Pérez-Rico, C., Pascual, G., Sotomayor, S., Montes-Mollón, M.Á, Trejo, C., Sasaki, T., Mecham, R., Bellón, J.M. & Buján, J. 2011, "Tropoelastin and Fibulin Overexpression in the Subepithelial Connective Tissue of Human Pterygium", *American Journal of Ophthalmology,* vol. 151, no. 1, pp. 44-52.

DOI: 10.1016/j.ajo.2010.07.012

(Article begins on next page)

Tropoelastin and Fibulin Overexpression in the Subepithelial Connective Tissue of Human Pterygium

CONSUELO PÉREZ-RICO, GEMMA PASCUAL, SANDRA SOTOMAYOR, MARÍA ÁNGELES MONTES-MOLLÓN, CYNTHIA TREJO, TAKAKO SASAKI, ROBERT MECHAM, JUAN MANUEL BELLÓN, AND JULIA BUJÁN

● PURPOSE: To evaluate possible changes in the collagen and elastic components of the subepithelial connective tissue of human pterygium.
● DESIGN: Immunohistochemical study.
● METHODS: Immunohistochemical staining using antitropoelastin, anti-fibulin-2, and anti-fibulin-3 antibodies was performed in 10 normal conjunctival and 20 pterygium specimens. Masson trichome staining also was performed to study subepithelial connective tissue. Sirius red staining was used to identify collagen type I and III components. Tropoelastin, fibulin-2, and fibulin-3 messenger ribonucleic acid (mRNA) expressions were analyzed in 9 conjunctival and 12 pterygium specimens by quantitative real-time polymerase chain reaction assay.
● RESULTS: The subepithelial connective tissue and vessels were more predominant in pterygium compared with the normal conjunctival tissue. Amorphous subepithelial zones were observed in the areas of the pterygium tissue, but not in normal conjunctiva. In- creased tropoelastin staining was seen in the pterygium tissue with areas of degenerative changes or immature formation of elastic fibers, as well an increase in tropoelastin mRNA, in contrast with fibulin-2 and fibulin-3 messenger levels. Fibulin-2 and fibulin-3 expression was colocalized in the subepithelial connective tissue and was distributed along blood and lymphatic vessels. Collagen type III, an immature form of collagen, was increased in the pathologic samples in association with a tissue remodeling process.
● CONCLUSIONS: Elastin metabolism is dysregulated in the pathogenesis of human pterygium with tropoelastin, fibulin-2, and fibulin-3 overexpression in the subepithelial connective tissue. (Am J Ophthalmol 2011;151:44 –52.

Accepted for publication July 23, 2010.

From the Department of Ophthalmology, University Hospital Principe de Asturias, University of Alcalá, Alcalá de Henares, Madrid, Spain (C.P.-R., M.A.M.-M.); the Department of Medical Specialities and Surgery, University of Alcalá, Alcalá de Henares, Madrid, Spain, and the Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain (G.P., S.S., C.T., J.M.B., J.B.); the Max-Planck-Institut für Biochemie, Martinsried, Germany (T.S.); and the Department of Cell Biology and Physiology, Washington University, School of Medicine, St. Louis, Missouri (R.M.).

Inquiries to Consuelo Pérez-Rico, Department of Ophthalmology, University Hospital Principe de Asturias, University of Alcalá, Carretera Alcalá-Meco s/n. 28805 Alcalá de Henares, Madrid, Spain; e-mail: cinta.perezrico@gmail.com

A pterygium is a triangular growth of fibrovascular tissue extending from the bulbar conjunctiva onto the cornea. Pterygium is more prevalent in patients living in regions closer to the equator.[1] Histologically, pterygium is characterized by an atrophic conjunctival epithelium and a highly vascularized mass of hypertrophic and elastotic degenerated connective tissue.[2] The extent and severity of this fibrovascular growth is a reliable morphologic index for predicting pterygium recurrence after excision.[3]

The pathogenesis of pterygium is uncertain. Many environmental factors such as ultraviolet (UV) irradiation, chronic irritation, and inflammation have been postulated to be causes.[4–9] Chronic exposure to UV light is the main predisposing environmental factor for pterygium formation.[10,11] Limbal stem cell deficiency resulting from chronic UV light exposure may result in concomitant breakdown of the limbal barrier and subsequent corneal conjunctivalization.[12–15]

