Development of Allele-Specific Gene-Silencing siRNAs for TGFBI Arg124Cys in Lattice Corneal Dystrophy Type I

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Purpose. This study aimed to investigate the potency and specificity of short-interfering RNA (siRNA) treatment for TGFBI-Arg124Cys lattice corneal dystrophy type I (LCDI) using exogenous expression constructs in model systems and endogenous gene targeting in an ex vivo model using corneal epithelial cell cultures.

Methods. A panel of 19 TGFBI-Arg124Cys–specific siRNAs were assessed by a dual-luciferase reporter assay. Further assessment using pyrosequencing and qPCR was used to identify the lead siRNA; suppression of mutant TGFBIp expression was confirmed by Western blot and Congo red aggregation assays. An ex vivo model of LCDI was established using limbal biopsies from corneal dystrophy patients harboring the Arg124Cys mutation. Treatment efficiency of the siRNA was assessed for the inhibition of the mutant allele in the primary patient's corneal epithelial cells using pyrosequencing, quantitative PCR (qPCR), and an ELISA.

Results. A lead siRNA was identified, and demonstrated to be potent and specific in inhibiting the TGFBI-Arg124Cys mutant allele at the mRNA and protein levels. Besides high allele specificity, siRNA treatment achieved a 44% reduction of the endogenous Arg124Cys allele in an ex vivo model of LCDI.

Conclusions. We have identified a lead siRNA specific to the TGFBI-Arg124Cys mutant allele associated with LCDI. Silencing of exogenous TGFBI was observed at mRNA and protein levels, and in an ex vivo model of LCDI with an efficient suppression of the endogenous mutant allele. This result indicates the potential of siRNA treatment as a personalized medicine approach for the management of heritable TGFBI-associated corneal dystrophies.

Keywords: allele discrimination, siRNA, lattice corneal dystrophy, TGFBI, RNA interference

RNA interference (RNAi) has proven to be a valid approach to therapeutic gene silencing. The use of short-interfering RNA (siRNA) molecules to silence the expression of a disease-causing mutant allele has been encouraging for a number of dominant negative conditions. Disorders treated by RNAi therapeutics in clinical trials include pachyonychia congenita, age-related macular degeneration, hepatitis C, and chronic myeloid leukemia,[1] with many more disease models currently undergoing siRNA efficacy assessment in the laboratory.[2–5]

Mutations in the TGF-β–induced gene (*TGFBI*, formerly *BIGH3*) cause a range of corneal dystrophies, including Reis-Bücklers and Thiel-Behnke corneal dystrophies.[6] One mutation in particular causes lattice corneal dystrophy type I (LCDI)[7,8] and follows a dominant negative inheritance pattern.[9] This point mutation is a C-to-T transition resulting in an amino acid change from arginine to cysteine at codon 124 (Arg124Cys). The LCDI was first characterized by the appearance of branching amyloid-like filaments in the anterior stroma.[9] Recent studies further identified considerable phenotypic variability of LCDI patients carrying the Arg124Cys mutation.[7,10,11] These potentially blinding filamentous aggregates have been reported in a number of *TGFBI*-Arg124Cys–caused LCDI patients.[8] The only currently available treatments for LCDI are either laser resurfacing keratectomy or surgical keratoplasty, an invasive procedure where pathologically-affected corneal tissue is excised (either full thickness [penetrating] or partial thickness [lamellar]) and replaced by transplanted donor tissue. This invasive type of treatment often

Table 1. Primer Sequences

Primer	Sequence
TGFBI FlagHA	5′-CTGTCTATCAAAAGTTATTAGAGAGGAGACTACAAGGACGACGATGACAAGC TCGATGGAGGATACCCATACGACGTCCCAGACTACGCTTGACTCGAGCTAAAT-3′
TGFBI StrepHA	5′-CTGTCTATCAAAAGTTATTAGAGAGGATGGAGCCACCCGCAGTTCGAAAAACTC GATGGAGGATACCCATACGACGTCCCAGACTACGCTTGACTCGAGCTAAAT-3′
TGFBI Pyro Fwd	5′-ATGAAAAGGTCCCTGGGGAGAA-3′
TGFBI Pyro Rev	5′-[Biotin]TATGGTAGCGGAGGGCATTG-3′
TGFBI Pyro Seq	5′-TCAGCTGTACACGGAC-3′

is only partially effective, requires long-term monitoring follow-up, can be associated with various morbidities, and usually subsequently results in disease recurrence within the grafted tissue or interface.[12,13] Both types of treatment can induce greater expression of mutant TGFBI due to the corneal injury incurred particularly early after laser keratectomy, as has been discovered subsequent to LASIK surgery.[14,15] This may be due to the induction of increased TGFBI expression as part of the wound healing process, in response to the corneal injury during penetrating keratoplasty.[15]

The use of allele-specific siRNA mutant gene silencing as a preventive therapeutic could offer a noninvasive treatment for TGFBI-related corneal dystrophies. The siRNAs are short RNA duplexes comprising 21 nucleotides that are designed to target the mutant allele specifically. We have described previously posttranscriptional down-regulation of mutant allele expression for a number of targets.[2–5,16] The RNAi pathway has been exploited previously by Yuan et al.[17] and Yellore et al.[18] to down-regulate total TGFBI protein, and we propose that this pathway could be hijacked in a more allele-specific nature to reduce the formation of dystrophic TGFBI aggregates.[18]

In this study, we identify a lead siRNA for the TGFBI-Arg124Cys mutation, and assess potency and specificity using exogenous constructs before translating this to an LCDI ex vivo model. We establish an LCDI model using corneal epithelial cells grown from a limbal biopsy of a patient with *TGFBI* Arg124Cys-related LCDI. This ex vivo model is necessary to show silencing of the endogenous gene; as currently to our knowledge no animal models exist that successfully replicate the disease phenotype of LCDI. In this ex vivo model the lead siRNA is found to be potent and allele-specific in silencing the endogenous expression of mutant TGFBI.

