

Alterations of epithelial stem cell marker patterns in human diabetic corneas and effects of *c-met* gene therapy

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Purpose: We have previously identified specific epithelial proteins with altered expression in human diabetic central corneas. Decreased hepatocyte growth factor receptor (*c-met*) and increased proteinases were functionally implicated in the changes of these proteins in diabetes. The present study examined whether limbal stem cell marker patterns were altered in diabetic corneas and whether *c-met* gene overexpression could normalize these patterns.

Methods: Cryostat sections of 28 ex vivo and 26 organ-cultured autopsy human normal and diabetic corneas were examined by immunohistochemistry using antibodies to putative limbal stem cell markers including ATP-binding cassette sub-family G member 2 (ABCG2), N-cadherin, Δ Np63 α , tenascin-C, laminin γ 3 chain, keratins (K) K15, K17, K19, β 1 integrin, vimentin, frizzled 7, and fibronectin. Organ-cultured diabetic corneas were studied upon transduction with adenovirus harboring *c-met* gene.

Results: Immunostaining for ABCG2, N-cadherin, Δ Np63 α , K15, K17, K19, and β 1 integrin, was significantly decreased in the stem cell-harboring diabetic limbal basal epithelium either by intensity or the number of positive cells. Basement membrane components, laminin γ 3 chain, and fibronectin (but not tenascin-C) also showed a significant reduction in the ex vivo diabetic limbus. *c-Met* gene transduction, which normalizes diabetic marker expression and epithelial wound healing, was accompanied by increased limbal epithelial staining for K17, K19, Δ Np63 α , and a diabetic marker α 3 β 1 integrin, compared to vector-transduced corneas.

Conclusions: The data suggest that limbal stem cell compartment is altered in long-term diabetes. Gene therapy, such as with *c-met* overexpression, could be able to restore normal function to diabetic corneal epithelial stem cells.

In pathological conditions, such as diabetes mellitus, the cornea is significantly affected and this can cause visual impairment. The most recognized diabetic complications in the cornea include neurotrophic corneal ulcers, filamentous keratitis, loss of corneal sensation, and a characteristic epithelial keratodystrophy, which is referred to as diabetic keratopathy [1-9]. Diabetic cornea exhibits basement membrane abnormalities, reduced numbers of hemidesmosomes, altered growth factor content and signaling, epithelial cellular enlargement, edema, and delayed wound healing resulting in persistent epithelial defects [2-4, 8-11]. Treatment for diabetic keratopathy remains symptomatic [2].

Corneal epithelial renewal and healing of epithelial wounds largely depend on corneal stem cells that, at least in humans, reside in the basal epithelial layer of the corneoscleral junction, limbus [12-21]. These cells represent less than 10%

of the total limbal basal epithelial cell population [22,23]. Deficiencies of or damage to these limbal epithelial stem cells (LESC) have serious implications for corneal function such as in-growth of conjunctival cells and neovascularization of the corneal stroma, which eventually lead to corneal opacity and vision loss [20,24-26]. These cells have a high capacity for self-renewal, which is retained throughout life. Corneal maintenance depends on LESCs as a source of epithelial proliferation and rapid renewal through generation of transient amplifying (TA) cells, which in turn differentiate into epithelial cells during their centripetal movement [21, 27-29].

Because of its role in epithelial renewal and wound healing, deficiency of the limbal niche and its residing LESCs may be responsible for abnormalities in diabetic corneal epithelium. In the present paper we examined various putative stem cell markers in ex vivo diabetic and normal epithelial limbal compartment, as well as in organ-cultured diabetic corneas upon overexpression of *c-met* proto-oncogene shown to normalize wound healing time and epithelial marker expression [30]. Immunostaining patterns of several putative stem cell markers were altered in the diabetic limbus, and some of these patterns could be normalized by *c-met*

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TABLE 1. DONOR CHARACTERISTICS.

Case number	Diabetes type	Age, sex	Diabetes duration, years	Cause of death
Ex vivo normal				
95-12	-	17, F	-	gunshot wound
95-15	-	59, M	-	cardiac arrest
95-44	-	68, M	-	massive hemorrhage
99-19	-	79, F	-	respiratory arrest
99-61	-	80, F	-	myocardial infarction
03-3	-	71, M	-	myocardial infarction
03-09	-	72, M	-	lung cancer
05-16	-	56, M	-	ruptured aortic dissection
05-25	-	60, F	-	COPD
05-26	-	60, F	-	COPD
05-45	-	10, M	-	exsanguination
05-46	-	10, M	-	exsanguination
05-56	-	45, M	-	cardiovascular accident
05-60	-	65, F	-	pneumonia
10-03	-	69, M	-	respiratory failure
Ex vivo diabetic				
95-17	IDDM	79, M	5	cardiac arrest
95-18	IDDM	79, M	5	cardiac arrest
96-06	IDDM	69, M	22	acute cardiac event
96-30	NIDDM	77, F	>5	cardiorespiratory arrest
96-46	IDDM, PDR	68, F	>48	cardiorespiratory arrest
96-47	IDDM, PDR	68, F	>48	cardiorespiratory arrest
96-95	IDDM	63, M	3	cardiovascular accident
96-96	IDDM	63, M	3	cardiovascular accident
99-08	IDDM, DR	67, F	30	myocardial infarction
99-79	IDDM, PDR	77, F	unknown	cardiac arrest
99-80	IDDM, PDR	77, F	unknown	cardiac arrest
01-47	IDDM, DR	64, F	unknown	ventricular arrhythmia
06-26	NIDDM	77, M	15	intracerebral hemorrhage
Organ-cultured diabetic				
07-27	IDDM	81, M	>10	acute renal failure
07-32	NIDDM	84, M	20	stroke
07-34	IDDM, DR	37, M	22	intracranial hemorrhage
08-35	IDDM, DR	88, M	38	cardiac arrest
08-36	NIDDM	82, M	15	stroke
08-38	IDDM	73 F	>10	diabetic ketoacidosis
08-40	NIDDM	59, M	20	cardiac arrest
08-44	IDDM, DR	71, M	15	cardiopulmonary arrest
08-49	NIDDM	59, F	28	intracranial hemorrhage
08-54	NIDDM	57, M	10	myocardial infarction
08-57	IDDM	78, F	15	respiratory failure
09-12	NIDDM, DR	61, F	unknown	myocardial infarction
09-16	NIDDM	61, F	>10	congestive heart failure

M, male; F, female; PDR, proliferative diabetic retinopathy; COPD, chronic obstructive pulmonary disease. In the ex vivo groups, each case number refers to one cornea; in organ-cultured diabetic group, each case number refers to a pair of fellow corneas.

TABLE 2. ANTIBODIES USED IN THE STUDY.

Antigen	Antibody	Source	Dilution	Immunostaining
ABCG2	Mouse mAb MAB4155	Millipore	1:50	-
ABCG2	Mouse mAb sc-58222	Santa Cruz Biotechnology	1:5	+
ABCG2	Rabbit pAb sc-25821	Santa Cruz Biotechnology	1:20	+
C/EBP δ	Rabbit pAb sc-636	Santa Cruz Biotechnology	1:20	-
Fibronectin	Mouse mAb 568	[72]	1:60	+
Integrin β 1	Mouse mAb MAB1959	Millipore	1:50	+
Integrin β 1	Mouse mAb MAB2000	Millipore	1:50	+
Keratin 15	Mouse mAb sc-47697	Santa Cruz Biotechnology	1:10	+
Keratin 17	Mouse mAb E3	[73]	straight	+
Keratin 17	Rabbit mAb #4543	Cell Signaling	1:50	+
Keratin 19	Mouse mAb MAB1607	Millipore	1:10	+
Keratin 19	Mouse mAb MA1-35554	Thermo Scientific	1:10	+
Keratin 19	Rabbit pAb PA1-38014	Thermo Scientific	1:20	+
Keratin 19	Mouse mAb MAB1608	Millipore	1:10	+
Laminin β 1	Rat mAb LT3	[74]	straight	+
Laminin β 2	Mouse mAb C4	Developmental Hybridoma Bank	straight	+
Laminin γ 1	Rat mAb A5	[75]	straight	+
Laminin γ 3	Rabbit pAb R96	[76]	1:500	+
Laminin γ 3	Rabbit pAb sc-25719	Santa Cruz Biotechnology	1:20	-
Laminin γ 3	Goat pAb sc-16601	Santa Cruz Biotechnology	1:10	+
N-Cadherin	Mouse mAb 3B9	Invitrogen	1:20	+
N-Cadherin	Rabbit pAb 12221	Abcam	1:50	-
N-Cadherin	Rabbit pAb sc-7939	Santa Cruz Biotechnology	1:20	+
Nidogen-1	Mouse mAb MAB2570	R&D Systems	1:50	+
Nidogen-2	Rabbit pAb 1080	[77]	1:200	+
Nidogen-2	Goat pAb sc-26132	Santa Cruz Biotechnology	1:25	-
Nidogen-2	Goat pAb sc-26133	Santa Cruz Biotechnology	1:25	-
Δ Np63	Goat pAb sc-8609	Santa Cruz Biotechnology	1:20	+
Δ Np63	Rabbit pAb 619001	Biologend	1:50	-
P63 α	Rabbit pAb	[23]	1:100	+
SOD2	Rabbit pAb sc-30080	Santa Cruz Biotechnology	1:10	+
Vimentin	Goat pAb sc-7558	Santa Cruz Biotechnology	1:20	+
Frizzled 7	Rat mAb 1981	R&D Systems	1:20	+
Tenascin-C	Mouse mAb BC2	[78]	straight	+