Elastodysplasia and elastodystrophy are 2 known manifestations present in the subepithelial connective tissue of the conjunctival portion of pterygium.[16] Pterygium may result from newly synthesized elastic fiber precursors and abnormal maturational forms of elastic fibers (elastodysplasia) that undergo secondary degeneration (elastodystrophy). These structures are presumed to be formed by actinically damaged fibroblasts of the conjunctival substantia propria. Immunohistochemical studies[17] confirmed that these fibers are elastic and do not result from elastotic degeneration of collagen fibers. Similar pathologic accumulation of abnormal elastic fibers is found in chronically photodamaged skin[18]; therefore, it is reasonable to associate the formation of elastoid degeneration in pterygium with UV irradiation.

Collagen and elastin, the major components of the extracellular matrix, are intrinsic indicators of physiologic and pathological states. The elastic function complements collagen fibrils, which impart tensile strength. However, to understand the healthy and diseased tissues, an investigation of the arrangement and modification of the major structural proteins, such as collagen and elastin, is crucial.

The elastic fibers are formed through the reticulation of an amorphous polymer composed of the protein elastin, known as tropoelastin, over a framework of fibrillin-rich microfibrils,[19] and latent transforming growth factor-β– binding proteins.[20,21] The fibulins are a family of proteins that are associated with basement membranes and elastic extracellular matrix fibers. Substantial evidence has implicated the fibulins in both elastic matrix fiber assembly and function (Figure 1).[22] Fibulin-1, fibulin-2, and fibulin-5 bind to tropoelastin.[23,24] Unlike fibulin-1, fibulin-2 is found at the interface between the microfibrils and the elastin core.[25] The ability of fibulin-2 to bind elastin and fibrillin-1 may indicate that fibulin-2 anchors fibrillin-containing microfibrils to elastin fibers. Fibulin-3 interacts with another basement membrane protein, extracellular matrix protein 1, and it also interacts with elastin monomer tropoelastin. These interactions likely contribute to the integrity of basement membrane zones and anchor other extracellular matrix structures such as elastic fibers to basement membranes.[26]

Several recent findings have indicated that fibulins are involved in human inherited ocular disorders like age-related macular degeneration and macular dystrophies.[27] However, they have never been tested in the pterygium pathogenesis. Therefore, this study focused on evaluating the expression of collagen and elastic components in human pterygium.

METHODS

● PATIENTS: Surgical specimens of primary pterygia and normal conjunctiva from age-matched cataract patients were obtained, according to the Ethics Committee of the University Hospital Principe de Asturias and conforming to the tenets of the Declaration of Helsinki of 1975. Written informed consent was obtained from all patients.

The following groups were established: for the immunohistochemical studies, control group (n = 10) specimens were obtained from normal conjunctiva from subjects with a mean age ± standard deviation (SD) of 53.2 ± 5.2 years, and pterygium group (n = 20) specimens were obtained from active pterygium from patients with a mean age ± SD of 49.3 ± 3.2 years. For quantitative real-time polymerase chain reaction assays (PCR), control group (n = 9) specimens had a mean age ± SD of 47.3 ± 14.6 years and pterygium group (n = 12) specimens had a ± SD of 47.3 ± 12.9 years. Immediately after harvesting the tissue samples, the conjunctival parts of pterygia specimens were placed in sterile minimal essential culture medium and transferred at 4 C to the laboratory.

● LIGHT MICROSCOPY: Tissue specimens were fixed in Bouin solution or paraformaldehyde 4%, dehydrated, embedded in paraffin, cut into 5-µm serial sections using a rotating microtome (Microm HM-325; MICROM International GmbH, Walldorf, Germany), deparaffinized, and rehydrated. These sections were used in the morphologic and immunohistochemical analyses.

Sections for histologic examination were stained using different techniques to examine the different components of the subepithelial connective tissue: hematoxylin and eosin, Masson trichrome (Goldner-Gabe variant), orcein, and Sirius red before observation under a light microscope (Zeiss Axiophot, Jena, Germany).

Sirius red was used to localize and assess the maturity of the collagen component of the subepithelial connective tissue from the normal conjunctival and pterygium specimens. This technique is based on the orientation and interaction between the sulphone groups of the dye and the amine groups of lysin and hydrolysin and guanidine groups of arginine in the collagen fibers; the colors differ depending on the degree of collagen maturity. Collagen type I (mature collagen) stains reddish- orange, and type III collagen (immature collagen) has a yellow-green hue. After staining, the tissue sections were examined by polarized light microscopy. Ten digitalized histologic images of Sirius red staining per patient were obtained using a digital camera fitted to the microscope and were analyzed using image analysis software (Axiovision AC 4.1; Carl Zeiss, Jena, Ger- many) to obtain staining intensity percentages.