Materials and Methods

Constructs

Full-length human *TGFBI* wild-type and Arg124Cys mutant coding sequences were cloned into a pcDNA3.1/V5-His vector producing pcDNA3.1-TGFBI-WT containing the wild-type sequence (ACTCAGCTGTACACGGACCGCACGGAGAAGCT GAGGC) and pcDNA3.1-TGFBI-Arg124Cys containing the mutant sequence (ACTCAGCTGTACACGGAC**T**GCACGGA GAAGCTGAGGC; mutation in bold). The full-length *TGFBI* wild-type and Arg124Cys cDNA fragments then were cloned into the psiTEST-Luc-target reporter plasmid (York Bioscience, York, UK) using restriction enzymes XhoI and BamHI to produce firefly luciferase reporter constructs. Cell viability and transfection efficiency were assessed using a *Renilla* luciferase expression construct (pRL-CMV; Promega, Southampton, UK). Epitope-tagged constructs were designed for Western blot analysis. Both *TGFBI* sequences underwent PCR amplification with the addition of either FlagHA or StrepHA epitope tags, and amplicons were cloned into a pCR3.1 vector (Life Technologies, Paisley, UK). The wild-type sequence was tagged with FlagHA (DYKDDDDK-LDGG-YPYDVPDYA) and the Arg124Cys sequence was tagged with StepHA (WSHPQFEK-LDGG-YPYDVPDYA). The equivalent nucleotide sequence for each tag was positioned directly 3′ of the stop codon for each sequence. These tags were introduced using primers TGFBI FlagHA and TGFBI StrepHA (Table 1). Plasmids were sequenced to confirm the presence of each epitope tag.

siRNA Design

A total of 19 siRNAs (19-nucleotide duplex with a 3′ dTdT overhang) was designed to target the TGFBI-Arg124Cys mutation, with the sense strand of each shown in Table 2. All siRNAs were synthesized by MWG Biotech AG (Ebersberg, Germany) along with siLuc, a positive control siRNA targeted against *firefly* luciferase mRNA, and NSC4, a nonspecific control siRNA targeted against an inverted bacterial β-galactosidase sequence. These siRNA sequences also are found in Table 2.

Cell Culture

The AD293 human embryonic kidney cells (Life Technologies) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with the addition of 10% fetal bovine serum (FBS; Life Technologies). All cell cultures were incubated at 37°C in the presence of 5% CO_2. Cells were cultured following normal laboratory protocols.

Table 2. siRNA Sequences

siRNA	Sequence
TGFBI-Arg124Cys-1	ACUCAGCUGUACACGGACUdTdT
TGFBI-Arg124Cys-2	CUCAGCUGUACACGGACUGdTdT
TGFBI-Arg124Cys-3	UCAGCUGUACACGGACUGCdTdT
TGFBI-Arg124Cys-4	CAGCUGUACACGGACUGCAdTdT
TGFBI-Arg124Cys-5	AGCUGUACACGGACUGCACdTdT
TGFBI-Arg124Cys-6	GCUGUACACGGACUGCACGdTdT
TGFBI-Arg124Cys-7	CUGUACACGGACUGCACGGdTdT
TGFBI-Arg124Cys-8	UGUACACGGACUGCACGGAdTdT
TGFBI-Arg124Cys-9	GUACACGGACUGCACGGAGdTdT
TGFBI-Arg124Cys-10	UACACGGACUGCACGGAGAdTdT
TGFBI-Arg124Cys-11	ACACGGACUGCACGGAGAAdTdT
TGFBI-Arg124Cys-12	CACGGACUGCACGGAGAAGdTdT
TGFBI-Arg124Cys-13	ACGGACUGCACGGAGAAGCdTdT
TGFBI-Arg124Cys-14	CGGACUGCACGGAGAAGCUdTdT
TGFBI-Arg124Cys-15	GGACUGCACGGAGAAGCUGdTdT
TGFBI-Arg124Cys-16	GACUGCACGGAGAAGCUGAdTdT
TGFBI-Arg124Cys-17	ACUGCACGGAGAAGCUGAGdTdT
TGFBI-Arg124Cys-18	CUGCACGGAGAAGCUGAGGdTdT
TGFBI-Arg124Cys-19	UGCACGGAGAAGCUGAGGCdTdT
siLuc	GTGCGTTGCTAGTACCAACdTdT
NSC4	TAGCGACTAAACACATCAAdTdT

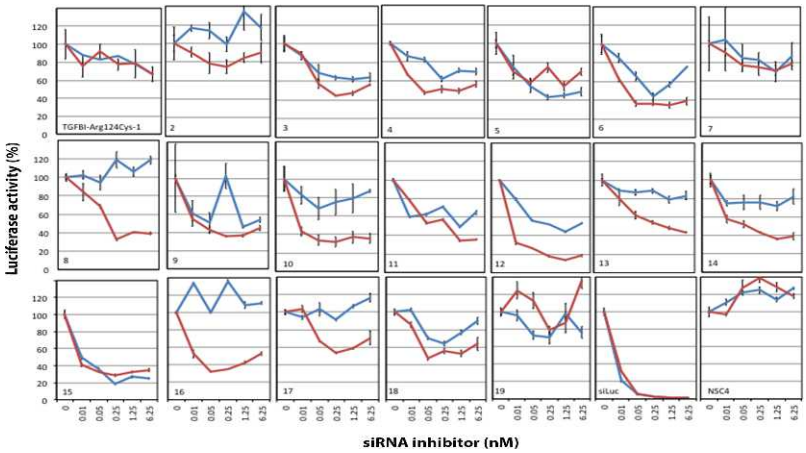

FIGURE 1. A dual luciferase reporter assay on a panel of 19 siRNAs specific to the TGFBI-Arg124Cys mutant allele. A dual luciferase reporter assay was carried out to assess a panel of 19 mutation-specific siRNAs, alongside a positive control siRNA (siLuc) and a nonspecific siRNA (NSC4). This assay represents a high throughput method of identifying potentially potent siRNAs for a target mutation. A total of 3 siRNAs, TGFBI-Arg124Cys-8, 14, and 16, displayed potency and allele specificity in preferentially knocking down the mutant allele (*red*) with minimal effect on the wild-type allele (*blue*). The SEM was calculated for each siRNA concentration where $n = 3$.

