Pachyonychia Congenita Project

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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.

2386 East Heritage Way, Suite B, Salt Lake City, Utah 84109 USA
Phone +1-877-628-7300 • Email—Info@pachyonychia.org
www.pachyonychia.org

Development of Quantitative Molecular Clinical End Points for siRNA Clinical Trials

Robyn P. Hickerson[1,7], Sancy A. Leachman[2,7], Lana N. Pho[2], Emilio Gonzalez-Gonzalez[3], Frances J.D. Smith[4], W.H. Irwin McLean[4], Christopher H. Contag[3], Devin Leake[5], Leonard M. Milstone[6] and Roger L. Kaspar[1,3]

RNA interference (RNAi) is an evolutionarily conserved mechanism that results in specific gene inhibition at the mRNA level. The discovery that short interfering RNAs (siRNAs) are selective, potent, and can largely avoid immune surveillance has resulted in keen interest to develop these inhibitors as therapeutics. A single nucleotide-specific siRNA (K6a_513a.12, also known as TD101) was recently evaluated in a phase 1b clinical trial for the rare skin disorder, pachyonychia congenita (PC). To develop a clinical trial molecular end point for this type of trial, methods were developed to: (1) isolate total RNA containing amplifiable mRNA from human skin and callus material; (2) quantitatively distinguish the single-nucleotide mutant mRNA from wild-type K6a mRNA in both patient-derived keratinocytes and patient callus; and (3) demonstrate that repeated siRNA treatment results in sustained inhibition of mutant K6a mRNA in patient-derived keratinocyte cultures. These methods allow noninvasive sampling and monitoring of gene expression from patient-collected shavings and may be useful in evaluating the effectiveness of RNAi-based therapeutics, including inhibitors that specifically target single-nucleotide mutations.

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INTRODUCTION

Pachyonychia congenita (PC) is a rare autosomal dominant genodermatosis characterized by thickened nails, leukokeratosis, and painful keratoderma with blistering primarily on the soles of the feet, which often necessitates ambulatory aids (Leachman *et al.*, 2005; Smith *et al.*, 2006). Detailed clinical data from a large PC case series will be published elsewhere (Eliason *et al.*). Mutations in at least four *keratin* (*KRT*) genes, including *KRT6A*, *KRT6B*, *KRT16*, and *KRT17*, are responsible for the observed symptoms (see Wilson *et al.*, this issue). These mutations, which often include single-nucleotide (nt) substitutions, cluster in the boundary motifs at the ends of the keratin rod domain. These motifs are associated with protein–protein interactions and disrupt proper intermediate filament formation (Letai *et al.*, 1993; Smith, 2003; Lane and McLean, 2004; Smith *et al.*, 2005). The discovery of RNA interference (RNAi) and the ability of short interfering RNAs (siRNAs) to selectively and potently block expression of specific mRNAs in a user-defined manner have opened up a new therapeutic field. A few siRNAs have now entered clinical trials for diseases including age-related macular degeneration and melanoma, as well as a number of viruses, with others in preclinical development (Dykxhoorn and Lieberman, 2006; de Fougerolles *et al.*, 2007; Kim and Rossi, 2007; Haussecker, 2008; Nguyen *et al.*, 2008; Novobrantseva *et al.*, 2008; Castanotto and Rossi, 2009; Whitehead *et al.*, 2009; Davis *et al.*, 2010; DeVincenzo *et al.*, 2010).

Gene-specific siRNAs aimed at the most commonly mutated keratin in PC, K6a, have been reported (Smith *et al.*, 2008). Moreover, potent siRNAs with single nt specificity for a K6a mutation have been identified (Hickerson *et al.*, 2008a). The K6a_513a.12 siRNA phosphate buffered saline-formulated good manufacturing practice quality inhibitor is known as TD101), which specifically targets the K6a p.Asn171Lys single nt mutation (Hickerson *et al.*, 2008a), has been evaluated for safety in a phase 1b clinical trial for treatment of PC by repeated intradermal injections (Leachman *et al.*, 2010). PC is an ideal disorder for evaluating the effectiveness of siRNA technology in

[1]*TransDerm, Santa Cruz, California, USA;* [2]*Department of Dermatology and Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah, USA;* [3]*Department of Pediatrics, Stanford University School of Medicine, Stanford, California, USA;* [4]*Division of Molecular Medicine, University of Dundee, Dundee, UK;* [5]*Thermo Fisher Scientific, Dharmacon Products, Lafayette, Colorado, USA and* [6]*Department of Dermatology, Yale University, New Haven, Connecticut, USA*

[7]*These authors contributed equally to this work.*

Correspondence: Roger L. Kaspar, TransDerm, 2161 Delaware Avenue, Santa Cruz, California 95060, USA. E-mail: Roger.Kaspar@TransDermInc.com

