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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.

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Use of Self-Delivery siRNAs to Inhibit Gene Expression in an Organotypic Pachyonychia Congenita Model

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Although RNA interference offers therapeutic potential for treating skin disorders, delivery hurdles have hampered clinical translation. We have recently demonstrated that high pressure, resulting from intradermal injection of large liquid volumes, facilitated nucleic acid uptake by keratinocytes in mouse skin. Furthermore, similar intradermal injections of small interfering RNA (siRNA; TD101) into pachyonychia congenita (PC) patient foot lesions resulted in improvement. Unfortunately, the intense pain associated with hypodermic needle administration to PC lesions precludes this as a viable delivery option for this disorder. To investigate siRNA uptake by keratinocytes, an organotypic epidermal model, in which pre-existing endogenous gene or reporter gene expression can be readily monitored, was used to evaluate the effectiveness of "self-delivery" siRNA (i.e., siRNA chemically modified to enhance cellular uptake). In this model system, self-delivery siRNA treatment resulted in reduction of pre-existing fluorescent reporter gene expression under conditions in which unmodified controls had little or no effect. Additionally, treatment of PC epidermal equivalents with self-delivery "TD101" siRNA resulted in marked reduction of mutant keratin 6a mRNA with little or no effect on wild-type expression. These results indicate that chemical modification of siRNA may overcome certain limitations to transdermal delivery (specifically keratinocyte uptake) and may have clinical utility for inhibition of gene expression in the skin.

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INTRODUCTION

Since the discovery that small interfering RNAs (siRNAs) can effectively silence gene expression in a number of mammalian systems, there has been increasing interest in developing these inhibitors as therapeutics to treat a variety of diseases, including those of the skin. Although siRNA-based therapeutics are currently in clinical trials for a number of targets, including the liver, kidney, lung, skin, and eye, as recently reviewed (Vaishnaw *et al.*, 2010), a number of inherent physical barrier properties of the skin represent formidable hurdles to siRNA delivery and progress into the clinic has been impeded.

To develop effective siRNA-based therapeutics for treatment of skin disorders, it is necessary to first identify potent and specific inhibitors and then develop effective delivery methods for these nucleic acid inhibitors to target cells in the skin. For the treatment of pachyonychia congenita (PC), we have identified inhibitors that are both selective and potent (Hickerson *et al.*, 2008; Smith *et al.*, 2008). The first reported siRNA clinical trial (Phase 1b) in skin used one of these inhibitors (TD101) to treat this rare skin disorder, and some efficacy was noted (Leachman *et al.*, 2008, 2010). PC is a dominant negative skin disorder caused by mutations in the inducible keratin genes, including *KRT6A*, *KRT6B*, *KRT16*, or *KRT17* genes, which often result in thickened dystrophic nails, leukokeratosis, and acanthosis histologically with painful blistering (Leachman *et al.*, 2005; Smith *et al.*, 2006). Detailed clinical and genetic analysis of PC is reported by Wilson *et al.*, this issue, and will be reported by Eliason *et al.* (personal communications). TD101 siRNA (also known as K6a_513a.12 siRNA, see Hickerson *et al.*, 2008) specifically targets the keratin 6a (K6a) single C to A nucleotide mutation, which results in the K6a p.Asn171Lys amino acid mutation.

Delivery of functional siRNA to skin (specifically to the epidermal keratinocytes) involves (i) stratum corneum penetration and (ii) keratinocyte uptake followed by incorporation into the RNA-induced silencing complex. Although hydrodynamic intradermal injection of unmodified, standard siRNA (i.e., siRNA without the modifications that allow for "self-delivery") to mouse skin resulted in target inhibition (Gonzalez-Gonzalez *et al.*, 2009), treatment methods that do not involve generation of

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Abbreviations: EGFP, enhanced green fluorescent protein; HPEKp, human primary epidermal keratinocyte progenitors; K, keratin protein; KRT, keratin gene; PC, pachyonychia congenita; tdTOM, tandem tomato fluorescent protein

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pressure, such as topical formulations or microneedles, do not result in target knockdown (Gonzalez-Gonzalez *et al.*, 2010b and unpublished data). In this study, we have evaluated keratinocyte uptake and demonstrate that chemically-modified self-delivery siRNAs can inhibit target gene expression in human organotypic epidermal cultures. These epidermal equivalents represent a keratinocyte siRNA uptake model to evaluate the effectiveness of modified siRNAs for their ability to inhibit endogenous or reporter gene expression when added to the organotypic culture medium. This allows the rapid assessment of keratinocyte uptake of functional siRNA, bypassing the complicating stratum corneum barrier.

RESULTS

Self-delivery siRNAs block reporter expression in primary human keratinocytes

In vivo imaging of fluorescent and bioluminescent reporters offers the opportunity to quantitatively and repeatedly monitor siRNA-mediated effects non-invasively, allowing spatiotemporal analyses to be performed. Use of these reporters in human primary keratinocyte and skin equivalent models serves to accelerate and refine the study of siRNA delivery and the effects of these agents.

