Heliyon 9 (2023) e13397

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Thrombospondin-1 induction and VEGF reduction by proteasome inhibition

Fawzia Bardag-Gorce [a,b,*], Carter Hoffman [a,b], Imara Meepe [a], Monica Ferrini [b], Richard H. Hoft [a], Joan Oliva [c], Yutaka Niihara [a,c]

[a] The Lundquist Institute at Harbor UCLA Medical Center, Torrance, CA, 90502, USA
[b] Charles R. Drew University of Medicine and Science, Los Angeles, CA 90059, USA
[c] Emmaus Medical, 21250 Hawthorne Blvd, Suite 800, Torrance, CA, 90505, USA

ARTICLE INFO

Keywords:
Corneal vascularization
Proteasome inhibition
Thrombospondin-1
VEGF

ABSTRACT

The present study focuses on investigating the expression of thrombospondin-1 (TSP-1), a natural inhibitor of neovascularization. Immunofluorescent staining was used to detect the expression of TSP-1 in rabbit corneal tissue with vascularization induced by limbectomy. TSP-1 was detected in healthy and Cultured Autologous Oral Mucosal Epithelial Cell Sheet (CAOMECS) grafted rabbit corneas. TSP-1 was not detected in diseased corneas. Rabbit and human primary oral mucosal and corneal epithelial cells were cultured and treated with proteasome inhibitor (PI) in vitro. Changes in the expression of TSP-1, HIF-1 alpha and 2 alpha, VEGF-A, and VEGF receptor were analyzed by Western blotting. Neovascularization developed in rabbits' corneas as early as 1 month after limbectomy and was stable for at least 3 months. HIF-1 alpha and VEGF-A expression was reduced in CAOMECS grafted corneas, as compared to sham corneas. While TSP-1 expression was decreased in injured corneas, it was expressed in CAOMECS grafted corneas, but still less expressed compared to healthy corneas. PI treatment, of human oral mucosal and corneal epithelial cells increased TSP-1 expression and reduced VEGF-A expression. The results showed that TSP-1 expression was lost in injured corneal surface and that CAOMECS grafting restored TSP-1 expression to certain extent. Proteasome inhibition treatment increased TSP-1 and decreased VEGF-A expression in human oral mucosal and corneal epithelial cells. The result suggests that corneal neovascularization could be managed with the inhibition of the proteasome after CAOMECS grafting and increase corneal transparency.

1. Introduction

The cornea is free of blood vessels and completely transparent. It refracts light onto the lens, which then refocuses the light onto the retina, allowing signal transmission and visual sense [1]. Corneal neovascularization occurs as the result of damage to the limbal stem cell niche, which causes the conjunctival epithelial cells to invade the corneal surface and promote angiogenic and inflammatory process. Without a clear and unobstructed cornea, as is the case with corneal neovascularization, a person's ability to refract light is severely impaired. Thus, visual acuity is drastically impacted and often results in corneal blindness [2].

Pro-angiogenic and anti-angiogenic factors are off-balance in vascularized cornea, with pro-angiogenic factors being amplified in

* Corresponding author. The Lundquist Institute at Harbor UCLA Medical Center, Torrance, CA, 90502, USA.
E-mail addresses: fgorce@lundquist.org (F. Bardag-Gorce), cj_hoffman5@outlook.com (C. Hoffman).

https://doi.org/10.1016/j.heliyon.2023.e13397
Received 8 November 2022; Received in revised form 27 January 2023; Accepted 30 January 2023
Available online 1 February 2023

what is known as an "angiogenic cascade" [3,4]. Corneal epithelial and endothelial cells in conjunction with macrophages are responsible for the production of pro-angiogenic factors such as fibroblast growth factors and vascular endothelial growth factor (VEGF) [5,6]. Increased production of VEGF leads to the endothelial cell-driven upregulation of matrix metalloproteinases (MMPs) in the vascular plexus of the limbus, ultimately forming immature blood vessels [5–7]. New blood vessel formation has been demonstrated to lead to chronic inflammation and scarring, which yields poor corneal transparency and visual acuity [1,8]. Correcting the balance of pro-angiogenic and anti-angiogenic factors is an essential process to inhibit corneal neovascularization. There is a growing interest in studying key pro-angiogenic factors such as TNF-α, MMP-2 & MMP-3, VEGF, and bFGF, as well as some significant anti-angiogenic factors such as TIMP-1 & TIMP-3, MMP-7 & MMP-13, COL8A-1, and endostatin [9–11]. Many other cytokines, interleukins and other metalloproteinases act as important angiogenic factors [12].

TSP-1 has been shown to be a potent anti-angiogenic factor. TSP-1 up regulation has been shown to be a potential candidate for alleviating corneal neovascularization as it functions to limit vessel density in normal tissues [13]. The thrombospondin protein family consists of five glycoproteins consisting of TSP-1 through TSP-5, which are derived from the thrombospondin genes [3,5]. TSP-1 has been found in corneal and conjunctival tissues, as well as within activated endothelium, within the spinal cord, and in healing wounds [5]. Functionally, TSP-1 possesses characteristics that allow it to play a role in angiogenic, immune, and lymphangiogenic functions [5, 13–15]. TSP-1 is an all-encompassing extracellular matrix protein that is expressed mainly by the corneal and conjunctival epithelium [5,13]. It is known to mainly interact with integrins, glycosaminoglycans, CD36/47, and other receptor proteins [5,13].

TSP-1 anti-angiogenic properties are believed to stem from its unique peptide sequence that activates the cytokine known as latent Transforming Growth Factor beta (TGF-b), which has supportive-immunomodulatory [13,16]. TGF-b not only modulates immune responses, but also aids in wound healing and cell proliferation [5,16]. We stipulated that the inhibition of TSP-1 would thus ultimately affect TGF-b activity as well. The authors [3] demonstrated the significance of TSP-1 in mice when comparing it to TSP-2 mouse models. The experiment showed how modulation of TSP-1 had a greater significance than TSP-2 in terms of corneal angiogenesis [3].

Understanding the balance between pro-angiogenic and anti-angiogenic factors is essential in managing corneal neovascularization. This study seeks to understand the expression of TSP-1 in healthy, diseased, and grafted corneas and the modulation of TSP-1 expression in oral mucosa and corneal epithelial cells.

2. Material & methods

2.1. Animal

New Zealand white rabbits male and female weighing 2.5–3 kg and 3–4 month old were used. They were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences published by the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council. The experimental protocol was approved by the IACUC and performed as previously reported [9]. The rabbits were briefly sedated, subjected to lamellar limbectomy, and followed for 3 months. A small biopsy of rabbit buccal tissue was then performed to produce CAOMECS. After 2–4 weeks, CAOMECS was fully grown as a multilayered cell sheet and was grafted back onto cornea. The ophthalmologist surgeon removed diseased corneal epithelia [9] that were used for Western blot analysis in comparison to healthy corneal epithelium. CAOMECS was then grafted back onto cornea. The control or sham group did not receive CAOMECS graft. CAOMECS grafted rabbits were then followed for 6 months to examine the therapeutic effects of CAOMECS grafting [9]. Corneas were then harvested, fixed, and examined with fluorescent immunostaining. Therefore, Western blot experiments were conducted on diseased corneal epithelia at 3 months. Fluorescent immunostaining experiments were conducted on sham corneas and CAOMECS grafted corneas at 9 months.

2.2. Oral mucosal epithelial cells isolation and CAOMECS engineering

Rabbit's buccal mucosa biopsies were dissociated using Dispase I (Roche Diagnostics GmbH, Mannheim, Germany), (as described in Ref. [9]. The epithelium was then peeled off from the lamina propria and subjected to trypsin digestion to isolate epithelial cells. The isolated epithelial cells were seeded on UpCell, a temperature-responsive culture ware (CellSeed Inc., Tokyo, Japan), in co-culture with Mitomycin C (MMC)-treated NIH/3T3 feeder cells [9]. After 2–4 weeks of cell culture, the cell sheet grew as a multilayered epithelial tissue-like and was grafted back onto cornea.

