Human Involucrin Promoter Mediates Repression-Resistant and Compartment-Specific LEKTI Expression

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Abstract

Gene-modified skin grafts, produced through gene transfer to human keratinocyte stem cells, offer the possibility of therapeutic benefit for inherited skin diseases. We have previously described efficient lentiviral vector–mediated gene transfer to keratinocyte stem cells and the generation of human skin grafts for the inherited skin disease, Netherton syndrome, which arises due to mutations in *serine protease inhibitor Kazal-type 5* (*SPINK5*). Vectors incorporating an internal murine retroviral–derived promoter [spleen focus-forming virus (SFFV)] in combination with a codon-optimized *SPINK5* transgene supported high levels of reconstitution and robust correction of skin architecture. Subsequent longer-term experiments have uncovered unanticipated silencing phenomena, with loss of *SPINK5* gene expression over time. The inadvertent introduction of CpG sites during codon optimization appears to have rendered vectors susceptible to silencing due to methylation across the promoter–transgene boundary. Substitution of the methylation-susceptible SFFV promoter with a 572-bp minimal human involucrin promoter (INVOp), which encodes very few CpG sites, prevented repression of the *SPINK5* transgene and resulted in durable and highly compartment-specific reconstitution of lympho-epithelial Kazal-type–related inhibitor (LEKTI) in human skin grafted onto immunodeficient mice. We conclude that skin grafts modified with lentiviral vectors encoding INVOp offer a suitable platform for therapeutic gene therapy in Netherton syndrome, and our experience highlights unanticipated effects of transgene codon optimization.

Introduction

NETHERTON SYNDROME (NS) is a life-threatening and debilitating inherited skin disorder caused by defective expression of lympho-epithelial Kazal-type–related inhibitor (LEKTI), a serine protease inhibitor encoded by the *SPINK5* (*serine protease inhibitor Kazal-type 5*) gene (Chavanas *et al.*, 2000; Bitoun *et al.*, 2002, 2003). LEKTI is expressed in the outer compartment of the epidermis and plays a critical role in the regulation of kallikreins 5 and 7, which hydrolyze the extracellular corneodesmosomes in the skin (Descargues *et al.*, 2005, 2006; Ishida-Yamamoto *et al.*, 2005). When control of kallikreins by LEKTI is lost, hyperactivated kallikreins can cause premature degradation of corneodesmosomes, resulting in overdesquamation and loss of skin-barrier function. LEKTI also inhibits elastase 2, an epidermal protease

involved in profilaggrin processing and lipid lamellae structure formation of the skin, by controlling kallikrein 5–mediated cleavage of proelastase 2 (Bonnart *et al.*, 2010). In the absence of curative therapies, we are developing *ex vivo* gene-therapy strategies based on lentiviral mediated gene correction of autologous keratinocyte stem cells for the generation of graftable bioengineered skin (Di *et al.*, 2011). We have previously reported that lentiviral mediated gene expression by a spleen focus-forming virus (SFFV) internal promoter of codon-optimized *SPINK5* (SPINK5co) supported sufficient LEKTI expression for architectural correction of NS skin in a skin-humanized mouse model. However, in contrast to normal LEKTI expression, transgene expression was not compartment-restricted within the epidermis, but was detectable throughout the basal and suprabasal layers. Subsequently, longer-term cultures have uncovered

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unanticipated silencing phenomena, which we now demonstrate are associated with methylation of CpG sites within the distal SFFV enhancer region and spreading across the transcription start site boundary within the SPINK5co transgene. We postulate that the process of codon optimization of the *SPINK5* transgene, intended to improve gene expression, resulted in the inadvertent introduction of a high number of CpG sites, which then rendered the SFFV-SPINK5co configuration susceptible to methylation-mediated silencing. We show that this phenomenon can be addressed by substitution of SFFV with a 572-bp human involucrin promoter (INVOp) element (Ghazizadeh *et al.*, 2002), which has few CpG, mediates highly compartment-specific *SPINK5* expression, and is not prone to repressive methylation.

Materials and Methods

Vector generation and keratinocyte transduction

SPINK5co was synthesized by GeneArt (Regensburg, Germany) and was cloned into a previously described replication-deficient self-inactivating (SIN) HIV-1 lentiviral vector. The vector encoded the HIV-1 central polypurine tract (cPPT), start site–mutated woodchuck postregulatory element (WPRE), and SFFV promoter (Demaison *et al.*, 2002) (Fig. 1). To generate INVOp-containing vectors, a 572-bp fragment of involucrin enhancer was cloned from the 2,500-bp full-length human INVOp (locus AF085346), using the method described by Ghazizadeh *et al.* (2002). Two DNA fragments containing a distal element (–2,473/–2,036) and a proximal region (–242/–1) of the INVOp were amplified from genomic DNA by PCR and cloned into pGEM-T vector (Promega, Southampton, UK). The two amplified fragments were ligated using restriction sites *Bgl*II (–2,102) and *Msc*I (–194), and the resulting 572-bp INVOp fragment was verified by sequencing. INVOp was then cloned in place of SFFV to generate INVOp-SP/GFP and INVOp-GFP. Vector stocks were pseudotyped with vesicular stomatitis virus envelope as previously described, concentrated by ultra-centrifugation, and titered on human 293T cells by flow cytometry. Keratinocytes were transduced by one round of exposure to SFFV-GFP, SFFV-SP/GFP, INVOp-GFP, or INVOp-SP/GFP vectors at a multiplicity of infection of 25.

