Enhanced Wound Healing, Kinase and Stem Cell Marker Expression in Diabetic Organ-Cultured Human Corneas Upon MMP-10 and Cathepsin F Gene Silencing

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Purpose. Diabetic corneas overexpress proteinases including matrix metalloproteinase-10 (M10) and cathepsin F (CF). Our purpose was to assess if silencing M10 and CF in organ-cultured diabetic corneas using recombinant adenovirus (rAV)-driven small hairpin RNA (rAV-sh) would normalize slow wound healing, and diabetic and stem cell marker expression.

Methods. Sixteen pairs of organ-cultured autopsy human diabetic corneas (four per group) were treated with rAV-sh. Proteinase genes were silenced either separately, together, or both, in combination (Combo) with rAV-driven *c-met* gene overexpression. Fellow control corneas received rAV-EGFP. Quantitative RT-PCR confirmed small hairpin RNA (shRNA) silencing effect. Ten days after transfection, 5-mm epithelial wounds were made with n-heptanol and healing time recorded. Diabetic, signaling, and putative stem cell markers were studied by immunofluorescence of corneal cryostat sections.

Results. Proteinase silencing reduced epithelial wound healing time versus rAV–enhanced green fluorescent protein (EGFP) control (23% for rAV-shM10, 31% for rAV-shCF, and 36% for rAV-shM10 + rAV-shCF). Combo treatment was even more efficient (55% reduction). Staining patterns of diabetic markers ($\alpha_3\beta_1$ integrin and nidogen-1), and of activated epidermal growth factor receptor and its signaling target activated Akt were normalized upon rAV-sh treatment. Combo treatment also restored normal staining for activated p38. All treatments, especially the combined ones, increased diabetes-altered staining for putative limbal stem cell markers, ΔNp63α, ABCG2, keratins 15 and 17, and laminin γ3 chain.

Conclusions. Small hairpin RNA silencing of proteinases overexpressed in diabetic corneas enhanced corneal epithelial and stem cell marker staining and accelerated wound healing. Combined therapy with c-met overexpression was even more efficient. Specific corneal gene therapy has a potential for treating diabetic keratopathy.

Keywords: diabetic cornea, limbal stem cell, wound healing, MMP-10, cathepsin F, EGFR, Akt, p-38, keratin, organ culture, c-met, gene therapy

Diabetic retinopathy (DR) is the major diabetic complication in the eye.[1] The most severe proliferative stage cannot be reproduced in animals. Vision loss from diabetes is mainly due to the retinal changes. However, other parts of the eye are also affected in up to 30% of cases: iris, with neovascularization and neovascular glaucoma; lens, with diabetic cataract; optic nerve, with glaucomatous neuropathy.[2–5] Also, 50% to 70% of patients have corneal abnormalities including recurrent erosions, delayed and incomplete wound healing, ulcers and edema, complications after vitrectomy, laser photocoagulation, and corneal surgery, as well as limbal epithelial stem cell (LESC) alterations.[6–14] Corneal neuropathy, manifested by loss of corneal sensation, and progressively reduced density of corneal stromal and subbasal nerves is also widespread in diabetic patients.[12,14–20] It has been suggested that diabetic neuropathy contributes to epithelial degenerative changes seen in diabetic keratopathy.[21–23] Diminished sensation in animal models and corneal nerve changes appear within the first 1 to 2 months of diabetes,[24,25] which supports the role of neuropathy in causing keratopathy.[26] However, corneal epithelial cells in culture exposed to high glucose to mimic the diabetic insult show altered signaling through epidermal growth factor receptor (EGFR) and slow wound healing,[27] similar to the in vivo situation.[24,28] Genetic or acquired limbal stem cell deficiency is also accompanied by nerve changes similar to those found in diabetics,[29–31] which suggests that epithelial alterations may in turn lead to the development of corneal neuropathy.

Our previous studies were focused on alleviating signs of diabetic keratopathy using gene therapy directed at the epithelial cells.[13,32–34] We have used a thoroughly validated

Table 1. Donor Characteristics

Case Number	Age, y	Sex	DM Type	Duration, y	Cause of Death	Culture Treatment
DR 09-29	60	F	NIDDM → IDDM	10 → 10	Coronary artery disease	shM10
DM 10-11	82	M	NIDDM	15	Respiratory failure	shM10
DM 11-1	73	M	NIDDM →IDDM	10 → 30	Ischemic stroke	shM10
DM 11-2	85	F	NIDDM	30	Cardiopulmonary arrest	shM10
DR 10-1	51	M	IDDM	35	Myocardial infarction	shCF
DM 10-4	58	F	NIDDM → IDDM	15 total	Sudden cardiac arrest	shCF
DM 10-27	81	F	NIDDM	10	Acute respiratory failure	shCF
DM 11-19	71	F	NIDDM	4	Intracerebral hemorrhage	shCF
DR 11-8	60	M	NIDDM	15	Myocardial infarction	shCF + shM10
DR 11-10	77	F	NIDDM	15	Cardiogenic shock	shCF + shM10
DM 11-12	83	M	NIDDM	10	Acute cardiac arrest	shCF + shM10
DM 11-22	72	M	NIDDM	13	Chronic obstructive pulmonary disease	shCF + shM10
DR 10-09	68	M	NIDDM	20	Myocardial infarction	Combo
DR 10-10	74	M	NIDDM	9	Pneumonia	Combo
DR 10-13	71	M	NIDDM	35	Intracerebral bleed	Combo
DR 10-14	70	M	NIDDM → IDDM	20 → 20	Subarachnoid hemorrhage	Combo