In order to take advantage of intravital fluorescence imaging systems, human primary epidermal keratinocyte progenitor (HPEKp) cells were transduced with two different lentiviral reporter constructs to generate a multi-reporter cell line expressing enhanced green fluorescent protein (EGFP) and a fusion reporter gene (Luc2/tdTOM) comprised of a highly modified luciferase gene (Luc2) and tandem tomato fluorescent protein (tdTOM), as shown in Figure 1a. This fusion construct contains the target site for an siRNA called CBL3 (Gonzalez-Gonzalez *et al.*, 2009). We have previously reported that unmodified and self-delivery (Accell) CBL3 siRNAs potently and specifically reduced reporter gene expression in human 293FT cells and in a transgenic

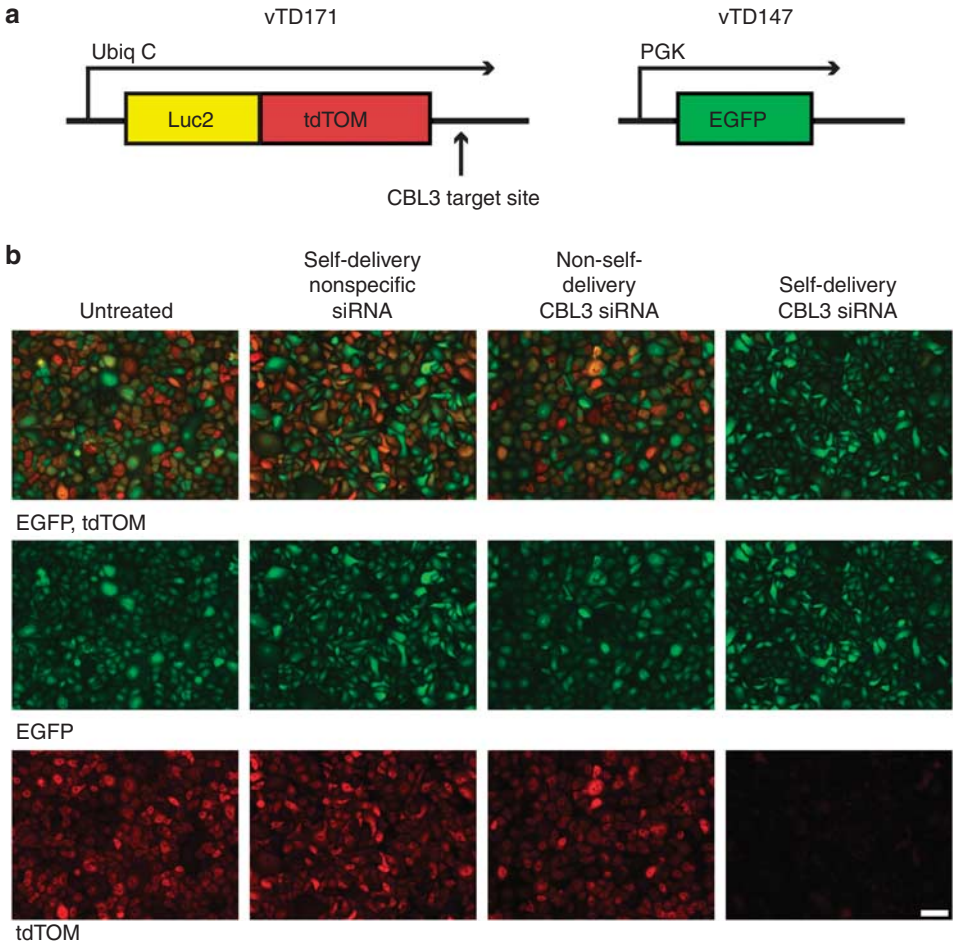
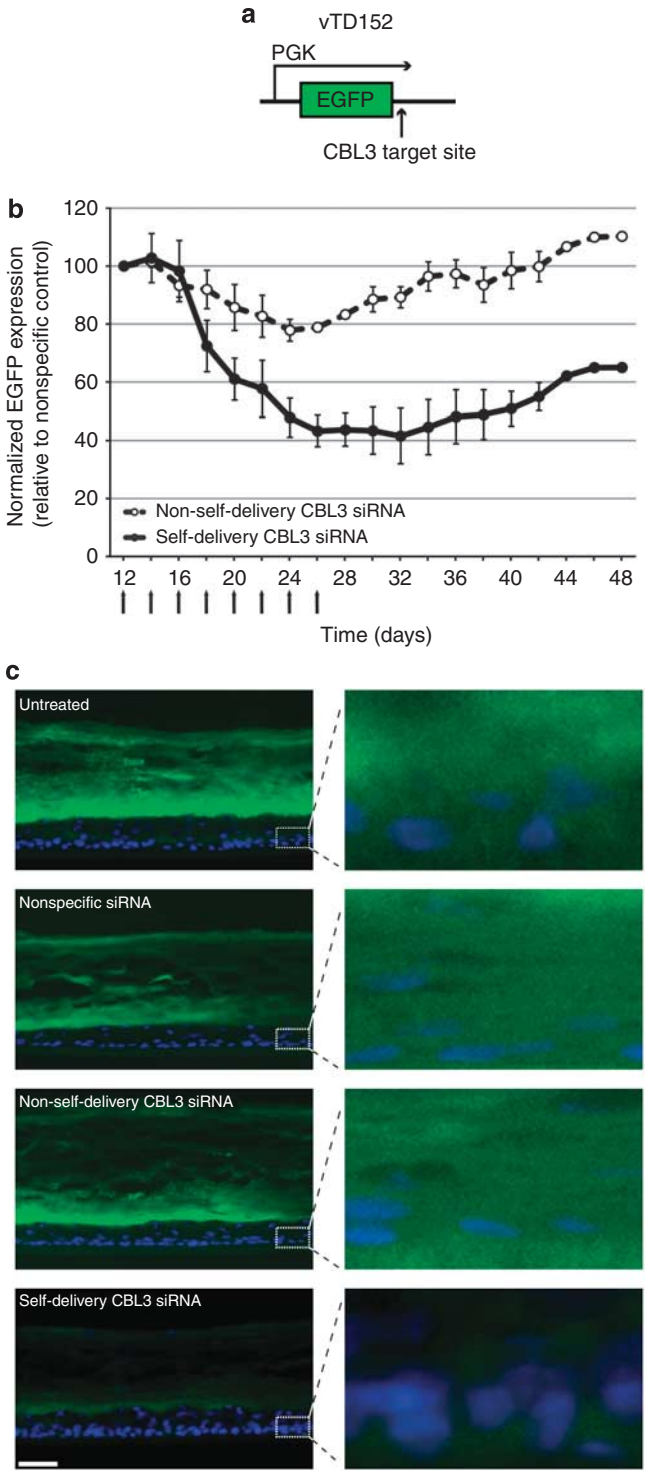