2.3. Fluorescent immunostaining

Paraffin-embedded tissue sections of rabbit corneas were used to conduct fluorescent Immunostaining analysis. Tissue sections were stained using HIF-1 alpha antibody (Abcam, Cambridge MA), HIF-2 alpha antibody (Novus Biological, Centennial CO), VEGF-A and VEGF receptor (Santa Cruz Biotechnology, Santa Cruz CA). TSP-1 antibody was purchased from Cell Signaling Technology, Danvers, MA). Alexa Fluor® 488 donkey anti-mouse fluorophore conjugated secondary antibodies was used. Cell nuclei staining was performed using propidium iodide (Invitrogen, Eugene, OR). Slides were analyzed using a Nikon 400 fluorescent microscope.

2.4. Proteasome inhibition experiments

Rabbit oral mucosa epithelial cells (5×10^5) were isolated and cultured as reported previously [9]. Cells were treated with proteasome inhibitor (PS341, MedChem Express LLC) was diluted in cell culture medium at 10 or 50 nM and incubated with the cell sheets

for 24 h and for 72 h. Control cells were treated with DMSO that was used to dissolve PI. Less than 0.1% of DMSO was used as the control for treated cells. Cell sheets were then washed and harvested to measure proteasome chymotrypsin-like activity and the expression levels of TSP-1.

Commercially available human primary oral epithelial cells (PCS-200-014) and corneal epithelial cells (PCS-700-010) were cultured as instructed by the supplier ATCC (using 5×10^5 at passage 4 and cell culture media provided by the supplier). Cells were treated with proteasome inhibitor at 50 nM for 24 h. Cells were then washed with cell culture medium and harvested for further biochemical analysis.

2.5. Western blot analysis

Two μg of total protein from sample homogenates were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 h in 25 mM Tris-HCl (pH = 8.3), 192 mM Glycine and 20% methanol. Membranes were probed with primary antibody against TSP-1, HIF-1 alpha, HIF-2 alpha, VEGF-A, VEGF receptor and Beta Actin (Millipore – Sigma). HRP-conjugated secondary antibody was used. Membranes were subjected to Chemiluminescence detection using Luminal according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

2.6. Statistics

Data were obtained from at least three different biological samples. Bars represent mean values ± SEM. P values were determined by one-way ANOVA and Student–Newman Keuls for multiple group comparisons (Sigma-Stat softdish, San Francisco, CA). Statistical significance was set at $p = \text{or} < \text{to } 0.05$. Bar graphs were shown as Mean ± SEM, n = 3–5.

3. Results

3.1. Expression of HIF-1 and VEGF factor

Lamellar limbectomy created corneal neovascularization (NV). NV developed in rabbits corneas as early as 1 month after limbectomy, and was stable for at least 3 months (Fig. 1A) as compared to the control healthy cornea (Fig. 1B) [9].

Diseased corneal epithelia were harvested and compared to healthy normal corneal epithelia that were collected from corneal surface before limbectomy. Fig. 2A–D showed that HIF-1 alpha, HIF-2 alpha, VEGF-A and VEGF receptor were up regulated in corneal epithelial cells harvested from the vascularized corneal surface. We found a significant difference for HIF-2 alpha.

Immunofluorescent staining showed that the expression of HIF-1 alpha and VEGF-A was up regulated in the diseased sham eye and down regulated in CAOMECS grafted corneas (Fig. 3A–F). This confirmed previously reported results from experiments made in our LSCD rabbit model that showed that CAOMECS grafting significantly reduces corneal vascularization [9]. HIF-1 alpha and VEGF-A expression was also reduced in CAOMECS grafted corneas, as compared to sham corneas, but still less expressed when compared to the healthy corneas.

3.2. Expression of TSP-1

The expression of TSP-1 in corneal epithelium and its role in maintaining corneal vascular homeostasis remains unknown. In the present study, we investigated the expression of TSP-1 in rabbit corneal surface with NV induced by surgical limbectomy. We also examined the effects of CAOMECS grafting in restoring a healthy expression of TSP-1 and preventing NV of corneal surface. TSP-1 was expressed in corneal epithelial cells (Fig. 4A), and had a much lower expression in conjunctival epithelial and limbal cells (Fig. 4B). In opposite to HIF-1 alpha expression, TSP-1 expression was decreased in corneal surface of injured and untreated corneas. While TSP-1 expression was decreased in injured corneas (Fig. 4C), it was expressed in CAOMECS grafted corneas (Fig. 4D), but still less expressed when compared to the healthy corneas (Fig. 4).

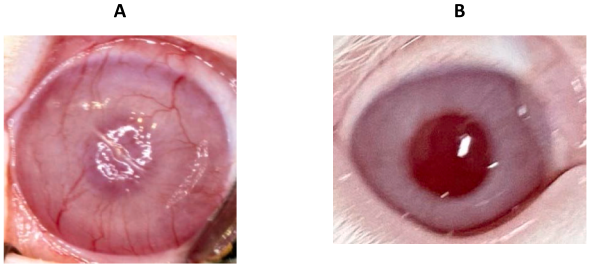

Fig. 1. **A** is a picture of rabbit corneas 3 months after limbectomy. Neovascularization is shown by the high number of blood vessels extending from conjunctiva toward central cornea. **B** is a healthy normal rabbit cornea.

