

# Stability Study of Unmodified siRNA and Relevance to Clinical Use

Robyn P. Hickerson,<sup>1</sup> Alexander V. Vlassov,<sup>2,6</sup> Qian Wang,<sup>3</sup> Devin Leake,<sup>5</sup> Heini Ilves,<sup>2</sup>  
Emilio Gonzalez-Gonzalez,<sup>3</sup> Christopher H. Contag,<sup>3,4</sup> Brian H. Johnston,<sup>2,4</sup> and Roger L. Kaspar<sup>1</sup>

RNA interference offers enormous potential to develop therapeutic agents for a variety of diseases. To assess the stability of siRNAs under conditions relevant to clinical use with particular emphasis on topical delivery considerations, a study of three different unmodified siRNAs was performed. The results indicate that neither repeated freeze/thaw cycles, extended incubations (over 1 year at 21°C), nor shorter incubations at high temperatures (up to 95°C) have any effect on siRNA integrity as measured by nondenaturing polyacrylamide gel electrophoresis and functional activity assays. Degradation was also not observed following exposure to hair or skin at 37°C. However, incubation in fetal bovine or human sera at 37°C led to degradation and loss of activity. Therefore, siRNA in the bloodstream is likely inactivated, thereby limiting systemic exposure. Interestingly, partial degradation (observed by gel electrophoresis) did not always correlate with loss of activity, suggesting that partially degraded siRNAs retain full functional activity. To demonstrate the functional activity of unmodified siRNA, EGFP-specific inhibitors were injected into footpads and shown to inhibit preexisting EGFP expression in a transgenic reporter mouse model. Taken together, these data indicate that unmodified siRNAs are viable therapeutic candidates.

## Introduction

RNA INTERFERENCE (RNAi) TECHNOLOGY, including use of small interfering RNAs (siRNAs), has been used extensively in target validation experiments and has generated intense activity in the development of these inhibitors as therapeutics (Behlke, 2006; Dallas and Vlassov, 2006; Kim and Rossi, 2007; Novobrantseva et al., 2008). Recently, several siRNAs have been evaluated in clinical trials with encouraging safety profiles and suggestions of efficacy (de Fougères et al., 2007). However, questions remain regarding siRNA stability *in vivo*, including whether modifications are needed for their development as therapeutic agents. One of our immediate goals is to develop siRNA-based therapeutics for dominant negative genetic skin disorders (Leachman et al., 2008), including pachyonychia congenita (PC), and we have therefore conducted this study on the stability of unmodified siRNAs under a variety of conditions with relevance to clinical use, including as topically delivered therapeutics.

Pachyonychia congenita is an ideal prototype skin disorder to investigate the effectiveness of therapeutic siRNAs (Leachman et al., 2008). PC is caused by mutations (often single nucleotide changes) in the inducible keratin genes encoding keratins 6a (K6a), K6b, K16, and K17 (Leachman et al., 2005; Smith et al., 2005, 2006). Common symptoms include thickened dystrophic nails, painful plantar hyperkeratosis with blistering, and follicular keratosis. The major complaints of patients center around the painful lesions that occur on or near the pressure points of the feet, presenting a localized defined area for siRNA treatment. We have previously shown that an unmodified mutation-specific siRNA (K6a\_513a.12, referred to in this paper as K6a siRNA) can specifically and potently target the C513A single nucleotide mutation in *KRT6A* (gene encoding K6a) and inhibit expression of the mutant keratin, which results in PC, with little or no effect on wildtype expression in both tissue culture (including PC patient-derived keratinocytes analyzed by quantitative real time PCR) and mouse models (Hickerson et al., 2008;

<sup>1</sup>TransDerm Inc., Santa Cruz, California.

<sup>2</sup>SomaGenics Inc., Santa Cruz, California.

<sup>3</sup>Molecular Imaging Program at Stanford (MIPS), and <sup>4</sup>Department of Pediatrics, Radiology, and Microbiology and Immunology, Stanford University School of Medicine, Stanford, California.

<sup>5</sup>Thermo Fisher Scientific, Dharmacon Products, Lafayette, Colorado.

<sup>6</sup>Present address: Applied Biosystems, Austin, Texas.

Leachman et al., 2008 and data not shown). This siRNA (known as TD101 following formulation) has been approved for a phase 1b clinical trial (Leachman et al., 2008). Chemically modified versions of this siRNA were tested in tissue culture cells and in mouse models and were shown to have similar potencies when compared with unmodified counterparts. In some cases, however, these chemical modifications altered the thermodynamic properties resulting in loss of single nucleotide specificity (unpublished data). These observations, coupled with the goals of developing siRNAs that would be degraded if they reached the bloodstream (i.e., resulting in little or no system exposure) as well as minimizing potential toxicities resulting from chemical modifications, led to the investigation of the suitability of using unmodified siRNA *in vivo*.

In the present study, the stability of unmodified siRNAs was examined under a variety of conditions pertinent to storage, delivery, and potential topical formulations relevant to conducting clinical trials for genetic skin disorders. The stability profiles of siRNAs targeting the internal ribosome entry site of hepatitis C virus (HCV IRES) and enhanced green fluorescent protein (EGFP) were determined in parallel with the K6a siRNA. The HCV siRNA has been shown previously to inhibit HCV IRES-mediated gene expression (Wang et al., 2005; Vlassov et al., 2007), and the EGFP siRNA has been shown to block EGFP-reporter gene expression (Wang et al., 2007), both *in vitro* and *in vivo*.

## Materials and Methods

### Design of siRNA

SiRNAs (19+2 format; 19 nucleotide duplex with two 3' uracyl nucleotide overhangs) were synthesized by Thermo Fisher Scientific, Dharmacon Products (Lafayette, CO, USA). The sense and antisense strands for each siRNA are as follows: SMARTselected™ EGFP-specific siRNA, 5' GCACCAUCUUCUUCAAGGAUU and 5' P-UCCUUGAAGAAGAUGGUGCUU; K6a(N171K)-specific siRNA, 5' CCCUCAAAACAAGUUUGCUU (site of C to A mutation resulting in the N171K mutant protein is shown in lowercase) and 5' P-GCAAACUUGUUUUUGAGGGUU; and HCV-specific siRNA, 5' GCACGAAUCCUAAACCUCUU and 5' P-UGAGGUUAGGAUUCGUGCUU. The non-specific control (NSC4) siRNA (inverted beta galactosidase sequence, Thermo Fisher Scientific, Dharmacon Products Catalog #D-001210) sense and antisense sequences are 5' UAGCGACUAAACACAUCUAAUU and 5' P-UUGAUGUGUUUAGUCGCUAAUU, respectively.

### SiRNA preparation

SiRNAs were dissolved in phosphate-buffered saline (PBS, 200  $\mu$ M final concentration) and 5  $\mu$ L aliquots were removed for analysis. Unless otherwise noted, all siRNAs were stored at  $-20^{\circ}\text{C}$  and discarded after the initial freeze/thaw cycle.

### Stability assays at varied temperatures and in biological fluids

SiRNAs (5  $\mu$ L at 200  $\mu$ M in PBS) were stored at  $4^{\circ}\text{C}$  or  $21^{\circ}\text{C}$  for 4 weeks or alternatively at  $37^{\circ}\text{C}$  for 5 and 24 hours or  $95^{\circ}\text{C}$

for 10, 30, 60, and 120 minutes. All heated incubations were conducted in a thermocycler with a heated lid to limit evaporation and prevent concentration of the sample. Ten freeze/thaw cycles were performed by alternating cycles of freezing at  $-20^{\circ}\text{C}$  for 12 hours, followed by thawing at  $21^{\circ}\text{C}$  for 30 minutes.

