siRNA Silencing of the Mutant Keratin 12 Allele in Corneal Limbal Epithelial Cells Grown From Patients With Meesmann's Epithelial Corneal Dystrophy

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Purpose. The aim of this study is to further assess our previously reported keratin 12 (K12)-Leu132Pro specific siRNA in silencing the mutant allele in Meesmann's Epithelial Corneal Dystrophy (MECD) in experimental systems more akin to the in vivo situation through simultaneous expression of both wild-type and mutant alleles.

Methods. Using *KRT12* exogenous expression constructs transfected into cells, mutant allele specific knockdown was quantified using pyrosequencing and infrared Western blot analysis, while the silencing mechanism was assessed by a modified rapid amplification of cDNA ends (5′RACE) method. Corneal limbal biopsies taken from patients suffering from MECD were used to establish cultures of MECD corneal limbal epithelial stem cells and the ability of the siRNA to silence the endogenous mutant *KRT12* allele was assessed by a combination of pyrosequencing, qPCR, ELISA, and quantitative-fluorescent immunohistochemistry (Q-FIHC).

Results. The siRNA displayed a potent and specific knockdown of K12-Leu132Pro at both the mRNA and protein levels with exogenous expression constructs. Analysis by the 5′RACE method confirmed siRNA-mediated cleavage. In the MECD cells, an allele-specific knockdown of 63% of the endogenous mutant allele was observed without effect on wild-type allele expression.

Conclusions. Combined with an effective delivery vehicle this siRNA approach represents a viable treatment option for prevention of the MECD pathology observed in K12-Leu132Pro heterozygous individuals.

Keywords: siRNA, MECD, keratin 12, ex vivo, allele discrimination

Keratins form the intermediate filament cytoskeleton in numerous epithelia including those of the ocular surface. The corneal epithelium–keratin, keratin 12 (K12), forms intermediate filaments with keratin 3 and these heterodimers provide structure and stability in corneal epithelial cells.[1,2] When a heterozygous missense mutation occurs within either of these genes, it is recognized that it will result in Meesmann's epithelial corneal dystrophy (MECD)[3,4] and a number of *KRT12* mutations have been reported with phenotypes of varying severity.[4–7] The *KRT12* mutation Arg135Thr is a common European mutation[4,8] while the Leu132Pro mutation, originally reported by this group[7] and recently confirmed by Hassan et al.,[6] results in a much more severe phenotype. Leu132Pro associated MECD can be differentiated from that caused by Arg135Thr, by the presence of microcysts and corneal scarring; this can lead to a severe loss in visual acuity requiring surgical intervention. If a central corneal penetrating graft or lamellar keratoplasty[9,10] is utilized to correct corneal scarring in MECD, a return to the disease phenotype occurs, due to the repopulation of the grafted tissue by mutation-containing host corneal epithelial cells. A personalized medicine approach, however, with familial screening and initiation of therapeutic intervention to reduce the amount of mutant allele, prior to the onset of symptoms, could be the safest and most effective therapy for this and similar conditions of the ocular surface.[11] One such personalized approach involves the exploitation of the RNA interference (RNAi) pathway.

The use of RNAi therapeutics in the form of short-interfering RNA (siRNA) is a promising gene silencing approach.[12–14] The specificity and efficiency of siRNA are unparalleled,[15] as they can discriminate between two alleles differing by just a single nucleotide, with implications for the treatment of many dominant negative disorders. We have previously demonstrated successful design of siRNAs for a number of keratin disor-

ders.[7,8,16–18] The effectiveness of siRNA-based therapies has recently seen their progression into clinical trials,[19–21] with research demonstrating a knockdown of 50% being sufficient to return to a normal phentoype.[22] The ocular surface, and in particular the corneal epithelium, is a prime candidate for the demonstration of gene silencing through siRNA application, due to the accessibility of the cornea. However, the advancement of siRNA molecular therapy for the eye is modest, possibly due to a lack of viable preclinical disease models.[23]

We previously reported mutant allele-specific silencing siRNAs for *KRT12* Leu132Pro[7] and Arg135Thr[8] in noncorneal cells. In the case of Leu132Pro, we identified a lead siRNA by in vitro assessment using only exogenous constructs, where wild-type or mutant *KRT12* was expressed separately. In this study, we confirm gene silencing of the mutant Leu132Pro allele is a result of cleavage of the K12 mRNA at the predicted cleavage site for our siRNA. In cells coexpressing wild-type and mutant *KRT12* constructs, we establish a technique for quantification of allelic discrimination using pyrosequencing and confirm silencing at the protein level using quantitative epitope-tagged infrared Western blot analysis. We established cultures of MECD corneal epithelial limbal stem cells using a corneal limbal biopsy from a number of patients heterozygous for the *KRT12* Leu132Pro mutation and demonstrated specific siRNA silencing of the endogenous mutant allele.

Methods

Constructs

The full-length human wild-type and Leu132Pro mutant *KRT12* coding sequence was PCR amplified, cloned into pCR2.1 (Invitrogen, Paisley, UK), and fully sequenced as previously described,[7,8] giving rise to pCR2.1-K12-WT (wild type) and pCR2.1-K12-L132P (Leu132Pro) expression constructs. Constructs used for the dual Flag/Strep Tag II quantitative infrared Western blot were cloned into pCR3.1 (Invitrogen) and sequenced as previously described,[8] generating a wild-type KRT12-FlagHA clone (pK12-WT/FlagHA) and a Leu132Pro KRT12-StrepHA clone (pK12-L132P/StrepHA).

siRNA Design

The K12-Leu132Pro specific siRNA used was previously identified by our group as having the most potent and specific mutant mRNA knockdown potential from a group of 19 siRNAs designed to be specific to the Leu132Pro mutation.[7] The sequence for this siRNA (K12-Leu132Pro-9) is 5′-AUG CAAAAUCCUAAUGAUAdTdT-3′ (mutation in bold) while the sequence for the nonspecific control siRNA (NSC4; an inverted bacterial β-galactosidase sequence) is 5′-UAGCGACUAAACA CAUCAAdTdT-3′.

