*Original article*

# C-MAF Expression in Adult Human Ocular Surface and its Implication in Pterygium Pathogenesis

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## Abstract

*Background:* c-MAF, a transcription factor that belongs to the b-Zip Maf transcription factor family, was found to be critical for lens development in vertebrates. It is a well-known fact that the adult human ocular surface expresses c-MAF, however, its role in the limbus, cornea and conjunctiva remains unknown. Thus, the present study aimed to investigate c-MAF expression within the human ocular surface, and its potential role in pterygium pathogenesis.

*Methods:* We performed immunohistochemical staining to detect c-MAF expression in frozen adult human tissue sections, including the limbus, cornea and conjunctiva, and cultured cells from eye cadavers. We then compared c-MAF expression to the expression of a known protein, P63. Lastly, we performed RT-PCR, and immunohistochemistry for c-MAF expression in healthy adult human conjunctiva and pterygium.

*Results:* We found differential c-MAF expression between adult human limbus, cornea and conjunctiva tissues. Further, we observed that c-MAF is downregulated in the pterygium compared to healthy conjunctiva.

*Conclusions:* Overall, our results suggest that c-MAF may play a context-specific role in maintaining limbal, corneal and conjunctival homeostasis, and may be critical for preventing pterygium development in humans.

*Keywords:* Conjunctiva, C-MAF Expression, Human Ocular Surface, Pterygium.

## Introduction

During the process of fetal ocular surface development, both the cornea and lens originate from the same cellular lineage. Moreover, the lens vesicle and the presumptive corneal epithelium are formed after pinching off from the overlying surface ectoderm, while the neural crest derived mesenchymal cells differentiate to form the corneal stroma and endothelium (1). To function effectively, the avascular cornea must withstand constant attrition from the external environment. Additionally, a continuous supply of corneal limbal epithelial cells is necessary to maintain surface transparency and refractivity (2). Both corneal stromal cells and lens fiber cells express water soluble crystallin proteins that are involved in maintaining transparency and refractive properties of the ocular surface (1). Like many other tissues, the development, maturation and commitment of the ocular surface requires a plethora of transcription factors (TFs) that are multifaceted in terms of gene regulation.

We and others observed that crystallin gene promoter transactivation by the transcription factor, c-MAF, is crucial for lens development in vertebrates (3). Though c-MAF expression and function has been extensively studied during lens fiber cell development, there is comparatively little knowledge regarding c-MAF function within the limbus, cornea or conjunctiva. Thus, the expression profile of this protein could help elucidate the role of c-MAF in these latter tissues. Based on cell type and spatiotemporal expression, c-MAF, a nuclear factor that belongs to the b-Zip family, may

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promote oncogenesis. According to the literature, c-MAF was found to be upregulated in multiple myeloma cells, and was, therefore, considered a potential target for various cancer therapies. Apart from cancer, uncontrolled c-MAF expression is likely involved in a number of pathological conditions (4). Pterygium, an ocular surface disorder characterized by hyperplasia of epithelial tissue, originates from the conjunctive, extends on cornea and progressively envelopes the pupil leading to visual impairment (5). Excessive exposure to UV light and dust has also been implicated in the pathogenesis of this benign tissue growth (6) but the exact pathophysiology remains unknown. In this study, we hypothesize that c-MAF may play a role in pterygium development. In this study, c-MAF expression was compared to the expression of P63, an ocular surface tissue protein that has been extensively studied by many groups (7, 8). Here, we found that c-MAF is expressed in ocular surface tissues in an aberrant fashion. Moreover, c-MAF expression is downregulated in pterygium tissue, suggesting that at peak concentrations, c-MAF may negatively regulate pterygium development.

## Materials and methods
### Preparation of human samples
We collected human conjunctival and pterygium biopsy samples with the approval of the Institutional Review Board of the Singapore National Eye Center, and informed consent from prospective surgery patients. All experimental procedures were performed according to the guidelines of the Declaration of Helsinki in Biomedical Research Involving Human Subjects.