Combo, shCF + shM10 + *c-met*. Arrows denote the number of years with a particular type of diabetes.

human diabetic corneal organ culture system that reproduces wound healing dynamics and diabetic marker distribution[35] due to the existence of epigenetic metabolic memory.[36,37] The epithelium of these corneas was transduced with recombinant adenoviruses (rAV) targeting genes that have altered expression levels in diabetic corneas, such as hepatocyte growth factor (HGF) receptor *c-met* proto-oncogene and two proteinases, *matrix metalloproteinase-10* (*M10*) and *cathepsin F* (*CF*). Overexpression of diabetes-downregulated c-met led to a faster wound healing as well as to a more normal expression of certain diabetic marker proteins in diabetic human organ-cultured corneas.[34] Interestingly, c-met upregulation also resulted in the concomitant normalization of several putative LESC markers that had a reduced expression in ex vivo diabetic corneas,[34] supporting the role of LESC alterations in diabetic keratopathy. Additionally, overexpression in normal corneas of *M10* and *CF* genes upregulated in diabetic corneas resulted in slower wound healing and decreased expression of several diabetic markers, similar to diabetic tissue.[33] The effects of overexpressed proteinases appeared to be mediated by reduced signaling from the EGFR-Akt axis.

In this study, it was examined whether silencing in diabetic corneas of *M10* and *CF* genes using small hairpin RNA (shRNA) driven by rAV vectors would exert a similar normalizing effect as seen with overexpression of *c-met* gene. We also studied the effects of combined treatment using proteinase gene silencing and *c-met* upregulation. The results showed that shRNA suppression of proteinases, especially when combined with c-met overexpression, normalized corneal epithelial wound healing and the expression levels of diabetic and limbal stem cell markers. These improvements seem to be due to activation of signaling pathways downregulated by diabetes.

Methods

Corneal Organ Cultures

Postmortem diabetic human eyes or corneas were acquired from the National Disease Research Interchange (NDRI, Philadelphia, PA). NDRI has a human tissue collection protocol approved by a managerial committee and subject to National Institutes of Health oversight. Corneas were from 16 donors (four pairs per each treatment group) in total. Fifteen donors had noninsulin-dependent diabetes mellitus (NIDDM), which in four cases progressed to insulin-dependent diabetes mellitus (IDDM), and one donor had IDDM (Table 1). Half of the donors had diagnosed DR. Donors included 6 women and 10 men, with mean age of 71 years, with no significant age differences among groups. All donors but one had long-term diabetes (average duration 20.4 years). Corneal organ cultures (four cases per group) were established as described[35] and were maintained in serum-free medium with insulin-transferrin-selenite, antibiotics and an antimycotic (Life Technologies, Carlsbad, CA).

shRNA Constructs

Two different shRNA constructs derived from each of the human *MMP-10* (shM10-S2 and shM10-S3) and *cathepsin F* (shCF-S2 and shCF-S3) coding sequences were designed based on the Ambion siRNA sequences as follows:

1. shM10-S2 (human MMP-10, or stromelysin-2) RefSeq number: NM_002425, Exon 1; MMP-10-S2: cat# 16708A, siRNA ID# 11404.
   Sense sequence (5′-3′): GGAGGACUCCAACAAGGAUtt; antisense sequence (5′-3′) AUCCUUGUUGGAGUCCUCCtc; shRNA sequence for MMP-10-S2, cloned into iLenti-EGFP vector: 5′GGAGGACTCCAACAAGGATttTTCAAGAGAAUCCTTGTTGGAGTCCTCCtcTTTTTT3.′
2. shM10-S3 (human MMP-10, or stromelysin-2) RefSeq number: NM_002425, Exon 7; MMP-10-S3: cat# 16708A, siRNA ID# 112912.
   Sense sequence (5′-3′) GCAGGGACACCGUUUUUAUtt; antisense sequence (5′-3′) AUAAAAACGGUGUCCCUGCtg; shRNA sequence for MMP-10-S3, cloned into iLenti-EGFPvector: 5′GCAGGGACACCGTTTTTAUttTTCAAGAGAATAAAAACGGTGTCCCTGCtgTTTTTT3.′
3. shCF-S2 (human cathepsin F) RefSeq number: NM_003793, Exon 3 & 4; CF-S2: cat# 16708A, siRNA ID# 13667.
   Sense sequence (5′-3′) GGACUUGCCUGUGAAGAUGtt; antisense sequence (5′-3′) CAUCUUCACAGGCAAGUCCtg; shRNA sequence CF-S2, cloned into iLenti-EGFP vector: 5′GGACTTGCCTGTGAAGATGttTTCAAGAGACATCTTCACAGGCAAGTCCtgTTTTTT3.′
4. CF-S3 (cathepsin F) RefSeq number: NM_003793, Exon 13; CF-S3: cat# 16708A, siRNA ID# 137213.
   Sense sequence (5′-3′) CCACCCUUUUCUAACAGCAtt; antisense sequence (5′-3′) UGCUGUUAGAAAAGG-

