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ADENOVIRUS-DRIVEN OVEREXPRESSION OF PROTEINASES IN ORGAN-CULTURED NORMAL HUMAN CORNEAS LEADS TO DIABETIC-LIKE CHANGES

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Abstract

Our previous data suggested the involvement of matrix metalloproteinase-10 (MMP-10) and cathepsin F (CTSF) in the basement membrane and integrin changes occurring in diabetic corneas. These markers were now examined in normal human organ-cultured corneas upon recombinant adenovirus (rAV)-driven transduction of *MMP-10* and *CTSF* genes.

Fifteen pairs of normal autopsy human corneas were used. One cornea of each pair was transduced with rAV expressing either *CTSF* or *MMP-10* genes. 1–2 × 10^8 plaque forming units of rAV per cornea were added to cultures for 48 hr with or without sildenafil citrate. The fellow cornea of each pair received control rAV with vector alone. After 6–10 days incubation without rAV, corneas were analyzed by Western blot or immunohistochemistry, or tested for healing of 5-mm circular epithelial wounds caused by topical application of n-heptanol.

Sildenafil significantly increased epithelial transduction efficiency, apparently through stimulation of rAV endocytosis through caveolae. Corneas transduced with *CTSF* or *MMP-10* genes or their combination had increased epithelial immunostaining of respective proteins compared to fellow control corneas. Staining for diabetic markers integrin $\alpha_3\beta_1$, nidogen-1, nidogen-2, and laminin γ2 chain became weaker and irregular upon proteinase transduction. Expression of phosphorylated Akt was decreased in proteinase-transduced corneas. Joint overexpression of both proteinases led to significantly slower corneal wound healing that became similar to that observed in diabetic ones.

The data suggest that MMP-10 and CTSF may be responsible for abnormal marker patterns and impaired wound healing in diabetic corneas. Inhibition of these proteinases in diabetic corneas may alleviate diabetic keratopathy symptoms.

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Keywords

diabetic cornea; organ culture; MMP-10; cathepsin F; Akt; sildenafil

Introduction

Diabetes mellitus type I (insulin-dependent, IDDM) and type II (non insulin-dependent, NIDDM) is a systemic disease characterized by hyperglycemia. According to the World Health Organization, in 2000 there were 171 million diabetics worldwide, which is likely to double by 2030 [50]. Diabetic eye complications referred to as retinopathy (DR) are generally considered as a disease of retinal microvasculature. DR affects at least 50% of type II diabetics and over 80% of type I diabetics after 15–20 years of disease [25]. Retinal changes contribute most significantly to vision loss, but other parts of the eye (cornea, lens, optic nerve, and iris) are also affected. According to the available data, up to 70% of diabetics suffer from corneal problems [13,49], although they are rather rarely diagnosed [23]. Clinically, delayed and abnormal epithelial wound healing, ulcers, edema, recurrent erosions, superficial punctate keratitis, abnormalities of the endothelium and corneal nerves are major manifestations of diabetic corneal disease [8,12,18,39,47,51].

It has been long recognized that diabetic corneal changes involved alterations of the epithelial basement membrane (BM) and of its interactions with corneal epithelium. These changes include increased BM fragility [2,16,18], decreased number of hemidesmosomes [2,54], altered epithelial adhesion, and BM reassembly after wounding [48]. Glycation of BM components [23], abnormalities of the opioid growth factor-receptor axis [33] and other growth factors and mediators [42,43,45,52] could contribute to diabetic corneal abnormalities. Our previous studies identified decreased immunostaining of specific BM components including laminins and entactin/nidogen-1 as well as of a laminin receptor, integrin $\alpha_3\beta_1$ in corneal epithelial BM [29]. These alterations were likely due to increased expression of specific proteinases, MMP-10 and cathepsin F, identified using immunohistochemistry, RT-PCR and gene microarrays [42,45]. The described diabetic changes could be readily reproduced in corneal organ cultures [22,45], apparently because of the diabetic memory phenomenon [57].

In the present paper the goal was to obtain a functional proof of principle for the involvement of these proteinases in diabetic corneal changes. To this end, normal corneas were transduced with recombinant adenoviruses (rAV) harboring full-length genes coding for MMP-10 and cathepsin F, and the expression of diabetes-associated markers and wound healing rates were examined. It is shown that overexpression of these two proteinases in normal corneas brings the patterns of diabetic markers and wound healing rates close to the diabetic ones. It may be thus suggested that MMP-10 and cathepsin F contribute to the abnormalities seen clinically in corneas of diabetic patients.

Material and Methods

Cell and organ culture

Human glioma U87MG, human embryonic kidney (HEK293), and Chinese hamster ovary (CHO) cell lines used for testing and optimizing rAV transduction were from the American Type Culture Collection (Rockville, MD). 293A cell line used for rAV production was from Invitrogen (Carlsbad, CA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) or a mixture of DMEM-Ham's F-12 (1:1) with 10% fetal calf serum, L-glutamine, antibiotics, and sodium pyruvate, in a humidified atmosphere with 5% CO_2 at 37° C.

Non-diabetic human autopsy corneas or whole globes were purchased from the National Disease Research Interchange (NDRI, Philadelphia, PA). NDRI human tissue collection protocol is approved by a managerial committee and is under National Institutes of Health oversight. A total of 31 corneas (Table 1) from 16 donors (4 females, 12 males, mean age 63.3 ± 15.5 years; one donor with insulin-dependent diabetes) were used. The whole corneas were cultured over agar-collagen gel [22] in 60-mm Petri dishes (BD Biosciences, Franklin Lakes, NJ), at a liquid-air interface (with medium covering the limbus), in serum-free medium with insulin-transferrin-selenite, antibiotics and antimycotic agent (Invitrogen, Carlsbad, CA). Two-three drops of medium were added one-two times a day to the top of the corneas for moistening the epithelium.

Culture treatments

25-mg sildenafil citrate tablets (Viagra®; Pfizer Corp., New York, NY) were crushed into powder and dissolved in culture medium to a concentration of 3.5 mg/ml. The stock solution was centrifuged and sterile-filtered through 0.2 μm syringe filters (Pall Corp., Ann Arbor, MI). Fresh sildenafil (half-life 3.5 hrs [59]) was added twice to the corneas at 75 μg/ml during the first six hours of adenoviral transduction that lasted 48 hr. Stock solution (10 mg/ml) of filipin III (Sigma-Aldrich, St. Louis, MO), a specific inhibitor of caveolae endocytosis pathway, was prepared in dimethylsulfoxide (DMSO) and was stored in −20°C. It was added to organ-cultured corneas in fresh medium (1 μl/2 ml; final concentration 5 μg/ml) together with sildenafil citrate during viral transduction. Control organ-cultured corneas received sildenafil citrate and DMSO (1 μl/2 ml).

Epithelial wound healing

Epithelial debridement was performed by placing a 5-mm paper disc soaked in n-heptanol on the central corneal surface for one min [10,22].Contrary to mechanical debridement, n-heptanol does not disrupt fragile diabetic epithelial BM [16]. Both healing corneas, target gene and control treated, were photographed every 24 hr until the epithelial defect was completely healed and healing time recorded. After healing, corneas were cut in half, one half embedded in OCT compound (Ted Pella, Inc., Redding, CA) and processed for indirect immunofluorescence on cryostat sections, and the other half or the epithelium was frozen in liquid nitrogen for Western blot analysis.

Adenoviruses

The used rAV were E1/E3-deleted type 5 with genes of interest under the control of major immediate early cytomegalovirus promoter [28]. They expressed full-length open reading frames (ORF) of genes coding for matrix metalloproteinase-10 (rAV-MMP10) or cathepsin F (rAV-CTSF), or green fluorescent protein (rAV-GFP; positive control), or an empty vector as a negative control. All viruses were generated based on the Gateway recombination technology as per manufacturer's instructions.

Briefly, the human MMP-10 and CTSF Ultimate™ full-length ORF clones in Gateway® entry vector (pENTR™221, Invitrogen) were transferred into rAV vectors (pAd/CMV/V5-DEST) using the ViraPower™ Adenoviral Gateway® Expression Kit and LR Clonase™ II enzyme mix (Invitrogen). pAd/CMV/V5/*lacZ* is included with each kit for use as a positive control in the ViraPower™ adenoviral expression system (rAV-gal). The reaction mixtures were transformed into DH5™ chemically competent E. coli to select for expression clones. The selected expression clones were sequenced at the UCLA core facility to confirm the correct orientation of the ORFs. The Pac I-digested vectors were used to transfect 293A cells to produce rAV stocks. The rAV were amplified by infecting the 293A producer cells with the crude viral lysates and purified using Vivapure® AdenoPACK™ kit (Sartorius Stedim SUS, Concord, CA). The titers of rAV stocks were twice determined using HEK-293 cells as described [53].

