Correction of hair shaft defects through allele-specific silencing of mutant *Krt75*

Ying Liu[1], **Elizabeth R. Snedecor**[2,3], **Xu Zhang**[1], **Yan-Feng Xu**[1], **Lan Huang**[1], **Evan Jones**[4], **Lianfeng Zhang**[1], **Richard A. Clark**[4], **Dennis R. Roop**[5], **Chuan Qin**[1,*], and **Jiang Chen**[1,2,4,5,*]

[1]Institute of Laboratory Animal Science, Chinese Academy of Medical Science and Comparative Medical Center, Peking Union Medical College; Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Beijing, China

[2]Department of Pathology, Stony Brook University, Stony Brook, NY

[3]Graduate Program in Genetics, Stony Brook University, Stony Brook, NY

[4]Department of Dermatology, Stony Brook University, Stony Brook, NY

[5]Department of Dermatology and Charles C. Gates Center for Regenerative Medicine and Stem Cell Biology, University of Colorado Anschutz Medical Campus, Aurora, CO

Abstract

Dominant mutations in keratin genes can cause a number of inheritable skin disorders characterized by intraepidermal blistering, epidermal hyperkeratosis, or abnormalities in skin appendages, such as nail plate dystrophy and structural defects in hair. Allele-specific silencing of mutant keratins through RNA interference is a promising therapeutic approach for suppressing the expression of mutant keratins and related phenotypes in the epidermis. However, its effectiveness on skin appendages remains to be confirmed *in vivo*. In this study, we developed allele specific siRNAs capable of selectively suppressing the expression of a mutant *Krt75*, which causes hair shaft structural defects characterized by the development of blebs along the hair shaft in mice. Hair regenerated from epidermal keratinocyte progenitor cells isolated from mutant *Krt75* mouse models reproduced the blebbing phenotype when grafted *in vivo*. In contrast, mutant cells manipulated with a lentiviral vector expressing mutant *Krt75*-specific shRNA persistently suppressed this phenotype. The phenotypic correction was associated with significant reduction of mutant *Krt75* mRNA in the skin grafts. Thus, data obtained from this study demonstrated the feasibility of utilizing RNA interference to achieve durable correction of hair structural phenotypes through allele-specific silencing of the mutant keratin genes.

*Correspondence: Jiang Chen, MD, Department of Pathology, Stony Brook University School of Medicine, Basic Science Tower, Level 9, Room 151, Stony Brook, NY 11794, Phone: 631-444-3199, Fax: 631-444-6487, jiang.chen@stonybrook.edu.
Chuan Qin, Ph.D., Institute of Laboratory Animal Science, Peking Union Medical College (PUMC), Chinese, Academy of Medical Science (CAMS), Beijing, 100021, China, Phone: 0086-10-6777-0815, Fax: 0086-10-6771-0812, qinchuan@pumc.edu.cn

CONFLICT OF INTEREST
None to declare.

Keywords

siRNA; hair follicle; keratin; gene therapy; regeneration; ex vivo

INTRODUCTION

Keratins are the most abundant form of intermediate filament proteins in keratinocytes. Mutations in keratins are responsible for the majority of inheritable epidermal disorders, such as epidermolysis bullosa (EBS), epidermolytic ichthyosis (EI, formerly known as epidermolytic hyperkeratosis (EHK)), Ichthyosis bullosa of Siemens (IBS), and pachyonychia congenita (PC), which are characterized by intraepidermal blistering and generalized or palmoplantar hyperkeratosis (Lane and McLean, 2004; Uitto *et al.*, 2007). Mutations in hair follicle related keratins can cause hair abnormalities, such as monilethrix, autosomal dominant woolly hair, and pseudofolliculitis barbae (Harel and Christiano, 2012; Schweizer *et al.*, 2007).

Most disease-causing mutations in keratins are dominant-negative mutations, in which the mutant keratin gene products adversely affect the integrity of the keratin intermediate filament network of affected keratinocytes (Harel and Christiano, 2012; Knobel *et al.*, 2015; McLean and Moore, 2011; Uitto *et al.*, 2007). Suppressing the production of mutant keratins through allele-specific inhibition of mutant keratin genes may be effective in correcting associated phenotypes (Knobel *et al.*, 2015; McLean and Moore, 2011).

RNA interference (RNAi) has been successfully utilized in achieving allele-specific inhibition of mutant keratin genes. For example, mutant-specific siRNAs against disease-causing mutations in the *KRT9*, *KRT5*, and *KRT6A* genes were demonstrated to be capable of inhibiting the expression of these mutant keratins *in vitro* and *in vivo* (Atkinson *et al.*, 2011; Hickerson *et al.*, 2011b; Hickerson *et al.*, 2008; Leslie Pedrioli *et al.*, 2012). Moreover, intraepidermal injection of siRNA was capable of suppressing plantar hyperkeratosis in pachyonychia congenita (Leachman *et al.*, 2010), confirming the therapeutic value of this approach for correcting phenotypes associated with keratin mutations. Whether this strategy can achieve lasting therapeutic benefit is yet to be determined *in vivo*.