Table 2. Antibodies Used in the Study

Antigen	Antibody	Source
MMP-10	Goat pAb sc-9941	Santa Cruz Biotechnology (Santa Cruz, CA)
Cathepsin F	Goat pAb AF2075	R&D Systems (Minneapolis, MN)
ABCG2	Mouse mAb MAB4155	EMD Millipore (Billerica, MA)
ABCG2	Mouse mAb sc-58222	Santa Cruz Biotechnology
Integrin $\alpha_3\beta_1$	Mouse mAb MAB1992	EMD Millipore
Keratin 15	Mouse mAb sc-47697	Santa Cruz Biotechnology
Keratin 17	Mouse mAb sc-58726	Santa Cruz Biotechnology
Laminin γ3	Rabbit pAb R96	Saghizadeh et al.[13]
Nidogen-1	Mouse mAb MAB2570	R&D Systems
ΔNp63	Goat pAb sc-8609	Santa Cruz Biotechnology
p-EGFR (Tyr845)	Rabbit pAb 44-784G	Life Technologies
p-Akt (Ser473)	Rabbit pAb 9271	Cell Signaling Technology (Danvers, MA)
p-p38 (Thr180/Tyr182)	Mouse mAb ab50012	Abcam (Cambridge, MA)

mAb, monoclonal antibody; pAb, polyclonal antibody.

GUGGtt; shRNA sequence CF-S3, cloned into iLenti-EGFP vector: 5′CCACCCTTTTCTAACAGCAttTTCAAGA GATGCTGTTAGAAAAGGGTGGtgTTTTTT3.′

The shRNA sequences (59-bp insert) were cloned by Capital Biosciences (Rockville, MD) into BbsI/XhoI cloning site of iLenti-EGFP vector (Applied Biological Materials, Inc., Richmond, BC, Canada) with a backbone size of 9200 bp, which contained hH1 promoter, GFP, and Kanamycin/Neomycin resistance genes. RefSeq is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD (www.ncbi.nlm.nih.gov/locuslink/refseq).

Adenovirus Constructs

rAVs were custom generated by Capital BioSciences by subcloning of shRNA sequences along with hH1 promoter and GFP tag sequence from iLenti-EGFP vector into a replication-incompetent (-E1/-E3) human rAV type 5 (Ad5) genome using a commercial expression system (Adeno-4; Applied Biological Materials, Inc.). After transformation and purification of rAV DNA, the *Pac* I-digested vectors were used to transfect HEK 293 cells to produce adenoviral stocks. The rAV were amplified by infecting the 293 producer cells with the crude viral lysates and purified using a purification kit. The titers of the rAV stocks were determined and used to transduce mammalian cell lines or organ-cultured corneas. Control rAV drove EGFP expression.

Adenovirus Transduction of Cell Cultures

The telomerase-immortalized diploid and nontumorigenic corneal epithelial cell line (HCEC) was obtained under a Material Transfer Agreement from Dr Dan Dimitrijevich (University of North Texas System; Dimitrijevich SD, et al. *IOVS* 2008;49:ARVO E-Abstract 4306). The HCEC are somewhat similar to LESC in that they express several putative LESC markers—keratins 14, 17, and 19 (but not 15), and ABCG2—but do not express differentiated corneal keratin 3 (data not shown here). The cells were used in preliminary experiments to optimize shRNA transduction and to confirm target gene downregulation. Cells were plated onto collagen type IV-coated plates in serum free medium (EpiLife; Life Technologies) supplemented with antibiotics and an antimycotic agent (Life Technologies), and human corneal growth supplement (HCGS; Life Technologies). Cells were grown in a humidified incubator with 5% CO_2 at 37°C. The cells were plated the day before transduction, and 70% confluent cells were transduced with rAV-sh at a multiplicity of infection of 50. The cells were incubated for 24 to 48 hours, and then collected and subjected to quantitative real-time RT-PCR (QPCR).

Adenovirus Transduction of Organ-Cultured Corneas

Sixteen pairs of age-matched autopsy diabetic organ-cultured corneas (four per group) were transduced with rAV-sh expressing siRNA against *MMP-10* (rAV-shM10) and/or *CTSF* (rAV-shCF) genes under human H1 (hH1) promoter. Recombinant adenovirus-driven small hairpin RNA were to either single target, both targets together, or both proteinases in combination (Combo) with rAV-expressing *c-met* gene (rAV-cmet). The other cornea of each pair was treated with control rAV expressing scrambled siRNA sequences (rAV-EGFP). The rAV particles were given at $1.25\text{-}2 \times 10^8$ plaque-forming units (pfu) per cornea in culture medium for 48 to 72 hours. To increase rAV transduction efficiency,[33] 75 μg/mL sterile sildenafil citrate (Viagra; Pfizer Corp., New York, NY) was added to the culture, together with the virus for 3 hours, after which another portion was added because of the short half-life of sildenafil in aqueous solutions.