Viral transduction

Cell lines U87MG and CHO were first transduced with the rAV vectors expressing target genes to confirm their overexpression. Non-diabetic organ-cultured corneas were transduced with rAV-CTSF or rAV-MMP10 constructs; in some experiments both constructs were used simultaneously. The other cornea of each pair was treated with control rAV-gal or rAV-vector. The rAV particles were given at $1–2 \times 10^8$ plaque forming units (pfu) per cornea along with 75 μg/ml sildenafil citrate in culture medium for 48 hr. rAV-GFP was added to the corneas at five or 15 μl of a stock solution containing $5–15 \times 10^7$ pfu/μl. To ensure better transduction, corneas were kept under the medium in 24-well plates (BD Biosciences) for the whole period of incubation with rAV. Corneas were then transferred to new dishes with a Corning round end spatula (Fisher Scientific, Pittsburgh, PA) and cultured in medium without rAV. After the additional four to eight days at the liquid-air interface, rAV-treated corneas were processed or tested for healing (see below). Marker expression was analyzed by immunohistochemistry and Western blot.

Immunohistochemistry and Western blot analysis

These were performed as described before [26,45]. The antibodies used and their reactivities in different assays are presented in Table 1. For each marker of fellow corneas, the same exposure was used when photographing stained sections of a vector-transduced one and a proteinase gene-transduced one. For Western blots, 8–16% gradient Tris-glycine SDS polyacrylamide gels were used (Invitrogen). Gel loading was normalized by β-actin content [26] (Table 2).

Statistical analysis

Data were analyzed using InStat 3 software program (GraphPad Software, San Diego, CA). Wound healing rates in pairs of control and proteinase-transduced fellow corneas were analyzed by paired two-tailed t test. Comparison with previous data obtained on organ-cultured normal and DR corneas [22] was done using unpaired two-tailed t test, Welch corrected. $P<0.05$ was considered significant.

Results

It was previously shown that vascular endothelial growth factor and inhibitors of phosphodiesterase 5 (PDE5) increased rAV delivery to the myocardium, presumably by enhancing vascular permeability via increased nitric oxide and cGMP [36]. A recent study using brain tumors showed that PDE5 inhibitors increased vascular permeability and adriamycin delivery through brain blood barrier [4]. We hypothesized that PDE5 inhibitors could also increase epithelial permeability to the rAV and tested this in organ-cultured corneas. As shown in Fig. 1A (left top row), addition of sildenafil during rAV-GFP infection of corneas did indeed significantly increase the number of GFP-positive epithelial cells. Because of the previous suggestion that PDE5 inhibitors may modulate endocytosis, the corneas treated with rAV-GFP with or without sildenafil were stained for the components of major endocytotic pathways. Distribution of clathrin (major protein of coated pits) did not appear to change in the epithelium upon sildenafil treatment (Fig. 1B, bottom row). Some decrease of stromal staining was variable and may not have contributed to the sildenafil effect because rAV in organ-cultured corneas do not transduce stromal cells [28]. However, caveolin-1 (major component of caveolae), which was predominantly seen in the basal epithelial cells in control corneas in accordance with previous data [1], changed its distribution upon sildenafil treatment with many suprabasal cells also becoming positive (Fig. 1B, top row). To test caveolae involvement more rigorously, corneas transduced with rAV-GFP were treated either with sildenafil or sildenafil with filipin III, an inhibitor of caveolae-mediated endocytosis. Filipin completely abrogated the effect of sildenafil on viral transduction (Fig. 1A, bottom row). These

data suggested that sildenafil acted by predominantly stimulating caveolae-dependent rAV endocytosis.

Before studying the effects of MMP-10 and cathepsin F transduction in human corneas, a confirmation of their overexpression was needed. Preliminary experiments with cells lines established this overexpression for both genes (data not shown here), and then the viruses were used in corneal organ cultures. Fairly efficient transduction of organ cultures could be achieved already within 24 hr when sildenafil was present (data not shown here) but without it, 48 hr ensured higher efficiency. Therefore, since some earlier experiments were done without sildenafil, 48 hr incubation time with the viruses was kept for all corneas. No deleterious effects were observed following this prolonged incubation with rAV and corneas always looked healthy and transparent. As shown in Fig. 2, both rAV-MMP10 (Fig. 2A) and rAV-CTSF (Fig. 2B) increased the expression of the transduced proteins as revealed by Western blot analysis of whole corneal lysates (top panels) and immunostaining of corneal sections (bottom panels). Staining for both proteins primarily increased in the epithelial cells. MMP-10 showed mostly cytoplasmic staining. Cathepsin F displayed cytoplasmic but also nuclear staining with several antibodies, in accordance with previous data [32].

Upon rAV-MMP10 transduction, there were changes in the patterns of diabetic corneal markers compared to rAV-vector. Staining for basement membrane laminin-332 γ2 chain, nidogen-2 (Fig. 3) and nidogen-1 (data not shown here) became weak and/or discontinuous, which was similar to diabetic corneas [22,29]. Similarly, the staining for laminin receptor, integrin $\alpha_3\beta_1$, became weaker and discontinuous. Essentially the same results were obtained following cathepsin F overexpression (Fig. 4), and when both proteinase genes were transduced together (data not shown here).

Major tight junction proteins, claudin-1 (Fig. 5), the main claudin type expressed in the corneal epithelium [61], and ZO-1 [3,19,58], did not reveal any staining pattern changes upon proteinase gene transduction. These data suggested that no significant compromise of epithelial barrier function occurred following proteinase transduction. As a physiological test for proteinase effects in organ-cultured corneas, wound healing dynamics was examined. Overexpression of either MMP-10 or cathepsin F did not produce a significant delay in wound healing compared to control. However, when viruses harboring genes for both proteinases were combined, transduced corneas healed significantly slower compared to vector controls. A typical time course picturing live corneas is shown on Fig. 6. On average, corneas transduced with rAV-MMP10 + rAV-CTSF healed twice as slowly as the fellow corneas transduced with rAV-vector (n=5, Fig. 7). Proteinase gene-transduced corneas healed on average in 5.2 ± 0.7 days (mean ± standard error) compared to 2.6 ± 0.4 days for vector controls ($p<0.003$). Comparison with our previous data on normal and DR corneas [22] showed that vector-transduced corneas healed similarly to normal corneas (2.6 days on average vs. 2.3 days, $p>0.05$, non-significant) and significantly faster than DR corneas (2.6 days vs. 4.5 days, $p<0.04$). Combined proteinase transduction made healing slower than in normal corneas (5.2 days vs. 2.3 days, $p<0.02$) effectively decreasing it to DR rates (5.0 days vs. 4.5 days, $p>0.05$, non-significant). It should be noted that time of wounding was important to show an effect. If wounds were made 3–6 days after transduction, the healing rates were not significantly changed in some experiments. More consistent results were obtained with wounds made 10 days after transduction or when the first wounds had been healed (within 4–5 days) and wounding was repeated. This could be due to a possible need for a certain time of proteinase action before functionally significant degradative changes occurred in the basement membrane and epithelial integrins. Wound healing delay upon proteinase transduction was more evident when the wounds became smaller, usually after the first two days (Fig. 6). During wound healing, the number of proliferating cells revealed by Ki-67 staining did not change upon proteinase

transduction. Cells expressing activated caspase 3 were extremely rare in control or proteinase-transduced corneas (data not shown here).

In order to gain insight into the possible mechanisms of the observed changes, patterns of major signaling intermediates in corneal wound healing were examined following proteinase gene transduction. No changes could be seen in the patterns of phosphorylated extracellular regulated kinase (p-ERK) or p38 mitogen-activated protein (MAP) kinase (p-p38) upon transduction of MMP-10, or cathepsin F (Fig. 8), or both together (data not shown here). However, overexpression of each proteinase alone or their combination led to decreased expression of phosphorylated Akt protein kinase (p-Akt) in the epithelial cells. Results were obtained by immunostaining (Fig. 9A) or Western blotting (Fig. 9B). It should be noted that immunostaining demonstrated the diabetic-like decrease in p-Akt [60] in non-wounded corneas or in those that had been wounded and then completely healed (6/7 cases). However, Western blot analysis was able to reveal decreased p-Akt expression only when corneas were actively healing epithelial wounds.

Because Akt is important for wound healing induced by epidermal growth factor (EGF) signaling [60] through its receptor tyrosine kinase (EGFR), we hypothesized that p-Akt decrease might be due to reduced activity of EGFR. Therefore, the expression of phosphorylated (activated) EGFR (p-EGFR) was also examined. As shown in Fig. 10, p-EGFR staining was indeed decreased in corneas overexpressing MMP-10, cathepsin F or both proteinases (6/7 cases) compared to vector-transduced fellow corneas.