SiRNAs (0.75  $\mu$ L of 200  $\mu$ M solution in PBS) were subjected to potential RNase degradation by incubation in various biological fluids (14.25  $\mu$ L) at  $37^{\circ}\text{C}$  for 10 minutes, 1 hour, 5 hours, and 48 hours. SiRNA stability in 95% blood serum was determined by incubation in fetal bovine serum (FBS; Hyclone, Logan, UT, USA, SH30070.03) or human serum (HS; Innovative Research, Southfield, MI, USA, IPLA-SERO). To test siRNA stability in contact with human skin, siRNA was incubated in PBS exposed to intact, live human skin (500  $\mu$ L PBS was collected from the palm of a volunteer hand after a 1 minute exposure with agitation). Similarly, siRNA was incubated in extract of human hair (10 hairs with roots were soaked in 500  $\mu$ L PBS for 24 hours at  $21^{\circ}\text{C}$ ) to test siRNA stability when in contact with human hair shafts and follicles. SiRNAs were digested in 95% fresh human saliva, at  $37^{\circ}\text{C}$  for 10 minutes, 1 hour, 5 hours, and 48 hours. As a control, siRNA was incubated in PBS with RNase A (Epicentre, Madison, WI) at  $10^{-3}$ ,  $10^{-2}$ ,  $10^{-1}$ , and 1 mg/mL final concentrations at  $37^{\circ}\text{C}$  for 10 minutes.

RNase and/or other proteins were removed from treated siRNAs by TE-saturated phenol extraction (one volume) followed by ethanol precipitation. Glycogen (1  $\mu$ L of 20 mg/mL solution from Roche, Basel, Switzerland) was added to facilitate quantitative recovery of RNA. An aliquot of each sample (50 pmoles per lane) was analyzed by 10% (19:1) native polyacrylamide gel electrophoresis (PAGE, Bio-Rad, Hercules, CA, USA) at 800V for 25 minutes. Gels were stained with ethidium bromide (0.1  $\mu$ g/mL) and visualized by phosphorimager (Bio-Rad, Molecular Imager FX).

### Firefly luciferase fusion constructs

Bicistronic constructs expressing both firefly luciferase (fLuc) and either EGFP (Wang et al., 2007), K6a(N171K) (Hickerson et al., 2008) or HCV (Wang et al., 2005) have been described previously.

### Cell culture

Human 293FT embryonic kidney cells (Invitrogen, Carlsbad, CA, USA) were maintained in DMEM (CAMBREX/BioWhittaker, Walkersville, MD, USA) with 10% FBS (HyClone, Logan, UT, USA), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate (CAMBREX/BioWhittaker, Walkersville, MD, USA) in a humidified 5%  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$ .

### Transient transfections of expression plasmids and siRNA

Functional siRNA inhibitory activity was determined, using an aliquot of the siRNA sample taken before electroporation, by cotransfection with reporter expression plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA)

according to the manufacturer's instructions. In brief, the day before transfection, 293FT cells were seeded at  $2.3 \times 10^4$  cells in 175  $\mu\text{L}$ /well in a 96-well plate, resulting in 80% cell confluency at the time of transfection. Cells were cotransfected (in triplicate) with a mixture of 16 ng expression plasmid, 1 nM siRNA (final concentration) and pUC19 (to give a final nucleic acid concentration of 160 ng per transfection), diluted to 13  $\mu\text{L}$  in optiMEM medium (Invitrogen). Lipofectamine 2000 (0.4  $\mu\text{L}$ ) was diluted in 12.6  $\mu\text{L}$  of optiMEM medium and incubated at room temperature for 5 minutes. This mixture was added to the nucleic acid mixture and incubated for 20 minutes at room temperature before addition to the plated cells.

Luciferase activity was measured at 48 hours following transfection by addition of 25  $\mu\text{L}$  of 3 mg/mL luciferin (BIOSYNTH International, Napperville, IL, USA; in PBS) to each well and incubation at room temperature for 5 minutes. The entire plate was imaged for 20 seconds using the IVIS<sup>TM</sup>50 *in vivo* imaging system (a Xenogen Product from Caliper Life Sciences, Alameda, CA, USA). FLuc activity was normalized to cells treated with nonspecific siRNA (i.e., the nonspecific control siRNA transfected with the EGFP expression plasmid was the HCV siRNA and the nonspecific control siRNA transfected with the K6a(N171K) or HCV plasmid was the EGFP siRNA).

### Immunohistochemistry

The immunocytochemistry procedure on a 10  $\mu\text{m}$  cryosection using an EGFP antibody directly conjugated to a fluorophore was performed as previously described (Cao et al., 2005). In brief, skin frozen in OCT medium was cryosectioned and OCT removed by washing with PBS for 5 minutes followed by incubation in 0.3% hydrogen peroxide for 2 minutes to quench endogenous peroxidase. Following a 5-minutes rinse in PBS, rabbit polyclonal antibody against GFP conjugated to Alexa Fluor 488, (Molecular Probes, cat# A21311, 1:200 dilution) was applied for 2 hours at room temperature. Following a 5-minutes PBS rinse, the sections were counterstained with DAPI and mounted with Gel-Mount aqueous media.

### Mice, footpad injections, and *in vivo* imaging

Transgenic L2G85 mice were obtained from a breeding colony at Stanford University. Animals were treated according to the Guidelines for Animal Care of both NIH and Stanford University.

*In vivo* imaging was performed on isoflurane-anesthetized mice using the Maestro Optical imaging system (CRi Inc., Woburn, MA, USA). Images were taken with an excitation filter of 445–490 nm and an emission filter of 515 nm (long-pass). Filter sets were set to capture images with 10 nm windows automatically from 500 to 850 nm using the Maestro software (exposure times were automatically calculated). Spectral un-mixing of the resulting TIFF image was performed using a user-defined EGFP library. Each spectrum was decided and set by un-mixing autofluorescence spectra and EGFP spectra manually selected using the computer mouse to select appropriate regions. Care was taken to

use the same settings for each image acquisition to allow the most quantitative analysis possible to enable comparison of data collected on different days. The un-mixed signal was pseudo-colored either green (Fig. 4) or white (Fig. 5). The white coloration allows better contrast facilitating inter-sample comparisons.