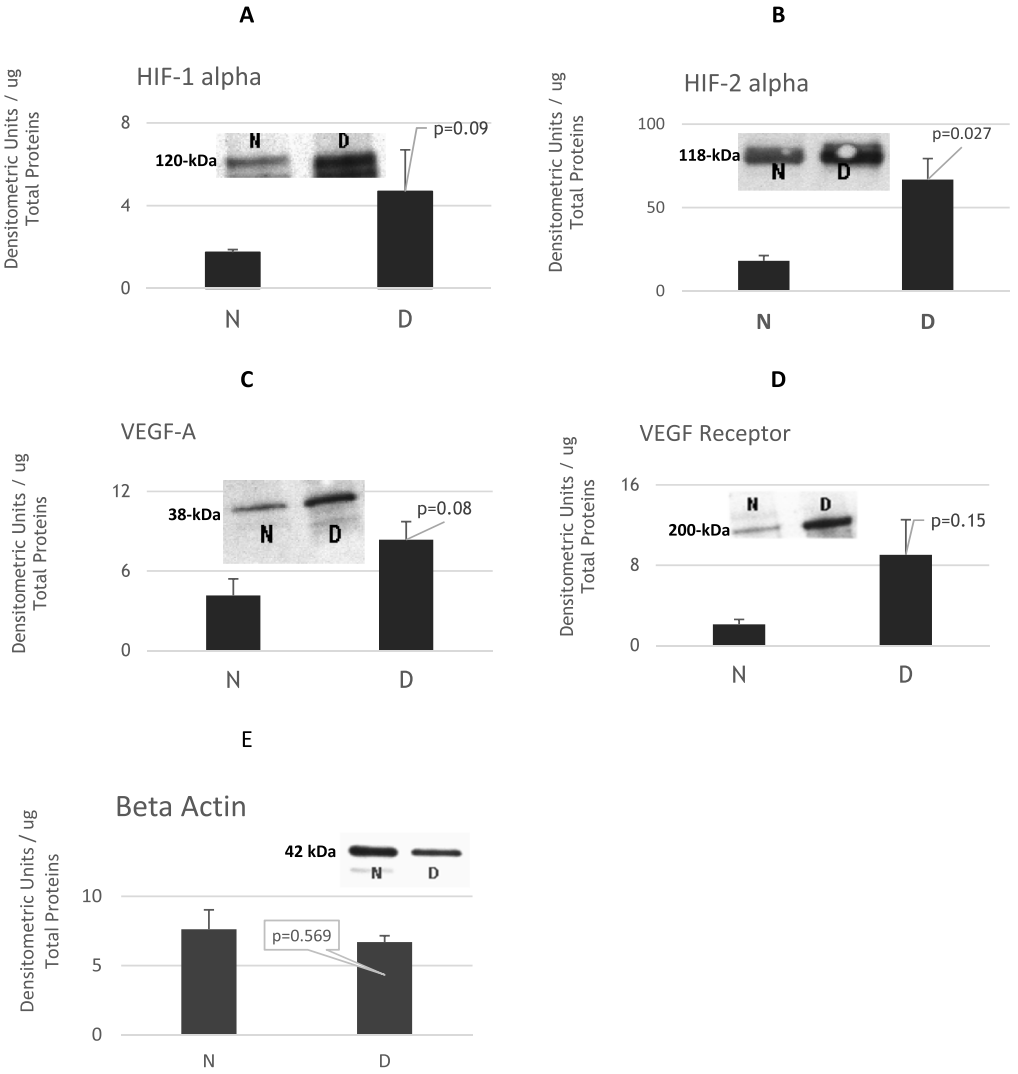

Fig. 2. Western blot analysis of HIF-1A, HIF2A, VEGF-A and VEGF receptor (respectively A to D). E showed the expression Beta Actin as a loading control. Corneal epithelial cells were sampled from the vascularized corneal surface of rabbits (diseased corneal epithelia (D) and healthy normal corneal epithelia (N). (Mean = /- SEM, n = 3).

3.3. TSP-1 induction and VEGF reduction by proteasome inhibition

To investigate the expression of TSP-1 cultured rabbit oral mucosal epithelial cells were treated with proteasome inhibitor at 10 nM and 50 nm concentrations for 24 and 72 h (Fig. 5A and B). Results showed that proteasome chymotrypsin-like activity was significantly decreased at both concentration and even more inhibited with 50 nM at 24hrs, reflecting the high specific potency of the drug toward its proteasome substrate (Fig. 5A and B). When proteasome activity was inhibited, the expression of TSP-1 was up regulated at both concentrations (Fig. 5C). Treatment with 50 nM concentration of proteasome inhibitor showed a greater induction of TSP-1. Experiments were then conducted with 50 nM of proteasome inhibitor for 24 and 72 h. Proteasome chymotrypsin-like activity was significantly inhibited at 24 h and recovered at 72 h (Fig. 5B). Results also showed a significant induction in the expression of TSP-1 at 24 h and a significant decrease at 72 h (Fig. 5D). Proteasome inhibitor is a reversible inhibitor that significantly inhibit chymotrypsin-like activity for only 24–48 h [17]. The results indicated that TSP-1 is up regulated when proteasome activity is inhibited. Fig. 5 E showed the levels of beta-actin as a loading control for Fig. 5C. F-actin in Figure E is the loading control for the results of Fig. 5D.

To further investigate the role of proteasome inhibition in up regulating the expression of TSP-1, human primary oral mucosa epithelial cells were cultured and treated with proteasome inhibitor at a concentration of 50 nM for 24 h. Fig. 6A and B showed that 50 nM proteasome inhibitor concentration did not affect the morphology of the cells. Results showed a significant decrease in the chymotrypsin-like proteasome activity (78% of inhibition (Fig. 6C). Proteasome inhibition increased in the expression of TSP-1, but without a significant difference (Fig. 6D). Results also showed that the proteasome inhibition significantly reduced VEGF-A expression

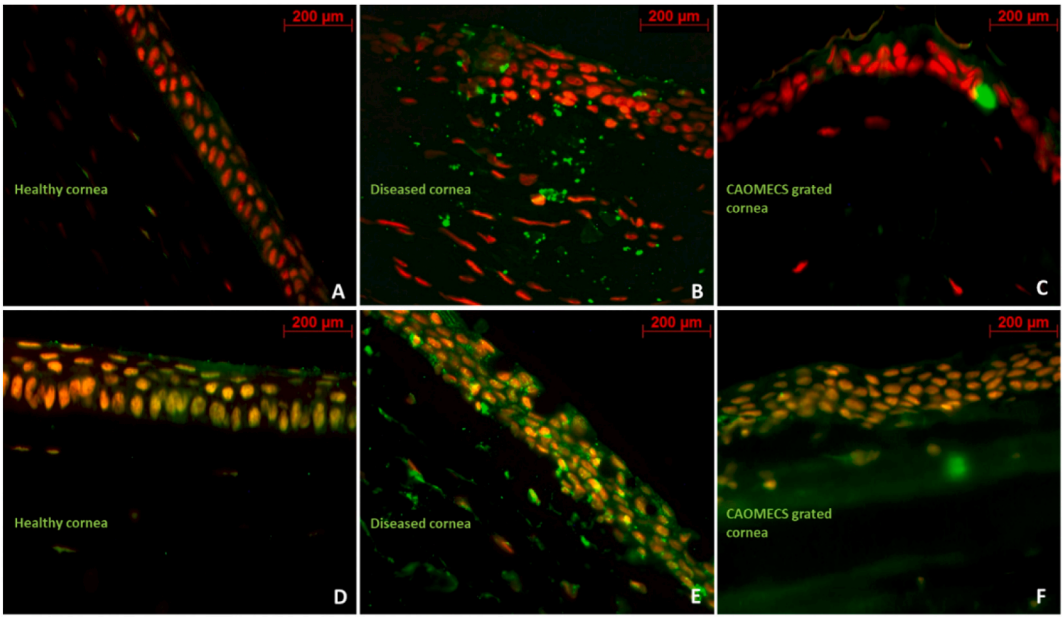

Fig. 3. Immunofluorescent staining (in green) of HIF- 1 alpha (A–C) and VEGF-A (D–F) in rabbit corneal tissue sections. Red is nuclear staining with propidium Iodide. (Mag. 40x). **A and D**: healthy cornea. **B and E**: Sham cornea (untreated). **C and F**: CAOMECS grafted cornea. Note that the expression of HIF-1 alpha and VEGF-A was reduced in CAOMECS grafted corneas as compared to sham or untreated cornea. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

levels (Fig. 6E).

Corneal epithelial cells (CEC) were also cultured and treated with proteasome inhibitor. Fig. 7A and B showed that proteasome inhibitor 50 nM concentration did not affect the morphology of the cells. Proteasome chymotrypsin-like activity was significantly decreased (70% of inhibition) (Fig. 7C) and the expression of TSP-1 was slightly increased without a significant difference (Fig. 7D). VEGF-A expression was also significantly reduced in CEC (Fig. 7E).

To summarize the main findings and results of the present study, Fig. 8 illustrates the potential beneficial effects of proteasome inhibition treatment in blocking corneal neovascularization. Proteasome inhibition up regulates TSP-1 levels, which consequently down regulates VEGF levels and may subsequently reduce corneal neovascularization.

4. Discussion

The first objective of this research was to investigate the expression of TSP-1 in rabbit vascularized corneas and in CAOMECS grafted corneas. Immunofluorescent staining of corneal tissue sections revealed different results between control, diseased, and CAOMECS grafted corneas. Staining of the normal eye (NE) tissue sample revealed that TSP-1 was greatly expressed in the corneal epithelium and had a limited expression in the limbus/conjunctiva. The expression of TSP-1 in central cornea is vital for an avascular and transparent cornea. However, since the limbus and conjunctiva are vascularized tissue, TSP-1 expression is consequently reduced. The a-vascularization of central cornea is required for transparency and a good visual acuity, hence high levels of TSP-1 expression.

Research on TSP-1 throughout the scientific community corroborates these results. At a foundational level, TSP-1 has been shown to be most expressed within the Bowman's layer, Descemet's membrane, and endothelium, but not within the stroma [5]. We observed a dominant expression of TSP-1 in corneal basal cells and some relatively minor expression in supra-basal cells. Previous research showed a positive staining for TSP-1 in human corneal epithelial basal cells [18].

