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Consecutive dosing of UVB irradiation induces loss of ABCB5 expression and activation of EMT and fibrosis proteins in limbal epithelial cells similar to pterygium epithelium

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ABSTRACT

Pterygium pathogenesis is often attributed to a population of altered limbal stem cells, which initiate corneal invasion and drive the hyperproliferation and fibrosis associated with the disease. These cells are thought to undergo epithelial to mesenchymal transition (EMT) and to contribute to subepithelial stromal fibrosis. In this study, the presence of the novel limbal stem cell marker ABCB5 in clusters of basal epithelial pterygium cells co-expressing with P63α and P40 is reported. ABCB5-positive pterygium cells also express EMT-associated fibrosis markers including vimentin and α-SMA while their β-catenin expression is reduced. By using a novel in vitro model of two-dose UV-induced EMT activation on limbal epithelial cells, we could observe the dysregulation of EMT-related proteins including an increase of vimentin and α-SMA as well as downregulation of β-catenin in epithelial cells correlating to downregulation of ABCB5. The sequential irradiation of limbal fibroblasts also induced an increase in vimentin and α-SMA. Taken together, these data demonstrate for the first time the expression of ABCB5 in pterygium stem cell activity and EMT-related events while the involvement of limbal stem cells in pterygium pathogenesis is exhibited via sequential irradiation of limbal epithelial cells. The later in vitro approach can be used to further study the involvement of limbal epithelium UV-induced EMT in pterygium pathogenesis and help identify novel treatments against pterygium growth and recurrence.

1. Introduction

The cornea, consisting of the epithelium, stroma and endothelium, is our window to the world. A healthy corneal epithelium is necessary for corneal transparency and precise refraction. It is constantly maintained by a regenerating limbal epithelial stem cell (LESC) population located

Abbreviations: LESC, Limbal Epithelial Stem Cell; ABCB5, ATP-binding cassette, sub-family B, member 5; EMT, Epithelial to Mesenchymal Transition; HLE, Human Limbal Epithelial; MMP, Matrix Metalloproteinase.

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in the niche of the basal layer of the limbus, the vascularised junction between the cornea and the conjunctiva. Recently ATP-binding cassette, sub-family B, member 5 (ABCB5) (Frank et al., 2003, Schatton et al., 2008) emerged as a highly specific novel LESC marker (Ksander et al., 2014). Ksander et al., demonstrated that ABCB5 marks LESCs and is required for LESC homeostasis, corneal development and regeneration while ABCB5 loss of function causes depletion of quiescent LESCs due to enhanced proliferation and apoptosis (Ksander et al., 2014).

Previously, we have shown that UV irradiation induced loss of putative stem cell characteristics (Notara et al., 2015, Notara et al., 2016) including the expression of ABCB5 (Notara et al., 2018a; Notara et al., 2018b). UVB irradiation is a key causing factor for pterygium, a non-cancerous triangular vascularised growth of the cornea, which invades the corneal periphery. It is characterised by destruction of Bowman's membrane, epithelial and fibroblast hyperproliferation as well as squamous and goblet cell hyperplasia (Bradley et al., 2010) (Notara et al., 2018a; Notara et al., 2018b). The pterygium tumour is opaque, vascularised, inflamed and apart from causing discomfort it may also disrupt vision when approaching the visual axis (Notara et al., 2018a; Notara et al., 2018b). The prevalence of pterygium ranges from 1 % to over 30 % with an estimated pooled prevalence of approximately 10 %. Risk factors for pterygium include age, male sex, outdoor activity, darker skin complexion and smoking; it is the most common in geographic latitude 40° around the equator which strongly supports the role of ultraviolet (UV) irradiation in the pathogenesis of pterygium, however the exact mechanism is not known (Shahraki et al., 2021).

There is evidence that in pterygium, the basal limbal epithelium exhibit aberrant expression of proteins such as matrix remodelling matrix metalloproteinases (MMPs) including MMP1, 13, 2 and 9 (Di Girolamo et al., 2000), along with putative stem cell markers (Chui et al., 2011) suggesting LESC may be involved in pterygium onset and progression as we had earlier postulated (Coroneo et al., 1991a; Coroneo et al., 1991b). In accordance with this theory, LESCs may also undergo epithelial to mesenchymal transition (EMT), a process by which epithelial cells take characteristics of mesenchymal cells. While being essential for development, EMT is also linked to fibrotic diseases and cancer (Chen et al., 2017). The EMT-undergoing LESCs may generate fibrosis in the limbal stroma and possibly trigger epithelial intrastromal invasion manifesting limbal barrier failure and LESC deficiency via the Wnt/β-catenin pathway (Kawakita et al., 2005). In a similar context, the intra-nuclear accumulation of β-catenin with expression of α-SMA and vimentin were identified in K14+ epithelial cells at the leading edge of epithelial invaginations of pterygia (Kato et al., 2007) while α-smooth muscle actin (α-SMA)-positive epithelial cell clusters exist within the basal pterygium epithelial indentations extending into the stroma of (Ando et al., 2011). Despite it having been postulated that limbal or conjunctival basal epithelial cells undergo EMT (Chui et al., 2008) to give rise to pterygium myo-fibroblasts this theory has not yet been tested and there are no pharmacological interventions against UVB-induced EMT (in the context of pterygium) in place.

Here we 1. used pterygium sections to investigate whether putative stem cell populations undergo EMT by assessment of ABCB5 and other putative stem cell markers including p63α and p40 in co-expression with vimentin, β-catenin and α-SMA. 2. *In vitro*, we used a novel model where primary human limbal epithelial cells and fibroblasts were sequentially UVB-irradiated and the activation of EMT signalling pathway was investigated by western blotting and immunofluorescence. Following up previous work where we demonstrated the loss of putative stem cell marker expression following limbal epithelial cells after UVB irradiation, we assessed the expression of ABCB5, first time observed in pterygium tissue and cultured cells, in order to correlate loss of stem cell character leading to EMT pathway activation. The overall aim was to elucidate further the mechanisms leading to pterygium pathogenesis and progression as well as to create an in vitro model of UV-induced stem cell damage leading to EMT activation. This in vitro platform will help towards studying the therapeutic potential of anti-EMT substances, particularly against pterygium recurrence.

2. Materials and methods

2.1. Ethics statement

Human cadaveric corneoscleral rims and buttons, a surplus of transplantation surgery, where only used in case of priory obtaining research consent and in accordance to the declaration of Helsinki. The tissue had ethics approval (UK Cologne local ethics committee, decision number 14–247) obtained from the Eyebanks of the department of ophthalmology in Cologne and from the Gewebebank Mecklenburg-Vorpommern in Rostock, Germany. Studies involving pterygium and ocular surface tissue obtained st the time of pterygium surgery were approved by the South-Eastern Sydney Area Health Service Human Research Ethics Committee (Eastern Section) 15/200, Sydney, Australia.

