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Desmin Deficiency is Not Sufficient to Prevent Corneal Fibrosis

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Abstract

The type III intermediate filament (IF) proteins vimentin and desmin are sequentially overexpressed in stromal myofibroblasts over the period when fibrosis sets in after corneal injury. Prior findings have revealed vimentin-deficient mice are significantly protected from corneal fibrosis after alkali injury, which has implicated this IF protein as an important regulator of corneal fibrosis. It has remained as yet unproven whether desmin contributes in any significant manner to corneal fibrosis. Here we have employed desmin-deficient (Des KO) mice in the corneal alkali injury model and show that injured Des KO mice develop fibrosis and show similar levels of corneal opacity at 14 days post-injury as wild type (WT) mice and retain this phenotype even at 30d post injury. Des KO corneas from injured mice show upregulation of vimentin and alpha-smooth muscle actin expression to equivalent levels as WT corneas, illuminating that desmin deficiency does not interfere with myofibroblast differentiation. Employing the small molecule withaferin A (WFA), an inhibitor of vimentin, we show that WFA treatment causes the decrease in steady state levels of vimentin and serine 38 phosphorylated vimentin, the latter a biomarker associated with corneal fibrosis, and improved corneal clarity through blockade of myofibroblast differentiation. To investigate further the mechanism of fibrosis in desmin deficiency, we examined keratin 8 expression in the epithelium, and found reduced levels of this cytokeratin in injured Des KO corneas compared to WT corneas. This finding also corroborates the decrease of cell proliferation in injured Des KO corneas compared to that in WT corneas. The fibrotic phenotype of Des KO corneas also features abundant vascularization, further exemplifying the magnitude of corneal pathology. Together, these findings illuminate that desmin does not contribute significantly to corneal fibrosis in this injury model.

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Author Contributions

Conceived and designed the experiments: PBM RM. Performed the experiments: AP CH SC LL GT SP-G PBM. Analyzed the data: AP CH SC LL GT MAS YC GT SP-G PMB RM. Wrote the paper: PBM RM

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Keywords

Desmin; corneal fibrosis; vimentin; phosphorylated vimentin; withaferin A; myofibroblast; keratin K8; cell proliferation

1. Introduction

After corneal injury, keratocytes differentiate under the influence of transforming growth factor-beta (TGF- β) into myofibroblasts [1]. Corneal keratocytes express low levels of the type III intermediate filament (IF) protein vimentin, whose expression and phosphorylated forms becomes increasingly abundant when differentiating into fibroblasts and myofibroblasts [2]. Vimentin plays an important role in wound healing having functions that are complex, such as involvement in cell migration, cell proliferation, epithelial-to-mesenchymal transition, signal transduction and myofibroblast generation [3,4,5,6,7,8]. Vimentin-deficient (Vim KO) mice remarkably show normal development and are fertile, but these mice exhibit, among others, deficits in dermal wound healing due to delayed appearance of myofibroblasts [9,10]. On the other hand, Vim KO mice subjected to corneal alkali injury regenerate their corneal epithelium and stroma and showing an attenuation of angiogenesis [11] and fibrosis [12] that leads to the recovery of corneal transparency. This anti-fibrotic phenotype was related to deficits in myofibroblast differentiation, as the activation of alpha-smooth muscle actin (α -SMA) expression in vimentin-deficient fibroblasts in response to TGF- β was found to be impaired [12]. Injured Vim KO corneas do however develop a milder pathology, as revealed by the development of corneal haze. The involvement of a second type III IF protein desmin, which is not expressed in uninjured cornea starts to increase in abundance in corneal myofibroblasts after injury in wild-type (WT) and is found at lower levels in injured Vim KO corneas. These findings have implicated that vimentin and desmin overexpression contribute to corneal fibrosis and haze, respectively [12].

