Delivery and Inhibition of Reporter Genes by Small Interfering RNAs in a Mouse Skin Model

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RNA interference offers the potential of a novel therapeutic approach for treating skin disorders. To this end, we investigated delivery of nucleic acids, including a plasmid expressing the reporter gene luciferase, to mouse skin by intradermal injection into footpads using *in vivo* bioluminescence imaging over multiple time points. In order to evaluate the ability of RNA interference to inhibit skin gene expression, reporter gene constructs were co-injected with specific or non-specific siRNAs and the *in vivo* effects measured. Our results revealed that specific unmodified and modified siRNAs (but not nonspecific matched controls) strongly inhibit reporter gene expression in mice. These results indicate that small interfering RNA, delivered locally as RNA directly or expressed from viral or non-viral vectors, may be effective agents for treating skin disorders.

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INTRODUCTION

Diseases of the skin with defined molecular targets are amenable to nucleic acid-based therapies due to tissue accessibility (Pfutzner and Vogel, 2000; Khavari *et al.*, 2002). Although normal skin (especially the stratum corneum) represents a formidable barrier to topical nucleic acid delivery, a number of methods have been used to deliver nucleic acids to skin (Hengge *et al.*, 1996; Vogel, 1999, 2000; Yu *et al.*, 1999; Mitragotri, 2000; Wraight and White, 2001; Khavari *et al.*, 2002; Raghavachari and Fahl, 2002; Prud'homme *et al.*, 2006). The availability of specific and potent gene inhibitors coupled with efficient localized delivery would be a boon to patients suffering from monogenic skin disorders such as epidermolysis bullosa and pachyonychia congenita (PC).

RNA interference is an evolutionarily conserved mechanism that results in specific gene inhibition. The recent discovery that small interfering RNAs (siRNAs) can effectively silence gene expression in a number of mammalian systems without inducing an immune response has resulted in an intense effort to develop these inhibitors as disease therapeutics (Dykxhoorn and Lieberman, 2005; Shankar *et al.*, 2005). In this study, we utilize *in vivo* bioluminescence imaging to reveal the spatiotemporal inhibition patterns of gene expression that are mediated by siRNAs targeting reporter genes expressed in mouse skin keratinocytes. The noninvasive analyses of gene expression afforded by this approach allows for the repeated monitoring of reporter gene expression over multiple time points in the same group of animals, minimizing the number of mice needed while refining the data sets and maximizing the amount of information obtained (McCaffrey *et al.*, 2002). These studies suggest that siRNAs developed against molecular targets in the skin may be effectively developed as therapeutics, especially for monogenic autosomal dominant skin disorders.

The work presented here is part of a larger effort aimed at developing siRNAs as novel therapeutics for skin disorders. The preclinical steps required for development of therapeutic siRNAs include: (i) identification of a specific and potent siRNA molecule that is active *in vivo*; (ii) development of a practical delivery system in which the siRNA is stable and active; (iii) identification of a specific siRNA that reverses a disease phenotype in an appropriate animal model, and (iv) demonstration of a lack of serious toxicity when siRNA is delivered at high concentrations in animals. Our initial efforts reported herein indicate that potent and specific enhanced green fluorescent protein (eGFP) siRNAs can be designed that are effective in mouse skin when co-delivered via intradermal injection with a bicistronic firefly luciferase (fLuc)/eGFP target. The use of the bicistronic reporter allows detection of both fLuc (easily quantitated but difficult to determine effects in individual cells) and eGFP (readily allows determination of transfection efficiency as well as siRNA effects on individual

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Abbreviations: β-gal, β-galactosidase; CMV, cytomegalovirus; EF1A, elongation factor 1A; eGFP, enhanced green fluorescent protein; fLuc, firefly luciferase; FMDV, foot and mouth disease virus; IP, intraperitoneal; KBM, keratinocyte basal medium; PC, pachyonychia congenita; SEAP, secreted alkaline phosphatase; siRNA, small interfering RNA

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cells). The eGFP siRNAs block expression of both fLuc and eGFP, due to degradation of the entire bicistronic mRNA. A single treatment of siRNA effectively blocks reporter gene expression for at least 5 days (transient reporter expression diminishes rapidly after peaking between one and two days), suggesting siRNA effects are long-lasting in the skin. Co-delivery of siRNA with target allows rapid determination of the ability of the siRNAs to silence target gene expression while minimizing complicating delivery issues. These studies will be extended to show that these same eGFP inhibitors can be used to inhibit pre-existing gene expression in transgenic mice expressing the same fLuc/eGFP bicistronic reporter system described in this paper, using various delivery technologies including intradermal injection of siRNAs.