KRT75, formerly known as *K6HF*, is expressed in the companion layer of the hair follicle and the medulla of the hair shaft (Winter *et al.*, 1998; Wojcik *et al.*, 2001). Heterozygous mutations in *KRT75* (A12T and E337K) are associated with the development of pseudofolliculitis barbae and loose anagen hair syndrome, respectively (Chapalain *et al.*, 2002; Winter *et al.*, 2004). Moreover, decreased expression of *KRT75* was also observed in cicatricial alopecia (Chapalain *et al.*, 2002; Sperling *et al.*, 2010). Genetically engineered mutant mice (*Krt75^{tm1Der}*) expressing a mutant form of *Krt75* (N159del) developed hair shaft blebbing (Chen *et al.*, 2008). Moreover, mutations in *Krt75* also contribute to the development of frizzle feathers in chicken (Ng et al., 2012) and altered enamel structure of human teeth (Duverger et al., 2014). These observations demonstrated an important role of *KRT75* in maintaining the structural integrity of the hair and other skin appendages.

In this study, we demonstrated that hair follicles regenerated with mutant *Krt75* epidermal keratinocyte progenitor cells were able to reproduce the hair shaft blebbing phenotype *in vivo*. Suppressing mutant *Krt75* expression by shRNA effectively suppressed the development of this hair shaft phenotype. Thus, this study established the feasibility of using *ex vivo* modified epidermal keratinocyte progenitor cells to prevent structural abnormalities of the hair.

RESULTS

Development of allele-specific siRNA for mutant *Krt75*

Small interfering RNAs (siRNAs) are capable of selectively silencing mutant keratin genes, such as *KRT6A*, *KRT5*, and *KRT9*, at the resolution of single base pairs. In this study, we examined whether siRNA is able to suppress a three base pair in-frame deletion (c. 545_547del (p.N159del)) mutation in the mouse *Krt75* gene (Chen *et al.*, 2008). Nineteen candidate siRNAs for the mutant *Krt75* were engineered and tested by allele-specific qRT-PCR (Figure 1a and Supplemental Figure S1).

Two candidate siRNAs, siN159D-5 and siN159D-6 (15 nM), demonstrated strong (> 70%) inhibition of mutant *Krt75*, but a moderate (< 45%) effect on wild-type *Krt75* (Figure 1b). In contrast, negative control siRNA (15 nM) had no effect on the expression of *Krt75* and a positive control siRNA (15 nM) indiscriminately suppressed the expression of both wild-type and mutant *Krt75* (Figure 1b). When evaluated at variable concentrations, both siN159D-5 and siN159D-6 demonstrated robust inhibitory effect on mutant *Krt75* (Figure 1c and d), but siN159D-6 is more selective for mutant, but not wild-type *Krt75* (Figure 1d). Thus, siN159D-6 was selected for future experiments. The scrambled sequence of siN159D-6 was used as negative control (siN159D-6S).

In order to achieve long-term inhibition in cells *in vivo*, siN159D-6 and siN159D-6S were cloned as hairpin shRNAs into pLVX-ShRNA2 lentiviral vectors (Supplemental Figure S2). Lentiviral vectors are not only able to efficiently infect large amounts of keratinocyte progenitor cells, but are also able to integrate into the cell's genome, thereby achieving continuous expression of shRNA. Lentiviral vectors expressing shN159D-6 or shN159D-6S were able to efficiently infect HEK293T and primary keratinocytes (greater than 83.6% and 82.0%, respectively) without noticeable adverse effects on cell growth and morphology *in vitro* (Supplemental Figure S2).

Mutant *Krt75*-specific shRNA suppresses hair shaft phenotype

To establish an *in vivo* model suitable for testing therapeutic effectiveness of RNAi, mutant *Krt75* keratinocyte progenitor cells were isolated from homozygous mutant *Krt75* mice (*Krt75^{tm1Der}*/*Krt75^{tm1Der}*) and grafted onto immune deficient nude mice to regenerate skin and hair follicles *in vivo*. Mutant cells efficiently regenerated skin and hair and, most importantly, the majority of hair shafts (85.2 ± 5.4%) contained the characteristic bleb phenotype as observed in the homozygous mutant *Krt75* mice (Figure 2a and Supplemental Figure S3). This result demonstrated the grafting of *ex vivo* cultured mutant keratinocyte progenitor cells can be used as a model to test therapeutic intervention.

To determine whether mutant *Krt75*-specific shRNA was able to suppress hair shaft defects regenerated in skin grafts, homozygous mutant *Krt75* keratinocytes were infected with lentiviral vectors prior to grafting. One month later, hair was regenerated. Analyses of hair regenerated with lentiviral vector-infected cells by light and transmission electron microscopy demonstrated that shN159D-6 was able to robustly suppress the formation of blebs in the hair shaft such that only $34.6 \pm 7.6\%$ of hair shafts contained bulbous lesions (Figure 2b and d, and Supplemental Figure S3). In contrast, scrambled shRNA (shN159D-6S) had no effect on suppressing the hair phenotype (Figure 2c and d, and Supplemental Figure S3) and the majority ($78.6 \pm 4.0\%$) of hair shafts regenerated with scrambled shRNA-treated cells contained defective hair shafts (Figure 2d).

