Published in final edited form as:
Exp Eye Res. 2014 December ; 129: 66–73. doi:10.1016/j.exer.2014.10.022.

Normalization of Wound Healing and Stem Cell Marker Patterns in Organ-Cultured Human Diabetic Corneas by Gene Therapy of Limbal Cells

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Abstract

Overexpression of *c-met* and suppression of *matrix metalloproteinase-10 (MMP-10)* and *cathepsin F* genes was previously shown to normalize wound healing, epithelial and stem cell marker patterns in organ-cultured human diabetic corneas. We now examined if gene therapy of limbal cells only would produce similar effects.

Eight pairs of organ-cultured autopsy human diabetic corneas were used. One cornea of each pair was treated for 48 hours with adenoviruses (Ad) harboring full-length *c-met* mRNA or a mixture (combo) of Ad with *c-met* and shRNA to *MMP-10* and *cathepsin F* genes. Medium was kept at the limbal level to avoid transduction of central corneal epithelium. Fellow corneas received control Ad with *EGFP* gene. After additional 5 (*cmet*) or 10 days (combo) incubation, central corneal epithelial debridement with nheptanol was performed, and wound healing times were determined microscopically. Corneal cryostat sections were immunostained for diabetic and putative limbal stem cell markers, $\alpha_3\beta_1$ integrin, nidogen-1, fibronectin, laminin γ3 chain, ΔNp63α, keratins 14, 15, and 17, as well as for activated signaling intermediates, phosphorylated EGFR, Akt, and p38.

Limbal c-met overexpression significantly accelerated healing of 8.5-mm epithelial wounds over EGFP controls (6.3 days vs. 9.5 days, $p<0.02$). Combo treatment produced a similar result (6.75

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Disclosure: M. Saghizadeh, None; **C.M. Dib,** None; **W.J. Brunken,** None; **A.V. Ljubimov,** None.

days vs. 13.5 days, p<0.03). Increased immunostaining vs. EGFP controls for most markers and signaling intermediates accompanied *c-met* gene or combo transduction.

Gene therapy of limbal epithelial stem cell compartment has a beneficial effect on the diabetic corneal wound healing and on diabetic and stem cell marker expression, and shows potential for alleviating symptoms of diabetic keratopathy.

Keywords

Cornea; Diabetes; Limbal gene therapy; C-met; Cathepsin F; MMP-10; Stem cell marker; Signaling pathways

1. INTRODUCTION

Diabetic retinopathy (DR) is the major complication of diabetes mellitus in the eye. It occurs both in type I (insulin-dependent) and type II (non-insulin-dependent) diabetes (Antonetti et al. 2012). Vision loss from diabetes is mainly due to the retinal changes but other parts of the eye are also affected. In the iris, it is neovascularization and neovascular glaucoma; in the lens, diabetic cataract; in the optic nerve, glaucomatous optic neuropathy (Nakamura et al. 2005; Blum et al. 2007; Helbig, 2007; Murta and Cavallerano, 2007). Depending on disease duration and retinopathy stage, these symptoms are encountered in less than 30% of diabetic patients. The diabetic corneal disease includes recurrent erosions, delayed and incomplete wound healing, ulcers and edema, complications after vitrectomy and corneal surgery, and neuropathy / loss of corneal sensation; up to 70% diabetics have corneal problems (Schultz et al. 1981; Herse, 1988; Didenko et al. 1999; Wylegała et al. 2006; Chen et al. 2009; Bikbova et al. 2012; Calvo-Maroto et al. 2014). The most severe proliferative stage of DR cannot be recapitulated in animal models. We have previously validated human diabetic corneal organ culture system that reproduces wound healing dynamics and diabetic marker distribution (Kabosova et al. 2003), most probably due to the existence of epigenetic metabolic memory (Roy et al. 1990; Grant et al. 1998; Kowluru et al. 2013). We have further developed specific gene therapy that effectively reversed slow diabetic corneal epithelial wound healing and restored similar to normal expression of several diabetic markers and putative stem cell-associated proteins. This therapy was based on overexpression in diabetic corneas of matrix metalloproteinase-10 (MMP-10) and cathepsin F that were knocked-down using adenovirus (Ad)-driven shRNA transduction (Saghizadeh et al. 2010a, 2013). Additionally, *c-met* proto-oncogene, receptor of hepatocyte growth factor (HGF), was reduced in diabetic corneas and its levels were successfully restored using its Ad-driven overexpression (Saghizadeh et al. 2005, 2010b). The best normalizing effect on diabetic corneas was obtained using combination therapy approach, upon changing the levels of all three targets (Saghizadeh et al. 2013). As stem cells are necessary for epithelial wound healing, and diabetes-altered stem cell marker patterns became much closer to normal after gene therapy, we hypothesized that gene therapy could be also efficient if applied only to the limbus that harbors corneal epithelial stem cells (Lehrer et al. 1998; Lu et al. 2001; Rama et al. 2010; Amitai-Lange et al. 2014). It was, therefore, examined whether gene therapy of the limbus, to increase expression of c-met and knock-down MMP-10 and cathepsin F only in limbal cells, would restore putative stem cell marker expression and

normalize wound healing in diabetic corneas. To ensure limbal involvement, healing of large 8.5 mm wounds was studied in organ-cultured corneas.