mAb, monoclonal antibody; pAb, polyclonal antibody.

overexpression. The data suggest that limbal compartment may play an important role in diabetic corneal alterations that can be corrected by gene therapy.

METHODS

Tissues: Age-matched normal, diabetic (with insulin-dependent [IDDM] or non-insulin-dependent [NIDDM] diabetes), and diabetic retinopathy (DR) autopsy human corneas were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA), within 24 (for ex vivo) to 48 h after death. NDRI has a human tissue collection protocol approved by a managerial committee and subject to National Institutes of Health oversight. In this study (Table 1), 15 normal (from 13 donors, mean age 57.8 \pm 21.8 years) and 13 diabetic (from 9 donors; mean age 71.2 \pm 6.3 years; 7 with IDDM, 2 with NIDDM, 4 with DR) ex vivo corneas, as well as 13 pairs of organ-cultured diabetic corneas (from 13 donors; mean age 68.5 \pm 14.4 years; 6 with IDDM, 7 with NIDDM, 4 with DR) were used. Mean ages in all groups as well as mean disease durations for known cases in ex vivo and organ culture diabetic groups did not differ significantly. The corneas were embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA)

and stored at -80°C for immunohistochemistry, or were processed for organ culture.

Corneal organ culture and viral transduction: As described previously [30,31], after filling the corneal concavity with warm agar-collagen mixture, corneas were cultured in serum-free medium with insulin-transferrin-selenite, antibiotics and antimycotic (Invitrogen, Carlsbad, CA), at a liquid-air interface with epithelium facing upwards. Organ-cultured diabetic corneas were transduced for 48 h with 1.0–2.0 $\times 10^8$ plaque-forming units of recombinant adenoviruses, rAV-cmet (harboring full-length *c-met* open reading frame) and the fellow corneas with rAV-vector (no gene inserted) as a control. Seventy-five $\mu\text{g/ml}$ of sterile sildenafil citrate (Viagra $^{\text{®}}$; Pfizer Corp., New York, NY) was added to the culture medium along with the viruses to increase rAV transduction efficiency [31]. Some transduced corneas were processed after 7–10 days in culture, some after wound healing experiments [30]. They were embedded in OCT and 5 μm cryostat sections cut for immunostaining on a Leica CM1850 cryostat (McBain Instruments, Chatsworth, CA).

Immunohistochemistry: The list of primary antibodies to putative stem cell markers is presented in Table 2. Different

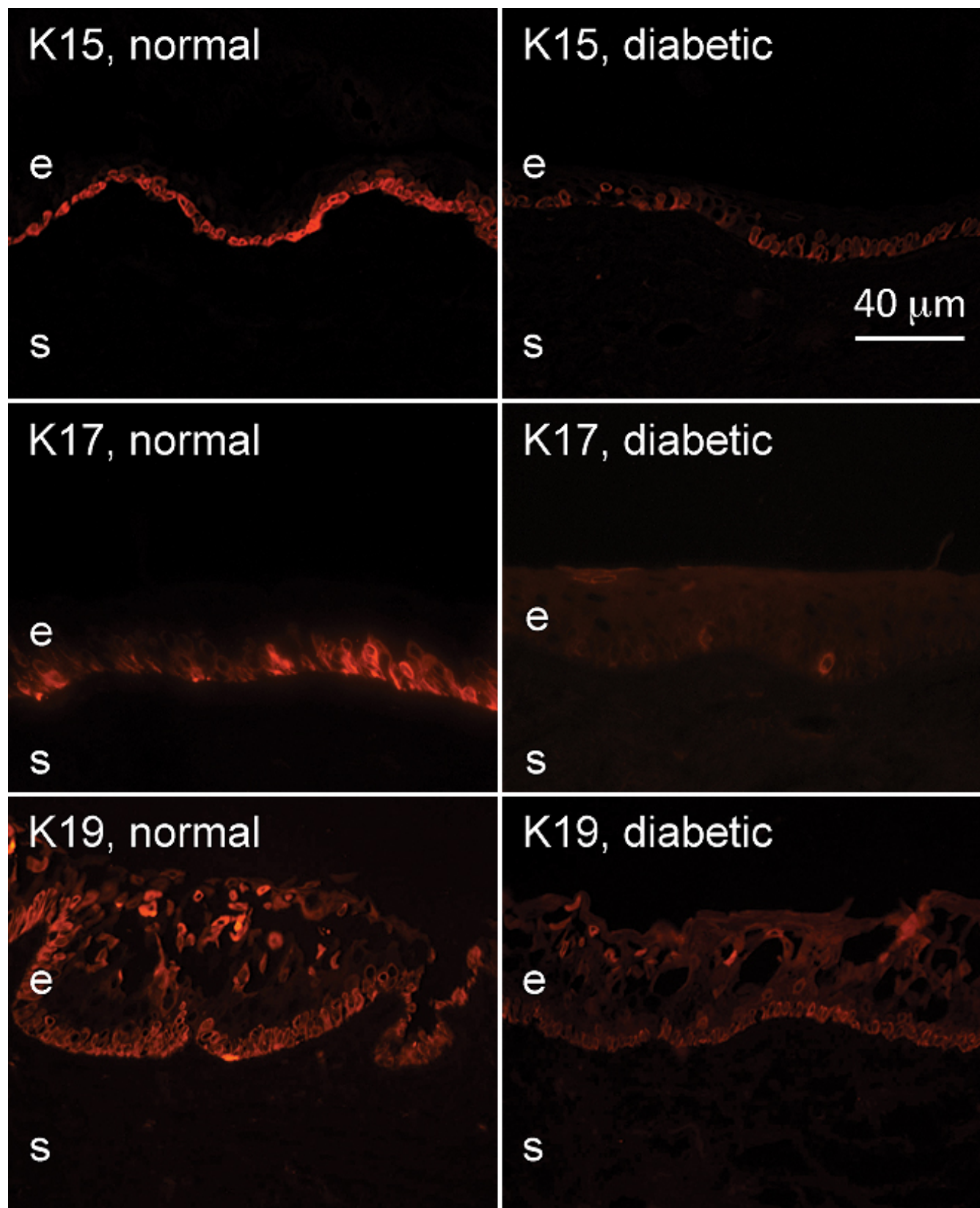


Figure 1. Keratin expression patterns in normal and diabetic ex vivo limbus. The staining intensity of K15, K17, and K19 was significantly decreased in the diabetic limbus. Note a reduction of K17-positive cells in the diabetic limbus as well. Here and in all other figures, each normal and diabetic pair was photographed at the same exposure times in the same staining experiments. e, epithelium, s, stroma. Bar=40 μm.

fixations such as 100% acetone at -20°C for 10 min, 100% methanol at -20°C for 10 min, 1% formalin (0.37% formaldehyde) in saline at room temperature for 5 min were used for different antibodies. For each marker the same exposure time was used when photographing stained sections of fellow corneas using a MicroFire digital camera (Optronics, Goleta, CA) attached to an Olympus BX40 microscope (Olympus USA, Melville, NY) and operated using PictureFrame software. Negative controls without a primary antibody were included in each experiment.