Because some hairs contain more than one bleb, the effectiveness of shRNA was also evaluated based on the number of bulbous lesions per hair shaft. Affected hair shafts regenerated with shN159D-6 lentiviral vector-infected cells contained 0.97 ± 0.11 bulbous lesions (Supplemental Figure S4), whereas affected hair shafts regenerated with non-infected cells and scrambled (shN159D-6S) lentiviral vector infected cells contained 1.43 ± 0.28 and 1.44 ± 0.23 blebs per hair shaft one month after grafting, respectively ($P < 0.01$, Supplemental Figure S4). Collectively, these findings demonstrated that the mutant *Krt75*-specific shRNA was not only able to suppress the frequency of defective hair shafts, but also ameliorate the severity of affected hair.

Mutant-specific shRNA suppresses the expression of mutant *Krt75 in vivo*

To determine whether suppression of the hair phenotype was associated with suppression of mutant *Krt75* expression, qRT-PCR was performed on skin grafts. The relative expression level of mutant *Krt75* was normalized to its level in non-infected control grafts. A marked reduction in the level of mutant *Krt75* transcripts ($37.3 \pm 6.9\%$) was observed in grafts regenerated with shN159D-6 lentiviral vector-infected cells (Figure 3a). In comparison, the expression level of mutant *Krt75* ($94.2 \pm 11.6\%$) in grafts regenerated with scrambled shRNA was almost identical to that in controls (Figure 3a).

The expression of a number of keratin genes that are expressed in the epidermis and hair follicles (*Krt5*, *Krt14*, and *Krt1*) or related to *Krt75* (*Krt6a* and *Krt17*) was evaluated. Results showed that the expression profiles of these genes were not affected in skin grafts regenerated with lentiviral vectors expressing either mutant Krt75-specific shRNA or scrambled shRNA. These results suggest that lentiviral-mediated transcription suppression of a mutant form of keratin is likely capable of achieving high specificity *in vivo*.

Lentiviral-mediated *ex vivo* modification of keratinocyte progenitor cells exhibit a sustained suppression of phenotype

To further determine whether phenotypic suppression was sustainable, skin grafts were analyzed three months after grafting. We estimate that hair follicles analyzed three months after grafting underwent two more hair cycles than those harvested at one month post-grafting. First, all experimental groups displayed no evidence of hair loss at three months (Supplemental Figure S5). Examination of hair shafts revealed that the proportion of phenotypic hair shafts in control and shRNA-treated groups was consistent with those

harvested at one month post-grafting (Supplemental Figure S5). Specifically, non-infected control and scrambled shRNA-infected keratinocytes regenerated hair shafts with 84.7 ± 3.1% and 81.2 ± 5.3% defects, respectively (Supplemental Figure S5). In contrast, hair regenerated with cells infected with shN159D-6 contained 37.0 ± 9.0% defective hair shafts (Supplemental Figure S5). Thus, hair regenerated with shN159D-6 treated cells harbored significantly reduced numbers of defective shafts ($P < 0.05$, Supplemental Figure S5). These results suggested that lentiviral vector-mediated manipulation of *ex vivo* cultured keratinocytes was able to maintain a lasting suppression of hair shaft phenotypes, likely due to the integration capability of lentiviral vectors and continuous expression of encoded shRNA.

RNAi can suppress phenotypes associated with heterozygous mutant *Krt75*

So far, the therapeutic effect of shN159D-6 was examined in homozygous mutant *Krt75* cells. However, disease-causing mutations in keratin genes are predominantly heterozygous mutations (Harel and Christiano, 2012; Lane and McLean, 2004; Uitto *et al.*, 2007), including those in *KRT75* (Chapalain *et al.*, 2002; Winter *et al.*, 1998). To determine whether the RNAi is capable of suppressing phenotypes associated with heterozygous mutations, hair was regenerated with epidermal keratinocyte progenitor cells obtained from heterozygous mutant *Krt75* mice ($Krt75^{+}/Krt75^{tm1Der}$).

Hair regenerated with heterozygous mutant *Krt75* cells contained 53.8 ± 10.4% bulbous hair shafts (Figure 4a and d). In contrast, hair regenerated with heterozygous mutant cells treated with shRNAD-6 contained only 20.9 ± 5.8% defective hair shafts, which was significantly reduced in comparison to non-infected and scrambled shRNA-treated (49.3 ± 8.9) groups ($P < 0.05$, Figure 4).

Heterozygous mutant cells express both wild-type and mutant *Krt75*, permitting the evaluation of the transcriptional levels of both transcripts. Quantitative RT-PCR on wild-type *Krt75* demonstrated comparable expression levels of wild-type *Krt75* in all experimental groups (non-infected control, 100.0 ± 6.7; shN159D-6, 89.2 ± 6.1; shN159D-6S, 88.1 ± 7.0, Figure 4e). In contrast, the expression of mutant *Krt75* was selectively suppressed in shRNAD-6 treated group (37.5 ± 3.3) but not in non-infected or scrambled controls (100.0 ± 8.4 and 87.4 ± 8.5, respectively, Figure 4e). These results suggest that suppressing the expression of mutant *Krt75* in heterozygous mutant cells is able to ameliorate the blebbing phenotype of the hair.