2.2. Primary human limbal epithelial cell harvesting and maintenance

Primary human limbal epithelial (HLE) cells were cultured in Cnt-57 medium provided by CELLnTEC Advanced Cell Systems (Bern, Switzerland). When used to HLE culture, this media is promoting a putative LESC phenotype. Sectioned corneo-scleral buttons or limbal rims were treated with a 1.2 U/ml dispase II solution (Sigma) for 2 h at 37 °C. Then, the epithelial cells were gently scraped by using a scalpel and following the limbal border in order to achieve an enriched LESC/progenitor population. The cells were then placed into a T-25 tissue culture flask (Nunc). The cultures were cultured at 37 °C and 5 % CO_2 in air; epithelial colonies emerged 3–5 days following isolation. The cells reached 80–90 % confluence in approximately 10 days and were always used at P0.

2.3. Isolation and culture of human limbal fibroblasts

Following the harvesting of epithelial cells, the scleral and corneal part of the rims was cut off to leave approximately 1 mm around the limbus. Then, the limbus was further cut in to smaller pieces. These pieces were placed epithelial side down on to 10 cm petri dishes and cultured in DMEM (Invitrogen) plus 10 % FBS and 1 % penicillin–streptomycin (Invitrogen) until fibroblasts appeared. The cells were subcultured at a ratio of 1:3 and the medium was exchanged every second day. The cells reached 80–90 % confluence in approximately 3 weeks and were always used at P0.

2.4. UVB-irradiation of cells

Limbal epithelial cells and limbal fibroblasts were plated into either 6-well plate wells (for protein collection) or in 8-well chambered slides (for immunostaining), one to be subjected to UVB irradiation and the other one to be used as a control. The cultures were cultured up to 80–85 % confluence. Before UVB treatment, the culture media was exchanged with PBS to prevent the production of photo-toxic substance of irradiated serum. The cells were placed in a Vilber Lourmat, Bio-Sun UV irradiator set at 265 nm and given a dose of at 30 mJ/cm^2. The same treatment was repeated after 48 h. After both irradiations, the PBS was replaced with normal culture medium. Chambered slides were fixed and protein samples were collected at day 4 post-treatment.

2.5. Immunocytochemistry of cells and tissue sections

For Cells: Eight-well permanox chambered slides from labtek, Nunc were used for immunocytochemistry of cultured cells. The cells were washed three times with PBS and treated with 4 % (wt/vol) paraformaldehyde for 10 min at room temperature. The samples were blocked for 1 h in PBS with added 5 % goat serum (Sigma) and 0.5 % Triton X (Sigma) followed by the primary antibody (refer to Table 1 for

Table 1
Primary antibodies used in this study for western blotting and immunostaining and their respective concentrations.

Antibody	Host	Dilution (WB)	Dilution (IF)	Manufacturer
ABCB5	Mouse	1:500	1:100	Ticeba GmbH, Heidelberg, Germany
p63α	Rabbit	N/A	1:200	Cell Signaling Technology, Danvers, USA
p40 (ΔNp63)	Mouse	N/A	1:100	Biocare Medical; Pacheco, USA
Vimentin monoclonal	Rabbit	1:500	1:200	Abcam, Cambridge, UK
α –SMA polyclonal	Rabbit	1:500	1:200	Abcam, Cambridge, UK
β -Catenin	Rabbit	1:2500	1:200	Abcam, Cambridge, UK
β-Actin	Mouse	1:300	N/A	Santa Cruz Biotechnology, Dallas, USA

primary and Table 2 for secondary antibodies used in this study) or blocking buffer without the primary antibody (negative control) overnight at 4 °C. Notably, the mouse anti-ABCB5 monoclonal antibody, clone 3C2-1D12, was provided by TICEBA GmbH (Frank et al., 2003, Ksander et al., 2014). Subsequently, the cells were incubated with their respective secondary antibody, washed and counterstained with DAPI for 5 min prior to mounting.

For tissue sections: De-paraffinization of paraffin-embedded formaldehyde-fixed tissue sections was carried out by washing in xylol (Fischer Scientific) for 10 min, then in 100 % ethanol, 96 % ethanol, 70 % ethanol, 30 % ethanol and tap water each step for 5 min and finally in distilled water for 10 min. The following antigen retrieval was used: the slides were transferred into a microwave-compatible coupling jar containing IHC Antigen Retrieval Solution with Ph6 (Sigma) and into a 95 °C water bath for 10 min. Subsequently, slides were washed with distilled water for 5 min followed by 5 min of washing with 1x PBS. After completion of antigen retrieval, the sections were permeabilized and blocked with 10 % goat serum (Sigma) and 0.5 % human FC block (BD Biosciences) in 0.3 % Triton (Sigma) in PBS for at least 1 h at RT. Subsequently, the sections -except for the negative control- were incubated overnight with the respective primary antibodies diluted in 10 % goat serum in PBS o/n at 4 °C. The different primary antibodies including their concentrations of optimal performance are listed in Table 1. Afterwards, the slides were washed with 1x PBS 3 times for 5 min each. Incubation with secondary antibodies which were diluted in 10 % goat serum in PBS (see Table 2) were performed for 1 h at room temperature. The negative control was incubated with a combination of all secondary antibodies used in the respective experiment. Slides were washed with 1x PBS for 5 min 3 times before sections were incubated with DAPI in PBS (1:2500) for 10 min. Mounting with Dako Fluorescence Mounting Medium (Dako) was performed and sections were dried at 4 °C.

An Olympus BX53 epifluorescence microscope was used for imaging of both cultured cells and tissue sections.

Table 2
Secondary antibodies used in this study for western blotting and immunostaining and their respective concentrations.

Antibody	Host	Dilution (WB)	Dilution (IF)	Manufacturer
Anti-Mouse Alexa 555	Goat	N/A	1:200	Sigma-Aldrich, St. Louis, USA
Anti-Rabbit Alexa 488	Goat	N/A	1:200	Sigma-Aldrich, St. Louis, USA
Anti rabbit HRP	Swine	1:3000	N/A	DAKO, Waldbronn, Germany
Anti mouse HRP	Goat	1:1000	N/A	DAKO, Waldbronn, Germany

2.6. Western blotting

Sub-confluent cell monolayers of each treatment group were lysed on ice with RIPA buffer (Sigma), followed by centrifugation of the lysates at 12.000 rpm for 10 min and storage of the supernatants at −85 °C until analysis. Protein extracts (1.5 mg/ml) were separated at a 10 % SDS-PAGE gel and then transferred at a PVDF membrane. The details of primary and secondary antibodies used are described in Tables 1 and 2 respectively. The membranes were developed using an enhanced chemiluminescence reagent (Bio-Rad, Munich, Germany) and the images were captured using a Bio-Rad Molecular Imager® Gel Doc™ XR System. Semi-quantification by densitometry was carried out using image J. Each group was normalised against beta actin and the control group (NO UV) was set as 1.