Statistics: Immunostaining results were analyzed by unpaired (for ex vivo corneas; $n=15$ for normal, $n=13$ for diabetic) or paired (for organ cultured corneas; $n=13$ pairs) Student's *t*-test (InStat, GraphPad Software, San Diego, CA). Staining intensity was scored arbitrarily as: 0 (negative), 0.5 (weak), 1 (distinct), 2 (moderate), 3 (strong), 4 (very strong). Most cases were stained at least twice with good reproducibility between experiments, and a mean intensity score from independent experiments was used for each case. The mean scores between groups (e.g., normal versus diabetic) were then compared. A *p*-value <0.05 was considered significant. Data are expressed as mean \pm standard error (SEM).

RESULTS

Distribution of putative stem cell markers in normal and diabetic ex vivo corneas: The immunostaining patterns of several limbal and/or putative stem cell markers were altered in the ex vivo diabetic limbus compared to the normal one. Figure 1, left column, shows the normal staining patterns of cytoskeletal structural proteins of epithelial cells, keratins 15, 17, and 19 (K15, K17 and K19). These three keratins were expressed in limbal compartment but not in central ex vivo corneal epithelium, in good agreement with previous data [17,32-34]. K15 and K19 were prominently expressed in the basal limbal cells and in lesser amounts in the suprabasal and superficial layers of the limbal epithelium (Figure 1). K17 protein was usually found in clusters of limbal basal cells (Figure 1), as was vimentin (not shown here). As shown in Figure 1, right column, in the diabetic limbus staining for all three keratins decreased by both intensity and number of positive cells; the latter was most pronounced for K17. Decreased staining for these keratins in the diabetic limbus reached significance. The most commonly used putative LESC markers [20,23,35-37], ATP-binding cassette transporter Bcrp1/ABCG2 and transcription factor $\Delta\text{Np}63\alpha$ isoform, were also significantly decreased in the diabetic limbus (Figure 2 and Figure 3). The same was true for another putative LESC marker [38], N-cadherin (Figure 3). β_1 Integrin [39] that is found in basal cells as well as the suprabasal and superficial layers of the corneal epithelium was expressed much less in the diabetic limbus (Figure 4), although its localization did not agree with the presence in LESC niche only. In the diabetic limbus, reduced and discontinuous immunostaining was observed for select ECM markers, such

as laminin $\gamma 3$ chain (Figure 4), expressed mostly in the limbal basement membrane [40,41]. Fibronectin staining was also significantly decreased in the diabetic limbal basement membrane (Figure 4). No significant changes were observed in the diabetic limbal cells for total tenascin-C (Figure 3), a ubiquitous laminin $\gamma 1$ chain, and putative LESC markers superoxide dismutase 2 (SOD2), vimentin, and a Wnt receptor, frizzled 7 (data not shown).

Normalization of putative stem cell marker patterns upon c-met overexpression: We have recently shown that the expression of certain markers altered in the ex vivo diabetic corneas including integrin $\alpha_3\beta_1$, some laminin chains, nidogen-1, nidogen-2, and phosphorylated p38 MAP kinase (p-p38) returned to almost normal patterns in the central region of diabetic organ-cultured corneas after c-met overexpression using rAV-driven c-met transduction [30]. As shown in Figure 5, c-met transduction brought about an increase in staining for some of these markers, such as integrin $\alpha_3\beta_1$ and p-p38, so that they became closer to normal (see [30]) in the diabetic limbus. We next examined if altered expressions of putative LESC markers could also be normalized by rAV-cmet transduction of diabetic organ-cultured corneas. Indeed, c-met overexpression was accompanied by increased limbal staining for K15 (did not reach significance), K17, and K19, as well as $\Delta\text{Np}63\alpha$ isoform compared to vector-transduced corneas (Figure 6; compare with Figure 1 and Figure 2), so that the staining became similar to normal corneas. Some of these increases were significant (Figure 7), although certain markers did not show an appreciable change in staining intensity. It should be mentioned that in organ-cultured diabetic corneas some K17 and K19 immunostaining could also be found in the suprabasal layers of the limbal epithelium as well as in the central cornea, whereas K15 was still expressed exclusively in the limbus.

DISCUSSION

As a systemic disease, diabetes has significant impact on all tissues. In the eye, the major vision-threatening effect is on retina (DR), whereas the other ocular parts are generally thought to be less affected [42,43]. However, more than a half of diabetics suffer from corneal problems related to neuropathy and epitheliopathy [44]. Various epithelial abnormalities are present in diabetic corneas that appear to be related to cell adhesion and basement membrane alterations, decreased innervation, and poor wound healing [44-46]. Using adenoviral gene therapy with overexpression of c-met that is downregulated in diabetic corneas [47], we were able to bring basement membrane protein patterns and wound healing times in organ-cultured diabetic corneas close to normal [30]. Other possible ways to normalize these corneas could be a restoration of normal signaling of the epidermal growth factor receptor (EGFR) axis that is downregulated by high glucose and diabetes [48,49], silencing of specific

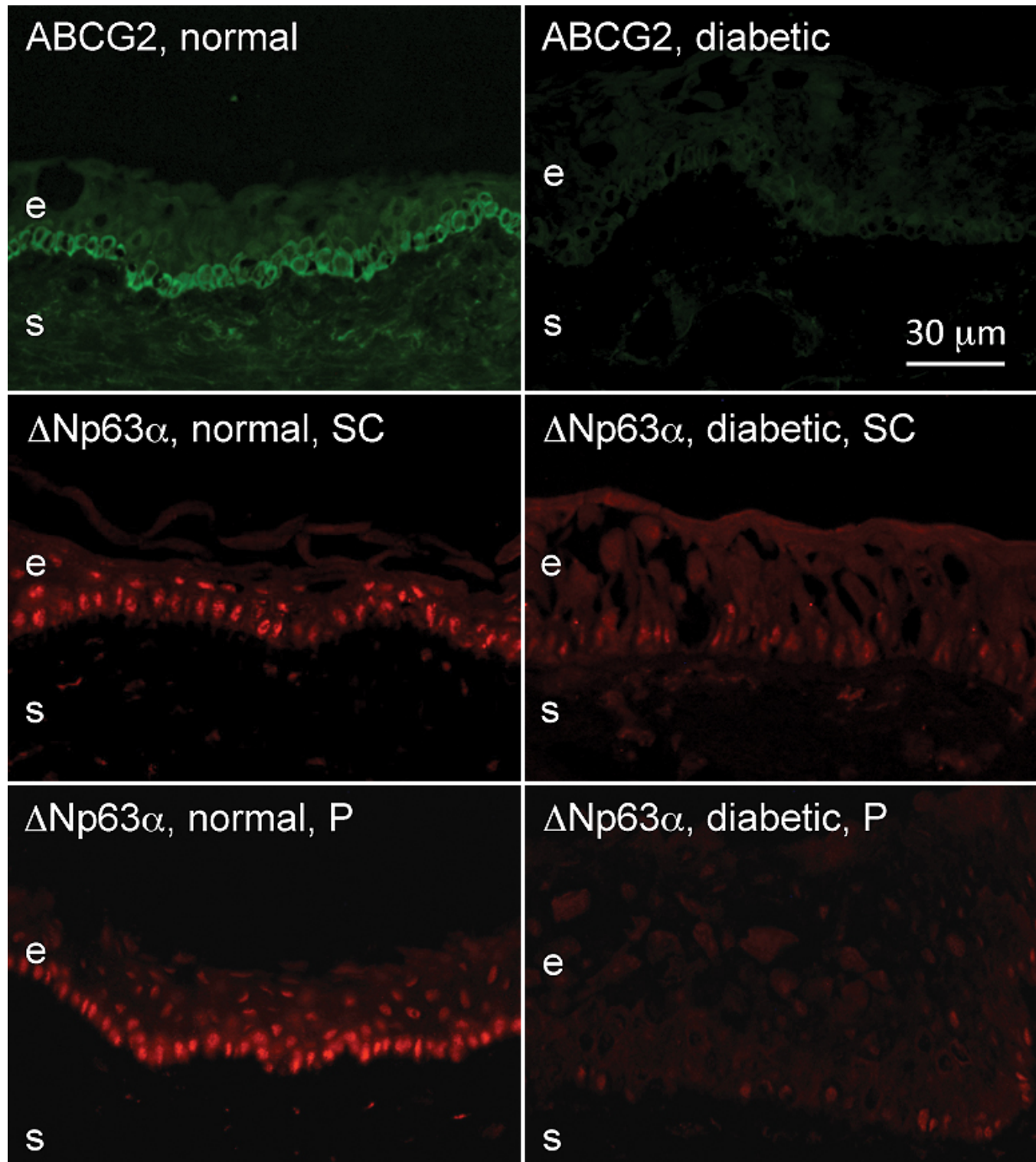


Figure 2. Putative LESC marker expression patterns in normal and diabetic ex vivo limbus. Note a dramatic decrease in staining intensity and the number of positive basal epithelial cells for ABCG2 and $\Delta Np63\alpha$ in the diabetic limbus. $\Delta Np63\alpha$ was revealed with two different antibodies (Santa Cruz, SC) and Pellegrini (P) with the same result. e, epithelium, s, stroma. Bar=30 μm .

proteinases [31] or a blockade of opioid growth factor - opioid growth factor receptor system with naltrexone [50].