● IMMUNOHISTOCHEMISTRY: The sections were deparaffinized, hydrated, and equilibrated in phosphate-buffered saline (pH 7.4). A rabbit polyclonal antitropoelastin antibody (1:500; donated by Dr Mecham) and rabbit monoclonal anti-fibulin-2 and anti-fibulin-3 antibodies (1:200; donated by Dr Sasaki) were used as primary antibodies. The antigen–antibody reaction was detected by alkaline phosphatase or peroxidase-labeled avidin–biotin procedures. The chromogenic substrate contained α-napthol and fast-red or diaminobenzidine. Nuclei were counterstained with Carazzi hematoxylin. After immunostaining, the tissue sections were examined by light microscopy. Using a computerized Microm image analyzer (MICROM International GmbH) on the stained sections, the labeling percentages were obtained for the different antibodies. For the quantitative analysis, 10 tissue sections per patient immunolabeled using each antibody were examined. A negative control of the technique was performed without the primary

antibody.

⬮ RIBONUCLEIC ACID EXTRACTED AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION: Total ribonucleic acid (RNA) isolated using Trizol (Invitrogen, Carlsbad, California, USA) and complementary deoxyribonucleic acid synthesis by reverse transcription with M-MLV reverse-transcriptase enzyme (Invitrogen) were carried out as described previously.[28] To detect genomic deoxyribonucleic acid contamination, another reverse transcription was carried out without enzyme. To quantify messenger RNA (mRNA) expression, quantitative real-time PCR was performed with iQ SYBR Green Supermix following the manufacturer's instructions (Bio-Rad Laboratoires, Hercules, California, USA), in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, California, USA). For quantification, we performed a standard curve experiment on the instrument. Negative control with UltraPure DNase/RNase-free distilled water (Invitrogen) was added in each reaction. The specific human primers amplified are the following: tropoelastin, 5=-CGA ACT TTG CTG CTG CTT TAG-3= (sense) and 5=-GTG TAT ACC CAG GTG GCG TG-3= (antisense); fibulin-2, 5=-CTC AGC CAT ATG CTC CTG TTT-3= (sense) and 5=-GAT GTC CAC ACA GTT GCC TTC-3= (antisense); fibulin-3, 5=-CAG GCT ACG AGC AAA GTG AAC-3= (sense) and 5=-ACA GTT GAG CCT GTC ACT GCT-3= (antisense); and glyceraldehyde 3-phosphate-dehydrogenase, 5=-GGA AGG TGA AGG TCG GAG TCA-3= (sense) and 5=-GTC ATT GAT GGC AAC AAT ATC CAC T-3= (antisense). The thermal cycling conditions were: an initial stage at 95 C for 10 minutes, followed by 40 cycles of 95 C for 15 seconds, 60 C (each primer) and 64 C (tropoelastin primers) for 30 seconds, and 72 C for 1 minute. Products were submitted to 2% agarose gel electrophoresis and were visualized with UV light. Gene expression was normalized against the expression of the constitutive gene glyceraldehyde 3-phosphate-dehydrogenase.

⬮ STATISTICAL ANALYSIS: All data were expressed as mean ± standard deviation (SD). Data analysis was performed using the Graph Pad Prism 4 package (GraphPad Software Inc., La Jolla, California, USA). Image analysis data were subjected to descriptive statistical analysis. Mean data were compared among the groups using the Mann–Whitney *U* test. The level of significance was set at $P < .05$.

RESULTS

⬮ LIGHT MICROSCOPY: Staining of the different samples showed that subepithelial connective tissue generally was more predominant in the pathologic tissue than in the normal conjunctiva. In these specimens, angiogenesis was more evident than in the control group and showed a large number of vessels in the subepithelial tissue (Figure 2). Subepithelial zones with amorphous and fibrillar material were not observed in normal conjunctiva (Figure 2, Top), but were observed in the areas of the pterygium tissue (Figure 2, Bottom). These amorphous or fibrillar zones failed to stain with Sirius red, indicating that these structures were not collagen fibers. The collagen fibers surrounding these amorphous areas were associated with some isolated fibers located within the structures (Figure 3, Right).