Vimentin and desmin share a common conserved binding site for the type III IF small molecule inhibitor withaferin A (WFA) [5]. Thus, WFA has been exploited as a pharmacological agent to target the overexpression of these IF proteins in corneal alkali injury showing it to be a potent inhibitor of corneal fibrosis in WT mice, as well as reducing corneal haze (in Vim KO mice), potentially through reduction of desmin levels as well [12]. As WFA targets soluble forms of type III IF proteins [5], the increased abundance of soluble serine 38 phosphorylated vimentin (p-Ser38Vim) that occurs during keratocyte-fibroblast-myofibroblast transition sensitizes soluble vimentin to WFA in differentiating fibroblasts leading to decreases in steady state levels of vimentin [2]. Although further details of the mechanism are still unclear, these initial effects of WFA on soluble forms of vimentin result in subsequent reduction in vimentin intermediate filaments *in vivo* [2]. This and other findings, made in other injury models, as well [13,14], have borne out the idea that overexpression of vimentin and desmin could contribute to corneal fibrosis/haze [15], yet the direct proof of whether desmin is a driver of corneal fibrosis remains elusive.

To directly test the hypothesis that desmin contributes to corneal fibrosis, we investigated mice that were genetically deficient in desmin (Des KO) [16] and employed the alkali injury model to ascertain their wound healing response. To our surprise, we found that Des KO mice lose corneal transparency and develop corneal fibrosis as readily as WT mice. Treatment of Des KO mice with WFA improved corneal transparency by downregulation of vimentin, identifying vimentin as the major driver of corneal fibrosis.

2. Materials and methods

2.1 Animals and in vivo corneal injury model

All animal experiments were conducted in accordance with the Declaration of Helsinki and Institutional Animal Care and Use Committees of the University of Connecticut Health Center approved procedures. For this study, we used both female and male 6–10 week-old WT mice. The desmin-deficient mice (Des KO) in 129 Svej background were obtained as breeding pairs from collaborating investigator's laboratory, who developed and characterized these mice extensively [16]. The Des KO mice were rederived at the University of Connecticut Health Center and maintained as homozygous breeding colonies. The corneal alkali injury model has been described in detail previously [12]. Briefly, mice were anesthetized, and 0.15 N NaOH was applied for 1 minute to the cornea, then the eyes were washed with buffered saline solution, and limbal and central corneal epithelium was gently removed by mechanical scraping. Mice were allowed to recover with application of topical antibiotics following the injury.

2.2 Drug treatment

Withaferin A (WFA) was purchased from Chromadex (ASB-00023250–250) and stored in aliquots at -20°C dissolved in dimethyl sulfoxide (DMSO). Injured mice were treated with vehicle (DMSO) or WFA (2 mg/kg solubilized in DMSO) on the day of injury, and every subsequent day, by intraperitoneal (i.p) injection for the duration of 7 or 14 days post injury [12].

2.3 Immunofluorescence

2.3.1 Analysis of frozen sections—At specific time points post-injury, WT and Des KO mice were sacrificed, eyes enucleated, and immediately frozen at -80°C or embedded in OCT compound and stored at -80°C . Whole eyes were subjected to cryosectioning at -20°C and tissue collected on glass slides, and fixed in 4% paraformaldehyde. Tissue slides from control, DMSO-treated, and WFA-treated samples were probed with the following primary antibodies: anti-rabbit phosphor-vimentin (pSer38Vim, 1:100, Santa Cruz Biotechnology, Cat# sc-16673R), anti-rat vimentin (1:200; R&D systems, Cat# MAB2105), or anti-rabbit α -Smooth Muscle Actin (α -SMA, 1:200, Abcam Cat# ab5694) overnight at 4°C , diluted in a background-reducing buffer (Dako, North America). After three washes with phosphate buffered saline (PBS), sections were probed with the respective secondary antibodies conjugated to Alexa Fluor 488 goat anti-rabbit IgG (1:1000, ThermoFisher Scientific Cat# A-11034), or Alexa Fluor 555 goat anti-rat (1:500, ThermoFisher Scientific Cat# A-21434) for 45 min at room temperature, and counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1 mg/ml in PBS) for 10 min. Digital images were acquired on an

Olympus IX81 fluorescence microscope equipped with MetaMorph software. Images were assembled using Adobe Photoshop Software.

2.3.2 Analysis of whole mount samples—For immunofluorescence studies, tissues were pooled for each time point and wound type and fixed immediately after euthanization in a paraformaldehyde-containing fixative (1× PBS, 1% formaldehyde, 2 mM MgCl₂, 5 mM ethylene-bis(oxyethylenitrilo)tetraacetic acid(EGTA), 0.02% octylphenoxypolyethoxyethanol (Nonidet NP-40) for 1 hour and 15 min at 4°C, followed by 2 washes for 10 min each in 1× PBS containing 0.02% Nonidet NP40 at room temperature. Tissues were placed in 4:1 methanol: DMSO for 2 hours at −20°C and then stored in 100% methanol at −20°C until used for whole mount staining studies.