Cell Culture

Human embryonic kidney cells (AD293; Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) enriched with 10% fetal bovine serum (Invitrogen). All cells were grown at 37°C with 5% CO_2 and passaged following normal laboratory procedures.

Study Participants and Isolation of Corneal Stem Cells

For the limbal biopsy and culture of MECD corneal epithelial stem cells ethical approval was gained from the East of Scotland Research Ethics Service and complied with all local research governance legislation. The research followed the tenets of the Declaration of Helsinki.

Subject recruitment inclusion criteria included subjects known to carry the Leu132Pro *KRT12* mutation. After informed consent, an experienced surgeon removed a small 2-mm^2 biopsy of corneal epithelial cells from the limbal region of a patient confirmed to carry the K12-Leu132Pro mutation. Cells were cultured under conditions previously described for isolated human corneal stem cells.[24] The colony-forming efficiency and p63 analysis of the human corneal stem cells was performed using methods as described in detail by Corradini et al.[24] The percentage of cells expressing K12 was assessed using standard immunocytochemistry methodology and was carried out using a goat polyclonal anti-K12 antibody (Clone N-16; Santa Cruz Biotechnology, Heidelberg, Germany), a donkey anti-goat fluorescein isothiocyanate conjugated antibody (Santa Cruz Biotechnology) or a donkey anti-goat tetramethyl rhodamine isothiocyanate antibody (Santa Cruz Biotechnology). Counting was performed in a masked manner. To ensure specificity of observed staining with this antibody, AD293 cells which are known to lack endogenous K12 expression were included as well as AD293 cells transfected with a GFP-K12 fusion protein and HaCaT cells known to express a wide range of keratin proteins including K5, K14, and K6a but not K12. Images were captured using an SP5 confocal/multiphoton microscope (Leica Geosystems, Ltd., Milton Keynes, UK).

Allelic Discrimination by Pyrosequencing

The AD293 cells were seeded at 4×10^5 cells per well and reverse transfections were performed using a transfection reagent (Lipofectamine 2000; Life Technologies, Paisley, UK). Each transfection contained 200 ng of pCR2.1-K12-WT and 200 ng of pCR2.1-K12-L132P, and either NSC4 or K12-Leu132Pro-9 siRNA with a final concentration of 5 nM. Transfections for each condition were performed in triplicate in 12 well plates. Messenger RNA (mRNA) was extracted at 24, 48, and 72 hours posttransfection. The RNA purification mini kit (Qiagen RNAeasy Plus; Qiagen, Manchester, UK) was used to extract the mRNA per manufacturer's instructions. Extracted mRNA was quantified and 1 μg was converted to cDNA using a high efficiency cDNA reverse transcription kit (Life Technologies). A pyrosequencing PCR using a commercial kit (PyroMark PCR kit; Qiagen), was used to amplify the region containing the mutation of interest with the following primers: Forward – 5′ CAATGATGGAGGCCTTCTT 3′, Reverse – 5′ [Biotin]TCTTC TAGAGCTTCGCACCTTATC 3′, Sequencing – 5′ AAAAGAAAC TATGCAAAATC 3′ (Sigma-Aldrich, Gillingham, UK).

Pyrosequencing was then performed using a commercial product (Pyromark Q24; Qiagen), according to the manufacturer's instructions. Complementary DNA from each sample was analyzed in duplicate by pyrosequencing. Pyrosequencing was also carried out on primary corneal epithelial cells, cultured in submerged conditions, treated with 5 nM of either NSC4 or K12-Leu132Pro-9 siRNA for 72 hours and transfected using a commercial reagent (Lipofectamine RNAiMax; Life Technologies), as described above.

Dual Flag/Strep Tag II Quantitative Infrared Immunoblot

Infrared quantitative Western blot analysis was carried out as previously described[8] on protein extracted from AD293 cells 48 hours posttransfection with pK12-WT/FlagHA, pK12-L132P/StrepHA, and either NSC4 or K12-Leu132Pro-9 siRNA at a concentration of either 0.5 nM or 5 nM.

5′RACE Analysis

A modified rapid amplification of cDNA ends (5′RACE) method was used to confirm that the K12 Leu132Pro mutant allele knockdown observed was caused by the introduction of the specific siRNA, as previously described.[8] This methodology allows for the determination of the mRNA cleavage site, which in this case should be at the phosphodiester bond positioned between nucleotides 10 and 11 from the 5′ end of the guide strand of the K12-Leu132Pro-9 siRNA. The gene specific primer used for K12 amplification was K12GSPrimer-5′ CATACTGCGCCCGCATATCATTG 3′ (MWG, Ebersberg, Germany).

Quantification of K12 by Quantitative-Fluorescent Immunohistochemistry (Q-FIHC)

Aliquots of cell suspensions obtained from cell culture, were transfected with 5 nM of either NSC4 or K12-Leu132Pro-9 siRNA using Lipofectamine RNAiMax, incubated for 72 hours, and were spun, methanol-fixed, and dried before staining. Spots of approximately 1000 cells were used for each antibody staining. Fluorescence intensity and cell size values were measured by microscope (Axio Observer.Z1; Zeiss, Jena, Germany), data analysis was performed with microscope software (Axiovision Rel.4.8; Zeiss) and on a worksheet containing the intensity of the fluorescence signal and the associated cell size as previously described by Di Iorio et al.[25] A two-axis matrix including fluorescence intensity and cell size, directly related to keratinocyte differentiation was obtained. The number of dots in the matrix matched with the number of cells analyzed on the cytology-spot. Samples of 174 and 180 cells were analyzed and a cutoff of 35 μm was set, to include only nonproliferating differentiated K12 positive cells.