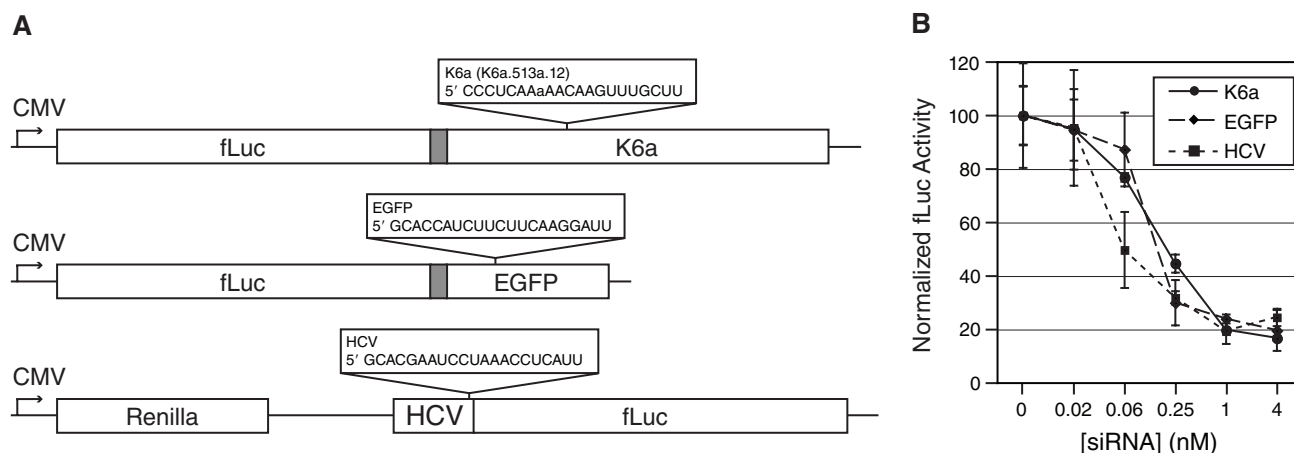
Mouse footpad injections were performed as recently described (Wang et al., 2007). EGFP-specific or NSC4 siRNAs were intradermally injected into footpads of L2G85 mice (three 50  $\mu\text{L}$  injections of 2  $\mu\text{g}/\mu\text{L}$  siRNA in PBS per mouse footpad spaced over 1 week) using a 28-gauge needle (Bectin Dickson and Company, Franklin Lakes, NJ, USA). At the indicated times, the mice were sedated using isoflurane, and live, anesthetized mice were imaged for EGFP expression using the CRi Maestro imaging system as described above.

## Results

To efficiently measure siRNA activity, a bioluminescence (luciferase)-based tissue culture assay was used. Bicistronic expression plasmids containing K6a (Hickerson et al., 2008), EGFP (Wang et al., 2007) or the HCV IRES (Wang et al., 2005; Vlassov et al., 2007) elements, linked to the fLuc reporter cDNA, were utilized (Fig. 1A). To determine the functional activity of the siRNAs designed to target each of these mRNAs, 293FT cells were cotransfected with a fixed amount of the appropriate expression plasmid and increasing concentrations of the corresponding inhibitor (Fig. 1B). As controls, K6a and HCV expression vectors were cotransfected with EGFP siRNA, while the EGFP plasmid was cotransfected with HCV siRNA. Each target was inhibited ~80% at 1 nM concentration of the specific siRNA, conditions under which no effect was observed following treatment with the nonspecific control siRNAs (data not shown). The 50% inhibitory concentration ( $\text{IC}_{50}$ ) values for each siRNA (K6a, EGFP, and HCV) were 200, 150, and 60 pM, respectively. On the basis of these results, all subsequent siRNA activity measurements were conducted at 1 nM final siRNA concentration.

To determine suitable storage conditions for siRNAs dissolved in PBS, stability assays were conducted at various temperatures and freeze/thaw conditions. Neither repeated freeze/thaw cycles (up to 10 cycles of slow freezing at  $-20^\circ\text{C}$  and thawing at  $21^\circ\text{C}$ ; Fig. 2A) nor extended incubations (up to 28 days at  $4^\circ\text{C}$  or  $21^\circ\text{C}$ ; Fig. 2B) were shown to have significant effects on siRNA integrity as measured by nondenaturing PAGE. Importantly, no loss of functional siRNA activity was detected when cotransfected with target expression plasmid into 293FT cells (Fig. 2, bar graphs). Following a 1-year incubation of K6a siRNA in PBS at  $4^\circ\text{C}$  or  $21^\circ\text{C}$ , there was no decrease in functional activity upon cotransfection with target plasmid (data not shown).

Formulation of siRNAs in topical creams or nanoparticles may require incubation at elevated temperatures for short periods of time. To determine the thermal stability of siRNAs (200  $\mu\text{M}$  in PBS), aliquots were incubated at  $37^\circ\text{C}$  for 24 hours or  $95^\circ\text{C}$  for 2 hours. EGFP, K6a, and HCV specific siRNAs incubated at  $37^\circ\text{C}$  for 24 hours showed no significant change in activity (i.e., EGFP siRNA activity decreased from 81% to 78% knockdown; both K6a siRNA and HCV siRNA activities slightly increased from 82% to 83% and from 76%



**FIG. 1.** Tissue culture-based assay for assessing functional siRNA activity. **(A)** Schematic representation of K6a, hepatitis C virus (HCV), and EGFP bicistronic reporter constructs [all linked to firefly luciferase (fLuc)] and the target sequences and locations for each siRNA. The K6a and EGFP coding regions are separated from the fLuc coding region by the foot and mouth disease virus (FMDV) 2A element [shaded gray, (Wang et al., 2007; Hickerson et al., 2008)]. **(B)** 293FT cells were cotransfected with each reporter plasmid and the indicated concentration of cognate siRNA. fLuc activity was measured following addition of luciferin substrate using the IVIS imaging system. The data were normalized and then corrected against cells transfected with a nonspecific control siRNA.

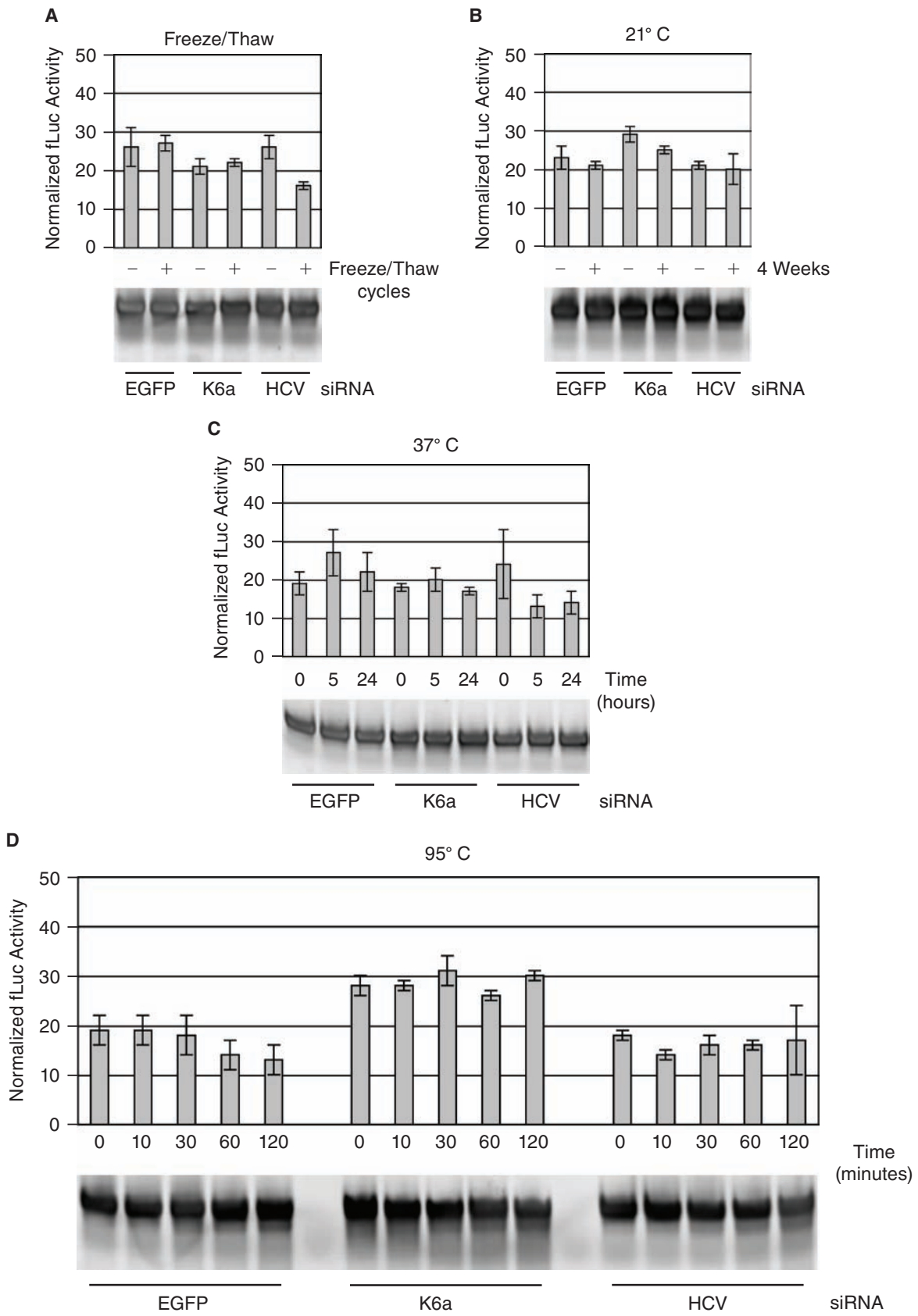
to 86%, respectively, Fig. 2C). Similarly, when these siRNAs were incubated at 95°C for 2 hours, no significant change in activity was observed (i.e., both EGFP and HCV siRNA activities increased from 81% to 87% and 82% to 83%, respectively; K6a siRNA activity slightly decreased from 72% to 70%, Fig. 2D). These data suggest, at least for time periods less than 2 hours, that unmodified siRNA is sufficiently stable for the majority of conditions that may be required for formulation.