Figure 1. Specific inhibition of reporter protein in a dual fluorescent reporter human primary keratinocyte cell line using self-delivery small interfering RNAs (siRNAs). (**a**) Schematic representation of the lentiviral expression plasmids used to generate the dual reporter cell line. vTD171 expresses tandem tomato fluorescent protein (tdTOM), containing the CBL3 siRNA target site in the 3′untranslated region, under the control of the human ubiquitin C promoter. vTD147 expresses enhanced green fluorescent protein (EGFP) under the control of the human phosphoglycerate kinase (PGK) promoter. (**b**) Human primary epidermal keratinocyte progenitor (HPEKp) cells were transduced with vTD171 and sorted for cells expressing tdTOM. The collected cells were then transduced with vTD147 and sorted for cells expressing EGFP. The resulting cell line was positive for both tdTOM and EGFP expression in >95% of the cells. Cells were treated 24 hours after seeding with 2 μM (final concentration) of the indicated siRNAs (self-delivery nonspecific control K6a_513a.12 siRNA, non-self-delivery CBL3 siRNA, and self-delivery CBL3 siRNA) and imaged 96 hours after treatment. Scale bar = 100 μm.

reporter mouse model (Gonzalez-Gonzalez *et al.*, 2009, 2010b).

Human PEKp cells were treated with 2 μM self-delivery CBL3 siRNA (Accell) and analyzed for reporter fluorescence 96 hours after treatment. Specific knockdown of tdTOM expression was visualized by fluorescence microscopy following treatment with the self-delivery CBL3 siRNA, while as expected, treatment with controls (including non-self-delivery CBL3) had little or no effect (Figure 1b). No relative EGFP inhibition was observed with control or specific siRNAs, as this construct does not contain the CBL3 siRNA target site (see Figure 1a).



Self-delivery siRNA knocks down reporter gene expression in skin equivalents

To evaluate the ability of self-delivery CBL3 siRNA to inhibit target reporter gene expression in a human epidermal model, HPEKp cells were transduced with vTD152, which contains the EGFP coding region followed by the CBL3 siRNA target site (Figure 2a). Epidermal equivalents prepared from EGFP-transduced cells readily differentiate, stratify, and maintain EGFP expression (Figure 2c). On day 12 following addition of the differentiation medium (skin equivalents fully differentiate by day 14; see Supplementary Figure S1 online), epidermal equivalents were treated every other day with self-delivery CBL3 siRNA and control siRNAs for 14 days (eight treatments total) and repeatedly imaged for EGFP expression before each treatment. EGFP reporter levels in epidermal equivalents treated with self-delivery CBL3 siRNA decreased 57 ± 5% by day 26 relative to treatment with nonspecific control siRNA (K6a_513a.12) and remained at that level for over a week before beginning to return to baseline levels (Figure 2b). Treatment with non-self-delivery CBL3 siRNA resulted in slight inhibition of reporter signal (21 ± 1%) that quickly returned to baseline once treatment ceased. On day 42, a representative epidermal equivalent from each treatment group was sectioned and imaged for EGFP expression. Skin equivalents treated with self-delivery CBL3 siRNA showed decreased expression of EGFP in all epidermal strata, including the live layers, which was not observed in the controls (Figure 2c).