Discussion

Major diabetic corneal complications including keratopathy and neuropathy are a recognized clinical problem. However, patients with clinically manifested corneal diabetes only receive topical symptomatic treatment including artificial tears, autologous serum possibly supplying adhesive and growth factors for healing, contact lenses as a corneal bandage, punctal occlusion to increase tear production, topical antibiotics to control infection in case of persistent epithelial defects [7,23,46]. Naltrexone (opioid growth factor antagonist), insulin, aldose reductase inhibitors, and substance P have been tested with varying degree of success for alleviating diabetic keratopathy, but they are still in various phases of preclinical and clinical testing [33,35,37].

Overall, there is a clear need for a more specific treatment approach for diabetic keratopathy, such as gene therapy targeting abnormally expressed corneal proteins. Corneal tissue is easily accessible to various vectors, is immune privileged, and in case of diabetes may need only a rather transient gene expression change, e.g., for accelerating of epithelial wound healing. All these qualities make cornea a very promising target for gene therapy [24,35].

We have exploited this approach using adenoviral vectors. Our previous studies have shown that diabetic corneas abnormally express several epithelial markers including specific proteinases [42,45]. Contrary to adeno-associated viruses, rAV has an advantage of transducing epithelium but not keratocytes in corneal organ culture [28] and was chosen as a gene therapy vector because of this property and its high transduction efficiency. In the present study genes coding for proteinases altered in diabetic corneas, MMP-10 and cathepsin F, were transduced into normal human organ-cultured corneas using rAV vectors. We then tested whether their overexpression would bring about diabetic-like changes in marker protein expression and wound healing. The experiments aimed at establishing functional importance of MMP-10 and cathepsin F in diabetic corneal changes.

Corneal epithelial cell transduction with rAV has a rather low efficiency at clinically relevant incubation times necessitating long exposure; in this work 48 hrs period was used to obtain a

functional effect. Therefore, there was a need to optimize rAV transduction, with increased efficacy at shorter incubation times. Previous work suggested that rAV-mediated gene transfer into myocardium was facilitated by PDE5 inhibitors [36], which also enhanced drug transport across tight-junction-controlled blood brain barrier [4]. Since we used whole corneas with epithelium containing tight junctions and rAV as a delivery vector, we tested sildenafil as the most water-soluble PDE5 inhibitor for possible increase in gene delivery to corneal cells. As expected, this reagent dramatically increased rAV-mediated gene transduction into organ-cultured corneas monitored by GFP expression. Using sildenafil it was also possible to reduce transduction time to 24 hrs still maintaining fairly high transduction efficiency.

Adenovirus usually uses clathrin-dependent coated pit pathway for cell entry [9]. However, in some cells it can also use caveolin-dependent lipid raft mechanism [9,38]. Clathrin staining of sections of corneas incubated with sildenafil did not reveal marked changes in expression or distribution. At the same time, in control corneas caveolin-1 staining was mainly observed in basal epithelial cells in accordance with previous data [1], but it was also seen in suprabasal cells after sildenafil reatment suggesting activation of this endocytotic pathway. This assumption was corroborated by incubating corneas with sildenafil in the presence of a specific caveolae-dependent pathway inhibitor, filipin. It dramatically reduced sildenafil-enhanced rAV-driven GFP transduction into cornea to levels below control (Fig. 1). These data strongly suggested that stimulation of caveolae-dependent endocytosis was the likely mechanism of sildenafil effect on rAV-mediated gene transfer to cornea. Our data, however, cannot completely exclude the involvement of clathrin-dependent pathway in adenoviral gene transfer and sildenafil effect on it.

Proteinase transduction apparently did not compromise epithelial barrier function as suggested from lack of changes in tight junction protein patterns. At the same time, overexpression of both proteinases in normal corneas (confirmed by Western blot and immunofluorescent staining) led to a change in the patterns of several markers [22] towards those observed in diabetic corneas. In particular, integrin $\alpha_3\beta_1$ staining became weak and disorganized. Basement membrane components, nidogens (Fig. 3,Fig. 4) and laminin β1 chain (data not shown here) also showed discontinuous patterns compared to rAV-vector transduced fellow corneas. For a secreted enzyme MMP-10 that can degrade basement membrane and extracellular matrix proteins [5,56], these data agree well with previous results on skin wound healing [27]. Overexpression of constitutively active MMP-10 mutant in skin also led to the disruption of laminin-5 (now called laminin-332) apparently by direct proteolysis of γ2 chain, and disorganized staining for β_1 chain-containing integrins. Although cathepsins are generally considered as lysosomal proteinases, accumulating evidence suggests that they may also exert their action outside of the cell and digest extracellular matrix [40]. Cathepsins including cathepsin F were found at sites of Bowman's layer rupture in keratoconus corneas [6], in corneas with keratectasia after failed LASIK procedure [30], and in the corneal stroma around implanted intracorneal rings [31]. Moreover, cathepsin F secretion was observed in macrophages stimulated by angiotensin II [20]. Therefore, one may suggest that alterations of basement membrane and integrin observed in rAV-CTSF transduced corneas could also be due to a direct action of extracellular cathepsin F.

An important functional consequence of proteinase transduction was a significant 2-fold delay in epithelial wound healing compared to vector-transduced corneas. This result may be puzzling because proteinases, especially MMPs are generally considered as facilitators of wound healing [11,17,55]. However, neither overexpression of MMP-10 in skin nor its knockout changed the skin or corneal wound healing rate, respectively [15,27]. Indirect data suggest that MMP-10 may even delay wound healing. Mice immunosuppressed by dexamethasone treatment demonstrated delayed cutaneous wound healing but MMP-10 was overexpressed. Reduction of MMP-10 expression by recombinant interleukin-6 was

accompanied by accelerated wound healing [14]. These data generally agree with our results showing that overexpression of either MMP-10 or cathepsin F alone did not change corneal epithelial wound healing rate, although degradative changes of the basement membrane and a laminin-binding integrin were induced. Apparently, the magnitude of the effect was not enough to delay wound healing in this system. Only when two proteinases were overexpressed together, there was a significant slowdown of wound healing. Joint overexpression appears to better mimic the situation in diabetic corneas that also show increased levels of both MMP-10 and cathepsin F [42,45] and slower than normal wound healing [22]. In this respect, proteinase-transduced corneas became similar to the diabetic ones suggesting that specific proteinase overexpression does contribute to diabetic corneal wound healing abnormalities.

Abnormal intracellular signaling would be a possible molecular mechanism of delayed wound healing upon proteinase gene transduction. Although phosphorylation/activation of major mediators, ERK and p38 MAPK, was unchanged, p-Akt showed a marked decrease compared to vector-transduced corneas. The p-Akt change was reproducibly observed by immunohistochemistry in healing or healed corneas, and by Western blot during active healing. These data are in agreement with previous evidence. In skin wounds of mice overexpressing MMP-10 in skin cells p-Akt was also depressed during healing [27]. Although short exposure of neutrophils to cathepsin G led to Akt stimulation, prolonged exposure (as in our experiments) led to Akt degradation and apoptosis activation [41]. In the cornea, where Akt mediated EGFR-dependent epithelial wound healing, a similar reduction of p-Akt was observed after high-glucose treatment mimicking diabetic conditions [60]. It may be suggested that decreased Akt activation upon proteinase gene transduction could lead to decreased cell migration translated in slower wound healing. Because Akt is downstream of several motogenic growth factors, especially EGF [60], the activation of EGFR was also examined. Interestingly, staining for p-EGFR also decreased upon MMP-10 and cathepsin F overexpression. These results suggest that MMP-10 and cathepsin F overexpression in normal corneas may interfere with EGFR signaling thereby affecting wound healing.

Overall, the data reported herein provide a proof of principle for a functional involvement of MMP-10 and cathepsin F overexpression in certain abnormalities associated with corneal diabetes including basement membrane alterations and wound healing slowdown. This makes them promising candidates for gene silencing therapy aimed at alleviating these signs of diabetic keratopathy. RNA interference and/or antisense treatment would be strategies of choice to inhibit MMP-10 and cathepsin F activity in order to restore normal structure and function to diabetic corneas. These treatments may be combined with overexpressing specific mediators decreased in diabetic corneas, such as c-met proto-oncogene, which can positively influence corneal markers and wound healing [44,45].