DISCUSSION

RNAi-mediated *ex vivo* therapy is among the most promising approaches for correcting phenotypes associated with dominant mutations in keratin genes in skin (Uitto, 2012). Although keratins are redundantly expressed in keratinocytes and considerable homology exists among keratin genes, recent progress has demonstrated the feasibility of engineering allele-specific siRNAs that are capable of suppressing mutant keratin expression (Atkinson et al., 2011; Hickerson et al., 2008; Leslie Pedrioli et al., 2012). More importantly, evidence obtained from a number of *in vitro* and *in vivo* models, and a single patient, split-body, vehicle-controlled phase I clinical trial support the usefulness of utilizing siRNA to suppress

mutant keratin expression (Hickerson *et al.*, 2011a; Leachman *et al.*, 2010; Leslie Pedrioli *et al.*, 2012). In this study, we developed siRNAs capable of specifically inhibiting a dominant mutant form of *Krt75*, and confirmed the effectiveness of RNAi-based *ex vivo* therapy at both the phenotypic and molecular levels in mice. Data obtained from this study not only demonstrated the feasibility of permanently suppressing the expression of mutant keratins, but also provided an example of correcting structural hair defects with *ex vivo* modified epidermal keratinocyte progenitor cells *in vivo*.

Models capable of mimicking skin disorders at both phenotypic and genetic levels may serve as important tools in testing novel therapeutics for inheritable skin disorders. The mouse model utilized in this study is a knock-in model. It contains a mutation analogous to one found in the *KRT6A* gene in pachyonychia congenita patients, in its endogenous *Krt75* locus (Chen and Roop, 2008). This mouse model represents genetic changes that occur in most keratin mutation-related disorders. Therefore, it may serve as an ideal pre-clinical model in performing proof-of-principle experiments pertinent to the development of therapeutics for disorders caused by keratin mutations.

The mutation targeted in this study is an in-frame three base-pair deletion mutation. It is analogous to a common mutation in the *KRT6A* gene (c.513_515del (p.N171del), which is also reported as (c.516_518del (p.N172del) as the codon 171 and 172 are identical) in pachyonychia congenita (PC) patients (Smith *et al.*, 2005; Wilson *et al.*, 2014). Because sequences flanking this site are almost identical between the mouse *Krt75* gene and human *KRT6A* gene, data obtained from this study strongly suggests that it is feasible to specifically target this mutation in human *KRT6A*. In fact, potent siRNAs were previously developed for a missense mutation (c.513C> A (p.N171K)) and the analogous c.513_515del mutation in *KRT6A* (Hickerson *et al.*, 2011b; Hickerson *et al.*, 2006). Interestingly, the most potent and selective siRNA for the analogous deletion in mouse *Krt6a* (N159del) or the human *KRT6A* (N171del) target the same region (D6 in Fig. 1c or (Hickerson *et al.*, 2006), respectively). Thus, these findings collectively support the rationale of using RNAi to suppress the disease-causing mutant allele.

Mutations in a number of genes involved in inheritable hair disorders cause structural defects in the hair shaft (Harel and Christiano, 2012). Pertinent to this study, mutations in *KRT75* are linked to loose anagen hair syndrome (Chapalain *et al.*, 2002) and pseudofolliculitis barbae (PFB) (Winter *et al.*, 2004). It is postulated that KRT75 is important for maintaining the integrity of the keratin intermediate filament network in companion layer cells where it is expressed. Mutations in *KRT75* likely destabilize endogenous keratin filaments. When compounded by mechanical stress, such as combing and shaving, mutant *KRT75* is likely to lead to the development of hair phenotypes. Data obtained from this study suggests that silencing mutant *KRT75* may ameliorate hair phenotypes through restoring the stability of the endogenous keratin intermediate filament network in companion layer keratinocytes.

Although siRNA is highly efficient and specific in silencing mutant keratins and, as this study demonstrated, *ex vivo* therapy may achieve long-term phenotypic improvement, safety concerns associated with lentiviral vectors prevented the use of lentiviral vector-mediated

delivery of shRNA in clinical settings. The development of robust delivery systems through which therapeutic siRNA oligonucleotides can be readily delivered to the site in need of treatment may provide a straight-forward and safe, albeit transient, solution for correcting associated phenotypes.

MATERIALS AND METHODS

Design of mutant *Krt75*-specific siRNA

A total of 19 possible siRNAs against the previously reported mutant *Krt75* (c.545_547 (p.N159del)) (Chen *et al.*, 2008) were designed and synthesized (Thermo Fisher, Shanghai, China) (Figure 1a). Synthetic siRNA duplexes are 21-mers containing two uracyl (U) nucleotide overhangs at the 3'-end of the target sequences, designated consecutively from siN159D-1 to siN159-19 (Figure 1a). A nonspecific siRNA that contains the inverted beta-galactosidase sequence was used as a negative control. A commercial siRNA (SASI_Mm01_00158646, Sigma-Aldrich, St. Louis, MO) directed against the mouse *Krt75* gene were used as positive control.

Cell culture and transient transfection

HEK293T cells, which do not express endogenous *KRT75*, were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a 5% CO_2 humidified incubator. The day before transfection, cells were seeded in 6-well plates at 2.5×10^5 cells per well. Equal amounts (0.5 μg) of wild-type and mutant *Krt75* expression plasmids (Chen *et al.*, 2008) were co-transfected with each siRNA (5 – 15 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 hours later for analyses.