Use of patient biopsies to generate a PC disease model

In order to determine if self-delivery siRNAs selectively inhibit mutant gene expression in a PC model, primary keratinocytes were isolated from two non-related PC patients harboring the K6a p.Asn171Lys mutation (IPCRR patients no. 10 and no. 799). Self-delivery K6a_513a.12 siRNA efficiently reduces mutant mRNA expression with little effect

Figure 2. Self-delivery enhanced green fluorescent protein (EGFP)-specific small interfering RNA (siRNA) inhibits reporter expression in transduced skin equivalents. (**a**) Human primary epidermal keratinocyte progenitor (HPEKp) cells were transduced with vTD152 lentivirus expressing EGFP containing the CBL3 siRNA target site in the 3′ untranslated region and used to generate epidermal equivalents (see Materials and Methods). (**b**) Assessment of self-delivery siRNA functional activity by fluorescence imaging. The skin equivalents were allowed to mature for ~2 weeks before treatment with 2 μM siRNA (unmodified nonspecific K6a_513a.12 siRNA, non-self-delivery CBL3 siRNA, or self-delivery CBL3 siRNA; in triplicate) every 48 hours. Skin equivalents were repeatedly imaged every 48 hours for EGFP fluorescence. (**c**) Sectioning of treated skin equivalents. On day 44, one of the three skin equivalents from each treatment group was frozen in optimal cutting temperature compound and sectioned (10 μm). The slides were mounted with Hydromount containing 4′,6-diamidino-2-phenylindole (DAPI) to counterstain nuclei. Sections were imaged using a fluorescence microscope equipped with GFP and DAPI filter sets. Scale bar = 50 μm.

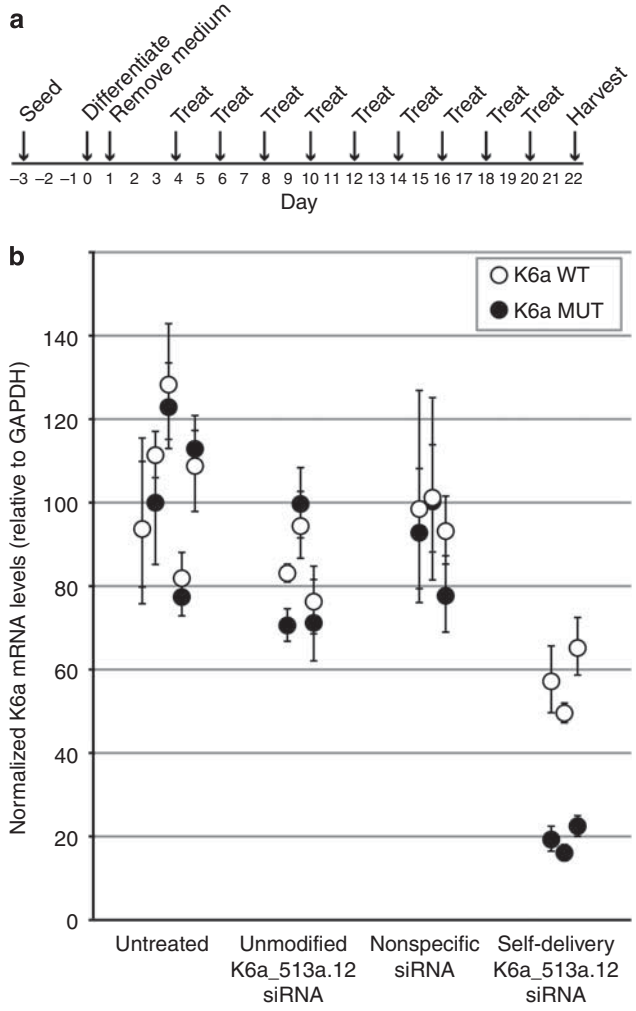


Figure 3. Self-delivery K6a_513a.12 small interfering RNA (siRNA) selectively inhibits mutant K6a mRNA expression in skin equivalents prepared from pachyonychia congenita (PC) patient primary keratinocytes. (**a**) Timeline summarizing treatment schedule. (**b**) Skin equivalents prepared from patient keratinocytes were treated with 2 μM self-delivery K6a_513a.12 and control siRNAs as indicated (non-self-delivery K6a_513a.12 siRNA, unmodified enhanced green fluorescent protein (EGFP)-specific siRNA, and self-delivery K6a_513a.12 siRNA; in triplicate). Total RNA was isolated and mutant (Mut; p.Asn171Lys) and wild-type (WT) K6a mRNA levels were determined by quantitative reverse transcription PCR relative to glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

on wild-type K6a expression in monolayer tissue culture experiments in both cell lines (data not shown). Epidermal equivalents were prepared from primary keratinocytes isolated from IPCRR patient no. 799. Beginning on day 4 post-differentiation, the epidermal equivalents were treated every other day (nine treatments total; Figure 3a). Two days following the final treatment, the equivalents were collected and half of each was used for immunohistochemical analysis and the remaining half for quantitative reverse transcription PCR. As expected, immunohistochemical staining showed expression of keratin 5 (K5) predominantly in the basal layer and keratin 10 (K10) in the suprabasal layers, similar to skin equivalents prepared from pooled normal HPEKp cells (Supplementary Figure S1 online). Mutant K6a mRNA levels were reduced 80% in epidermal equivalents treated with self-delivery K6a_513a.12 siRNA compared with 42% reduction of wild-type mRNA levels relative to untreated controls. Mutant and wild-type mRNA levels remained unchanged relative to untreated controls in epidermal equivalents treated with control siRNAs (Figure 3b).