2.7. Statistical analysis

Statistical analysis of the experimental data was carried out by using Prism 6.0 software (GraphPad). A T-test with Mann-Whitney post-test was applied. Results producing a p value lower than 0.05 were defined as statistically significant. We used a minimum of 3 experimental triplicates and repeated experiments at least three times. Cells from at least three cadaveric tissue donors were used to account for biological variability. Error bars displayed in graphs correspond to standard deviation.

3. Results

3.1. Differential expression of putative LESC markers in normal limbus and pterygium

The localisation of putative LESC markers in human limbus sections was compared to surgically excised pterygium. The putative stem cell markers ABCB5, P63α and P40 were expressed in the basal and suprabasal layers of normal limbal epithelium (Fig. 1A and B). Specifically, bright clusters ABCB5 (Fig. 1A red channel) and P63α (Fig. 1A green channel) co-localised in the limbal crypts and crypt-surrounding limbal basal layer (Fig. 1A highlighted by magenta and yellow dotted ovals respectively) while bright patches of cells co-expressing P63α (Fig. 1B green channel) and P40 (Fig. 1B red channel) were also observed in the basal layer of the both the limbal crypts as well as the basal layer of the crypt-surrounding limbus (Fig. 1B highlighted by magenta and yellow dotted ovals respectively).

Similarly to the limbus, ABCB5 and P63α as well as P40 and P63α were also co-localised in bright tightly packed clusters of epithelial cells throughout the basal epithelial layer of pterygium (Fig. 1D and E highlighted by yellow ovals and white arrows respectively).

3.2. Expression of EMT markers in normal limbus and pterygium

To understand the EMT phenomena involved in pterygium pathogenesis especially in relation to LESC, the expression of EMT related proteins including α-SMA, Vimentin and β-catenin was examined in both normal limbus and pterygium sections by using immunofluorescence. These proteins where co-localised with the putative stem cell marker ABCB5. In the normal limbus, there was expression of ABCB5, especially in the basal layers of the limbal crypts (Fig. 2 A-C, red channel). The cells in all layers exhibited low expression of α-SMA (Fig. 2A, green channel). While limbal fibroblasts expressed Vimentin (Fig. 2B, green channel), β-Catenin was localized at the membrane of the limbal epithelial cells of basal, suprabasal and epithelial layers (Fig. 2C, green channel).

ABCB5 expression in pterygium epithelium was localized in bright clusters of the basal and suprabasal layers (Fig. 3A-C, red channel) while, conversely to the normal limbal epithelium, it exhibited α-SMA expression which was reduced in the basal layer (Fig. 3A, green channel, highlighted by dotted line) and increased in the suprabasal and superficial layers. Vimentin on the other hand was strongly expressed by