For the whole mount procedure, the back of the eye was cut, retina, lens, and iris removed before staining. Tissues were transferred to a graded methanol-TritonX-100 series (75%, 50%, and 25% methanol:TritonX-100 for 15, 15 and 10 min respectively). All incubations were performed with gentle shaking and at room temperature, unless otherwise specified. The eyes were washed twice in PBS, for 30 min each, followed by incubation with blocking buffer for 2 hours. Blocking buffer was made as follows: to 100 ml 1× PBS, 1g of bovine serum albumin (BSA) was added, the mixture was stirred for 10 min, 1 ml of horse serum was added, and the mixture was stirred for an additional minute. The tissues were then incubated overnight with primary antibody diluted in blocking buffer at 4°C. The next day, the tissues were washed five times with PBS and 0.02% Tween 20 (PBST) for 1 hour each, blocked for 2 hours, and then incubated with secondary antibody diluted in blocking buffer overnight at 4°C. The following day, eyes were washed three times with PBST for 1 hour each, followed by nuclear staining with DAPI (Thermo fisher; #46190) for 5 min, and washed with distilled water. To achieve the best flattening, the corneas were placed epithelial side-up with mounting media (Fluoromount G; Electron Microscopy Sciences; #17984–25) and coverslipped. Corneal flatmounts were stained with the following antibodies: keratin K8 (K8) (DSHB, University of Iowa, #TROMA-1), keratin K18 (K18) (RDI, #CBL236), Ki67 (Abcam, #ab16667). Appropriate secondary DyLite 488, 594, and 647 antibodies from Jackson ImmunoResearch were used for immunolabeling.

2.3.3 Confocal Microscopy—Confocal microscopy was performed at the GW Nanofabrication and Imaging Center at the George Washington University Medical Center. For high-resolution immunofluorescence imaging, a confocal laser-scanning microscope (Zeiss 710) was used to image the localization of Alexa Fluor 488 (argon laser; 488 nm laser line excitation; 495/562 emission filter), Alexa Fluor 594 (561 diode laser; 594 nm laser line excitation; 601/649 emission filter) and Alexa Fluor 647 (633 Diode laser; 647 nm laser line excitation; 671/759 emission filter). Optical sections ($z=0.5\ \mu\text{m}$ or $1\ \mu\text{m}$) were acquired sequentially with a 63× objective lens.

3D images were obtained using the XYZ option in Volocity software (Version 6.3, Perkin Elmer). High-resolution images are presented in their XY, XZ and YZ planes. 10× images for K8 and Ki67 were obtained using the Nikon Eclipse E600. Data analysis for Ki67 was done using ImageJ using the cell counting module. The analysis of K8 data was subjected to nonparametric two-tailed Mann-Whitney *U* test using GraphPad Prism 7 statistical software.

2.4 Transmission electron microscopy

Samples from uninjured, and 14d-injured Des KO mice were collected for transmission electron microscopy analysis (TEM) analysis as previously described for WT and Vim KO mice [12]. Briefly, the central part of the mouse cornea was excised, and processed for TEM. Three different corneas were analyzed from Des KO mice.

2.5 Western blot analysis

At specific time points post-injury, WT and Des KO mice were sacrificed, and corneas were collected. Three corneas for each treatment condition were pooled for protein extraction. Protein extraction was carried out following a previously described protocol [12]. Briefly, for total protein extraction, corneas were lysed in an ice-cold buffer containing 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 20 mM HEPES, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 400 mM NaCl, 1 mM EGTA, 0.1% Nonidet P-40, and a protease inhibitor mixture cocktail (Roche Applied Science, cat # 11–836–153–001). 2% sodium dodecyl sulphate (SDS; w/v) was added, and after flash freezing and thawing, samples were centrifuged for 10 min at 14,000 rpm, and supernatants were collected. For soluble protein extraction, corneas were lysed in an ice-cold buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Nonidet NP-40, 5 mM NaF, 1 mM PMSF, 1 mM DTT, and a protease inhibitor mixture (Roche Applied Science, Cat # 11–836–153–001). Extracts were left on ice for 45 min, and then centrifuged for 10 min at 14,000 rpm at 4°C to collect the soluble fraction. Equal amounts of proteins from both experimental protocols (total and soluble extractions) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins transferred to polyvinylidene difluoride (PDVF) membranes, Bio-Rad, Hercules, CA, USA). Protein blots were probed with anti-rabbit phospho-vimentin (pSer38Vim, 1:400, Santa Cruz Biotechnology, Cat# sc-16673-R), anti-mouse vimentin (clone V9, 1:400, Santa Cruz Biotechnology, Cat# 6260), and anti-rabbit GAPDH (1:1000, Abcam, Cat# ab8245). Blots were scanned, and band intensities were quantified using NIH ImageJ software, and normalized to GAPDH bands.