Flow cytometry analysis

The proportion of green fluorescent protein (GFP)-positive cells and GFP intensity were monitored by flow cytometry (Becton Dickinson, San Jose, CA), and data were collected using CellQuest (Becton Dickinson; V3.0). Values were normalized against initial time points and analyzed by non-parametric tests using GraphPad Prism 5.

Skin biopsies and keratinocyte cultures

Skin biopsies were obtained by punch biopsy from a patient with NS (confirmed null homozygous mutations at c. 2200delAA in *SPINK5* gene) under a protocol approved by our Local Ethics Committee and with informed consent from parents. Primary keratinocytes were isolated from skin biopsies by incubation with 0.25% trypsin-EDTA for 3 hr. Primary keratinocytes and keratinocyte cell line NTERT cells (Dickson *et al.*, 2000) were seeded with lethally irradiated 3T3 cells and grown in the keratinocyte culture medium (Rheinwald, 1989). Transduced and untransduced control NTERT cells were treated with keratinocyte medium containing 5 μM azacitidine (5'-azacytidine; Pharmion, Hillingdon, UK), as described previously (Chien *et al.*, 2005), with medium being replaced every 3 days for up to 40 days. During the period of 40 days, cells were passaged when they reaching confluence. Transduced and nontransduced cells without azacitidine treatment were cultured in parallel.

Pyrosequencing and methylation analysis

DNA methylation analysis was carried out by EpigenDx (Worcester, MA). In brief, genomic DNA from transduced cells cultured in the presence or absence of azacitidine was denatured and treated with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen, Crawley, UK). Targeted sequences, which included a 341-bp boundary region of SFFV-SPINK5co (13 CpGs for SFFV and 8 CpGs for SPINK5co), a 246-bp boundary region of INVOp-SPINK5co (7 CpGs for INVOp and 8 CpGs for SPINK5co), a 138-bp boundary region of SFFV-eGFP (8 CpGs for SFFV and 2 CpGs for eGFP), and a 126-bp boundary region of INVOp-eGFP (3 CpGs for INVOp and 2 CpGs for eGFP), were amplified from bisulfite-treated DNA samples using specific forward and biotinylated reverse primers, followed by pyrosequencing with specific sequencing
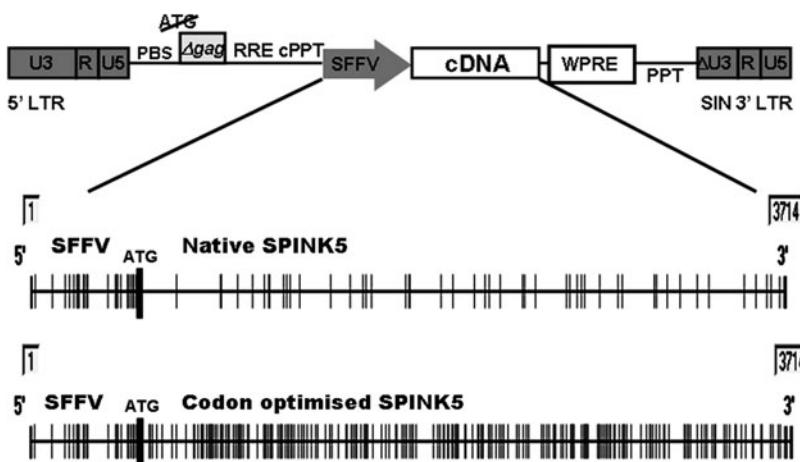

FIG. 1. Schematic view of the lentiviral vector and of the SFFV promoter–transgene sites susceptible to CpG methylation. The upper panel shows SIN HIV-1-based viruses, encoding the SFFV-LTR promoter, the HIV-1 cPPT element, and a mutated WPRE. Following codon optimization, multiple novel CpG sites were introduced throughout the SPINK5co transgene compared with the native *SPINK5* gene (MethylPrimer Express Software v1; Applied Biosystems, Foster City, CA).

primers for each amplicon. Quantification of methylation at all individual CpG sites within the amplified PCR product was performed using PyroQ-CpG software.

Bioengineered skin preparation and grafting to immunodeficient mice

The methods for preparing and grafting bioengineered skin in nude mice have been previously described (Larcher et al., 2007). In brief, primary NS keratinocytes infected with lentiviral vectors encoding eGFP or SPINK5-eGFP cDNA linked to SFFV or INVOp were seeded on top of a fibrin matrix populated with live primary human fibroblasts (dermal equivalent). After keratinocytes reached confluence, the bioengineered skin constructs were grafted onto a dorsum of 6-week-old female immunodeficient nude mice (NMRI strain; Elevage-Janvier, Le Genest-St Isle, France). Three mice were grafted for each condition. Successfully grafted animals were anesthetized, and the presence of eGFP fluorescent grafts in the whole animals was monitored using a Kodak ISO 2000MM live imaging apparatus (Kodak, Rochester, NY) under appropriate illumination/filter pairs. Close-up photographs of fluorescent grafted areas were obtained in a stereomicroscope equipped with a GFP fluorescent illumination and digital camera (Olympus, Barcelona, Spain). Eight weeks after grafting, skin samples from grafts were taken post mortem, embedded in OCT or paraffin, and sectioned for histological and immunohistochemical examinations.

