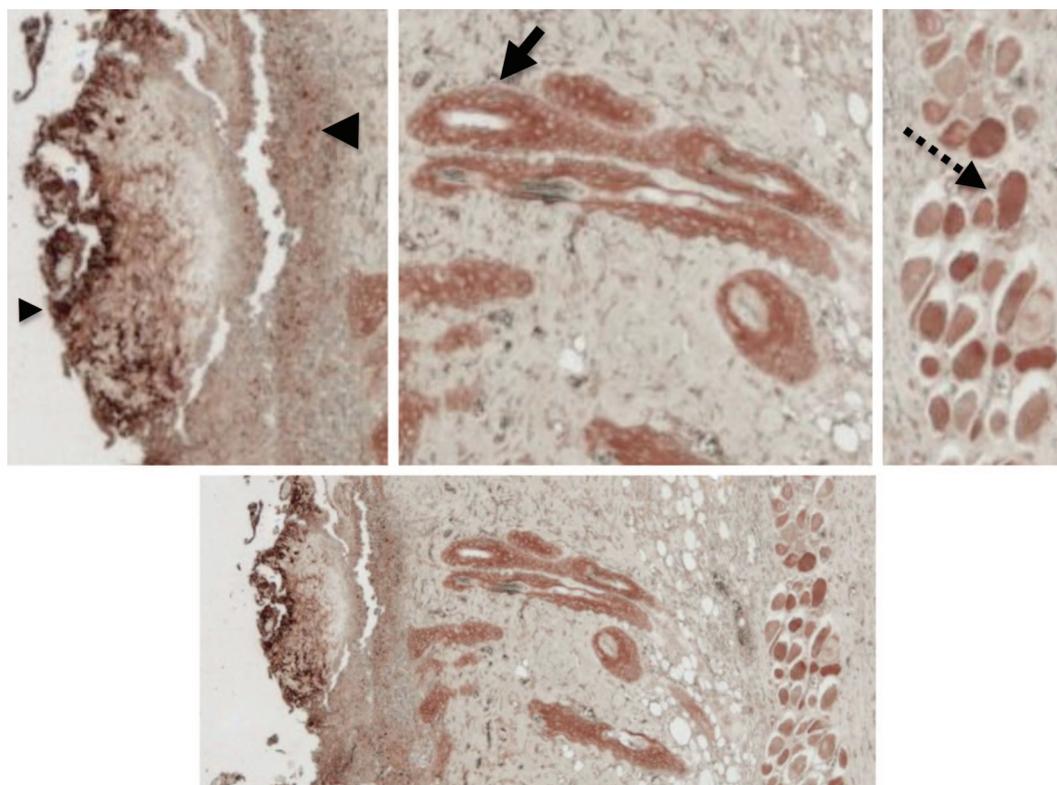


Fig. 2. eGFP staining of superficial inferior epigastric artery flap injected with lentivirus vector intradermally (hematoxylin and eosin stain) (*below*). Expression of eGFP is exhibited in epidermis (*small arrowhead*), dermis (*large arrowhead*) (*above, left*), adnexa (hair follicle, *large arrow*) (*above, center*), and muscle (*dashed arrow*) (*above, right*).

traarterially or intravenously (data not shown). Incorporation of the *eGFP* gene in the host genome was confirmed by polymerase chain reaction (Fig. 3).

Transgene Expression Is Limited to Flap Architecture

Optical imaging analysis of successfully transduced superficial inferior epigastric artery flaps (animals in group 3) proved stable and long-term transgene expression of luciferase (Fig. 4). The flaps displayed widespread and reproducible bioluminescence activity throughout the entire sur-

face area of the flap, independent of the vascular composite allografts performed (Fig. 5). No luciferase expression was observed in the areas adjacent to and underneath the flap.