2. METHODS

2.1. Human Tissue and Ethics Statement

Postmortem diabetic human donor eyes were purchased 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. This work was covered by an approved Cedars-Sinai Medical Center exempt IRB protocol EX-1055.

2.2. Organ Culture, Viral Transduction and Wound Healing

Eight pairs of age-matched diabetic corneas (4 per group; Table 1) were organ-cultured in serum-free DMEM with insulin-transferrin-selenite (Kabosova et al. 2003). Ad harboring shRNA to *MMP-10* and *cathepsin F* genes (Saghizadeh et al. 2013) were obtained from Capital Biosciences (Gaithersburg, MD), and Ad overexpressing full-length *c-met* cDNA was engineered in the laboratory (Saghizadeh et al. 2010b). Control Ad (Capital Biosciences) drove enhanced green fluorescent protein (EGFP) expression. Virus incubation with corneas was performed for 48 hours with medium level at the limbus to ensure that central corneas were not transduced (Saghizadeh et al. 2010a, 2010b, 2013). This was verified by EGFP expression in live corneas showing EGFP signal only in the limbal compartment (Fig. 1A). Details of the procedure have been published. After 5 days of additional incubation with Ad-c-met (Saghizadeh et al. 2010b) or 10 days with Ad-c-met and Ad-shRNAs (combo treatment; Saghizadeh et al. 2013), central corneal epithelial wounds were made for 1 min with round paper disks soaked in n-heptanol. Fellow corneas of each pair received Ad-EGFP and were also wounded.

Wound closure was monitored microscopically as described (Saghizadeh et al. 2013), and expressed as the number of days to complete healing. In a pilot experiment, Ad-cmet transduced cornea and fellow Ad-EGFP control had a 5-mm wound made as described before (Saghizadeh et al. 2010a, 2010b, 2013). All other corneas had 8.5-mm wounds made to ensure limbal cell involvement in the healing process.

2.3. Immunostaining

Upon completion of the healing process, corneas were cut in half and embedded in O.C.T. compound (Sakura Finetek USA, Torrance, CA). Cryosections were immunostained using specific antibodies for a variety of markers (Table 2; Saghizadeh et al. 2013). These included proteins used for transduction (c-met, MMP-10, and cathepsin F), proteins with reduced expression in diabetic corneas ($\alpha_3\beta_1$ integrin, nidogen-1, laminin γ3 chain, and fibronectin), putative limbal epithelial stem cell (LESC) markers (ΔNp63α, keratins 14, 15, and 17), as well as phosphorylated (p-) activated signaling intermediates (p-p38, p-EGFR, and p-Akt). Controls without primary antibodies were routinely included and were negative. Pictures were taken in an Olympus BX40 microscope (Olympus USA, Melville, NY), equipped with a high-sensitivity MicroFire 2-megapixel color digital camera (Imaging

Planet, Goleta, CA). In each experiment, the same exposure times were used across all cases for each marker. The results were assessed by two independent observers upon immunostaining in three independent experiments. Although there was some typical staining variability among individual cases, possibly due to differences in donor ages and/or disease duration and severity, the presented pictures are representative of most studied cases.

2.4. Statistical Analysis

Wound healing times in transduced corneas vs. controls were statistically compared with two-tailed Student t test using Prism5 program (GraphPad Software, San Diego, CA), with $p<0.05$ considered significant. Data are presented as the mean ± standard error of mean (SEM).

3. RESULTS

3.1. Epithelial wound healing acceleration by gene therapy

Limbal transduction of organ-cultured diabetic corneas with Ad-c-met caused increased immunostaining for the transgene in the limbal epithelial cells in comparison with Ad-EGFP (Fig. 2A). Such an increase was also noted when corneas were transduced with a mixture of three Ad harboring *c-met* gene and shRNA for *MMP-10* and *cathepsin F* genes (Fig. 2B), suggesting that the additional viruses did not negatively impact the expression of respective transgenes. In the latter case (combo treatment), immunostaining for MMP-10 and cathepsin F in the limbal epithelium was reproducibly and markedly reduced. These results were in complete accordance with previous results obtained using quantitative RT-PCR after transduction of whole corneas (Saghizadeh et al. 2010b, 2013).