The Sirius red staining in healthy specimens showed that collagen types I and III were present in similar proportions (Table and Figure 3, Top left). Sirius red staining in the pterygium samples showed that collagen types I and III were presented in the subepithelial areas; however, in these pathologic specimens, the immature form of

collagen (collagen III) increased compared with the normal conjunctival specimens (P = .032) and showed a tissue remodeling process (Table and Figure 3, Top right).

In the normal conjunctiva, most connective tissue was collagenous amid large areas of connective tissue of lower density (Figure 3, Left panel). By light microscopy, we observed greater quantities of lymphatic vessels in the healthy samples compared with pathologic specimens. The epithelium of both healthy and pathologic samples did not stain positively for the different types of collagens with the Sirius red staining (Figure 3).

- IMMUNOHISTOCHEMISTRY**:** In the control conjunctival tissue, large areas of low density with no staining were observed in the subepithelial connective tissue. Tropoelastin staining was observed mainly in the epithelial area. The subepithelial connective tissue showed very little expression for tropoelastin (Figure 4, Top left). The tissue showed relative staining for fibulin-2 in the epithelial and subepithelial areas (Figure 4, Top middle). The expression of fibulin-3 was higher than the other 2 antibodies in the healthy samples. Epithelium showed a slight staining and the areas of the subepithelial connective tissue in contact with the basal epithelium showed the greatest intensity (Figure 4, Top right).

Significantly increased (P = .0018) tropoelastin staining (Table) compared with the control group was observed in the pterygium tissue with large areas of degenerative changes or immature formation of elastic fibers (Figure 4, Bottom left). The staining was localized in the thickened and tortuous fibers, characteristic of elastin fibers of the subepithelial connective tissue, and was absent in the epithelium. Increased fibulin-2 and fibulin-3 expression (P = .0096 and P .021) was seen in the pterygium compared with the control samples (Table; Figure 4, Bottom middle and Bottom right). The labeling of both antibodies was colocalized in the subepithelial connective tissue and was distributed along blood and lymphatic vessels. There was no expression for fibulins in the epithelial tissue. The 3 proteins were overexpressed significantly in the pathologic tissue compared with normal conjunctiva (Table).

- QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ASSAY: Tropoelastin, fibulin-2, and fibulin-3 mRNA expression were detected in both conjunctival and pterygium specimens. As shown in Figure 5, PCR products revealed a band at 357 bp for tropoelastin, at 395 bp for fibulin-3, at 101 bp for glyceraldehyde 3-phosphate-dehydrogenase, and at 340 and 481 bp that correspond to a short and long isoforms for fibulin-2, respectively. Tropoelastin mRNA showed a significant increase (P <.0001) in pterygium compared with conjunctiva, raising the expression 2.8 times in active pterygium (Figure 5). However, fibulin-2 and fibulin-3 expression were not augmented in pathologic specimens as compared with the control group. Both types of fibulins showed similar expression in the control group, only levels of fibulin-3 decreased significantly (P = .0209) at 1.5 times in active pterygium tissue.

DISCUSSION

The mechanisms of pterygium development are unknown, but its relationship with chronic sun exposure is well established. Chronic exposure to UV light is considered the main predisposing environmental factor for pterygium formation.[10–14] Pterygium pathologic features are characterized by an atrophic conjunctival epithelium and a highly vascularized mass of hypertrophic and elastotic degenerated connective tissue.[2]

Some authors have reported an elastin overexpression in sun-damaged skin. They showed an increase of elastin mRNA level and enhanced elastin promoter activity.[29] However,

other studies showed that the elastin overexpression results from a posttranscriptional mechanism rather than increased mRNA synthesis.[30] Currently, the literature directly related with the elastic component and the pterygium pathogenesis is very scarce. For this reason, this study was focused on examining morphologic alterations and elastin and fibulin expression in human pterygium compared with normal conjunctival tissue.