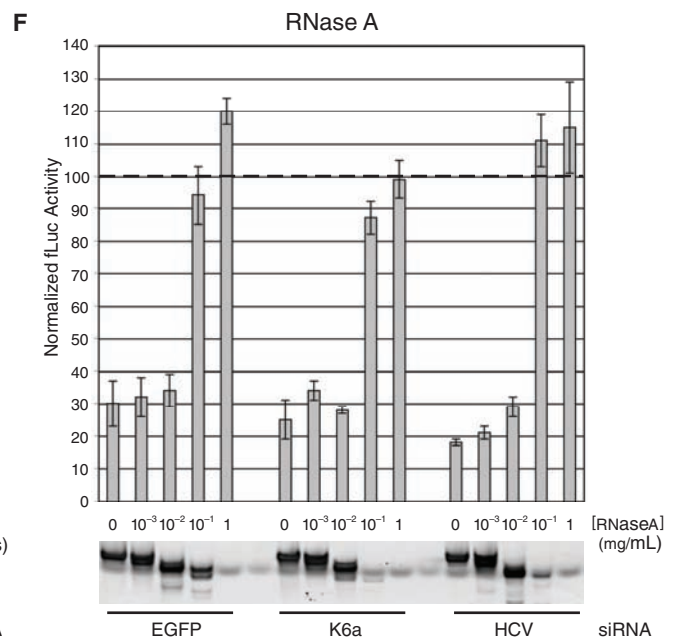
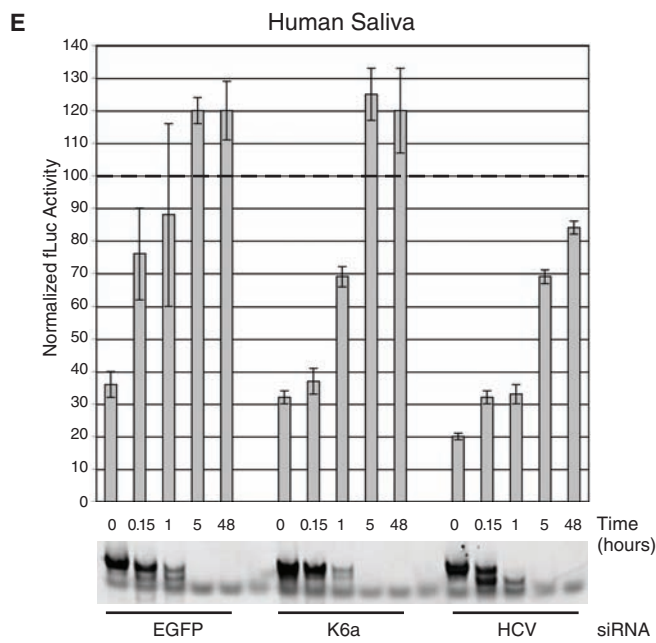
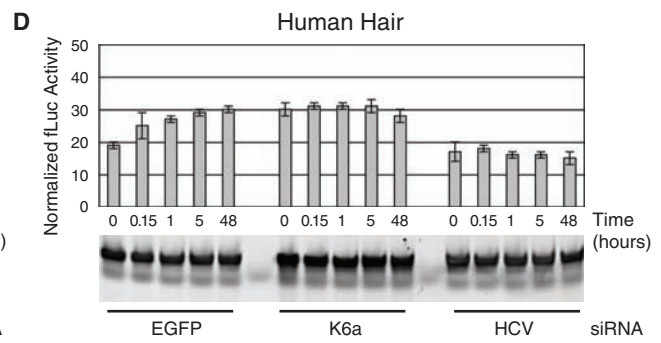
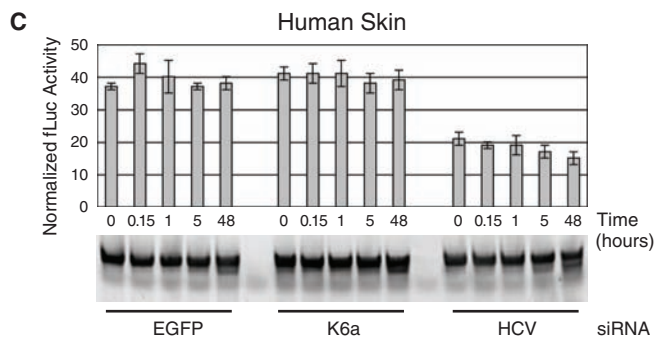
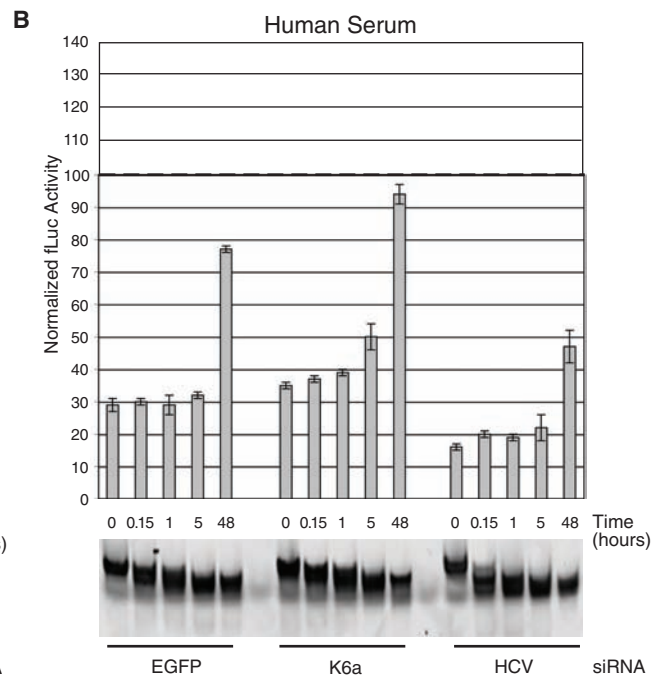
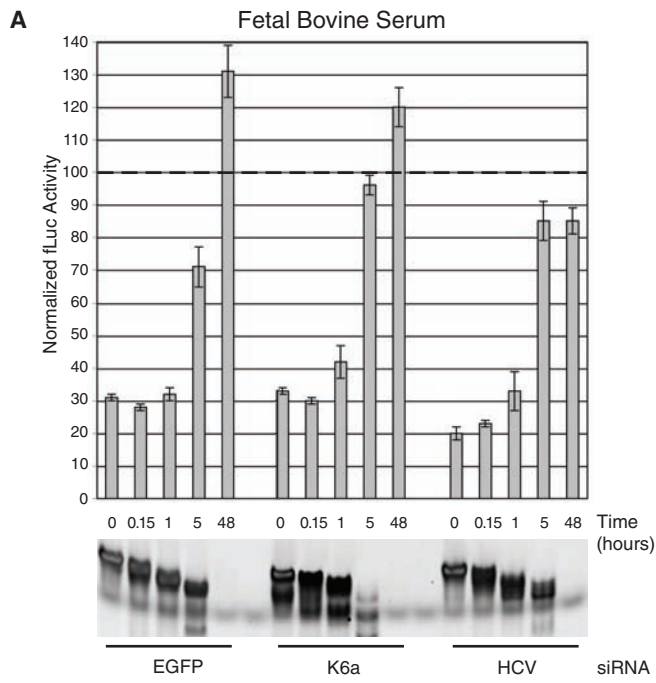
To evaluate the stability of siRNAs when exposed to blood, the inhibitors were incubated in 95% FBS or HS for various time periods up to 48 hours at 37°C. Incubation in FBS showed rapid degradation and loss of activity (Fig. 3A), whereas incubation in HS showed only partial degradation, even after 48 hours (Fig. 3B). Incubation of siRNAs in PBS exposed to human skin or hair for 48 hours at 37°C showed minor siRNA degradation but no change in functional activity (Fig. 3C and D). Strikingly, incubation of siRNA in 95% human saliva showed degradation of all three siRNAs with the EGFP siRNA being the most sensitive (62% loss of functional activity observed within 10 minutes Fig. 3E). As a positive control, siRNAs were incubated with increasing concentrations of RNase A (Fig. 3F). No degradation was observed at low RNase A concentrations and very little loss of activity (i.e., treatment with 10 µg/mL RNase A for 10 minutes at 37°C resulted in the following slight decrease