Abbreviations: cDNA, complementary DNA; C_t, PCR cycle threshold; K, keratin protein; KRT, *keratin gene; nt, nucleotide; PC, pachyonychia congenita; PC-10 cells, immortalized keratinocytes derived from a PC patient with the K6a p.Asn171Lys mutation; PC-2 cells, immortalized keratinocytes derived from a PC patient with the K16 p.Asn125Asp mutation; QRT-PCR, quantitative reverse transcription-PCR; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; siRNA, small interfering RNA*

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clinical trials because: (1) no effective treatment exists that addresses the underlying cause of PC (Leachman *et al.*, 2005, 2008); (2) the most problematic symptoms are localized on or near the pressure points of the feet and are readily accessible (Leachman *et al.*, 2005); (3) the genetic mutations are known and potent and specific siRNAs have been developed (Smith, 2003; Smith *et al.*, 2005; Hickerson *et al.*, 2008a); and (4) a central recruitment and dedicated international patient registry has been developed and is actively maintained by the non-profit PC patient support organization, PC Project (see www.pachyonychia.org).

There are multiple ways in which treatment outcomes for skin disorders, including PC, can be monitored and measured, such as clinical assessment and subjective patient responses, including the validated Dermatology Life Quality Index (Finlay and Khan, 1994; Lewis and Finlay, 2004; see also www.dermatology.org.uk). However, the ability to quantitatively and efficiently measure whether a mutant mRNA is inhibited *in vivo* would be beneficial in objectively evaluating the effectiveness of the proposed treatment (e.g., siRNA). One way to measure the effectiveness of a mutation-specific siRNA in a clinical trial would be to use quantitative reverse transcription-PCR (QRT-PCR) to monitor the absolute amount of target or alternatively the ratio of mutant to wild-type K6a mRNAs present in tissue samples. To this end, a method to isolate amplifiable mRNA from PC patient callus shavings coupled with a QRT-PCR assay that quantitatively discriminates mutant p.Asn171Lys from wild-type K6a is reported in this study. The utility of this QRT-PCR approach was demonstrated by showing specific knockdown of mutant K6a mRNA following repeated siRNA treatment of patient-derived keratinocytes.

RESULTS

Isolation of amplifiable RNA from patient plantar calluses

Isolation of intact RNA from human skin biopsies, but not from noninvasive human skin callus shavings, has been previously reported (Trost *et al.*, 2007). In this study we present a method to isolate RNA, not only from skin, but also from patient skin callus. Various RNA isolation procedures were compared using both shavings (large callus pieces removed with a razor) and pumiced material (smaller extractions from debridement with a pumice stone; data not shown). The most reliable and reproducible procedure involved collection of pumiced samples in RNALater (Applied Biosystems, Foster City, CA), the mechanical disruption of the tissue in a ''bead-beater''-type homogenizer followed by isolation of total RNA (Supplementary Figure S1 online). It should be noted that the majority of the RNA isolated from certain patient samples appear to be of bacterial origin (Supplementary Figure S1c online).

Development and validation of allele-specific QRT-PCR

The QRT-PCR strategy was designed to allow single nt discrimination of mutant and wild-type K6a mRNAs (Figure 1). Primers were designed to flank the K6a p.Asn171Lys mutation site in positions exhibiting the greatest divergence from other highly related type II keratins. The wild-type and mutant-specific probes were designed to discriminate the K6a single nt change at position 513 and to avoid genomic DNA amplification by flanking an intron.

In order to determine the ability of the QRT-PCR assay to discriminate between mutant and wild-type K6a targets, total RNA from cell lines that contain both K6a p.Asn171Lys mutant and wild-type alleles (heterozygous PC-10 keratinocytes,

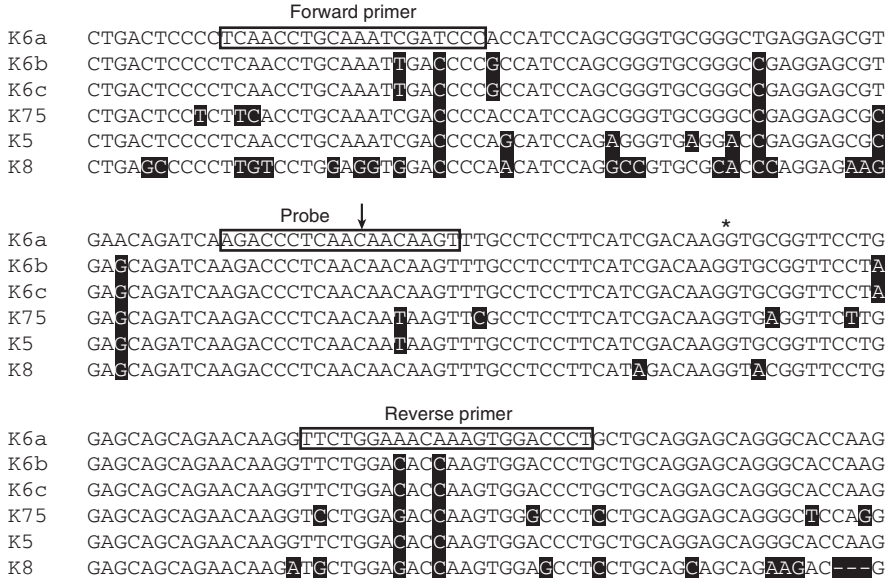


Figure 1. Design of quantitative reverse transcription-PCR (QRT-PCR) assays to allow K6a allelic discrimination. A sequence alignment of K6a, K6b, K6c, K75, K5, and K8 complementary DNAs (cDNAs) is shown. Primers were designed to hybridize to K6a in regions that are distinct from the homologous K6b and other related keratins and to flank a 1 kb intron (location noted with an asterisk). Wild-type- and mutant-specific probes were designed to anneal such that the single-nucleotide (nt) difference at position 513 (designated with arrow) was near the middle of the hybridized region.