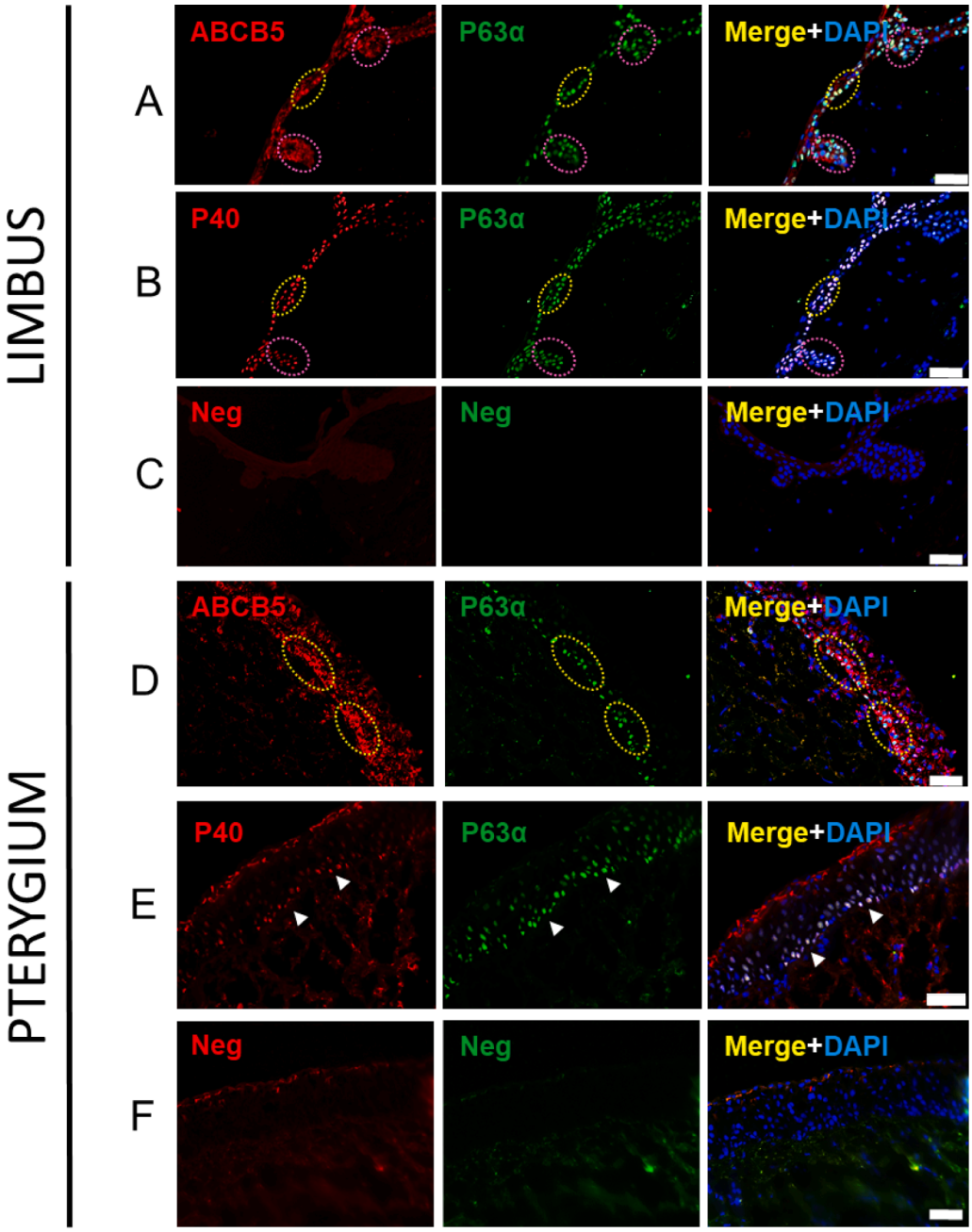

Fig. 1. Immunofluorescence of limbus (A-C) and pterygium (D-F) sections showing distribution of LESC markers. Dotted ovals highlight limbal crypts (magenta) and basal epithelial compartment (yellow). Co-localisation of ABCB5 and *P*63-α (A limbus, D Pterygium, red channel and green channel respectively) as well *P*63-α and P40 (B limbus, E Pterygium, red channel and green channel respectively) showed that in both limbus and pterygium there are clusters of basal epithelial cells brightly stained for these markers. Panels C and F depict negative controls for limbus and pterygium respectively (C and F). Five and eight donors were used for limbus and pterygium respectively. Scale bars correspond to 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stromal cells in close proximity to the basal epithelium (indicated by white arrows in Fig. 3B, green and merged channel) as well as by cells in the basal and suprabasal epithelial compartment (highlighted by white circle in Fig. 3B, red and merged channel). β-Catenin was absent in many cells of the pterygium epithelium (indicated by white arrows in Fig. 3C, green and merged channel) while the epithelial cell morphology and stratification appeared dysregulated compared to the limbal epithelium, by featuring variant cell size and shape (Fig. 3C, green channel). Some basal epithelial cells, also expressing ABCB5, featured signs of nuclear translocation of β-Catenin, as depicted in the inlets of Fig. 3C (Fig. 3C, red and green channel respectively, highlighted by yellow dotted boxes and white arrows).

3.3. Differential expression of α-SMA and vimentin in normal limbus and pterygium stroma

The localisation of α-SMA and vimentin in stromal cells of normal limbus and pterygium was examined. In the limbal stroma, α-SMA-was rarely expressed while the epithelium was negative (Fig. 4A, stroma/epithelium border defined by dotted white line). Conversely, the pterygium epithelium expressed α-SMA while numerous clusters of stromal cells (Fig. 4B, highlighted by white arrows) also produced it. Similarly, in the limbus, Vimentin was predominantly expressed in the stromal compartment while it was strongly expressed in clusters of stromal cells present in the pterygium stroma (Fig. 4C and D pterygium stromal cells highlighted by white arrows (D), stroma/epithelium border defined by dotted white line).

3.4. UV-induced alterations in the expression of ABCB5 and EMT-related proteins of corneal epithelial cells

In order to obtain UV-induced alterations related to EMT, we have irradiated cultured corneal epithelial cells with two doses of UVB at 0.03 J/cm^2 with an interval of 48 h (Fig. 5A). Subsequently, the cells