Elastin is the polymeric protein responsible for the properties of extensibility and elastic recoil of the extracellular matrix in a variety of tissues. Although proper assembly of the elastic matrix is crucial for durability, the process by which this assembly takes place is not well understood. Recent data have suggested that the complex interaction of tropoelastin, the monomeric form of elastin, with a number of other elastic matrix-associated proteins, including fibrillins, fibulins, and matrix-associated glycoprotein, is important for achieving the proper architecture of the elastic matrix.[31] Tropoelastin is a common ligand for fibulin family proteins that have unique and partially overlapping expression pattern. In our immunohistochemical analysis, we also demonstrated that the protein expression of fibulin-2 and fibulin-3 overlaps in the pterygium samples. Genetic linkage and molecular studies also have associated several fibulin genes with various human heritable disorders that affect a wide range of organs, including the eye.[27] Other authors have demonstrated that fibulins may be key proteins in supramolecular organization of the corneal stroma, affirming that these molecules and its binding partners, synthesized by corneal cells, surround fibroblasts with tight matrix mesh that stabilize corneal structure.[32]

Tissue remodeling occurs constantly in almost all tissues, and a balance between the degradation and synthesis of matrix components is required for homeostasis. In several diseases, however, this balance seems to be upset. The enzymes that degrade elastin, such as elastase and matrix metalloproteinases, are thought to be involved in reducing elastin expression in different diseases, and thus contribute to degradation of the extracellular matrix and disease progression. Dushku and associates stated that pterygia are tumors of altered limbal basal cells and stromal fibroblasts that secrete transforming growth factor-β, synthesize abnormal elastic material, and produce various types of matrix metalloproteinases similar to other invasive tumours.[33]

The gene and protein expression study is a useful tool for clarifying the mechanisms of pterygium development. Data reported by microarray analyses[34] have shown that there are many genes that are significantly upregulated or downregulated in pterygium versus conjunctiva. Genes encoding for cell adhesion, extracellular matrix components (included different types of collagen and enzymes implicated in the reticulation of elastin as LOXL, lysyl oxidase-like), and structural proteins, including wound-healing–related proteins, were upregulated significantly in pterygium. This fact is not always correlated with the upregulation or down- regulation of protein. Finally, these authors concluded from this study that aberrant wound healing is a key process in pterygium pathogenesis.

This study showed that human pterygium presented higher tropoelastin, but not fibulin-2 and fibulin-3, mRNA levels than conjunctival tissue. However, these elastic component protein levels all were increased in the pathologic specimens, as was the immature form of collagen (collagen III).

Our results agree with those of Wang and associates who reported strong tropoelastin protein levels in the conjunctival part of pterygium by immunohistochemical staining.[17] These investigators reported that high tropoelastin expression does not result from an

increase in mRNA levels, but rather from posttranscriptional modification of tropoelastin in cultured UV-irradiated fibroblasts from pterygium. Our results showed that the pterygium specimens presented higher tropoelastin mRNA expression as compared with normal conjunctiva by quantitative real-time PCR analysis. So, this mRNA overexpression is associated with the high protein levels obtained in pterygium by immunohistochemical studies. The incoherence with the results of Wang and associates may be the result of 2 different factors.[17] The first is that our RNA isolation was developed in fresh tissue instead of cultured fibroblasts. The second is that different techniques were used: reverse-transcriptase PCR versus northern hybridization and quantification of radiolabeled tropoelastin.

This is the first report in the literature that evaluates fibulin-2 and fibulin-3 expression in the pterygium tissue. Both protein levels were increased in pathologic specimens, but there was not an increase in the mRNA level. We did not find differences in the amounts of total fibulin-2 mRNA in pterygium versus conjunctival specimens, and in the case of fibulin-3 mRNA, the expression even decreased in pterygium specimens. This may be the result of mRNA degradation by a negative feedback. So, the protein overexpression may be the result of posttranscriptional mechanisms.[17]

In summary, one manifestation of elastodysplasia in the human pterygium is shown by an increase of tropoelastin and not of fibulin-2 and fibulin-3 mRNA levels, while present an overexpression in the 3 protein levels, showing dysregulation of the elastin metabolism in this type of pathologic condition. In conclusion, elastin metabolism is upregulated in the pathogenesis of human pterygium with tropoelastin, fibulin-2, and fibulin-3 overexpression in the subepithelial connective tissue.