When healing of large epithelial wounds was examined, it was noted that at least in some corneas, centripetally oriented streaks of EGFP-positive cells were seen extending to the central cornea (Fig. 1B), in accordance with published evidence (Amitai-Lange et al. 2014; Di Girolamo et al. 2014). Statistical analysis of epithelial wound healing showed significant decrease in healing times both for *c-met* and combo treatments vs. vector controls (Fig. 3). Limbal transduction of *c-met* gene reduced healing time by 33% (6.3 days vs. 9.5 days, $p<0.02$), whereas combo treatment caused a reduction of 51% (6.75 days vs. 13.5 days, $p<0.03$), which was very similar to the 55% decrease obtained using transduction of the whole corneal epithelium (Saghizadeh et al. 2013). Therefore, limbal gene therapy was able to normalize healing of large epithelial wounds in diabetic corneas.

3.2. Normalization of diabetic and stem cell markers by limbal gene therapy

The effects of limbal gene therapy were next examined on the patterns of diabetic markers, $\alpha_3\beta_1$ integrin and nidogen-1/entactin, that were previously shown to have a markedly decreased expression in diabetic corneas (Ljubimov et al. 1998; Kabosova et al. 2003). $\alpha_3\beta_1$ integrin staining was consistently irregular in the limbal epithelium of vector-transduced corneas, whereas the staining became strong and more uniform upon both treatments (Fig. 4A). Nidogen-1 staining was weak and discontinuous in limbal basement membrane of vector-transduced corneas. Upon both treatments, strong and nearly continuous staining was consistently observed across cases (Fig. 4B). Staining for fibronectin and laminin γ3 chain

was discontinuous or absent over large areas in the limbal basement membrane of vector-transduced corneas. It became nearly continuous upon *c-met* and combo treatments (Fig. 5A, B).

In our previous study we observed marked reduction of immunostaining for several putative LESC markers in diabetic *ex vivo* and organ-cultured corneas (Saghizadeh et al. 2011). C-met and combo transduction of whole corneal epithelium was accompanied by restoration of normal patterns of these markers (Saghizadeh et al. 2011, 2013). Therefore, it was further examined whether limbal gene therapy would also normalize the expression patterns of such markers. As expected, both *c-met* and combo gene therapies were able to markedly increase limbal immunostaining for keratin 14 (Fig. 6A) and keratin 15 (Fig. 6B). Additionally, ΔNp63, a putative limbal stem cell marker enriched in limbal epithelial holoclones (Pellegrini et al. 2001, 2014; Rama et al. 2010; Joe and Yeung, 2014) was also increased both in staining intensity and the number of positive/bright cells upon both treatments (Fig. 7). Because of the much higher abundance of the α isoform of ΔNp63 in the cornea (Robertson et al. 2008), it may be assumed that this result pertains mostly to the ΔNp63α. Thus, the treatments apparently provided functional normalization of limbal stem cell compartment. Of the tested markers, only keratin 17 did not show appreciable changes in staining intensity upon treatments (data not shown here).

3.3. Increased activation of signaling intermediates in limbal cells by gene therapy

In previous experiments it was shown that c-met normalization of epithelial wound healing in diabetic corneas was apparently due to activation of p38 mitogen-activated protein kinase (MAPK) (Saghizadeh et al. 2010b). At the same time, suppression of proteinase expression in the whole corneal epithelium was accompanied by the activation of wound healing-promoting EGFR-Akt axis (Saghizadeh et al. 2010a, 2013). Therefore, we verified whether limbal gene therapy also caused activation of respective signaling pathways. *C-met* transduction of the diabetic limbus was associated with limbal upregulation of p-p38, and the same effect was observed after combo treatment (Fig. 8A). Additionally, in accordance with previous results, limbal p-EGFR and p-Akt were also upregulated following combo treatment, as evidenced by consistently increase in staining intensity and in the numbers of positive cells (Fig. 8B). Therefore, both pathways important for corneal wound healing, through EGFR-Akt (Xu et al. 2009; Xu and Yu, 2011; Saghizadeh et al. 2010a, 2013) and p38 (Sharma et al. 2003; Saika et al. 2004; Saghizadeh et al. 2010b) apparently mediated the observed effects of limbal gene therapy.