Quantification of K12 by ELISA

An ELISA was performed on protein extracts from primary corneal epithelial cells treated with 5 nM of either NSC4 or K12-Leu132Pro-9 siRNA for 72 hours and transfected using a commercial reagent (Life Technologies), as described above. Protein was extracted in 50 μL RIPA buffer containing a cocktail of protease inhibitors and quantified using a Bradford assay prior to loading. Each sample was diluted by a factor of 1:50 and 4 μg of total protein was loaded per well. The ELISA was performed following the manufacturer's protocol (antibodies-online GmbH, Aachen, Germany).

Quantitative Real-Time PCR (qPCR) Analysis

The cDNA samples from MECD patient cells treated with either NSC4 or K12-Leu132Pro-9 siRNA were also analyzed by qPCR. A commercially available assay (assay Id 140679; Roche Diagnostics, Ltd., West Sussex, UK) for *K12* quantification was used following the manufacturer's protocol along with a *HPRT* assay (assay Id 102079; Roche Diagnostics, Ltd.), where *HPRT* was used as the reference gene. A *KRT19* assay (assay Id 103579; Roche Diagnostics, Ltd.); *ABCG2* assay (Roche, West Sussex, UK); and a *KRT14* assay (assay Id 117459; Roche Diagnostics, Ltd.) were used to assess variations in limbal stem cell marker expression.[26] All cDNA samples were run in triplicate with relative expression levels of each gene calculated using the ΔΔCT method. In each case, gene expression levels from cells treated with the K12-Leu132Pro-9 siRNA were compared with those observed in the NSC4-treated cells.

Transfection Efficiency of MECD cells

Experiments were carried out to assess transfection efficiency in the MECD cells. Small interfering RNA with a fluorescent tag (siGlo; Thermo Fisher Scientific, Epsom, UK) was used to calculate transfection efficiency; this was measured by flow cytometry or manual counting of immunofluorescence cells. Cells were transfected with 5 nM siGlo for 24 hours and then were viewed or detached with trypsin and resuspended in 500 μL of PBS. Using flow cytometry (Gallios; Beckman Coulter, Inc., Brea, CA, USA) transfection efficiency was calculated.

Cell Viability Assay

Primary corneal epithelial cells seeded at 1.5×10^4 cells per well of a 96-well plate were transfected with a commercial reagent (Life Technologies) and 5 nM of either NSC4 or K12-Leu132Pro-9 siRNA. After 72 hours, a cell viability reagent (PrestoBlue; Life Technologies) was added and incubated for half an hour before the signal was read on a plate reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). All treatment readings were normalized to media only controls.

Statistics

A Student's *t*-test was used to calculate significance for pyrosequencing and qPCR data. Statistical analysis of the infrared immunoassay data included an analysis of variance using Levene's followed by a Brown-Forsythe test. All error bars represent the standard error of the mean unless otherwise stated. For all statistical analysis significance was set at ≤ 0.05.

Results

siRNA-Mediated Inhibition of Mutant K12 in the Presence of Wild-Type K12

In this study, pyrosequencing was developed as a method to quantify cDNA strands that differ by just a single nucleotide, as is the case with the *KRT12* wild-type versus Leu132Pro alleles. We initially optimized the technique using RNA extracted from AD293 cells cotransfected with wild-type and mutant *KRT12* expressing constructs, and treated with either NSC4 or K12-Leu132Pro-9 siRNA. Time points of 24, 48, and 72 hours post siRNA transfection were assessed. Quantification of the ratio of the amount of mutant mRNA: wild-type mRNA (Fig. 1) demonstrated a reduction of 83% (±1.8%), after 48 hours of K12-Leu132Pro-9 siRNA treatment. The pyrogram shown demonstrates visually the reduction in the mutant allele (C) represented by the circled peak, while the wild-type allele (T) remains the same (Fig. 1).

Using epitope tagged infrared Western blot analysis the siRNA silencing of mutant K12 was observed at the protein level while simultaneously the specificity of the siRNA was assessed in relation to wild-type K12 expression. The wild-type K12 Flag-HA and mutant K12 Strep-HA proteins were imaged using anti-Flag monoclonal and anti-Strep-tag II polyclonal antibodies, respectively, to confirm siRNA allele–specific silencing of mutant K12 protein.[8,17] Cells transfected with the NSC4 siRNA showed no discernable knockdown of either wild-type (red bands in Fig. 2 representing α-flag) or mutant (green bands in Fig. 2 representing α-strep) K12 protein. With quantification of protein expression (Fig. 2), the K12-Leu132-Pro-9 siRNA demonstrated a significant and specific reduction of mutant strep-tagged protein at both 0.5 nM and 5 nM concentrations of 91% (±50.2%) and 89% (±24.4%), respectively. The lower green band (Fig. 2) represents β-actin, used here to confirm uniform loading.

