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15 March 2005

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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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INVITED REVIEW ARTICLE

Therapeutic siRNAs for dominant genetic skin disorders including pachyonychia congenita

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Summary The field of science and medicine has experienced a flood of data and technology associated with the human genome project. Over 10,000 human diseases have been genetically defined, but little progress has been made with respect to the clinical application of this knowledge. A notable exception to this exists for pachyonychia congenita (PC), a rare, dominant-negative keratin disorder. The establishment of a non-profit organization, PC Project, has led to an unprecedented coalescence of patients, scientists, and physicians with a unified vision of developing novel therapeutics for PC. Utilizing the technological by-products of the human genome project, such as RNA interference (RNAi) and quantitative RT-PCR (qRT-PCR), physicians and scientists have collaborated to create a candidate siRNA therapeutic that selectively inhibits a mutant allele of *KRT6A*, the most commonly affected PC keratin. *In vitro* investigation of this siRNA demonstrates potent inhibition of the mutant allele and reversal of the cellular aggregation phenotype. In parallel, an allele-specific

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quantitative real-time RT-PCR assay has been developed and validated on patient callus samples in preparation for clinical trials. If clinical efficacy is ultimately demonstrated, this "first-in-skin" siRNA may herald a paradigm shift in the treatment of dominant-negative genetic disorders.
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1. Introduction

The human genome project has provided accessible and comprehensive documentation of the human genome. This has greatly facilitated the development of new gene discovery technologies. It has also spawned the field of functional genomics, which attempts to assign functional relevance to copious sequence data. One of the most successful functional genomics technologies yet developed involves the use of small interfering RNAs (siRNAs) to interrogate the function of human genes. The capacity of siRNA to specifically and potently block gene expression *in vitro* has led to the consideration of siRNA as a candidate therapeutic agent as well. The continuing rapid pace of discovery and development in genetics and genomics portends the advent of individualized medicine, in which (1) patient genetic information can be rapidly analyzed, (2) disease mutations can be identified and (3) mutation-specific siRNAs can be selected, synthesized, tested for safety and efficacy, and efficiently delivered as novel therapeutics. Emergence of a variety of sequence-specific therapies [1] for ultra-rare, non-lethal, dominant-negative skin disorders, such as pachyonychia congenita (PC), would have been unthinkable without the rapid and relatively inexpensive synthetic and analytic technologies that developed along with the genome project.

A major task in the years ahead is the development of treatments for all dominant-negative disorders using findings from structural, functional and genetic basic science investigation. One such disease-targeted treatment involves the use of siRNAs. SiRNAs are a new class of RNA inhibitors that act via the RNA-induced silencing complex (RISC) to specifically degrade target RNAs. Inhibition of gene expression by siRNA is mediated by hybridized RNAs, typically containing a 19 bp complementary region with two nucleotide 3′ overhangs (19 + 2 design), that are sufficiently small so as to avoid immune surveillance [2]. This mechanism is distinct from the classical antisense activity of single-stranded oligonucleotides mainly with respect to the involvement of RISC, which catalytically cleaves the target mRNA and thereby exerts activity for a period of time dictated by RISC turnover. Unlike antisense oligonucleotides, persistence of the siRNA within cells outside of the RISC is theoretically not required for continued RNAi activity. Therefore, an intermittent dosing schedule for the siRNA can be rationalized in clinical trials.

2. Pachyonychia congenita is an ideal "proof of principle" model for siRNA therapeutics

To date, 54 functional keratin genes have been identified and mutations in 20 keratin genes have been associated with human genetic disorders [3–5]. Typically, the sites of these mutations lie in the alpha helical domains involved in protein–protein interactions. Since all known keratins act in pairs, mutation of one member of the pair also affects the function of the partner protein, resulting in disruption of higher-order intermediate filament formation or assembly kinetics [6]. Significant basic

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scientific data regarding the molecular etiology of keratinizing disorders have been available since the mid-1990s. Although some therapeutic strategies have been suggested from experimental work, no therapeutic agents until now have reached the point of clinical trial.

Pachyonychia congenita is a well-characterized genetic disorder predominantly affecting nails and skin, which is caused by mutations in keratins *KRT6A* or *KRT16* (PC-1, OMIM 167200) and *KRT6B* or *KRT17* (PC-2, OMIM 167210). The physical findings most commonly include grossly thickened nails coupled with palmoplantar hyperkeratosis [7,8]. The thickened fingernails are disfiguring and hinder fine fingertip actions used in a multitude of tasks. Importantly, PC patients experience severe incapacitating pain associated with the plantar keratoderma (Fig. 1). This necessitates significant lifestyle modification including the use of wheelchairs or crutches, and regular pain medication.