Study Participants and Isolation of Corneal Epithelial Cells

Ethical approval was gained from the East of Scotland Research ethics service and complied with all local research governance legislation, for the limbal biopsy and culture of LCDI corneal epithelial cells. The research followed the tenets of the Declaration of Helsinki.

The inclusion criterion for subjects recruited for this research was the presence of the c.C417T mutation in the *TGFBI* gene. Informed consent was given before the removal of a limbal biopsy, 2 mm^2 in size, by an experienced ophthalmic surgeon. Cells were isolated from limbal biopsies and cultured under previously described growth conditions for human corneal epithelial cells[19] and extracted DNA was sequenced to confirm the presence of the c.C417T mutation in the *TGFBI* gene. Colony-forming efficiency of the human corneal epithelial cells was performed using methods as described in detail by Corradini et al.[19]

Dual Luciferase Reporter Assay

The AD293 cells were seeded at a concentration of 6.5×10^3 cells/well in a 96-well plate and incubated for 24 hours. The TGFBI psiTEST-Luc-target reporter constructs (1 wild-type and 1 mutant) were transfected separately into these cells at 20 ng per well using Lipofectamine 2000 (Life Technologies), following the manufacturer's protocol. Cells also were co-transfected with 1 ng of pRL-CMV per well and with concentrations of siRNA between 0 and 6.25 nM before being incubated for 24 hours. All 19 mutation-specific siRNAs, as well as siLuc and NSC4 were tested by this method, separately gauging their knockdown of wild-type or mutant *TGFBI* by assessment of *firefly* luciferase activity. Transfections were

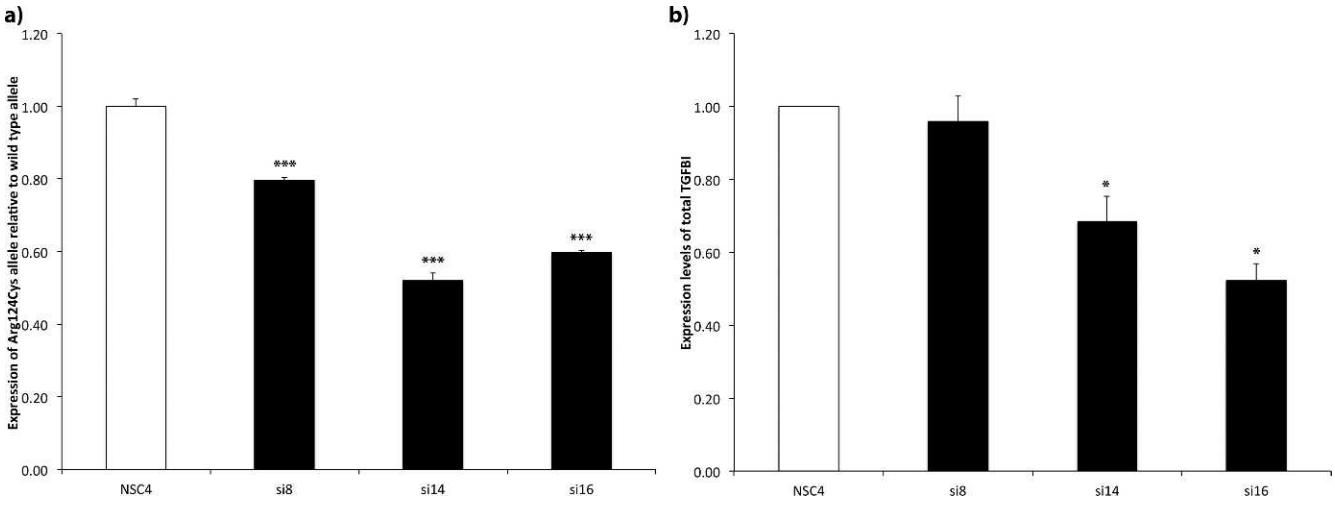

FIGURE 2. Pyrosequencing analysis to determine the lead siRNA against the TGFBI-Arg124Cys mutant allele. The 3 mutation-specific siRNAs identified through the dual luciferase reporter assay were assessed by pyrosequencing and qPCR. Mutant allele expression was established relative to wild-type expression by pyrosequencing, and compared against NSC4 treated cells (**a**). The qPCR was used to assess total knockdown of *TGFBI*, so as to determine the allele specificity of each siRNA (**b**). Cells were treated for 48 hours with the siRNAs to elicit a response before RNA was extracted and mRNA levels quantified. TGFBI-Arg124Cys-14 was deemed to be the most potent siRNA based on the 48% mutant knockdown obtained by pyrosequencing. The SEM was calculated for each condition where $n = 6$ and a Student's *t*-test was used to calculate significance ($^*P < 0.05$, $^{***}P < 0.001$).