4. DISCUSSION

Compared to many types of therapies, gene therapy is unique because it affects primarily one disease-associated target with high specificity and can result either in a gain or a loss of function of the targeted gene. This property largely determined huge interest in this approach and led to the development of various genetic drugs, delivery vehicles and clinical trials. The cornea is a very attractive tissue for gene therapy because of superficial location that facilitates gene delivery by various means and follow-up of the effects and possible adverse reactions. Major advances in corneal gene therapy have been recently reviewed in

detail (Mohan et al. 2013). Despite successful use in different experimental models, clinical development of corneal gene therapy has been hampered by insufficient information about genetic causes of most common diseases and targets for intervention.

Our previous work has identified several markers reproducibly altered in human corneas from long-term diabetic patients (Saghizadeh et al. 2001, 2005, 2011) and, hence, amenable to gene therapy. Because these markers were related to epithelial basement membrane structure, as well as growth factors, proteinases and stem cell-associated proteins, it was suggested that their alterations could contribute to the poor cell migration and slow wound healing clinically observed in diabetic patients. Therefore, wound healing and marker expression were chosen as functional tests to monitor gene therapy efficacy applied to diabetic organ-cultured corneas. Organ-cultured diabetic corneas reproduce changes in wound healing seen in diabetic patients and marker expression alterations typical for *ex vivo* diabetic corneas (Kabosova et al. 2003; Saghizadeh et al. 2011). The feasibility of using corneal organ cultures was corroborated by the existence of metabolic memory, most probably related to stable epigenetic changes elicited by long-term hyperglycemic insult (Kowluru et al. 2013). For this reason, there was no need to keep organ-cultured corneas in high glucose. Using this model system and adenoviral gene delivery we showed that overexpression of *cmet* proto-oncogene and/or downregulation of specific proteinase genes (*MMP-10* and *cathepsin F*) in the whole corneal epithelium normalized epithelial wound healing and the expression of several markers in diabetic corneas (Saghizadeh et al. 2010b, 2013). It was also shown that many putative LESC markers had significantly reduced immunostaining in diabetic corneas, which could be corrected by the same gene therapy (Saghizadeh et al. 2011).

It is well established that LESC concentrated at the corneal periphery are important for epithelial maintenance, renewal and wound healing (Notara et al. 2010; Mort et al. 2012; Joe and Yeong, 2014; Ksander et al. 2014). As they become altered with diabetes, judged by significantly reduced expression of a number of their putative markers (Saghizadeh et al. 2011), this dysfunction could cause epithelial abnormalities typical for diabetic keratopathy. Therefore, targeting gene therapy to limbal cells could alleviate critical problems in diabetic corneal epithelium. This hypothesis was tested here using limbal gene therapy with subsequent healing of large corneal wounds, in which there is known participation of limbal stem cell compartment (Amitai-Lange et al. 2014).

As expected, limbal gene therapy produced similar effects on the diabetic corneas as gene therapy of whole corneal epithelium (Saghizadeh et al., 2010b, 2013). Wound healing times were reduced in half and the expression patterns of several diabetic ($\alpha_3\beta_1$ integrin, nidogen-1, fibronectin, and laminin γ3 chain) and LESC markers (keratins 14 and 15, and ΔNp63α) became similar to normal. Combination therapy with overexpression of *c-met* gene and shRNA-mediated suppression of *MMP-10* and *cathepsin F* genes produced the strongest effect. Therefore, targeted gene therapy of corneal stem cell compartment is sufficient to reverse and normalize important alterations of the diabetic cornea.

The effects of specific gene therapy involved activation of major signaling pathways in the limbal cells that could constitute a molecular mechanism of wound healing and marker

normalization. Key signaling intermediates previously associated with corneal wound healing (Sharma et al. 2003; Saika et al. 2004; Xu et al. 2009; Saghizadeh et al. 2010a, 2010b, 2013; Xu and Yu, 2011; 2013), including phosphorylated/activated EGFR, Akt, and p38 MAPK, had higher expression in treated corneas. It should be noted that EGFR-Akt axis is important for expansion of undifferentiated LESC (He et al. 2006). Additionally, p38 can influence LESC marker expression including ΔNp63α, and their growth factor-induced self-renewal (Cheng et al. 2009; Ho et al. 2013). In case of combined gene therapy these two pathways (EGFR-Akt and ERK-p38) could act in concert because of existing cross-talk between HGF/c-met system and EGFR (Spix et al. 2007; Xu and Yu, 2007). The data suggest that activation of these pathways following gene therapy could have dual effect on LESC maintenance/proliferation and LESC-driven epithelial wound healing.