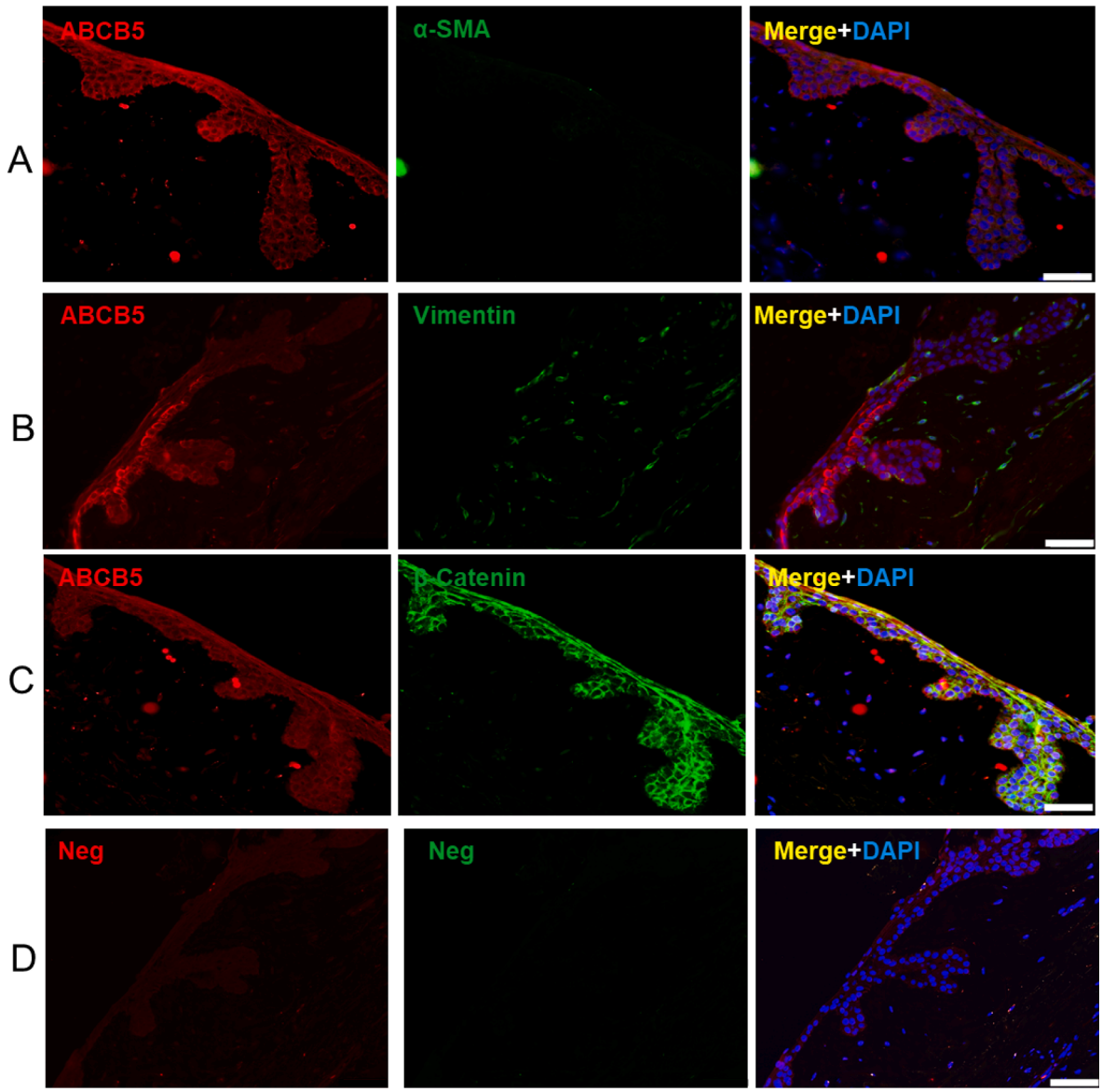

Fig. 2. Immunofluorescence of limbus sections depicting the localisation of ABCB5 and EMT and fibrosis markers. ABCB5 and α-SMA were limited in the epithelium and stromal compartments respectively (A, red channel and green channel). Vimentin was expressed by stromal cells throughout the limbal stromal while there was minimal co-localisation with ABCB5-positive cells in the epithelium (B, green channel and red channel respectively). β-Catenin was expressed throughout the limbal epithelium co-localising with ABCB5-positice cells (C, green and red channel). Panel D depicts negative controls for limbus and pterygium respectively (C and F). Tissue specimens from 5 donors were used. Scale bars correspond to 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were either fixed or protein was isolated after 4 days. The immunostaining data exhibited a significant UV-induced reduction in ABCB5 staining (Fig. 5B and F) also confirmed by western blotting (Fig. 5J, densitometry results shown in K). Moreover, enlarged cells positive for α-SMA and vimentin were present in UV treated cultures (Fig. 5G, I respectively) compared to their irradiated counterparts (Fig. 5C, E). UV–treatment induced an enlargement of cells and reduced expression of β-catenin compared to the non-treated culture (Fig. 5H and D respectively). Western blotting data confirmed that α-SMA and vimentin were significantly upregulated following UV irradiation (western blot shown in Figure J and densitometry plots in Figure L and N respectively) while the levels of β-catenin were significantly reduced upon UV treatment (Fig. 5J and M).

3.5. UV-treatment-initiated upregulation of α-SMA and vimentin in limbal fibroblasts

In order to obtain UV-induced alterations related to EMT and fibrosis phenomena similar to pterygium, we irradiated cultured limbal fibroblasts with two doses of UVB at 0.03 J/cm2 with an interval of 24 h. Subsequently, either the cells were fixed at 5 days or protein was isolated after 4 days. The immunostaining data exhibited a UV-induced increased in both α-SMA and vimentin signal (Fig. 6A-D). This was confirmed by western blotting data (E). Specifically, densitometry analysis demonstrated that there was statistically significant upregulation of both α-SMA and vimentin (Fig. 6F and G respectively).