Quantitative RT-PCR

Total RNA was extracted from cells or graft tissues with Trizol (Invitrogen). After DNase (RQ1, Promega, Madison, WI) treatment, 250 ng mRNA was reverse transcribed to cDNA with SuperScript First-Strand Synthesis System (Invitrogen). 15 ng cDNA was used in quantitative PCR. Quantitative real-time PCR for wild-type and mutant *Krt75* was performed with an identical primer pair (GeneCore BioTechnologies, Shanghai, China) (Supplemental Figure S1). Two minor groove binder (MGB) probes for allelic-specific assays for wild-type and N159del *Krt75* were designed with Primer Express Software (Applied Biosystems v2.0, Thermo Fisher, Waltham, MA) and labeled with FAM and HEX, respectively (GeneCore BioTechnologies) (Supplemental Figure S1). Quantitative real-time PCR was carried out on an ABI 7500 thermal cycler (Applied Biosystems 7500 Fast Dx, Thermo Fisher). Mouse beta-actin (β-actin, FAM-MGB, Life Technologies, Grand Island, NY) was used as endogenous control. All experiments were carried out in triplicates in a minimum of three independent experiments. Data were analyzed with the standard curve method. Quantification of *Krt1*, *Krt5*, *Krt6a*, *Krt14*, and *Krt17* were performed with specific TaqMan assays (*Krt1*, Mm00492992_g1; *Krt5*, Mm01305291_g1, *Krt6a*, Mm00833464_g1; *Krt14*, Mm00495207_m1; *Krt17*, Mm00495207_m1, Life Technologies). *Gapdh* (Mm99999915_g1, Life Technologies) was used as an internal control. The Ct method was used for analyses.

Lentiviral vector construction and production

The shRNAs encoding siN159D-6 and scrambled siRNA (siN159D-6S) were cloned into pLVX-shRNA lentiviral vectors (Clontech, Mountain View, CA) between Pst I and BamH I sites (Supplemental Figure S2). The lentiviral vectors were produced in 293T cells as described elsewhere (Yasuda *et al.*, 2013). Viral vectors were dissolved in serum-containing medium, aliquoted in single-use vials, and stored at −80°C.

Primary keratinocyte culture

Primary keratinocytes were isolated from newborn mice as described previously (Lichti *et al.*, 2008). Specifically, skins of control or mutant *Krt75* pups (Chen *et al.*, 2008) were incubated with dispase II (Roche, Indianapolis, IN) to separate the epidermis. Epidermal sheets were then digested with 0.25% trypsin (Life Technologies) to release keratinocytes. The keratinocyte suspension was then cleared through filtration and centrifugation. Keratinocytes were then plated in 10 cm dishes with fibroblast-conditioned medium (Yuspa *et al.*, 1986).

HEK293T or primary keratinocytes were infected with lentiviral vectors as described previously (Yasuda *et al.*, 2013). Briefly, cells growing at approximately 20–30% confluency were treated with 8 μl/ml polybrene (Sigma-Aldrich) and infected with concentrated lentiviral vectors overnight. Subsequently, cells were cultured in complete growth medium until harvested for analyses or grafting.

Keratinocyte grafting and hair regeneration

Keratinocyte grafting was performed as described previously (Dai *et al.*, 2011). Specifically, approximately 1×10^6 keratinocytes infected with lentiviral vector were mixed with 2×10^6 primary fibroblasts freshly isolated from newborn pups, and seeded in grafting chamber on the backs of nude ($Foxn1^{-/-}$) mice. Three weeks later, hair grew out from the graft site. All procedures related to mice were approved by IACUC of the Institute of Laboratory Animal Science and Stony Brook University.

Microscopy

Hair plucked from skin grafts or clipped with scissors was used for light microscopy examination. A minimum of one hundred hairs of each experimental group were examined under stereoscope. A hair shaft containing one or more characteristic blebs was recorded as defective. Scanning electron microscopy was carried out as described previously (Chen *et al.*, 2008). Skin grafts were processed without any manipulation on the hair. TM1000 scanning electron microscope (Hitachi High-Technologies, Tokyo, Japan) was used for imaging.

Statistical analyses

All quantifications are presented as mean ± SD. Student's t-test was used for statistical analysis. $P<0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

Atkinson SD, McGilligan VE, Liao H, et al. Development of allele-specific therapeutic siRNA for keratin 5 mutations in epidermolysis bullosa simplex. J Invest Dermatol. 2011; 131:2079–86. [PubMed: 21716320]

Chapalain V, Winter H, Langbein L, et al. Is the loose anagen hair syndrome a keratin disorder? A clinical and molecular study. Arch Dermatol. 2002; 138:501–6. [PubMed: 11939812]

Chen J, Jaeger K, Den Z, et al. Mice expressing a mutant Krt75 (K6hf) allele develop hair and nail defects resembling pachyonychia congenita. J Invest Dermatol. 2008; 128:270–9. [PubMed: 17851587]

Chen J, Roop DR. Genetically engineered mouse models for skin research: Taking the next step. J Dermatol Sci. 2008

Dai D, Zhu H, Wlodarczyk B, et al. Fuz controls the morphogenesis and differentiation of hair follicles through the formation of primary cilia. J Invest Dermatol. 2011; 131:302–10. [PubMed: 20962855]

Duverger O, Ohara T, Shaffer JR, et al. Hair keratin mutations in tooth enamel increase dental decay risk. J Clin Invest. 2014; 124:5219–24. [PubMed: 25347471]

Harel S, Christiano AM. Genetics of structural hair disorders. J Invest Dermatol. 2012; 132:E22–6.