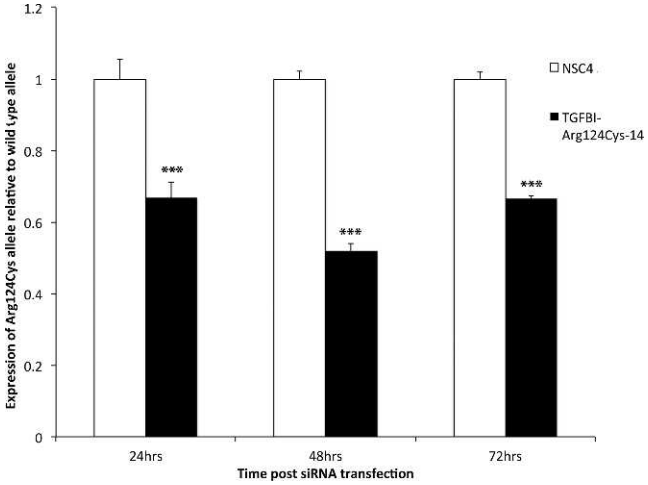

FIGURE 3. Determination of the optimal time point for siRNA mediated knockdown of TGFBI-Arg124Cys. Pyrosequencing was used to gauge the optimal time point for siRNA mediated silencing in regard to the TGFBI-Arg124Cys mutant allele. Time points of 24, 48, and 72 hours post siRNA treatment were assessed. The 48-hour time point demonstrated the greatest knockdown where the mutant allele displayed a 48% reduction in mRNA expression, compared to that of cells treated with the NSC4 siRNA. The SEM was calculated for each time point where $n = 6$ and a Student's *t*-test was used to calculate significance (***$P < 0.001$).

carried out in triplicate with media removal 24 hours after transfection; cells then were washed in PBS before the addition of passive lysis buffer (Promega). *Firefly* luciferase and *Renilla* luciferase activities were assessed using the LUMIstar OPTIMA (BMG Labtech, Aylesbury, UK).

Allelic Discrimination by Pyrosequencing

The AD293 cells were seeded at 8×10^4 cells per well of a 12-well plate and transfected using Lipofectamine 2000 with 200 ng of pcDNA 3.1-TGFBI-WT and 200 ng of pcDNA 3.1-TGFBI-Arg124Cys expression constructs, and siRNA at a final concentration of 5 nM. Each condition was repeated in triplicate. The Qiagen RNAeasy Plus kit (Qiagen, Venlo, The Netherlands) was used to extract RNA from these cells at a number of time points after transfection. Extracted mRNA was quantified before 500 ng was converted to cDNA using a high efficiency cDNA reverse transcription kit (Life Technologies). Each cDNA sample underwent a pyrosequencing PCR using the PyroMark PCR kit (Qiagen), using primers TGFBI Pyro Fwd and TGFBI Pyro Rev (Table 1; Sigma Aldrich, Dorset, UK), which amplified a small region of *TGFBI* containing codon 124, the location of the Arg124Cys mutation. Products then were pyrosequenced using the Qiagen Pyromark Q24, according to manufacturer's instructions using the sequencing primer TGFBI Pyro Seq (Table 1); cDNA from each transfection was analyzed in duplicate by pyrosequencing.

The LCDI ex vivo model was assessed in a manner similar to the AD293 method. The TGFBI-Arg124Cys–positive corneal epithelial cells were seeded at 2.5×10^5 cells per well in a 6-well plate. At the same time, each well was treated with 5 nM of either NSC4 or TGFBI-Arg124Cys-14 siRNA and transfected using Lipofectamine RNAiMax (Life Technologies) before being incubated for 72 hours. Cells then were removed and mRNA extracted using the Qiagen miRNAeasy kit; 500 ng was converted to cDNA using the Superscript Vilo cDNA synthesis kit (Life Technologies) before undergoing analysis by pyrosequencing following the same protocol as described previously.

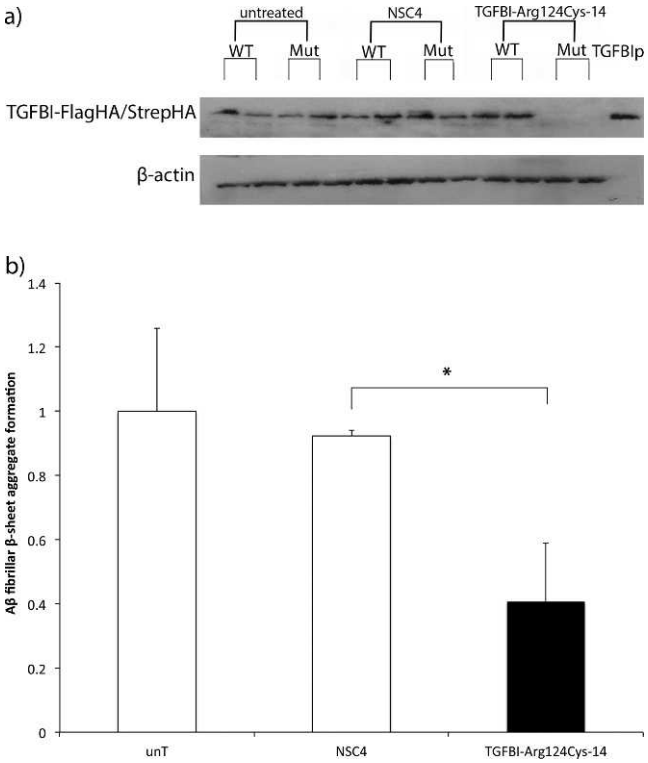

FIGURE 4. Evaluation of the silencing ability of the TGFBI-Arg124Cys-14 siRNA at the protein level. The knockdown capabilities of the TGFBI-Arg124Cys-14 siRNA were assessed further at the protein level. Western blot analysis demonstrated a reduction in TGFBI-Arg124Cys protein, while wild-type TGFBI protein levels remained stable. β-actin was used as a loading control, while recombinant human TGFBI was used as a positive control (**a**), $n = 2$. A Congo red aggregation assay was then used to confirm a functional change caused by the introduction of the TGFBI-Arg124Cys-14 siRNA by quantifying amyloid aggregate formation. The Congo red aggregation assay demonstrated a 52% decrease in amyloid aggregate formation between NSC4 and TGFBI-Arg124Cys-14 treated cells (**b**), confirming the knockdown observed by Western blotting. The SEM was calculated for each time point where $n = 9$ and a Student's *t*-test was used to calculate significance (*$P < 0.05$).