Promoting the expression of anti-angiogenic biomolecules should ideally cause a limitation on vessel density and growth, especially in the case of a potent anti-angiogenic factor such as TSP-1 [12]. Evidenced by previous research and data, our results encompassing TSP-1 expression would indicate that increasing the expression levels should yield decreased neovascularization of the corneal epithelium. The vascularized corneas of the sham eye (SE) showed a marked decrease in overall TSP-1 expression in the corneal epithelium. Regarding angiogenic elements, the SE corneas showed a great increase in the relative amount of neovascularization that was taking place in the corneal tissues, as compared to the NE cornea. Another striking observation was the vast quantitative increase in goblet cells in the SE cornea of the cornea as compared to both NE corneas for the cornea and conjunctiva.

Our results also showed that TSP-1 expression was recovered within the epithelium of CAOMECS grafted cornea versus the non-grafted cornea. Not only does the result of CAOMECS grafting show the restoration of TSP-1 expression as compared to the non-grafted cornea, but electron microscopy imaging also showed a decreased count of goblet cells and decreased vascularization. As seen in the NE corneal tissue section, epithelial cells are essentially layered or stacked squamous cells. This layering disappears almost entirely in the SE tissue cornea as the corneal epithelium is experiencing erosion. When looking back at the CAOMECS grafted cornea,

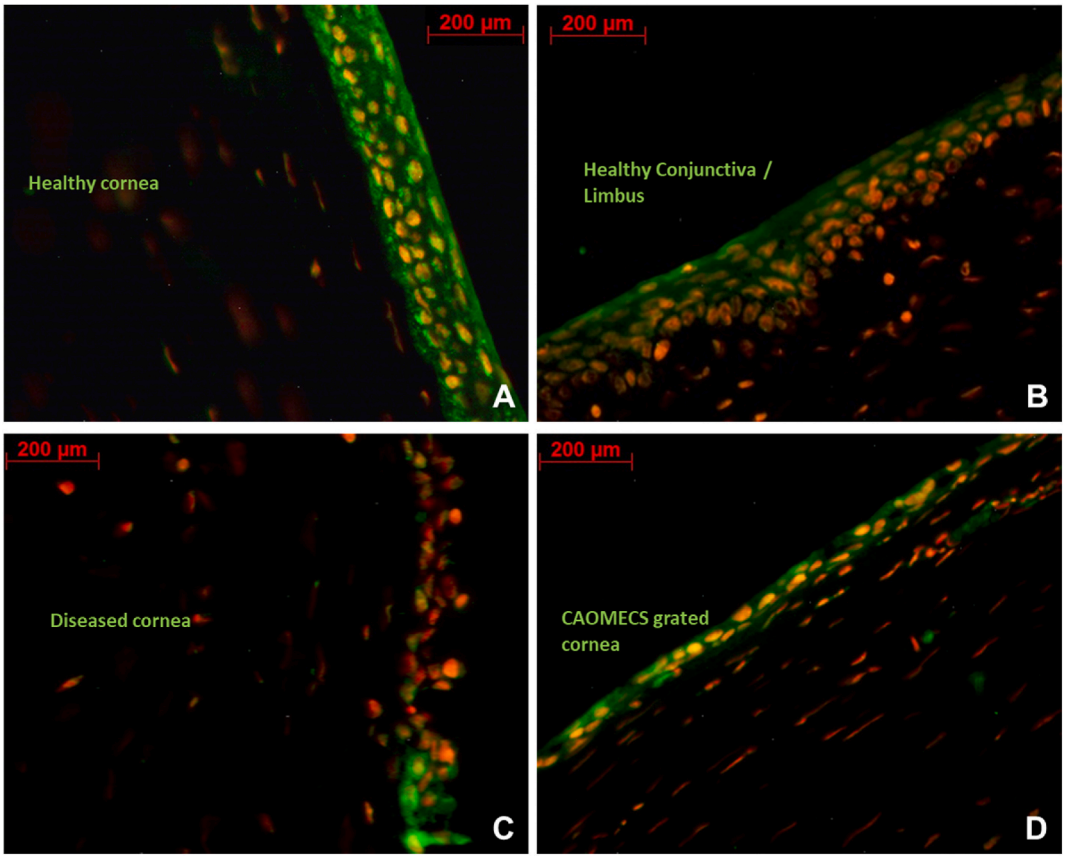

Fig. 4. Rabbit cornea TSP-1 immunofluorescent staining in green. Nucleus is stained in red. TSP-1 expression for normal corneal tissue section (**A**), conjunctival tissue section (**B**), diseased corneal tissue section (**C**), and CAOMECS grafted corneal tissue section (**D**). Note that TSP-1 expression was predominantly expressed in the healthy corneal epithelium (**A**), and was decreased in the limbus and conjunctival epithelium (**B**). In the injured and untreated cornea, there was a marked decrease in TSP-1 expression along with corneal epithelium erosion. CAOMECS grafted cornea showed a recovery in TSP-1 expression as compared to the diseased cornea. Magnification is x40. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

there is in fact a measure of restoration regarding the layered nature of these squamous corneal epithelial cells. A previous study [9] showed that the number of blood vessels invading central cornea was significantly decreased after CAOMECS grafting. In CAOMECS-grafted corneal tissue section, TSP-1 expression was recovered to a certain extent.

Activating the expression of TSP-1 may be enough to limit or even fend off the angiogenic effects seen in vascularized corneas. We hypothesized that proteasome activities may play a major role in corneal vascularization and that modulating its activities may alleviate corneal NV especially by increasing the expression of TSP-1 and decreasing the expression of VEGF-A. In an in vitro study, we tested our hypothesis by treating cultured cells with proteasome inhibitor in a dose and time response study. The expression of TSP-1 was increased in both type of cells – oral mucosa and corneal epithelial cells treated with proteasome inhibitor. How does proteasome inhibition modulate the expression of TSP-1 remains obscure.

During inflammatory conditions showing high levels of cytokines resulting from the injury caused to corneal tissue, Hif-1 alpha is activated. Proteasome inhibitor Velcade or Bortezomib has been reported to inactivate Hif-1 alpha by inhibiting the PI3K/Akt/mTOR pathway [19]. In addition, Bortezomib treatment suppresses HIF-1 transcriptional activity by inhibiting the interaction of HIF-1α with the acetyltransferase p300 required for Hif-1 alpha transcriptional activities [20,21]. Consequently, if Hif-1 alpha is inactivated, VEGF is also inactivated and inhibition of pro-angiogenic factors is accomplished [22–24]. Logically, proteasome inhibition would result in the accumulation of Hif-1 alpha and thus the accumulation of VEGF. Our results showed that VEGF was significantly down regulated after treating the cells with proteasome inhibitor. It is possible that another molecular mechanism than Hif-1 alpha signaling takes place linking the down regulation of VEGF to proteasome inhibitor. TSP-1 has potent anti-angiogenic effects via multiple mechanisms.