Mechanisms responsible for the epithelial changes in diabetic corneas are still not well understood. One possibility

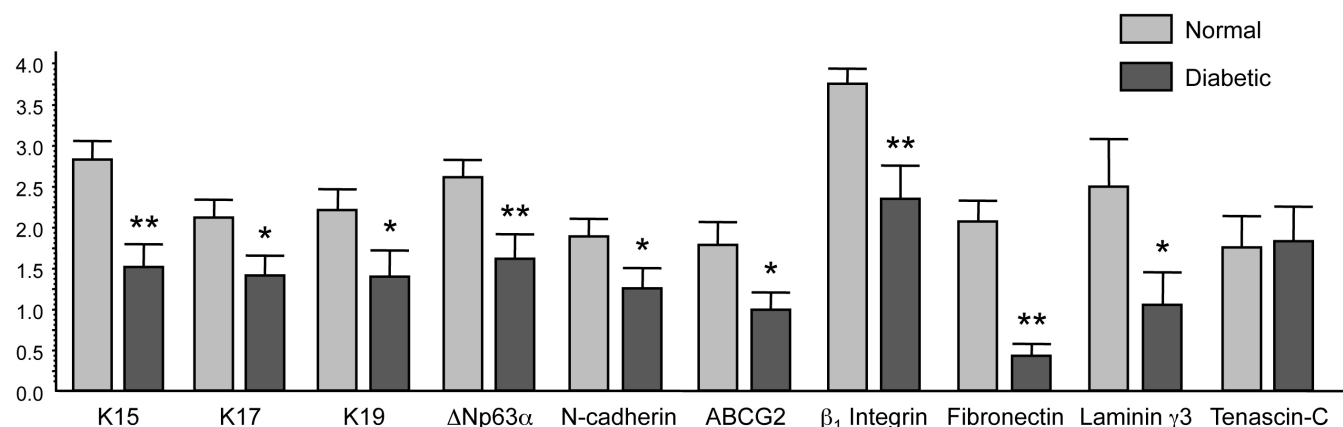


Figure 3. Statistical analysis of changes in the staining for various markers in diabetic versus normal ex vivo limbus. Significant staining decrease was observed for K15, K17, K19, ΔNp63α, N-cadherin, ABCG2, fibronectin, β₁ integrin, and laminin γ3 chain. Data are mean±SEM. Normal, n=15; diabetic, n=13. *p<0.05; **p<0.01. Details are in the Methods section.

is that epithelial alterations could be triggered or exacerbated by abnormal innervation, which may be the case with ulcers [51]. However, several lines of evidence support direct detrimental action of hyperglycemia in diabetes on corneal epithelium. In diabetic rabbits, corneal wound healing is not delayed [52], although corneal neuropathy develops [53]. In human corneal organ culture where corneas are denervated, delayed diabetic epithelial wound healing persists [30]. In normal organ-cultured porcine corneas, high glucose causes delayed epithelial wound healing [48]. Because LESC and their immediate progeny (TA cells) play a key role in the epithelial maintenance and renewal, these data support the hypothesis that LESC and/or TA cells may be altered in the course of diabetes. Using a large panel of antibodies to putative LESC/limbal basal epithelial markers we tested this hypothesis using ex vivo human corneas, as well as organ-cultured diabetic corneas upon viral-induced *c-met* overexpression.

In the ex vivo corneas, many tested markers including keratins 15, 17 and 19, as well as Bcrp1/ABCG2, ΔNp63α isoform, N-cadherin, laminin γ3 chain, and β₁ integrin were significantly downregulated in diabetic compared to normal limbus. In some cases, such as with integrin β₁ or laminin γ3 chain, the immunostaining intensity was diminished. In other cases, such as with K17, ABCG2, or N-cadherin, the number of positive cells was markedly reduced in the diabetic limbus. This is the first demonstration of changes in LESC marker expression in a common disease that does not involve LESC deficiency. It may be suggested that the observed differences in marker expression between normal and diabetic corneal limbus relate to functional abnormalities of stem cell niche in diabetes. At present one can only speculate on what kind of dysfunction such reduced marker expression would be related. A plausible candidate would be impaired cell migration translating into slower and incomplete wound healing in diabetic corneas. The data support the idea that stem cell niche

alterations may underlie poor wound healing and other epithelial abnormalities typical for diabetic corneas. It would also be important to understand whether diabetes changes marker expression in LESC, TA cells or both. The generally even distribution of most studied putative LESC markers in the limbal basal cells (comprised by LESC and TA cells) would favor the hypothesis that reduced expression of these markers in diabetic corneas is applicable to both LESC and TA cells.

It was interesting to examine whether gene therapy that can bring diabetic corneas closer to normal in terms of specific protein expression and wound healing rates would also change the expression of putative LESC markers in diabetic corneas toward normal patterns. To this end, we used organ-cultured diabetic corneas following gene therapy with *c-met*, which had significantly improved epithelial wound healing and expression of basement membrane markers and signaling intermediates [30].

Compared to vector treatment, *c-met* treated corneas displayed enhanced staining for several putative LESC markers, which became similar to normal ex vivo limbus. These data attest to the feasibility of using specific gene therapy to normalize the functions of LESC in diabetic corneas, which may be useful for LESC transplantation in diabetics. However, not all the markers altered in diabetic ex vivo corneas showed increased staining upon *c-met* gene transduction (e.g., β₁ integrin) suggesting that one-gene therapy was not enough for corneal normalization.

A partial effect of *c-met* upregulation on LESC marker expression could be related to the influence of this gene therapy only on certain cell signaling pathways. As we showed before [30], *c-met* overexpression in diabetic corneas causes normalization of epithelial wound healing by restoring signaling through p38. However, overexpression of proteinases cathepsin F and matrix metalloproteinase-10 (MMP-10) in diabetic corneas or incubation of normal