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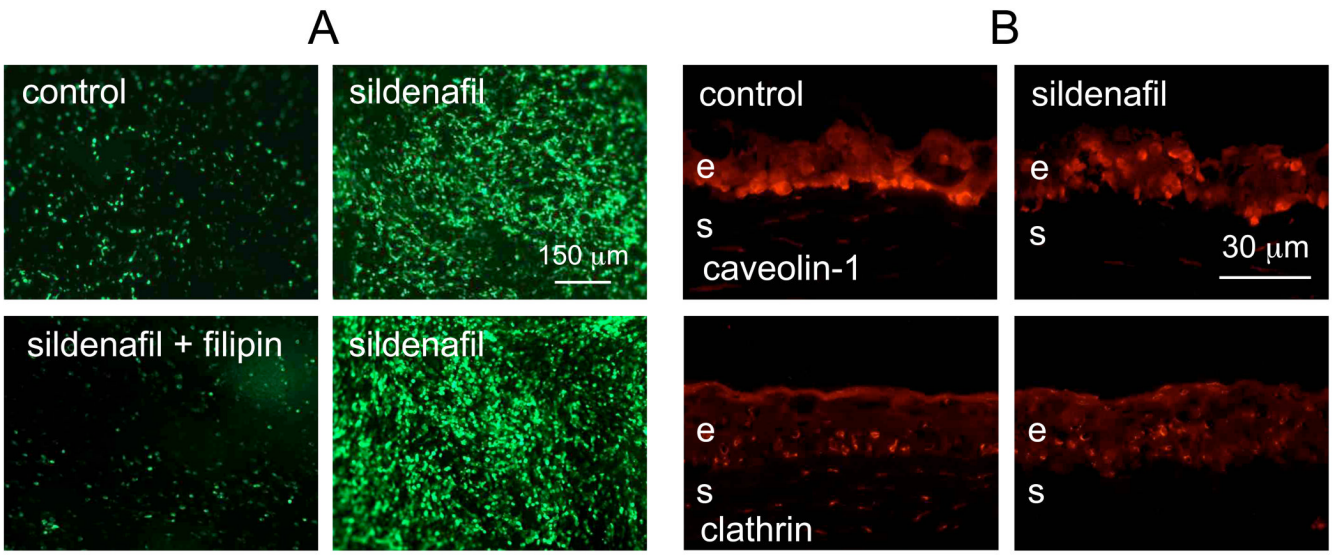

Figure 1.

Effect of sildenafil on rAV transduction of organ-cultured corneas. A, live corneas. Top row: left panel, cornea transduced with rAV-GFP, 6 days after the addition of the virus; right panel, a fellow cornea transduced with the same virus in the presence of 75 μg/ml sildenafil. Note significant increase in transduction efficiency. Bottom row: left panel, addition of caveolae-dependent endocytosis inhibitor, filipin III (right panel), completely abolished the effect of sildenafil (left panel) on rAV-GFP transduction. The data suggest the importance of caveolae endocytosis in sildenafil-mediated increase in rAV transduction efficiency. Pictures of live corneas are shown. Scale bar = 150 μm.

B, immunofluorescence for markers of major endocytotic pathways. Top row: left panel, staining for caveolin-1 is most pronounced in the basal epithelial layer of a control cornea; right panel, after sildenafil incubation of a fellow cornea, caveolin-1 becomes also prominent in suprabasal layers. Bottom row: left panel, clathrin immunoreactivity is seen in basal and suprabasal cells in a control cornea; right panel, addition of sildenafil does not alter clathrin pattern in the epithelium of a fellow cornea. Pictures of corneal sections are shown. E, epithelium; s, stroma. Scale bar = 30 μm.

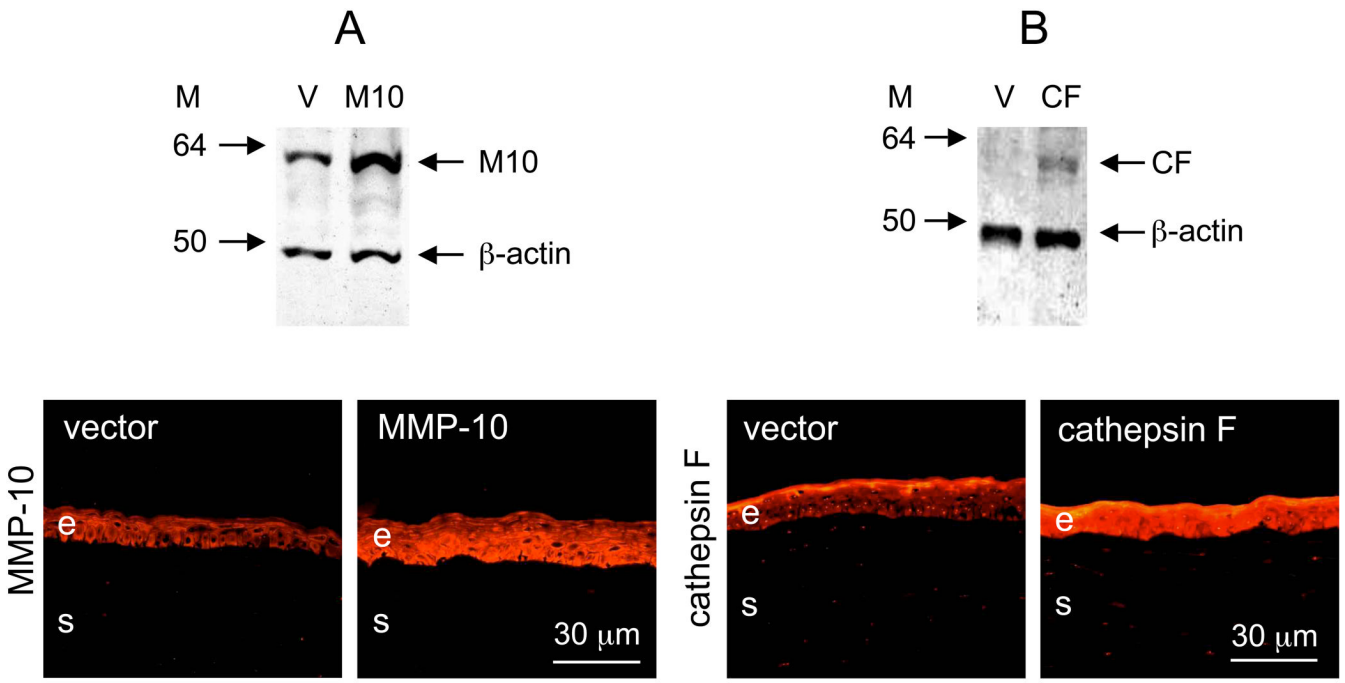

Figure 2.

Overexpression of MMP-10 and cathepsin F after rAV transduction of respective genes. A, MMP-10 (M10); B, cathepsin F (CF). Top panel: Western blots; bottom panel: immunofluorescence on corneal sections (A, stained for M10; B, stained for CF). V, transduction with vector alone (rAV-vector). Note increased expression of M10 and CF after rAV transduction compared to vector alone by both methods. M, markers in kDa. The gel loading was normalized by β-actin content [26]. Bar = 30 μm.