In vivo pseudo-three-dimensional animated reconstruction generated using diffuse light imaging tomography technology allowed us to localize the source of the photon emission as the dermis and to assess the photon emission in layers deeper than the dermis, by measurement of the exact width and thickness of luciferase expression. (See Video, Supplemental Digital Content 2, which is a video demonstrating in vivo pseudo-

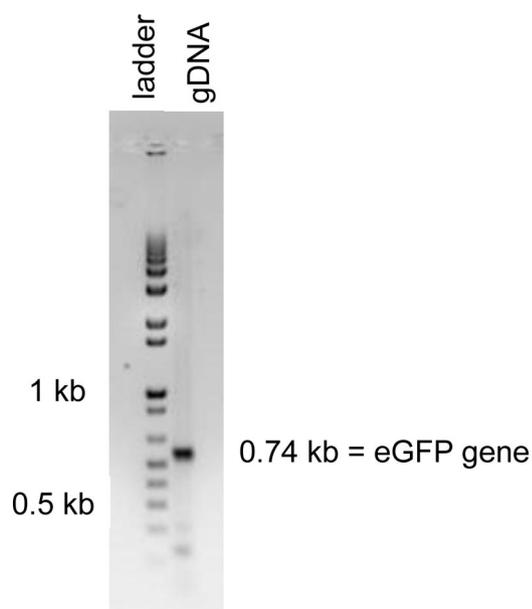


Fig. 3. Polymerase chain reaction analysis demonstrated integrated copies of the eGFP reporter gene at 2 weeks after intradermal injection. Genomic DNA was extracted from a biopsy specimen containing skin and subcutaneous tissue from the intradermal injection site.

three-dimensional animated reconstruction generated using diffuse light imaging tomography, <http://links.lww.com/PRS/A451>.) Noticeably, luciferase expression was confined to the flap architecture, and was measured in all planes (coronal, sagittal, and axial). No signal was reported outside of the flap (Fig. 6). All flaps injected intradermally, independent of their anatomical location, displayed comparable gene expression, which lasted long-term up to 150 days.

DISCUSSION

Gene therapy has received negative attention because of highly publicized reports of toxicity in clinical trials. Lentiviral vectors in particular have come under significant scrutiny. However, numerous human trials using lentiviral vectors are currently underway, and many have recently produced clinically safe and effective results in various clinical settings.

The use of lentiviral vector allows packaging of relatively large cDNAs that can transduce several different tissue types, including skin, hepatic, muscle, and neural tissues.⁵⁻⁷ In addition, the generation of self-inactivating vectors minimizes risks and allows safe transduction of both dividing and nondividing cells. The new generation of vectors allows the coexpression of two genes in the same transcriptional unit (bicistronic vectors) or three

genes in independent transcriptional units, allowing simultaneous long-term expression of multiple genes.⁸ The possibility of inducing or suppressing gene expression by using tetracycline-inducible lentiviral vectors allows fine regulation of transgene expression when required and supports the versatility of their use.

The use of lentiviral vectors to achieve immunomodulation has been applied successfully in other transplant models in which treatment of the *recipient* cells, rather than the allograft itself, was used. In these studies, the lymphohematopoietic system of the recipient was essentially made fully chimeric, with all lymphohematopoietic cells expressing the class I and class II major histocompatibility complex antigens of the *donor*, thereby permitting tolerance of a renal allograft from a donor with the same class I and class II genes expressed.⁹⁻¹¹

Models in which full hematopoietic chimerism is achieved essentially create the same immunomodulated system through hematopoietic stem cell engraftment following ablation of the *recipient's* lymphohematopoietic system (bone marrow ablation by chemotherapy or irradiation). In this setting, donor allografts are tolerated, as they share major histocompatibility complex antigens with the *donor* lymphohematopoietic system present because of the state of full chimerism.¹²

Unfortunately, the converse of this approach—engineering the expression of recipient class I and class II genes on donor cells within the allograft—is unlikely to produce similar tolerance induction, as alloreactive T-cell clones would continue to proliferate against the *nonself* major histocompatibility complex antigens present on the allograft despite the concomitant presence of *self* major histocompatibility complex antigens. This hypothesis is yet unproven, however, as there is some evidence that a large enough T-regulatory cell pool may be generated in the recipient by recipient class I and class II expression on the donor allograft to suppress alloreactivity. Immunomodulatory proteins may provide an attractive alternative to transduction with class I and class II genes (a number of attractive candidate proteins are listed in Table 2).