Quantitative Real-Time PCR (qPCR)

The cDNA samples from the previously described pyrosequencing transfections in AD293 cells and in our LCDI ex vivo models were analyzed by qPCR. A commercially available assay for *TGFBI* quantification (assay Id 104720; Roche Diagnostics Ltd., West Sussex, UK) was used following the manufacturer's protocol alongside an *HPRT* assay (assay Id 102079; Roche Diagnostics Ltd.). The *TGFBI* expression levels were normalized against *HPRT*. The gene expression of corneal epithelial cell differentiation markers also was assessed by qPCR in LCDI samples; *ABCG2* (Roche Diagnostics Ltd.), *KRT14* (assay Id 117459; Roche Diagnostics Ltd.), and *KRT19* (assay Id 103579; Roche Diagnostics Ltd.).[20] Each cDNA sample was run in triplicate for each assay and relative gene expression was calculated using the ΔΔCT method. Expression levels of each gene in cells treated with TGFBI-Arg124Cys-14 were compared to that of NSC4 treated cells or untreated cells.

Western Blot Analysis

Transfections were carried out in 6-well plates, with each condition being repeated in duplicate. The AD293 cells were seeded at 4×10^5 cells per well and transfections were carried

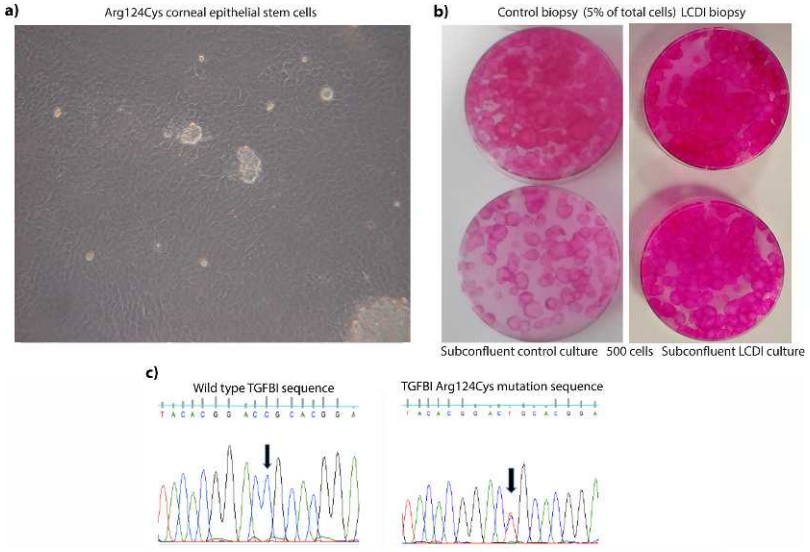

Figure 5. Establishment of an ex vivo model of TGFBI-Arg124Cys LCDI from a patient's limbal biopsy. A limbal biopsy from a LCDI patient known to carry the TGFBI-Arg124Cys mutation was used to establish an ex vivo model of LCDI. It was noted that cell morphology was comparable across LCDI and non-LCDI cell cultures ([**a**] image of LCDI patient's corneal epithelial cells in culture). After 12 days in culture the colony-forming efficiency of these cells was assessed (**b**), while DNA was extracted from LCDI cultures and sequencing confirmed the presence of the TGFBI-Arg124Cys mutation (**c**).

out simultaneously using Lipofectamine 2000. Each transfection contained 2 μg of either TGFBI-WT-FlagHA or TGFBI-Arg124Cys-StrepHA and left untreated or treated with 5 nM NSC4 or TGFBI-Arg124Cys-14. After 72 hours total protein was extracted from cell pellets using Lysis M buffer (Roche Diagnostics Ltd.) containing protease inhibitors (Sigma Aldrich) and protein was quantified using a Bradford assay before loading. Western blotting was carried out according to a previously published method.[4] The TGFBI protein was stained using an anti-TGFBI rabbit polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and a secondary polyclonal horseradish peroxide (HRP) conjugated swine anti-rabbit antibody (Dakocytomation, Ely, UK).

Detection was carried out by enhanced chemiluminescence (Thermo Scientific, Epsom, UK). Recombinant human TGFBI protein (100 ng; R&D Systems, Abingdon, UK) was run alongside as a positive control. The β-actin was used as a loading control, and was detected using a mouse anti-human monoclonal antibody (Sigma Aldrich) and a HRP conjugated goat anti-mouse antibody (Dakocytomation).

Congo Red Aggregation Assay

A method similar to that described by Yam et al.[21] was used to assess the formation of amyloid aggregates in conditioned media of cells transiently expressing TGFBI-Arg124Cys. AD293 cells were seeded at a density of 8×10^4 cells/well in a 12-well plate and transfected with pcDNA3.1-TGFBI-Arg124Cys, and co-transfected with 5 nM NSC4 or TGFBI-Arg124Cys-14. All transfections were carried out in triplicate using Lipofectamine 2000 according to the manufacturer's protocol. Cells were incubated for 72 hours before conditioned media was collected. Conditioned media was placed in a fresh 12-well plate and incubated with 10 μM Aβ(1-40) peptides (>97% purity; rPeptide, Bogart, GA) at 37°C for a further 12 hours. Conditioned media then were concentrated into approximately 300 μL using an Amicon Ultracel YM-30 centrifugal filter (Millipore, Watford, UK). Congo red (20 μM) and 100 μL of concentrated conditioned media then were mixed in 1 well of a 96-well plate. This was repeated in triplicate for each transfection. The plate was agitated for 4 hours before spectrophotometric absorbance was recorded at 540 and 630 nm wavelengths.