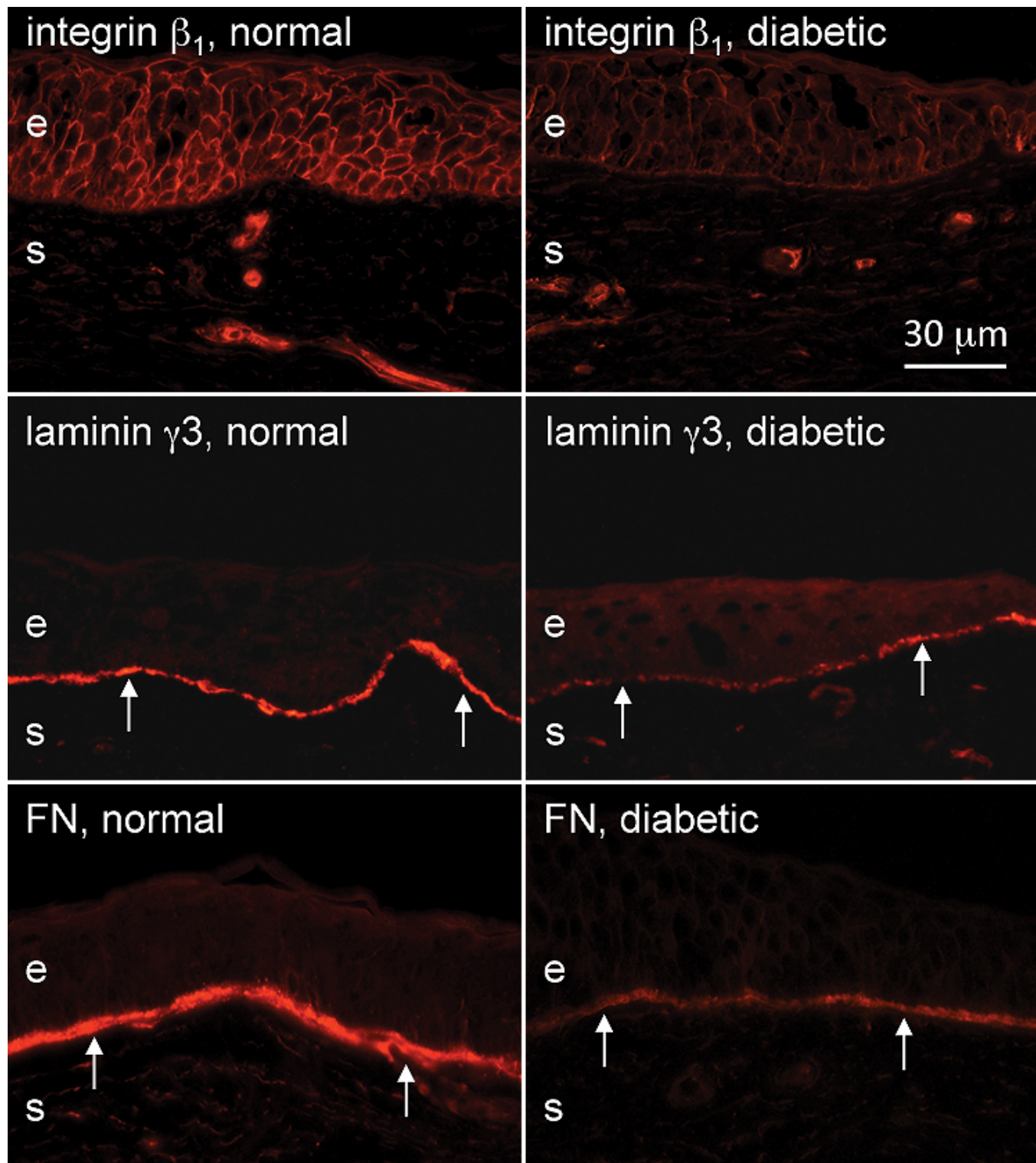


Figure 4. Integrin and basement membrane protein expression patterns in normal and diabetic ex vivo limbus. Integrin β_1 staining is markedly reduced in the diabetic limbus, which occurs in all epithelial layers. A limbal-specific laminin γ_3 chain staining is weak and discontinuous in the diabetic limbal epithelial basement membrane (arrows). This is also true for fibronectin. e, epithelium, s, stroma. Bar=30 μm .

corneas in high glucose appear to reduce migration-promoting EGFR signaling through Akt phosphorylation [31,48]. Our

preliminary data showed that a combined gene therapy with c-met overexpression and shRNA silencing of cathepsin F and

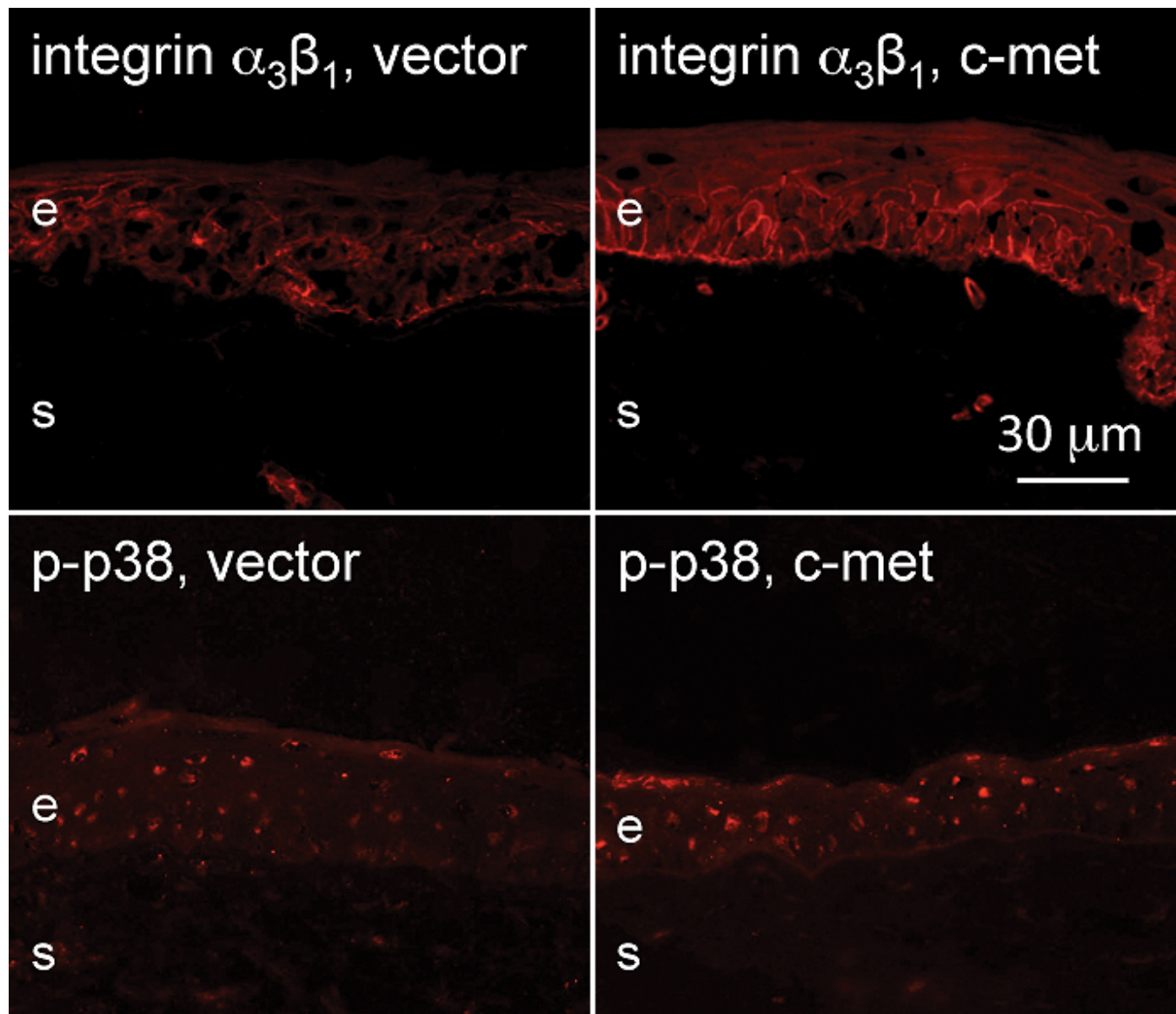


Figure 5. Increased diabetic marker expression in the diabetic limbus in organ culture upon *c-met* overexpression. Both integrin $\alpha_3\beta_1$ and p-p38 staining in the limbal epithelium is increased upon *c-met* gene transduction and becomes similar to normal. e, epithelium, s, stroma. Bar=30 μm .

MMP-10 brought diabetic corneas significantly closer to normal in terms of epithelial protein expression, p38 and Akt phosphorylation, and wound healing time than *c-met* upregulation alone. Therefore, by a concerted acting on several key signaling pathways, this combination could possibly exert a greater positive effect on putative LESC marker expression in the diabetic limbus.

Based on limbal location, little or no expression in the central cornea, preferential expression in basal limbal epithelial cells, various putative LESC markers have been proposed, such as K8, K15, K17, K19, Bcrp1/ABCG2, $\Delta\text{Np63}\alpha$, N-cadherin, laminin γ_3 chain, β_1 integrin, TCF4, frizzled 7, SOD2, epiregulin, Notch-1, α -enolase, vimentin,

C/EBP δ , SPON1, and nectin-3 [23,32,33,35,36,41,54-65]. However, despite numerous attempts, no single and reliable LESC marker has been identified so far. This is in part due to the fact that unambiguous identification of LESC has been difficult. These cells are generally considered as largely non-proliferating, or slow cycling. Based on this criterion many authors agree that if corneal cultures or animal corneas in vivo are labeled with tritiated thymidine or bromodeoxyuridine and then chased for a while (at least several weeks), the few corneal cells that retain the label should be considered LESC. This promising strategy has been used to examine which markers are expressed by these cells. They were found to stain for K14, K15, CDH3 (P-cadherin), Wnt-4 [61], as well as to