Downstream signaling by TSP-1 demonstrates that TSP-1-induced inhibition of various metalloproteinases results in the suppression of VEGF release [5,13]. Previous work in the field has highlighted a down regulation of MMP3, a proangiogenic factor and an up regulation of TIMP3, an anti-angiogenic factor in CAOMECS grafted rabbit corneas. The expression of these factors reflected a preventive characteristic of CAOMECS grafting for the further progression of corneal neovascularization [9]. Semi-quantitative measurements of TIMP-1 & TIMP-3 showed a higher expression in engineered oral mucosal cell sheets compared to control oral

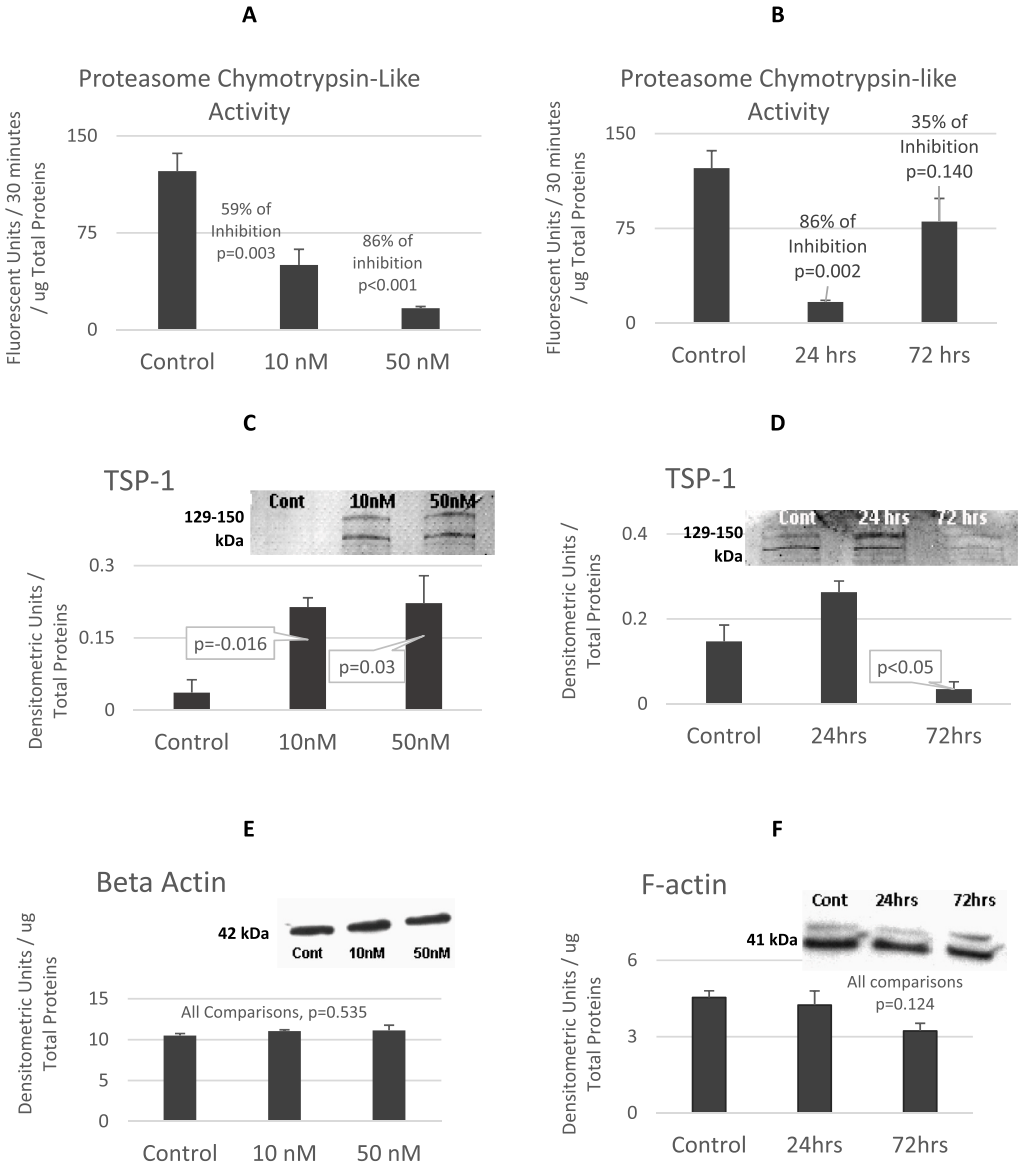

Fig. 5. Rabbit cultured oral mucosal epithelial cells treated with proteasome inhibitor to activate the expression of TSP-1. **A** and **C** showed the measurements of proteasome chymotrypsin-like activity. **B** and **D** showed the expression levels of TSP-1 in the dose and time response studies. **E** and **F** respectively showed the expression of Beta Actin and F-Actin as a loading controls. (Mean ± SEM, n = 3).

epithelial cells [25]. With the verification of these MMPs and TIMPs as being anti-angiogenic, promoting TSP-1 expression is then thought to have an effect on these biomolecules. As noted previously, TSP-1 has a unique signaling role on many biomolecules associated within the scope of angiogenesis and lymphangiogenesis. Thereby signaling an increase in its expression levels will also increase other anti-angiogenic factors to an extent. Another example of TSP-1 impeding on the angiogenic pathway is by altering or inhibiting protein-receptor activity such as VEGF receptor 2 [5]. Evidently, TSP-1 has potent downstream effects on various pathways, mechanisms, and biomolecules, which need further investigated. TSP-1 is known to be degraded through Low-density lipoprotein receptor–related protein-1 (LRP1) endocytosis and lysosomal degradation [26]. Our results showed that TSP-1 is stabilized by proteasome inhibition, which indicates that proteasome activity is also involved in the degradation of TSP-1. The specific mechanism by which proteasome clear out TSP-1 is not known and needs further investigation. The effects of proteasome inhibitor Bortezomib in preventing angiogenesis by repressing the release of VEGF and thus angiogenesis has been reported [27]. It is possible that proteasome inhibition suppresses VEGF release through TSP-1 stabilization as TSP-1 is also involved in the suppression of VEGF release via various mechanisms [28–30].

Blockade of VEGF has been investigated for decades in cancer treatment [31,32]. The combinatory treatment utilizing VEGF antibody (bevacizumab) and immune checkpoint inhibitors (atezolizumab) is currently the treatment for hepatocellular carcinoma