### Preparation of human limbal and corneal tissues
Human limbal rims were obtained from the Singapore Eye Bank following a central corneal button transplant procedure. The rims were washed with phosphate-buffered saline (PBS) and embedded in Tissue-Tek OCT compound for cryosectioning. Cryosections, at a thickness of 10 microns, were subjected to immunostaining.

### Preparation of human conjunctival tissue
After receiving proper informed consent from patients undergoing routine surgery for pterygium or cataract, small conjunctival and pterygial biopsy samples were collected. The biopsied conjunctival and pterygial tissues were transported to the laboratory within the medium (Leibovitz L-15; Invitrogen-Gibco), washed with PBS and embedded in Tissue-Tek OCT compound for cryosectioning. Cryosections, at a thickness of 10 microns, were subjected to immunostaining.

### Feeder cell preparation
3T3 cells were maintained in DMEM with 10% fetal bovine serum (FBS). At 70% to 80% confluency, the cells were treated with 4 µg/µl mitomycin C for 2 hours at 37 °C under 5% $CO_2$ and 95% air to arrest cell growth. After incubation, the cells were washed with PBS for 5 minutes (x3), and trypsinized using 0.25% trypsin and 0.02% EDTA for 5 minutes, and replated at a density of $2.4x10^4$ cells/cm$^2$ on glass slides.

### Cultivation of primary human limbal epithelial cells
Human limbal rims were collected, washed with PBS, exposed to 1.2 U/ml dispase and incubated at 37 °C for 2 hours. The epithelial cell sheet was then separated into single cells using 0.25% trypsin and 0.02% EDTA for 8 minutes. Limbal cells were plated at $3-4×10^4$ cells/cm$^2$ in glass slides containing mitomycin-C treated 3T3 feeder cells. The media was changed every 2-3 days.

### Cultivation of primary human conjunctival epithelial cells
After obtaining proper informed consent from patients undergoing routine surgery for pterygium or cataracts, small conjunctival and pterygial biopsy samples were collected. The biopsied conjunctival and pterygial tissues were washed with PBS for 5 minutes (x3), and antibiotic solution containing penicillin 200 IU/mL, streptomycin 200 ng/mL, and amphotericin B 200 ng/ml. Collected tissues were cultured as explants in keratinocyte growth medium (KGM; Gibco). Conjunctival

and pterygium cells were removed using 0.25% trypsin and 0.02% EDTA for 8 minutes. The cell pellets collected after centrifugation were plated on glass slides for subculturing.

### Immunohistochemistry and immunocytochemistry

Primary tissues were fixed in 4% paraformaldehyde (PFA), while cultured epithelial cells on glass slides were fixed in cold methanol for 15 minutes at room temperature (RT) prior to blocking and permeabilizing with 2% BSA in PBS and 0.4% Triton X-100. The primary antibodies, including mouse anti-KI67 (Sigma-Aldrich), mouse anti-P63 (Abcam, Cambridge, UK), mouse anti-c-MAF, mouse anti-E-CAD and mouse anti-CK15 (Millipore) were incubated in 100× diluted blocking buffer at 4 °C overnight. The cells were then washed with PBS and incubated for one hour at RT with the appropriate fluorophore conjugated secondary antibodies (Alexa Fluor; Invitrogen) followed by counterstaining with DAPI (Vectashield, Vector Laboratories, Burlingame, CA). Zeiss Axioplan 2 microscope (Carl Zeiss Meditec, Inc.) was used to detect immunofluorescence.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from the limbus, cornea, conjunctiva and pterygium tissues using RNeasy mini kit (Qiagen, Valencia, CA, USA) and respective cDNA was generated using SuperScript III First Strand Kits (Invitrogen). Polymerase chain reaction (PCR) was performed using a PCR Master mix (2X) (Fermentas Life Sciences). PCR samples were denatured for 5 minutes at 94 °C followed by 30 PCR cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 58 °C, extension for 45 seconds at 72 °C, and termination for 7 minutes at 72 °C. The PCR products were verified by 2% agarose gel electrophoresis. The following forward and reverse oligonucleotide primers were used: *GAPDH:* Forward, 5'-GCCAAGGTCATCCATGACAAC-3', Reverse, 5'-GTCCACCACCCTGTTGCTGTA-3'; *c-MAF:* Forward, 5'-CCCAGGACTTCGCTATTTTG-3', Reverse 5'-CGCTCTCTACCTCTGTGCAA-3'.