Publication of this article was supported by grant RD07/0062/0008 from the Fundación para la Investigación Biomédica del Hospital Universitario Príncipe de Asturias (Alcalá de Henares, Spain), and Instituto de Salud Carlos III (Madrid, Spain). The authors indicate no financial conflict of interest. Involved in Design and conduct of study (C.P-R., G.P., J.B.); Collection, management, analysis, and interpretation of data (G.P., S.S., M.A.M-M., C.T., C.P-R.); Preparation of article (C.P-R., G.P.); and Review and approval of the manuscript (J.M.B., T.S., R.M., J.B.). The study protocol was approved by the Ethics Committee of the University Hospital Principe de Asturias, and all study procedures adhered to the recommendations of the Declaration of Helsinki. Written consent was obtained from all participants.

REFERENCES

1. Hilgers JH. Pterygium: its incidence, heredity and etiology. Am J Ophthalmol 1960;50(Oct):635– 644.
2. Cameron ME. Histology of pterygium: an electron microscopic study. Br J Ophthalmol 1983;67(9):604 – 608.
3. Tan DT, Chee SP, Dear KB, Lim AS. Effect of pterygium morphology on pterygium recurrence in a controlled trial comparing conjunctival autografting with bare sclera excision. Arch Ophthalmol 1997;115(10):1235–1240.
4. Wong WW. A hypothesis on the pathogenesis of pterygiums. Ann Ophthalmol 1978;10(3):303–308.
5. Coroneo MT, Di Girolamo N, Wakefield D. The pathogenesis of pterygia. Curr Opin Ophthalmol 1999;10(4):282–288.
6. Hill JC, Maske R. Pathogenesis of pterygium. Eye 1989; 3(Pt2):218 –226.

7. Cilova-Atanasova B. On the pathogenesis of pterygium. Folia Med (Plovdiv) 1971;13(2):67–74.
8. Jaros PA, DeLuise VP. Pingueculae and pterygia. Surv Ophthalmol 1988;33(1):41–49.
9. Mackenzie FD, Hirst LW, Battistutta D, Green A. Risk analysis in the development of pterygia. Ophthalmology 1992;99(7):1056–1061.
10. Maloof AJ, Ho A, Coroneo MT. Influence of corneal shape on limbal light focusing. Invest Ophthalmol Vis Sci 1994; 35(5):2592–2598.
11. Coroneo MT. Pterygium as an early indicator of ultraviolet insolation: a hypothesis. Br J Ophthalmol 1993;77(11):734–739.
12. Taylor HR, West SK, Rosenthal FS, Munoz B, Newland HS, Emmett EA. Corneal changes associated with chronic UV irradiation. Arch Ophthalmol 1989;107(10):1481–1484.
13. Coroneo MT. Albedo concentration in the anterior eye: a phenomenon that locates some solar diseases. Ophthalmic Surg 1990;21(1):60–66.
14. Kwok LS, Coroneo MT. A model for pterygium formation. Cornea 1994;13(3):219–224.
15. Dushku N, Reid TW. Immunohistochemical evidence that human pterygia originate from an invasion of vimentin-expressing altered limbal epithelial basal cells. Curr Eye Res 1994;13(7):473–481.
16. Austin P, Jakobiec FA, Iwamoto T. Elastodysplasia and elastodystrophy as the pathologic bases of ocular pterygia and pinguecula. Ophthalmology 1983;90(1):96–109.
17. Wang IJ, Hu FR, Chen PJ, Lin CT. Mechanism of abnormal elastin gene expression in the pinguecular part of pterygia. Am J Pathol 2000(4);157:1269–1276.
18. Montagna W, Kirchner S, Carlisle K. Histology of sun- damaged human skin. J Am Acad Dermatol 1989;21(5Pt1): 907–918.
19. Mecham RP, Heusar JE. The elastic fibre. In: Hay ED, ed. Cell biology of the extracellular matrix. 2nd ed. New York: Plenum Press; 1991.
20. Saharinen J, Hyytiäinen M, Taipale J, Keski-Oja J. Latent transforming growth factor-beta binding proteins (LTBPs)-structural extracellular matrix proteins for targeting TGF-beta action. Cytokine Growth Factor Rev 1999;10(2):99– 117.
21. Shipley JM, Mecham RP, Maus E, et al. Developmental expression of latent transforming growth factor beta binding protein 2 and its requirement early in mouse development. Mol Cell Biol 2000;20(13):4879–4887.
22. Liu X, Zhao Y, Gao J, et al. Elastic fiber homeostasis requires lysyl oxidase-like 1 protein. Nat Genet 2004;36(2):178–182.
23. Nakamura T, Lozano PR, Ikeda Y, et al. Fibulin-5/DANCE is essential for elastogenesis in vivo. Nature 2002;415(16868): 171–175.
24. Sasaki T, Göhring W, Miosge N, Abrams WR, Rosenbloom J, Timpl R. Tropoelastin binding to fibulins, nidogen-2 and other extracellular matrix proteins. FEBS Lett 1999;460(2): 280–284.
25. Reinhardt DP, Sasaki T, Dzamba BJ, et al. Fibrillin-1 and fibulin-2 interact and are colocalized in some tissues. J Biol Chem 1996;271(32):19489–19496.
26. Zhang Y, Marmorstein LY. Focus on molecules: fibulin-3 (EFEMP1). Exp Eye Res 2010;90(3):374–375.
27. Stone EM, Lotery AJ, Munier FL, et al. A single EFEMP1 mutation associated with both Malattia Leventinese and Doyne honeycomb retinal dystrophy. Nat Genet