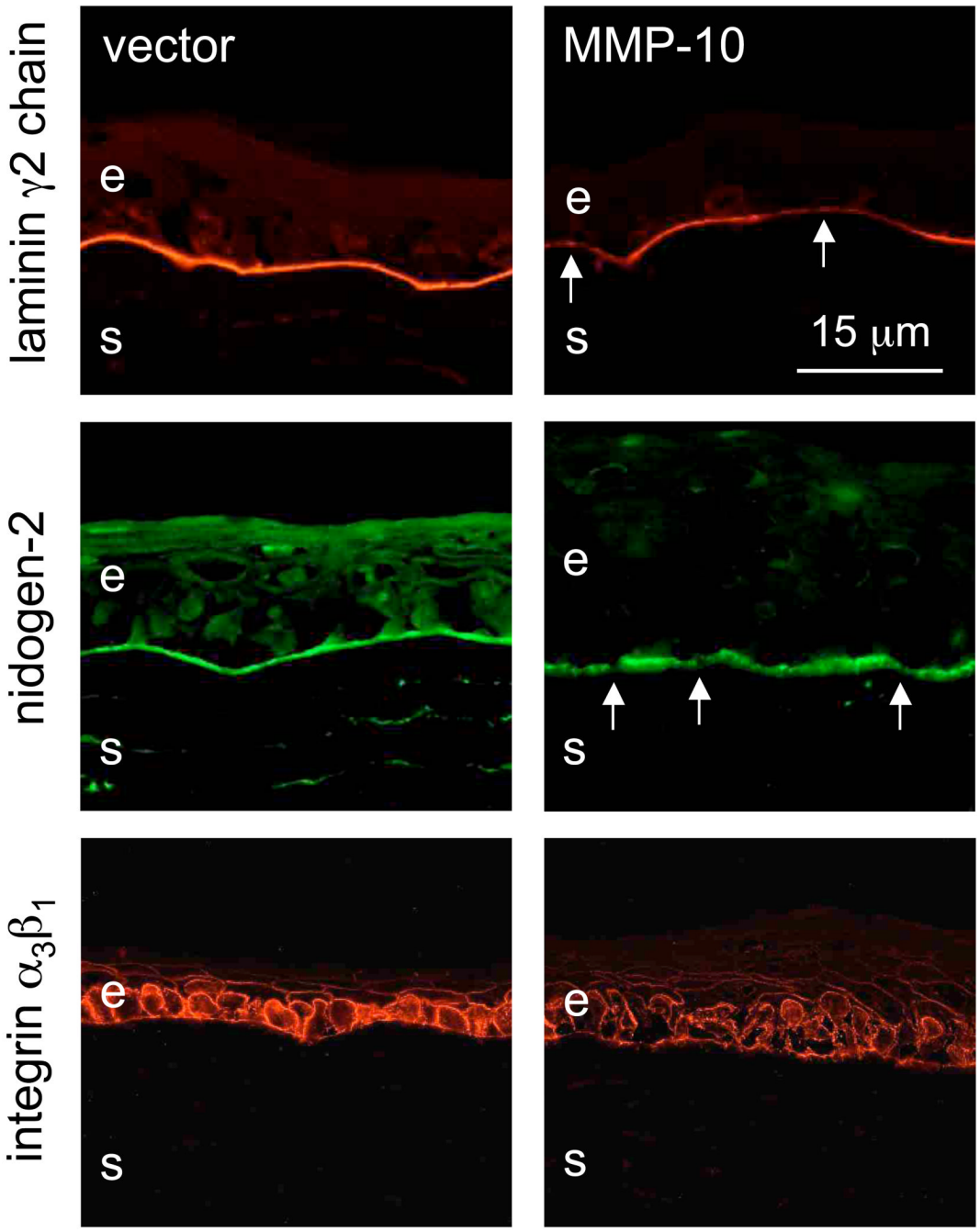

Figure 3.
Altered expression of diabetic markers after rAV-MMP10 transduction. Top row: laminin γ2 chain, middle row: nidogen-2. Note discontinuous (arrows) and markedly weaker (γ2 chain) staining of the epithelial basement membrane for both markers upon MMP-10 transduction. Bottom row: integrin $\alpha_3\beta_1$. Note decreased and discontinuous staining upon MMP-10 transduction. E, epithelium; s, stroma. Immunofluorescence. Bar = 15 μm.

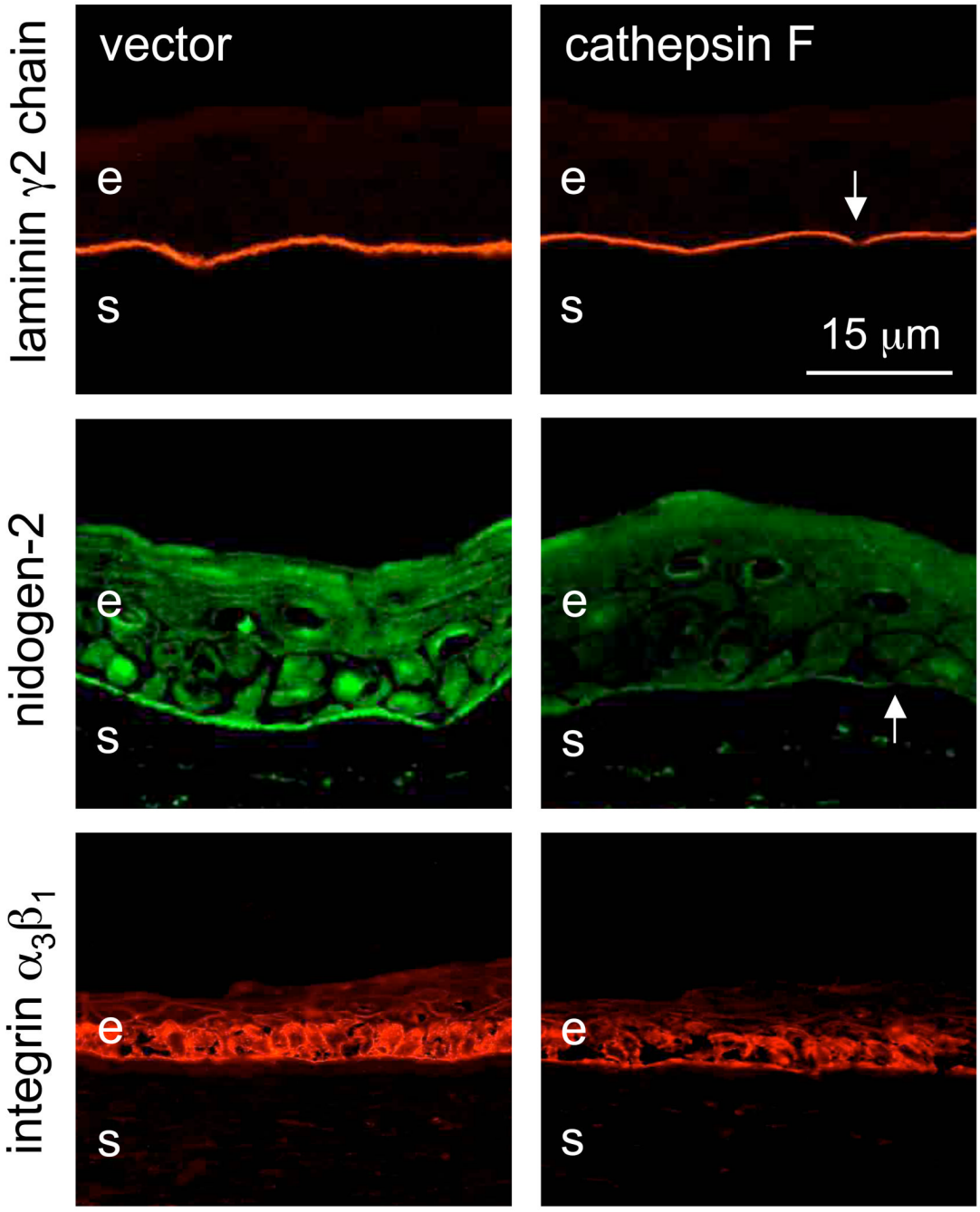

Figure 4.
Altered expression of diabetic markers after rAV-CTSF transduction. Top row: laminin γ2 chain, middle row: nidogen-2. Note markedly decreased (nidogen-2) and discontinuous (arrows) staining of the epithelial basement membrane for both markers after cathepsin F transduction. Bottom row: integrin $\alpha_3\beta_1$. Note disorganized and weaker staining upon cathepsin F transduction. E, epithelium; s, stroma. Immunofluorescence. Bar = 15 μm.