Some limitations in lentiviral vectors are inherent in the type of virus itself used. We found that the lentiviral vectors did not migrate through the endothelium and that transduction was limited to the vascular endothelium. This property may at first appear disadvantageous but in reality

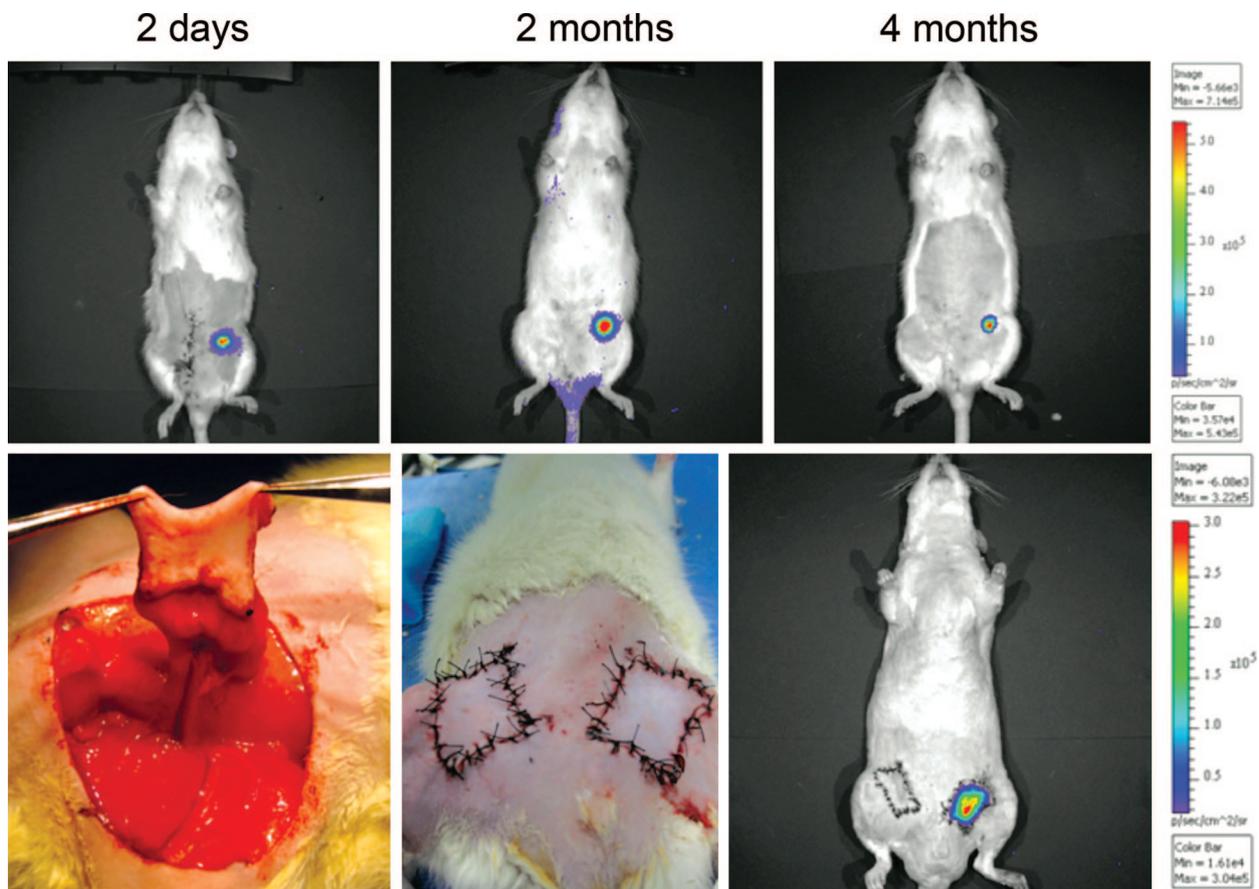


Fig. 4. Intradermal groin injections and superficial inferior epigastric artery flap as a preliminary transduction model for composite tissue grafts. Intradermal injections in the groin of the first animal served as a proof of concept. Photographs at 48 hours, 2 months, and 4 months are shown here (*above*). To translate these results to surgical flaps, the superficial inferior epigastric artery flap was chosen (*below*), as it may include tissues of several types (epidermal, dermal, connective, adipose, muscular, neural, and endothelial). (*Left*) Two flaps, measuring 2.5×2.5 cm, were marked on the groin skin and elevated as an island flap based on the superficial inferior epigastric vessels. (*Center*) At the end of the procedure, the flap was sutured back in place. In the animals from group 3, transduction of one flap was achieved by intradermal injection of lentiviral vector. Contralateral intradermal injections with phosphate-buffered saline served as controls in all experimental animals. (*Right*) Luciferase expression was assessed at 48 hours after transduction following intravenous injection of D-luciferin. Rats displaying signs of successful *luc* gene transduction were followed up on days 7, 15, and 30 and monthly thereafter up to the study endpoint (day 150).

may be advantageous in the transplantation setting, as the endothelium is the initial site of antigen-antibody interaction.

Initially, intravascular perfusion was attempted to simulate a scenario in which a flap could be injected on the back table to achieve homogeneous transduction of all cell types of the vascular composite allograft. However, intradermal delivery of the transgenes proved superior to intravascular perfusion, probably because of the capacity of the lentiviral vector to efficiently transduce the vessel endothelial cells and therefore limit the number of viral particles reaching the flap parenchyma. Nevertheless, intradermal injections were easy to administer in this experimental rat model and

allowed widespread, consistent, and long-term transduction of the entire flap in all treated animals. Direct dermal injection of a vector into a face or limb allotransplant may appear cumbersome as a clinical approach; however, reconstructive surgeons commonly use injection techniques with local anesthesia for hydrodissection or even tumescence without problematic sequelae. Indeed, this intradermal approach has proven very successful in vivo in clinical studies of the treatment of epidermolysis bullosa with intradermal lentiviral vector injection. When lentiviral vectors were injected intradermally, dermal fibroblasts were shown to be the primary targets of the vector.^{13,14}