## Results

### c-MAF and P63 expression in human limbus and cornea

The immunoreactivity of c-MAF was examined in adult human limbus and cornea tissue sections collected from three healthy donors. The limbal epithelial crypt is considered a rich source for limbal stem cells. In our study, we detected a strong reactivity of c-MAF in limbal crypt cells (Fig. 1A & D). The resultant c-MAF expression pattern overlapped with P63 expression (Fig. 1B & D). We found that there was no c-MAF reactivity in the papilla-like columns of the palisades of Vogt in the superior limbus (Fig. 1E), whereas, as consistent with the previous findings, P63 expression was detected in the smaller densely packed basal cells, and in some larger cells packed within the columns (Fig. 1F & H) (9). In the peripheral cornea, we observed that c-MAF expression (Fig. 1I & L) and P63 expression (Fig. 1J & L) was strong in all cells of the basal epithelium. When we examined the corneal stroma, there were more c-MAF-positive cells located near the endothelial layer compared to P63-positive cells (Fig. 1M & N & P). Interestingly, endothelial cells had noticeable c-MAF expression (Fig. 1Q & S), however, we detected no P63 reactivity (not shown). To confirm the expression of c-MAF at the RNA level, RT-PCR was performed using the total amount of RNA collected from the limbus and cornea. Our RT-PCR results show a similar c-MAF expression profile (Fig. 5B).

### Expression of c-MAF and other proteins in cultured primary human limbal epithelial cells
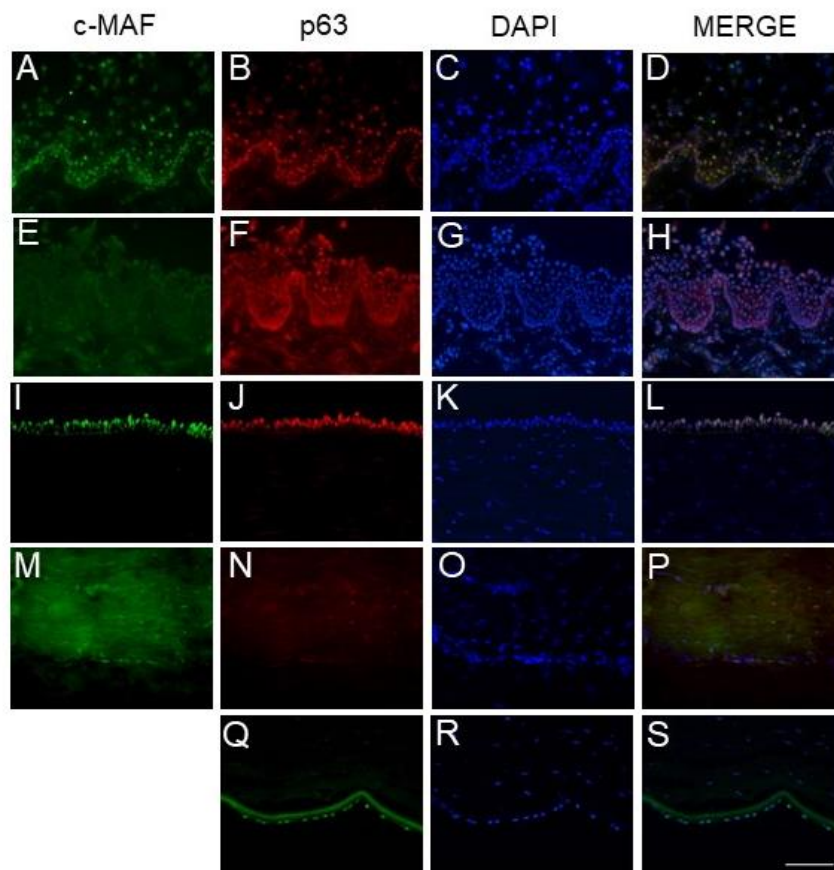
Primary human limbal epithelial cells were cultured on mitomycin-C treated feeder cells. A representative colony of cultured limbal epithelial cells exhibited a healthy morphology in the co-culture system (Fig. 2D). Immunostaining against the c-MAF antibody revealed that most of the cells located in the center of the colony strongly expressed c-MAF