3. Results

3.1 Des KO mice develop corneal opacity and tissue abnormalities upon alkali injury

We subjected mice to corneal alkali injury and found that both WT and Des KO mice developed corneal opacity that became quite prominent at day 14 (14d) post-injury (Figure 1A). To determine whether corneal opacity was also retained at the later time points, we injured another cohort of mice and examined them at 30d post-injury. Corneal opacity was found to be apparent also at this later time point (Figure 1A), revealing that injury-induced loss of corneal transparency was not temporary. Furthermore, biomicroscopic analysis by blinded scoring revealed similar levels of corneal opacity between the Des KO mouse line and WT mice at the 14d or 30d post-injury time points (Figure 1B). Hematoxylin and eosin staining of corneal tissue sections from control Des KO mice further revealed that there were no apparent abnormalities due to desmin deficiency (Figure 1C). Next, we compared the ultrastructure of corneas from control uninjured and injured Des KO corneas by transmission electron microscopy (TEM) analysis. We found that 14d injured Des KO mice present with several corneal abnormalities compared to the uninjured Des KO controls (Figure 1D

and 1E). In uninjured Des KO corneas, an intact basement membrane (Figure 1D, panel ii) separated the corneal epithelium (Figure 1D, panel i) from the stroma containing keratocyte(s) (Figure 1D, panel iii) embedded in a well-organized collagen network (Figure 1D, panel iv). This finding confirmed that there were no apparent corneal abnormalities due to desmin deficiency also at the ultrastructural level. As previously reported with alkali injury in 14d-injured WT corneas [12], the presence of numerous goblet cells in the epithelium was similarly observed also in injured Des KO corneas at this time point (Figure 1E, panel ii), and the basement membrane showed the absence of the regenerated lamina densa (Figure 1E, panel iii). Myofibroblasts containing numerous mitochondria (Figure 1E, panel iv) and large amounts of rough endoplasmic reticulum (Figure 1E, panel v, arrows) were also embedded in a disorganized collagen matrix (Figure 1E, panel vi). Together, these results revealed that while uninjured Des KO mice showed the anticipated cellular and ultrastructural elements of a normal transparent cornea, the injured Des KO corneas revealed several hallmark structural changes consistent with the phenotype of significantly reduced corneal transparency seen in this model of injury as previously reported for WT mice [12].

3.2 Alkali injury induces expression of fibrotic markers in Des KO mice

Next, we examined the expression of fibrotic markers by immunohistochemistry in corneas from WT and Des KO mice at 14d post-injury (Figure 2). In control uninjured corneas from WT and Des KO mice, the resident keratocytes, identified by their spindle-like cell shape, expressed constitutive low levels of vimentin, and the serine 38 phosphorylated isoform of vimentin (p-ser38Vim) (Figure 2, panels C and F, iii). The stroma of WT and Des KO corneas were negative for α -SMA expression (Figure 2, panels B and E, i). At 14d post-injury, a prominent number of myofibroblasts (α -SMA⁺) were present in the stroma of both WT and Des KO corneas. These differentiated α -SMA⁺ cells in injured Des KO corneas displayed an enlarged cytoplasm (Figure 2, panel E, iii), and similar to injured WT corneas, injured Des KO corneas also expressed high levels of vimentin (Figure 2, compare panels A and D, ii) and p-Ser38Vim (Figure 2, compare panels C and F, ii). To corroborate these data western blot analysis was performed from injured and non-injured corneas (Figure 3). Western blot analysis also showed increased expression of vimentin and p-Ser38Vim in injured WT corneas, showing a 2.3-fold and 4.9-fold increase in vimentin and p-Ser38Vim expression at 14d post-injury, respectively. Importantly, injured Des KO corneas also showed increased expression of vimentin as well as p-Ser38Vim at 14d post-injury (2.6 and 3.4-fold, respectively). This increased expression of vimentin and p-Ser38Vim was maintained also at 30d post-injury in both WT mice (2.3 and 3.6-fold, respectively) as well as in Des KO corneas (2.1 and 3.2-fold, respectively) over their respective uninjured controls.