1999;22(2): 199–202.
28. Pascual G, Rodríguez M, Gomez-Gil V, García-Honduvilla N, Buján J, Bellón JM. Early tissue incorporation and collagen deposition in lightweight polypropylene meshes: bioassay in an experimental model of ventral hernia. Surgery 2008;144(3):427–435.
29. Bernstein EF, Chen YQ, Tamai K, et al. Enhanced elastin and fibrillin gene expression in chronically photodamaged skin. J Invest Dermatol 1994;103(2):182–186.
30. Schwartz E, Feinberg E, Lebwohl M, Mariani TJ, Boyd CD. Ultraviolet radiation increases tropoelastin accumulation by a post-transcriptional mechanism in dermal fibroblasts. J Invest Dermatol 1995;105(1):65–69.
31. Cirulis JT, Bellingham CM, Davis EC, et al. Fibrillins, fibulins, and matrix-associated glycoprotein modulate the kinetics and morphology of in vitro self-assembly of a recombinant elastin-like polypeptide. Biochemistry 2008; 47(47):12601–12613.
32. Ducros E, Berthaut A, Mirshahi P, et al. Expression of extracellular matrix proteins fibulin-1 and fibulin-2 by human corneal fibroblasts. Curr Eye Res 2007;32(6):481–490.
33. Dushku N, John MK, Schultz GS, Reid TW. Pterygia pathogenesis: corneal invasion by matrix metalloproteinase expressing altered limbal epithelial basal cells. Arch Ophthalmol 2001;119(5):695–706.
34. Tong L, Chew J, Yang H, Ang LP, Tan DT, Beuerman RW. Distinct gene subsets in pterygia formation and recurrence: dissecting complex biological phenomenon using genome wide expression data. BMC Med Genomics 2009;2:14.

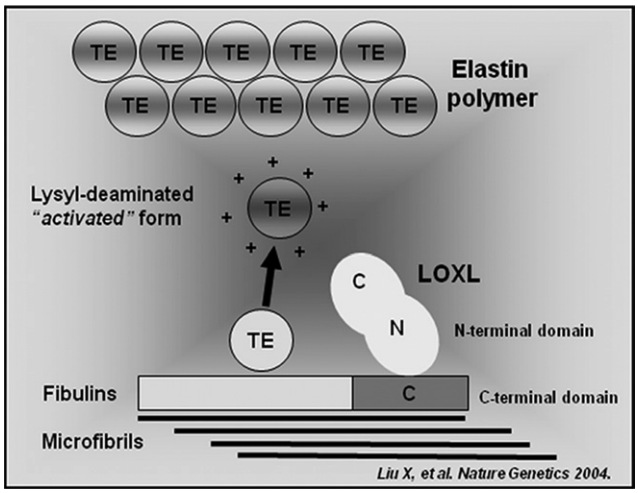

FIGURE 1. Model for development of the elastic fiber. Tissue-specific variations and partial functional redundancies are not considered. Binding of fibulin to both tropoelastin and LOXL1 brings the enzyme and substrate into juxtaposition for efficient and spatially restricted polymer formation. LOXL1 converts tropoelastin into a lysyl-deaminated form, and the 'activated' tropoelastin associates with one another or deposits onto the existing polymer through coacervation, followed by spontaneous covalent cross-linking. LOXL1 = lysyl oxidase like1; TE = tropoelastin. Figure modified from Liu X and associates.[22] Reprinted by permission from Macmillan Publishers Ltd.