Immunostaining and immunoblotting

Immunofluorescence staining and histochemistry were performed on frozen or paraffin tissue sections (6-μm thickness) using methods previously described (Di et al., 2009). A custom-made affinity-purified rabbit polyclonal antibody (Eurogentec, Southamton, UK) directed against the C-terminus of LEKTI was used at 580 ng/ml. For immunoblotting, cells were lysed in a cooled buffer composed of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, cocktail protease inhibitors, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1% Triton X-100 for 15 min at 4°C. Samples were then centrifuged at 12,000 rpm for 10 min to pellet the insoluble material. The total protein concentration in the supernatant was determined using the Bio-Rad protein assay kit (Bio-Rad, Hertfordshire, UK). Samples from the supernatant were further diluted in 5× sample buffer containing 100 mM dithiothreitol, 10% sodium SDS, 30% glycerol, 0.001% bromphenol blue, and 0.5 mM Tris-HCl, pH 6.8. Equal quantities of total protein were loaded in 10% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and incubated with LEKTI antibody overnight at room temperature with shaking. On the following day, membranes were incubated with mouse anti-rabbit IgG conjugated with horseradish peroxidase secondary antibody (Sigma, Poole, UK), and signals were detected using the ECLplus system (GE Healthcare, Buckinghamshire, UK).
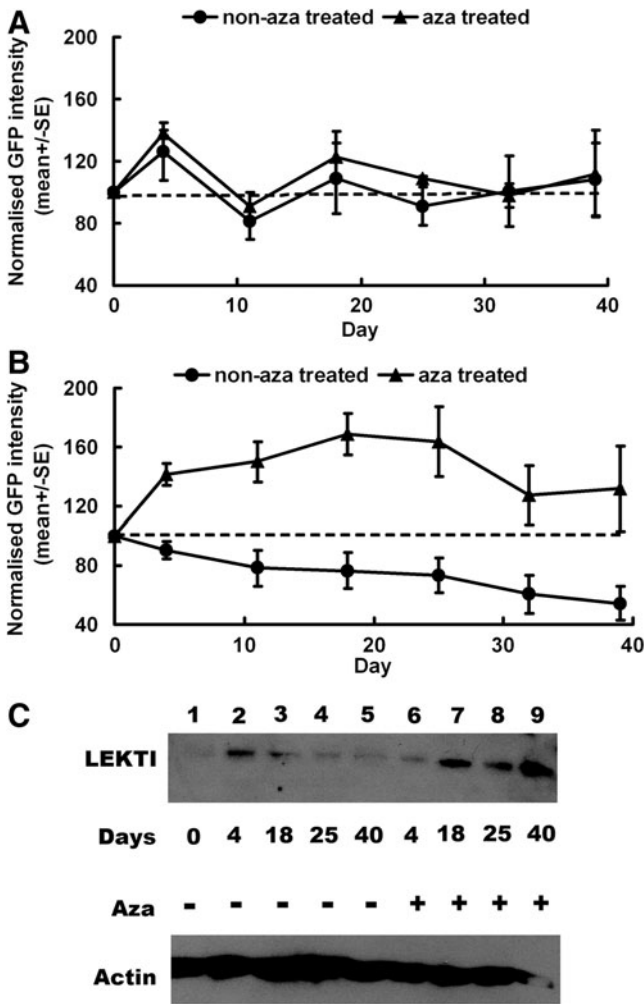
Results

Methylation-mediated silencing of SPINK5 gene expression

We previously reported that SIN lentiviral vectors derived from HIV-1 and incorporating an internal SFFV promoter upstream of SPINK5co/GFP (SFFV-SP/GFP) mediated high levels of LEKTI expression in primary keratinocytes, whereas protein expression from a non–codon-optimized SPINK5 transgene had been barely detectable (Di et al., 2011). We reasoned that because the native SPINK5 cDNA encodes a high proportion of rare codons and has a relatively low GC content, mRNA turnover was likely to be rapid and protein expression levels may not have been sufficient for therapeutic benefit. Therefore, gene optimization was undertaken using a proprietary algorithm (GeneArt) (Fig. 1). This removed potentially repressive sequences and increased GC content from 43% in native SPINK5 cDNA to 58% in SPINK5co. The process also included the replacement of inhibitory sites (cryptic splice sites, polyA sites, and RNA instability motifs) and the introduction of a Kozak sequence to increase translational initiation (Fath et al., 2011). Codon usage was adapted for expression in human cells, resulting in a very high "codon adaptation value" (0.93). Thereafter, vectors encoding SPINK5co mediated LEKTI expression sufficient to normalize human skin architecture in grafts derived from patients with NS (Di et al., 2011). However, we noted a decline in the expression of reporter gene eGFP in long-term cultured cells with serial propagation, suggesting repression of transgene expression. To test this, keratinocyte cell line NTERT cells were transduced with SFFV-SP/GFP and SFFV-GFP lentiviral vectors, and the intensity of eGFP expression in these cells was monitored by flow cytometry over a 40-day period. Interestingly, although eGFP intensity in cells transduced with the control SFFV-GFP vector did not change over time (Fig. 2A), it was markedly decreased in cells transduced with SFFV-SP/GFP vector ($p < 0.01$) (Fig. 2B). Western blot analysis of LEKTI expression revealed reduction in the expression of LEKTI protein in SFFV-SP/GFP transduced cells (Fig. 2C). Quantitative PCR for integrated vector copy number confirmed the presence of stable integrants, excluding the possibility of vector-mediated toxicity, which may have resulted in the loss of gene-modified populations (data not shown). As the DNA sequences in SPINK5co had increased CpG motifs after gene codon optimization compared with those in native SPINK5 (Fig. 1), we suspected that DNA methylation might be the cause of SPINK5 transgene silencing. To investigate the possibility of methylation-mediated silencing, transduced cells were cultured in the presence of the DNA methyltransferase inhibitor, azacitidine. Flow cytometry analysis showed greater stability of GFP expression for SFFV-SP/GFP transduced populations (Fig. 2B), and western blot analysis revealed increased levels of LEKTI expression in cells cultured in the presence of azacitidine (Fig. 2C). In contrast, there were no significant changes in SFFV-GFP cells treated with azacitidine (Fig. 2A). These results supported the hypothesis that methylation-mediated silencing was compromising transgene expression from vectors expressing SP/GFP in combination with the SFFV promoter.