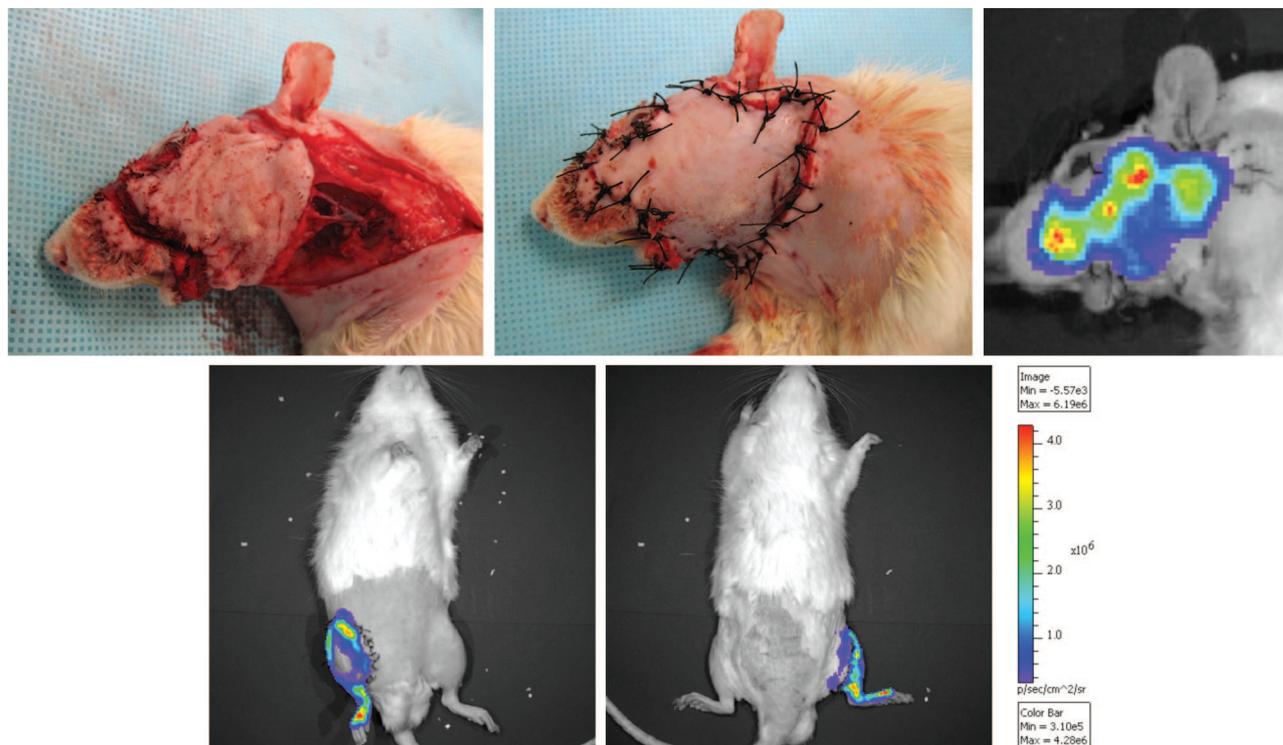
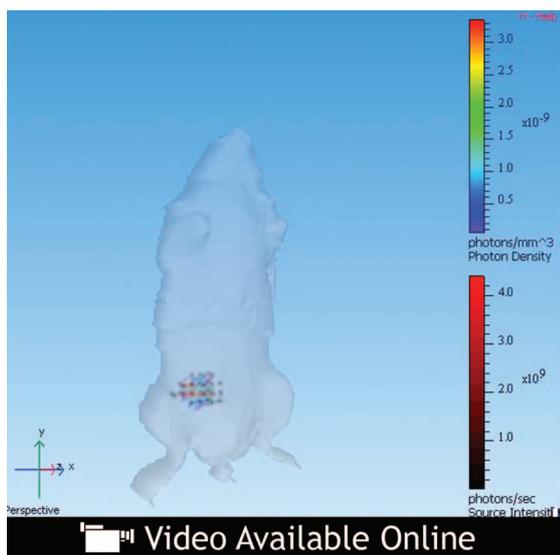


Fig. 5. Transgene expression was achieved following intradermal injection in different flaps. Hemifacial flaps (*above*) and limbs (*below, left and right*) were isolated and injected with lentiviral vector. Long-term luciferase expression was visible starting 48 hours after transduction.



Video. Supplemental Digital Content 2 demonstrates in vivo pseudo-three-dimensional animated reconstruction generated using diffuse light imaging tomography, <http://links.lww.com/PRS/A451>.

To evaluate the ability of lentiviral vectors to produce stable gene expression in the epidermis, Ghazizadeh et al. demonstrated that lentiviral vectors are highly efficient in gene transfer and in-

tegration in epidermal progenitor cells in vitro and in vivo.^{15,16} Several studies indicate that among different gene transduction techniques, the lentiviral vector–based system achieves the highest transduction levels of skin cells (>90 percent) after a single injection.^{16–18}

In our study, a self-inactivating human immunodeficiency virus was used. The use of self-inactivating long-terminal repeats and the removal of accessory genes such as *tat*, *nef*, *vpu*, *vpr*, and *vif* minimizes the risk of reconstituting replication-competent viral particles.¹⁹ Therefore, this vector can be considered relatively safe. Pseudotyping of the vector using the vesicular stomatitis virus glycoprotein broadens viral tropism beyond human cells expressing CD4, allowing transduction of several cell types.