within their nuclei (Fig. 2A & C), whereas, P63 expression was almost equal in all the cells of the colony (Fig. 2E & G). The expression patterns for c-MAF and P63 were similar to those observed in the limbal epithelium in vivo. In this context, we further checked the expression of a proliferation marker Ki67 and found its reactivity to be strongest in most cells of the colony (Fig. 2I & K). Furthermore, the expression of CK15, a specific marker of limbal basal epithelium or corneal progenitor (10, 11), was stronger in peripheral colony cells, in contrast to c-MAF expression (Fig. 2H). Finally, the cell adhesion molecule, E-cadherin, was expressed in the central region of limbal epithelial colony similar to c-MAF (Fig. 2L).

### *c-MAF and P63 expression in human conjunctiva*
Human conjunctival tissues were obtained from donors undergoing eye surgery. Paraffin

sections showed distinct c-MAF expression within a subset of the population containing small cell nuclei located in the basal layer (Fig. 3A & D, arrows). Similarly, a large number of cells in the superficial and suprabasal layer expressed c-MAF (Fig. 3A & D, arrowheads). Moreover, we observed c-MAF-positive staining in dispersed subepithelial stromal cells (Fig. 3A). In contrast, basal cells strongly expressed P63, in comparison to the superficial layer (Fig. 3B & D). However, few cells of the suprabasal layer showed reduced P63 expression (Fig. 3B & D, arrowheads). Our findings regarding P63 expression aligns with previous reports (12). To further confirm the expression of c-MAF at the RNA level, we performed RT-PCR on the conjunctiva, and found that our RT-PCR data was consistent with our present observations (Fig. 5A).



**Fig. 1.** Immunofluorescent staining of c-MAF and p63 on frozen sections of human limbal epithelial crypt (A-D), palisades of Vogt of limbus (E-H), peripheral cornea (I-L), corneal stroma (M-P) and endothelial cells (Q-S). DAPI stains cell nuclei (3rd column). Limbal epithelial crypt shows equal expression of c-MAF (A, D) and p63 (B, D). Cells in the palisades of Vogt express p63 (F, H) but not c-MAF (E, H). Only the basal layers of peripheral cornea show c-MAF (I, L) and p63 (J, L) expression. Some stromal cells are also positive to c-MAF (M, P) and p63 (N, P) reactivity. Endothelial cells clearly exhibit c-MAF expression (Q, S).

DAPI    MERGE
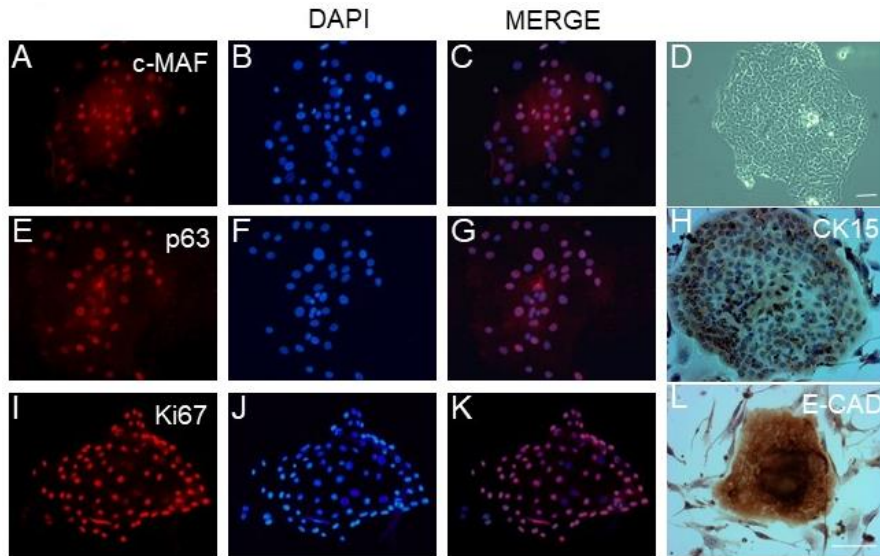
**Fig. 2.** Gene expression profiling in cultured human limbal epithelial cells. Strong expression of c-MAF (A-C) and p63 (E-G) is found in central cells of the colonies. Ki67 is detected in almost all cells (I-K). CK15 is strongly expressed in peripheral cells (H). E-Cadherin expression is strong in central cells of the colonies (L). Phase contrast image shows cell morphology of the clone (D). DAPI depicts cell nuclei (B, F, & G).