The discovery of PC as a keratin disorder followed a genetic linkage analysis study of a large Scottish pedigree. This linkage study showed co-segregation of the disease with polymorphic markers within the type I keratin gene cluster at 17q12–q21 [9]. Subsequently, a heterozygous missense mutation was identified in the family in *KRT17* [10]. Simultaneously, a missense mutation was detected in *KRT16* in a sporadic case of PC-1 [10]. A second gene was shown to be associated with PC-1 when mutations were identified in *KRT6A* [11]. Some time later a mutation was detected in *KRT6B* in an extended family with the PC-2 phenotype [12].

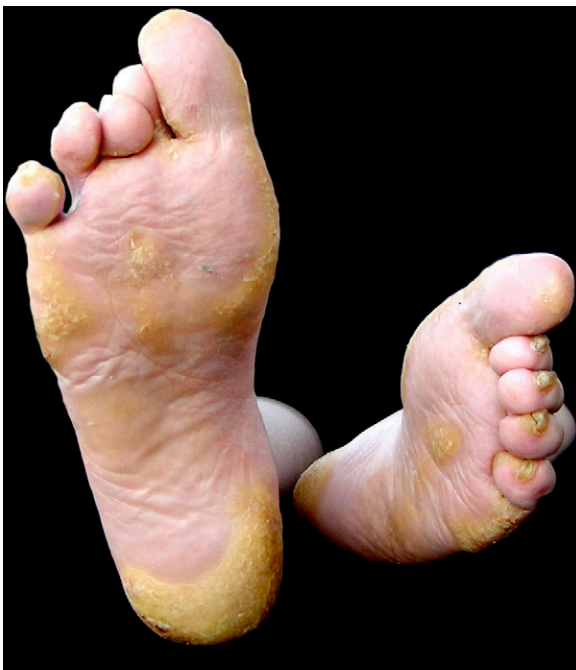

Fig. 1 Typical painful and debilitating plantar hyperkeratosis observed in a pachyonychia congenita patient harboring the K6a N171K mutation. Treatment with siRNA will be by injection into the calluses.

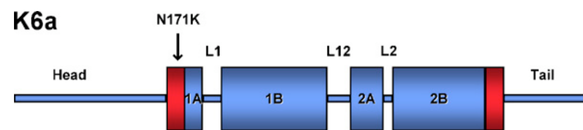

Fig. 2 A schematic representation of the protein domain organization common to all keratins. Four coiled coil domains, 1A, 1B, 2A, 2B, are separated by non-helical linkers, L1, L12 and L2. Shaded in red are the helix boundary domains that are highly conserved in sequence between all keratins. The majority of mutations identified in PC (in K6a, K16, K6b, K17) fall within these domains. The position of the most common amino acid mutated in K6a, N171, is shown.

To date, there are published reports of 55 causative mutations responsible for PC in 108 independently ascertained families (www.interfil.org). The gene most commonly mutated is *KRT6A*, and the most common site of mutation is at position 171 in the amino acid sequence (Fig. 2).

PC serves as a prototype indication for siRNA-based therapy. The molecular basis of PC is known and the defective genes responsible have been identified. In addition, many different keratins are expressed in the epidermis and there is probable functional redundancy. SiRNA technology permits selective targeting of the dominant mutant allele, potentially eliminating only the functionally disruptive version of the keratin. A further advantage is the external location and focal nature of the plantar skin lesions permitting minimally invasive localized treatments to be performed on patients. Localized siRNA therapy for a skin disease also has a major advantage over other tissue targets since the results of the therapeutic intervention can be directly observed and if necessary sampled, a feature which recently facilitated the first-in-man gene therapy grafting of a junctional EB patient with a laminin mutation [13].

Any advance in the development of siRNA-based therapeutics for PC will likely be directly applicable to treatment of other dominant genodermatoses, as well as indirectly applicable to a much larger number of human disorders that have dominant-negative etiology. Further, development of methods for delivery of siRNA-based therapeutics may have downstream benefit for treatment of unrelated genetic disorders that also affect the skin.

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3. Development of mutation and gene-specific siRNAs

In PC, two approaches to the development of siRNAs have been undertaken. The first is the development of mutation specific siRNAs, which have the disadvantage of limiting the number of families who could be treated but are highly specific. The second approach was directed at developing gene specific siRNAs that simultaneously target both wildtype and mutant genes.