To investigate this phenomenon further, we quantified the levels of DNA methylation at individual CpG sites across the boundary region between the SFFV promoter and SPINK5co, including the transcription start site. DNA from cells transduced with the SFFV-SP/GFP and cultured in the presence or absence of azacitidine was extracted after 5, 12, and 40 days and treated with bisulfite before pyrosequencing and analysis. DNA methylation was quantified, focusing on 13

FIG. 2. SFFV promoter in combination with SPINK5co results in methylation-mediated DNA silencing. NTERT keratinocytes transduced with lentiviral vectors encoding SFFV-GFP **(A)** or SFFV-SP/GFP **(B)** were cultured in the presence or absence of $5 \mu M$ azacitidine (aza) for a period of 40 days. GFP intensity in transduced cells cultured over time was monitored by flow cytometry. **(A)** GFP intensity in cells transduced with SFFV-GFP remained stable, and azacitidine treatment had no significant effect on these cells. **(B)** GFP intensity in cells transduced with SFFV-SP/GFP decreased over time and was rescued by azacitidine treatment. **(C)** LEKTI expression in NTERT cells transduced with SFFV-SP/GFP detected by western blot decreased over time (lanes 2–5) and were rescued by azacitidine treatment (lanes 6–9).

CpG sites within 174 bases of distal SFFV promoter and 8 CpG sites within 163 bases of proximal *SPINK5* cDNA. The proportion of methylated CpG sites increased significantly over time in the absence of azacitidine treatment from around 6% for both SFFV and SPINK5co regions at day 5 to 15% for SFFV and 19% for SPINK5co at day 40 ($p < 0.001$) (Fig. 3A). Moreover, by day 40, azacitidine treatment resulted in a significant reduction of DNA methylation within both SFFV and SPINK5co regions (SFFV, $9.1 \pm 3.6\%$; *SPINK5*, $11.2 \pm 4.1\%$; $p < 0.001$) (Fig. 3A). Interestingly, methylation within the SFFV promoter of control groups transduced with vectors expressing eGFP alone (SFFV-GFP) was much lower
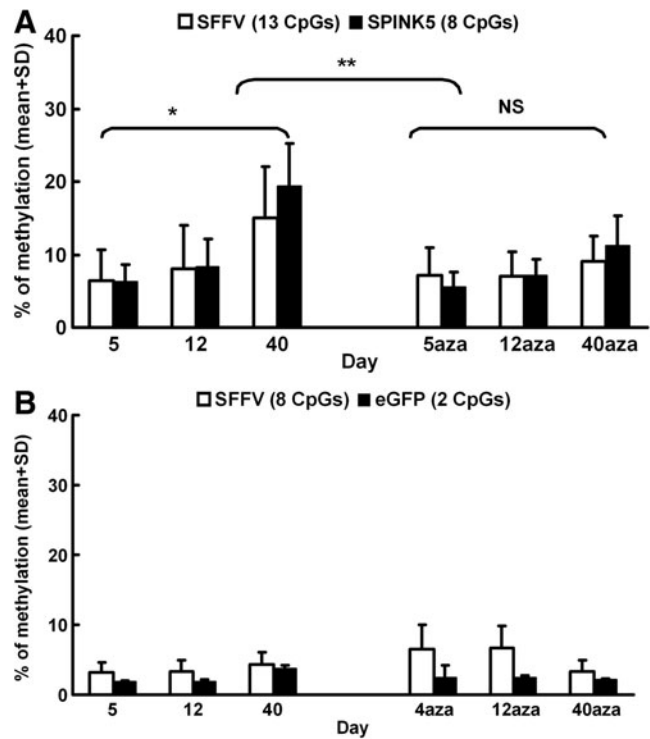