Table 1. Single-nucleotide discrimination of wild-type and mutant targets is observed only with mutant and wild-type probes in the same qPCR reaction

	Probes separate		Probes together	
Cell line	C_t (WT)	C_t (MUT)	C_t (WT)	C_t (MUT)
PC-10	20.3 ± 0.1	20.1 ± 0.1	20.6 ± 0.1	20.4 ± 0.1
HaCaT	20.8 ± 0.1	23.7 ± 0.3	21.0 ± 0.1	None detected

Abbreviations: C_t, PCR cycle threshold; PC-10, pachyonychia congentia-10; WT, wild-type; qPCR, quantitative PCR.

immortalized keratinocytes derived from a PC patient with the K6a p.Asn171Lys mutation) or homozygous wild-type K6a alleles (HaCaT cell line) was analyzed. As expected, approximately equal C_t values for wild-type and mutant K6a were obtained from PC-10 cells, when probes were used together or separately. HaCaT cells do not express p.Asn171Lys mutant K6a mRNA; however, QRT-PCR analysis of HaCaT total RNA with each probe separately unexpectedly resulted in signal for both the wild type and mutant. When both probes were used together, no mutant K6a was detected, indicating that quantitative discrimination of mutant and wild-type K6a mRNAs is achievable only if both the wild-type and p.Asn171Lys mutant probes are simultaneously present during the amplification reaction (Table 1). Similar results were obtained using mutant and wild-type plasmid DNA (data not shown).

To further investigate the robustness and accuracy of the assay, known amounts of *in vitro*-transcribed mutant and wild-type K6a mRNAs were measured by QRT-PCR in a complex environment (Figure 2). Total RNA, isolated from PC-2 cells (immortalized keratinocytes derived from a PC patient with an irrelevant K16 p.Asn125Asp mutation) or HaCaT cells, was ''spiked'' with *in vitro*-transcribed K6a p.Asn171Lys mutant mRNA. Neither PC-2 nor HaCaT cells express p.Asn171Lys mutant K6a mRNA; however, both cell lines express wild-type K6a. 293FT cells, which do not express K6a (see Supplementary Figure S2 online), were used as a non-keratinocyte control. A constant amount of *in vitro*-transcribed wild-type K6a mRNA (empirically determined to generate C_t values similar to those observed from RNA isolated from HaCaT and PC-2 cells) was added to total RNA isolated from 293FT cells. To mimic the expected results of siRNA treatment, decreasing amounts of p.Asn171Lys mutant mRNA were added to each total RNA sample. The results obtained in the background of homologous keratin mRNAs (i.e., RNA isolated from PC-2 or HaCaT cells, which express K5 and K6b among other keratins) show the same trend as in the non-keratinocyte background (i.e., RNA from 293FT cells, which express neither K5 nor K6b). These results indicate that even in the presence of homologous keratins, the amount of mutant K6a detected by QRT-PCR is directly and linearly dependent on the amount present, indicating that this assay can quantitatively distinguish mutant and wild-type K6a mRNAs.