Hickerson RP, Flores MA, Leake D, et al. Use of self-delivery siRNAs to inhibit gene expression in an organotypic pachyonychia congenita model. J Invest Dermatol. 2011a; 131:1037–44. [PubMed: 21248764]

Hickerson RP, Leachman SA, Pho LN, et al. Development of quantitative molecular clinical end points for siRNA clinical trials. J Invest Dermatol. 2011b; 131:1029–36. [PubMed: 21191405]

Hickerson RP, Smith FJ, McLean WH, et al. SiRNA-mediated selective inhibition of mutant keratin mRNAs responsible for the skin disorder pachyonychia congenita. Ann N Y Acad Sci. 2006; 1082:56–61. [PubMed: 17145926]

Hickerson RP, Smith FJ, Reeves RE, et al. Single-nucleotide-specific siRNA targeting in a dominant-negative skin model. J Invest Dermatol. 2008; 128:594–605. [PubMed: 17914454]

Knobel M, O'Toole EA, Smith FJ. Keratins and skin disease. Cell Tissue Res. 2015

Lane EB, McLean WH. Keratins and skin disorders. J Pathol. 2004; 204:355–66. [PubMed: 15495218]

Leachman SA, Hickerson RP, Schwartz ME, et al. First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder. Mol Ther. 2010; 18:442–6. [PubMed: 19935778]

Leslie Pedrioli DM, Fu DJ, Gonzalez-Gonzalez E, et al. Generic and personalized RNAi-based therapeutics for a dominant-negative epidermal fragility disorder. J Invest Dermatol. 2012; 132:1627–35. [PubMed: 22402445]

Lichti U, Anders J, Yuspa SH. Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. Nat Protoc. 2008; 3:799–810. [PubMed: 18451788]

McLean WH, Moore CB. Keratin disorders: from gene to therapy. Hum Mol Genet. 2011; 20:R189–97. [PubMed: 21890491]

Ng CS, Wu P, Foley J, et al. The chicken frizzle feather is due to an alpha-keratin (KRT75) mutation that causes a defective rachis. PLoS Genet. 2012; 8:e1002748. [PubMed: 22829773]

Schweizer J, Langbein L, Rogers MA, et al. Hair follicle-specific keratins and their diseases. Exp Cell Res. 2007; 313:2010–20. [PubMed: 17428470]

Smith FJ, Liao H, Cassidy AJ, et al. The genetic basis of pachyonychia congenita. J Investig Dermatol Symp Proc. 2005; 10:21–30.

Sperling LC, Hussey S, Sorrells T, et al. Cytokeratin 75 expression in central, centrifugal, cicatricial alopecia--new observations in normal and diseased hair follicles. J Cutan Pathol. 2010; 37:243–8. [PubMed: 19614992]

Uitto J. Molecular therapeutics for heritable skin diseases. J Invest Dermatol. 2012; 132:E29–34.

Uitto J, Richard G, McGrath JA. Diseases of epidermal keratins and their linker proteins. Exp Cell Res. 2007; 313:1995–2009. [PubMed: 17531221]

Wilson NJ, O'Toole EA, Milstone LM, et al. The molecular genetic analysis of the expanding pachyonychia congenita case collection. Br J Dermatol. 2014; 171:343–55. [PubMed: 24611874]

Winter H, Langbein L, Praetzel S, et al. A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. J Invest Dermatol. 1998; 111:955–62. [PubMed: 9856802]

Winter H, Schissel D, Parry DA, et al. An unusual Ala12Thr polymorphism in the 1A alpha-helical segment of the companion layer-specific keratin K6hf: evidence for a risk factor in the etiology of the common hair disorder pseudofolliculitis barbae. J Invest Dermatol. 2004; 122:652–7. [PubMed: 15086549]

Wojcik SM, Longley MA, Roop DR. Discovery of a novel murine keratin 6 (K6) isoform explains the absence of hair and nail defects in mice deficient for K6a and K6b. J Cell Biol. 2001; 154:619–30. [PubMed: 11489919]

Yasuda M, Claypool DJ, Guevara E, et al. Genetic manipulation of keratinocyte stem cells with lentiviral vectors. Methods Mol Biol. 2013; 989:143–51. [PubMed: 23483393]