The presented data attest to the feasibility of limbal gene therapy for alleviating important abnormalities of diabetic keratopathy including slow wound healing and LESC dysfunction. For potential future clinical use, gene therapy of the limbus only could be easier performed and would require smaller amounts of virus than the therapy of the whole cornea. In severe diabetic keratopathy with persistent epithelial defects and recurrent erosions or abnormal healing after vitrectomy, there may be a need for replacing ailing limbal epithelium. In these cases, expansion of these cells in culture and normalization with gene therapy could pave the way for future transplantation purposes to diabetic patients with advanced diabetic keratopathy. Additionally, our approach could be expanded using silencing of other promising diabetes-altered corneal targets, such as microRNAs (Funari et al. 2013) and opioid growth factor receptor (McLaughlin et al. 2012).

CONCLUSIONS

1. Specific adenoviral gene therapy of limbal compartment of organ-cultured diabetic corneas was sufficient to normalize defective epithelial wound healing.
2. Combined therapy with overexpression of *c-met* gene (downregulated in diabetic cornea) and shRNA silencing of *MMP-10* and *cathepsin F* genes (upregulated in diabetic cornea) was more efficient than *c-met* therapy in restoring normal limbal expression patterns of diabetic and putative limbal epithelial stem cell markers.
3. The effects of gene therapy are apparently mediated by activation of EGFR-Akt and p38 signaling pathways.
4. Our data provide an experimental proof of principle on the importance and feasibility of limbal stem cell gene therapy for normalization of diabetic corneas.

ACKNOWLEDGMENTS

The study was supported by NIH R01 EY13431 (AVL), CTSI grant UL 1RR033176 (AVL), Cedars-Sinai Regenerative Medicine Institute (MS), and Department of Surgery (AVL); RPB Challenge Grant to Department of Ophthalmology and NIH R01 EY12676 (WJB). Results have been presented in part at the Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Orlando, FL, May 2014.

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HIGHLIGHTS

– adenovirus-driven gene therapy of limbal cells in human organ-cultured diabetic corneas is sufficient to restore normal epithelial wound healing time

– overexpression of *c-met* gene and silencing of *MMP-10* and *cathepsin F* genes led to normalization of the expression of diabetic and putative stem cell markers in treated corneas

– effects of gene therapy appear to be mediated by wound-healing related signaling pathways involving EGFR-Akt and p38 axes

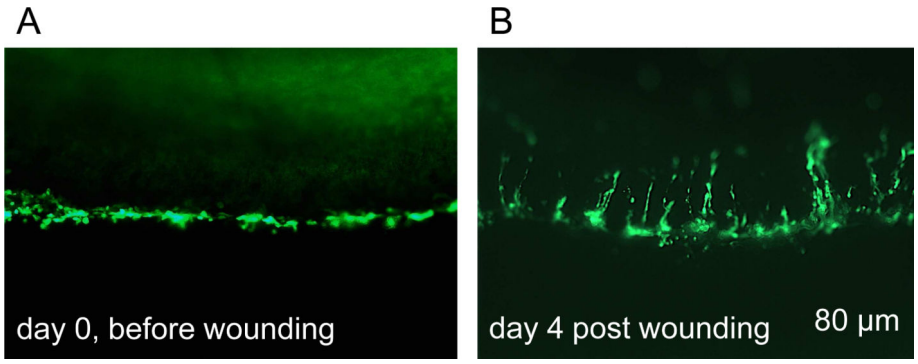

Figure 1.

Transduction of organ-cultured diabetic cornea with Ad-EGFP (live images). A, only limbal region is transduced (EGFP+ cells, green). B, during healing of a large 8.5 mm wound, limbal cell migrate centripetally. Days are relative to wound healing study. e, epithelium, s, stroma. Bar = 80 μm.

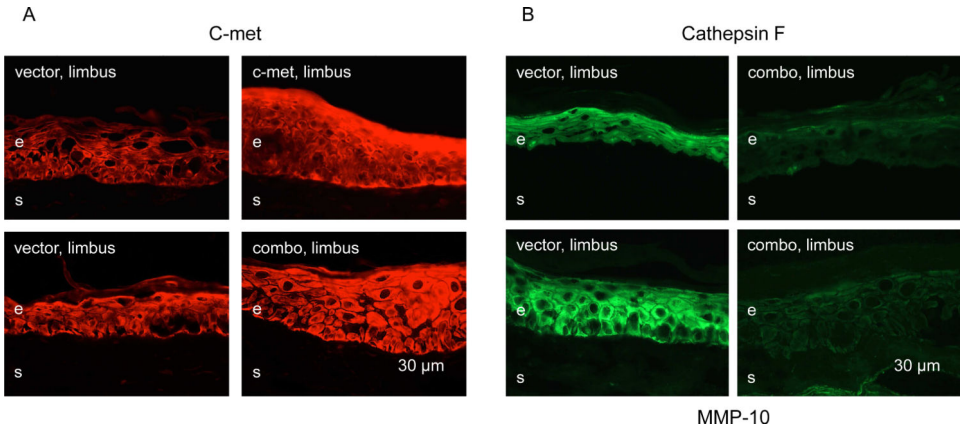

Figure 2.