RESULTS

Dose response and time-course analyses of reporter gene expression in mouse skin

Balb/c mouse footpads were intradermally injected with a reporter gene plasmid (pL2G; Figure 1a) encoding a bicistronic mRNA comprised of the fLuc and eGFP open reading frames separated by the foot and mouth disease virus (FMDV) 2A oligopeptide sequence to facilitate expression of equal amounts of fLuc and eGFP (Donnelly *et al.*, 2001; Cao *et al.*, 2005; de Felipe *et al.*, 2006). The mice were imaged for fLuc expression at 12, 24, 36, 48, 96, and 400 hours post gene transfer. The 24-hour time point is shown in Figure 1b. Peak expression was observed between 24 and 48 hours, with 1–2 μg of injected expression plasmid (data not shown; see also Figure 4b, pUC19 data set). Mouse footpad skin was chosen over other mouse skin in part due its greater thickness, the absence of hair follicles, ease of access, and relevance to certain skin diseases. Comparable results were observed following intradermal injection into mouse back skin (data not shown).

To determine the cell type and percentage of cells expressing reporter gene following intradermal injection, a β-galactosidase (β-gal) expression vector was introduced into mouse footpads (Figure 2). eGFP expression from pL2G was not readily detectable in mouse footpads, presumably due to low expression and high autofluorescence of the skin (data not shown). Following an incubation period of 36 hours, the footpad was removed, stained for β-gal activity (panel a), and sectioned and stained for microscopic analysis (panels b and c). Figure 2c shows that ~20% of epidermal keratinocytes within 2–3 mm of the injection site express β-gal at the higher (20 μg) plasmid dose. No non-keratinocyte LacZ-positive cells were observed. These results are consistent with previous studies showing that intradermally injected DNA diffuses into the epidermis and is expressed by keratinocytes (Hengge *et al.*, 1995, 1996; Sawamura *et al.*, 2002).

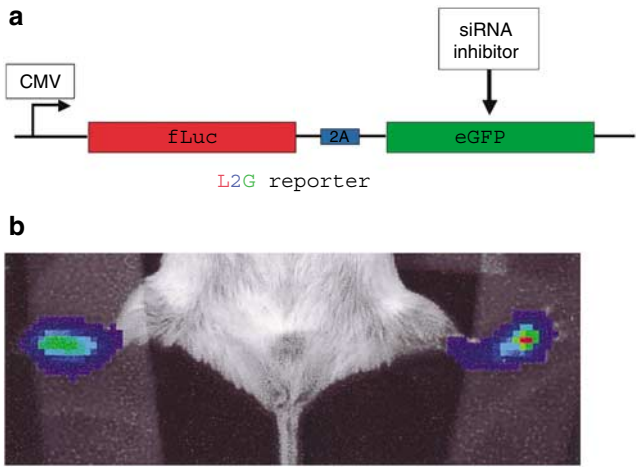

Figure 1. Luciferase expression in mouse footpad following intradermal injection of a reporter plasmid. (**a**) Schematic representation of the pL2G expression plasmid (expresses a hybrid fLuc-2A-eGFP mRNA) with target region of eGFP siRNA inhibitor noted. The bicistronic nature of this reporter is conferred by the 2A sequence from foot and mouth disease virus (see Materials and Methods). (**b**) The pL2G plasmid was injected intradermally into the footpads of mice at concentrations of 1 μg (right paw) and 2 μg (left paw). Luciferase expression levels were assessed by whole-body bioluminescence imaging at 12, 24, 36, 48, 96, and 400 hours post gene transfer. The representative image is of a mouse imaged at 24 hours. The pseudocolor image superimposed over the gray scale reference image represents signal intensity with red indicating highest signal and purple the lowest.

Stabilized and unstabilized gene inhibitors block reporter gene expression in human tissue culture cells and mouse skin