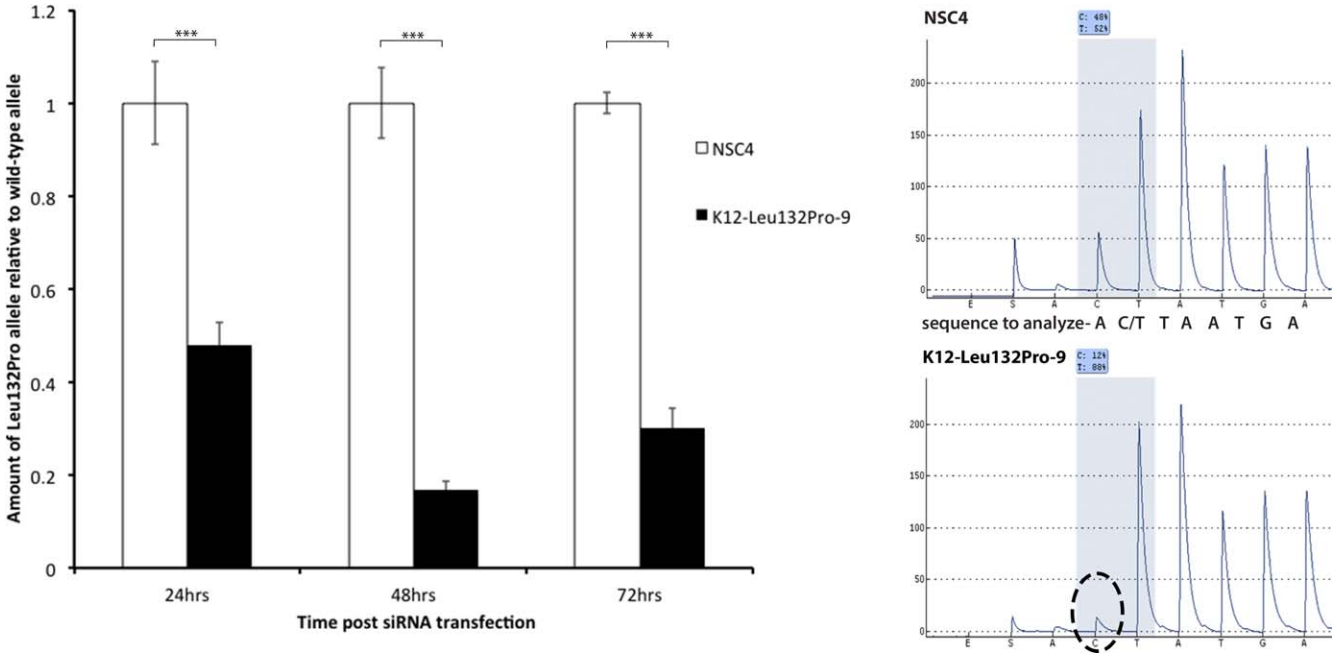

FIGURE 1. Small interfering RNA knockdown of the mutant K12 allele quantified by pyrosequencing. Pyrosequencing data demonstrated effective silencing of the mutant allele by the K12-Leu132Pro-9 siRNA. Mutant allele expression was calculated relative to that of the wild type. In cells treated with K12-Leu132Pro-9 for 48 hours a knockdown of 83% of K12-Leu132Pro expression was observed compared with no knockdown in the NSC4-treated cells. The pyrograms (on the right-hand side) demonstrate wild-type (T) and mutant (C) mRNA levels in NSC4- (*top*) and K12-Leu132Pro-9–treated cells (*bottom*). Peaks indicate the presence of bound nucleotide, where larger peaks indicate consecutive nucleotide binding in the sequence. For each condition; $n = 6$. $^{***}P < 0.001$.

K12 mRNA Cleavage Site Confirmed by 5′RACE Analysis

To confirm that silencing of mutant *KRT12* was by a siRNA RNA-induced silencing complex (RISC)–mediated mechanism of action, a modified 5′RACE method was used, as previously described.[8,27] Rapid amplification of cDNA ends was carried out on total RNA extracted from AD293 cells transfected with mutant *KRT12* and K12-Leu132Pro-9 siRNA. As shown in Figure 3, the cleavage site on the mutant K12 mRNA was found to be between the 10th and 11th nucleotides from the 5′ end of the siRNA antisense strand, as predicted (positions 393 and 394 in the *KRT12* cDNA sequence from the start codon).

Isolation and Culture of MECD Patients' Corneal Epithelial Limbal Stem Cells

The use of exogenous expression constructs for assessment of *KRT12* expression levels with and without siRNA treatment provides a simple artificial test system. Ultimately, we need to confirm siRNA silencing of the endogenous mutant allele. Here we demonstrate this using cells cultured from human corneal limbal biopsies taken from three MECD patients carrying the *KRT12* Leu132Pro mutation. Primary corneal epithelial cells were isolated from 2-mm^2 biopsies for MECD patients and three non-MECD cell samples were isolated from normal control donors (within 24 hours after death); cell yield from

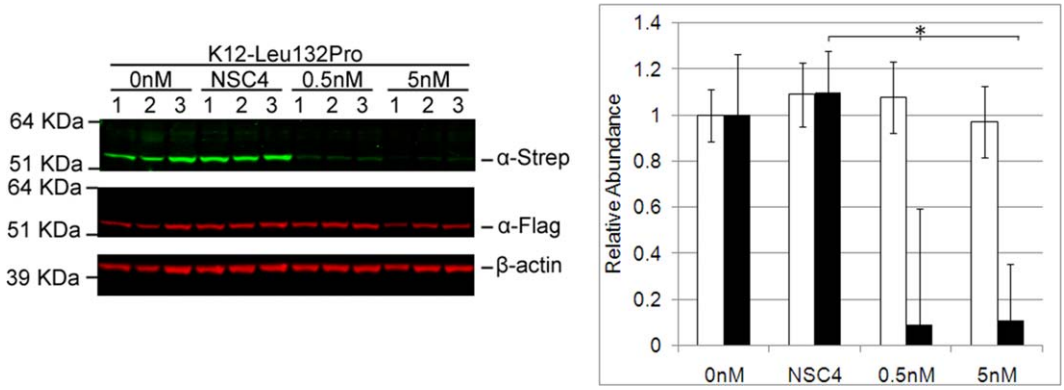

FIGURE 2. Dual Flag/Strep Tag II quantitative infrared immunoblot analysis confirming allelic discrimination of K12-Leu132Pro-9 siRNA. Infrared immunoblot analysis confirmed the knockdown of the Leu132Pro mutant K12 by K12-Leu132Pro-9 in the presence of wild-type K12, in AD293 cells transiently expressing pK12-WT/FlagHA and pK12-L132P/StrepHA. Knockdown of mutant protein of 91% and 89% was observed at the 0.5 nM and 5 nM concentrations respectively, with no significant knockdown of either wild-type or mutant expression observed in cells treated with NSC4. Both wild-type and mutant protein was standardized against β actin levels before being normalized to untreated cell protein levels. Mean relative abundances were calculated with error bars indicating standard deviations. Knockdown was deemed significant ($^*P < 0.05$) where $n = 9$.