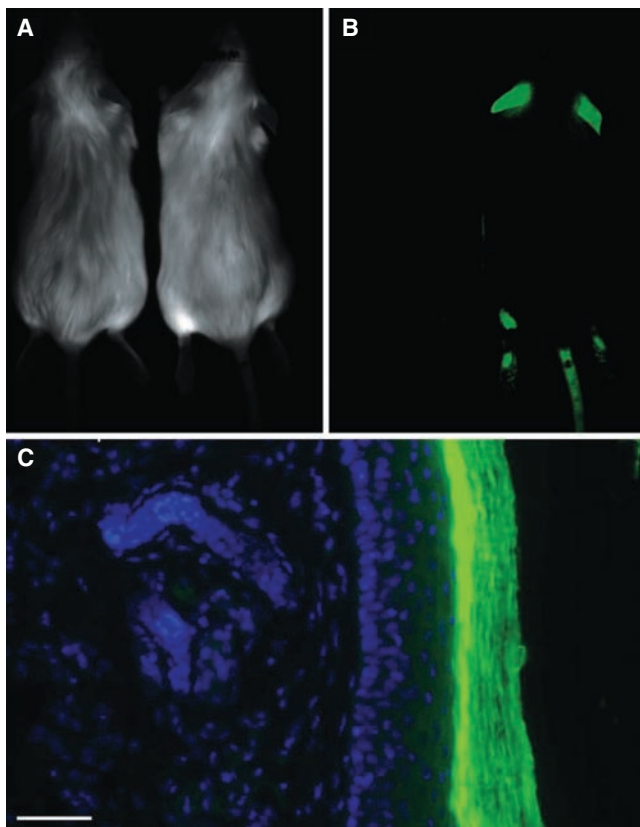
in activity for each siRNA when compared to untreated samples: EGFP siRNA, 70→66% inhibition; K6a siRNA, 75→72% inhibition; and HCV siRNA, 82→71% inhibition), suggesting that siRNAs are partially resistant to cleavage by this enzyme. Interestingly, the degradation observed by gel electrophoresis did not always correlate with loss of activity, suggesting that partially degraded siRNAs may retain full functional activity (e.g., comparison of the lanes treated with 10<sup>-2</sup> mg/mL RNase A with the untreated control lanes shows a clear change in electrophoretic mobility; however, no change in functional activity was observed, Fig. 3F). The change in mobility may be due to cleavage of the single-stranded 3' UU overhangs, as it has been reported that the 3' overhangs are not necessary for siRNA activity (Czauderna et al., 2003; Rose et al., 2005).

To evaluate whether unmodified siRNAs can inhibit pre-existing gene expression in an animal model, EGFP expressing transgenic mice [L2G85 line (Cao et al., 2005)] were treated with specific EGFP or control nonspecific siRNAs on opposing paws. EGFP has been reported to be expressed in the epidermis and stratum corneum in L2G85 mouse ears (Cao et al., 2005). EGFP is also expressed in mouse paws and readily visualized by *in vivo* fluorescence imaging and also immunostaining of skin footpad sections (Fig. 4). As the major complaint of PC patients is the painful blisters on the soles of the feet, the L2G85 mouse footpad was used to mimic

**FIG. 2.** SiRNA stability under conditions relevant to clinical use, including incorporation into cream formulations. Ten freeze/thaw cycles **(A)** were performed by alternating cycles of freezing at -20°C for 12 hours, followed by thawing at 21°C for 30 minutes. SiRNAs were stored at 21°C for 28 days **(B)** or alternatively at 37°C **(C)** or 95°C **(D)** for the indicated times. An aliquot of each sample (50 pmoles siRNA per lane) was analyzed by native 10% PAGE. Gels were stained with ethidium bromide and visualized by phosphorimager. Functional activity was determined following cotransfection of 1 nM siRNA (final concentration) with 16 ng fLuc fusion construct in 293FT cells as described in Figure 1.







**FIG. 4.** L2G85 EGFP transgenic mouse model. L2G85 mice (which express EGFP under the control of the modified chicken beta actin promoter; Cao et al., 2005) were assayed for GFP expression using the CRi Maestro *in vivo* imaging system. (A) Image following illumination with full-spectrum light. (B) EGFP-specific emission following excitation with blue light is pseudo-colored green (following un-mixing from background spectra, see Materials and Methods). Left mouse: nontransgenic control mouse. Right mouse: L2G85 mouse (expresses EGFP). Note that the fur blocks detection of fluorescence. Shaved L2G85 mice show EGFP expression in shaved regions (data not shown). (C) EGFP expression (antibody staining, see Materials and Methods) of a skin section prepared from the footpad of an L2G85 mouse (bar = 50  $\mu$ m). The section was counterstained with DAPI to allow visualization of nuclei (purple).

foot sole treatment. Comparison of skin sections from the footpad and from the ear of L2G85 mice showed similar localization of EGFP expression (Fig. 4; Cao et al., 2005 and data not shown). Spectral analysis of L2G85 mouse footpads using the CRi Maestro *in vivo* imaging system showed an emission spectrum indicative of EGFP expression (data not shown). We have previously shown that the EGFP-specific siRNA