4. Discussion

Exposure to UV irradiation is one mechanism known to compromise

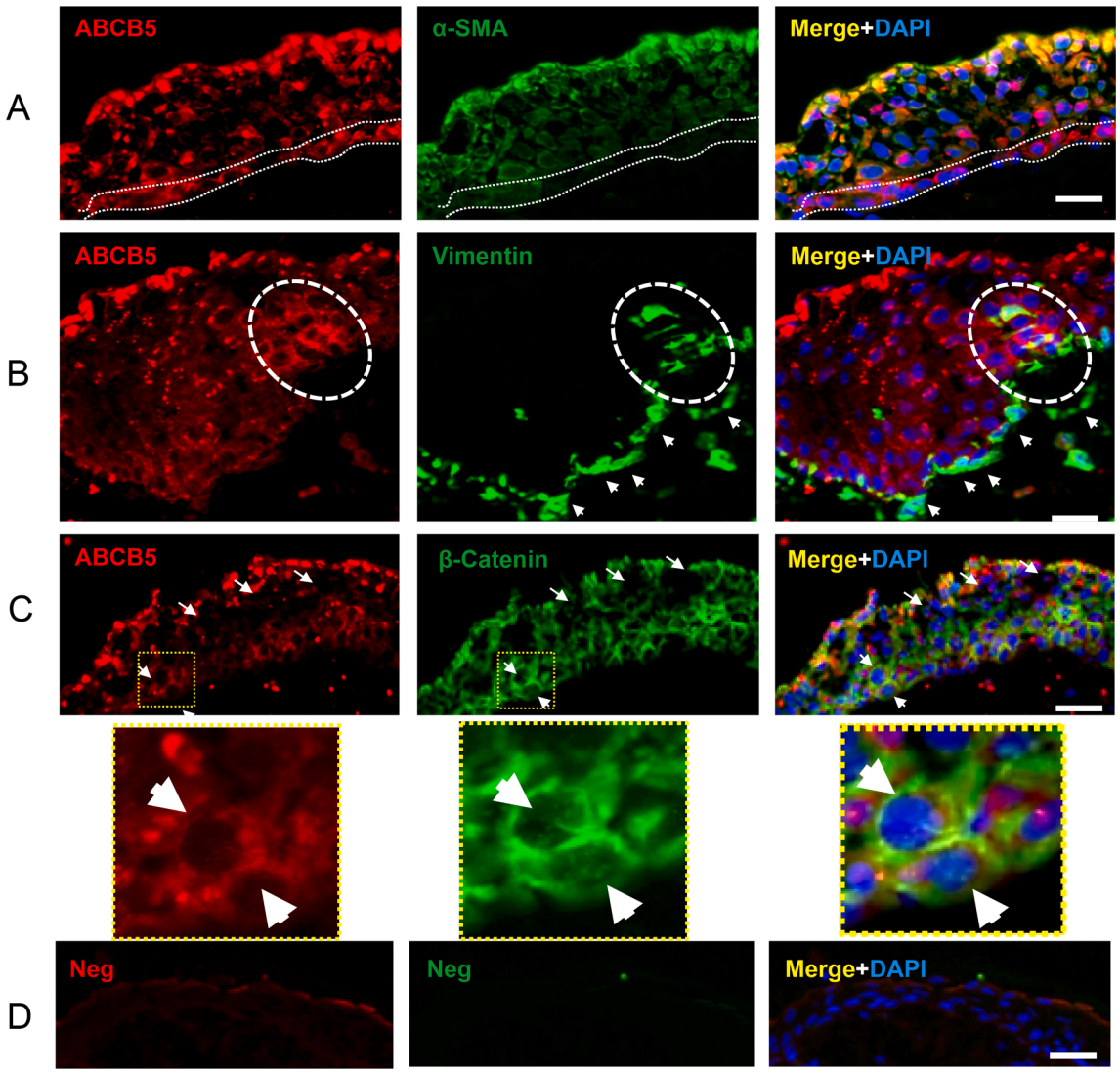

Fig. 3. Immunofluorescence of pterygium sections depicting the localisation of ABCB5 and EMT and fibrosis markers. ABCB5 expression in pterygium epithelium was localized in the basal and wing cells (A-C, red channel) while α-SMA signal was reduced in the basal layer (A, green channel, highlighted by dotted line). Vimentin was strongly expressed by stromal cells proximal to the basal epithelium (B, green channel, indicated by white arrows) and by basal and wing epithelial cells (B, highlighted by white circle). β-Catenin expressed whithin the pterygium epithelium was partially lost (C, green channel indicated by white arrows). Some basal epithelial cells, also expressing ABCB5, featured signs of nuclear translocation of β-Catenin, (C, inlet red and green channel respectively, highlighted by yellow dotted boxes and white arrows). Panel D depicts a negative control where the primary antibody step was omitted. Tissue specimens from 7 donors were used. Scale bars correspond to 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the LESC niche and is associated with pterygium. The cornea is particularly susceptible to UV irradiation due to its inherent transparency as well as to its shape, which contributes to a peripheral light focusing effect that affects the nasal limbus, where UV irradiation is strongest by a 20-fold (Coroneo et al., 1991, Maloof et al., 1994). This is the predominant and characteristic site for the onset of pterygium, as well as the habitat of limbal epithelial stem cells, the population responsible for corneal epithelium regeneration. The pterygium breaks the limbal barrier separating the cornea from the conjunctiva and centripetally invades the corneal surface. It is characterised by squamous and goblet cell hyperplasia. In advanced cases, the visual axis may be covered by vascularised opaque tissue, thus leading to discomfort and decrease or loss of vision (Bradley et al., 2010).

There is strong histological evidence that what is responsible for pterygium onset is altered limbal epithelial cells able to express both MMPs and basal limbal markers, thus suggesting that the condition may indeed be a limbal stem cell disorder as previously postulated (Davanger and Evensen 1971, Coroneo et al., 1991, Chui et al., 2011). It is logical to suggest that since stem cells are long-cycling and live through most of the lifetime of an individual, they can accumulate UV-related damage over time, which can lead to benign or even malignant growths.

Our data demonstrated that the putative LESC markers expressed in the basal compartment of the limbus including the limbal crypts were also found in the basal epithelial and wing layers of pterygium. Previous reports demonstrated the presence of P63 (Sakoonwatanyoo et al., 2004, Ramalho et al., 2006, Atkinson et al., 2008, Nubile et al., 2013) and P63α-expressing pterygium basal epithelial cells, the latter consisting pterygium stem cell micro-clusters also positive for cytokeratin 15 (Chui et al., 2011). Our data demonstrated that P63α was largely co-expressed with P40 (directed against the ΔN domain of the ΔNp63 isoform of p63, both isoforms linked to LESC phenotype (Di Iorio et al., 2005)) in tightly packed clusters of basal epithelial cells in both limbus and pterygium sections. While low expression of P40 was previously reported in cultured pterygium cells (Josifovska et al., 2017), the presence of this marker in situ is a novel finding. Since P40 was linked to cancer stem cell activity and adverse prognosis in malignancies including prostate and

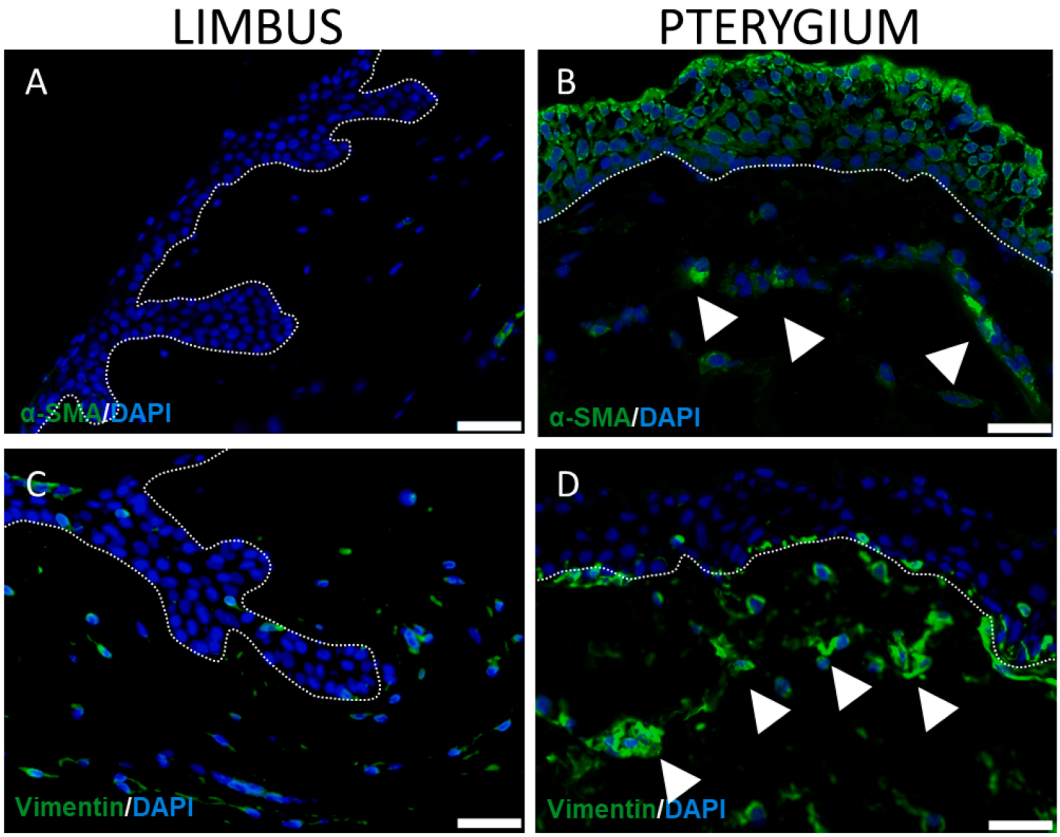

Fig. 4. Fibrosis markers in limbal versus pterygium stroma: α-SMA-was scarcely expressed in the limbal stroma (A) while, whithin the pterygium stroma α-SMA was strongly expressed in numerous cells clusters (B, highlighted by white arrows). In the limbus, Vimentin was mainly expressed in the stroma (C) while it was strongly expressed in stromal cells clusters (D highlighted by white arrows). The stroma/epithelium border was defined by dotted white line. Tissue specimens from 5 donors were used. Scale bars correspond to 50 μm.

breast cancer (Liu et al., 2020, Galoczova et al., 2021), its potential role in pterygium pathogenesis and progression merits further investigation. We have previously termed pterygium, a stem cell disorder with pre-malignant features. In these series, features of ocular surface squamous neoplasia (OSSN) and primary acquired melanosis and stem cells arranged in micro-clusters in pterygium basal epithelium in 12 % of cases were reported (Chui et al., 2011).