FIG. 3. Methylation analysis of the promoter–transgene boundary in NTERT cells transduced with SFFV-containing vectors. NTERT keratinocytes transduced with lentiviral vectors encoding SFFV-GFP or SFFV-SP/GFP were cultured in the presence or absence of $5 \mu M$ azacitidine (aza) for a period of 40 days. DNA was extracted after 5, 12, and 40 days and treated with bisulfite before pyrosequencing and analysis. **(A)** Average percentages of methylation within a 341-bp boundary region of SFFV-SPINK5co including 13 CpGs for SFFV (white bars) and 8 CpGs for SPINK5co (black bars) show increasing methylation over time (*$p < 0.001$). Methylation was significantly reduced by day 40, following azacitidine treatment compared with cells without azacitidine treatment (**$p < 0.001$). There was no significant (ns) change within the azacitidine-treated group over time. **(B)** Low methylation levels within a 138-bp boundary region including 8 CpGs for SFFV (white bars) and 2 CpGs for eGFP (black bars) were detected in cells transduced with SFFV-GFP.
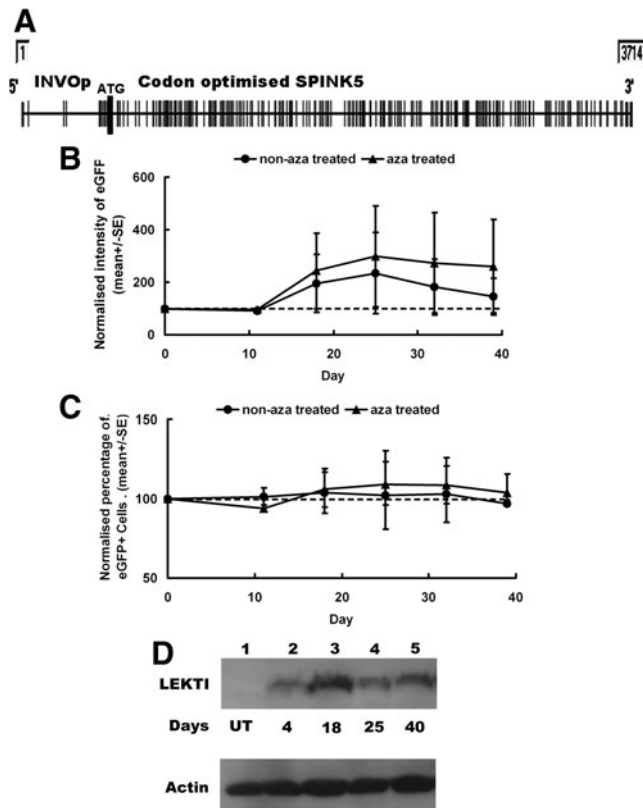
($4.3 \pm 1.8\%$ at day 40 for SFFV-GFP versus $15.1 \pm 7.0\%$ at day 40 for SFFV-SP/GFP; $p < 0.001$), with overall levels ranging from 1.6% to 6.6% (Fig. 3B). Thus, CpG sites within the distal region of SFFV were more susceptible to methylation when the promoter was used in combination with codon-optimized, GC-enriched *SPINK5*, suggesting spreading of methylation across the promoter–cDNA boundary.

The human INVOp addresses the silencing of SPINK5co

Involucrin is an epidermal protein expressed in the suprabasal layers of stratified squamous epithelium during keratinocyte differentiation. We investigated whether a 572-bp regulatory element derived from the full-length human INVOp could mediate SPINK5co transgene expression and support compartment-specific LEKTI expression. As INVOp has very few CpG sites (a total of 7 CpGs in INVOp
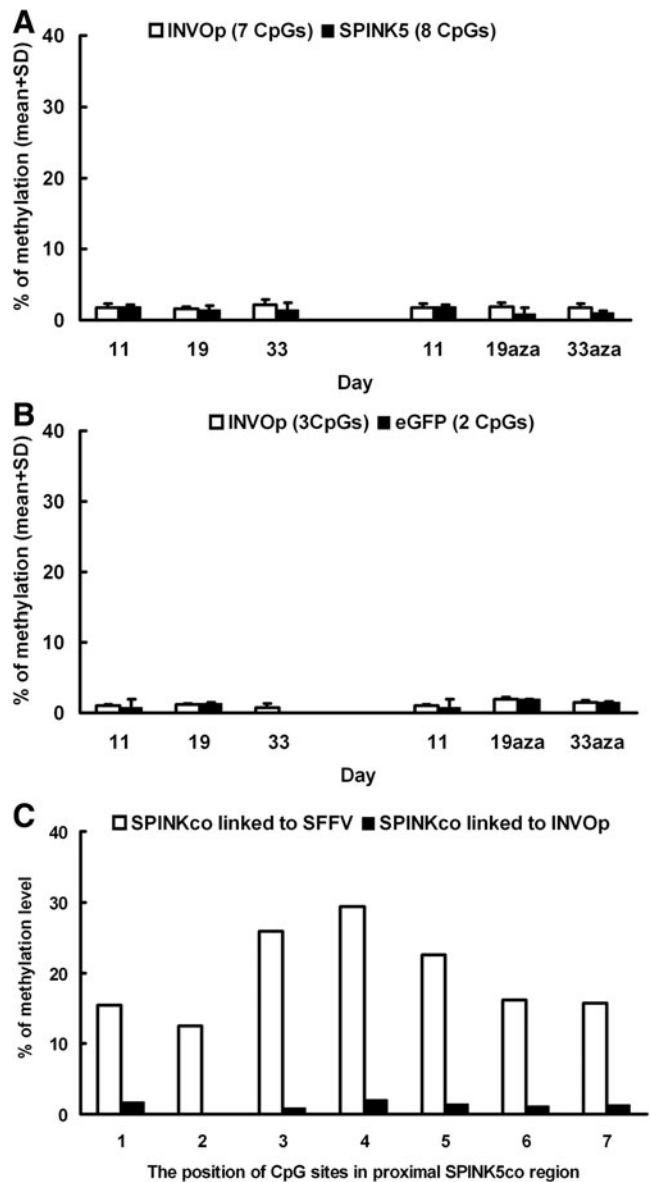
compared with 26 CpGs within SFFV) (Fig. 4A), we hypothesized that using this promoter might prevent methylation-mediated silencing of SPINK5co transgene. We incorporated INVOp into the lentiviral constructs by substituting SFFV to generate INVOp-SP/GFP and INVOp-GFP vectors. NTERT keratinocytes were transduced and cultured with or without azacitidine, for up to 40 days. Flow cytometry analysis revealed no significant reduction in the proportion of eGFP-expressing cells or in the intensity of GFP expression in cells transduced with INVO-SP/GFP over time (Fig. 4B and C). In addition, western blot analysis showed stable LEKTI expression over the analyzed period (Fig. 4D).