Verification of viral transduction by immunostaining. A, limbal c-met expression is increased upon limbal transduction with Ad-c-met or combined Ads with *MMP-10* and *cathepsin F* shRNA and full-length *c-met* (combo). B, both MMP-10 and cathepsin F expression in the limbus is markedly decreased upon combo treatment. Here and below, representative pictures were taken with the same exposure times for each row and each marker. Here and below, the transduced components are indicated in white on the panels, and the markers revealed by immunostaining of corneal sections are indicated in black above and below the respective panels. e, epithelium, s, stroma. Bar = 30 μm.

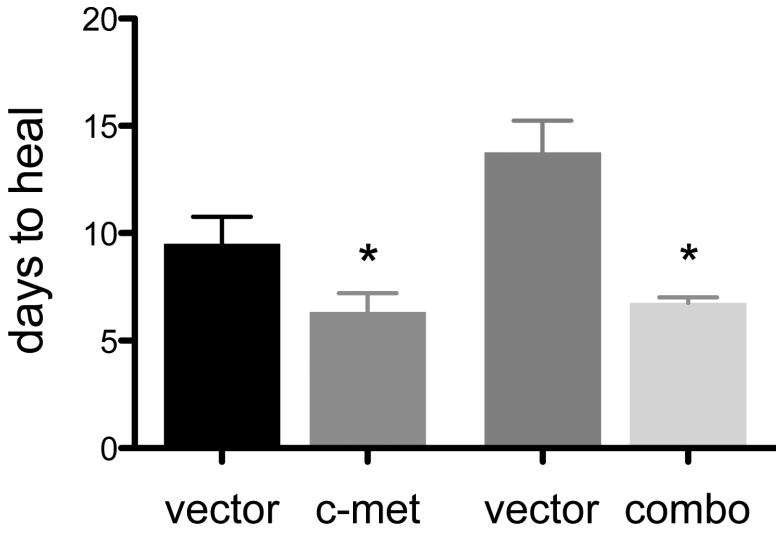

Figure 3.

Epithelial wound healing after various treatments. C-met overexpression significantly reduced healing time (by 33%). Combo treatment resulted in complete normalization of epithelial wound healing time (51% healing time reduction). *p<0.05.

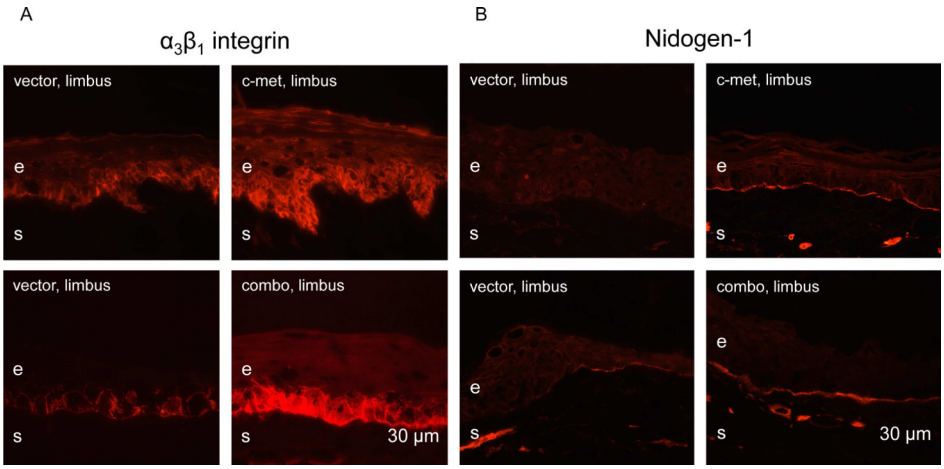

Figure 4.

Normalization of patterns of diabetic markers upon limbal gene therapy. A, epithelial $\alpha_3\beta_1$ integrin immunostaining becomes markedly stronger and more regular in the limbus following c-met overexpression and combo treatment. B, staining for nidogen-1 in the limbal basement membrane after vector transduction is weak and discontinuous over large areas (arrows). It becomes bright and nearly continuous following both treatments. e, epithelium, s, stroma. Bar = 30 μm.