Protein Quantification by ELISA

An ELISA was performed on conditioned media from LCDI cells treated with the NSC4 siRNA, TGFBI-Arg124Cys-14 siRNA, or RNAiMax only to quantify levels of total TGFBI protein. The TGFBI-Arg124Cys–positive corneal epithelial cells were seeded at 2.5×10^5 cells per well in a 6-well plate. At the same time, each well was left untreated, or treated with 5 nM of either NSC4 or TGFBI-Arg124Cys-14 siRNA and transfected using Lipofectamine RNAiMax (Life Technologies) before being incubated for 72 hours. Conditioned media were removed from each well and the ELISA was performed. Each sample underwent a 1:100 dilution before the ELISA was performed following the manufacturer's protocol (Abcam, Cambridge, UK).

Statistics

Statistical significance was set at $P < 0.05$. A Student's *t*-test was used to calculate statistical significance for pyrosequencing, the Congo red aggregation assay and qPCR. All error bars are representative of the standard error of the mean. Pearson coefficients were calculated for qPCR reference genes to confirm a strong correlation in expression levels between NSC4 and siRNA treated cells ($P < 0.05$).

Results

siRNA Induced Knockdown of Mutant TGFBI in Exogenous Constructs

A reporter assay employing *firefly* and *Renilla* luciferases was used to assess allele-specific knockdown of TGFBI Arg124Cys in the presence of mutation specific siRNAs (Fig. 1). Three siRNAs; 8, 14, and 16, demonstrated allele-specific knockdown