Next, to analyze the methylation status of INVOp and SPINK5co, we carried out pyrosequencing and analysis of bisulfite-treated DNA. DNA from cells transduced with the INVOp-SP/GFP and INVOp-GFP cultured in the presence or absence of azacitidine was extracted after 11, 19, and 33 days in culture and treated with bisulfite before pyrosequencing. DNA methylation was quantified, focusing on the distal region of INVOp (7 sites within 106 bp) and the proximal region of SPINK5co (8 CpG sites within 163 bp). Our results

revealed low levels of methylation within the INVOp region over time in cells transduced with INVOp-SP/GFP or INVOp-GFP vectors, ranging from around 0% to 2.2% (Fig. 5A and B). Azacitidine treatment did not change the methylation status in cells transduced with either vector (Fig. 5A and B).

FIG. 4. INVOp supports stable LEKTI expression. (A) Schematic view of the INVOp-SPINK5co sites susceptible to CpG methylation. (B) NTERT cells transduced with INVOp-SP/GFP were cultured in the presence or absence of 5 μM azacitidine (aza) for a period of 39 days. GFP intensity did not change in the first 11 days, but was increased following day 11. This is likely to be a result of enhanced involucrin expression during keratinocyte differentiation. (C) The proportion of GFP-positive cells remained stable in the presence/absence of azacitidine treatment. (D) Western blot analysis with anti-LEKTI antibody shows stable LEKTI expression in NTERT cells transduced with INVOp-SP/GFP over time.

FIG. 5. Methylation in vectors incorporating the INVOp. NTERT cells transduced with INVOp-SP/GFP were cultured in the presence or absence of 5 μM azacitidine (aza) for over 30 days. Methylation across the promoter–transgene boundary was quantified by pyrosequencing following bisulfite treatment of genomic DNA. (A) There was low-level methylation within the 246-bp boundary region of INVOp-SPINK5co (white bars, INVOp; black bars, transgene SPINK5co). (B) The relevant control vector configuration also showed low-level methylation within a 126-bp boundary region of INVOp-eGFP (white bars, INVOp; black bars, eGFP). (C) Methylation at seven individual CpG sites within the proximal SPINK5co region at the end of the culture period was found to be significantly lower in cells transduced with INVOp-SP vectors (black bars) compared with cells transduced with SFFV-SP constructs (white bars).

Importantly, methylation levels within the proximal SPINK5co were significantly lower than methylation levels within the same region in vectors incorporating SFFV promoter, reaching 1.5±1.0% for INVOp-SP/GFp versus 19.4±5.9% for SFFV-SP/GFP ($p < 0.001$). This difference was evident at each individual site analyzed (Fig. 5C). We conclude that substitution of INVOp in place of SFFV supports stable LEKTI expression *in vitro* and results in reduced susceptibility to transgene repression.