To validate the RNA-based gene inhibitors, correlative cell culture studies were performed in keratinocytes and other cells. The pL2G expression plasmid was cotransfected with eGFP-directed siRNAs, both with and without chemical modifications (siSTABLE modifications without or with a cholesterol (chol) derivative on the sense strand (Soutschek *et al.*, 2004), into both human 293FT embryonic kidney cells and E6/E7-immortalized human keratinocytes (Figure 3b). An expression vector encoding secreted alkaline phosphatase (SEAP) was cotransfected to control for transfection efficiency and nonspecific effects. No SEAP activity was detected in the keratinocyte cell line, presumably due to the lower transfection efficiency of these cells. (The amount of luciferase expression per cell in the keratinocyte cell line is ~3,000-fold less than the levels observed in the 293 cells; data not shown.) Figure 3 shows that both unmodified and modified eGFP inhibitors potently inhibit L2G expression, as measured by both fLuc activity (Figure 3) and eGFP fluorescence (data not shown), with an $IC_{50} \leqslant 1$ nM for all eGFP siRNAs tested in the 293FT cells (Figure 3a) and the keratinocyte cell line (Figure 3b and data not shown). Little or no effect was observed with the irrelevant nonspecific control (NSC4) siRNA inhibitors. The decrease at 25 nM for NSC4 siSTABLE plus cholesterol in Figure 3a was not observed in other similar experiments (data not shown). To confirm that the siRNAs were acting by degrading target mRNA, a Northern blot analysis was performed (Figure 3c). Equal amounts of total RNA, isolated from cells transfected without (lane 7) or with pL2G plasmid, alone (lane 1) or with siRNAs (lanes 2–6), were separated by gel electrophoresis. The separated RNAs were transferred to a membrane and hybridized to probes specific for fLuc, SEAP, and translation elongation factor 1A (EF1A). Both unmodified (lane 2) and modified (lane 4) eGFP siRNAs specifically inhibited L2G mRNA accumulation (76% and 79% inhibition, respectively; quantitation by

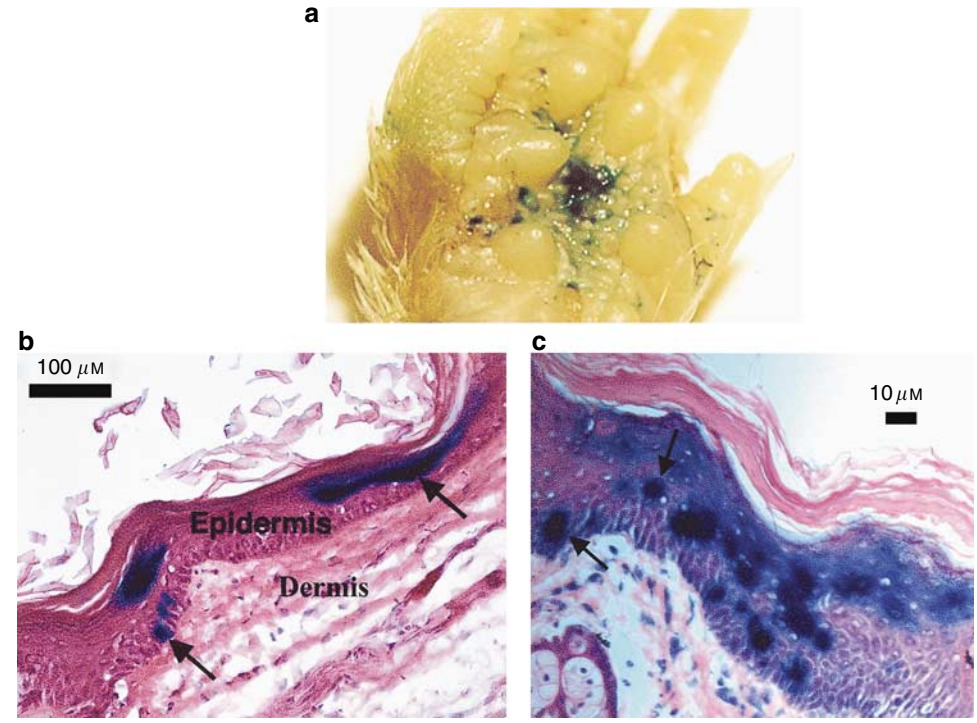

Figure 2. Expression of β-gal in mouse footpad keratinocytes. (**a**) The β-gal expression plasmid was injected intradermally into a mouse footpad and 36 hours later the mouse was killed and the paw removed and tissues stained for β-gal activity. (**b**) Footpads that had been injected with 2 μg plasmid were removed, fixed, sectioned, and stained with hematoxylin and eosin. (**c**) Footpads that had been injected with 20 μg β-gal plasmid were removed, fixed, sectioned, and stained with hematoxylin and eosin. The micrographs, shown at 100 (**b**) and 200 (**c**)-fold magnifications, reveal gene expression in keratinocytes (arrows).

phosphorimager) compared to the corresponding NSC4 controls (lanes 3 and 5) when corrected for SEAP and endogenous EF1A mRNA levels. Little or no inhibition was observed using an irrelevant (hepatitis C virus) siRNA (lane 6). pSEAP2 plasmid was included in each of the transfections (lanes 1–7). No fLuc or SEAP mRNA was detected in untransfected cells (data not shown).