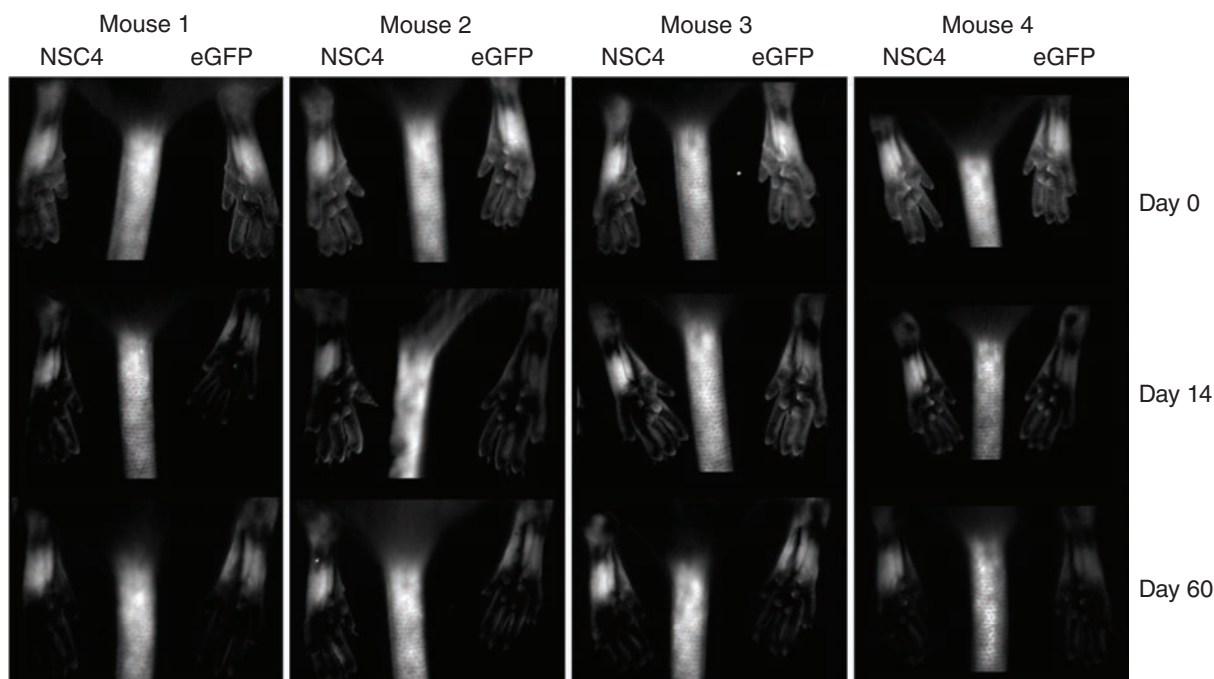
can specifically and potently block EGFP gene expression in tissue culture and mouse models when codelivered with a target vector (Wang et al., 2007). To determine whether intradermal injection of EGFP-specific siRNA can inhibit preexisting EGFP gene expression in a mouse model, three intradermal footpad injections were performed in L2G85 mice over a 7-day period and the amount of fluorescence was monitored over a 3-month period (Fig. 5). Mice treated with specific EGFP siRNA showed inhibition of fluorescence beginning at day 10 and reached maximum inhibition at day 14 (Fig. 5 and data not shown). This inhibition persisted for 2 months without additional treatment before EGFP signal began to reappear. In contrast, minor inhibition was observed in the paw treated with nonspecific siRNA (most likely due to the variability in the concentration of siRNA delivered to some skin cells due to the intradermal injection delivery method utilized; high levels of this siRNA have been shown to nonspecifically knock down reporter gene expression in tissue culture cells (data not shown)). These data suggest that inhibition can be long-lasting. Additional experiments are needed to determine if sustained inhibition, perhaps for years can be maintained by periodic administration.

## Discussion

In the past several years, there has been intense interest in developing siRNAs as therapeutics. The capacity of siRNAs to specifically and potently block gene expression offers unprecedented potential for treatment of congenital disorders (particularly dominant negative genetic mutations) and portends the advent of individualized medicine, in which siRNAs theoretically can be designed to target any given gene mutation. The unmodified K6a siRNA used in this study (also known as TD101) has been tested in a double-blind phase 1b clinical trial using escalating TD101 doses to locally treat PC foot lesions (by intradermal injection) of all known, eligible, and willing U.S. PC patients harboring the K6a N171K mutation (Leachman et al., 2008). This clinical trial is the "first-in-man" for siRNA skin treatment as well as the first siRNA trial to target a mutant gene. To date, only a few other siRNA therapeutics (including those developed for macular degeneration, respiratory syncytial virus, and acute renal failure) have entered clinical trials (Novobrantseva et al., 2008). If efficacy in the clinic is proven and delivery obstacles overcome, siRNA agents represent a new class of drugs with the potential to revolutionize treatment of a large number of disorders (de Fougerolles, 2008).

The siRNAs that are currently in clinical trials include both unmodified and chemically modified forms; the latter were designed to enhance nuclease resistance and greatly increase serum half-lives (Chiu and Rana, 2003; Czauderna et al., 2003; Layzer et al., 2004; de Fougerolles et al., 2005; Zhang et al., 2006; De Paula et al., 2007; Novobrantseva et al., 2008). Modifications that enhance serum stability

**FIG. 3.** Stability of siRNA exposed to skin, hair, saliva, and sera. SiRNAs (10  $\mu$ M final concentration) were subjected to potential RNase degradation by incubation in 95% fetal bovine serum (A), 95% human serum (B), PBS exposed to human skin (C), or hair (D), or 95% saliva (E) for the indicated times, or RNase A (F) for 10 minutes at the indicated concentrations. Treated siRNAs were analyzed as in Figure 2.



**FIG. 5.** SiRNA-mediated inhibition of preexisting gene expression in an EGFP transgenic mouse model. Treatment of L2G85 mice with unmodified EGFP-specific siRNA leads to decreased fluorescence. EGFP-specific or nonspecific control (NSC4) siRNAs were intradermally injected (100  $\mu$ g/injection) into footpads of L2G85 mice (three injections per mouse footpad spaced over 1 week,  $n = 4$ ). At the indicated times, the mice were imaged for EGFP expression using the Maestro (CRi) imaging system (EGFP expression is pseudo-colored white in these images; fluorescence is not observed in areas of fur) as described in Figure 4. This experiment has been independently repeated three times.

include backbone modifications such as phosphorothioate or boranophosphate linkages and 2'-OH modifications such as 2'-fluoro, 2'-O-methyl, 2'-O-(2-methoxyethyl), 2'-O-(2,4-dinitrophenyl), and locked nucleic acids and modifications of the termini such as 5'-phosphate, 5'-O-methyl, and 3'-deoxythymidine. Although chemical modifications can greatly enhance stability, the increase in half-life does not always result in a corresponding increase in functional activity (Layzer et al., 2004). In addition, it should be noted that many of the *in vivo* studies demonstrating disease protection were performed using unmodified inhibitors (Song et al., 2003a, 2003b; Bitko et al., 2005; Wesche-Soldato et al., 2005; Palliser et al., 2006), suggesting that serum stability is only one consideration in developing an effective therapeutic. Once taken up by cells and incorporated into the RNA-induced silencing complex (RISC), modified and unmodified siRNAs may have similar functional half-lives due to protection by binding to proteins in the RISC complex. In addition, siRNAs are rapidly cleared by the renal system [an elimination half-life of 6 minutes has been reported (Soutschek et al., 2004)]; therefore, the identification of modifications that allow directed delivery might be of greater importance than enhanced stability for development of therapeutics. Conjugation of lipids (particularly cholesterol) has been shown to not only increase thermodynamic and nuclease stability, but more importantly improve the biodistribution and pharmacokinetic profiles of siRNAs by targeting specific cell types [reviewed in (De Paula et al., 2007)].