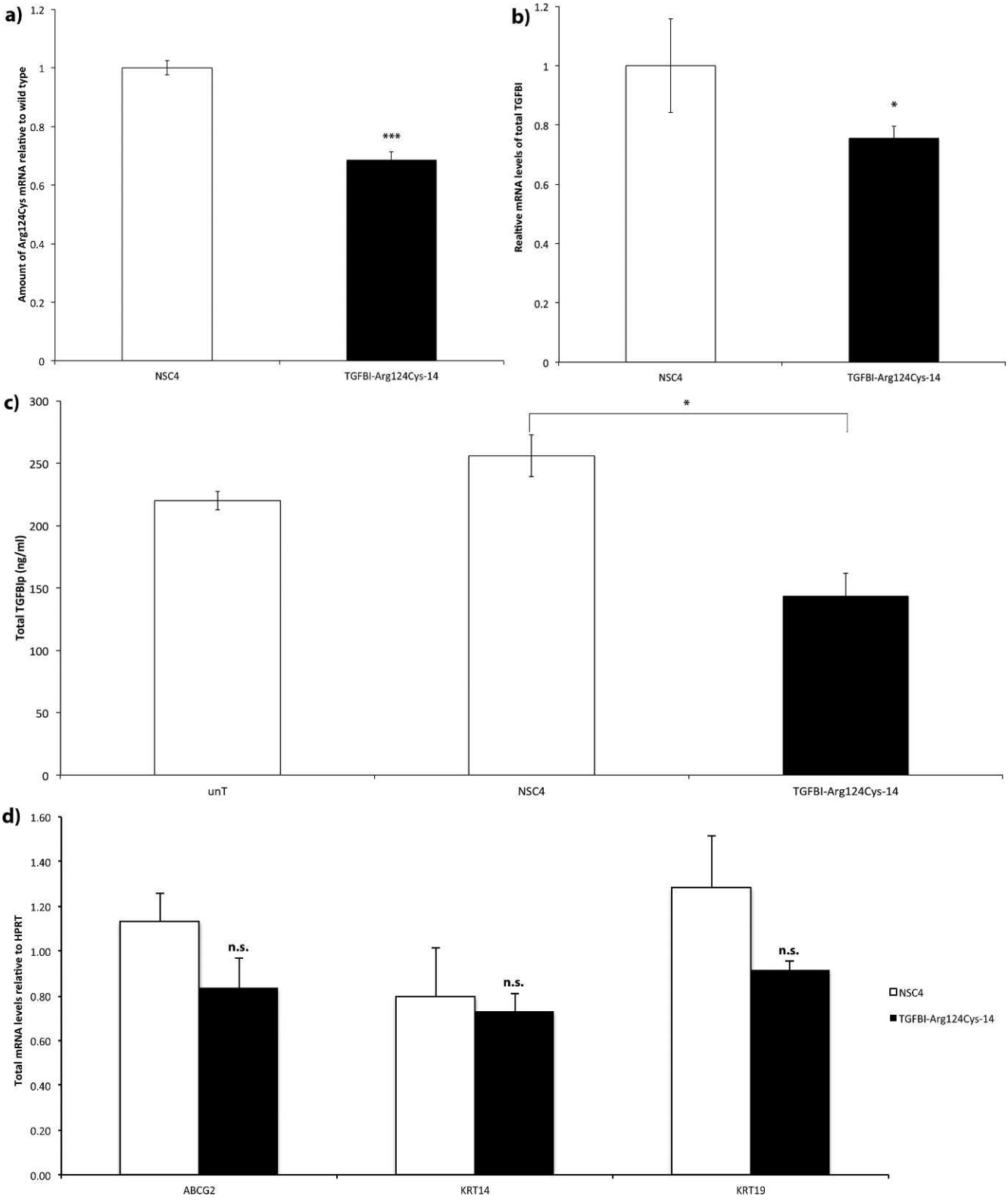

Figure 6. Quantification of the allele-specific silencing abilities of the TGFBI-Arg124Cys-14 siRNA in an ex vivo model of LCDI. The allele specificity of the lead siRNA, TGFBI-Arg124Cys-14, was assessed in an LCDI ex vivo model. Wild-type and mutant TGFBI mRNA levels were quantified by pyrosequencing, while total *TGFBI* expression levels were measured by qPCR. All cDNA samples were run in triplicate with expression levels compared to those of NSC4 treated cells. Pyrosequencing (**a**) displayed a knockdown of 44% of mutant allele expression, where $n = 6$ (***$P < 0.001$). This would equate to a 22% reduction in total *TGFBI* if the siRNA is allele-specific. Quantitative PCR analysis (**b**) demonstrated a total *TGFBI* mRNA knockdown of 24%, where $n = 9$ (*$P < 0.05$), which strongly correlates with the knockdown observed by pyrosequencing. An ELISA was performed to confirm knockdown of TGFBI at the protein level 72 hours after the introduction of the siRNA (**c**). The total decrease of TGFBI in the conditioned media of the LCDI cells treated with the TGFBI-Arg124Cys-14 siRNA when compared to NSC4 treated cells was found to be 43%, further

establishing the potency of the TGFBI-Arg124Cys-14 siRNA, where $n = 9$ (*$P < 0.05$). The qPCR analysis of corneal epithelial cell differentiation markers *ABCG2*, *KRT14,* and *KRT19* also was carried out to confirm that no adverse effects were elicited by the introduction of the TGFBI-Arg124Cys-14 siRNA (**d**). No significant variations in gene expression were observed, where $n = 9$. The SEM was calculated for both techniques and a Student's *t*-test was used to establish significance.

of mutant TGFBI and, therefore, were assessed further by pyrosequencing.

For pyrosequencing, AD293 cells transiently expressing wild-type and mutant TGFBI were transfected with 5 nM of either NSC4 or a TGFBI-Arg124Cys–specific siRNA. The RNA was extracted from cells 48 hours after siRNA transfection, and relative levels of wild-type and mutant *TGFBI* mRNA were determined (Fig. 2a) with mutant mRNA levels expressed relative to wild-type levels and compared to cells treated with NSC4. As pyrosequencing measures mutant mRNA levels relative to wild-type, qPCR also was carried out to determine the levels of total *TGFBI* (wild-type and mutant, Fig. 2b). After assessing the expression levels observed by pyrosequencing and qPCR, the TGFBI-Arg124Cys-14 siRNA was deemed to be the most potent and allele-specific, and, therefore, was selected as the lead siRNA. Silencing with the TGFBI-Arg124Cys-14 siRNA was assessed at 24, 48, and 72 hours after transfection, with maximal knockdown of mutant mRNA observed at 48 hours (Fig. 3).

To confirm specific mutant allele knockdown at the protein level, AD293 cells were transfected transiently with TGFBI-WT-FlagHA or TGFBI-Arg124Cys-StrepHA and 5 nM of either TGFBI-Arg124Cys-14 or NSC4, or were left untreated. Cells expressing mutant TGFBI that were untreated or treated with NSC4 displayed comparable levels of TGFBI protein, while cells treated with TGFBI-Arg124Cys-14 showed a potent knockdown of mutant protein (Fig. 4a).

An amyloid aggregation assay was developed[21] to assess the effect of TGFBI-Arg124Cys-14 at a functional level by quantifying the formation of amyloid aggregates in the conditioned media of cells transiently expressing TGFBI-Arg124Cys. The amyloid aggregate formation observed in cells left untreated was normalized to 1, with the aggregation observed in NSC4- and TGFBI-Arg124Cys-14–treated cells compared to this value. A 52% decrease in amyloid aggregate formation between NSC4- and TGFBI-Arg124Cys-14–treated cells was observed (Fig. 4b). Results from the Western blot analysis and the Congo red aggregation assay concurred with the potency and specificity of the TGFBI-Arg124Cys-14 siRNA previously observed at the mRNA level.

Establishment of an Ex Vivo Model of LCDI From Patient Corneal Epithelial Cells

The siRNA-mediated knockdown of the TGFBI-Arg124Cys allele described above was carried out using exogenous constructs as an artificial disease model. We wanted to translate this knockdown into an ex vivo model of LCDI using cells cultured from a limbal biopsy taken from a patient with LCDI and the TGFBI-Arg124Cys mutation. Cells were grown alongside normal non-LCDI cells from 3 independent wild-type individuals. Corneal epithelial cells were isolated from 2 mm^2 biopsies from LCDI patients and from normal control donors (within 24 hours of death). The LCDI cells generated large colonies with a smooth and regular perimeter similar to those generated by control cells (Fig. 5a). Subconfluent primary cultures of LCDI cells had a mean colony forming efficiency (CFE) of 16.8% and were frozen for further analysis (Fig. 5b, after 12 days in culture). The CFE and growth rate of LCDI cells were similar to control primary cultures.

DNA and RNA were extracted from human LCDI cells to confirm the presence of the *TGFBI* Arg124Cys mutation (Fig. 5c).

Silencing of the Endogenous Mutant Allele in an Ex Vivo Model of LCDI

Primary cultures of LCDI corneal epithelial cells were used to confirm the potency of the TGFBI-Arg124Cys-14 siRNA. Cells were treated with either NSC4 or TGFBI-Arg124Cys-14 at a concentration of 5 nM, and incubated for 72 hours, after which time RNA was extracted. Pyrosequencing was performed to determine expression levels of endogenous wild-type and mutant *TGFBI* mRNA. In the presence of TGFBI-Arg124Cys-14, siRNA mutant allele mRNA levels reduced by 44% (± 2.4%) compared to that of the NSC4 treated cells (Fig. 6a).