These positive cell culture results supported the translation from culture to a murine skin model (Figure 4). Balb/c mouse footpads were co-injected with the pL2G reporter gene plasmid and unmodified or modified eGFP or non-specific (NSC4 or HCV) siRNAs. At the indicated time points, an intraperitoneal (IP) injection of luciferin was given and the mice were imaged (Figure 4a) as described in Figure 1. Substantial mouse-to-mouse variability was observed in all of the animal experiments, presumably due to variability in the thickness and elasticity of the skin of the footpads and/or the difficulty of injecting the nucleic acid at precisely the same location for each mouse. Nevertheless, compared to the control paws injected with NSC4 and HCV siRNAs, the paws injected with each of the eGFP siRNAs showed robust inhibition of reporter gene expression (Figure 4b; e.g. 94 and 97% inhibition by unmodified and modified eGFP siRNAs as compared to their matched NSC4 control siRNAs at the 48 hours time point). No significant decrease was observed using the control HCV siRNA; a slight decrease was observed using NSC4 control siRNAs, possibly due to the excess of siRNA to plasmid (2,000-fold more siRNA on a molar basis) injected into the footpads. These results strongly suggest that these RNA-based inhibitors can be used to potently inhibit endogenous gene expression. The observation that unmodified eGFP siRNAs had similar activity to stabilized ones in tissue culture cells and co-injection animal experiments bodes well for obtaining sustained inhibition of endogenous genes in animal and human studies.

DISCUSSION

RNA interference holds tremendous potential as a powerful and robust therapeutic strategy for specifically blocking gene expression. In the present report, we show that siRNAs specifically block reporter gene expression in mouse keratinocytes under conditions in which delivery is not limiting (due to co-delivery of siRNA and target plasmids to the same cells). The strong cytomegalovirus (CMV) promoter utilized results in transient high reporter mRNA (and protein) expression, likely at levels comparable to constitutively-expressed abundant mRNAs such as translation elongation factor EF1A (see Figure 3c), glyceraldehyde-3-phosphate dehydrogenase, or endogenous keratins (data not shown). The results presented here indicate that these RNA-based inhibitors can be used to potently inhibit the expression of ectopically expressed genes, suggesting that targeting of endogenous genes is also feasible. siRNAs containing modifications for stabilization against nucleases and targeting to specific cell types (in the case of cholesterol), as well as unmodified siRNAs, were all effective in blocking gene

expression, indicating that these modifications did not appear to block activity in these assays. The inhibitory activity of the siRNAs lasted for the duration of these experiments (5 days) without noticeable increase (restoration) of reporter gene expression. The observation that unmodified eGFP siRNAs had similar activity to stabilized derivatives in cell culture indicates that modified siRNAs retain potency for sustained inhibition in animal and human studies.

One of the main unresolved issues of using siRNAs as therapeutics is efficient delivery to appropriate cells. Mouse skin keratinocytes can be readily transduced by lentiviral vectors (Kuhn *et al.*, 2002). However, issues of safety, real or perceived, have limited acceptance of viral vectors in the clinic. An *in vitro* synthesized small hairpin RNA directed against the same eGFP target site utilized in this study showed similar activity to the eGFP siRNA inhibitors in cultured human keratinocytes (data not shown) and could be readily expressed from a viral (or plasmid) vector if this route of administration were shown to be safe. We have also shown that small hairpin RNAs delivered directly to the liver are highly effective at inhibiting gene expression (Wang *et al.*, 2005).

Skin delivery techniques based on ballistic methods (Nanney *et al.*, 2000; Oshikawa *et al.*, 2001), injection (Choate and Khavari, 1997; Baek *et al.*, 2001), ultrasound and iontophoresis (Mitragotri, 2000), and chemical depilation-induced anagen for hair follicles (Domashenko *et al.*, 2000) successfully deliver nucleic acids to skin cells. Direct topical application has also been used with mixed results (Mehta *et al.*, 2000; Raghavachari and Fahl, 2002; White *et al.*, 2002; Meykadeh *et al.*, 2005). Several published reports (and our unpublished data) indicate that electroporation may increase delivery following injection or topical delivery by as much as 1,000-fold, with little or no tissue damage by disrupting the membrane structure and increasing permeability of the stratum corneum, suggesting that electroporation may be combined with other methods to increase delivery (Brand, 2001; Zhang *et al.*, 2002; Denet *et al.*, 2004; Prud'homme *et al.*, 2006). It should be noted that siRNAs, due to their smaller size and the site of siRNA activity (cytoplasm), may be delivered more efficiently compared with expression plasmids, which require nuclear entry (Herweijer and Wolff, 2003). Here, direct injection of siRNAs provided delivery sufficient to inhibit reporter gene expression.