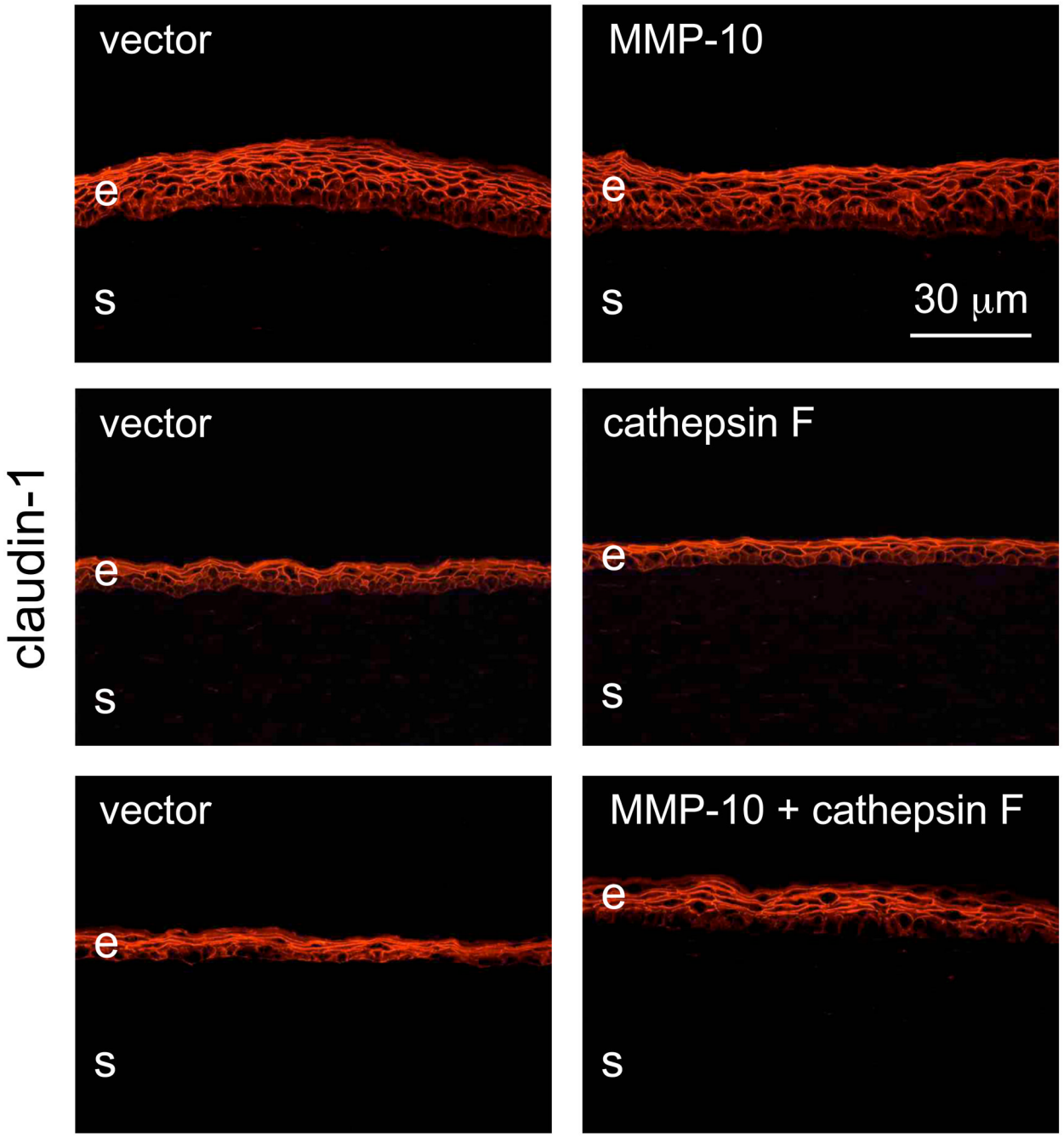

Figure 5.
Staining of transduced corneas for a tight junction component claudin-1. Transduction with rAV-MMP10, rAV-CTSF or their combination does not cause any changes in claudin-1 patterns compared to rAV-vector (vector). E, epithelium; s, stroma. Immunofluorescence. Bar = 30 μm.

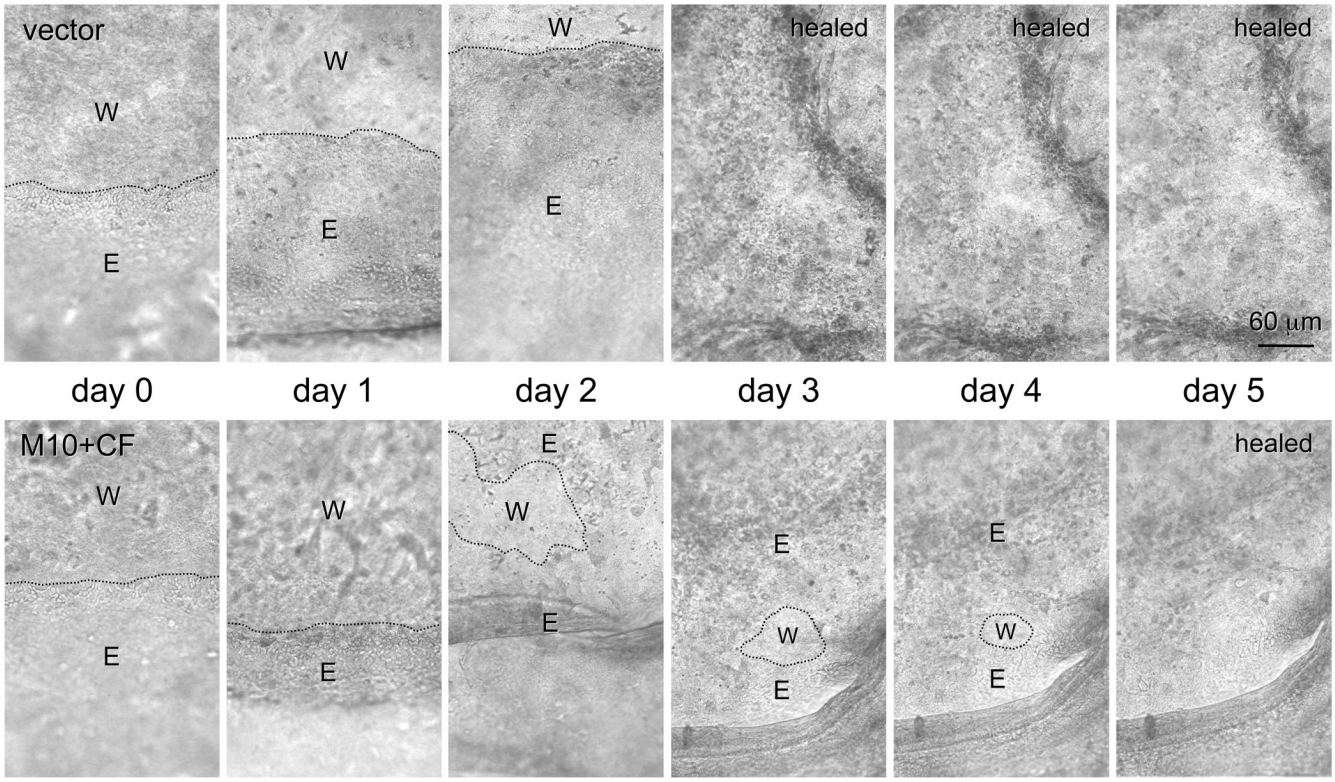

Figure 6.

Representative pictures of live healing corneas. One cornea was transduced with rAV-vector (vector), whereas the fellow corneas was transduced by rAV-MMP10 + rAV-CTSF (M10+CF). Note slower healing upon proteinase gene transduction (5 days) compared to rAV-vector (3 days), especially when the wound became smaller. E, epithelium; W, wound. Dotted lines denote wound margins. Day 0, the day when the wounds are made. Bar = 60 μm.