As pyrosequencing demonstrated a mutant knockdown of 44%, this should translate to a total *TGFBI* reduction of 22%, where $0.44 \times 50\%$ (mutant allele) = 22% if the siRNA is allele-specific. The qPCR analysis of the siRNA treated ex vivo LCDI model demonstrated a knockdown of total *TGFBI* of 24% (± 4.11%, Fig. 6b), which concurs with the knockdown observed by pyrosequencing and, thus, confirms the allele-specific nature of the TGFBI-Arg124Cys-14 siRNA.

An ELISA specific to human TGFBI was used to quantify TGFBI protein levels in the conditioned media of LCDI cells 72 hours after treatment with the NSC4 siRNA, TGFBI-Arg124Cys-14 siRNA, or RNAiMax only. The concentration of total TGFBI in NSC4-treated cells was 255.9 ng/mL (± 16.7), while in TGFBI-Arg124Cys-14–treated cells it was 143.7 ng/mL (± 18.2, Fig. 6c). This represents a decrease in total TGFBI protein of 44% demonstrating potent knockdown capabilities of the TGFBI-Arg124Cys-14 siRNA.

To confirm no adverse effects arose from the introduction of our siRNA, the expression levels of 3 corneal epithelial cell differentiation markers were assessed by qPCR. As shown in Figure 6d, no significant variations were observed in the expression of ABCG2, KRT19, or KRT14 in cells untreated or treated with NSC4 or TGFBI-Arg124Cys-14. Introduction of siRNA TGFBI-Arg124Cys-14 at a concentration of 5 nM has no effect on corneal epithelial cell features, apart from the down regulation of the TGFBI-Arg124Cys allele.

Discussion

The TGFBI-related dystrophies comprise some of the most common corneal dystrophies worldwide.[6] The current treatment options of laser or surgical replacement keratoplasty are ineffective in a number of cases,[12,13] and may even induce a more severe phenotype in others.[14,15] Consequently, alternative treatment options must be explored and developed. Due to the dominant negative pathomechanism of TGFBI-related dystrophies,[9] TGFBI-Arg124Cys–related LCDI is an ideal candidate for the establishment of a personalized medicine approach. This study is centered around siRNA-mediated silencing, a treatment pathway already investigated for a number of other dominant negative disorders[22–24] and antiviral therapeutics.[25,26] Further research currently is focusing around various other applications for exploiting the RNAi pathway.[27–29] Before the work on *TGFBI*, we developed and

assessed siRNAs for two additional mutations: KRT12-Leu132-Pro[4] and KRT12-Arg135Thr,[2] both associated with Meesmann's epithelial corneal dystrophy.[4,30]

Currently, there is a lack of an LCDI animal model that successfully recapitulates the disease. This possibly may be due to the fact that TGFBI-related dystrophies comprise a group of extremely slow onset conditions.[31–33] Therefore, the establishment of this ex vivo model for LCDI is important in the continued assessment of future treatment options. Importantly, it is necessary to note that, although LCDI is classed as a stromal dystrophy, it has been shown that TGFBI has an enhanced expression level on the extracellular surface of the corneal epithelial cells[34] before the mutant form of the protein appears to translocate to the stroma.[35] Presently, our model system is limited to corneal epithelial cells where TGFBI is expressed maximally.

In this article, we focused on TGFBI-Arg124Cys, one of the causative mutations for LCDI,[7,8] and identified a lead siRNA from a panel of 19 prospective siRNAs. A dual luciferase reporter assay was employed as a high throughput method of identifying potential lead siRNA candidates with 3 siRNAs; 8, 14, and 16, demonstrating significant potency and specificity to be assessed further by pyrosequencing and qPCR. Pyrosequencing allows for the assessment of siRNAs in an environment more closely resembling that of the patient's corneal epithelium, where cells are expressing wild-type and mutant *TGFBI* alleles. In using this methodology, mutant mRNA expression is calculated with respect to wild-type expression, though any effect of the siRNA on wild-type expression is undetectable. For this reason, a combination of pyrosequencing and qPCR was employed to assess mutant knockdown, while also confirming unaltered expression levels of wild-type *TGFBI*. The establishment of this combined analytical technique was crucial for assessing expression levels in our ex vivo model of LCDI, where endogenous *TGFBI* alleles are not labelled, unlike the dual luciferase reporter assay employed previously.

Once the lead siRNA was identified as TGFBI-Arg124Cys-14 by pyrosequencing and qPCR it was assessed further at the protein level in transiently expressing TGFBI cell cultures. By Western blotting the potency of the TGFBI-Arg124Cys-14 siRNA and its allele specificity was confirmed. To support these data, a Congo red aggregation assay also was performed and resulted in a 52% decrease in amyloid aggregate formation between NSC4- and TGFBI-Arg124Cys-14–treated cells. The silencing ability of TGFBI-Arg124Cys-14 then was evaluated in an LCDI ex vivo model. Knockdown of the mutant allele was quantified by pyrosequencing and allele specificity was confirmed by qPCR, demonstrating a potent knockdown of 44% of mutant *TGFBI* expression. This was supported at the protein level, where quantification of total TGFBI by an ELISA demonstrated a reduction of 44% in the conditioned media of LCDI cells treated with the TGFBI-Arg124Cys-14 siRNA when compared to those treated with the NSC4 siRNA. Due to the well-accepted difficulties in transfecting primary cells, this 44% knockdown represents promising mutant allele silencing.[36] Future work surrounding this siRNA will concentrate on developing alternative platforms for validating this therapeutic approach for TGFBI-related dystrophies.

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