### *c-MAF expression in cultured primary human conjunctival epithelial cells*

Human conjunctival biopsies obtained from three donors were cultured and used for immunostaining against c-MAF and P63 antibodies. All cultured cells exhibited nuclear staining of c-MAF to varying degrees (Fig. 3E & H). We then examined the expression of P63 in these cultured cells, however, there was no positive signal upon detection (Fig. 3F & H).
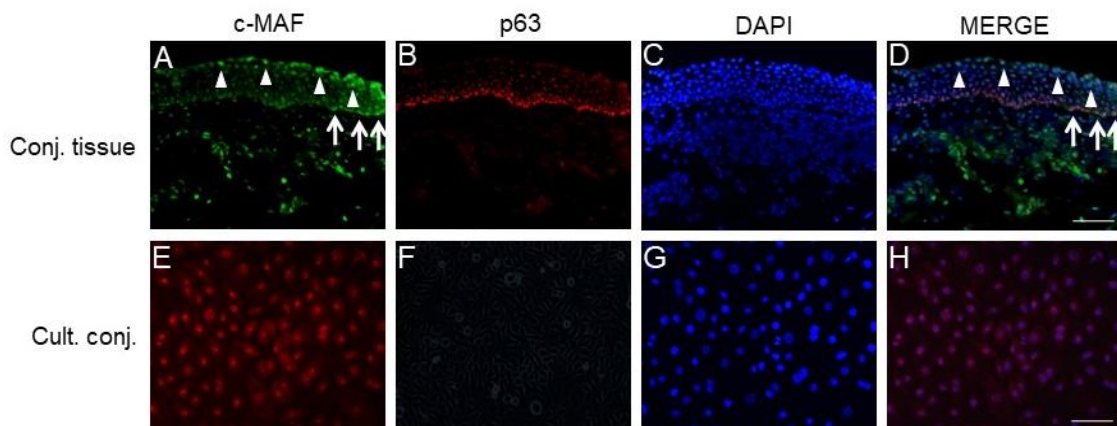


**Fig. 3.** Expression pattern of c-MAF and p63 in human conjunctival tissue (A, D) and cultured cells (E-H). c-MAF is expressed in some cells in the most upper layer (A, D, arrowheads) while weakly expressed in a subset of population with small nuclei in the basal layer (A, D, arrows). p63 in strongly expressed in basal layer (B, D). Although c-MAF is expressed in cultured conjunctival cells (E, H), p63 is absent (F). DAPI depicts cell nuclei (C, G).
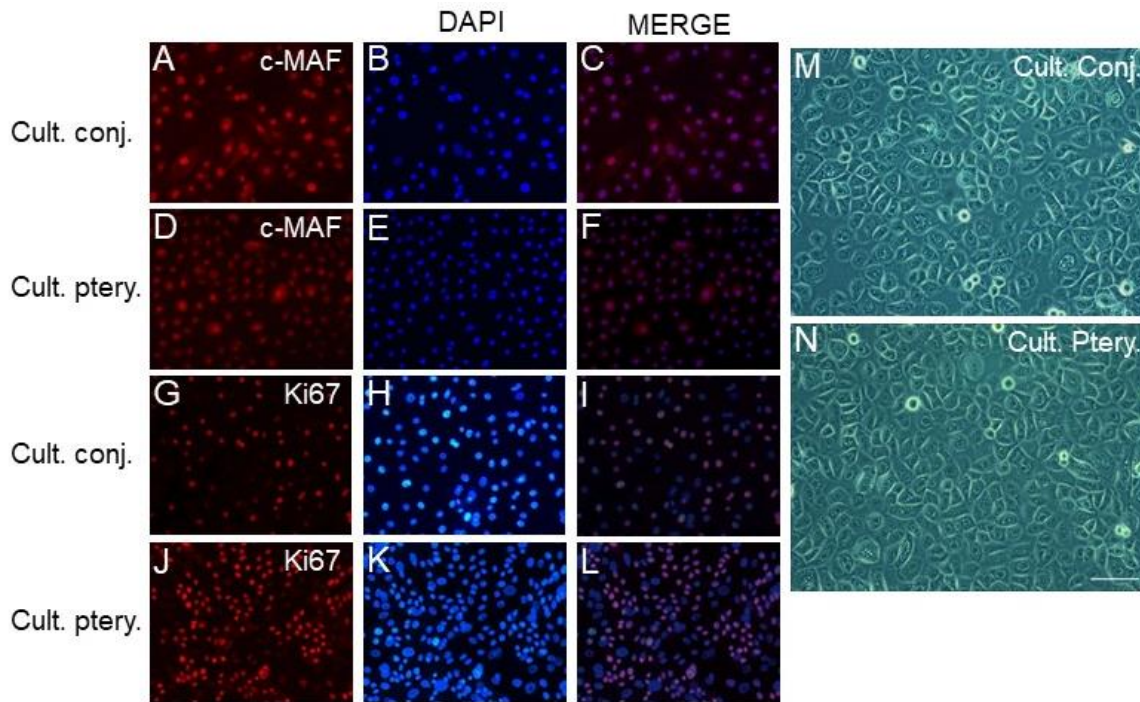
### *Altered expression of c-MAF and Ki67 in cultured conjunctival and pterygial cells*