DISCUSSION

RNA interference has therapeutic potential for treating a variety of disorders, including those of the skin. Skin is an attractive target for siRNA-based therapeutics, as it is readily accessible for localized treatment (minimizing potential problems associated with systemic administration) and monitoring (visually, with intravital imaging, and/or by biopsy). The ability to design, screen and identify potent, selective, and stable siRNAs is now relatively straightforward. We and others have developed siRNAs that have single-nucleotide specificity in tissue culture cells and mice (Schwarz *et al.*, 2006; Hickerson *et al.*, 2008). Unfortunately the ability to deliver these potential therapeutics to appropriate tissues and cells has not kept pace.

Two of the major obstacles to siRNA delivery to skin are penetration through the stratum corneum barrier and efficient functional siRNA uptake by keratinocytes, including incorporation into the RNA-induced silencing complex. The stratum corneum barrier, composed of terminally differentiated squamous cells containing a hydrophobic intercellular lipid matrix, prevents efficient penetration of large (>500 Da), charged molecules (e.g., siRNA is highly anionic and ~13,000 Da) that are topically administered. The advantages and disadvantages of a number of methods to facilitate stratum corneum penetration have been summarized in a recent review (Geusens *et al.*, 2009), including tape stripping, ballistic delivery, microneedle application, intradermal injection, sonophoresis, electroporation, jet injection, iontophoresis, chemical enhancers, lipid-based systems, and chemical depilation.

The methods used for efficient nucleic acid (including siRNA) delivery across the stratum corneum barrier (e.g., by intradermal injection or microneedles) do not necessarily result in efficient uptake and functional activity. A number of methods to facilitate cellular siRNA uptake in skin have been reported. High pressure (''pressure fection''), resulting from intradermal injection of relatively large volumes of liquid results in enhanced delivery and expression of plasmid constructs (Gonzalez-Gonzalez *et al.*, 2010a) and likely uptake of functional siRNA (Gonzalez-Gonzalez *et al.*, 2009, 2010b) by keratinocytes in mouse skin. Electroporation has been shown to increase membrane permeability and enhance delivery of molecules to tissues *in vivo* (Heller and Heller, 2006), including siRNA in a skin xenograft mouse model (Takei *et al.*, 2008). Furthermore, Kigasawa *et al.* (2010) showed that iontophoresis facilitates siRNA penetration through the stratum corneum and subsequent silencing of an endogenous gene (IL-10) in rats.

A variety of chemical methods allowing cellular uptake has also been reported. Lipid-based formulations are highly

effective *in vitro*, and some efficacy *in vivo* has been reported, as recently reviewed (Whitehead *et al.*, 2009). Binding of peptide transduction domains or cell penetrating peptides non-covalently to siRNA via a double-stranded RNA binding domain facilitates siRNA delivery, as recently reviewed (Meade and Dowdy, 2009). Administration of these peptide–siRNA complexes has been shown to reduce target gene expression in a mouse xenograft brain tumor model (Michiue *et al.*, 2009). Modified siRNAs containing lipophilic groups have also been shown to specifically inhibit target gene expression without the use of carrier molecules or transfection reagents. Alnylam Pharmaceuticals demonstrated that conjugation of lipid and steroid groups increases delivery efficiency both *in vitro* and *in vivo* (Lorenz *et al.*, 2004; Soutschek *et al.*, 2004). More recently, Wu *et al.* (2009) demonstrated that intravaginal administration of cholesterol-conjugated siRNA allowed protection from HSV-2 in mice. Other self-delivery siRNAs are being developed by RXi Pharma (sd-rxRNA, http://www.rxipharma.com).

In this study, we have evaluated Accell self-delivery siRNAs (CBL3 and K6a_513a.12) and demonstrate specific target inhibition in epidermal skin equivalents expressing reporter proteins or skin equivalents prepared from PC patient-derived keratinocytes by repeated and non-invasive imaging over the course of the experiment. Recently, self-delivery siRNAs delivered by microneedle arrays to transgenic reporter mice have been shown to silence target gene expression (Gonzalez-Gonzalez *et al.*, 2010b), suggesting that this approach may have clinical utility, in which direct, local administration is feasible. Owing to the proprietary nature of the self-delivery siRNAs (at least for the Dharmacon and RXi inhibitors), it is unknown if these inhibitors are working through a similar mechanism(s) such as conjugations of lipids or steroid moieties, as demonstrated by Alnylam Pharmaceuticals or through a distinct mechanism.