3.1. Identification of K6a N171K mutation-specific siRNAs

Several viable approaches are available to identify optimal siRNAs for targeting mutant mRNAs in dominant-negative diseases. A rational approach, taking advantage of existing algorithms that predict good target sites as well as the site most likely to yield discrimination can be employed. Alternatively, all possible siRNAs targeting the mutation site can be prepared (sequence walk) and tested, assuring that all possible effective siRNAs are identified. Both approaches have been used with success [14–16] and a combination of the two may be most effective, eliminating those sequences that are known to be ineffective or non-discriminating. Efficient and effective screening requires an assay that will quickly and accurately identify suitable candidates. One approach is to make bicistronic constructs, in which the mutant (and wildtype as control) is linked to a reporter construct such as a fluorescent protein or firefly luciferase and cloned into a plasmid expression vector ([14] and data not shown). The effectiveness of the candidate siRNAs can then be scored by fluorescence or luciferase assay following plasmid co-transfection with candidate siRNAs. Using this type of approach, we identified K6a.513a.12, an siRNA that has no effect on wildtype K6a expression in tissue culture or animal studies, but silences the mutant form containing a single nucleotide change [14].

The single nucleotide specificity of siRNA treatment can be readily observed by linking wildtype and mutant targeted cDNAs to tags such as fluorescent proteins. Fig. 3A shows the intermediate filaments formed following transfection of expression plasmids containing *KRT6A* wildtype cDNA linked to plum fluorescent protein as well as mutant *KRT6A* linked to yellow fluorescent protein (YFP), which results in aggregates of mutant protein. Co-transfection with differentially tagged wildtype (plum) and mutant (YFP) K6a expression constructs allows visualization of the distinctly tagged wildtype or mutant keratin 6a (K6a) proteins, which can be selectively inhibited with siRNAs that are specific to either the wildtype or mutant forms of *KRT6A* mRNA (Fig. 3B), thereby demonstrating the single nucleotide specificity of the siRNA agents. The specificity of the K6a.513a.12 siRNA is further demonstrated by inhibition of mutant *KRT6A* mRNA and not wildtype mRNA in immortalized keratinocytes derived from a K6a N171K patient biopsy (Fig. 4).

While effective siRNAs targeting the N171K mutation were readily identified, other mutations may be more problematic and effective siRNAs may be more difficult to identify. The rapid progress of siRNA development, however, suggests that even difficult sites may be amenable to targeting, by modification of one of both strands in the siRNA molecule. For example, modification of the antisense strand to facilitate stronger hybridization to the target may allow tuning to specific mutation sites [15].

3.2. Identification of gene-specific siRNAs

The overlapping structure and function of the *KRT6A* and *KRT6B* genes, as well as studies of *KRT6A* knockout mice [17–19], strongly suggest that reduction or elimination of K6a expression can be compensated for by K6b or other keratins. Thus, siRNAs targeting both the mutant and wildtype forms of K6a, may be a viable approach to treating PC. We have recently identified siRNAs that specifically block expression of both wildtype and mutant forms of K6a with no effect on homologous keratin gene expression such as K6b [16].

Comparison of the DNA sequences of the cDNAs encoding human *KRT6A* (GenBank RefSeq accession no. NM_005554) and *KRT6B* (GenBank RefSeq accession no. NM_005555) revealed that only a few isolated bases can distinguish these two genes in terms of their protein-encoding sequences. However, these two genes do differ significantly in their non-coding 3′UTR UTR sequences. Using the Dharmacon siDESIGN center, four inhibitors were designed within the 3′UTR of K6a that were predicted to inhibit *KRT6A* expression without affecting the expression of *KRT6B* or other type II keratin genes due to significant sequence differences. Using a variety of biochemical assays, three of these siRNA reagents were shown to potently and specifically inhibit K6a expression in cell culture systems, where they could essentially knock out K6a at very low concentrations [16]. One of these inhibitors was also shown to inhibit K6a reporter gene expression in vivo using an animal model system [16].

4. Animal models relevant to studying dominant-negative disorders

The parallel mouse and human genome projects have greatly facilitated logical construction of animal