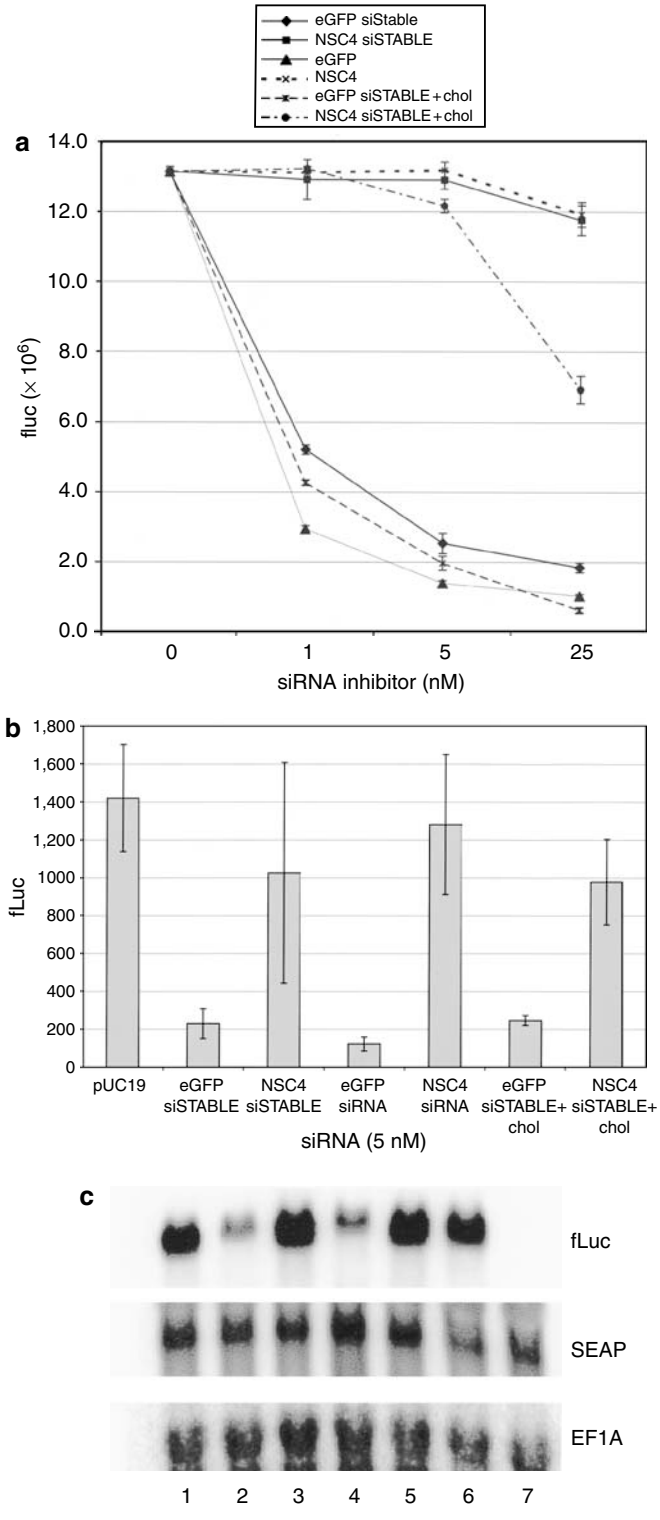

Figure 3. eGFP siRNAs potently inhibit expression of a luciferase/eGFP bicistronic mRNA in tissue culture cells. (**a**) The pL2G expression plasmid was cotransfected into 293FT cells with secreted alkaline phosphatase plasmid (SEAP, to control for transfection efficiency and nonspecific effects) and eGFP- or nonspecific (NSC4) siRNAs that were either unmodified (siRNA) or chemically stabilized (siSTABLE or siSTABLE + chol, which contains a cholesterol derivative at the 5′ end of the sense strand). Each pair of eGFP and NSC4 siRNAs contains the same chemical modifications (e.g. unmodified, siSTABLE or siSTABLE + chol). Forty-eight hours following transfection, cells were lysed and the amount of fLuc activity determined (see Materials and Methods). (**b**) Human HPV E6/E7-transformed keratinocytes were cotransfected with target plasmid and siRNAs and analyzed 72 hours later in a manner similar to 293FT cells (see panel a). (**c**) Northern blot analysis of 293FT cells cotransfected as in panel a. A 10 μg weight of total RNA, isolated from cells transfected without (lane 7) or with pL2G, alone (lane 1) or cotransfected with siRNAs (lanes 2–6), was separated by denaturing gel electrophoresis, transferred to membrane, and hybridized initially to radiolabeled fLuc cDNA and subsequently to SEAP and EF1A probes. The RNA blot was exposed to a phosphorimager screen (see Materials and Methods). Lanes 2 and 4: unmodified and siSTABLE + cholesterol eGFP siRNAs. Lanes 3 and 5: unmodified and siSTABLE + cholesterol NSC4 siRNAs. Lane 6: hepatitis C virus siRNA.