ABCB5 is considered a specific LESC marker (Ksander et al., 2014) while its presence in skin mesenchymal stem cells has shown therapeutic potential (Tietze et al., 2018, Riedl et al., 2021); this is tested in ongoing clinical trials including treatment of chronic ulcers (Ballikaya et al., 2020, Kerstan et al., 2021). ABCB5 is also a marker for cancer stem cells associated with poor prognosis and increase metastatic activity in glioblastoma (Lee et al., 2020a; Lee et al., 2020b), colorectal cancer (Gasser et al., 2017, Guo et al., 2018), ocular surface squamous neoplasia (Jongkhajornpong et al., 2016a; Jongkhajornpong et al., 2016b), and skin melanoma (Frank et al., 2005, Lin et al., 2013, Wang et al., 2017) as well as conjunctival (de Waard et al., 2015) and uveal (Thill et al., 2011) melanomas. Immunofluorescence results confirmed the presence of ABCB5 and its co-localisation with P63α in the basal compartment of the limbus. Notably, an increased expression in the limbal crypts which are putative niche structures containing a higher number of stem cells (Dziasko et al, 2014) was also observed. The previously unreported, ABCB5 expression and its co-expression with p63α were also observed in basal pterygium epithelium indicating that ABCB5 may mark a stem cell population within pterygium. While in normal limbus α-SMA and vimentin expression in the limbal stroma was limited, their expression was increased in pterygium stroma confirming previous reports associating these markers to the fibrosis associated with the condition (Touhami et al., 2005, Kato et al., 2007, Engelsvold et al., 2013). Specifically, α-SMA activity is linked to an activated fibroblast phenotype with increased contractility which is mediating tissue fibrosis during wound healing (Hinz et al., 2001) while it is considered an EMT marker (Zeisberg and Neilson 2009). On the other hand, vimentin intermediate microfilaments actively change the shape of epithelial cells to the characteristic elongated mesenchymal morphology featuring during EMT (Mendez et al., 2010). The presence of these markers in pterygium epithelium is aberrant (Kato et al., 2007) and their co-localisation within bright clusters of ABCB5-positive epithelial cells suggests that EMT phenomena which are suggested to play a key role in pterygium pathogenesis and propagation of the associated fibrosis. A similar epithelial phenotype has been previously observed in limbal stem cell deficiency-induced pannus where p63α-positive basal epithelial cells also expressed α-SMA and vimentin whereas β-catenin localisation was cytoplasmic and weak, suggesting the involvement of EMT to subepithelial tissue fibrosis (Kawashima et al., 2010). In pterygium, the co-expression of these markers with ABCB5 is also an indication that EMT may be initiated in the stem cell compartment thus driving fibrosis correlating with previous findings of p63α-expressing basal limbal pterygium cells thought to be involved in pterygium pathogenesis (Dushku and Reid 1994).

Previous own study indicated that ABCB5 expression in limbal epithelial cells was downregulated upon UVB treatment, which also correlated with loss of other stem cell markers including P63α, as well as integrin β1 and a drop of the colony forming efficiency attributed to stem cell differentiation as well as a dysregulation of secreted angiogenic and pro-inflammatory cytokines (Notara et al., 2018a; Notara et al., 2018b). In this current study, the reduction of ABCB5 following a double UVB insult was confirmed and coincided with an upregulation of α-SMA and β-Catenin suggesting that UVB treatment may induce pro-fibrotic changes involving EMT. Previous reports have linked UVB-