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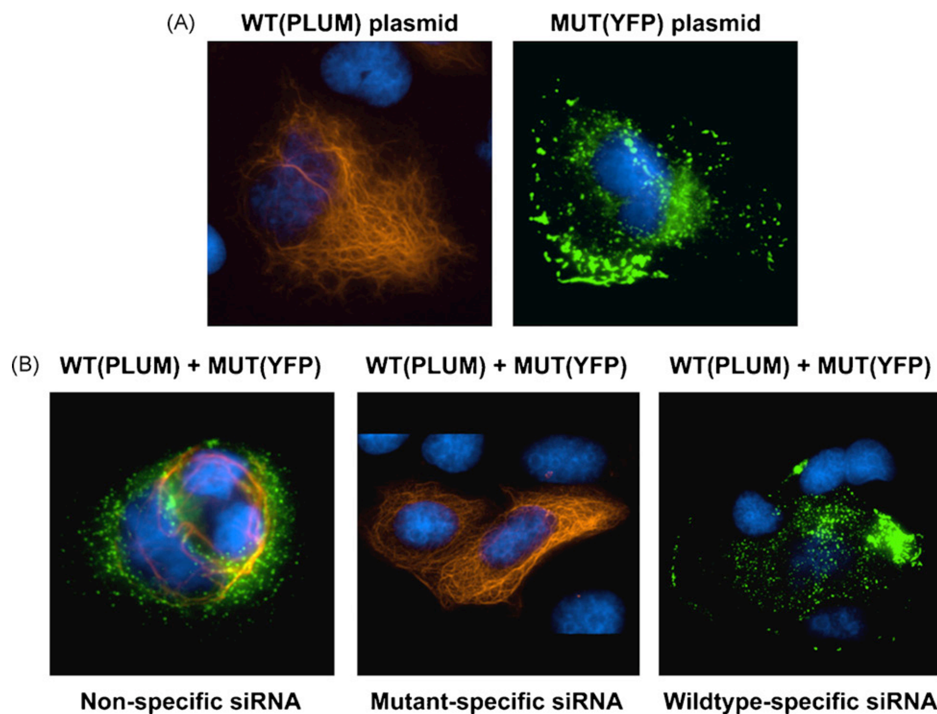

Fig. 3 Ability of siRNAs to specifically target the single nucleotide *KRT6A* N171K mutation responsible for the dominant disorder pachyonychia congenita. (A) Human PLC hepatoma cells were transfected with wildtype *KRT6A* fused to plum fluorescent protein or alternatively N171K mutant *KRT6A* fused to yellow fluorescent protein (YFP) and visualized by fluorescence microscopy [14]. (B) Co-transfection of tagged mutant (YFP) and wildtype (plum) *KRT6A* expression plasmids with siRNA. Co-transfection with non-specific control (NSC4) siRNA had no effect on plasmid expression with both plum-colored filaments (wildtype K6a) or yellow/green aggregates (N171K mutant K6a) observed. Addition of mutant-specific siRNA (K6a.513a.12) blocked mutant K6a expression along with its YFP tag, resulting in only wildtype expression, which leads to intermediate filament formation (plum coloration). As a further control, cells were treated with siRNA specific to the wildtype form (K6a.513c.12), resulting in no filaments being formed and only yellow/green (from YFP) aggregates observed.

models. Comparison of the human and mouse genome sequences revealed that only about 300 human genes do not have a murine ortholog [20], facilitating the rapid generation of knockout or knock-in mutant mice corresponding to most human genes. In the case of the keratin-related genodermatoses, the most useful and realistic models are mice in which dominant-negative mutations, equivalent to those commonly found in human patients, can be activated in the epidermis by topical application of a small-molecule inducer [21,22]. These revolutionary mice allow induction of epidermal fragility phenotypes in small regions of the skin to allow the study of pathogenetic mechanisms and therapy systems, without major distress to the animal or lethality. Analogous inducible PC mice are under development currently.

5. PC clinical trial utilizing siRNA

As discussed above, PC is an ultra-rare disorder and current treatment modalities primarily center on symptomatic relief. Recent registry data suggests that the incidence of PC is likely to be on the order of a few thousand individuals worldwide (see www.pachyonychia.org and [23]).

The development of the potent and exquisitely selective siRNA targeting N171K mutant *KRT6A mRNA* discussed above has created the opportunity to undertake the first siRNA clinical trial (initiated early 2008) for any skin disorder. This first trial investigates the safety and tolerability of intralesional injections of siRNA into PC patient calluses. The study will be a split-body and double-blinded investigation, injecting drug into one foot and vehicle into a matched callus on the other foot of a PC patient. The primary purpose of the study is to assess dose safety and tolerance of an increasing volume and concentration of siRNA.

A secondary objective is to evaluate patients for any signs of efficacy at the injection site (and elsewhere on the skin). Multiple measures of efficacy will be assessed including clinical examination, subjective patient scoring systems of pain and quality of life, and a state-of-the-art real-time RT-PCR assay that quantitatively distinguishes wildtype and mutant keratin mRNAs in callus shavings (Hickerson, Leachman, et al., in preparation).