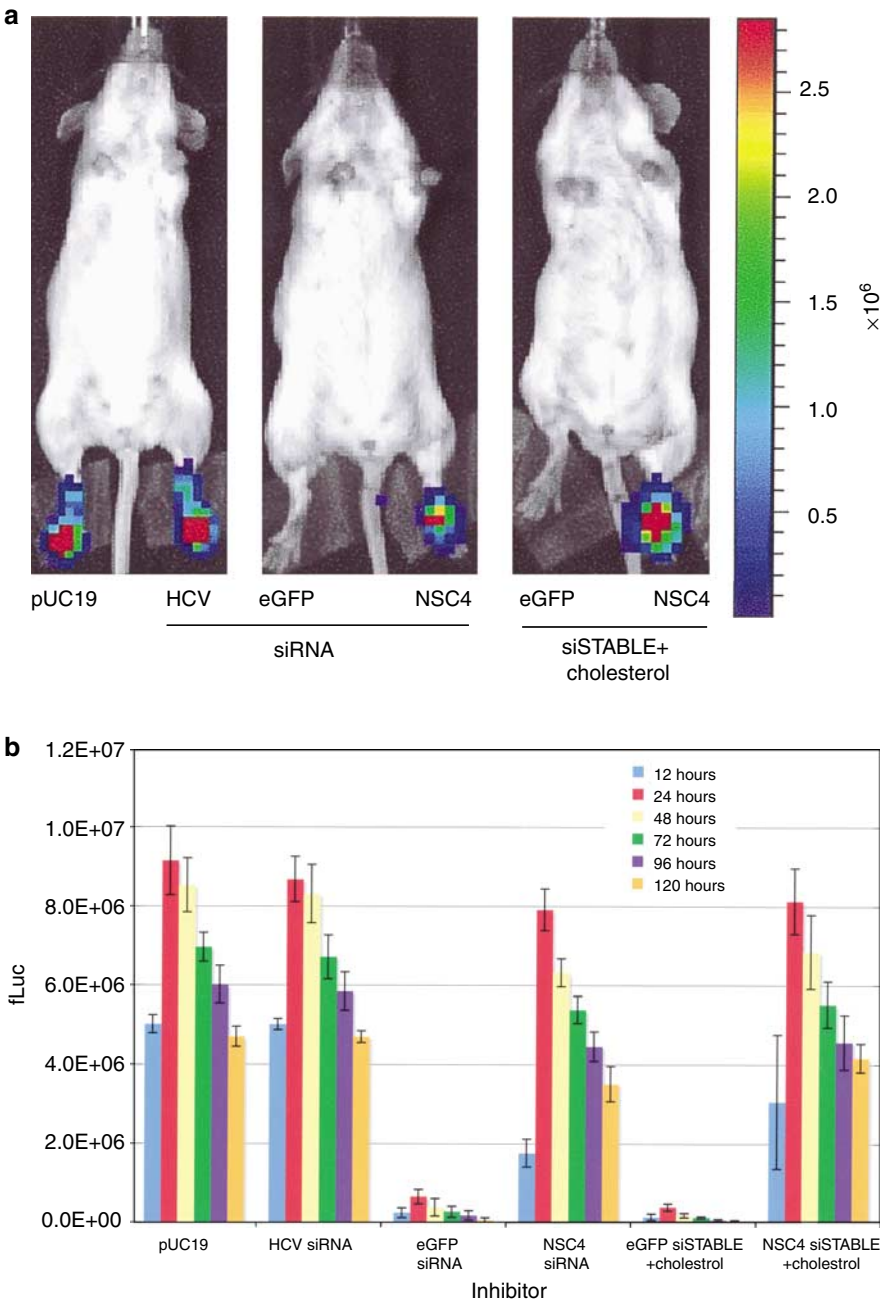

Figure 4. Inhibition of target gene expression by specific siRNAs in a murine footpad skin model. The pL2G expression plasmid was co-injected with either specific eGFP (right paw) or irrelevant NSC4 (left paw) siRNAs (unmodified or siSTABLE + chol), or hepatitis C nonspecific siRNA. Compensatory pUC19 plasmid DNA was added to the injection to achieve the same amount of nucleic acid delivered to each mouse footpad. At the indicated time points, luciferase expression in the footpads was determined following IP luciferin injection by imaging as described in Figure 1. (**a**) Mice were imaged using the IVIS200 *in vivo* imaging system (a representative mouse is shown for each inhibitor at the 72 hours time point). Red color represents highest luciferase expression and purple lowest. (**b**) Quantitation of luciferase activity in treated mice (three mice per group) was performed using LivingImage software and demonstrates reduction in signals in paws treated with siRNA (eGFP) specific to the target plasmid (pL2G).

The results presented in this study indicate that siRNAs can robustly inhibit gene expression in murine skin keratinocytes. The use of *in vivo* bioluminescence imaging allows rapid and repeated quantitation of reporter gene expression in the skin and assessment of the effects of siRNA inhibitors, without tissue removal or harm to the animals. Further experiments are needed to determine the effectiveness of siRNAs targeting disease-specific gene expression in animal models and to assess the feasibility of using siRNAs in a clinical setting.

Autosomal dominant skin disorders resulting from expression of mutant keratins may be good targets for initial siRNA therapies. One such disease is PC. This is a rare skin disorder characterized by thick and dystrophic nails, oral leukoplakia, and skin defects including palmoplanter hyperkeratosis with blistering at the pressure points. This disorder