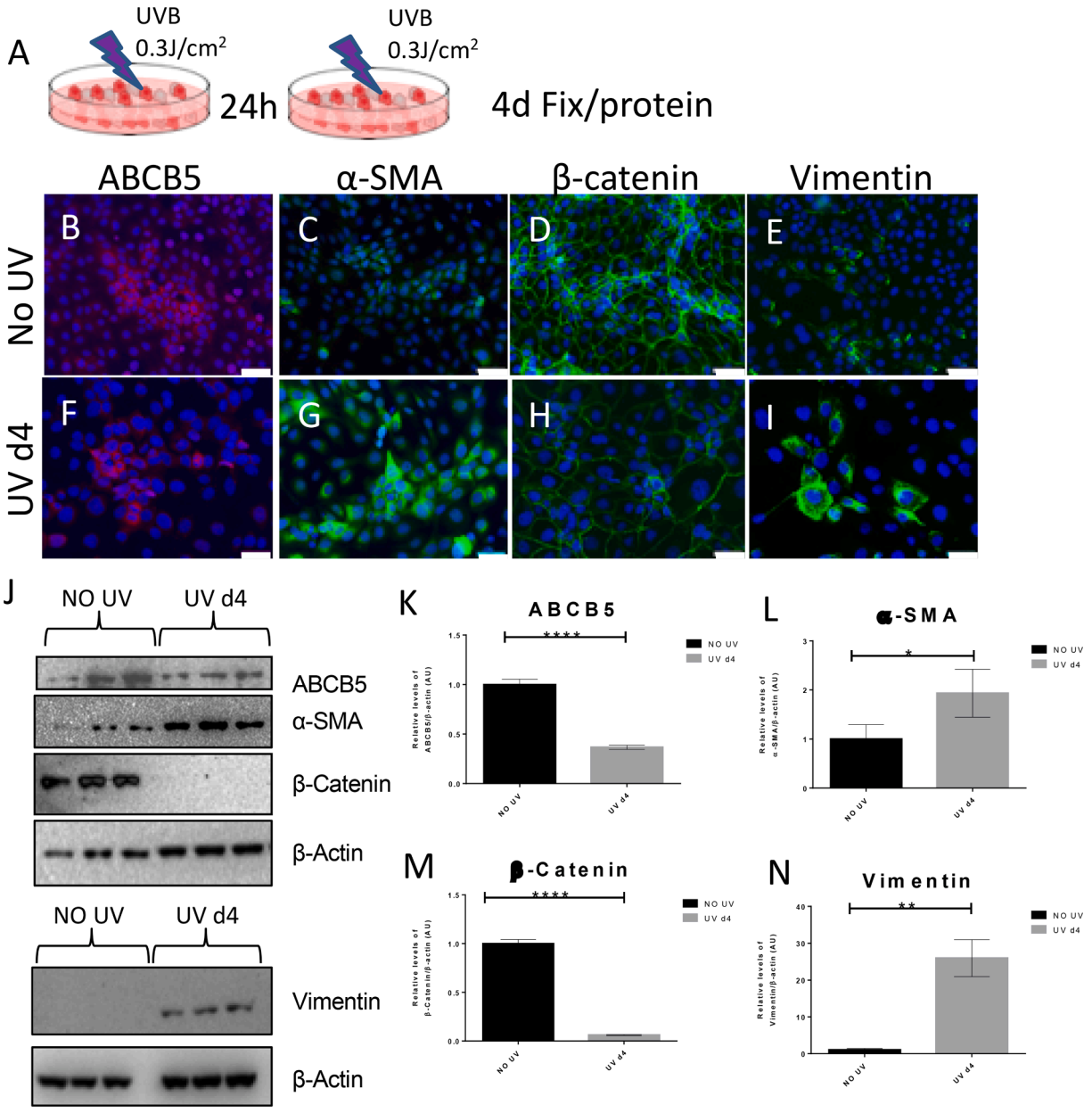

Fig. 5. Effects of repetitive UVB irradiation in cultured corneal epithelial cells. Schematic representation of the sequential irradiation of corneal cell cultures (A). Corneal epithelial cells featured alterations including reduction of ABCB5 (B, F), upregulation of α-SMA (C, G), upregulation of β-Catenin (D, H) and increase of Vimentin (E, I) following repetitive UVB treatment. Results were confirmed by western blotting, were protein isolated by non-treated cells compared to cells 4 days post-UV-treatment was analysed (J). Densitometry analysis was also carried out and showed significant differences in all markers (K-N for ABCB5, α-SMA, β-Catenin and Vimentin respectively). **** correspond to $p < 0{,}0001$, ** to $p < 0{,}01$ and * to $p < 0{,}05$ (*t*-test). A minimum of 5 donors with 3 technical replicates were used. Scale bars correspond to 100 μm.

induced oxidative damage to the upregulation of α-SMA and EMT in cultured lens cells contributing to cataractogenesis (Yoshitomi et al., 2019) while in dermal endothelial cell cultures UVB treatment induced an increase of vimentin expression linked to senescence (Shin et al., 2014). Other studies demonstrated that UVB caused downregulation of β-catenin via proteolytic cleavage in HaCaT keratinocytes (Hung et al., 2006) whereas UVB-induced downregulation of β-catenin in epidermal stem cells has been linked to deactivation of Wnt/β-catenin pathway, a key stemness-maintaining signalling machinery (Zhang et al., 2019). The in vitro changes observed in UVB-treated limbal epithelial cells correlate to the in vivo phenotype observed in pterygium epithelium where epithelial cells featured α-SMA and vimentin positivity as well as loss of β-Catenin.

Taken together, these data indicate a link of UV-induced ABCB5 loss to EMT activation and pterygium pathogenesis. As ABCB5 is also expressed in other UV-induced cancers including ocular surface squamous neoplasia (Jongkhajornpong et al., 2016a; Jongkhajornpong et al., 2016b), conjunctival melanoma (de Waard et al., 2015) and uveal melanoma (Broggi et al., 2019) while it is regarded as a cancer stem cell marker (Grimm et al., 2012, Setia et al., 2012, Kozovska et al., 2016, Lee et al., 2020a; Lee et al., 2020b), the use of EMT-inhibitors including rapamycin may be considered as a therapeutic strategy against tumour recurrence in pterygium as well as in malignant tumours.

5. Conclusions

Our data indicate that the sequential irradiation of limbal epithelial and fibroblast cultures is a new in vitro model for the study of EMT and

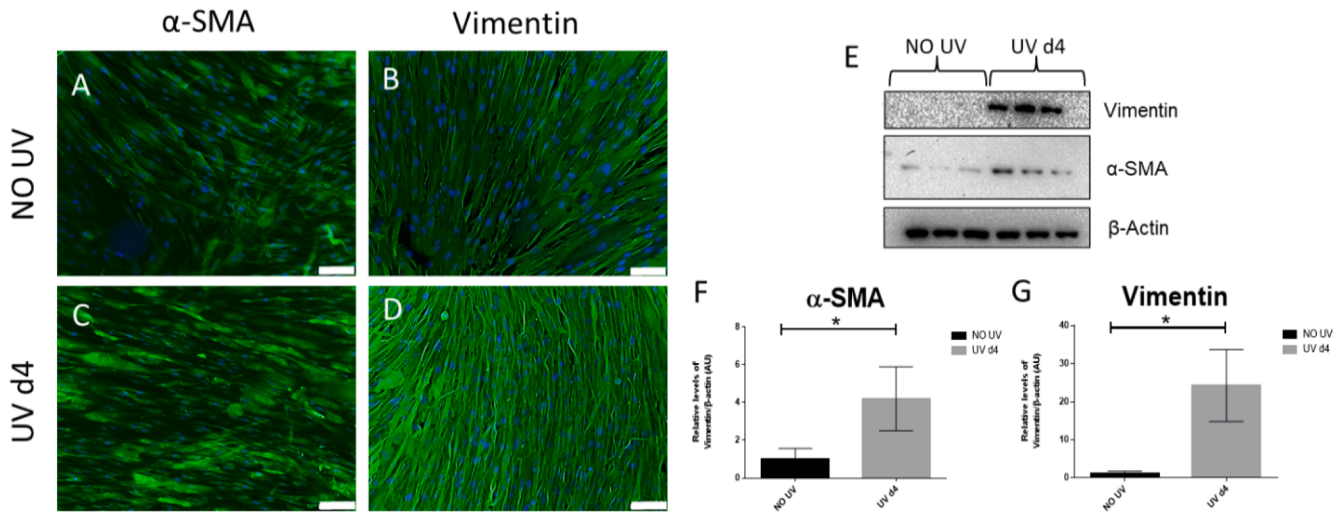

Fig. 6. Immunofluorescence staining of limbal fibroblasts depicting an increase in α-SMA (A, C) and Vimentin (B, D) expression following repetitive UVB irradiation. Scale bars corresponding to 50 μm. All cells were fixed 4 days following treatment and were stained on the same day. Results were confirmed by western blotting, were protein isolated by non-treated cells compared to cells 4 days post-UV-treatment was analysed (E). Densitometry analysis was also carried out and showed significant differences in both markers (F and G for α-SMA and Vimentin respectively). A minimum of 5 donors with 3 technical replicates were used. * corresponds to $p < 0,05$ (*t*-test). Error bars correspond to 100 μm.

fibrosis mechanisms in the human limbus linked to stem cell-initiated pterygium pathogenesis as well as serve as an in vitro platform for the assessment of EMT-inhibitory substances to be used against pterygium recurrence. The novel finding of ABCB5 and fibrosis marker expression as well as UVB induction in pterygium may allow for future novel therapeutic interventions against pterygium development and recurrence.

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