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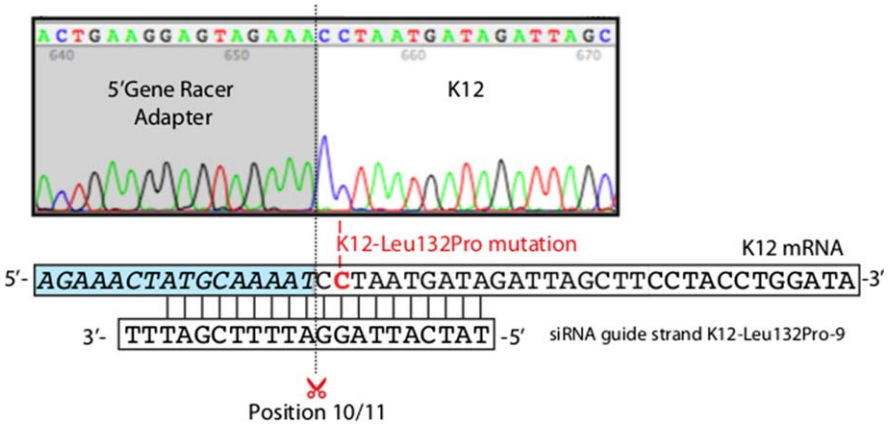

Figure 3. Rapid amplification of cDNA ends analysis confirming siRNA-mediated cleavage of K12 Leu132Pro mRNA. Keratin 12 mRNAs cleaved with siRNA K12-Leu132Pro-9 were ligated to a 5′ gene racer adapter and converted to cDNA. Keratin 12–specific products were isolated by two rounds of PCR with a K12 gene specific reverse primer (K12GSPrimer) and Generacer 5′ (GR5′) and nested Generacer 5′ forward primers. The PCR products were ligated into the PCR4.0 sequencing plasmid, amplified by bacterial transformation and colonies were sequenced with primers M13R and M13F. Sequencing confirmed that the 5′generacer adapter had ligated to the free end of the K12 mRNA at nucleotide 393 from the start codon, the site predicted by the standard model for siRNA cleavage, namely between nucleotides 10 and 11 counting from the 5′ end of the siRNA guide strand.

tissues ranged between 9000 to 14,000 cells/mm^2 for MECD biopsies, whereas cell yield from control unaffected human biopsies was between 12,000 to 18,000 cells/mm^2. Meesmann's epithelial corneal dystrophy limbal cells generated large colonies (1% of cells from biopsy) with a smooth and regular perimeter (Fig. 4A) and a similar morphology when compared with colonies generated by control cells. Subconfluent primary cultures of MECD limbal keratinocytes had a