Although several studies have described successful delivery of siRNA to skin, improvements are likely needed for successful translation to the clinic (for a review, see Geusens *et al.*, 2009). For example, although intradermal injection delivers functional unmodified siRNA to skin keratinocytes in mouse models (Wang *et al.*, 2007; Hickerson *et al.*, 2008; Smith *et al.*, 2008; Gonzalez-Gonzalez *et al.*, 2009), this delivery is likely mediated through high pressure as described above (Gonzalez-Gonzalez *et al.*, 2009, 2010a) and may result in unacceptable pain levels in some indications, especially where large areas of skin require treatment. For example, in the recently completed Phase 1b PC clinical trial using TD101 siRNA to target a mutant keratin 6a mRNA, intradermal injection resulted in significant improvements in PC symptoms; however, the pain associated with administration to the exquisitely painful PC calluses necessitated oral pain medication and regional nerve blocks to make treatment bearable (Leachman *et al.*, 2010).

In light of the limitations of the currently available siRNA skin delivery technologies, the field would greatly benefit from the development of patient-friendly technologies or refinements of existing technologies that allow siRNA to be efficiently delivered across the stratum corneum and allow functional uptake by keratinocytes. At a meeting held last year in Montreal entitled ''Achieving Successful Delivery of Nucleic Acids to Skin'' the attendees concluded that effective siRNA-based therapeutics will likely require a combination of methodologies that facilitate stratum corneum penetration and keratinocyte uptake (Kaspar *et al.*, 2009; Gonzalez-Gonzalez *et al.*, 2010b). Furthermore, a consensus arose that in order to make rapid progress toward development of patient-friendly nucleic acid delivery technologies, standardized *in vitro* and *in vivo* models are needed as well as common pools of nucleic acids (including siRNAs) to allow a comparative ''apples to apples'' evaluation. To this end, a National Institutes of Health Grand Opportunities (GO Delivery!) proposal was recently funded with the aim of (i) bringing together researchers with expertise in nucleic acid delivery; (ii) developing reagents, model assay systems and analysis tools that can be used in a validated and transferable algorithm for testing delivery of nucleic acids to cells in the epidermis; and (iii) providing a mechanism to share reagents, skin models, results, and ideas (Kaspar *et al.*, 2009). This consortium is beginning to bear fruit as a number of delivery technologies have been tested (the results of these studies will be reported elsewhere).

The availability of self-delivery siRNAs that are readily taken up by skin keratinocytes, coupled with a stratum corneum penetration strategy (e.g., microneedles or topical formulations), may result in patient-friendly delivery of sufficient quantities of inhibitor to result in clinical efficacy. We have recently reported that dissolvable microneedle arrays can deliver self-delivery siRNAs and inhibit reporter gene expression in a transgenic mouse model (Gonzalez-Gonzalez *et al.*, 2010 b). The ability of these siRNAs to block expression in a human skin equivalent model, under conditions in which non-self-delivery siRNAs have little or no effect, bodes well for use of this class of inhibitors in patient skin and the future for RNA interference-based skin therapeutics.

MATERIALS AND METHODS

Design of siRNA

SiRNAs (Accell and non-Accell) were synthesized by Thermo Fisher Scientific, Dharmacon Products (Lafayette, CO). K6a_513a.12 ((Hickerson *et al.*, 2008); also known as TD101 (Leachman *et al.*, 2010)), EGFP (Hickerson *et al.*, 2008), and CBL3 (Gonzalez-Gonzalez *et al.*, 2009) siRNA sequences have been previously reported.

Preparation and maintenance of primary keratinocyte cell lines

Human PEKp cells were obtained from CELLnTEC Advanced Cell Systems AG (Bern, Switzerland) and maintained according to the manufacturer's instructions. Briefly, cells were thawed and transferred into CnT-57 medium at 4×10^3 cells cm^{-2}. Once 70–80% confluent, cells were collected by trypsinization using TrypLE Select (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and passaged at the same density. PC-10 primary keratinocytes were isolated as described in Hickerson *et al.*, 2010. PC-799 primary

keratinocytes (immortalized keratinocytes derived from a PC patient with a K6a p.Asn171Lys mutation) were isolated from an epidermal biospy taken at the University of Utah from the thigh of a patient harboring the K6a p.Asn171Lys mutation (IPCRR patient no. 799). The study was conducted according to the Declaration of Helsinki Principles under Western Institutional Review Board (IRB) approval (Western IRB Study no. 1057496), and participants gave their written informed consent. All patients were genotyped as part of the IRB-approved registry maintained by Pachynychia Congenita Project (http://www.pachyonychia.org, see also Wilson *et al.*, this issue). The IPCRR no. 799 biopsy was shipped overnight on ice in Epilife medium (Invitrogen) containing antibiotic/antimycotic (Invitrogen; contains 10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of amphotericin B per ml). The biopsy was incubated in 12 ml CnT-57 medium containing 10 mg ml^{-1} Dispase II (Roche, Indianapolis, IN) and 2 × antibiotic/antimycotic for 15 hours at 4 °C. The epidermis was removed from the dermis and incubated in a 500 μl ''drop'' of TrypLE Select on a Petri plate for 30 minutes at 21 °C. The tissue was disrupted by gentle rubbing on the surface of the Petri plate. Cells were diluted with at least 2.5 volumes of CnT-57 medium, collected by centrifugation at 160 × *g* for 5 minutes at 21 °C, seeded at 4×10^4 cells cm^{-2} in CnT-57 medium and expanded as described above.