The lack of reported signal beyond the flap architecture provides encouraging preliminary data regarding the procedure's safety and possible future clinical applicability. However, more tests are needed to assess the rate and extent of insertional mutagenesis and of the infectivity of the vector before the transition to clinical application. The lentiviral vectors used in this study allowed luciferase expression for over 150 days. No de-

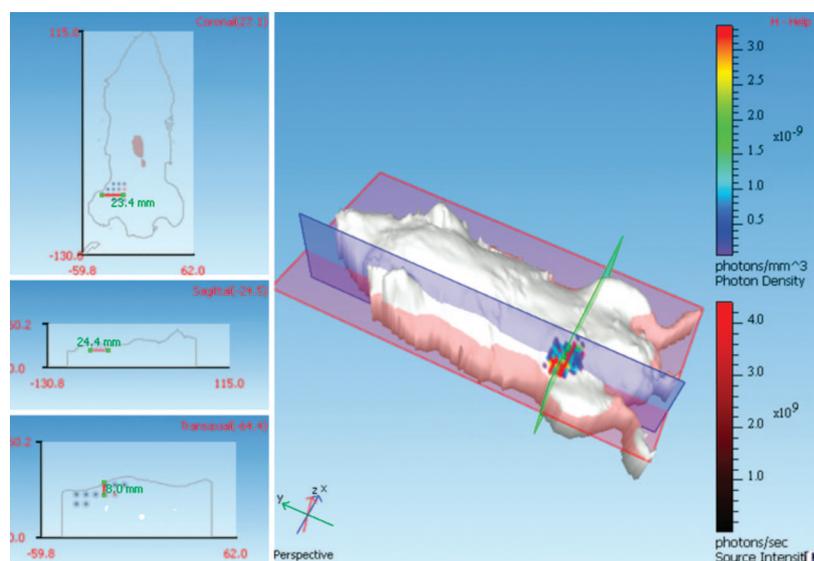


Fig. 6. Three-dimensional image reconstruction of experimental rat. Diffuse intensity light tomography was used to obtain three-dimensional images of the bioluminescence emitted 48 hours after lentiviral vector injection. The bioluminescent signal was measured in photons per second per square centimeter per steradian. Noticeably, luciferase expression was confined to the transduced area within the flap, as measured in all planes (coronal, sagittal, and axial). No signal was reported outside of the flap.

Table 2. Candidate Immunomodulatory Genes for Future Applications

CTLA4 Ig
CD40 Ig
MHC class I
MHC class II
siRNA against MHC I/II

Ig, immunoglobulin; MHC, major histocompatibility complex.

crease in bioluminescence was noted at the time the animals were euthanized (study endpoint).

The adenoviral model described in superficial inferior epigastric artery flaps in rats by Agrawal et al.²⁰ has several advantages, as follows: it has a high transduction efficiency when administered intraarterially; transduction is limited to the injected flap without systemic effects; and the short-term transgene expression is ideal in clinical scenarios where permanent gene product is not needed or even harmful.²¹ To make this model clinically relevant for transplant surgery and reconstructive transplantation with vascular composite allografts, however, long-term immunomodulatory gene expression would be required, allowing localized and permanent transgene product in the allograft, eliminating or reducing the need for chronic immunosuppression or facilitating tolerance induction.

CONCLUSIONS

In this proof-of-principle experiment, we demonstrate that the introduction of a transgene into the cutaneous component of a superficial inferior epigastric artery flap face or limb allograft in the rat can be achieved using a human immunodeficiency virus-1–based lentiviral vector, leading to long-term transgene expression. We hypothesize that in the future, by introduction of immunomodulatory gene constructs in a lentiviral vector, this technique may be used to improve the risk-to-benefit ratio in vascular composite allografts by abrogation of the immunogenicity of the skin component of a face or hand allograft, thereby decreasing the risk of rejection and/or permitting the induction of immunologic tolerance. Optimization of this gene-delivery approach may allow long-term, constitutive expression of immunomodulatory proteins in face and hand allografts. Future goals include replacement of the luciferase and eGFP reporter genes with key immunomodulatory proteins.

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