In circumstances where localized delivery is preferred, it may be advantageous to use unmodified siRNA to limit unintended side effects resulting from siRNAs reaching the bloodstream. For example, in skin tissue, similar functional activities have been observed for both unmodified and modified siRNAs (Wang et al., 2007 and unpublished data), suggesting that stabilized siRNAs may not be necessary. [It should be noted, however, that skin has been reported (Probst et al., 2006) to contain RNase activity.] Therefore, in the case of skin delivery, there may be safety advantages in using unmodified siRNAs, as intact siRNAs that are cleared from the skin would then presumably be susceptible to increased degradation following entry to the bloodstream or other tissues with higher RNase activities. Indeed, the present results suggest that siRNAs are more quickly degraded in serum than in contact with skin (compare panels B and C in Fig. 3).

The data presented in this report suggest that unmodified siRNAs are quite stable in a variety of conditions and may be viable candidates for drug development, if tissues containing high levels of nucleases active against siRNAs are avoided. For example, exposure of unmodified siRNAs to hair or skin resulted in little, if any, reduction in activity, suggesting that topical application may be feasible. Furthermore, neither brief exposures to high temperature (up to 95°C) nor long incubation time at lower temperatures (i.e., 1 year at 4°C and 21°C) had any effect on activity. Taken together, these observations suggest that the conditions



required for formulating unmodified siRNAs into creams or nanoparticles or other delivery methodologies are unlikely to significantly inactivate the siRNA. Furthermore, the stability demonstrated following extended incubations at 4°C and 21°C suggest that siRNAs are sufficiently stable for formulation in saline solutions and likely in other formulations and will have adequate shelf lives in a clinical setting. Alternatively, the lack of any observable effects following repeated freeze/thawing suggests that even longer shelf lives are possible when stored in frozen form.

Unmodified siRNAs typically have half-lives of less than 20 minutes in serum as analyzed by PAGE (Czaderna et al., 2003; Morrissey et al., 2005), although longer-lived RNA duplexes (remarkably stable in serum for days) have been reported (Braasch et al., 2003). This variation in reported half-lives may be due to the biophysical properties of the siRNA duplex or to batch variability in serum. We have also observed that siRNAs are relatively resistant to degradation in fetal bovine and human sera. The changes in gel mobility seen upon treatment with serum and RNase A (Fig. 3A, B, and F) are both suggestive of initial “nibbling” of the ends with little loss of functional activity, followed by loss of integrity of the duplex band accompanied by loss of functional activity (e.g. see Fig. 2F). [Consistent with these observations, several laboratories (Czaderna et al., 2003; Rose et al., 2005) have reported similar activities for “blunt-end” siRNAs as compared to siRNAs that contain 3′ overhangs.] There is evidence that inactivation of siRNAs in serum is due to RNase A-like enzyme activity, as degradation of siRNAs in the presence of serum can be inhibited by RNaseOUT, a protein inhibitor of pancreatic ribonucleases such as RNase A, B, and C (Haupenthal et al., 2006, 2007). Moreover, mass-spectral analysis of degradation products of siRNAs exposed to serum shows that cleavage occurs particularly at UpA sequences near one end, suggestive of an RNase A-like activity (Turner et al., 2007). (It should be noted that the assay for RNA integrity used here, nondenaturing PAGE analysis with ethidium staining, is sensitive to loss of the full-length double-stranded molecule but may not reveal single strand breaks that do not cause the duplex to fall apart.)

Intradermal injection of siRNAs into footpads of an EGFP transgenic mouse model results in specific inhibition of reporter expression for 2 months (Fig. 5 and data not shown), suggesting that these unmodified inhibitors are sufficiently stable to reduce preexisting gene expression. Coupled with the increasing number of literature reports showing the effectiveness of unmodified (i.e., not chemically stabilized) siRNAs to achieve desirable outcomes in animal models (Song et al., 2003b; Palliser et al., 2006; Wang et al., 2007), these data suggest that unmodified siRNAs are viable candidates for clinical use.

## Acknowledgments

We thank Robert Reeves, Jonathan Hardy, Pauline Chu, Shay Keren, and Tim Doyle for excellent technical assistance with the *in vivo* imaging systems, and Peggy Kemper in the Contag lab for managing the L2G85 mouse colony (all from Stanford University). We thank Sergei Kazakov (Somagenics)

and Anne Dallas (Somagenics) for critical comments, Robert Kaspar (TransDerm) for assistance with data analysis, and Manuel Flores (TransDerm) for assistance in preparation of figures. The contributions of A.V.V., H.I., and B.H.J. were partially supported by NIH grant IR43AR054301. The contributions of R.L.K. and R.P.H. were partially supported by NIH grant IR43AR055881. E.G.G. is the recipient of a PC Project fellowship. We are grateful for the generous support, collegiality, and useful suggestions from members of The International Pachyonychia Congenita Consortium (IPCC) and PC Project, a 501(c)(3) public charity focused on finding a cure for PC.

## Disclosure Statement

R.L.K., R.P.H., B.H.J., and A.V.V. have filed patents related to using siRNAs as therapeutics. C.H.C. has financial interests in Caliper Life Sciences (maker of the IVIS imaging system).

## References

- BEHLKE, M.A. (2006). Progress towards *in vivo* use of siRNAs. *Mol. Ther.* **13**, 644–670.
- BITKO, V., MUSIYENKO, A., SHULYAYEVA, O., and BARIK, S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* **11**, 50–55.
- BRAASCH, D.A., JENSEN, S., LIU, Y., KAUR, K., ARAR, K., WHITE, M.A., and COREY, D.R. (2003). RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* **42**, 7967–7975.
- CAO, Y.A., BACHMANN, M.H., BEILHACK, A., YANG, Y., TANAKA, M., SWIJNENBURG, R.J., REEVES, R., TAYLOR-EDWARDS, C., SCHULZ, S., DOYLE, T.C., FATHMAN, C.G., ROBBINS, R.C., HERZENBERG, L.A., NEGRIN, R.S., and CONTAG, C.H. (2005). Molecular imaging using labeled donor tissues reveals patterns of engraftment, rejection, and survival in transplantation. *Transplantation* **80**, 134–139.
- CHIU, Y.L., and RANA, T.M. (2003). siRNA function in RNAi: a chemical modification analysis. *RNA* **9**, 1034–1048.
- CZADERNA, F., FECHTNER, M., DAMES, S., AYGUN, H., KLIPPEL, A., PRONK, G.J., GIESE, K., and KAUFMANN, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* **31**, 2705–2716.
- DALLAS, A., and VLASSOV, A.V. (2006). RNAi: a novel antisense technology and its therapeutic potential. *Med. Sci. Monit.* **12**, RA67–74.
- DE FOUGEROLLES, A., MANOHARAN, M., MEYERS, R., and VORNLOCHER, H.P. (2005). RNA interference *in vivo*: toward synthetic small inhibitory RNA-based therapeutics. *Methods Enzymol.* **392**, 278–296.
- DE FOUGEROLLES, A., VORNLOCHER, H.P., MARAGANORE, J., and LIEBERMAN, J. (2007). Interfering with disease: a progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov.* **6**, 443–453.
- DE FOUGEROLLES, A.R. (2008). Delivery vehicles for small interfering RNA *in vivo*. *Hum. Gene Ther.* **19**, 125–132.
- DE PAULA, D., BENTLEY, M.V., and MAHATO, R.I. (2007). Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. *RNA* **13**, 431–456.
- HAUPENTHAL, J., BAEHR, C., KIERNMAYER, S., ZEUZEM, S., and PIIPER, A. (2006). Inhibition of RNase A family enzymes prevents degradation and loss of silencing activity of siRNAs in serum. *Biochem. Pharmacol.* **71**, 702–710.