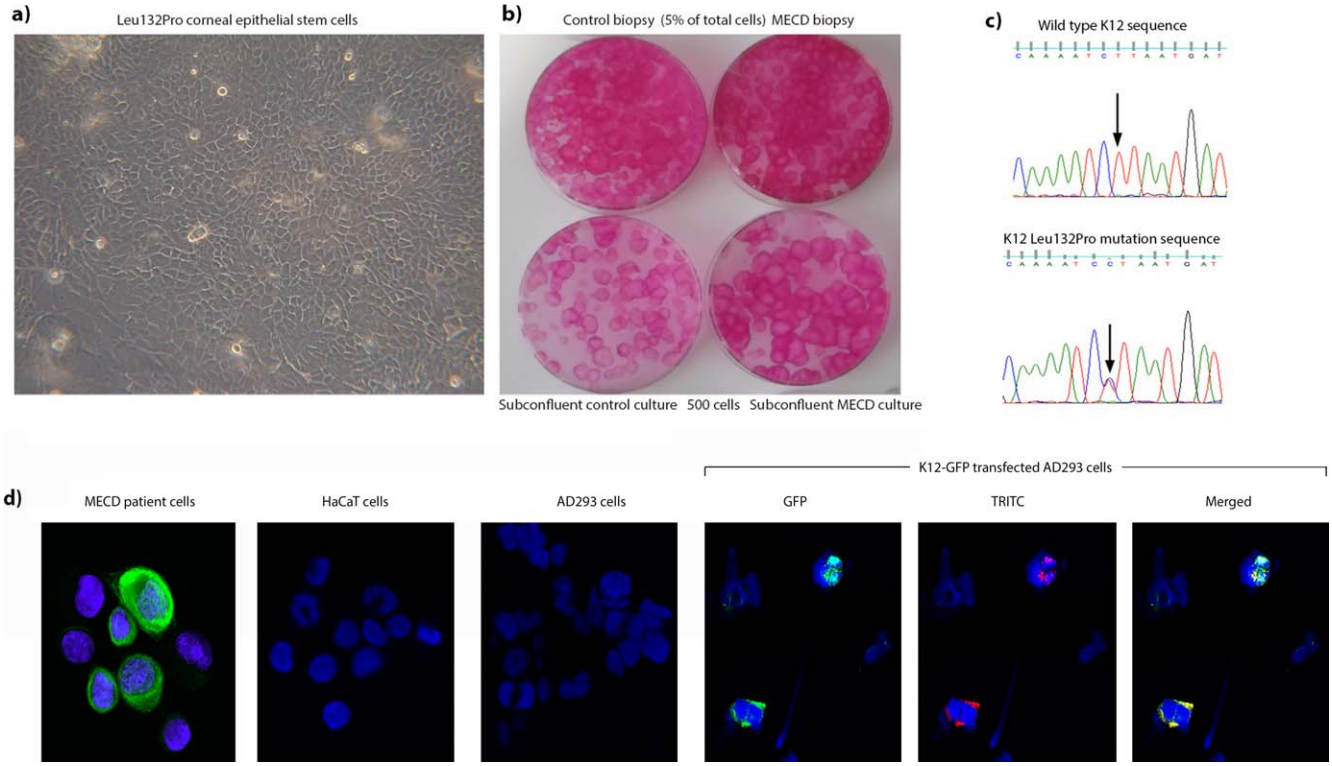

Figure 4. Cell cultures of MECD established from a patient's limbal biopsy. A limbal biopsy was taken from MECD patients heterozygous for the *KRT12* Leu132Pro mutation. (**A**) Cells were cultured alongside non-MECD cells from wild-type control donors. (**B**) The colony-forming efficiency was assessed after 12 days in culture and it was noted that a similar cellular morphology was exhibited across both MECD and non-MECD cultures. (**C**) DNA was extracted from MECD cultures and sequencing confirmed the presence of the *KRT12* Leu132Pro mutation. (**D**) Immunocytochemistry confirmed the presence of K12 protein in the MECD patient cells. Control IgG2a antibody substitution for the anti K12 antibody proved specificity through lack of staining (image not shown). No staining was apparent in HaCaT cells and AD293 cells, both known to lack endogenous K12 expression. AD293 cells transfected with an exogenous construct that expresses a K12-GFP fusion protein were used as a positive control, which showed a localization (*yellow*) of anti K12 TRITC staining (*red*) with the GFP fused K12 (*green*). Nuclei were counterstained with DAPI; to enable visualization of cells.

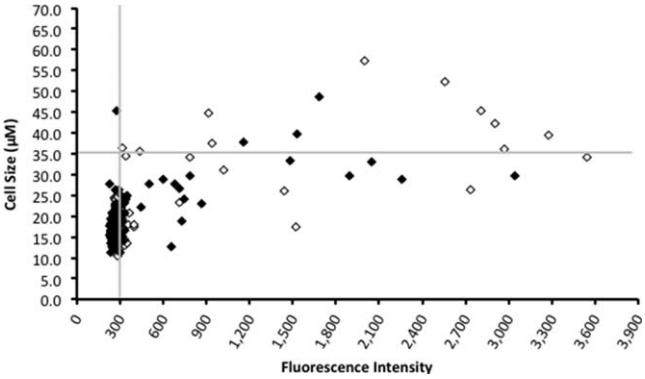

FIGURE 5. Quantitative-fluorescent immunohistochemistry of MECD cells treated with the K12-Leu132Pro-9 siRNA. The Q-FIHC was undertaken to confirm silencing by K12-Leu132Pro-9 siRNA at the protein level in the cultured MECD cells. A cell size of greater than 35 μm was used as a cutoff; to include only the larger nonproliferating differentiated K12 positive cells. A reduction of 59% in K12 positive cells was noted between control cells (*white fill*) and those cells treated with 5 nM K12-Leu132Pro-9 siRNA (*black fill*).

mean of 16.8% colony forming cells and were frozen for further analysis. Colony-forming efficiency (Fig. 4B, after 12 days in culture) and growth rate were similar to control primary cultures generated from unaffected limbal biopsies. Similarly, cultured MECD and control keratinocytes contained a comparable amount of limbal stem cells, measured as the number of p63 positive bright cells.[25] However, after one further passage in culture, MECD limbal cells showed a higher value proportion of p63 positive bright cells compared with normal controls (5.2% vs. 2%). The DNA and RNA extracted from the human MECD cells were used to confirm the presence of the *KRT12* Leu132Pro mutant allele (Fig. 4C). The MECD cultures were assessed for the presence of K12 protein (corneal differentiation marker) and the number of cells positive for K12 was assessed. The MECD cultures contained a higher number proportion of K12 positive cells compared with normal controls (32.5% vs. 15.5% in total cell population, and 5.6% vs. 1.9% in large nonproliferating differentiated cells), quantified by immunocytochemistry (Fig. 4D). Antibody specificity was confirmed by the lack of staining in a number of cell types known not to express K12 as shown in Figure 4D.

The stage of keratinocyte terminal differentiation is closely related to its size, this is true for all keratinocytes in culture; as well as those within the epidermis and ocular epithelia. The keratinocyte enlarges progressively as it moves between the basal layer and the upper layers. On this basis, we analyzed the percentage of large nonproliferating, differentiated cells (>35 μm) showing positive staining for K12, in NSC4 or K12-Leu132Pro-9 siRNA-treated samples from the same patient, using Q-FIHC.[25] Quantitative-fluorescent immunohistochemistry combines cell observer microscopy and advanced analysis software, allowing high-definition image processing. Compared with conventional analysis systems such as flow cytometry or quantitative Western blotting, which requires a high number of cells, this assay allows one to determine and localize protein signals from a low number of cells (as low as 500–1000/sample). Using this methodology, we show a 59% decrease in large, K12 positive cells in those samples treated with the K12-Leu132Pro-9 siRNA (Fig. 5).