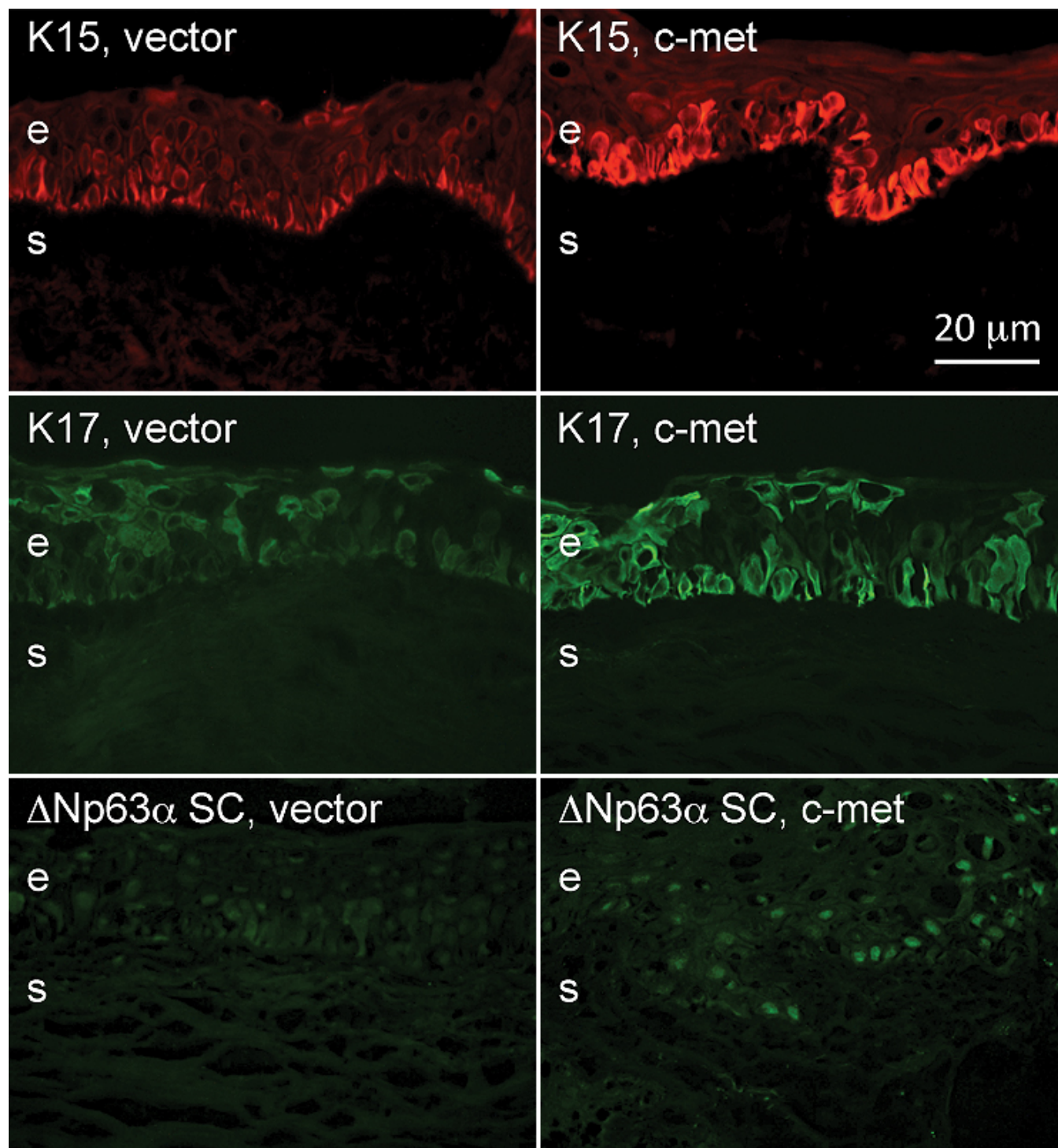


Figure 6. Increased putative LESC marker expression in the diabetic limbus in organ culture upon *c-met* overexpression. *c-Met* gene transduction leads to elevated expression of K15, K17, and Δ Np63 α in the limbus of organ-cultured diabetic corneas. The staining intensity and regularity appear more normal (compare with Figure 1 and Figure 2). Note that in organ cultures keratins (especially K17) can also be seen in suprabasal epithelial layers. e, epithelium, s, stroma. Bar=20 μ m.

contain high levels of integrins β_1 and β_4 [60]. However, these markers are expressed not only in putative LESC but also in other limbal cells, as well as in central corneal cells (e.g., both

integrins) and thus cannot be considered specific for LESC. Additional experiments with label-retaining cells using a large panel of antibodies are definitely needed to establish which

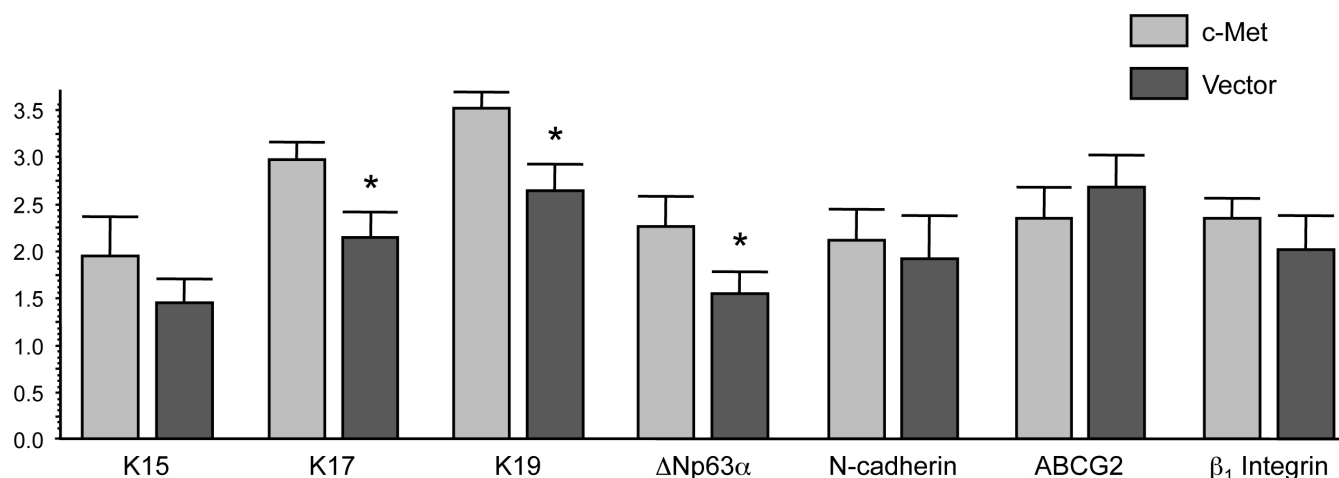


Figure 7. Statistical analysis of changes in the staining for various markers in the diabetic limbus in organ culture upon c-met overexpression. Significant staining increase after *c-met* gene transduction was observed for K17, K19, and Δ Np63 α . Changes in the expression levels of K15, N-cadherin and β_1 integrin did not reach significance. Data are mean \pm SEM. Thirteen pairs of c-met or vector treated organ cultured diabetic corneas were used. * p <0.05. Details are in the Methods section.

existing markers are more specific for LESC. Currently, it is generally agreed that a combination of several markers should be used to characterize the presence of LESC in tissues and cultures.

The problem of LESC markers has gained wide attention because of recent success in transplantation of cultured limbal epithelium to patients with LESC deficiency [26,66-71]. Unfortunately, not all such cultures, especially when only small amounts of biopsied tissue were available for autologous transplantation, have been characterized as to the expression of putative LESC markers. Some authors, however, to standardize the cultures for successful transplantation, did examine one to several markers, e.g., p63 and K19 [69,70], confirming the presence of LESC-like cells in the transplanted cultures. In line with low content of LESC in corneal tissue, successful transplantations could be achieved when the fraction of p63-positive cells exceeded 3% [70].

In summary, we provide here the first account of significant alterations of limbal stem cell compartment in human diabetic corneas with respect to several commonly used putative LESC markers. These abnormalities may lead to diabetic LESC dysfunction and to clinically observed epithelial problems in diabetics including poor wound healing. Partial normalization of these pathological changes by c-met overexpression may offer a possibility of improving LESC function and general corneal health in diabetes by specific gene therapy. Another promising approach could be autologous transplantation of limbal epithelial cells to diabetic patients with advanced disease after prior normalization of their marker expression levels by gene therapy during culture.