Pterygium is an ocular surface disease caused by a multitude of environmental and genetic factors, however, the exact pathogenesis of pterygium remains unclear. A number of genes are suggested to play role in pterygium formation including proliferation and tumor suppression genes (13). Here, we investigated the involvement of a proto-oncogene as well as differentiation factor, c-MAF, in pterygium (14). Immunodetection using cultured epithelial cells from both conjunctiva and pterygium, revealed that c-MAF is strongly expressed in normal conjunctival epithelial cells (Fig. 4A & C) in comparison to pterygial cells (Fig. 4D & F). At

the mRNA level, c-MAF exhibited a similar expression pattern (Fig. 5A). Furthermore, we examined the expression of Ki67, a proliferation marker, in these cells (15). As expected, Ki67 showed greater reactivity in pterygial cells compared to conjunctival cells (Fig. 4J & L and G & I respectively). Both cultured conjunctival and pterygial cells showed a healthy morphology (Fig. 4M & N). These results indicate that despite the rapid expansion of the pterygium tissue, decreased c-MAF expression could protect against oncogenesis.



**Fig. 4.** Expression of c-MAF and Ki67 in cultured conjunctival as well as pterygial cells. c-MAF expression is stronger in normal conjunctival cells (A-C) than pteyrigium cells (D-F). Differentiation marker Ki67 is strongly expressed in most cells of the pterygium (J-L) but weakly expressed in conjunctiva (G-I). Cell morphology appears same in both cases (M, N). DAPI stains cell nuclei (2nd column).
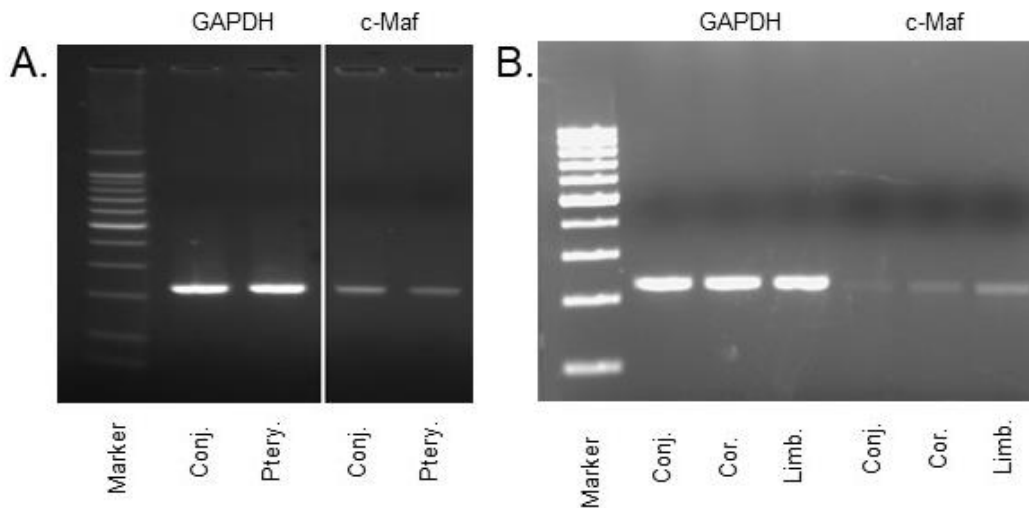