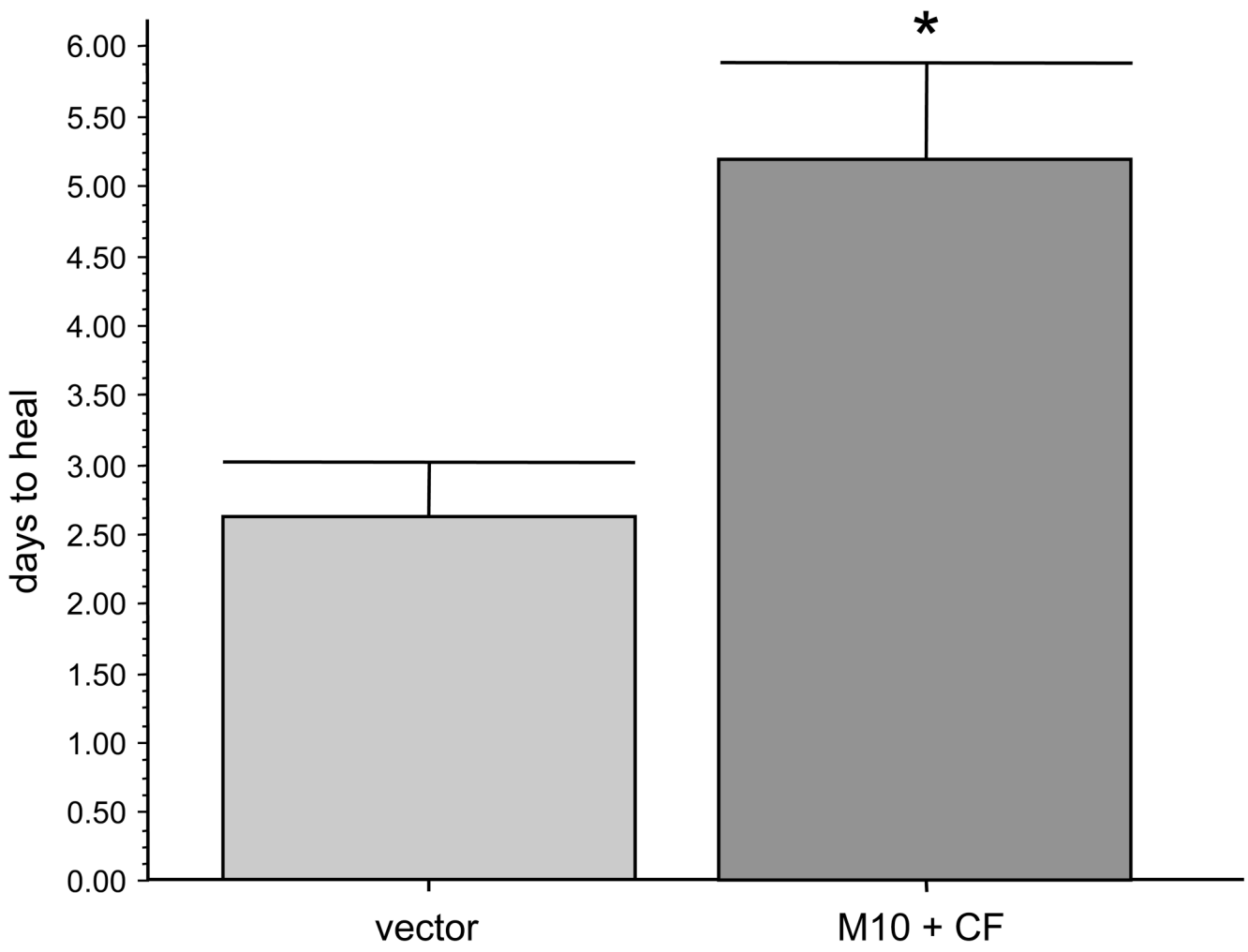

Figure 7.
Quantitation of healing in transduced corneas. Transduction with rAV-MMP10 + rAV-CTSF (M10+CF) slows down wound healing twofold compared to rAV-vector (vector). * $p<0.003$ by paired two-tailed t test.

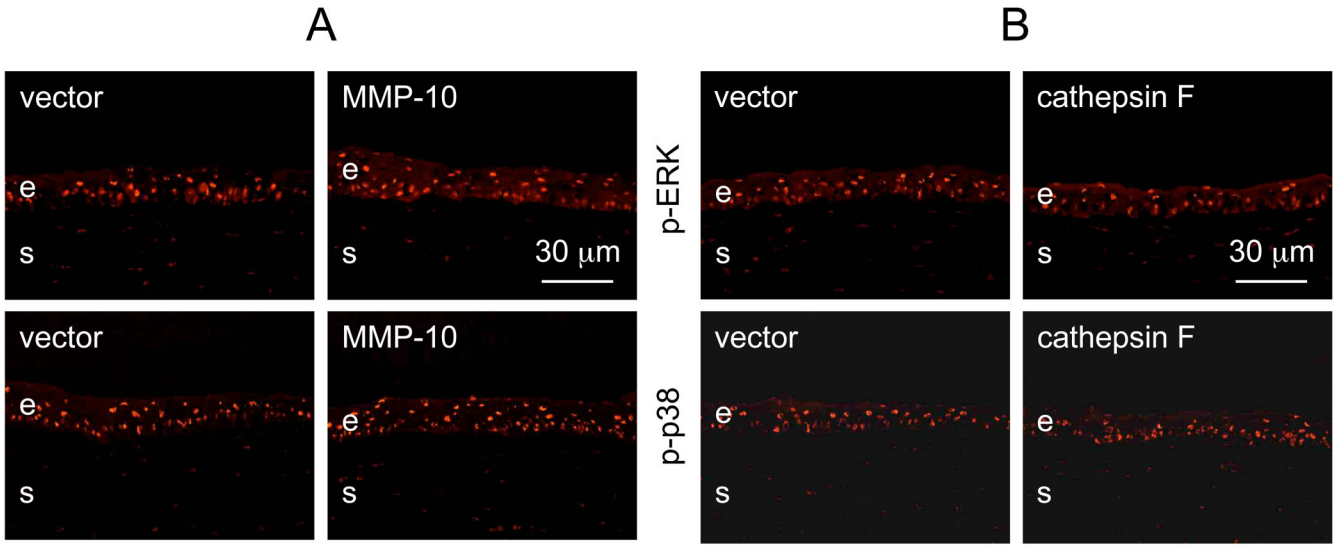

Figure 8.
Patterns of phosphorylated ERK (p-ERK) and p38 (p-p38) kinases in transduced corneas. A, MMP-10 transduction; B, CTSF transduction. There is no significant difference between corneas transduced with MMP-10 or CTSF compared to vector alone. E, epithelium; s, stroma. Immunofluorescence. Bar = 30 μm.

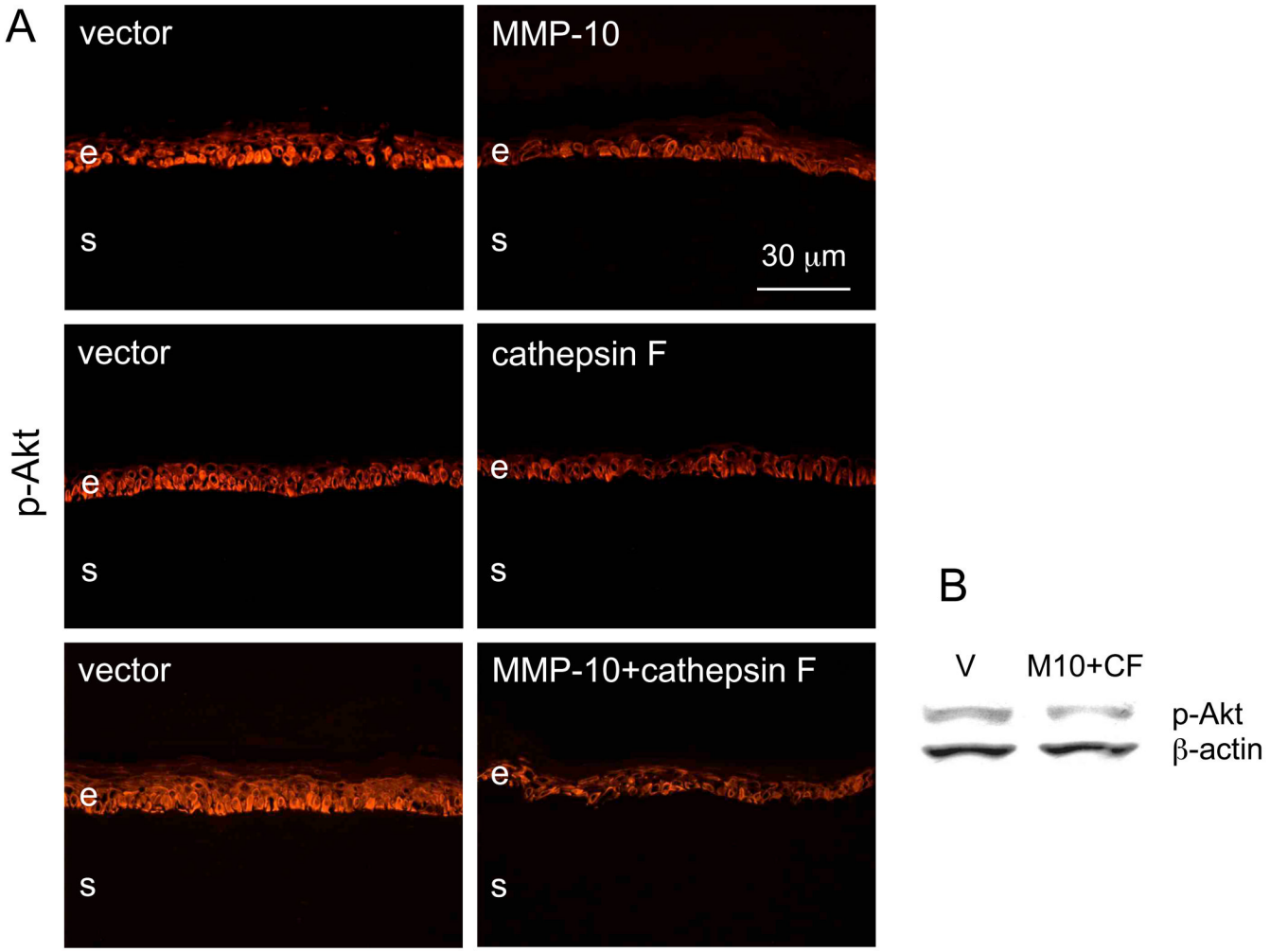

Figure 9.

Phosphorylated Akt (p-Akt) kinase in transduced corneas. A: immunofluorescent staining of corneal sections. Upon transduction with rAV-MMP10, rAV-CTSF or their combination, the epithelial staining for p-Akt (using pAb 9271) is noticeably decreased and becomes less regular in the basal cell layer. B: Western blot analysis. Upon transduction with rAV-MMP10 + rAV-CTSF, p-Akt is decreased as revealed by pAb 9271; pAb sc-7985-R gave the same result (data not shown here). The gel loading was normalized by β-actin content [26]. E, epithelium; s, stroma; V, vector; M10, MMP-10; CF, cathepsin F. Bar = 30 μm.