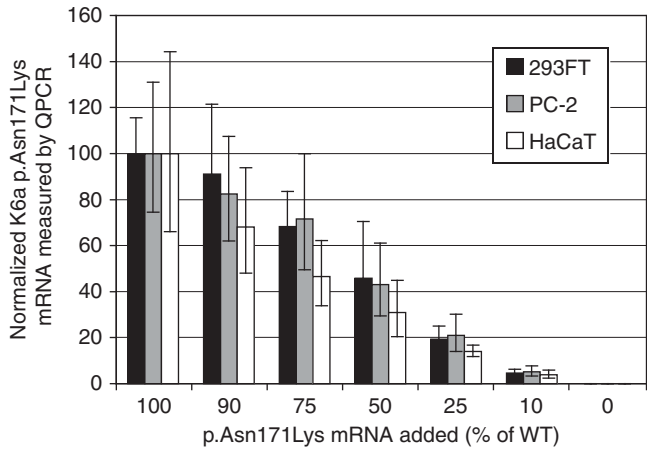


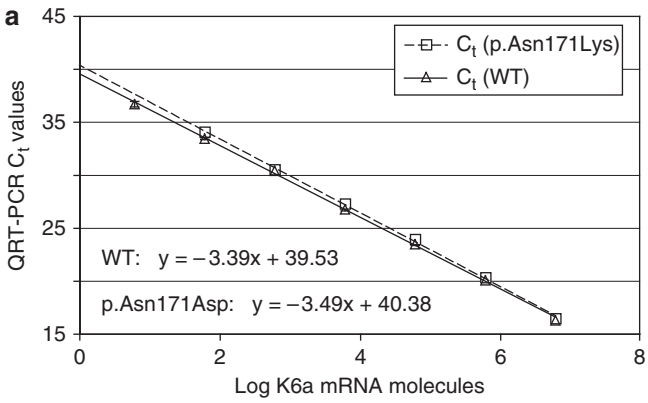
Figure 2. Quantitative reverse transcription-PCR (QRT-PCR) assay discriminates between p.Asn171Lys and wild-type K6a regardless of keratin background. Decreasing amounts of p.Asn171Lys mutant *in vitro*-transcribed mRNA were added to total RNA isolated from the indicated cell lines (mimicking the expected result of small interfering RNA (siRNA) treatment) to demonstrate the specificity and sensitivity of the QRT-PCR assay (a constant amount of *in vitro*-transcribed wild-type K6a mRNA was added to total RNA from 293FT cells). The RNA samples were subjected to QRT-PCR using the combined K6a wild-type/p.Asn171Lys assay (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene). The amount of mutant K6a detected under the indicated conditions was normalized to the 100% mutant data point for each cell line. The data are reported as the ratio of normalized p.Asn171Lys to normalized wild-type mRNA levels. Error bars represent standard error.

Patient-derived keratinocytes and calluses express equal amounts of mutant and wild-type mRNAs

In order to determine the absolute K6a wild-type and p.Asn171Lys mutant mRNA copy numbers in keratinocytes and callus samples, standard curves were generated (Figure 3) in which known amounts of *in vitro*-transcribed mutant or wild-type K6a mRNAs were plotted against C_t values generated by QRT-PCR. Based on these curves, absolute amounts of wild-type and mutant K6a mRNAs were determined in HaCaT cells and PC-10 cells, as well as patient and control callus samples. Notably, the PC-10 cells contain approximately equal amounts of K6a wild-type (820 molecules per cell) and mutant (1,100 molecules per cell) mRNAs. Slightly lower levels of K6a wild-type mRNAs were found in the human HaCaT keratinocyte line (490 molecules per cell) and, as expected, no mutant K6a mRNA was detected. RNA isolated from callus samples of three patients harboring the p.Asn171Lys mutation contained equal levels of mutant and wild-type K6a mRNAs (data shown only for patient IPCRR no. 10), whereas no mutant K6a mRNA was detected in callus samples from a non-PC volunteer or ten PC patients with mutations in K6a (other than K6a p.Asn171Lys), K6b, K16, or K17 (e.g., Callus no. 2 from IPCRR Patient no. 2 harbors the K16 p.Asn125Asp mutation).

siRNA treatment results in sustained inhibition of mutant K6a

To investigate the potential utility of this assay for monitoring the effectiveness of siRNA treatment in which the

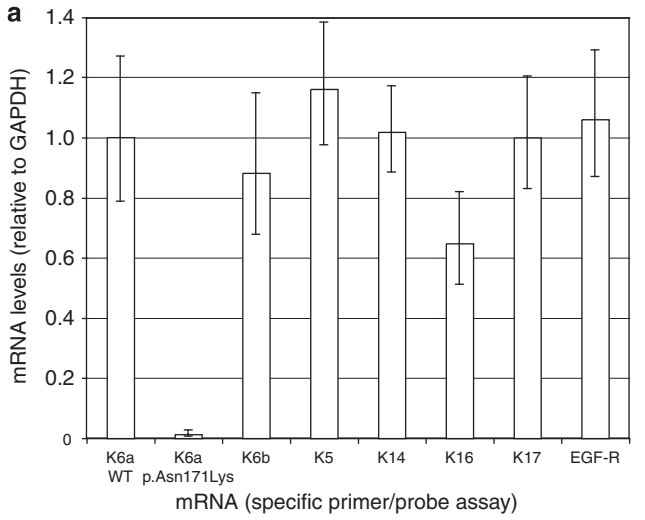


b

	Total RNA (ng per cell)	C_t		K6a mRNA (molecules per mg)		K6a mRNA (molecules per cell)	
		WT	p.Asn171Lys	WT	p.Asn171Lys	WT	p.Asn171Lys
Callus no. 10 (K6A p.Asn171Lys)	-*	25.3 (3.6)	25.7 (3.7)	3.3×10^4	3.4×10^4	-	-
Callus no. 2 (K16 p.Asn125Asp)	-*	30.7 (1.0)	None detected	1.7×10^3	None detected	-	-
Non-PC callus	-*	32.9 (1.5)	None detected	3.7×10^2	None detected	-	-
PC-10 cells	0.022	24.0 (0.2)	23.9 (0.1)	1.3×10^7	1.8×10^7	8.2×10^2	1.1×10^3
HaCaT cells	0.016	24.3 (0.1)	None detected	1.8×10^7	None detected	4.9×10^2	None detected