**Fig. 5.** RT-PCR shows the differential expression of c-MAF in conjunctival and pterygial tissues (A) and in normal ocular surface (B, limbus, cornea, conjunctiva).

## Discussion

While the expression of c-MAF and lens development has been reported in a wide range of species, its function was recently found critical for lens differentiation. Moreover, c-MAF has been characterized as a regulator of tissue-specific gene expression for various

biological processes (16). Apart from the lens, its role in other regions of the eye has not been fully elucidated, including the ocular surface tissue. A previous study investigated the expression of c-MAF at the mRNA level in the basal cells of developing epidermis and keratinocytes of embryonic rats (17). However, the corneal epithelial cells are different from epidermal keratinocytes due to its avascularity, non-keratinizing and transparent morphology. To our knowledge, no data concerning c-MAF expression in the corneal epithelia exists in the literature. Our study specifically investigated the expression pattern of c-MAF in different tissues of the human ocular surface such as the cornea, limbus and conjunctiva, and the pterygium, to elucidate a possible role for pterygium pathogenesis.

We studied c-MAF expression and compared it to the expression of putative limbal stem/progenitor or proliferative cell marker P63, which has been widely studied in the context of ocular surface development and homoeostasis (18). We observed both overlapping and independent expression patterns of c-MAF, in comparison to P63, in different tissues of the ocular surface. For example, in the cornea and limbal epithelial crypts, and cultured limbal epithelial cells, c-MAF expression mostly overlaps with P63 expression. Limbal epithelial crypts are limbocorneal projections that harbor limbal stem cells for both cornea and conjunctiva (19, 20). However, cells within the palisades of Vogt did not express c-MAF suggesting that c-MAF and P63 exhibit a co-operative function in maintaining the stemness of the adult stem cells in limbal epithelial crypts via unknown mechanisms. This assumption aligns with a previous study demonstrating that MafB and c-MAF are highly expressed in basal keratinocytes and hair follicles, which are two major stem cell niche sites during the late embryo stage (17).

E-cadherin is strongly expressed in the superficial layer, but not the basal layer of the limbal epithelium (11). Interestingly, our study detected high c-MAF and E-cadherin expression within central colony cells while CK15 (Fig. 2H), a specific marker of basal epithelium (21), was highly detected in peripheral colony cells.

These findings suggest that both c-MAF and E-cadherin can act on cells to promote differentiation processes during passaging. However, the absence of c-MAF and the presence of P63 in cells located within the papilla-like limbal structure suggests that c-MAF and P63 have distinct site-specific activity that are controlled by separate genes.

In the peripheral cornea, both c-MAF and P63 were expressed in the basal layer of the epithelium (Fig. 1I & J). Specifically, a strong c-MAF reactivity was detected in stromal cells of the cornea, however, we found little to no P63 reactivity (Fig. 1N). These results suggest that c-MAF and P63 may function separately in this tissue, and that it is possible that corneal basal cells undergo limited proliferation by P63 activity, and these cells differentiate, because of increased c-MAF activity, on demand later on. Another remarkable finding during this study was the detection of intense nuclear staining of c-MAF in endothelial cells, an important focus for future investigation.