After additional 4 days incubation, some rAV-sh–treated corneas were processed for immunohistochemistry and QPCR,[34] to confirm shRNA-silencing effect.

Immunohistochemistry

Various diabetic, signaling, and putative stem cell markers ($\alpha_3\beta_1$ integrin, nidogen-1, laminin γ3 chain, p-p38, p-EGFR, p-Akt, N-cadherin, ΔNp63α, ABCG2, keratins 15, and 17) were studied by immunostaining of corneal cryostat sections.[13,33,34] The antibodies are listed in Table 2. To assess the immunostaining results, the slides were always evaluated by two independent observers upon immunostaining in two to three independent experiments, and only the data on markers where the concordance was complete are presented. Such a concordance was not always obtained for certain markers and these are not mentioned. Due to individual variations in age and disease duration and severity, the changes are presented only if they had the same trend in more than 50% of studied cases.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed essentially as described.[34] Briefly, total RNA was extracted from shRNA-transduced HCEC or separated corneal epithelium with an

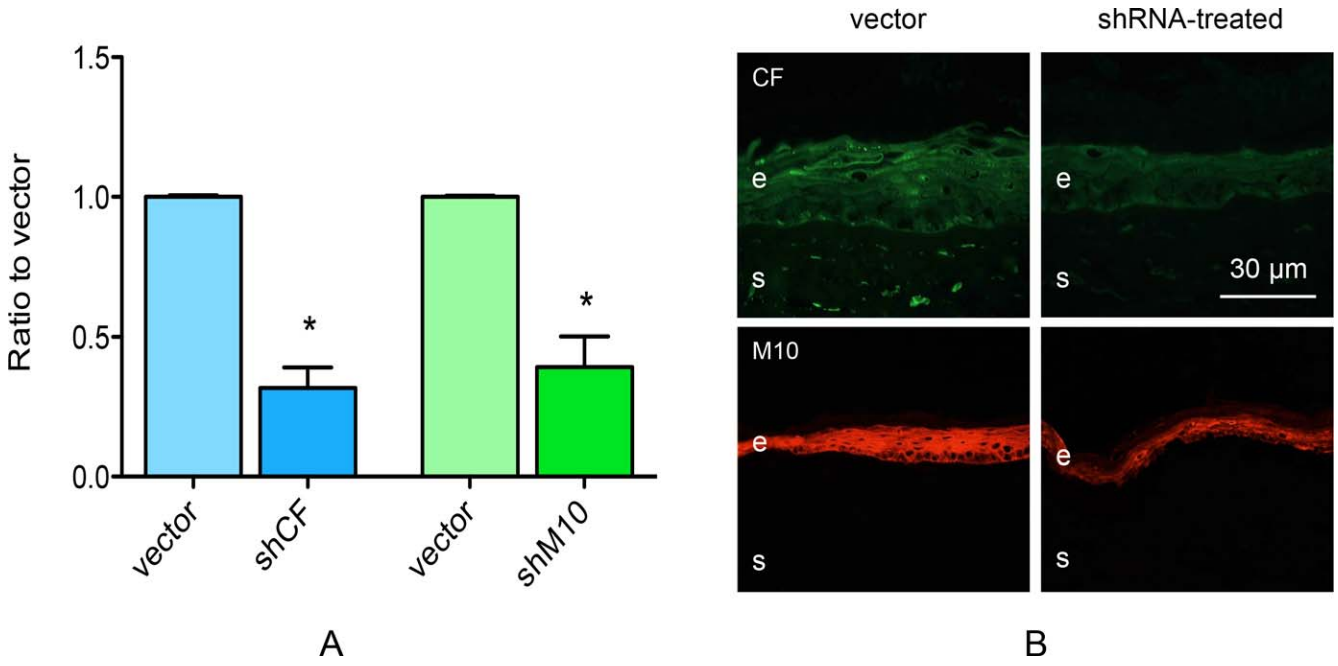

Figure 1. Recombinant adenovirus-shM10 and rAV-shCF transduction into organ-cultured human diabetic corneas leads to a decrease in the expression of their respective target genes and proteins, M10, and CF. (**A**) Quantitative real-time RT-PCR of epithelial cell layers. (**B**) Immunofluorescent staining of corneal sections; exposure times were the same for each control and treated cornea. *$P < 0.003$. *Scale bar*: 30 μm. e, epithelium; s, stroma.

RNA kit (RNeasy Mini Kit; Qiagen, Valencia, CA) treated with DNase I (Qiagen) to eliminate possible genomic DNA contamination, and purified on mini-columns (Qiagen). The RNA yield was determined using a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE). Total RNA (0.5 μg) was reverse transcribed into first-strand cDNA (High Capacity cDNA Reverse Transcription Kit; Life Technologies). Quantitative real-time RT-PCR was performed using a sequence-detection system (Prism 7900HT; Life Technologies) in 384-well plates, to evaluate the expression levels of the *MMP-10* and *cathepsin F* genes and of the endogenous control gene, *β_2-microglobulin* (*Taq*Man Gene Expression Assays; Life Technologies). The following gene-specific assay kits were used per Life Technologies: matrix metallopeptidase-10 (MMP-10, stromelysin 2) assay ID Hs00233987_m1 (GenBank RefSeq NM_002425.2, exon boundary 9-10, assay location, 1370; amplicon size, 82 bp); cathepsin F (CTSF) assay ID Hs00186901_m1 (GenBank RefSeq NM_003793.3, exon boundary 4-5, assay location, 700; amplicon size, 105 bp); and β_2-microglobulin assay ID Hs99999907_m1 (GenBank RefSeq NM_004048.2, exon boundary 2-3; assay location, 413; amplicon size, 75 bp). Quantitative real-time RT-PCR conditions were as described, and the comparative threshold cycle (Ct) method (ΔΔCt) was used, which corrects for any difference in the expression of the internal normalization gene.[34]