Preparation of lentiviral expression plasmids

All enzymes were purchased from New England Biolabs (Ipwich, MA) and all final constructs were verified by DNA sequencing. Lentiviral expression constructs pCCL-PGK-EGFP (clone 169) and pCG419 were generously provided by Heini Ilves (Cell Genesys). The IgK-mar sequence (Yi *et al.*, 1999) from CG419 was inserted upstream of the central poly-purine tract region of pCCL-PGK-EGFP to generate pTD147 as follows. pCG419 was digested with *Hpa*I and *Xho*I followed by Klenow treatment to generate a 428 bp fragment containing ''blunt ends'', which was ligated into *Hpa*I-linearized, calf intestinal phosphatase-treated pCCL-PGK-EGFP.

The CBL3 siRNA target site was inserted into the 3′ untranslated region of EGFP in pTD147 to generate pTD152. EGFP was PCR amplified from pTD147 with forward primer 239 (5′-CGACCTGC ATCCCCCGGGTCGCCACC**ATG**GTGAGC) and reverse primer 238 (5′-AAGCAGATCTTGTCTTCGTTGGGAGTGAATTAGCCCTTCCAG TCCCCCCTTTTCTTTTAAAAAGTGGCTAAGATCTACAGCTGCCTTG TAAGTCATTGGTCTTAAAGCTTAAAGTCGAGGCCGCTGAATTCGT TTAAACCTTG*TAACGATCCACGACGTAAA*AATGCGGTACC**TTA**CT TGTACAGCTCGTCCATG) introducing an *Xma*I restriction site (underlined) upstream of the ATG translation start codon (bold) and the CBL3 target site (italicized) flanked by *Kpn*I, *Pme*I, and *Eco*RI restriction sites (underlined) downstream of the TAA translation stop codon (bold). The resulting 918-bp PCR fragment was cloned into pCRII-TOPO vector (Invitrogen), the product of which was digested with *Bbs*I and *Bfu*AI in the presence of 20 ng μl^{-1} duplex DNA containing the *Bfu*AI restriction site (oligo 240: 5′-ACTGATACC TGCTCGACATGTTCATT and oligo 241: 5′-AATGAACATGTCGAGC AGGTATCAGT) to release the 898-bp fragment containing EGFP and the CBL3 siRNA target site. This fragment was ligated into *Bbs*I/*Bfu*AI-digested pTD147.

To generate pTD171, the CBL3 target site was inserted into the 3′ untranslated region of tdTOM in pFULT, a lentiviral vector that expresses Luc2 and tdTOM under the control of the ubiquitin C promoter (Liu *et al.*, 2010). Oligos (oligo-274: 5′-GTAGTCTAGATTTTT ACGTCGTGGATCGTTACAATCTAGAATCG and oligo-275: 5′-CGA TTCTAGATTG*TAACGATCCACGACGTAAAA*ATCTAGACTAC) containing the CBL3 target site (italicized) flanked by *Xba*I restriction sites (underlined) were synthesized, annealed, and digested with *Xba*I to generate a 26-bp fragment that was inserted into *Xba*I-digested pFULT.

Preparation of lentivirus

Human 293T embryonic kidney cells (Invitrogen) were maintained in DMEM (CAMBREX/BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (HyClone, Logan, UT), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate (growth media). The day before transfection, 293T cells were seeded at 5 to 8×10^6 cells per 10 cm plate resulting in 50–80% cell confluency at the time of transfection. The medium was replaced with fresh medium 0.5–3 hours before transfection. Cells were transfected with 17 μg transfer vector (pTD147, pTD152, or pTD171), 8 μg pMDLg/p-RRE (packaging construct), 3.5 μg pRSVrev–*rev* complementary DNA-expression plasmid (Dull *et al.*, 1998), and 3.5 μg pMD2.G–vesicular stomatitis virus envelope-expressing construct (Follenzi *et al.*, 2000), using the CalPhos Mammalian Transfection Kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Virus was collected at 24 and 36 hours after transfection and centrifuged for 10 minutes at 2,000 r.p.m. to remove cellular debris. Virus was either stored at 4 °C and used within 48 hours or aliquoted and stored at −80 °C.