Allele-Specific Knockdown of the Endogenous Mutant K12 Allele in MECD Cells

Pyrosequencing was carried out on RNA extracted from MECD cells to determine if K12-Leu132Pro-9 siRNA could silence the endogenous mutant K12 allele expression. Cells were treated with NSC4 or K12-Leu132Pro-9 siRNA for a number of time points. A significant knockdown of the endogenous mutant allele of 63% (±19.7%) was observed after 72 hours; as demonstrated in Figure 6A. Quantification of total K12 expression was performed by qPCR and confirmed that only mutation-specific knockdown was being observed with the wild-type K12 allele unaffected. The expression of total K12 was found to be 68% (±11.1%) in K12-Leu132Pro-9–treated cells, where in cells treated with NSC4 K12 expression was unaffected (Fig. 6B). The total K12 expression concurs with the knockdown of mutant allele demonstrated by pyrosequencing, indicating that the K12-Leu132Pro-9 siRNA is highly specific to the Leu132Pro mutant K12 allele. Silencing of endogenous K12 protein was quantified by ELISA. Protein was extracted from MECD cells 72 hours after treatment with either NSC4 siRNA or K12-Leu132Pro-9 siRNA. A 23% knockdown of total K12 protein was observed in the cell lysates from cultures treated with the K12-Leu132Pro-9 siRNA, when compared with the K12 expression in NSC4 treated cultures (Fig. 6C). This reduction in total K12 is in agreement with the K12 knockdown observed at mRNA level and reflects approximately 46% silencing of the mutant K12 allele. We also assessed the effect of K12-Leu132Pro-9 siRNA on limbal stem cell differentiation by quantifying expression of limbal stem cell markers KRT19, ABCG2, and KRT14 by qPCR in cells either untreated or transfected with NSC4 or K12-Leu132Pro-9 siRNA. The mRNA levels from all three stem cell markers were standardized against *HPRT* expression and normalized to the gene expression in NSC4 treated cells. As shown in Figure 6B no significant variations were observed in the expression of KRT19, ABCG2 or KRT14, indicating the introduction of siRNA K12-Leu132Pro-9 at a concentration of 5 nM appears to have no effect on markers of limbal stem cell differentiation. Transfection efficiency of siRNAs was assessed using a fluorescently tagged siRNA, siGlo. Both flow cytometry and counts of siGLO positive cells confirmed a transfection efficiency of approximately 60%. Cell viability was not found to be affected by treatment with NSC4 or K12-Leu132Pro-9 siRNA (Fig. 6E).

DISCUSSION

Ribonucleic acid interference represents a promising field of therapeutics based on personalized genetic manipulation.[12] Small interfering RNAs have demonstrated an unparalleled specificity in their ability to distinguish between two alleles that differ by just a single nucleotide, a quality that is advantageous in the treatment of heritable dominant negative disorders due to mutations in a single nucleotide.[7,8,16,17,28] Target disorders include pachyonychia congenita[29] and cystic fibrosis,[30] with many more conditions of varying severities. A number of siRNAs have already been demonstrated to be effective in humans, while some have recently entered clinical trials targeting diseases such as age-related macular degeneration.[19,20,31]

We are interested in the development of new therapeutic approaches for corneal dystrophies such as MECD, or in the *TGFBI*-related dystrophies.[4,5,7,32–35] We have reported a number of potent siRNAs for the treatment of various keratin disorders, with their effectiveness demonstrated in immortalized cell lines or in transgenic animal models.[7,8,16,17,36] We have also developed a potent siRNA for one of the *TGFBI* related corneal dystrophies.[37]

Within this study, we further demonstrate the potency and specificity of the previously identified K12-Leu132Pro-9 siRNA[7] using exogenous constructs before we assess knockdown of