Wound Healing in Organ Cultures

To study wound healing, 5-mm epithelial wounds were made with n-heptanol 10 days after rAV transduction as we have described previously,[33,34] and healing times were recorded. The wound healing process was monitored daily by two independent observers, and pictures were taken from different parts of the wound. The morphological monitoring of healing was based upon the detection of keratocytes that are known to undergo apoptosis after the epithelial debridement wounds.[38] Such dead keratocytes look like black spider-shaped cells in transmitted light. In nonhealed wounds many of these cells are seen just at the level of the advancing epithelial sheet. Healing was judged complete when a layer of epithelial-looking cells was detected above the dead keratocytes that were no longer visible when the microscope lens was focused on the uppermost cell layer. The presented data refer to the number of days to complete healing.

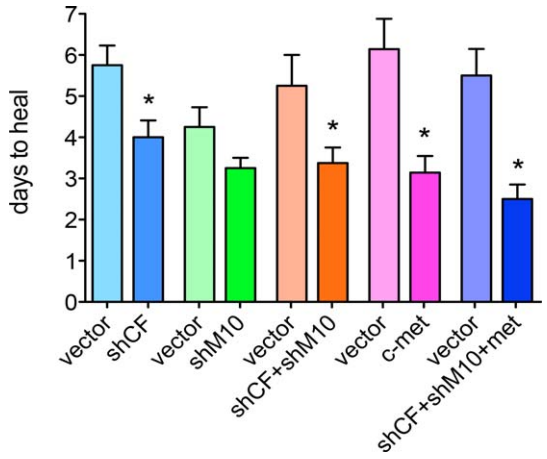

Figure 2. Various gene therapy treatments in organ-cultured diabetic corneas reduce epithelial wound healing time. *M10* gene silencing slightly accelerates healing, whereas *CF* gene silencing results in significant acceleration. Wound healing time is further decreased when both proteinase genes are knocked down. Complete normalization of wound healing time is achieved with combined proteinase gene silencing by shRNA and c-met overexpression. Four pairs of corneas in each group were used, except for c-met (seven pairs were used). Data for c-met overexpression are from Saghizadeh et al.[34] *$P < 0.03$.

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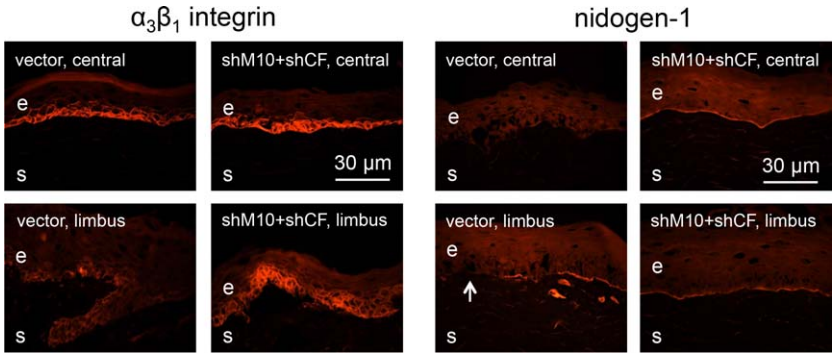

FIGURE 3. Combined proteinase silencing (shM10 + shCF) in organ-cultured diabetic corneas normalizes the patterns of select diabetic markers, epithelial $\alpha_3\beta_1$ integrin, and basement membrane nidogen-1, in the central cornea (*top row*) and limbus (*bottom row*). *Arrow* indicates interrupted limbal basement membrane staining in rAV-EGFP-treated cornea. Immunofluorescent staining of corneal sections. *Scale bar*: 30 μm.

Statistical Analysis

Times to complete wound healing after chemical debridement of the epithelium in pairs of vector and shRNA or *c-met*-transduced corneas were analyzed by paired two-tailed Student's *t*-test (all statistical analyses were done using the Prism 5 software program; GraphPad Software, San Diego, CA). Comparison of times to complete wound healing with an additional group of *c-met*-transduced corneas[34] was performed by ordinary ANOVA with the Bonferroni multiple-comparisons post test. Patient ages in different groups were compared by ordinary ANOVA. Data are presented as the mean ± SEM.