3.3 Withaferin A downregulates the corneal fibrotic phenotype in Des KO mice

We next investigated whether the corneal fibrosis in Des KO mice was sensitive to the activity of WFA, as vimentin and p-Ser38Vim expression were increased in fibrotic corneas. Des KO mice were subjected to corneal alkali injury and examined at both 7d and 14d post-injury with cohorts treated with vehicle or WFA (2 mg/kg/d) by i.p. injections. Mice treated with vehicle showed presence of corneal haze at the 7d time point that was found to convert to fibrosis by 14d post-injury (Figure 4). WFA treatment improved corneal clarity at both the 7d and 14d time points significantly ($P < 0.05$). Immunohistochemical analysis of fibrotic

markers at 14d post-injury revealed that WFA treatment caused a reduction in protein levels of vimentin, pSer38Vim, as well as, α -SMA (Figure 4, panels C, D, E). Vehicle-treated corneas of Des KO mice showed that the differentiated resident cells displayed an enlarged cytoplasm typical of cells undergoing transition from wound fibroblasts into myofibroblasts, as represented by the strong expression of α -SMA. Next, we investigated by western blot analysis the expression levels of vimentin and p-Ser38Vim in injured corneas from vehicle and WFA-treated Des KO mice. We confirmed that vimentin expression and p-Ser38Vim levels were increased by injury in Des KO corneas in both the soluble and cytoskeletal (insoluble) protein fractions (Figure 5). As anticipated from previous findings [2,5,6], multiple p-Ser38Vim species with increased abundance were detected in response to injury compared to uninjured controls. Interestingly, many of these p-Ser38Vim antigen-immunoreactive bands that are seen increased in the soluble and insoluble fractions from injured corneas were differentially reduced in WFA treated corneas. The 75 kDa pSer38Vim species was not consistently altered by injury and WFA treatment, therefore, only the bands below this size (marked by the bracket, Figure 5) were quantified. Taken together, our findings show that while desmin loss does not interfere with corneal fibrosis, the injury-induced overexpression of vimentin remains sensitive to WFA and was associated with improved phenotype of Des KO mice.

3.4 Altered keratin K8 and K18 expression in injured corneas of Des KO mice

In previous studies, we had reported that Vim KO corneas are protected from fibrosis, revealing that an intact epithelium was restored after injury in this alkali injury model [12]. However, the presence of goblet cells in the epithelium of injured Des KO corneas demonstrated by TEM analysis, similar to that previously reported in WT corneas [12], prompted us to examine the epithelium for keratin staining. In tissues from 14d-injured corneas, keratin 18 staining was not restricted to cells alone as additional extracellular immunostaining was detected (Figure 6). This made it challenging to make comparisons with Des KO corneas and draw conclusions. However, K8 immunostaining was strongly detected specifically in goblet cells of injured WT corneas (Figure 6). Therefore, we quantified the levels of K8+ cells in WT and Des KO injured corneas using different quadrants of the cornea for this analysis by confocal microscopy. We found that injured Des KO corneas showed significantly fewer K8+ cells, being 3.9-fold lower than that compared to injured WT corneas ($p = 0.0002$) (Figure 7). This finding revealed that K8 expression is aberrantly regulated upon injury in Des KO corneal epithelium compared to WT mice.