INVOp supports robust,compartmentalized LEKTI expression *in vivo*

To evaluate the efficacy of the INVOp-SP/GFP combination in a skin-humanized mouse model, we transduced primary keratinocytes obtained from an NS patient with INVOp-SP/GFP and INVOp-GFP control vectors. Cells transduced with SFFV-SP/GFP and SFFV-GFP were included for comparison. Clonogenicity of keratinocytes transduced with SFFV-GFP, INVOp-SP/GFP, and INVOp-SP/GFP evaluated using colony-forming assay did not reveal significant differences among cell groups (data not shown). Transduced cells were expanded and cultured as bioengineered skin equivalents and grafted onto immunodeficient nude mice as previously described (Di *et al.*, 2011). NS grafts transduced with INVOp-GFP showed histological features of NS, including psoriasiform changes and hypogranulosis with a parakeratotic stratum corneum and defective keratinization (Fig. 6D). LEKTI expression was not detected in grafts transduced with SFFV-GFP or INVO-GFP control vector (Fig. 6F and H). Although the initial gene-transfer efficiency monitored by flow cytometry for GFP-positive populations was lower in keratinocytes transduced with INVOp vectors (25%) compared with SFFV vectors (40%), the level of LEKTI expression and the extent of architectural correction were greatest in grafts generated from INVOp-SP/GFP (Fig. 6G and I). In addition, the localization of suprabasal LEKTI in NS grafts driven by INVOp closely mirrored the compartmentalization of native LEKTI expression (Fig. 6I). Thus, vectors incorporating INVOp supported durable SPINK5co transgene expression leading to compartment-specific LEKTI reconstitution and correction of skin architecture.

Discussion

Gammaretroviral vectors, incorporating long terminal repeat (LTR) promoters, have been widely used in early-phase clinical studies (Qasim *et al.*, 2009). The ability of strong enhancer elements within the LTRs to mediate transactivation of proto-oncogenes, resulting in insertional mutagenesis, has been a major concern associated with conventional retroviral configurations (Hacein-Bey-Abina *et al.*, 2003, 2008; Ott *et al.*, 2006; Howe *et al.*, 2008) and, more recently, loss of transgene expression has been documented in clinical studies (Stein *et al.*, 2010). This has been attributed to various causes, including positional variegation effects, promoter methylation, and histone modification. For example, in a phase 1 study of retroviral correction of chronic granulomatous disease, methylation of CpG sites within the SFFV promoter led to extinction of transgene expression and loss of therapeutic effects, whereas intact enhancer function mediated clonal expansion through transactivation effects. Next-generation SIN vectors, with deleted U3 LTR promoter activity, use
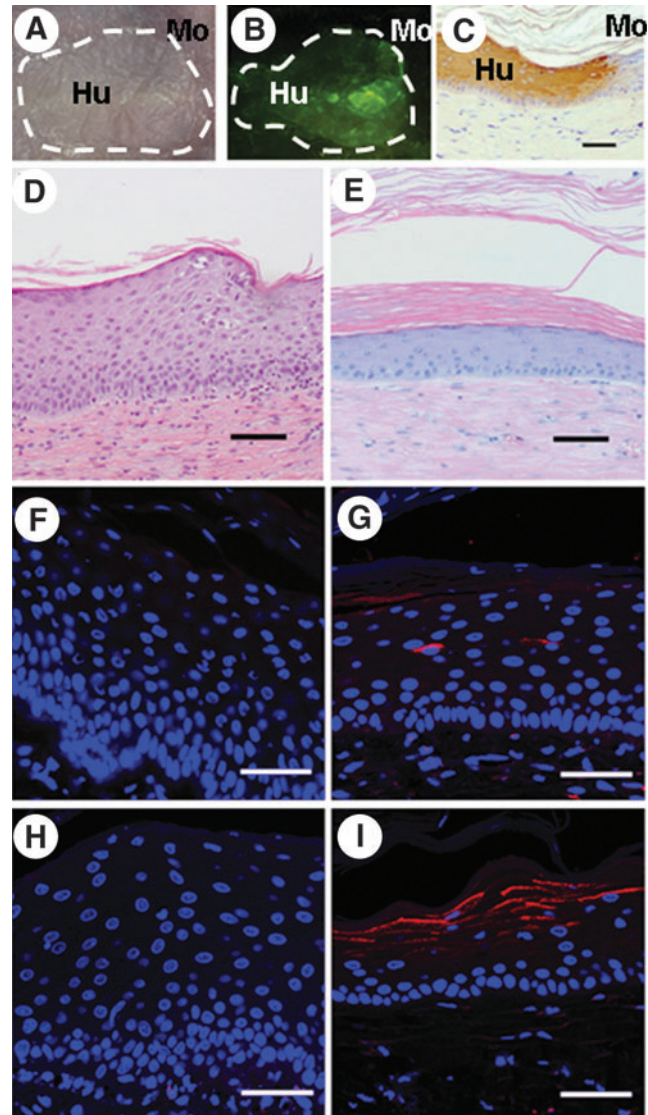
FIG. 6. INVOp supports robust, compartment-specific LEKTI expression in NS skin grafts. NS keratinocytes were transduced with INVOp-GFP (**D, H**) or INVOp-SP/GFP (**A-C, E, I**) or SFFV-GFP (**F**) or SFFV-SP/GFP (**G**) and grafted onto nude mice. Skin grafts were examined 8 weeks after grafting. (**A**) Macroscopic appearances of the grafts under transmitted light. (**B**) Expression of eGFP within the skin grafts visible under 488-nm (blue) light. (**C**) Anti-human involucrin antibody staining, revealing the histological demarcation of murine (Mo) and human (Hu) skin boundary. (**D, E**) Histological appearance of grafts by hematoxylin and eosin staining. Results showed corrected epidermal structures with a thinner, well differentiated epidermis in the INVOp-SP/GFP grafts (**E**), compared with the control graft transduced with INVOp-GFP (**D**), which shows typical features of NS with thickened epidermis and incomplete terminal differentiation and hypogranulosis. LEKTI expression (shown in red) was absent in control groups transduced with SFFV-GFP (**F**) or INVOp-GFP (**H**), but was detectable in grafts transduced with SFFV-SP/GFP (**G**) and INVOp-SP/GFP (**I**). High-level LEKTI expression with suprabasal compartment localization was shown in the INVOp-SP/GFP graft (**I**), compared with the SFFV-SP/GFP graft (**G**) in which only limited numbers of LEKTI-positive cells were detected. Scale bar = 50 μm for F–I and 200 μm for C–E.