RESULTS

We have previously shown that M10 and CF were significantly elevated in diabetic corneas, and that their overexpression in normal organ-cultured corneas led to a delayed epithelial wound healing.[33,39,40] Therefore, it was reasonable to attempt their silencing in diabetic cornea in order to normalize wound healing and marker expression. Proteinase gene silencing was achieved by transduction of organ-cultured human diabetic corneas with adenoviral constructs harboring preselected shRNAs to *M10* and/or *CF*. Preliminary experiments obtained on HCEC line confirmed a decrease of both targets upon rAV-sh transduction. In the transduced corneas, the rAV-sh constructs

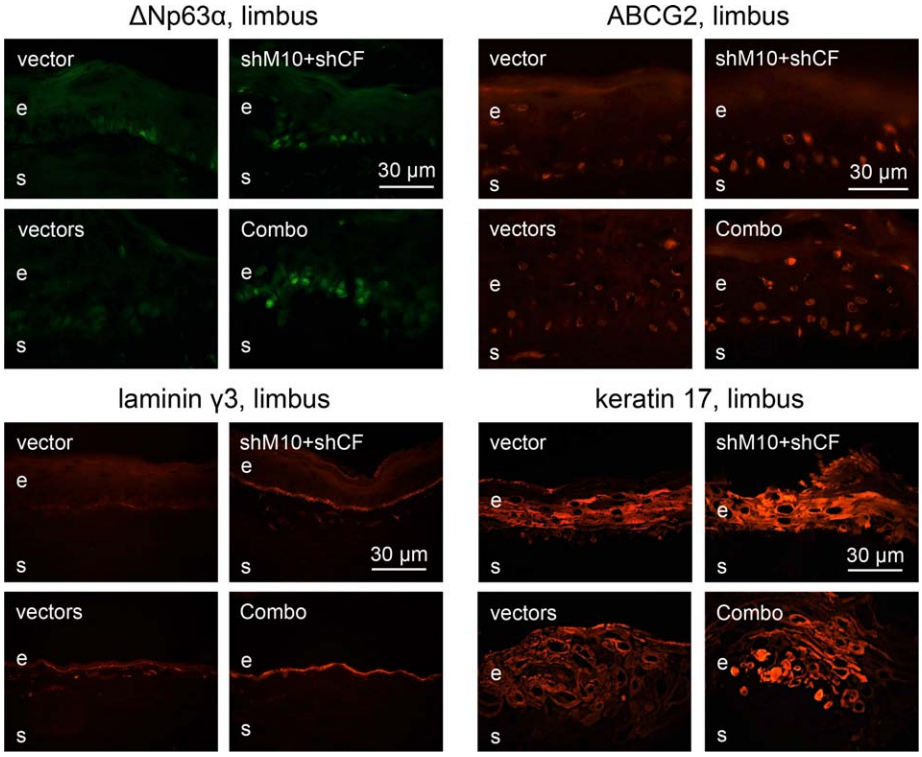

FIGURE 4. Increased expression of limbal markers upon combined treatments in organ-cultured diabetic corneas. *Top left*: ΔNp63α. Both shM10 + shCF, and shM10 + shCF + *c-met* overexpression (Combo) treatments increase staining intensity and the number of positive basal epithelial cells. The same is true for ABCG2 (*top right*). *Bottom left*: laminin γ3 chain. Treatments markedly increase staining intensity and continuity. *Bottom right*: keratin 17. Both treatments increase staining intensity. Immunofluorescent staining of limbal corneal sections. *Scale bar*: 30 μm.

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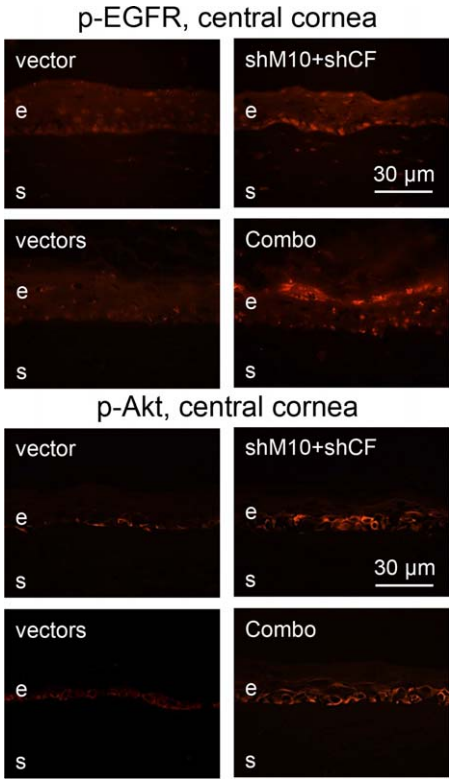

Figure 5. Combined proteinase silencing (shM10 and shCF) leads to increased expression (*top rows*) of activated EGFR (p-EGFR) and Akt (p-Akt). The same effect is seen when proteinase silencing is combined with c-met overexpression (Combo, *bottom rows*). Immunofluorescent staining of central corneal sections. *Scale bar*: 30 μm.