3.5 Reduction of cell proliferation in injured corneas of Des KO mice

As failure of the corneal epithelium to regenerate the basement membrane can promote stromal fibrosis [15], we next examined the state of cell proliferation in epithelium of injured WT and Des KO mice. WT and Des KO mice were subjected to corneal alkali injury and eyes from non-injured and 14d post-injured mice were subjected to whole mount corneal immunostaining for Ki67 and examined by confocal microscopy (Figure 8, panel A). Although control unwounded Des KO corneas had an apparent small increase in number of cells undergoing proliferation compared to unwounded corneas from WT controls, this was not significant ($P > 0.05$; non-parametric ANOVA Kruskal-Wallis test). This pattern of Ki67 staining was reversed in the injury state. The injured WT corneas recovered levels of Ki67

staining to that observed in control unwounded WT corneas, but an almost 6-fold-lower level of Ki67 staining was observed in injured Des KO corneas compared to injured WT corneas ($P < 0.05$) (Figure 8 B). This pattern of reduced cell proliferation in the injured Des KO corneas was consistently observed across the peripheral, peripheral-central region and central regions of the cornea.

4. Discussion

The major finding of this study is that desmin is not required for corneal fibrosis in the setting of alkali burn injury. Des KO mice when injured develop corneal opacity to similar levels as their WT counterparts. This phenotype is evidenced by sustained increases in the expression of vimentin and α -SMA-expressing myofibroblasts that were abundant in corneas of Des KO injured mice, showing similar equivalent levels to that in WT injured mice.

The type III IF protein desmin is not normally expressed in any layers of the cornea in the adult mouse [12,13]. After corneal injury, desmin expression is *de novo* induced in myofibroblasts, at a time point somewhat later than vimentin and α -SMA expression becoming prominent in stromal myofibroblasts during the course of wound healing. Thus, it has been hypothesized that desmin could be a biomarker of the mature myofibroblasts associated with corneal haze in the rodent PRK model [15]. In the alkali injury model, desmin expression becomes significantly increased considerably earlier (by 7d post injury) in WT corneas, but has a delayed expression in Vim KO corneas (at 14d post injury), and also is produced at lower levels in Vim KO corneas [12]. These previous findings which relate injury-induced expression of desmin with corneal pathology (fibrosis or haze) led to the suggestion that desmin may be produced temporally later in myofibroblast development to boost their contractile function [12]. Desmin and vimentin can form heteropolymeric IFs forming stronger cytoplasmic filaments [17] and promote scar formation [18]. However, despite this logical model, vimentin+/ α -SMA+ overexpressing myofibroblasts accumulate in injured Des KO corneas and contribute to corneal fibrosis similarly to injured WT corneas. Thus, a role for desmin in corneal fibrosis is not supported by our data. Moreover, detection of several p-Ser38Vim antigenic species reflecting a complex pattern of vimentin phosphorylation that we previously associated with myofibroblast differentiation [2], were also observed in fibrotic corneas of Des KO mice, and shown to be decreased along with reduction in total vimentin levels by WFA treatment. Also, another study using shRNA strategy in a suture-based corneal injury model demonstrated vimentin downregulation was sufficient to block corneal fibrosis [19]. Taken together, these findings identify vimentin as a major driver of corneal fibrosis in the setting of injuries.

The mechanism of corneal fibrosis in desmin deficiency is intriguing considering vimentin deficiency promotes regenerative healing in alkali-injured corneas [12]. Prior studies have extensively documented the development of fibrosis in Des KO heart [20,21]. It was recently shown that cardiac fibrosis in Des KO mice can be rescued through genetic overexpression of TNF- α , which leads to NF- κ B activation and the ectopic expression of K8/K18 expression in cardiomyocytes [22]. The inflammatory signaling pathway linking TNF- α to keratins appears to be mediated by desmin deficiency and likely plays a role in governing

the outcome in alkali-injured corneas. TNF- α and NF- κ B activation occurs in alkali injured corneas of WT mice [23,24,25]. Our findings show that K8 is expressed similarly in unwounded Des KO corneal epithelial cells but downregulated relative to WT corneas after wounding. In addition, we found that after wounding, epithelial cell proliferation is reduced in Des KO corneas relative to WT wounded corneas. After wounds that affect the entire ocular surface, the limbal stem cells proliferate and differentiate into corneal epithelial and goblet cells that express K8 [26]. Reduced K8 expression may be secondary to the fact that Des KO corneal epithelial cells are less proliferative after injury. In WT mice, activation of NF- κ B/TNF- α signal transduction could impact differentiation of limbal epithelial cells into K8+ goblet cells. It is known that NF- κ B activation is important for corneal wound healing, as the deletion of I κ B kinase β exacerbates corneal fibrosis in the alkali injury model [27]. Keratin K8 null-mice develop fibrosis in the liver [28], suggesting the K8 deficiency, or also decreased expression of K8 as in the case of injured Des KO corneas, can be pathological. Taken together with prior findings [12], it is clear that vimentin and desmin do not reciprocally compensate for the loss of each other's functions in the injured cornea, and as such, other novel mechanisms to overcome type III IF protein deficiency are elicited to either attenuate or drive corneal fibrosis in their respective deficiencies.