Preparation of single- and dual-reporter primary keratinocyte cell line

Human PEKp cells were transduced by spinoculation (Berkowitz *et al.*, 2001). Cells (500,000) were seeded 24 hours before transduction in a T25 flask in CnT-57 medium. Lentivirus (10 ml in DMEM containing 10% fetal bovine serum) was concentrated by ultracentrifugation in a SW28 rotor at 20,000 × *g* for 90 minutes at 4 °C. The pellet was resuspended in 4 ml ice-cold CnT-57 before transduction. Polybrene (4 μl of 8 mg ml^{-1} phosphate-buffered saline solution) was added and mixed before addition to the T25 flask. The cap was sealed with parafilm and the flask was centrifuged in a swinging bucket rotor at 800 × *g* for 20–90 minutes at 32 °C. After incubation in a CO_2 incubator at 37 °C for 4–6 hours, the medium was changed and the cells were expanded in CnT-57 medium as described above. Once 70–80% confluent, the transduced HPEKp cells (at least 4×10^6) were trypsinized using Tryple Select (Invitrogen) according to the manufacturer's protocols and resuspended in phosphate-buffered saline containing 0.1% BSA and antibiotic-antimicotic at 1×10^6 cells ml^{-1}. The suspension was kept on ice until sorting. Sterile cell sorting was performed on the basis of EGFP + fluorescence using a BD FACSAria II (BD Biosciences) with a 100 μm nozzle. EGFP signal was excited with a 488 nm laser and detected with a 530/30 nm band-pass emission filter. An initial forward scatter and side scatter gate was set to include viable homogeneous cells and to exclude debris. The gated cells exhibiting the top 25% of EGFP expression were sorted, collected in CnT-57 medium, and plated at 4×10^3 cells cm^{-2} in a T25 flask. For the dual-reporter HPEKp cell line, once the EGFP-transduced and sorted cells reached 60–80% confluency, a second transduction with vTD171 was performed as described above. These cells were sorted on the basis of tdTOM signal using

the same 488 nm excitation laser and a 585/42 nm band-pass emission filter.

Preparation of skin equivalents

Human skin equivalents were prepared using the three-dimensional epidermal culture system (CELLnTEC) following the manufacture's instructions. HPEKp cells were maintained in CnT-57 medium and seeded (200,000 cells in 400 µl) in 12-mm inserts lined with a 0.4 µm porous membrane (Millipore, Billerica, MA). The inserts were maintained in a 6-cm plate containing 10.5 ml CnT-57 medium. After 2–3 days, the CnT-57 medium in both the insert and the plate was replaced with differentiation medium (CnT-02-3DP); this is referred to as ''day 0''. After 15–16 hours, the medium in the insert was removed, and the medium in the plate was replaced with 3 ml fresh differentiation medium to generate an air-liquid interface.

Treatment of three-dimensional and monolayer epidermal cultures with siRNAs

Human PEKp cells (20,000 cells in each well of a 24-well plate in CnT-57 medium) were seeded 24 hours before treatment. Three-dimensional epidermal cultures were seeded 1–2 weeks before treatment. siRNA was added to a final concentration of 2 µM (optimal concentration determined by dose–response studies in primary human keratinocytes, data not shown; this concentration is also consistent with the manufacture's (Dharmacon) recommendations) directly to the medium in the lower chamber, leaving the surface of the epidermal equivalent dry. EGFP levels were measured by fluorescence imaging in the IVIS Lumina II *in vivo* imaging system (Caliper Life Sciences, Hopkinton, MA) with the 465 nm excitation and GFP emission filters. Messenger RNA knockdown was measured by quantitative reverse transcription PCR as described in (Hickerson *et al.,* this issue) by lysis in Qiazol (Qiagen, Valencia, CA) and purified using the RNeasy kit (Qiagen), which includes an on-column DNase digestion. Total RNA was reverse transcribed from random hexamer primers using the First Strand Synthesis Kit containing Superscript III (Invitrogen). Target gene knockdown was analyzed using a custom TaqMan gene expression assay for K6a p.Asn171Lys mutant and wild-type mRNA (Hickerson *et al.*, 2010) and an inventoried GAPDH TaqMan gene expression assay (Hs_99999905_m1) as the reference gene (Applied Biosystems, Foster City, CA).

Immunohistochemistry

Immunohistochemical staining was performed using K5-specific (Santa Cruz Biotech, Santa Cruz, CA) and K10-specific (Lab Vision, Freemont, CA) antibodies. Skin equivalents were frozen in optimal cutting temperature compound medium and sectioned (10 µm). Sections were fixed in acetone at −20 °C for 15 minutes, dried and blocked with 10% heat-inactivated goat serum, 1% BSA, 0.025% Triton X-100 in phosphate-buffered saline for 1 hour at 21 °C and incubated overnight in a 1:500 dilution of the primary antibody in the same solution used for blocking. Rinsed slides were incubated for 1 hour with the appropriate Alexa-546-conjugated secondary antibody (1:2,000 dilution; Invitrogen) and mounted with Hydromount (National Diagnostics, Atlanta, GA) containing 4′,6-diamidino-2-phenylindole (DAPI, 1 µg ml^{-1}) to counterstain nuclei. Sections were imaged using a Zeiss Axio Observer Inverted Fluorescence Microscope equipped with DsRed and DAPI filter sets (Carl Zeiss Microimaging, Thornwood, NY).

CONFLICT OF INTEREST

RLK and RPH have been issued a patent for use of siRNA to treat PC. CHC is the scientific founder of Xenogen, which has been acquired by Caliper Life Biosciences.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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