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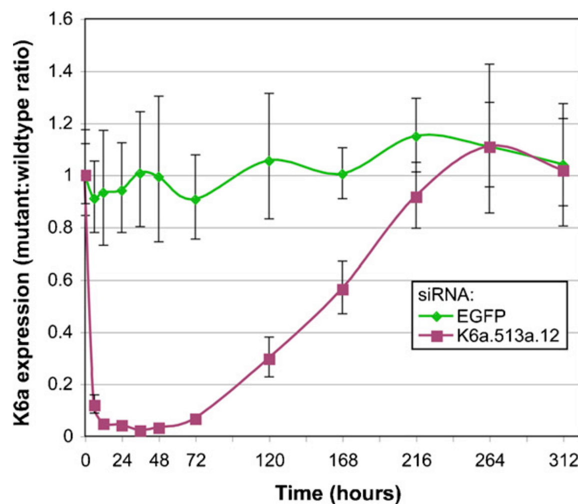

Fig. 4 Mutant-specific siRNA potently reduces mutant K6a mRNA levels without affecting wildtype levels in immortalized keratinocytes prepared from a PC patient. PC-10_K6a_N171K cells (immortalized keratinocytes prepared from a K6a N171K PC patient skin biopsy) were treated with K6a.513a.12 (targets N171K mutant mRNA) or control irrelevant siRNA (targets EGFP) at time 0 h. At the indicated timepoints, RNA was isolated, reversed transcribed and the resulting cDNA subjected to real-time qRT-PCR analysis (Hickerson, Leachman, et al., manuscript in preparation) using custom gene expression assays for wildtype and mutant K6a (GAPDH gene expression assay was used as the endogenous control). Normalized mutant K6a expression divided by wildtype K6a expression is plotted for each sample.

6. Future perspectives

The transition of siRNA agents into routine clinical use is on the horizon. To date, only a handful of siRNA therapeutics (including those developed for macular degeneration and respiratory syncytial virus) have entered clinical trials and none has yet obtained FDA approval [24–26]. Here we report on the progress of a new siRNA entering clinical trials in PC patients with the *KRT6A* N171K mutation, with a gene-specific *KRT6A* siRNA study possibly to follow. This is the first-in-man siRNA therapeutic trial for a skin indication and the first siRNA to target a gene mutation. In the case of PC, the rapid trajectory of the siRNA inhibitors into clinical trials has only been made possible by the strength of the basic scientific knowledge and technologies resulting from the human genome project (and related efforts). The clinical trial with this agent will represent an important proof-of-principle human experiment, rigorously designed with quantifiable endpoints to test whether this siRNA therapeutic is not only safe, but also holds promise in the treatment of this disorder. The potential for specificity in the design of these siRNA agents offers unprecedented potential in the field of tailored and individualized medicine. If this siRNA proves effective in the treatment of PC, it may herald the onset of tailored siRNA therapeutics for any number of dominant skin diseases as well as other disorders. If efficacy is proven, siRNA agents may be a new class of drug with the potential to cause a paradigm shift in the treatment of dominant-negative genetic disorders.

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The Pachyonychia Congenita Project (PC Project, see www.pachyonychia.org) has been critically instrumental in effectively organizing an international group of PC patients, patient advocates, and the International PC Consortium (IPCC), a group of physicians and scientists who have agreed to work together to develop PC therapeutics. The authors thank PC Project, and in particular the director, Mary Schwartz, for unwavering support of this project and for providing the patient photograph. We are grateful to PC patients for their strong support and also thank IPCC members for stimulating discussion and insights. Finally, we thank Roger Tsien (UC San Diego) for providing the plum fluorescent protein construct and Devin Leake (Thermo Scientific, Dharmacon Products) for providing siRNAs.

Conflict of interest

Drs. Smith, McLean, Kaspar, and Hickerson have filed a patent related to treatment of PC with siRNA therapeutics. Dr. Leachman is independent of any commercial funder and has had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Roger L. Kaspar, PhD, is the CEO and scientific founder of TransDerm, a company focused on developing novel therapeutics, including inhibitors based on RNA interference (RNAi) technology, for skin disorders. Dr. Kaspar received his doctorate from the University of Washington (Seattle) in biochemistry (David Morris) and performed post-doctoral work at M.I.T. (Lee Gehrke), Stanford University (Helen Blau), and Chiba University (Tomohito Kakegawa). After serving on the faculty at Brigham Young University (Utah) in the Department of Chemistry and Biochemistry, he left academia to work at SomaGenics, prior to founding TransDerm. Drawing on his expertise in the area of post-transcriptional gene regulation, his current efforts are focused on designing highly potent and selective therapeutic siRNAs that target disease-causing genes in skin disorders, including pachyonychia congenita, and investigating methods to efficiently deliver agents such as siRNAs to appropriate skin cells.

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