Goblet cell accumulation in the repairing epithelial layer of Des KO corneas was similar to that of WT mice [26]. However, Vim KO mice subjected to alkali injury recover an almost-native epithelium, revealing the absence of goblet cells in their regenerated corneas [12]. Thus, the reduced barrier function caused by the presence of goblet cells on the corneal surface of Des KO corneas coupled with their secretion of mucins and other proteins would induce corneal neovascularization [29]. While we did not pursue the quantitation of corneal neovascularization because this was not a focus of the present study, nevertheless, numerous corneal blood vessels were observed by biomicroscopy in alkali-injured corneas of Des KO mice (see Figure 1). In essence, desmin deficiency does not alter the complex pathological outcome in this model of alkali injury on several levels. It would be interesting to examine other types of injuries that do not produce limbal cell deficiency or neovascularization [30,31] as more subtle roles for desmin in these other injury scenarios might become apparent.

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Highlights

- Corneal fibrosis occurs in desmin deficiency
- Desmin deficient corneas when injured show similar high levels vimentin and serine-38 phosphorylated vimentin as wild type corneas
- Desmin deficient corneas when injured show reduced epithelial cell proliferation compared to wild type mice
- Desmin deficient corneas when injured have lower levels of keratin K8 compared to wild type mice
- Withaferin A targets vimentin and downregulates corneal fibrosis in desmin deficient mice

Figure 1. Corneal wound healing in Des KO mice.

WT and Des KO mice were subjected to corneal alkali injury. A) Representative images of mouse eyes photographed at 14d and 30d post-injury ($n = 8$ samples/group). B) Quantification of corneal opacity by biomicroscopy at 14d and 30d post-injury using an opacity scale of 0 (clear) to 4 (opaque) ($n = 8$ samples/group). $P > 0.05$ was non-significant (ns). C) Hematoxylin and eosin stained corneal sections from uninjured WT and Des KO mice. Corneas from control and 14d post-injured Des KO mice were prepared for transmission electron microscopy (TEM). D) In control samples, the basement membrane (BM, panel D, ii), including the lamina densa (asterisks, panel D, ii), separated the regular epithelium from the stroma, in which keratocytes (panel D, iii) were embedded surrounded by a well-formed collagen network (panel D, iv). E) In injured samples, goblet cells are present in the disrupted epithelium (panel E, ii), and there was no evidence of BM between the epithelium and stroma (panel E, iii). Myofibroblasts containing large number of mitochondria (panel E, iv), and rough endoplasmic reticulum (panel E, v), were surrounded by disorganized extracellular matrix (panel E, vi).

Figure 2. Alkali injury induces corneal fibrosis in Des KO mice.

WT and Des KO mice were subjected to alkali injury ($n = 8$ animals/group). Mice were sacrificed at 14d and tissue sections of corneas were processed for immunofluorescence analysis. Representative images of WT (A-C) and Des KO (D-F) samples stained with antibodies against either vimentin (panel A, D, *red*), α -SMA (panel B, E, *green*), or p-Ser38Vim (panel C, F, *red*), and all counterstained with DAPI (*blue*). Inset panels provide improved visualization of different cell morphologies in injured samples compared to uninjured (control). Bar scale = 105 μ m. Images were taken at 20X magnification.

Figure 3. Vimentin expression is sustained in injured Des KO mice.

WT and Des KO mice were subjected to alkali injury ($n = 8$ animals/group). Corneal buttons from 14d and 30d post-injury were processed for western blot analysis. Equal amount of total protein extracts were fractionated by SDS-PAGE, and blots were probed with antibodies against vimentin (clone V9), and p-Ser38Vim. GAPDH was used as loading control. A) Representative images of western blots from WT corneas with densitometric quantification of V9 (panel B) and p-Ser38Vim (panel C), normalized to GAPDH. D) Representative images of western blots from Des KO corneas with densitometric quantification of V9 (panel E) and p-Ser38Vim (panel F), normalized to GAPDH. Each lane represents protein lysate pooled from 3 corneas, and the western blot is a representative of three independent experiments. The closely-spaced bands for p-Ser38Vim marked by the brackets (panels A and D) were used for quantitation. The 75 kDa band was not consistently altered by injury. $P < 0.05$ was considered significant and marked by * and **.