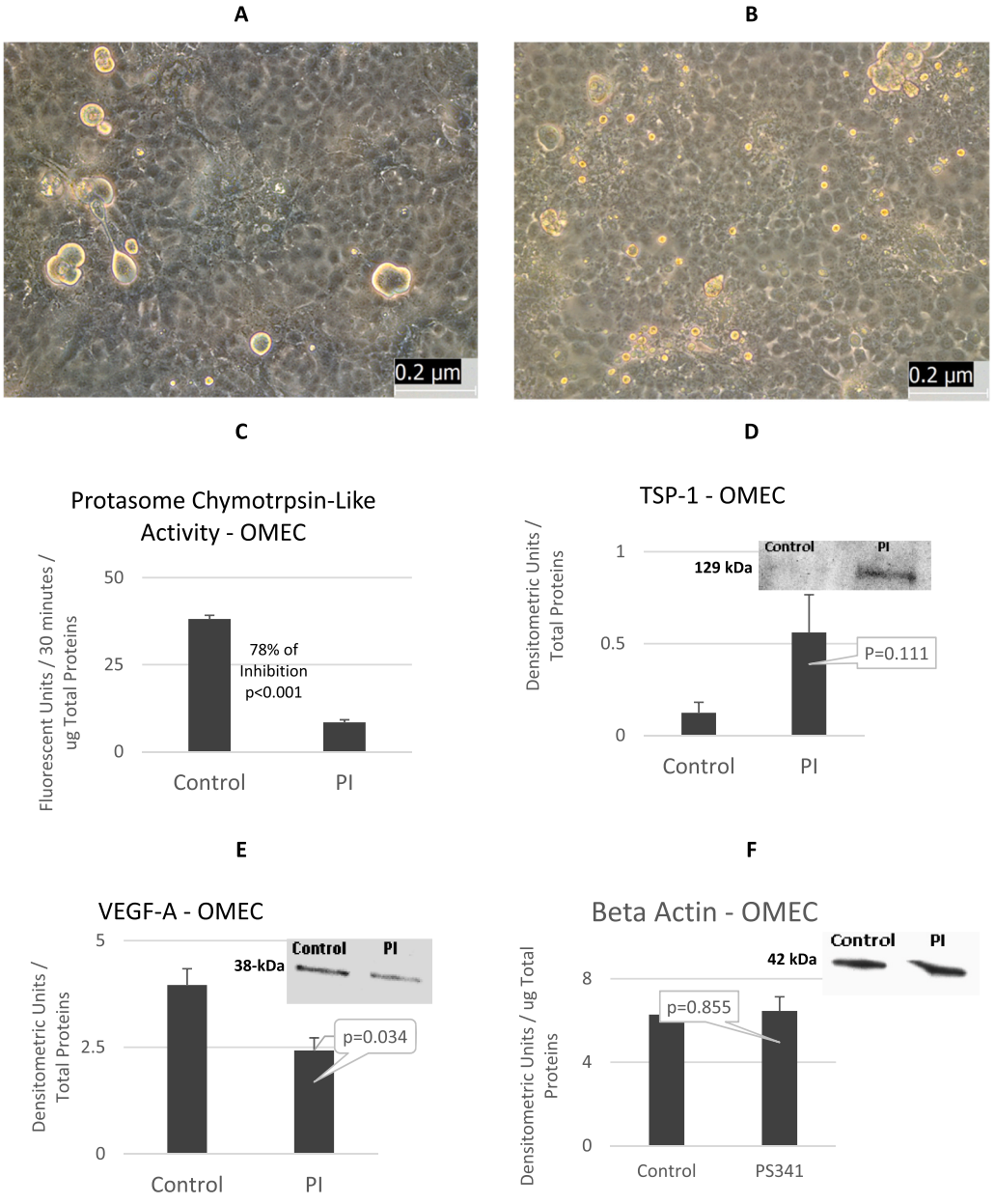

Fig. 6. TSP-1 expression levels in human cultured oral mucosal epithelial cells after treatment with 50 nM proteasome inhibitor. **A** showed control untreated cells. **B** showed treated cells with proteasome inhibitor (Magnification is x20). **C** showed proteasome chymotrypsin-like activity and **D** showed the expression levels of TSP-1. **E** showed the expression levels of VEGF-A and **F** showed the expression of Beta Actin as a loading control (Mean = /- SEM, n = 3).

[33]. When used to treat corneal neovascularization, bevacizumab caused significant corneal thinning [34]. Other antibodies against different forms of VEGF seemed to be effective in inhibiting corneal neovascularization in rat model of corneal neovascularization [35]. Anti-VEGF were found to be efficient in reducing immune reactions during corneal transplantation [36]. However, in attempt to achieve complete regression of neovascularization, successful corneal transplantations or allograft survival, and to increase visual acuity, more potent anti-angiogenic factors are needed. TSP-1 is a potent anti-angiogenic factors that need further investigations to alleviation of corneal neovascularization.

5. Conclusion

In conclusion, the results showed that TSP-1 expression was lost in the injured and vascularized corneal surface and that CAOMECS

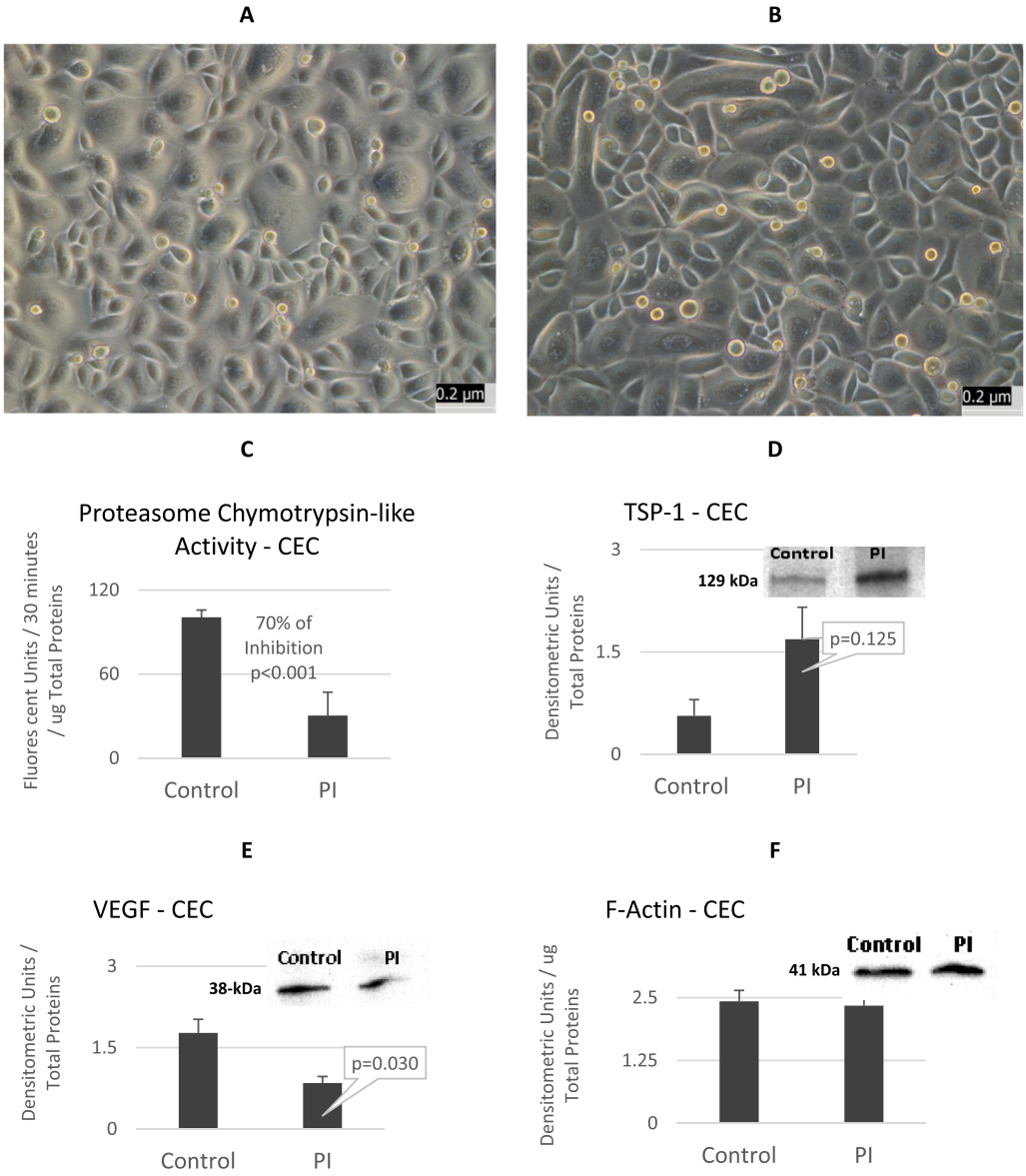

Fig. 7. TSP-1 expression levels in human cultured corneal epithelial cells (CEC) after treatment with 50 nM proteasome inhibitor. **A** showed control untreated cells. **B** showed cells treated with proteasome inhibitor (Magnification is x20). **C** showed proteasome chymotrypsin-like activity and **D** showed the expression levels of TSP-1. **E** showed the expression levels of VEGF-A and **F** showed the expression of F-Actin as a loading control (Mean = /- SEM, n = 3).

grafting restored TSP-1 expression to certain extent. Proteasome inhibitor treatment of cultured oral mucosal epithelial cells stabilized TSP-1 expression, which could be used to induce TSP-1 expression after CAOMECS grafting to manage corneal vascularization. By understanding the molecular mechanisms that control neovascularization, it may be possible to design therapeutic strategies to selectively prevent or halt pathologic vascular growth. The anti-angiogenic thrombospondin-1 (TSP-1) has been shown to be a natural inhibitor of neovascularization. An increase in TSP-1 expression might decrease corneal vascularization.