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REFERENCES

- Herse PR. A review of manifestations of diabetes mellitus in the anterior eye and cornea. *Am J Optom Physiol Opt* 1988; 65:224-30. [PMID: 3284372]
- Cavallerano J. Ocular manifestations of diabetes mellitus. *Optom Clin* 1992; 2:93-116. [PMID: 1504481]
- Saini JS, Khandalavla B. Corneal epithelial fragility in diabetes mellitus. *Can J Ophthalmol* 1995; 30:142-6. [PMID: 7627899]
- Sánchez-Thorin JC. The cornea in diabetes mellitus. *Int Ophthalmol Clin* 1998; 38:19-36. [PMID: 9604736]
- Malik RA, Kallinikos P, Abbott CA, van Schie CH, Morgan P, Efron N, Boulton AJ. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia* 2003; 46:683-8. [PMID: 12739016]
- Negi A, Vernon SA. An overview of the eye in diabetes. *J R Soc Med* 2003; 96:266-72. [PMID: 12782689]
- Saito J, Enoki M, Hara M, Morishige N, Chikama T, Nishida T. Correlation of corneal sensation, but not of basal or reflex tear secretion, with the stage of diabetic retinopathy. *Cornea* 2003; 22:15-8. [PMID: 12502941]
- Gekka M, Miyata K, Nagai Y, Nemoto S, Sameshima T, Tanabe T, Maruoka S, Nakahara M, Kato S, Amano S. Corneal epithelial barrier function in diabetic patients. *Cornea* 2004; 23:35-7. [PMID: 14701955]
- Wylegała E, Moćko L, Woyna-Orlewicz A, Teper S, Orzechowska-Wylegała B. Diabetic complications within ocular surface. *Pol Merkur Lekarski* 2006; 21:495-7. [PMID: 17345849]

10. Ben Osman N, Jeddi A, Sebai L, Zghal I, Kaouech M, Gaigi S, Ayed S. The cornea of diabetics. *J Fr Ophtalmol* 1995; 18:120-3. [PMID: 7738303]
11. Inoue K, Kato S, Ohara C, Numaga J, Amano S, Oshika T. Ocular and systemic factors relevant to diabetic keratoepitheliopathy. *Cornea* 2001; 20:798-801. [PMID: 11685054]
12. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986; 103:49-62. [PMID: 2424919]
13. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989; 57:201-9. [PMID: 2702690]
14. Tseng SC. Concept and application of limbal stem cells. *Eye (Lond)* 1989; 3:141-57. [PMID: 2695347]
15. Lauweryns B, van den Oord JJ, Missotten L. The transitional zone between limbus and peripheral cornea. An immunohistochemical study. *Invest Ophthalmol Vis Sci* 1993; 34:1991-9. [PMID: 8387976]
16. Majo F, Rochat A, Nicolas M, Jaoudé GA, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature* 2008; 456:250-4. [PMID: 18830243]
17. Secker GA, Daniels JT. Corneal epithelial stem cells: deficiency and regulation. *Stem Cell Rev* 2008; 4:159-68. [PMID: 18622724]
18. Sun TT, Tseng SC, Lavker RM. Location of corneal epithelial stem cells. *Nature* 2010; 463:E10-1. [PMID: 20182462]
19. Castro-Muñozledo F, Gómez-Flores E. Challenges to the study of asymmetric cell division in corneal and limbal epithelia. *Exp Eye Res* 2011; 92:4-9. [PMID: 21056036]
20. Pellegrini G, Rama P, De Luca M. Vision from the right stem. *Trends Mol Med* 2010; 17:1-7. [PMID: 21075055]
21. Di Girolamo N. Stem cells of the human cornea. *Br Med Bull* 2011 [PMID: 21680602]
22. Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005; 89:529-32. [PMID: 15834076]
23. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of $\Delta Np63$ and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA* 2005; 102:9523-8. [PMID: 15983386]
24. Dua HS, Saini JS, Azuara-Blanco A, Gupta P. Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management. *Indian J Ophthalmol* 2000; 48:83-92. [PMID: 11116520]
25. Hatch KM, Dana R. The structure and function of the limbal stem cell and the disease states associated with limbal stem cell deficiency. *Int Ophthalmol Clin* 2009; 49:43-52. [PMID: 19125063]
26. Nakamura T, Kinoshita S. New hopes and strategies for the treatment of severe ocular surface disease. *Curr Opin Ophthalmol* 2011; 22:274-8. [PMID: 21537182]
27. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci* 1983; 24:1442-3. [PMID: 6618809]
28. Wiley L, SundarRaj N, Sun TT, Thoft RA. Regional heterogeneity in human corneal and limbal epithelia: an immunohistochemical evaluation. *Invest Ophthalmol Vis Sci* 1991; 32:594-602. [PMID: 1705924]
29. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 1998; 111:2867-75. [PMID: 9730979]
30. Saghizadeh M, Kramerov AA, Yu FS, Castro MG, Ljubimov AV. Normalization of wound healing and diabetic markers in organ cultured human diabetic corneas by adenoviral delivery of c-met gene. *Invest Ophthalmol Vis Sci* 2010; 51:1970-80. [PMID: 19933191]
31. Saghizadeh M, Kramerov AA, Yaghoobzadeh Y, Hu J, Ljubimova JY, Black KL, Castro MG, Ljubimov AV. Adenovirus-driven overexpression of proteinases in organ-cultured normal human corneas leads to diabetic-like changes. *Brain Res Bull* 2010; 81:262-72. [PMID: 19828126]
32. Yoshida S, Shimmura S, Kawakita T, Miyashita H, Den S, Shimazaki J, Tsubota K. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* 2006; 47:4780-6. [PMID: 17065488]
33. Lyngholm M, Vorum H, Nielsen K, Østergaard M, Honoré B, Ehlers N. Differences in the protein expression in limbal versus central human corneal epithelium - a search for stem cell markers. *Exp Eye Res* 2008; 87:96-105. [PMID: 18571161]
34. Merjava S, Neuwirth A, Tanzerova M, Jirsova K. The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva. *Histol Histopathol* 2011; 26:323-31. [PMID: 21210345]
35. Kawasaki S, Tanioka H, Yamasaki K, Cannon CJ, Kinoshita S. Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. *Exp Eye Res* 2006; 82:293-9. [PMID: 16115622]
36. Kubota M, Shimmura S, Miyashita H, Kawashima M, Kawakita T, Tsubota K. The anti-oxidative role of ABCG2 in corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2010; 51:5617-22. [PMID: 20538997]
37. Echevarria TJ, Di Girolamo N. Tissue-regenerating, vision-restoring corneal epithelial stem cells. *Stem Cell Rev* 2011; 7:256-68. [PMID: 21069583]
38. Hayashi R, Yamato M, Sugiyama H, Sumide T, Yang J, Okano T, Tano Y, Nishida K. N-cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells* 2007; 25:289-96. [PMID: 17008425]
39. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 2004; 22:355-66. [PMID: 15153612]
40. Kabosova A, Azar DT, Bannikov GA, Campbell KP, Durbeej M, Ghohestani RF, Jones JC, Kenney MC, Koch M, Ninomiya Y, Patton BL, Paulsson M, Sado Y, Sage EH, Sasaki T, Sorokin LM, Steiner-Champlaud MF, Sun TT, Sundarraj N, Timpl R, Virtanen I, Ljubimov AV. Compositional differences between infant and adult human corneal basement membranes. *Invest Ophthalmol Vis Sci* 2007; 48:4989-99. [PMID: 17962449]