Yuspa SH, Morgan D, Lichti U, et al. Cultivation and characterization of cells derived from mouse skin papillomas induced by an initiation-promotion protocol. Carcinogenesis. 1986; 7:949–58. [PubMed: 2871947]
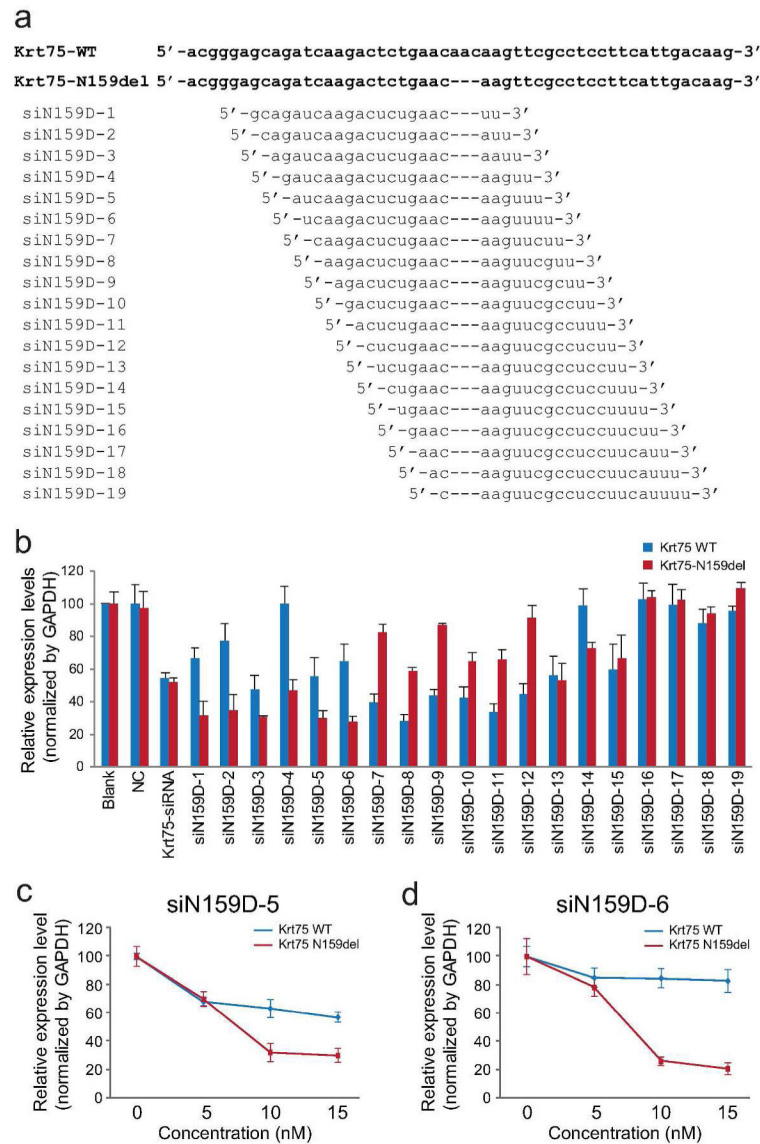
Figure 1. Mutant *Krt75*-specific siRNA