results from mutations (usually single-nucleotide mutations) in genes encoding keratin 6a/b, 16, or 17 proteins in epidermal keratinocytes (Smith, 2003; Leachman *et al.*, 2005). Efficient delivery of potent and PC disease-specific siRNAs to the pressure points on the soles may alleviate the sole blistering and debilitating pain in these patients. These PC-specific mutant keratins are attractive siRNA targets, as inhibitor discrimination between mutant and wild-type PC genes is probably unnecessary due to the compensatory effect of other keratins. Redundancy and overlapping activities of keratin proteins have been demonstrated in transgenic and knockout mouse experiments (Wojcik *et al.*, 2001; Wong and Coulombe, 2003; Wong *et al.*, 2005). Thus, an effective treatment may result from inhibition of both the mutant (perhaps by as little as 50% reduction; (Cao *et al.*, 2001; Wong *et al.*, 2005)) and wild-type genes with potentially no deleterious effects due to decreased expression of the wild-type gene.

MATERIALS AND METHODS

Design of siRNAs

Chemically synthesized siRNAs were generated by Thermo Fisher Scientific, Dharmacon Products (Lafayette, CO). The SMARTselected™ eGFP sequences for the sense and antisense strands are 19 + 2 format (21-mer), sense 5′-GCACCAUCUUCUUCAAGGAUU and antisense 5′-P-UCCUUGAAGAAGAUGGUGCUU. These siRNAs were also synthesized with the siSTABLE™ *in vivo* modification pattern (Watanabe *et al.*, 2005) to enhance the siRNA's nuclease resistance. Further, in one set of the siSTABLE™ siRNAs, cholesterol was placed on the 5′ sense strand. The sequences used for the NSC4 (inverted beta galactosidase sequence) siRNAs are sense 5′-UAGCGACUAAACACAUCAAUU and antisense 5′-P-UUGAUGUGUUUAGUCGCUAUU. The hepatitis C-specific siRNA sequences and their utility against a specific target *in vivo* have been previously reported (Wang *et al.*, 2005).