*Not reported due to bacterial RNA contamination

Figure 3. Quantitative determination of p.Asn171Lys mutant and wild-type K6a mRNA amounts in callus samples obtained from pachyonychia congenita (PC) patients. (**a**) A standard curve was generated by reverse transcription of known quantities of *in vitro*-transcribed wild-type and mutant p.Asn171Lys K6a mRNAs. Decreasing amounts of reverse-transcribed material were analyzed by quantitative PCR (QPCR). Error bars represent standard error (SE). (**b**) Determination of absolute amounts of wild-type and mutant K6a in callus and cultured keratinocytes. C_t values obtained from PC patient callus complementary DNA (cDNA) or 1 ng cDNA from PC-10 and HaCaT cells are reported (SE are reported in parentheses). Molecules per mg of callus sample or tissue culture cells and molecules per tissue culture cell are also reported using the standard curve generated in **a**.



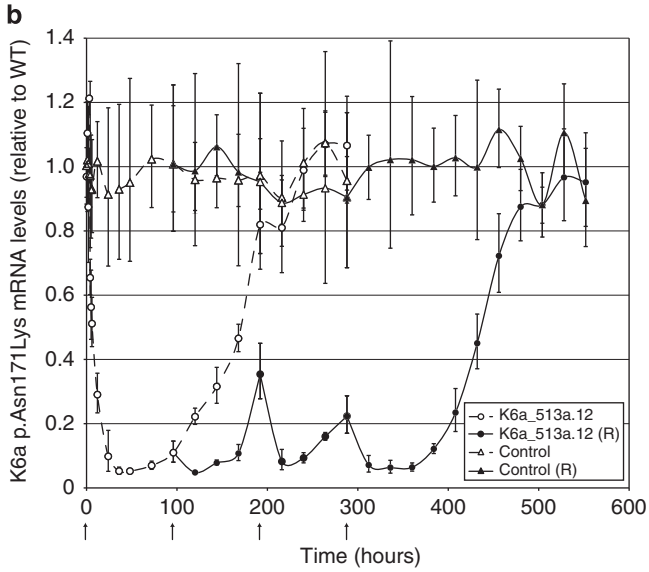


Figure 4. Single-nucleotide (nt)-specific small interfering RNA (siRNA) reduces mutant K6a levels in the patient cell line. (**a**) PC-10 cells were treated with K6a_513a.12 (targets p.Asn171Lys mutant K6a mRNA) or control siRNA (targets enhanced green fluorescent protein (EGFP)) at the indicated time points (arrows). Following a 24-hour incubation, RNA was isolated and subjected to quantitative reverse transcription-PCR (QRT-PCR) analysis using the indicated primer/probe assays (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene). All data generated were normalized to EGFP siRNA-treated cells. (**b**) PC-10 cells were repeatedly treated (labeled ''R'' in the figure insert) with K6a_513a.12 or control EGFP siRNA at 0, 96, 192, and 288 hours. At the indicated time points, samples were processed as described in **a**. Normalized mutant K6a expression relative to wild-type (WT) K6a expression is plotted for each sample showing specific and sustained knockdown. Error bars represent standard error.

p.Asn171Lys mutation is targeted, PC-10 cells were treated with mutant-specific siRNA (K6a_513a.12). Following a 24-hour incubation, total RNA was isolated and analyzed by QRT-PCR. Figure 4a shows that K6a_513a.12 siRNA potently and specifically inhibits mutant K6a mRNA levels (98% reduction), with no effect on K6a wild-type mRNA levels. Furthermore, little or no effect was observed on other related keratin mRNAs, including K6b, or on the unrelated EGFR. The modest inhibition observed for K16 is reproducible and has been seen in other laboratories (Wong and Coulombe, 2003; FJD Smith, unpublished data) and may result from feedback inhibition following downregulation of its binding partner K6a. These data indicate that p.Asn171Lys mutant-specific siRNA potently targets mutant K6a mRNA with little or no nonspecific effects on related keratin gene expression in patient-derived keratinocytes, and that the effectiveness of the siRNAs can be readily and quantitatively measured by QRT-PCR.

As repeated siRNA administration will be necessary in treating dominant genetic diseases, PC-10 cells were repeatedly treated (every 4 days) with the mutant-specific siRNA (K6a_513a.12) and control siRNA (targets enhanced green fluorescent protein (EGFP), which is not present in these cells). Total RNA was isolated and analyzed by

QRT-PCR every 24 hours. The levels of mutant mRNA decreased relative to wild-type within a few hours following treatment with the mutant-specific siRNA, whereas there was no change when treated with the control siRNA (Figure 4b).

Determination of mRNA cleavage site

To demonstrate that the observed mutant K6a knockdown was due to siRNA-mediated cleavage, K6a mRNA cleavage products from total RNA isolated from K6a_513a.12 siRNA-treated PC-10 cells were characterized using a modified 5′ RACE (rapid amplification of complementary DNA (cDNA) ends) technique (Soutschek *et al.*, 2004). As shown in Supplementary Figure S3 online, the cleavage site is between residues 10 and 11 of the siRNA antisense strand (between positions 514 and 515 in the K6a cDNA), consistent with previously published RISC (RNA-induced silencing complex)-mediated cleavage data (Elbashir *et al.*, 2001).