It is a well-known fact that the lens and cornea are both avascular transparent tissues responsible for the maintenance of ocular transparency through the expression of crystallin proteins. Studies, pertaining to lens fiber cells, demonstrate that MAF proteins may transactivate the crystallin gene promoter to stimulate vertebral lens development. Accordingly, c-MAF null-mice showed microphthalmia and impaired lens development (3). Several studies have shown that the corneal stroma express crystallin-like proteins abundantly, which contributes to the development of corneal transparency (22). In our study, we observed a strong c-MAF expression in corneal stromal cells (Fig. 1M). We postulate that a possible link between c-MAF and crystallin gene expression in corneal stromal tissue may exist to maintain ocular transparency (23).

There are conflicting expression patterns between c-MAF and P63 in conjunctival tissues and cultured conjunctival cells. Our immunostaining data revealed that c-MAF was present in a subset of smaller nuclei in the basal layer of conjunctival epithelium. These cells were also positive for P63 expression (Fig. 1A &

B). A recent study demonstrates that c-MAF binds directly to pluripotent related gene promoters in human adipose tissue-derived adult mesenchymal stem cells, disruption of which by reactive oxygen species, led to decreased proliferation and self-renewal capacity and altered differentiation bias, indicating that c-MAF has multiple roles in different tissues and environmental contexts (24). Taken together, it is tempting to speculate that double-positive (c-MAF+P63+) cells are more likely to act as conjunctival progenitor cells compared to single P63-positive cells. Similarly, c-MAF+P63+ cells in the suprabasal layer may represent transient amplifying cells. We propose that weak c-MAF expression and moderate P63 expression in smaller cells along the bottom layer of human conjunctival epithelium represent progenitor-like cells that play a role in cell regeneration during homeostasis in adults. Surprisingly, P63 was undetectable in cultured conjunctival cells. To analyze this difference, we postulate that the culture medium may not contain upstream factors that critically regulate P63 expression, warranting future investigation. Nevertheless, our study indicates that c-MAF expression was strongest in the superficial layer of the conjunctiva, suggesting that c-MAF, as a differentiation factor, may play a significant role during the terminal differentiation of these cells. Taken together, c-MAF and P63 have distinct tissue-specific roles within the conjunctiva, and that further experiments are necessary to define their individual roles in this tissue.

In our study, c-MAF expression was higher in healthy conjunctival tissue compared to the pterygial tissue (Fig. 4A & D and Fig. 5A). A previous study suggests that abnormal epithelial differentiation in the pterygial epithelium is associated with a lower level of Pax-6 (25). Pax-6 is a known regulator of c-MAF expression in both precursor and developing lens cells (26) and is expressed in the conjunctival epithelium (27). In addition, we found that Pax-6 expression is decreased in the pterygial tissue compared to healthy conjunctiva (HMR, unpublished data). Hence, we conclude that lower Pax-6 levels in the pterygial tissue downregulates c-MAF

expression, which in turn, results in abnormal pterygial epithelial differentiation, and the indirect promotion of pterygial cell proliferation.

We detected upregulated Ki67 in cultured pterygial cells (Fig. 2J). This was expected, since the pterygium requires the proliferative function of specific molecules to maintain its proliferative nature. Our findings are consistent with a previous study demonstrating an increased expression of Ki67 in the pterygium (28). It is possible that the upregulation of proliferative molecules and the downregulation of differentiation factors are important in promoting pterygium development. Further study of adhesion molecules, MMPs and tumor suppressor proteins, which could provide additional insight for possible mechanisms involving c-MAF and pterygium growth is warranted.

Overall, this study extensively examined c-MAF expression within the human ocular surface context to specifically elucidate its role in the limbus, cornea and conjunctiva. Our findings suggest that c-MAF could assume a context-specific role that is dependent on the type of tissue. We found that c-MAF maintains stem cell properties within limbal epithelial crypts, whereas, during conjunctival tissue homeostasis, c-MAF serves a dual role. In the conjunctiva, c-MAF may negatively control the differentiation of smaller basal epithelial cells, while positively regulate the differentiation of cells located in the superficial layer. Furthermore, we demonstrate a possible link between c-MAF expression and pterygium, since a lower level of c-MAF was detected in the pterygial tissue when compared to healthy conjunctiva. Future studies are required to determine the precise mechanisms of how c-MAF regulates downstream pathways during pterygium pathogenesis in order to develop targeted therapies for pterygium management.

## Acknowledgment

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