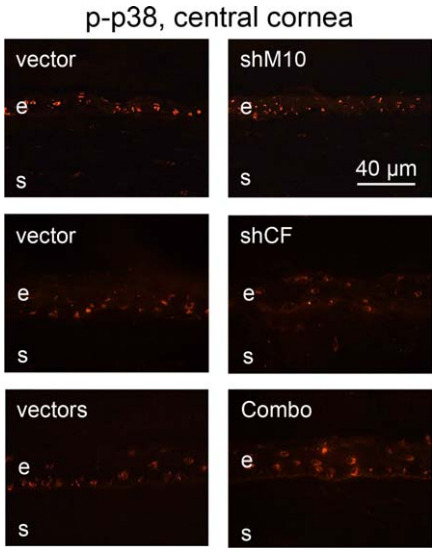

Figure 6. Proteinase silencing does not cause p38 MAP kinase activation (p-p38, *top two rows*). However, when this silencing is combined with c-met overexpression (Combo, *bottom row*), the expression of p-p38 increases. This result is in accordance with our previous data linking normalizing c-met effects on diabetic corneal wound healing with p38 activation. Immunofluorescent staining of central corneal sections. *Scale bar*: 40 μm.

reduced the respective gene expression in the epithelial cell layer by 60% (*M10*) to 70% (*CF*) compared with vector (Fig. 1A) as identified by QPCR. Immunostaining for respective antigens also showed markedly decreased expression of each proteinase upon shRNA transduction (Fig. 1B).

Transduction of long-term diabetic corneas with shRNA to *CF* significantly decreased wound healing times (31% reduction in healing time) compared with rAV-EGFP vector control (Fig. 2). Small hairpin RNA to *M10* also accelerated wound healing (23% reduction in healing time), but significance was not achieved, in line with previous data on slight influence of overexpressed M10 on normal corneal wound healing.[33] Combination of two shRNAs produced a further acceleration of epithelial wound healing (36% reduction in healing time), but still not achieving the magnitude of the effect observed upon c-met overexpression (49% reduction in healing time resulting in normal healing[34]). Upon Combo treatment, however (Fig. 2), the corneas healed even faster than normal ones (55% decrease in healing time versus vectors). Overall, proteinase gene silencing had a functional beneficial effect on diabetic corneal epithelial wound healing. The highest rAV-sh effect was achieved by a combination of shRNA to *M10* and *CF*; therefore, most data below on marker expression are presented using this combination.

Small hairpin RNA treatment reversed the reduced expression of diabetic markers, epithelial $\alpha_3\beta_1$ integrin and basement membrane nidogen-1, both in the central cornea and limbus (Fig. 3). The integrin staining became stronger and more uniform upon shRNA treatment. The nidogen-1 staining also became stronger and markedly more continuous than in the vector-treated fellow corneas. Some of the putative LESC markers also showed higher and almost similar to normal expression in the limbus upon shRNA treatment. Fig. 4 illustrates this point for ΔNp63α, ABCG2, laminin γ3 chain, and keratin 17. With all markers, both shRNA combination and Combo treatment with c-met overexpression produced similar results, with slightly higher marker expression with Combo. Interestingly, in our previous study, c-met overexpression alone also caused similar changes in putative LESC marker expression.[13]

In order to test the mechanisms of proteinase shRNA action, staining was performed for various signaling intermediates. Our previous data suggested the involvement of EGFR-Akt pathway inhibition in mediating the effects of proteinase overexpression.[33] In line with this evidence, the expression of activated phosphorylated EGFR (p-EGFR) and Akt (p-Akt) was markedly enhanced upon shRNA treatment (Fig. 5, top panels). This was also seen with single shRNA treatment (not shown here), and with Combo treatment (Fig. 5, bottom panels). Therefore, EGFR-Akt signaling appeared to be improved upon proteinase gene knockdown. Our previous data suggested that c-met overexpression in diabetic corneas accelerated wound healing through activation of p38.[34] In accordance with these data, immunostaining for p-p38 was increased upon Combo treatment, although it remained unchanged upon rAV-sh treatment (Fig. 6).

Discussion

Gene therapy is emerging as a promising new treatment modality for various corneal diseases. Different delivery systems, both viral and nonviral, have been used in preclinical studies with reasonable success, targeting epithelial, stromal, and endothelial corneal cells.[41–45] In our previous work, we have used rAV-driven gene therapy for corneal diabetes, focusing on diabetic keratopathy. Recombinant adenovirus—contrary to adeno-associated virus,[32] for example—does not penetrate into the corneal stroma, and mostly surface cells (epithelial and endothelial) are transduced. As corneal epithelial cell alterations may underlie diabetic keratopathy, especially, slow wound healing, rAV was chosen as a more specific

epithelium-transducing vector than adeno-associated virus. Using this approach, we have demonstrated the efficacy of c-met overexpression in normalizing diabetic corneal wound healing and marker expression, including, for the first time, putative LESC markers.[13,34] Conversely, it was also possible to slow down wound healing to nearly diabetic levels by overexpressing in normal organ-cultured corneas two diabetes-increased proteinases, M10 and CF, using rAV-driven gene therapy.[33]