- HAUPENTHAL, J., BAEHR, C., ZEUZEM, S., and PIIPER, A. (2007). RNase A-like enzymes in serum inhibit the anti-neoplastic activity of siRNA targeting polo-like kinase 1. *Int. J. Cancer* **121**, 206–210.
- HICKERSON, R.P., SMITH, F.J., REEVES, R.E., CONTAG, C.H., LEAKE, D., LEACHMAN, S.A., MILSTONE, L.M., MCLEAN, W.H., and KASPAR, R.L. (2008). Single-nucleotide-specific siRNA targeting in a dominant-negative skin model. *J. Invest Dermatol.* **128**, 594–605.
- KIM, D.H., and ROSSI, J.J. (2007). Strategies for silencing human disease using RNA interference. *Nat. Rev. Genet.* **8**, 173–184.
- LAYZER, J.M., MCCAFFREY, A.P., TANNER, A.K., HUANG, Z., KAY, M.A., and SULLENGER, B.A. (2004). In vivo activity of nuclease-resistant siRNAs. *RNA* **10**, 766–771.
- LEACHMAN, S., KASPAR, R., FLECKMAN, P., FLORELL, S., SMITH, F., MCLEAN, W., LUNY, D., MILSTONE, L., VAN STEENSEL, M., DIGIOVANNA, J., EPSTEIN, E., MUNRO, C., O'TOOLE, E., CELEBI, J., KANSKY, A., and Lane E.B. (2005). Clinical and pathological features of Pachyonychia congenita. *J. Invest. Derm. Symp.* **P. 10**, 3–17.
- LEACHMAN, S.A., HICKERSON, R.P., HULL, P.R., SMITH, F.J.D., MILSTONE, L.M., LANE, E.B., BALE, S.J., ROOP, D.R., MCLEAN, W.H.I., and KASPAR, R.L. (2008). Therapeutic siRNAs for dominant genetic skin diseases including pachyonychia congenita. *J. Dermatol. Sci.* **51**, 151–157.
- MORRISSEY, D.V., BLANCHARD, K., SHAW, L., JENSEN, K., LOCKRIDGE, J.A., DICKINSON, B., MCSWIGGEN, J.A., VARGESE, C., BOWMAN, K., SHAFFER, C.S., POLISKY, B.A., and ZINNEN, S. (2005). Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* **41**, 1349–1356.
- NOVOBRANTSEVA, T.I., AKINC, A., BORODOVSKY, A., and DE FOUGEROLLES, A. (2008). Delivering silence: advancements in developing siRNA therapeutics. *Curr Opin Drug Discov Devel* **11**, 217–224.
- PALLISER, D., CHOWDHURY, D., WANG, Q.Y., LEE, S.J., BRONSON, R.T., KNIPE, D.M., and LIEBERMAN, J. (2006). An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* **439**, 89–94.
- PROBST, J., BRECHTEL, S., SCHEEL, B., HOERR, I., JUNG, G., RAMMENSEE, H.G., and PASCOLO, S. (2006). Characterization of the ribonuclease activity on the skin surface. *Genet Vaccines Ther.* **4**, 4.
- ROSE, S.D., KIM, D.H., AMARZGUIOUI, M., HEIDEL, J.D., COLLINGWOOD, M.A., DAVIS, M.E., ROSSI, J.J., and BEHLKE, M.A. (2005). Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res.* **33**, 4140–4156.
- SMITH, F.J.D., KASPAR, R.L., SCHWARTZ, M.E., MCLEAN, W.H.I., and LEACHMAN, S.A. (2006). Pachyonychia congenita. *GeneReviews*: [www.genetests.org/profiles/pc](http://www.genetests.org/profiles/pc).
- SMITH, F.J.D., LIAO, H., CASSIDY, A.J., STEWART, A., HAMILL, K.J., WOOD, P., JOVAL, I., VAN STEENSEL, M.A.M., BJORCK, E., CALLIF-DALEY, F., PALS, G., COLLINS, P., LEACHMAN, S.A., MUNRO, C.S., and MCLEAN, W.H.I. (2005). The genetic basis of Pachyonychia congenita. *J. Investig. Dermatol. Symp. Proc.* **10**, 21–30.
- SONG, E., LEE, S.K., DYKXHOORN, D.M., NOVINA, C., ZHANG, D., CRAWFORD, K., CERNY, J., SHARP, P.A., LIEBERMAN, J., MANJUNATH, N., and SHANKAR, P. (2003a). Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. *J. Virol.* **77**, 7174–7181.
- SONG, E., LEE, S.K., WANG, J., INCE, N., OUYANG, N., MIN, J., CHEN, J., SHANKAR, P., and LIEBERMAN, J. (2003b). RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347–351.
- SOUTSCHEK, J., AKINC, A., BRAMLAGE, B., CHARISSE, K., CONSTIEN, R., DONOGHUE, M., ELBASHIR, S., GEICK, A., HADWIGER, P., HARBORTH, J., JOHN, M., KESAVAN, V., LAVINE, G., PANDEY, R.K., RACIE, T., RAJEEV, K.G., ROHL, I., Toudjarska, I., WANG, G., WUSCHKO, S., BUMCROT, D., KOTELIANSKY, V., LIMMER, S., MANOHARAN, M., and VORNLOCHER, H.P. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178.
- VLASSOV, A.V., KORBA, B., FARRAR, K., MUKERJEE, S., SEYHAN, A.A., ILVES, H., KASPAR, R.L., LEAKE, D., KAZAKOV, S.A., and JOHNSTON, B.H. (2007). shRNAs targeting hepatitis C: effects of sequence and structural features, and comparison with siRNA. *Oligonucleotides* **17**, 223–236.
- WANG, Q., CONTAG, C.H., ILVES, H., JOHNSTON, B.H., and KASPAR, R.L. (2005). Small hairpin RNAs efficiently inhibit hepatitis C IRES-mediated gene expression in human tissue culture cells and a mouse model. *Mol. Ther.* **12**, 562–568.
- WANG, Q., ILVES, H., CHU, P., CONTAG, C.H., LEAKE, D., JOHNSTON, B.H., and KASPAR, R.L. (2007). Delivery and inhibition of reporter genes by small interfering RNAs in a mouse skin model. *J. Invest. Dermatol.* **127**, 2577–2584.
- WESCHE-SOLDATO, D.E., CHUNG, C.S., LOMAS-NEIRA, J., DOUGHTY, L.A., GREGORY, S.H., and AYALA, A. (2005). In vivo delivery of caspase-8 or Fas siRNA improves the survival of septic mice. *Blood* **106**, 2295–2301.
- ZHANG, H.Y., DU, Q., WAHLESTEDT, C., and LIANG, Z. (2006). RNA interference with chemically modified siRNA. *Curr. Top Med. Chem.* **6**, 893–900.

Address reprint requests to:  
 Dr. Roger L. Kaspar  
 2161 Delaware Ave. Suite D  
 Santa Cruz, CA 95060

E-mail: [Roger.Kaspar@TransDermInc.com](mailto:Roger.Kaspar@TransDermInc.com)

Received for publication May 31, 2008; accepted after revision August 6, 2008.