41. Schlötzer-Schrehardt U, Dietrich T, Saito K, Sorokin L, Sasaki T, Paulsson M, Kruse FE. Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. *Exp Eye Res* 2007; 85:845-60. [PMID: 17927980]
42. Khan ZA, Farhangkhoe H, Chakrabarti S. Towards newer molecular targets for chronic diabetic complications. *Curr Vasc Pharmacol* 2006; 4:45-57. [PMID: 16472176]
43. Jackson GR, Barber AJ. Visual dysfunction associated with diabetic retinopathy. *Curr Diab Rep* 2010; 10:380-4. [PMID: 20632133]
44. Abdelkader H, Patel DV, McGhee CNJ, Alany RG. New therapeutic approaches in the treatment of diabetic keratopathy: a review. *Clin Experiment Ophthalmol* 2011; 39:259-70. [PMID: 20973888]
45. Messmer EM, Schmid-Tannwald C, Zapp D, Kampik A. In vivo confocal microscopy of corneal small fiber damage in diabetes mellitus. *Graefes Arch Clin Exp Ophthalmol* 2010; 248:1307-12. [PMID: 20490534]
46. Ljubimov AV, Huang ZS, Huang GH, Burgeson RE, Gullberg D, Miner JH, Ninomiya Y, Sado Y, Kenney MC. Human corneal epithelial basement membrane and integrin alterations in diabetes and diabetic retinopathy. *J Histochem Cytochem* 1998; 46:1033-41. [PMID: 9705969]
47. Saghizadeh M, Kramerov AA, Tajbakhsh J, Aoki AM, Wang C, Chai NN, Ljubimova JY, Sasaki T, Sosne G, Carlson MR, Nelson SF, Ljubimov AV. Proteinase and growth factor alterations revealed by gene microarray analysis of human diabetic corneas. *Invest Ophthalmol Vis Sci* 2005; 46:3604-15. [PMID: 16186340]
48. Xu KP, Li Y, Ljubimov AV, Yu FS. High glucose suppresses epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt signaling pathway and attenuates corneal epithelial wound healing. *Diabetes* 2009; 58:1077-85. [PMID: 19188434]
49. Xu K, Yu FS. Impaired epithelial wound healing and EGFR signaling pathways in the corneas of diabetic rats. *Invest Ophthalmol Vis Sci* 2011; 52:3301-8. [PMID: 21330660]
50. McLaughlin PJ, Sassani JW, Klocek MS, Zagon IS. Diabetic keratopathy and treatment by modulation of the opioid growth factor (OGF)-OGF receptor (OGFr) axis with naltrexone: a review. *Brain Res Bull* 2010; 81:236-47. [PMID: 19683562]
51. Lockwood A, Hope-Ross M, Chell P. Neurotrophic keratopathy and diabetes mellitus. *Eye (Lond)* 2006; 20:837-9. [PMID: 16215544]
52. Zagon IS, Sassani JW, Carroll MA, McLaughlin PJ. Topical application of naltrexone facilitates reepithelialization of the cornea in diabetic rabbits. *Brain Res Bull* 2010; 81:248-55. [PMID: 19853024]
53. Li J, Mai C, Hu Y. A study on histochemistry and electron microscopy of corneal nerves in experimental diabetic rabbits. *Zhonghua Yan Ke Za Zhi* 1996; 32:258-9. [PMID: 9590839]
54. Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 2005; 118:1715-24. [PMID: 15811951]
55. Barbaro V, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M. C/EBP δ regulates cell cycle and self-renewal of human limbal stem cells. *J Cell Biol* 2007; 177:1037-49. [PMID: 17562792]
56. Kusanagi R, Umemoto T, Yamato M, Matsuzaki Y, Nishida K, Kobayashi Y, Fukai F, Okano T. Nectin-3 expression is elevated in limbal epithelial side population cells with strongly expressed stem cell markers. *Biochem Biophys Res Commun* 2009; 389:274-8. [PMID: 19716807]
57. Mei H, Nakatsu MN, Ng M, Truong TT, Lee G, Deng SX. Differential expression of frizzled 7 in human corneal epithelial stem cells. *ARVO Annual Meeting*; 2011 May 1-May 5; Fort Lauderdale (FL).
58. Zhou M, Li XM, Lavker RM. Transcriptional profiling of enriched populations of stem cells versus transient amplifying cells. A comparison of limbal and corneal epithelial basal cells. *J Biol Chem* 2006; 281:19600-9. [PMID: 16675456]
59. Bian F, Liu W, Yoon KC, Lu R, Zhou N, Ma P, Pflugfelder SC, Li DQ. Molecular signatures and biological pathway profiles of human corneal epithelial progenitor cells. *Int J Biochem Cell Biol* 2010; 42:1142-53. [PMID: 20363357]
60. Pajoohesh-Ganji A, Pal-Ghosh S, Simmens SJ, Stepp MA. Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. *Stem Cells* 2006; 24:1075-86. [PMID: 16282441]
61. Figueira EC, Di Girolamo N, Coroneo MT, Wakefield D. The phenotype of limbal epithelial stem cells. *Invest Ophthalmol Vis Sci* 2007; 48:144-56. [PMID: 17197527]
62. Thomas PB, Liu YH, Zhuang FF, Selvam S, Song SW, Smith RE, Trousdale MD, Yiu SC. Identification of Notch-1 expression in the limbal basal epithelium. *Mol Vis* 2007; 13:337-44. [PMID: 17392684]
63. Schlötzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005; 81:247-64. [PMID: 16051216]
64. Takács L, Tóth E, Losonczy G, Szanto A, Bähr-Ivacevic T, Benes V, Berta A, Vereb G. Differentially expressed genes associated with human limbal epithelial phenotypes: new molecules that potentially facilitate selection of stem cell-enriched populations. *Invest Ophthalmol Vis Sci* 2011; 52:1252-60. [PMID: 21071743]
65. Merjava S, Brejchova K, Vernon A, Daniels JT, Jirsova K. Cytokeratin 8 is expressed in human corneal conjunctival epithelium, particularly in limbal epithelial cells. *Invest Ophthalmol Vis Sci* 2011; 52:787-94. [PMID: 20926822]
66. Ahmad S, Kolli S, Lako M, Figueiredo F, Daniels JT. Stem cell therapies for ocular surface disease. *Drug Discov Today* 2010; 15:306-13. [PMID: 20149892]
67. Kolli S, Lako M, Figueiredo F, Ahmad S. Corneal epithelial stem cells and their therapeutic application. In: Bharavand H, editor. *Trends in Stem Cell Biology and Technology*. Dordrecht (The Netherlands): Humana Press, a Part of Springer Science + Business Media, LLC; 2009. p. 319-65.
68. Burman S, Sangwan V. Cultivated limbal stem cell transplantation for ocular surface reconstruction. *Clin Ophthalmol* 2008; 2:489-502. [PMID: 19668747]
69. Shortt AJ, Secker GA, Rajan MS, Meligoni G, Dart JK, Tuft SJ, Daniels JT. Ex vivo expansion and transplantation of limbal epithelial stem cells. *Ophthalmology* 2008; 115:1989-97. [PMID: 18554721]
70. Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal

- regeneration. *N Engl J Med* 2010; 363:147-55. [PMID: 20573916]
71. Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S. 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. *J Cell Biochem* 2011; 112:993-1002. [PMID: 21308743]
72. Ljubimov AV, Burgeson RE, Butkowski RJ, Michael AF, Sun TT, Kenney MC. Human corneal basement membrane heterogeneity: Topographical differences in the expression of type IV collagen and laminin isoforms. *Lab Invest* 1995; 72:461-73. [PMID: 7723285]
73. Guelstein VI, Tchypysheva TA, Ermilova VD, Litvinova LV, Troyanovsky SM, Bannikov GA. Monoclonal antibody mapping of keratins 8 and 17 and of vimentin in normal human mammary gland, benign tumors, dysplasias and breast cancer. *Int J Cancer* 1988; 42:147-53. [PMID: 2456993]
74. Ljubimov AV, Afanasjeva AV, Litvinova LV, Senin VM. Basement membrane components produced by a mouse ascites teratocarcinoma TB24. Analysis with monoclonal and polyclonal antibodies. *Exp Cell Res* 1986; 165:530-40. [PMID: 3522258]
75. Ljubimov AV, Bartek J, Couchman JR, Kapuller LL, Veselov VV, Kovarik J, Perevoshchikov AG, Krutovskikh VA. Distribution of individual components of basement membrane in human colon polyps and adenocarcinomas as revealed by monoclonal antibodies. *Int J Cancer* 1992; 50:562-6. [PMID: 1371500]
76. Aisenbrey S, Zhang M, Bacher D, Yee J, Brunken WJ, Hunter DD. Retinal pigment epithelial cells synthesize laminins, including laminin 5, and adhere to them through α 3- and α 6-containing integrins. *Invest Ophthalmol Vis Sci* 2006; 47:5537-44. [PMID: 17122146]
77. Kohfeldt E, Sasaki T, Göhring W, Timpl R. Nidogen-2: a new basement membrane protein with diverse binding properties. *J Mol Biol* 1998; 282:99-109. [PMID: 9733643]
78. Balza E, Siri A, Ponassi M, Caocci F, Linnala A, Virtanen I, Zardi L. Production and characterization of monoclonal antibodies specific for different epitopes of human tenascin. *FEBS Lett* 1993; 332:39-43. [PMID: 7691659]