DISCUSSION

A clear demonstration of the clinical efficacy of therapeutic siRNAs in PC patients should pave the way for treatment of other dominant negative genodermatoses including epidermolysis bullosa simplex, epidermolytic palmoplantar keratoderma, bullous congenital ichthyosiform erythroderma, and many other dominant skin disorders (Lane and McLean, 2004). The single nt specificity of the siRNAs that we (Hickerson *et al.*, 2008a) and others (Brummelkamp *et al.*, 2002; Ding *et al.*, 2003; Miller *et al.*, 2003; Dykxhoorn *et al.*, 2006; Hengge, 2006; Schwarz *et al.*, 2006; Klootwijk *et al.*, 2008; Pfister *et al.*, 2009) have demonstrated suggests that this approach may be useful, particularly for dominant negative disorders, if efficient delivery can be achieved. The ability to quickly identify appropriate siRNAs coupled with the ability to assay their effectiveness using approaches, such as the QRT-PCR protocol described in this study, may usher in an era of individualized medicine. Offending dominant genes could be identified (perhaps by gene array technology or high-throughput sequencing), followed by design, preparation, and selection of potent and mutation-specific siRNAs that, following appropriate testing for safety and efficacy, would be administered to patients. The effectiveness of these inhibitors could be monitored through subjective observation as well as using molecular end points including QRT-PCR assays, such as the one described in this study.

Quantitative molecular end points are of crucial importance for objectively evaluating the effectiveness of test agents in clinical trials. In preparation for future clinical trials in which PC patients will be treated with mutant-specific siRNAs (e.g., K6a_513a.12 targeted to the K6a p.Asn171Lys mutation; Leachman *et al.*, 2008, 2010; Hickerson *et al.*, 2008a), a method to measure mutant versus wild-type mRNA levels by QRT-PCR was developed.

In designing the QRT-PCR assay, we sought to satisfy the National Clinical Laboratory criteria utilized by ARUP Laboratories (http://www.clsi.org/Source/Custom/Currentdocs.cfm?Section=Current_Versions_of_CLSI_Documents&CFID=3081615&CFTOKEN=53505146) and the NYC Department of Health (http://www.wadsworth.org/labcert/TestApproval/submitguide.htm) for clinical QRT-PCR assays, namely: confirmation of appropriate analytical specificity and sensitivity (Figure 2), minimal ''between-run'' and ''within-run'' variation (Supplementary Table S1 online), defined limits of detection (Figure 3), and appropriate clinical sensitivity (Supplementary Figure S4 online).

The single nt specificity of the QRT-PCR assay was demonstrated by assaying total RNA from PC-10 cells that contain equal amounts of wild-type and p.Asn171Lys mutant K6a mRNA and from HaCaT keratinocytes that express only wild-type K6a. As shown in Table 1, both probes must be used simultaneously in order to achieve the expected result. The wild-type probe was also able to discriminate mutant and wild-type plasmid under all conditions tested. However, the mutant probe was able to discriminate mutant and wild-type plasmid only in the presence of the wild-type probe (data not shown). The ability of the wild-type probe to differentiate between mutant and wild-type targets may result from the formation of a C:G base pair with the wild-type cDNA, whereas hybridization of the mutant probe to the mutant cDNA results in an A:T base pair. In addition to the increased stability of the wild-type canonical base pair, the wild-type probe mismatch with the mutant cDNA results in a sterically bulky G:A mismatch as opposed to the more easily tolerated C:T mismatch resulting from the mutant probe mismatch with the wild-type cDNA. The unexpected observation that complete discrimination was achieved only when both the wild-type and mutant probes were used simultaneously suggests that competition for the target is necessary to achieve the desired single nt specificity.

The usefulness of the single nt-specific QRT-PCR assay is dependent on the ability to extract sufficient amounts and quality of RNA from patient samples (skin or callus) that can be quantitatively analyzed. The specific and reproducible amplification of a variety of keratin mRNAs (K6a, K6a p.Asn171Lys, K6b, K5, K14, K16, and K17) and non-keratin RNAs (18S rRNA; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin, and lamin A/C mRNAs) from RNA extracted from PC patient callus samples suggests that sufficiently intact mRNA can be isolated that can be evaluated in a quantitative fashion (Supplementary Figure S2 online). This work shows that QRT-PCR can be performed on RNA isolated from patient shavings (obtained by noninvasive means). To our knowledge, this is previously unreported. It should be noted that amplifiable RNA can also be obtained from skin by tape harvesting and mRNA expression levels determined by DNA microarray or QRT-PCR (Wong *et al.*, 2004; Benson *et al.*, 2006). In addition to the ability to measure relative amounts of mRNAs in human tissue and cell lines, wild-type and mutant K6a absolute mRNA copy numbers in both patient callus samples and keratinocyte cell lines (HaCaT and PC-10) were determined using the single nt-specific QRT-PCR assay described in this study.