Author contribution statement

Fawzia Bardag-Gorce: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Carter Hoffman: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Imara Meepe: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or

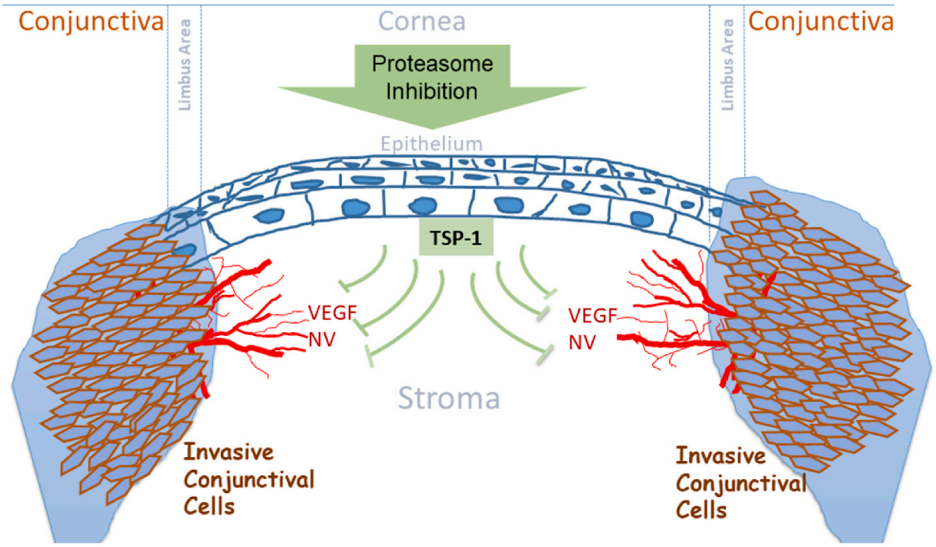

Fig. 8. Schematic illustration of proteasome inhibition effects in blocking injury-induced corneal neovascularization.

data.

Monica Ferrini, Yutaka Niihara: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Richard H. Hoft, Joan Oliva: Performed the experiments; Analyzed and interpreted the data.

Funding statement

Dr Fawzia Bardag-Gorce was supported by Emmaus Medical Inc [ELS-002].

Data availability statement

No data was used for the research described in the article.

Declaration of interest's statement

The authors declare the following conflict of interests: Research is fully supported by Emmaus Medical Inc. The salaries of Dr. Fawzia Bardag Gorce and Dr. Joan Oliva are fully supported by Emmaus Medical Inc. Dr. Yutaka Niihara is the CEO and the Chairman of Emmaus Medical, Inc. The rest of the authors has no support from Emmaus Medical, Inc. and no conflict of interest.

References

[1] A. Di Zazzo, D. Gaudenzi, J. Yin, M. Coassin, M. Fernandes, R. Dana, S. Bonini, Corneal angiogenic privilege and its failure, Exp. Eye Res. 204 (2021), 108457, https://doi.org/10.1016/j.exer.2021.108457.

[2] Michel Haagdorens, Sara Ilse Van Acker, Veerle Van Gerwen, Sorcha Ní Dhubhghail, Carina Koppen, , Marie-José Tassignon, Nadia Zakaria, Limbal stem cell deficiency: current treatment options and emerging therapies, Stem Cell. Int. 2016 (2016), 9798374, https://doi.org/10.1155/2016/9798374.

[3] C. Cursiefen, S. Masli, T.F. Ng, M.R. Dana, P. Bornstein, J. Lawler, J.W. Streilein, Roles of thrombospondin-1 and -2 in regulating corneal and Iris angiogenesis, Investig. Ophthalmol. Vis. Sci. 45 (4) (2004) 1117–1124, https://doi.org/10.1167/iovs.03-0940.

[4] Elizabeth A. Logsdon, Stacey D. Finley, S Popel Aleksander, Feilim Mac Gabhann, A systems biology view of blood vessel growth and remodeling, J. Cell Mol. Med. 18 (8) (2014 Aug) 1491–1508, https://doi.org/10.1111/jcmm.12164.

[5] W. Foulsham, T.H. Dohlman, S.K. Mittal, Y. Taketani, R.B. Singh, S. Masli, R. Dana, Thrombospondin-1 in ocular surface health and disease, Ocul. Surf. 17 (3) (2019) 374–383, https://doi.org/10.1016/j.jtos.2019.06.001.

[6] A. Mukwaya, L. Jensen, B. Peebo, N. Lagali, MicroRNAs in the cornea: role and implications for treatment of corneal neovascularization, Ocul. Surf. 17 (3) (2019) 400–411, https://doi.org/10.1016/j.jtos.2019.04.002.

[7] Y. Tatematsu, Q. Khan, T. Blanco, J.A. Bair, R.R. Hodges, S. Masli, D.A. Dartt, Thrombospondin-1 is necessary for the development and repair of corneal nerves, Int. J. Mol. Sci. 19 (10) (2018) 3191.

[8] M.P. Nicholas, N. Mysore, Corneal neovascularization, Exp. Eye Res. 202 (2021), 108363, https://doi.org/10.1016/j.exer.2020.108363.

[9] F. Bardag-Gorce, J. Oliva, A. Wood, R. Hoft, D. Pan, J. Thropay, A. Makalinao, S.W. French, Y. Niihara, Carrier-free cultured autologous oral mucosa epithelial cell sheet (CAOMECS) for corneal epithelium reconstruction: a histological study, Ocul. Surf. 13 (2) (2015) 150–163, https://doi.org/10.1016/j.jtos.2014.12.003.

[10] H.-C.J. Chen, L.-K. Yeh, Y.-J. Tsai, C.-H. Lai, C.-C. Chen, J.-Y. Lai, C.-C. Sun, G. Chang, T.-L. Hwang, J.-K. Chen, D.H.-K. Ma, Expression of angiogenesis-related factors in human corneas after cultivated oral mucosal epithelial transplantation, Investig. Ophthalmol. Vis. Sci. 53 (9) (2012) 5615–5623, https://doi.org/10.1167/iovs.11-9293.

[11] C. Cursiefen, K. Maruyama, F. Bock, D. Saban, Z. Sadrai, J. Lawler, R. Dana, S. Masli, Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes, J. Exp. Med. 208 (5) (2011) 1083–1092, https://doi.org/10.1084/jem.20092277.

[12] Jie Meng, Bingying Lin, Siyu Huang, Yangyang Li, Pengsen Wu, Fan Zhang, Ke Yu, Xiangqing Hei, Danping Huang, Melatonin exerts anti-angiogenic and anti-inflammatory effects in alkali-burned corneas, Ann. Transl. Med. 10 (8) (2022 Apr) 432, https://doi.org/10.21037/atm-21-4927.

[13] B. Jiménez, O.V. Volpert, S.E. Crawford, M. Febbraio, R.L. Silverstein, N. Bouck, Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1, Nat. Med. 6 (1) (2000) 41–48, https://doi.org/10.1038/71517.

[14[X. Tan, Y. Chen, W. Foulsham, A. Amouzegar, T. Inomata, Y. Liu, S.K. Chauhan, R. Dana, The immunoregulatory role of corneal epithelium-derived thrombospondin-1 in dry eye disease, Ocul. Surf. 16 (4) (2018) 470–477, https://doi.org/10.1016/j.jtos.2018.07.005.

[15] K. Uno, H. Hayashi, M. Kuroki, H. Uchida, Y. Yamauchi, M. Kuroki, K. Oshima, Thrombospondin-1 accelerates wound healing of corneal epithelia, Biochem. Biophys. Res. Commun. 315 (4) (2004) 928–934, https://doi.org/10.1016/j.bbrc.2004.01.146.

[16] C. Anastasi, P. Rousselle, M. Talantikite, A. Tessier, C. Cluzel, A. Bachmann, N. Mariano, M. Dussoyer, L.B. Alcaraz, L. Fortin, A. Aubert, F. Delolme, N. El Kholti, J. Armengaud, P. Fournié, C. Auxenfans, U. Valcourt, S.V.-L. Goff, C. Moali, BMP-1 disrupts cell adhesion and enhances TGF-β activation through cleavage of the matricellular protein thrombospondin-1, Sci. Signal. 13 (639) (2020) eaba3880, https://doi.org/10.1126/scisignal.aba3880.

[17] Fawzia Bardag-Gorce, Proteasome inhibitor treatment in alcoholic liver disease, May 28, World J. Gastroenterol. 17 (20) (2011) 2558–2562, https://doi.org/10.3748/wjg.v17.i20.2558.