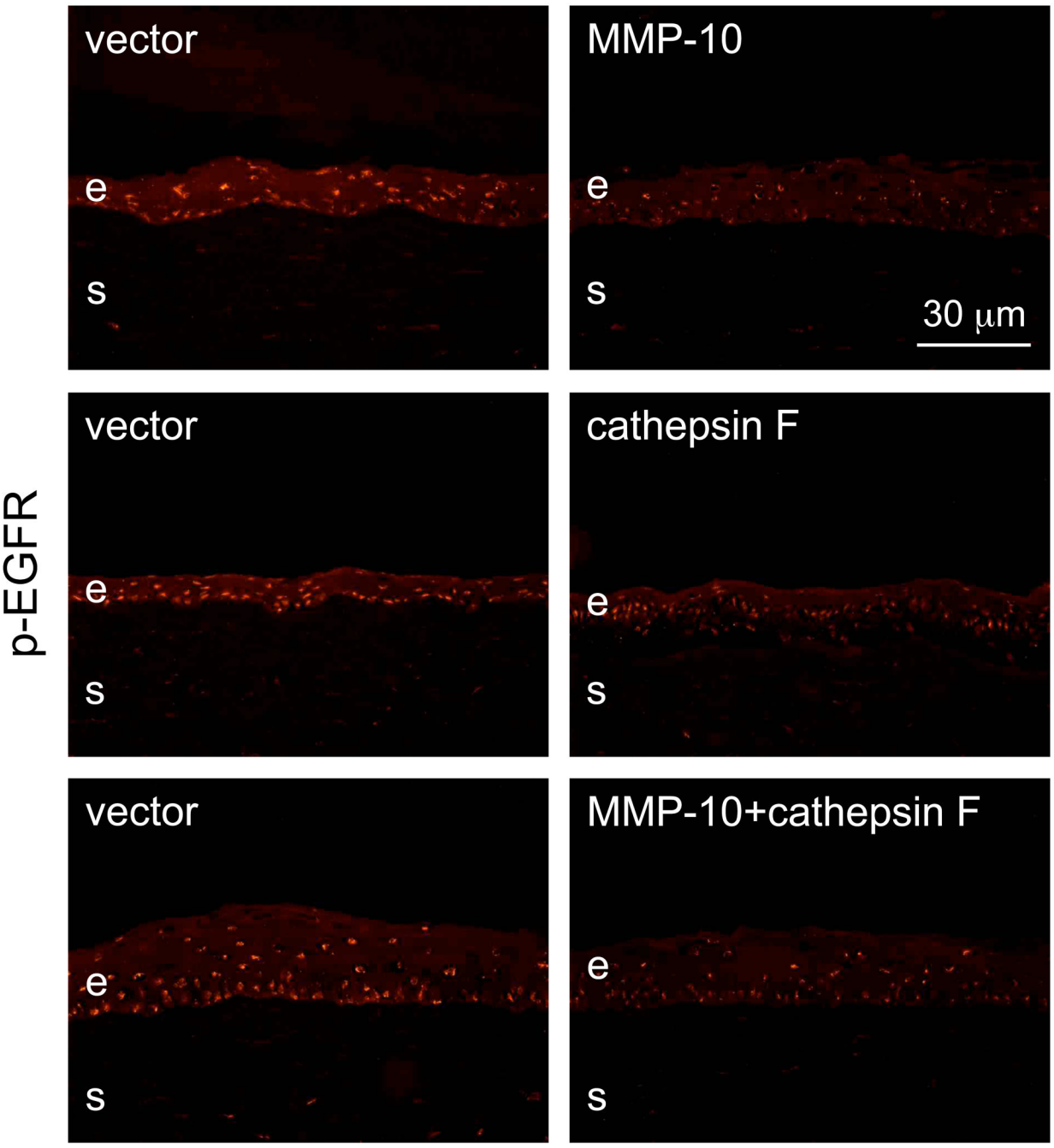

Figure 10.
Staining patterns of transduced corneas for phosphorylated EGFR (p-EGFR). Note decreased epithelial nuclear staining (both by intensity and number of positive cells) upon transduction with rAV-MMP10, rAV-CTSF or their combination. E, epithelium; s, stroma. Immunofluorescence. Bar = 30 μm.

Table 1

Donor characteristics

Case number	Age	Gender	Cause of death	Culture treatment
07-25	47	F	natural causes	CF
07-29	66	M	sub-arachnoid bleed	M10
07-31, IDDM	52	M	sudden death during surgery	CF
07-28	49	M	heart disease	M10
08-45	64	M	multi-organ failure	M10+CF
08-58 (1)	61	F	congestive heart failure	GFP
08-60	94	M	cardiac arrest	M10+CF
08-62	79	M	myocardial infarction	GFP
09-2	41	F	brain tumor	M10+CF
09-4	69	M	metastatic cancer	M10+CF
09-9	61	M	congestive heart failure	M10+CF
09-10	80	M	heart disease	GFP
09-15	76	M	cardiac arrest	M10+CF
09-18	75	M	respiratory failure	M10+CF
09-19	62	F	heart disease	M10+CF
09-20	37	M	electrocution	M10+CF

M10, MMP-10; CF, cathepsin F; M, male; F, female; IDDM, type I diabetes. In case 08-58 one cornea was analyzed.

Table 2

Antibodies used in this study

Antigen	Antibody & Cat. No.	Works in	Source/reference
Cathepsin F	goat pAb sc-9633	IHC#	Santa Cruz Biotechnology
Cathepsin F	goat pAb AF2075	IHC	R&D Systems
Cathepsin F	rabbit pAb sc-13987	WB	Santa Cruz Biotechnology
Cathepsin F	rabbit pAb 11055-1-AP	IHC#	Proteintech Group
Cathepsin F	mouse mAb sc-73774		Santa Cruz Biotechnology
MMP-10	goat pAb sc-9941	IHC	Santa Cruz Biotechnology
MMP-10	mouse mAb MAB9101		R&D Systems
MMP-10	mouse mAb MAB9102		R&D Systems
MMP-10	goat pAb sc-26694	IHC#	Santa Cruz Biotechnology
MMP-10	rabbit pAb 29580	IHC	Anaspec
MMP-10	mouse mAb NCL-MMP10	WB	Novocastra
Nidogen-1	mouse mAb A9	IHC	[21]
Nidogen-1	mouse mAb MAB2570	IHC	R&D Systems
Nidogen-2	rabbit pAb 1080	IHC	[21]
Laminin γ1 chain	rat mAb A5	IHC	[21]
Laminin γ2 chain	mouse mAb D4B5	IHC	Millipore
Integrin $\alpha_3\beta_1$	mouse mAb MAB1992	IHC	Millipore
Akt	mouse mAb 610860	WB	BD Transduction Labs
p-Akt (Ser473)	rabbit pAb sc-7985-R	IHC, WB	Santa Cruz Biotechnology
p-Akt (Ser473)	rabbit pAb 9271	IHC, WB	Cell Signaling
ERK1/2	rabbit mAb 4695	IHC, WB	Cell Signaling
p-ERK1/2 (Thr202/Tyr204)	rabbit mAb 4370	WB	Cell Signaling
p-ERK1/2 (Thr185/Thr202)	rabbit pAb ab4819	IHC	Abcam
p38 MAPK	rabbit pAb 9212	WB	Cell Signaling
p-p38 (Thr180/Tyr182)	rabbit pAb AB3828	WB#	Millipore
p-p38 (Thr180/Tyr182)	rabbit mAb 9215	WB#	Cell Signaling
p-p38 (Thr180/Tyr182)	mouse mAb ab50012	IHC	Abcam
p-EGFR (Tyr845)	rabbit pAb 44-784G	IHC	Invitrogen
p-EGFR (Tyr845)	rabbit mAb 2342-1		Epitomics
ZO-1	rabbit pAb 40-2300	IHC^	Invitrogen
Claudin-1	rabbit pAb 51-9000	IHC^	Invitrogen
Clathrin heavy chain	mouse mAb 610499	IHC*	BD Transduction Labs
Caveolin-1	rabbit pAb 610059	IHC	BD Transduction Labs
Ki-67	mouse mAb sc-101861	IHC*	Santa Cruz Biotechnology
Activated caspase-3	rabbit pAb G7481	IHC	Promega

Antigen	Antibody & Cat. No.	Works in	Source/reference
β-actin	mouse mAb A5316	WB	Sigma

mAb, monoclonal antibody; pAb, polyclonal antibody; IHC, immunohistochemistry; WB, Western blot; p-, phosphorylated;

weak reactivity;

* acetone fixation needed;

^ 95% ethanol followed by acetone fixation needed