The mode of TD101 administration during the recently completed TD101 Phase Ib clinical trial (Leachman *et al.*, 2010) was via intradermal injection (twice per week; Leachman *et al.*, 2010). Intradermal injection(s) of reporter-specific

siRNA was previously shown to inhibit reporter gene expression in mouse skin models (Wang *et al.*, 2007; Smith *et al.*, 2008; Hickerson *et al.*, 2008a, b; Gonzalez-Gonzalez *et al.*, 2009). Although no adverse events were noted, and signs of efficacy were observed, intradermal injections were determined to be unacceptable because of intolerable pain. Therefore, alternative ''patient-friendly'' delivery methods are currently under development. The ability to repeatedly and reproducibly knock down mutant K6a mRNA levels (with no effect on wild-type; see Figure 4b) with sequential siRNA treatments in PC patient-derived keratinocytes bodes well for repeated treatments in a clinical setting if patient-friendly delivery technologies can be developed. The demonstration that quantifiable mRNA can be isolated from skin and skin callus and single nt differences between K6a wild-type and p.Asn171Lys mutant mRNAs can be discriminated by QRT-PCR suggests that these methods may be useful for measurement of molecular end points in future clinical trials.

MATERIALS AND METHODS

Plantar skin shavings and skin explants

Affected callused areas on the plantar surface of PC patients were collected with institutional review board approval (Western IRB no. 1057496), and callused regions were obtained from a volunteer control. The study was conducted according to the Declaration of Helsinki Principles, and the participants gave written informed consent. Each sample was collected during routine grooming by PC participants and included the outer layers of the callus that would normally be discarded. Shavings were immediately placed in RNALater (Applied Biosystems) and stored at 4 °C. After 24 hours, samples were transferred to −20 °C for no longer than 2 weeks, at which time samples were transferred to −80 °C for long-term storage until total RNA extraction was performed.

De-identified human skin (from abdominoplasty procedures) was obtained with informed consent from a local surgeon and used within 1–4 hours following surgical removal. Adipose tissue was removed before RNA isolation.

RNA isolation

Total RNA was prepared using a FastPrep instrument (FastPrep-24, FP24, from MP Biomedicals, Solon, OH) to mechanically lyse the cells and the RNeasy Mini Kit (Qiagen, Valencia, CA) to isolate pure RNA. Frozen callus samples (in RNALater) were thawed at 4 °C and ~30 mg of sample was placed in a 2 ml tube prefilled with 1.2 g ''D'' lysing matrix (MP Biomedicals). The skin explant tissue was similarly processed. Qiazol (1 ml; Qiagen) was added and the samples were immediately processed in the FP24 homogenizer, three times at a setting of 6.0 m s^{-1} for 40 s with a 2-minute incubation on ice between each round. To remove debris, samples were centrifuged twice for 5 minutes each at 12,000 × *g* at 21 °C, followed by addition of 200 µl chloroform. Samples were vortexed, incubated at 21 °C for 5 minutes and then centrifuged at 12,000 × *g* for 15 minutes at 4 °C. The aqueous layer was transferred to a clean tube and an equal volume of 70% ethanol was added. The sample was loaded on an RNeasy column provided with the RNeasy Mini Kit, treated with DNase, and eluted with 30 µl RNase-free water as described by the manufacturer.

Reverse transcription

RNA isolated from skin or plantar callus samples or tissue culture cells was reverse transcribed using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, 1–2 µg of total RNA isolated from skin samples or tissue culture cells was incubated with random hexamer primers (5 ng µl^{-1}) and dNTPs (1 mM) at 65 °C for 5 minutes in a total volume of 10 µl and then placed on ice for at least 1 minute. Owing to bacterial contamination, it was not possible to quantify the amount of RNA isolated from PC patient callus samples; therefore, 8 µl of a total RNA sample (30 µl from ~30 mg callus) was added to the reverse transcription reaction. The reverse transcription reaction was performed by addition of 1 µl Superscript III reverse transcriptase (200 U µl^{-1}) and 1 µl RNase OUT (40 U µl^{-1}) in 1 × buffer (20 µl final volume) at 25 °C for 10 minutes, followed by 50 °C for 30 minutes. The enzyme was heat denatured at 85 °C for 5 minutes.

In vitro-transcribed RNA

K6a wild-type or p.Asn171Lys mutant mRNA was *in vitro*-transcribed from 1.4 µg *Xho*I-linearized plasmid (pTD103 and pTD104; Hickerson *et al.*, 2008a) using the Ampliscribe T_7-Flash Transcription Kit (Epicentre Biotechnologies, Madison, WI). The *in vitro*-transcribed RNA was purified using a G-50 gel filtration column (Bio-Rad, Hercules, CA).