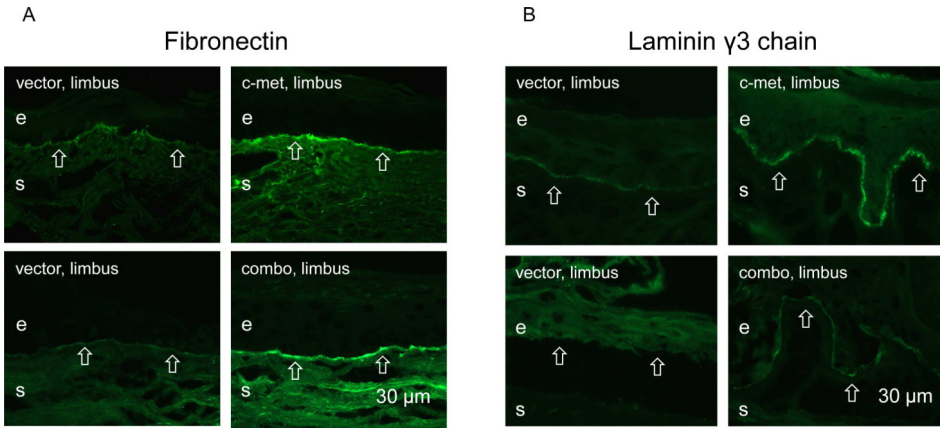

Figure 5.

Normalization of basement membrane protein patterns after gene therapy. Staining of the limbal basement membrane (open arrows) for fibronectin (A) and laminin γ3 chain becomes strong and nearly continuous following both treatments. e, epithelium, s, stroma. Bar = 30 μm.

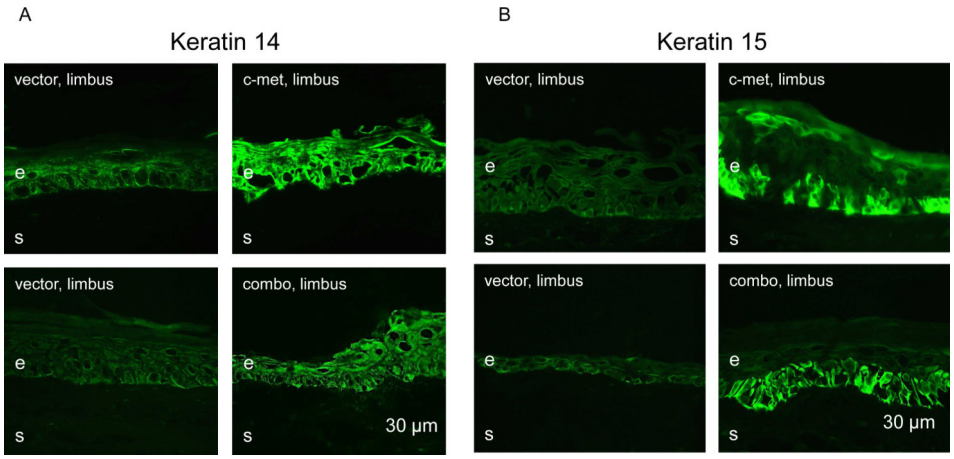

Figure 6.

Increased expression of putative limbal stem cell markers upon limbal gene therapy. Both treatments dramatically increase limbal staining for keratin 14 (A) and keratin 15 (B). e, epithelium, s, stroma. Bar = 30 μm.

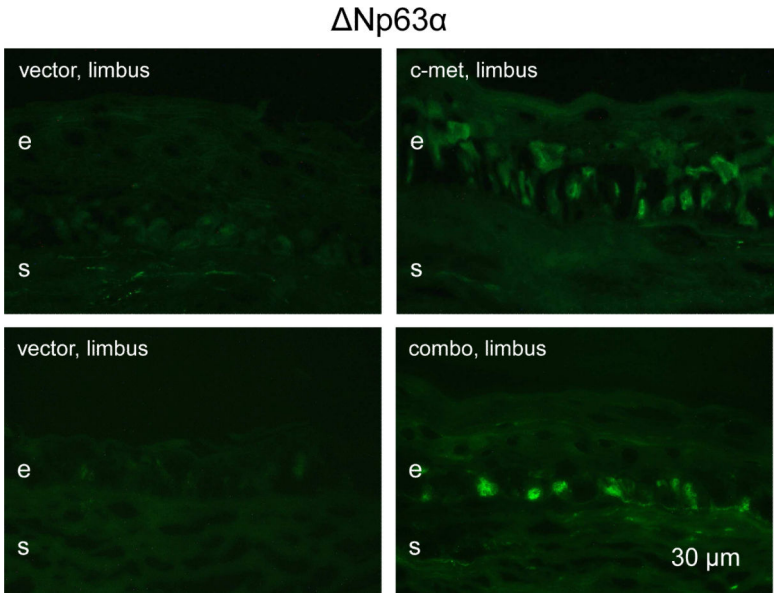

Figure 7.