Figure 4. WFA downregulates corneal opacity in injured Des KO mice.

Des KO mice were subjected to corneal alkali injury and groups of mice received daily i.p. injections of either DMSO (Veh) or WFA (2 mg/kg/d) for 7 or 14 days. A) Representative images of mouse eyes photographed at 7d and 14d post-injury ($n = 8$ animals/group). B) Quantification of corneal opacity by biomicroscopy analysis at 7d and 14d post-injury using an opacity scale of 0 (clear) to 4 (opaque) ($n = 8$ animals/group). $P < 0.05$ was considered significant and marked by * and **. Representative images of Des KO corneal samples at 14d post-injury stained with antibody against either vimentin (panel C, red), α -SMA (panel

D, green), or p-Ser38Vim (panel E, red), and all counterstained with DAPI (*blue*). Scale bar = 105 μm . Images were taken at 20X magnification.

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Figure 5. WFA attenuates corneal fibrosis in the Des KO mouse by reducing vimentin protein levels.

WT and Des KO mice were subjected to corneal alkali injury. Mice received daily i.p. injections of DMSO (Veh) or WFA (2 mg/kg/d) for 14 days. Mice were then sacrificed, and corneas were processed for western blot analysis. A) Equal amount of soluble protein extracts were fractionated by SDS-PAGE, and blots were probed with antibodies against vimentin (clone V9) and p-Ser38Vim. GAPDH was used as loading control. B, C) Quantitation of band density for soluble vimentin (panel A) and soluble p-Ser38Vim (bands marked by bracket, panel A) normalized to GAPDH. D) Cytoskeletal insoluble protein fractions were solubilized and fractionated by SDS-PAGE, and blots were probed with antibodies against vimentin (clone V9) and p-Ser38Vim. GAPDH was used as loading control. E, F) Quantitation of band density for insoluble vimentin and insoluble p-Ser38Vim (panel D). Each lane represents protein lysate pooled from 3 corneas, and the western blot is a representative of three independent experiments. The closely-spaced bands for p-Ser38Vim marked by the bracket (panels A and D) were used for quantitation. The 75 kDa band was not consistently altered by injury or WFA treatment. $P < 0.05$ was considered significant and marked by * and **.

Figure 6. Keratin K8 and K18 expression in Des KO mouse corneas.

WT and Des KO mice (n=8/group) were subjected to corneal alkali injury and mouse eyes analyzed for K8 and K18 expression by immunostaining. Confocal microscopy was performed on corneal whole mount immunostained tissues. DAPI counterstaining was used to mark cell nuclei. Representative *en face* images and orthogonal views of flat-mounted corneas are shown. Note the significant extracellular staining for K18.

Figure 7. Keratin K8 expression is decreased in Des KO mouse corneas.

WT and Des KO mice (n= 8/group) were subjected to corneal alkali injury and mouse eyes analyzed for expression of K8 expression by immunostaining. Confocal microscopy was performed on corneal whole mount immunostained tissues and four quadrants from each cornea were imaged. NIH ImageJ software was employed using the cell counts module for analysis. The scatter plot with bar represents the distribution of cell counts measured from each of 4 quadrants derived from the groups of eight eyes/genotype showing the means $\pm$ SEM. A significant difference ($P=0.0002$) was observed.

Figure 8. Reduced cell proliferation in injured Des KO mouse corneas.

Wild type (WT) and Des KO mice were subjected to corneal alkali injury. Unwounded controls and 14d injured corneas from WT and Des KO mice (n=8 mice) were subjected to immunostaining for Ki67 expression on corneal whole mounts and staining analyzed by confocal microscopy. A) Representative images from the central and peripheral cornea are shown. B) The numbers of Ki67+ cells/field in the central, peripheral and central-peripheral regions of the cornea were counted and graphed. $P < 0.05$ was considered significant and marked by *, ** and ***; ns= non-significant. Scale bar = 20 μ m.