Reverse transcription of *in vitro*-transcribed RNA added to total RNA

A fixed amount of *in vitro*-transcribed wild-type K6a RNA (0.1 ng) and decreasing amounts of *in vitro*-transcribed mutant p.Asn171Lys RNA (from 100% (i.e., 0.1 ng) to 0% of the wild-type K6a RNA added) were added to 1 µg of total RNA from 293FT cells. Decreasing amounts of *in vitro*-transcribed K6a mutant RNA (0.1–0 ng) were added to 1 µg of total RNA isolated from HaCaT or PC-2 keratinocytes. Samples were reverse transcribed as described above.

QRT-PCR

The K6a TaqMan assay (Applied Biosystems) consists of forward and reverse primers (5′-TCAACCTGCAAATCGATCCC-3′ and 5′-AGGGTCCACTTTGTTTCCAGAA-3′ respectively) and 6-FAM-labeled wild-type-specific and VIC-labeled mutant-specific TaqMan MGB probes (5′-AGACCCTCAACAACAAGT-3′ and 5′-AGACCCTCAAAAACAAGT-3′, respectively) (underline shows single nt change). Inventoried TaqMan assays for K5 (Hs00361185_m1), K6b (Hs00749101_s1), K14 (Hs00559328_m1), K16 (Hs00373910_g1), K17 (Hs01588578_m1), EGF-R (Hs01076073_m1), 18S rRNA (Hs99999901_s1), β-actin (Hs99999903_m1), GAPDH (Hs99999905_m1), and lamin A/C (Hs00153462_m1) were purchased from Applied Biosystems. A typical QPCR reaction was prepared as follows: 1.25 µl 20 × primer/probe mix, 6.25 µl H_2O, and 12.5 µl 2 × master mix were combined such that the final primer and probe concentrations were 200 and 120 nM, respectively. A total of 5 µl of cDNA (0.2 ng µl^{-1} for samples prepared from skin samples or tissue culture cells or the maximum possible concentration of cDNA prepared from each callus sample) was then added to each well and analyzed using the ABI standard 7500 procedure. The data were analyzed using the Applied Biosystems Sequence Detection software (version 1.4) and reported as the number of PCR cycles required to

achieve a threshold fluorescence for a specific cDNA, defined as the "C_t" value or as the relative quantitation using GAPDH as the reference gene. All data points reported are the mean of three replicate assays and error is reported as the standard error.

Preparation of immortalized keratinocyte cell lines

Skin biopsy specimens from previously genotyped patients were collected with institutional review board approval (Western IRB no. 1057496) and were shipped overnight at 4 °C in culture medium. Keratinocytes obtained from epidermal sheets from those biopsies were initially grown on 3T3 feeder cells and then expanded in low calcium medium, as described previously (Schwartz *et al.*, 1992). Second passage cells were transduced with high-titer supernatants containing *human papilloma virus* (*HPV*) *16 E6/E7* genes in the LXSN retroviral vector (Halbert *et al.*, 1991). Cells were split twice a week at a ratio of 1:3 for 3 weeks and then once a week at 1:10 thereafter. Immortalization was assumed after the tenth passage, the time at which normal cells show distinct slowing of growth. Keratinocyte lines (PC-10 and PC-2) for the experiments described were used after the twelfth passage.

siRNA transfection

PC-10 immortalized keratinocytes were transfected with RNAiMAX (Invitrogen) according to the manufacturer's instructions for "reverse transfection." Mutant-specific (K6a_513a.12; Hickerson *et al.*, 2008a) and nonspecific control (SMARTselected EGFP-specific siRNA; Thermo Fisher Scientific, Dharmacon Products, Lafayette, CO; Wang *et al.*, 2007) siRNAs were diluted in 25 µl optiMEM medium in a well of a 24-well plate (15 nM final concentration after addition of cells). A total of 1 µl of RNAiMAX lipofectamine (Invitrogen) was diluted in 25 µl of optiMEM medium and immediately added to the nucleic acid solution and left for 10–15 minutes at 21 °C. Trypsinized PC-10 cells (55,000 cells in 450 µl) were then added to the well and gently mixed before incubation. Cells were lysed with 1 ml Qiazol, and RNA was isolated using the RNeasy Mini Kit. The resulting RNA samples were subjected to QRT-PCR as described above.

5′ RACE analysis

Total RNA was isolated as described above from 500,000 PC-10 keratinocytes treated with 3 nM mutant-specific K6a_513a.12 siRNA for 6 hours. mRNA was isolated with the Oligotex mRNA Mini Kit (Qiagen) and subjected to a modified 5′ RACE procedure as described (Soutschek *et al.*, 2004) using the K6a gene-specific primer K6a-sp242 (5′-GGGTGCTCAGATGGTATAGAG-3′). The resulting PCR product was ligated into the pCRII-TOPO vector and confirmed by sequencing.

CONFLICT OF INTEREST

Drs Kaspar, Hickerson, McLean, and Smith have filed patents relating to siRNA therapy for pachyonychia congenita. Dr Leake is the Director of Research and Development for Thermo Fisher Scientific's RNA Technologies division (formerly Dharmacon).

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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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