Increased expression of putative stem cell marker ΔNp63α following limbal gene therapy. The number of positive cells in the limbus and staining intensity are markedly increased upon both treatments. e, epithelium, s, stroma. Bar = 30 μm.

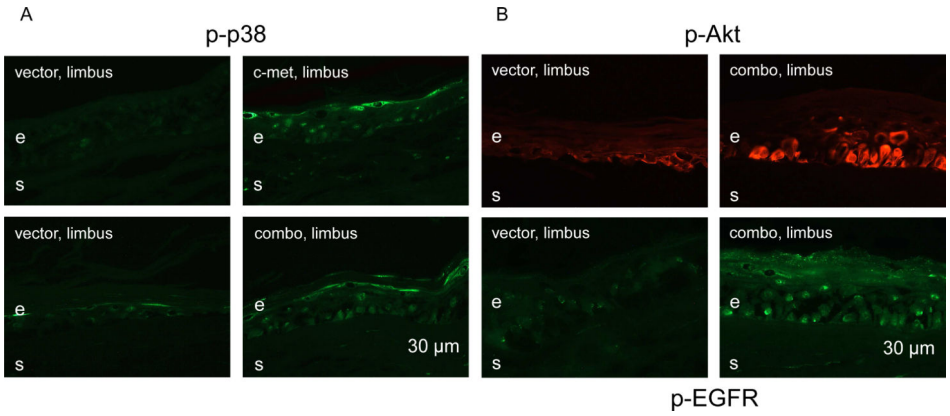

Figure 8.

Increased limbal expression of signaling intermediates after gene therapy. A, limbal p-p38 staining (intensity and number of positive cells) is markedly enhanced upon both treatments. B, limbal staining for p-EGFR and p-Akt is dramatically increased following combo treatment. e, epithelium, s, stroma. Bar = 30 μm.

TABLE 1

Donor Characteristics

Case number	Age, years	Sex	DM type	Duration, years	Cause of death	Culture treatment
DR 11-20	62	M	NIDDM	10	Congestive heart failure	*c-Met*
DM 11-21	73	M	NIDDM	10	Pulmonary edema	*c-Met*
DM 11-28	71	F	NIDDM	8	Myocardial infarction	*c-Met*
DM 11-29	84	M	NIDDM	30	Congestive heart failure	*c-Met*
DM 12-19	79	M	NIDDM	Over 10	Hyperkalemic cardiac arrest	Combo
DM 12-21	84	F	NIDDM	20	Alzheimer's disease	Combo
DM 12-22	60	M	NIDDM	10	Cardiovascular disease	Combo
DM 12-23	71	F	NIDDM → IDDM	14 → 1	Anoxic encephalopathy	Combo

Combo, sh-cathepsin F + sh-MMP-10 + *c-met*. Arrows denote the number of years with a particular type of diabetes.

TABLE 2

Antibodies Used in the Study

Antigen	Antibody	Source	Dilution
MMP-10	Goat pAb sc-9941	Santa Cruz Biotechnology (Dallas, TX)	1:10
Cathepsin F	Goat pAb AF2075	R&D Systems (Minneapolis, MN)	1:25 of 0.5 mg/ml stock
c-Met	Goat pAb sc-161	Santa Cruz Biotechnology	1:10
Keratin 14	Mouse mAb NB100-65109	Novus Biologicals (Littleton, CO)	Undiluted
Keratin 15	Mouse mAb sc-47697	Santa Cruz Biotechnology	1:10
Keratin 17	Mouse mAb sc-58726	Santa Cruz Biotechnology	Undiluted
ANp63	Goat pAb sc-8609	Santa Cruz Biotechnology	1:200
Fibronectin	Mouse mAb 618	Ugarova et al. 1996	Undiluted
Laminin γ3 chain	Rabbit pAb R96	Saghizadeh et al. 2011	1:500
Nidogen-1	Mouse mAb MAB2570	R&D Systems	1:50
Integrin $\alpha_3\beta_1$	Mouse mAb MAB1992	EMD Millipore (Billerica, MA)	1:50
p-EGFR (Tyr845)	Rabbit pAb 44-784G	Thermo Fisher Scientific (Waltham, MA)	1:50
p-Akt (Ser473)	Rabbit pAb 9271	Cell Signaling Technology (Danvers, MA)	1:50
p-p38 (Thr180/Tyr182)	Rabbit pAb 05-1059	EMD Millipore	Undiluted

mAb, monoclonal antibody; pAb, polyclonal antibody. Undiluted antibodies refer to straight hybridoma supernatants.