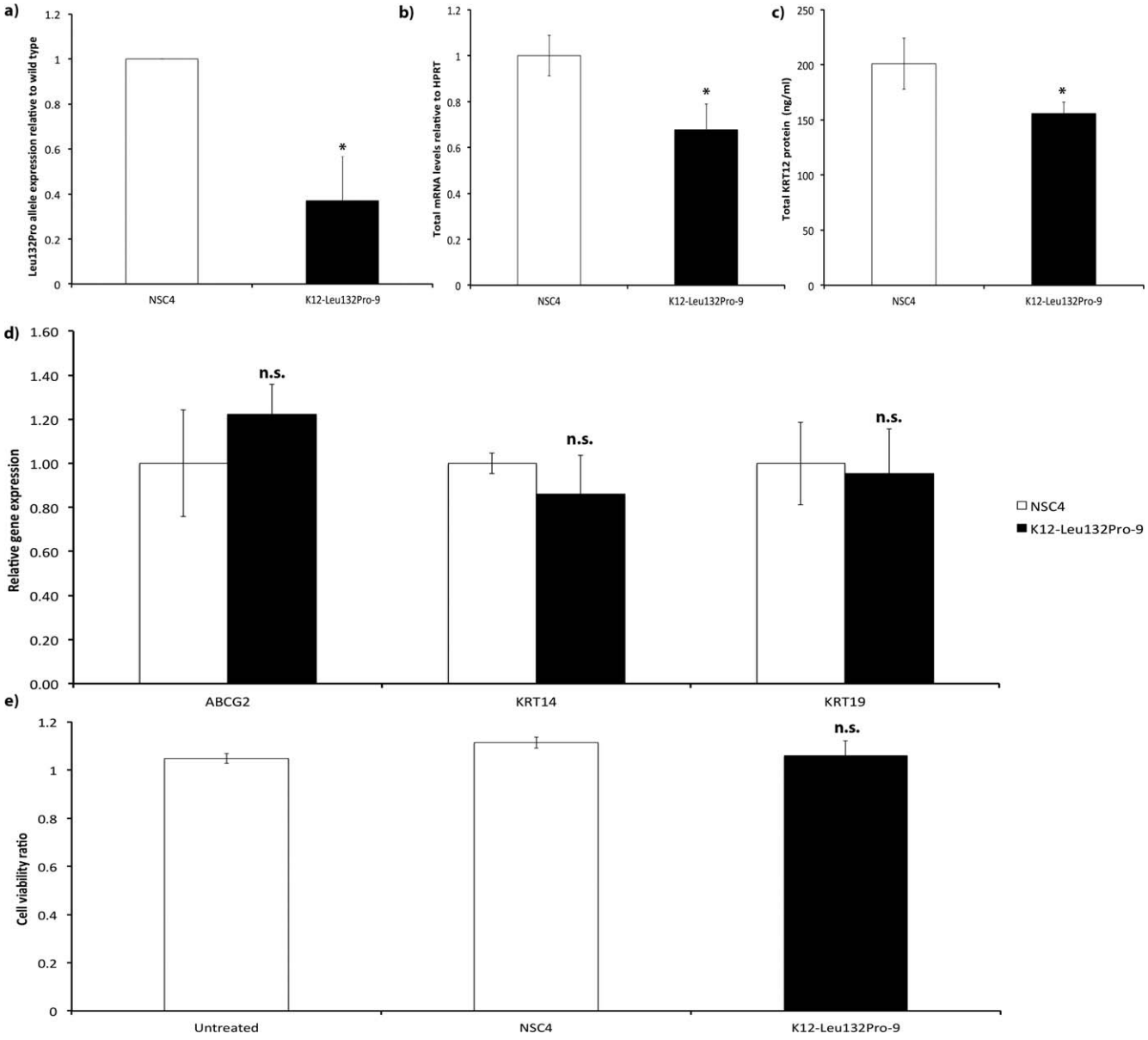

FIGURE 6. Allele-specific knockdown of endogenous K12 in MECD cells by siRNA intervention. A combination of pyrosequencing and qPCR analysis confirmed that an effective and specific knockdown of Leu132Pro mutant K12 mRNA resulted from the treatment of MECD cells with the K12-Leu132Pro-9 siRNA at a concentration of 5 nM. (**A**) Pyrosequencing demonstrated a reduction in mutant allele levels by 63% relative to that of wild-type allele expression when cells were treated with 5 nM K12-Leu132Pro-9, where $n = 6$. Wild-type and mutant allele expression was normalized against cells treated with 5 nM NSC4. (**B**) Quantitative PCR analysis was then used to confirm the allele specific nature of the knockdown induced by K12-Leu132Pro-9, a 32% knockdown of total K12 was observed, where $n = 9$. (**C**) Quantification of K12 by ELISA in MECD cells treated with the K12-Leu132Pro-9 siRNA also revealed a 23% knockdown of total K12 protein when compared with cells treated with the NSC4 siRNA, further establishing the silencing capabilities of the K12-Leu132Pro-9 siRNA, where $n = 4$. (**D**) The expression level of three limbal stem cell markers was also assessed to confirm that no off target effects were elicited by the introduction of the K12-Leu132Pro-9 siRNA. Expression of KRT19, ABCG2, and KRT14 showed no significant change when compared with MECD cells treated with the NSC4 siRNA, where $n = 9$. (**E**) A cell viability assay was used to confirm that introduction of siRNA into the MECD cells did not affect normal cell viability, where $n = 9$. $^*P < 0.05$. n.s., not significant.

the endogenous mutant K12 allele in our MECD corneal limbal epithelial cells.

Both pyrosequencing (Fig. 1) and epitope-tagged infrared Western blot analysis (Fig. 2) methodologies allow silencing of the mutant allele to be assessed in the presence of the wild-type allele, a situation more akin to that in the diseased cells in MECD. Pyrosequencing quantifies the amount of mutant mRNA expression with respect to the wild-type allele; with the drawback that changes in wild-type expression cannot be detected. However, by combining the pyrosequencing data with qPCR for total K12 mRNA levels, the mutant knockdown can be deduced while also demonstrating unchanged levels of wild type expression in cells treated with mutant specific siRNAs. Development of this combined pyrosequencing and qPCR approach is essential in the quantification of endogenous mutant and wild type alleles in patient cells as K12 alleles are not tagged in any way to allow discrimination by the researcher. Analysis of 5′ RACE (Fig. 3) demonstrated that

mutant K12 mRNA was cleaved between nucleotides 10 and 11 from the 5′ end on the siRNA antisense strand; the cleavage site initially postulated for RISC-mediated cleavage.[38] The efficacy of the K12-Leu132Pro-9 siRNA in its ability to silence the endogenous mutant allele was assessed in our MECD cells. The Q-FIHC analysis of K12 expression in large, nonproliferating MECD cells showed a 59% knockdown of K12 positive cells by the K12-Leu132Pro-9 siRNA (Fig. 5). Moreover mutation-specific knockdown in these cells was also confirmed by a combination of pyrosequencing and qPCR (Figs. 6A, 6B). This knockdown (63%) was less than that observed in cells transiently expressing K12 (83%); however, primary culture patient cells are notoriously difficult to transfect, which most likely explains the reduced silencing of the mutant allele in the MECD patient cells.[39] An ELISA showed 23% knockdown of total K12 protein by the K12-Leu132Pro-9 siRNA when compared with cells treated with the NSC4 siRNA (Fig. 6C). This would constitute a 46% reduction in mutant K12 protein if the wild-type expression remains unaffected.

Evaluation of any off-target effects caused by this siRNA has already been addressed in our previous work[7]; however, in MECD cells we also assessed the effect siRNA had on limbal stem cell differentiation markers; specifically, KRT19, ABCG2 and KRT14 (Fig. 6D) and noted K12-Leu132Pro-9 siRNA had no significant effect on the expression level of these three differentiation markers. Currently, RNA interference strategies such as the one outlined within this study can be limited by confounding off-target effects. These are not a significant problem here or in prior studies[18] possibly due to the care we take in selecting the most potent and specific siRNAs through performing a gene walk of the target to be silenced. Small interfering RNA targeting a K6a mutant allele, recently used for a Phase 1b clinical trial,[31] had an excellent rodent toxicity profile and no toxicity issues in humans. The in-depth analysis of off-target effects will need to be fully assessed in the appropriate animal models; to confirm corneal treatment with siRNA does not lead to a separate clinical phenotype.

Importantly, artificial models and human disease are very different and multiple evaluations are needed before any attempt to move therapy into patients.

One hurdle in the advancement of these siRNAs into clinical trials is the current lack of a suitable delivery vehicle to overcome cellular phospholipid bilayers as well as neutralizing the polyanionicity of the siRNA spine. Research into altered siRNA complexes remains ongoing throughout both academia and industry with a number of promising methods highlighted to date.[40–42]

We have shown that it is possible to knockdown both exogenous and endogenous mutant K12 using a mutation specific siRNA. In conjunction with a suitable siRNA delivery vehicle that can be incorporated into a simple eye drop formulation, this siRNA would be most beneficial for effective treatment for ocular surface disorders such as the corneal dystrophies described within.

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