[18] E. Sekiyama, T. Nakamura, L.J. Cooper, S. Kawasaki, J. Hamuro, N.J. Fullwood, S. Kinoshita, Unique distribution of thrombospondin-1 in human ocular surface epithelium, Investig. Ophthalmol. Vis. Sci. 47 (4) (2006) 1352–1358, https://doi.org/10.1167/iovs.05-1305.

[19] Christina D. Befani, Panagiotis J. Vlachostergios, Eleana Hatzidaki, Patrikidou Anna, Sophia Bonanou, Simos George, Christos N. Papandreou, Panagiotis Liakos, Bortezomib represses HIF-1α protein expression and nuclear accumulation by inhibiting both PI3K/Akt/TOR and MAPK pathways in prostate cancer cells, J. Mol. Med. (Berl.) 90 (1) (2012 Jan) 45–54, https://doi.org/10.1007/s00109-011-0805-8.

[20] D.H. Shin, Y.S. Chun, D.S. Lee, L.E. Huang, J.W. Park, Bortezomib inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated repression of hypoxia-inducible factor-1, Blood 111 (2008) 3131–3136.

[21] Haejin Yoon, Ji-Hong Lim, Chung-Hyun Cho, L Eric Huang, Jong-Wan Park, CITED2 controls the hypoxic signaling by snatching p300 from the two distinct activation domains of HIF-1α, Biochim. Biophys. Acta 1813 (12) (2011 Dec) 2008–2016, https://doi.org/10.1016/j.bbamcr.2011.08.018.

[22] Di Wang, Qin Qin, Qin-Juan Jiang, Da-Fei Wang, Bortezomib sensitizes esophageal squamous cancer cells to radiotherapy by suppressing the expression of HIF-1α and apoptosis proteins, J. X Ray Sci. Technol. 24 (4) (2016 Apr 13) 639–646, https://doi.org/10.3233/XST-160571.

[23] Diana C. Birle, David W. Hedley, Suppression of the hypoxia-inducible factor-1 response in cervical carcinoma xenografts by proteasome inhibitors, Feb 15, Cancer Res. 67 (4) (2007) 1735–1743, https://doi.org/10.1158/0008-5472.CAN-06-2722.

[24] Gerald S. Falchook, Jennifer J. Wheler, Naing Aung, Edward F. Jackson, Janku Filip, David Hong, Chaan S. Ng, Nizar M. Tannir, Kristie N. Lawhorn, Mei Huang, Laura S. Angelo, Deeksha Vishwamitra, Kenneth Hess, Adrienne N. Howard, Kristin L. Parkhurst, Hesham M. Amin, Razelle Kurzrock, Targeting hypoxia-inducible factor-1α (HIF-1α) in combination with antiangiogenic therapy: a phase I trial of bortezomib plus bevacizumab, Nov 15, Clin. Trial Oncotarg. 5 (21) (2014) 10280–10292, https://doi.org/10.18632/oncotarget.2163.

[25] Fawzia Bardag-Gorce, Joan Oliva, Andrew Wood, Niihara Hope, Andrew Makalinao, Sean Sabino, Derek Pan, Jacquelyn Thropay, Hiroyuki Sota, Yutaka Niihara, Microarray analysis of oral mucosal epithelial cell sheet, Tiss. Engin. Regen. Med. 10 (2013) 362–370.

[26] H. Chen, J. Sottile, D.K. Strickland, D.F. Mosher, Binding and degradation of thrombospondin-1 mediated through heparan sulphate proteoglycans and low-density-lipoprotein receptor-related protein: localization of the functional activity to the trimeric N-terminal heparin-binding region of thrombospondin-1, Pt 3, Biochem. J. 318 (Pt 3) (1996 Sep 15) 959–963, https://doi.org/10.1042/bj3180959.

[27] Yimei Liu, Meiling Feng, Jingjing Cai, Shifeng Li, Xufeng Dai, Shan Ge, Shengzhou Wu, Repurposing bortezomib for choroidal neovascularization treatment via antagonizing VEGF-A and PDGF-D mediated signaling, Exp. Eye Res. 204 (2021 Mar), 108446, https://doi.org/10.1016/j.exer.2021.108446.

[28] J.C. Rodriguez-Manzaneque, T.F. Lane, M.A. Ortega, R.O. Hynes, J. Lawler, M.L. Iruela-Arispe, Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor, Proc. Natl. Acad. Sci. USA 98 (2001) 12485–12490, https://doi.org/10.1073/pnas.171460498.

[29] J. Greenaway, J. Lawler, R. Moorehead, P. Bornstein, J. LaMarre, J. Petrik, Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1), J. Cell. Physiol. 210 (2007) 807–818, https://doi.org/10.1002/jcp.20904.

[30] X. Zhang, S. Kazerounian, M. Duquette, C. Perruzzi, J.A. Nagy, H.F. Dvorak, et al., Thrombospondin-1 modulates vascular endothelial growth factor activity at the receptor level, Faseb. J. 23 (2009) 3368–3376, https://doi.org/10.1096/fj.09-131649.

[31] Alessandro Di Federico, Alessandro Rizzo, Riccardo Carloni, Andrea De Giglio, Riccardo Bruno, Dalia Ricci, Giovanni Brandi, Atezolizumab-bevacizumab plus Y-90 TARE for the treatment of hepatocellular carcinoma: preclinical rationale and ongoing clinical trials, Expet Opin. Invest. Drugs 31 (4) (2022 Apr) 361–369, https://doi.org/10.1080/13543784.2022.2009455.

[32] Alessandro Rizzo, , Angela Dalia Ricci, Alessandro Di Federico, Giorgio Frega, Andrea Palloni, Simona Tavolari, Giovanni Brandi, Predictive biomarkers for checkpoint inhibitor-based immunotherapy in hepatocellular carcinoma: where do we stand? Front. Oncol. 11 (2021 Dec 17), 803133 https://doi.org/10.3389/fonc.2021.803133 eCollection 2021.

[33] Alessandro Rizzo, , Angela Dalia Ricci, Gennaro Gadaleta-Caldarola, Giovanni Brandi, First-line immune checkpoint inhibitor-based combinations in unresectable hepatocellular carcinoma: current management and future challenges, Expet Rev. Gastroenterol. Hepatol. 15 (11) (2021 Nov) 1245–1251, https://doi.org/10.1080/17474124.2021.1973431.

[34] Kristie J. Sun, S Jun Albert, Kelley Bohm, Daniel Daroszewski, Samir Jabbour, Corneal thinning following bevacizumab intrastromal injection for the treatment of idiopathic lipid keratopathy, Case Rep. Am. J. Ophthalmol. Case Rep. 27 (2022 Jun 24), 101618, https://doi.org/10.1016/j.ajoc.2022.101618.

[35] M.T. Eski, Kuddusi Teberik, Pembe Oltulu, Handan Ankaralı, Murat Kaya, Merve Alpay, The effects of subconjunctival bevacizumab, ranibizumab, and aflibercept on corneal neovascularization, Hum. Exp. Toxicol. 41 (2022 Jan-Dec), 9603271221084674, https://doi.org/10.1177/09603271221084674.

[36] Thomas H Dohlman, Matthew McSoley, Francisco Amparo, Tatiana Carreno-Galeano, Mengyu Wang, Mohammad Dastjerdi, Rohan Bir Singh, Giulia Coco, Antonio Di Zazzo, Hasanain Shikari, Ujwala Saboo, Kimberly Sippel, Jessica Ciralsky, Sonia H. Yoo, Matheus Sticca, Tais H. Wakamatsu, Somasheila Murthy, Pedram Hamrah, Ula Jurkunas, Joseph B. Ciolino, Jose A.P. Gomes, Victor L. Perez, Yin Jia, Reza Dana, Bevacizumab in high-risk corneal transplantation: a pilot multicenter prospective randomized control trial, Random. Contr. Trial Ophth. 129 (8) (2022 Aug) 865–879, https://doi.org/10.1016/j.ophtha.2022.03.024.