(a) Sequences of wild-type and c.545_547del (p.N159del) *Krt75* and candidate siRNAs for mutant *Krt75*. (b) Relative expression levels of wild-type and mutant *Krt75* in HEK293T cells co-transfected with wild-type and mutant *Krt75* and siRNA (15 nM) by quantitative RT-PCR. Blank, cells transfected with *Krt75* expression plasmids without siRNA; NC, cells transfected with negative control (a fragment of inverted beta-galactosidase sequence) siRNA; Krt75-siRNA, a commercially available siRNA against *Krt75*. (c, d) Relative expression levels of wild-type and mutant *Krt75* in cells transfected with 0 – 15 nM siN159D-5 and siN159D-6 siRNAs, as normalized to GAPDH. All experiments were carried out in triplicates in a minimum of three independent experiments.
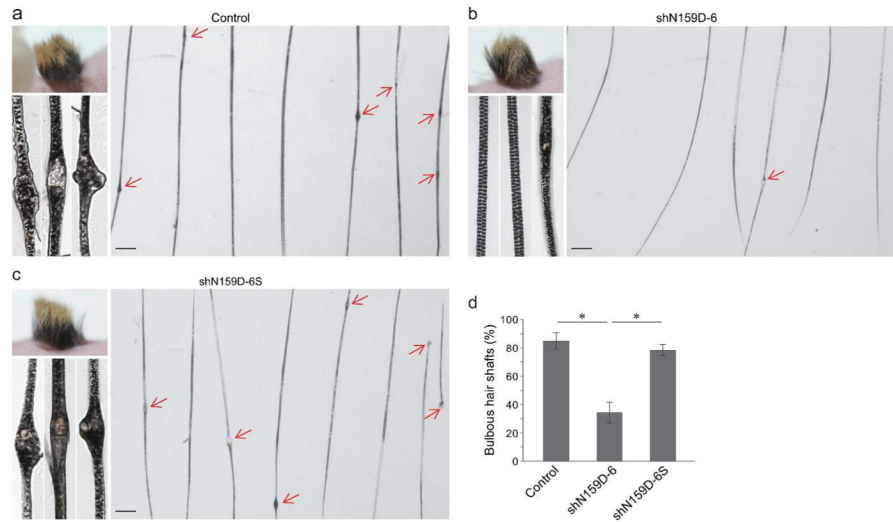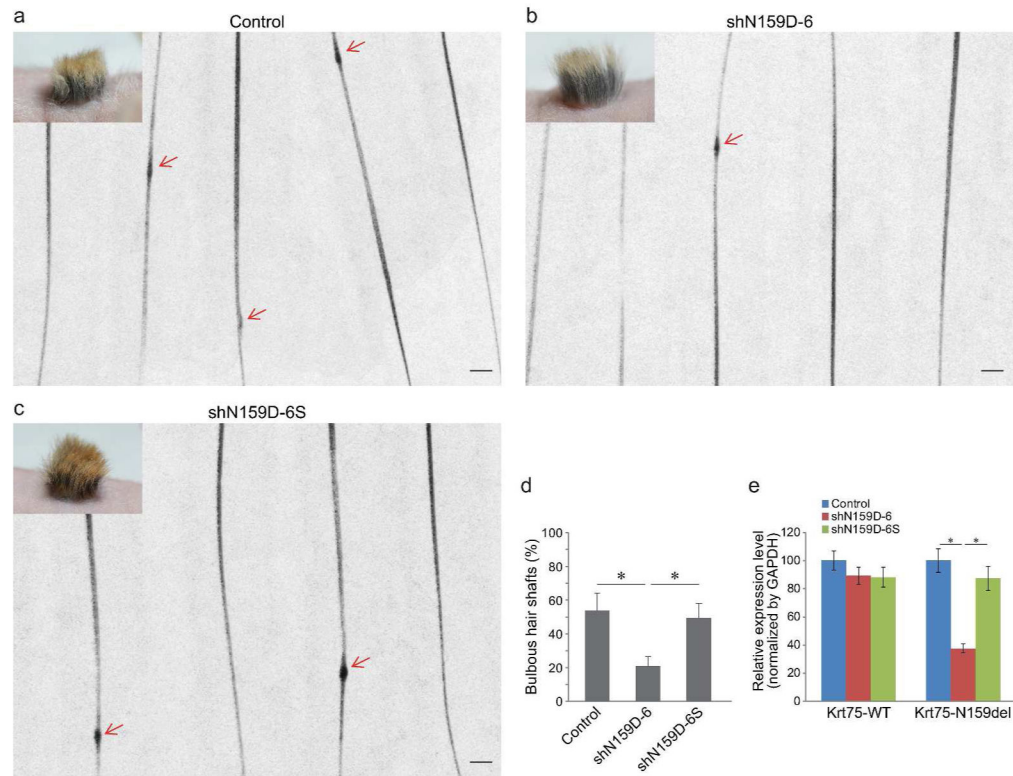
Figure 2. Phenotypes of hair regenerated with shRNA-modified homozygous mutant *Krt75* keratinocyte progenitor cells

(a–c) Representative gross appearance, low and high power images of hair regenerated with non-infected cells. Control (a), shN159D-6 lentiviral vector infected cells (b), and scrambled (shN159D-6S) lentiviral vector infected cells (c) at one month of grafting. Arrows point to bulbous lesions (blebs) along the hair shaft. (d) Quantification of hair shafts containing blebs. Asterisk, *P* < 0.05. Scale bar: 250 μm.
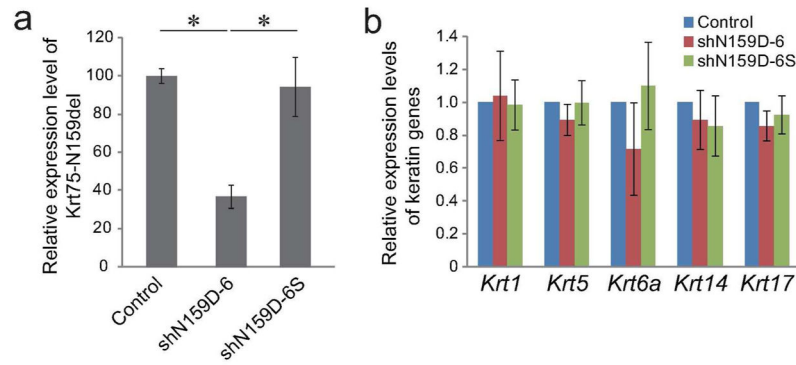
Figure 3. Gene expression in skin grafts regenerated with shRNA modified homozygous mutant *Krt75* keratinocyte progenitor cells

(a) Relative expression level of mutant *Krt75* (Krt75-N159del) in grafts regenerated with *ex vivo* cultured cells (as described in Figure 2) by qRT-PCR one month after grafting . (b) Relative expression levels of *Krt1*, *Krt5*, *Krt6a*, *Krt14*, and *Krt17* by qRT-PCR in skin grafts described in (a). All experiments were carried out in triplicates in a minimum of three independent experiments. Asterisk, $P < 0.05$.

Figure 4. Phenotype and gene expression in skin grafts regenerated with shRNA-modified heterozygous mutant *Krt75* keratinocyte progenitor cells

(a–c) Representative hair phenotypes of hair regenerated with control and lentiviral vector-infected cells (as described in Figure 2) at one month of grafting. Arrows point to bulbous lesions (blebs) along the hair shaft. (d) Quantification of hair shafts containing blebs. (e) Relative expression level of wild-type (Krt75-WT) and mutant *Krt75* (Krt75-N159Del) in skin grafts described in (a). Asterisk, $P < 0.05$. Scale bar: 250 μm.