Transfections and reporter gene assays

Human 293FT cells (Invitrogen, Carlsbad, CA) were maintained in DMEM (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (HyClone, Logan, UT), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate. HPV16 E6/E7-immortalized keratinocytes (Fsk1.T.E6/E7 (Krueger *et al.*, 1999), kindly provided by Gerald Krueger, University of Utah) were cultured in keratinocyte basal medium (KBM) basal medium supplemented with KGM SingleQuots and Growth Factors (CAMBREX/BioWhittaker). The day before transfection, 293FT cells were seeded at 0.85×10^5 cells/well in a 48-well plate (for keratinocytes 0.5×10^5 cells/well were used), resulting in ~80% cell confluency at the time of transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For the inhibition experiments, 293FT cells were cotransfected (in triplicate) with 30 ng of a bicistronic reporter gene plasmid (pL2G) that expresses fLuc and eGFP under the control of the constitutive and ubiquitously expressed CMV promoter (Cao *et al.*, 2005), 25 ng pSEAP2-control plasmid (BD Biosciences Clontech, Mountain View, CA) as a transfection control, and the indicated amounts of synthetic siRNAs (typical amount, 1 pmole) supplemented with pUC19 to give a final nucleic acid concentration of 400 ng per transfection. In the pL2G plasmid, the FMDV 2A sequence is used to create the bicistronic message. This sequence results in pseudotermination of the polypeptide encoded in the first open reading frame (fLuc), and then without disengaging from the mRNA, the second open reading frame (eGFP) is translated (Donnelly *et al.*, 2001; de Felipe *et al.*, 2006). For the keratinocytes, 125 ng pL2G, the indicated amounts of inhibitors, 125 ng pSEAP, and sufficient pUC19 to give a final concentration of 400 ng total nucleic acid were used for each transfection. Forty-eight hours later (72 hours for the keratinocytes), the supernatant was removed and heated at 65°C for 30 minutes (min). A 5–10 μl volume of the supernatant was added to 150 μl of the *p*-nitrophenyl phosphate liquid substrate system (pNPP; SIGMA, St Louis, MO). After 30–60 minutes of incubation at room temperature, samples were read (405 nm) on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) and quantitated using SOFTmax software (Molecular Devices). Due to the low level of transfection, no SEAP activity was detected in the keratinocytes. The remaining cells were lysed and luciferase activity measured using the Dual-Luciferase Reporter assay system (Promega, Madison, WI) and MicroLumat LB 96 P luminometer (Berthold Technologies, Bad Wildbad, Germany).

RNA isolation and Northern blot analysis

Total cellular RNA was extracted from 293FT cells (from two wells of a six-well tissue culture plate) 48 hours following transfection (240 ng pL2G, 5 nM siRNA, 200 ng pSEAP, and sufficient pUC19 plasmid to give 3.2 μg nucleic acid per well) with RNAzol (Cinna/Biotecx, Houston, TX). The RNA (10 μg of each) was fractionated on a 1.2% agarose formaldehyde denaturing gel, transferred to a membrane, and sequentially hybridized to ^{32}P-radiolabeled probes specific to fLuc, SEAP, or EF1A and visualized/quantitated by phosphorimager as previously described (Wang *et al.*, 2005).

Mice

Six-week old female Balb/c mice were obtained from the animal facility of Stanford University. Animals were treated according to the Guidelines for Animal Care of both NIH and Stanford University.

Mouse footpad injections and *in vivo* imaging

Mouse footpad injections were performed as described (Hengge *et al.*, 1996; Zhu *et al.*, 2001). In a typical experiment, a total volume of 50 μl phosphate-buffered saline containing 20 μg siRNA inhibitor and 2 μg of pL2G plasmid was injected into the mouse footpad. All animals were imaged 10 minutes after IP injection of luciferin (100 μl of 30 mg/ml luciferin; 150 mg/kg body weight; Contag and Bachmann, 2002). Mice were sedated using isoflurane and live anesthetized mice were imaged using the IVIS200 imaging system (Xenogen Corp., Alameda, CA). The resulting light emission was quantitated using LivingImage software (Xenogen), written as an overlay on Igor image analysis software (WaveMetrics Inc., Lake Oswego, OR). Raw values are reported as photons per second and standard errors of the mean for each group ($n = 3$ animals) are shown.

β-Galactosidase staining and microtome sectioning. β-gal activity was assayed in intact mice footpads. Thirty-six hours following intradermal footpad injection of β-gal expression plasmid

(pCMVSport β-gal; Invitrogen), mice were killed by CO_2 asphyxiation and the paw was surgically removed. After fixation in 0.5% paraformaldehyde for 12 hours and equilibration in 20% sucrose overnight, the skin was rinsed three times with phosphate-buffered saline and incubated overnight at 37°C in phosphate-buffered saline containing 400 μg/ml X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 4 mM potassium ferricyanide, 4 mM $MgCl_2$, and 0.1% Nonidet P-40 as previously described (Smeyne *et al.*, 1991). Following β-gal staining, the skin was embedded in OCT compound (VWR, West Chester, PA), cut into 10-μm serial sections, and collected on coated glass slides (VWR). Sections were counterstained with hematoxylin and eosin (SIGMA) and visualized by microscopy.

CONFLICT OF INTEREST

Mr Ilves and Dr Johnston are employees of Somagenics Inc., which could benefit potentially over the long term from this publication. Dr Contag is the founder of Xenogen Corp.

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