heterologous promoters, which could address concerns relating to transactivation. However, in many preclinical studies, internal promoters derived from murine retroviral LTR sequences have remained popular, as they support high levels of transcriptional activity in a wide variety of cell types (Demaison *et al.*, 2002). We have previously described robust and durable gene expression from SIN HIV-1 vectors incorporating an internal SFFV-derived promoter in a variety of cell types, including hematopoietic stem cells, lymphocytes (Qasim *et al.*, 2007), fibroblasts (Fassone *et al.*, 2010), and keratinocytes (Di *et al.*, 2011). Evidence is accumulating that, in susceptible cell lines such as the P19 embryonic carcinoma line and hematopoietic stem cells, gene expression mediated by LTR-derived elements is rapidly repressed through CpG methylation. Notably, the SFFV-LTR includes methylation-susceptible CpG motifs within its Sp1 transcription binding site, enhancer core, and CAAT/TATA box elements (Zhang *et al.*, 2007, 2010).

Several approaches are being investigated to address DNA methylation and histone modification, which can result in silencing of vector-mediated transgene expression. One strategy has been to incorporate chromatin insulators such as the chicken β-globin locus control region HS4 element (cHS4) into retroviral LTR regions to reduce expression variegation, but their inclusion may compromise vector titer (Gaszner and Felsenfeld, 2006), and a recent clinical report has raised questions about their inclusion in therapeutic vectors (Cavazzana-Calvo *et al.*, 2010). Alternatively, enhancerless ubiquitous chromatin opening element (UCOE) sequences, derived from the human HNRPA2B1-CBX3 locus (A2UCOE), have been shown to confer stable transgene expression in a variety of different cell types, including hematopoietic stem cells used for the correction of murine severe combined immunodeficiency (Zhang *et al.*, 2007, 2010). UCOEs exhibit a dominant chromatin opening capability and encode methylation-free CpG islands that potently resist DNA methylation. Although we considered substitution of SFFV with A2UCOE in vectors expressing SPINK5co, preliminary experiments using previously described A2UCOE-GFP lentiviral vectors suggested that these vectors may not support sufficiently high levels of gene expression in keratinocytes. Similarly, gene expression derived from vectors with alternative human promoter elements derived from elongation factor 1-α gene and phosphoglycerate kinase promoter were notably reduced in comparison with SFFV (data not shown). In contrast, we found that minimal (572 bp) human INVOp, with low CpG content, mediated both compartment-specific and repression-resistant transgene expression.

A general model of epigenetic repression of vector-mediated gene expression envisages methylation of enhancer-promoter elements that may spread to nearby CpG sites within the transgene (Bird and Wolffe, 1999; Curradi *et al.*, 2002). Our observations are consistent with a model whereby, once initiated, DNA methylation is perpetuated *in cis* and spreads to neighboring CpG sites, breaching a threshold beyond which transcription is repressed. We conclude that juxtaposition of the SFFV and SPINK5co resulted in clustering of methylation-susceptible CpG sites, predisposing to transgene repression over time. Further investigations will be required to fully elucidate additional mechanisms, such as chromatin-mediated repression, but we have demonstrated that substituting of the CpG-rich SFFV promoter with

INVOp circumvents susceptibility to methylation-mediated silencing of SPINK5co. Deployment of the INVOp is particularly attractive in the context of inherited skin diseases such as NS, where gene expression is highly compartmentalized. In the normal skin, to maintain a constant thickness of epidermis, corneocytes within the uppermost layers of the epidermis are shed by desquamation and are continuously replaced by keratinocytes undergoing differentiation. Correct epidermal assembly requires compartmentally restricted gene expression; for example, both involucrin and LEKTI proteins are expressed in the uppermost layers of the epidermis. In the absence of a well-defined promoter associated with the endogenous *SPINK5* locus, INVOp provides a valuable alternative and supports LEKTI expression in suprabasal layers of the epidermis. Interestingly, essential proximal regulatory regions of human INVOp have previously been shown to encode binding sites for lens epithelium–derived growth factor (Kubo *et al.*, 2002), a well-defined transcriptional coactivator and a critical host-cell factor that interacts with HIV-1 integrase during proviral integration. It is not know whether this association could influence vector integration or function, and detailed integration-site characterization will be required to determine whether the integration profile of lentiviral vectors encoding INVOp differs from other HIV-1-derived vectors.

The lentiviral vector system described here has addressed unanticipated problems associated with our previous proof-of-principle constructs. Further refinements are under way to produce a third-generation, four-plasmid, vector system (Dull *et al.*, 1998) for translational studies and phase I clinical testing in a clinical trial of gene-modified skin grafts in NS.

Acknowledgments

Author Disclosure Statement

The authors have no conflict of interest to declare.

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