In the present study, the expression of these proteinases was suppressed in organ-cultured human diabetic corneas using specific rAV-delivered shRNAs. This treatment caused acceleration of corneal epithelial wound healing impaired in diabetes and a common sign of diabetic keratopathy, and normalized the patterns of certain diabetes-associated corneal markers, $\alpha_3\beta_1$ integrin and nidogen-1, similar to the effect of c-met overexpression.[34] Another similarity with c-met treatment was observed in the limbal compartment, which harbors corneal epithelial stem cells. Silencing of M10 and CF at least partially normalized the patterns of several putative stem cell markers. Importantly, combined silencing of both M10 and CF produced stronger effects on the tested parameters than suppression of each proteinase individually. Overall, the diabetic corneas upon proteinase silencing became more similar to normal corneas. The results suggest that both proteinases, in agreement with previous data obtained using their overexpression in normal corneas,[33] have a functional role in diabetes-induced impairment of corneal wound healing and downregulation of specific adhesive and stem cell markers.[13,35]

The involvement of proteinases in extracellular matrix and integrin degradation that also occurs in diabetic corneas has been well established.[46–48] Matrix metalloproteinase-10 and CF are upregulated in various cancers and are implicated in tumor invasion and metastasis.[49–51] In the cornea, M10 is localized during wound healing behind the leading edge of the migrating epithelium.[52] However, in M10 knockout mice, the slowing down of corneal epithelial wound healing did not reach significance.[52] Our data on the modest effect of *M10* shRNA knockdown on corneal wound healing (Fig. 2) corroborate this previous work. At the same time, exposure of corneal epithelial cells in culture to M10 resulted in the degradation of $\alpha_3\beta_1$ integrin and reduced cell adhesion.[47] Moreover, overexpression of constitutively active M10 in skin caused proteolysis of basement membrane γ2 chain of laminin-332 and disorganized staining for β_1 chain-containing integrins.[53] These effects of M10 could explain our data on increased and more organized staining of diabetes-downregulated $\alpha_3\beta_1$ integrin and basement membrane nidogen-1 upon M10 knockdown by shRNA.

Cathepsins including CF are typically considered as lysosomal cysteine proteinases. However, high CF expression has been associated with aggressive behavior of tumors.[50] Cathepsin F can be secreted (e.g., by macrophages in an angiotensin II-dependent manner) and can digest extracellular matrix.[54–56] Therefore, the ability of *CF* shRNA to restore nearly normal patterns of basement membrane and integrin components in diabetic corneas could be due to the suppression of extracellular CF.

Overexpression of M10 and/or CF in normal human corneas resulted in slowdown of wound healing, with marked decrease of activated EGFR and Akt[33]; a similar effect was seen in skin wound healing.[53] In line with these results, silencing of these proteinases in diabetic corneas resulted in increased staining for both p-EGFR and p-Akt (Fig. 5). The EGFR/Akt signaling axis is known to stimulate corneal epithelial wound healing, and is downregulated in diabetes in vivo and by high glucose in vitro.[27,28,57] Secreted proteinases could possibly regulate intracellular signaling through their action on integrins, including $\alpha_3\beta_1$, that can interact with growth factor receptors, such as EGFR.[58,59]

Increased staining for putative LESC markers upon proteinase silencing as a sign of normalization of limbal stem cell compartment could also involve EGFR. This receptor has higher expression in basal limbal cells (site of LESC localization) compared with suprabasal cells and central cornea.[60,61] Moreover, the survival signaling pathway involving EGFR, and PI3-kinase/Akt is important in LESC expansion, and proliferation without inducing cell differentiation.[62,63]

We have also shown here that epithelial wound healing and marker expression in diabetic corneas were further normalized when M10 and CF silencing was combined with *c-met* overexpression. This Combo treatment accelerated wound healing in diabetic corneas even slightly beyond the normal range, and had an additive effect to both shRNA and *c-met* treatments alone (Fig. 2). Our previous data have implicated activation of p38 MAP kinase in c-met effects, which was corroborated here (Fig. 6). In fact, p38 has been previously shown to mediate corneal epithelial migration and wound healing.[64,65] Activation of p38 by c-met overexpression could also have beneficial effects on LESC compartment, because p38 is involved in LESC marker expression (e.g., ΔNp63α), and their self-renewal induced by growth factors.[66,67] These data show that the additional effect of c-met overexpression in our system may have resulted from activation of p-38 on top of the EGFR-Akt pathway. This suggestion is further supported by data on the cross talk between HGF/c-met system and EGFR.[68,69]

In summary, shRNA silencing of diabetes-elevated proteinases, M10 and CF, in organ-cultured diabetic corneas produced normalizing effects on marker expression, prosurvival signaling activation (EGFR-Akt axis) and epithelial wound healing. Combination of two shRNAs to both proteinases increased the effect, primarily on wound healing. The most significant effect was achieved when shRNAs to *M10* and *CF* were combined with c-met overexpression. This led to a complete normalization of wound healing times compared with fellow corneas treated with control vectors. The additive effect of c-met treatment appears to be mediated by the additional activation of p38. The Combo treatment may be, therefore, the most effective because of simultaneous activation of EGFR-Akt and p38 pathways to promote wound healing and increase diabetes-downregulated marker expression. The presented data suggest that our combination gene therapy dramatically ameliorates wound healing rates and the expression of specific epithelial markers, including stem cell markers. This therapy may prove to be beneficial to long-term diabetics with nonhealing corneal lesions (see also Bikbova et al.[70]), as well as to those with limbal stem cell problems caused by diabetes.

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