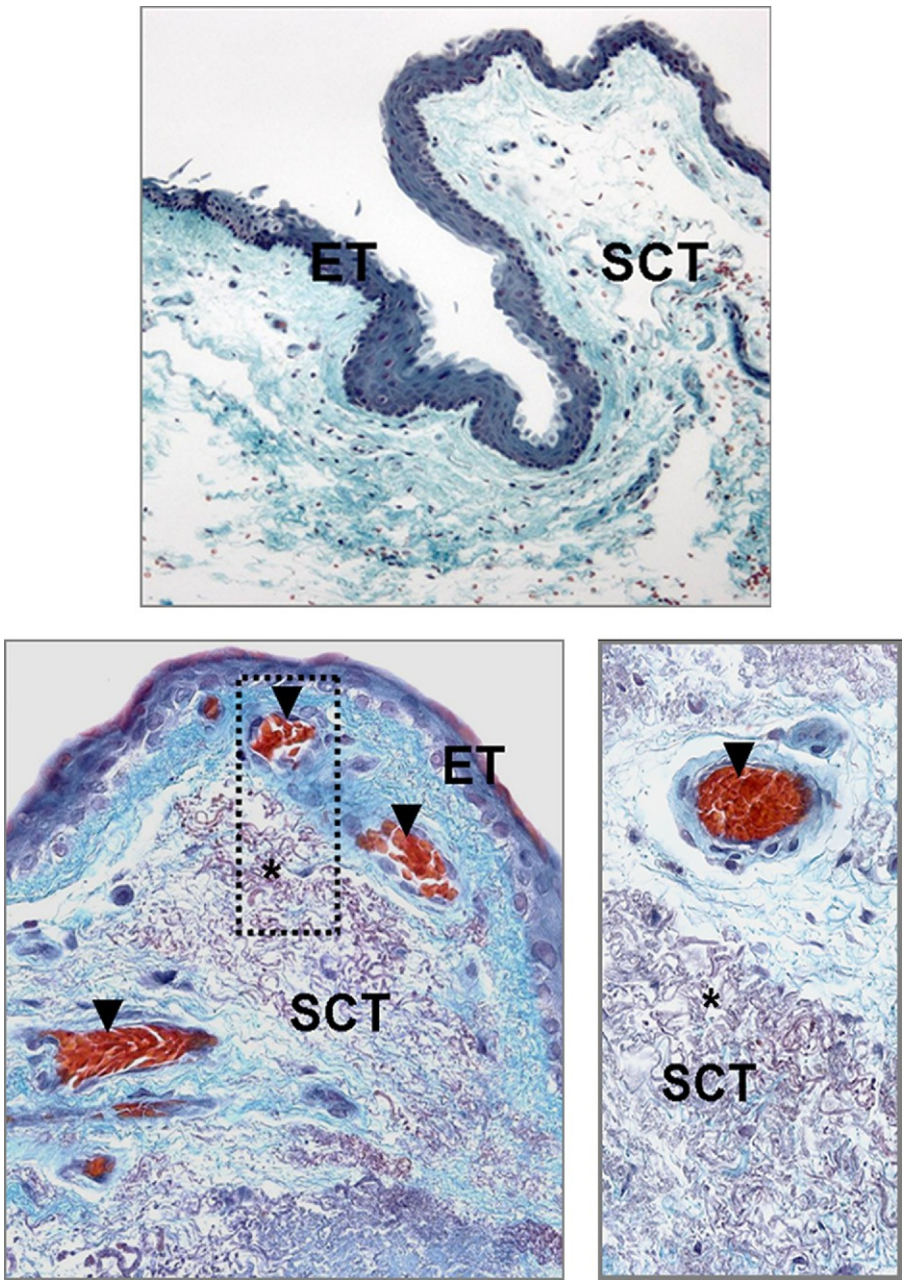

FIGURE 2. Photomicrographs showing Masson trichrome staining of conjunctival and pterygium tissue. (Top) Conjunctival tissue from healthy patients. (Bottom left) Tissue from patients with a pterygium (light microscopy [LM], ×200 magnification). (Bottom right) Magnification of the rectangular area from the bottom left image showing different fibers in the subepithelial connective tissue. Subepithelial zones with amorphous and fibrillar material (*) seen in the pterygia are not seen in normal conjunctival tissue (LM, ×630 magnification). ET = epithelial tissue; SCT = subepithelial connective tissue; ► = blood vessels.

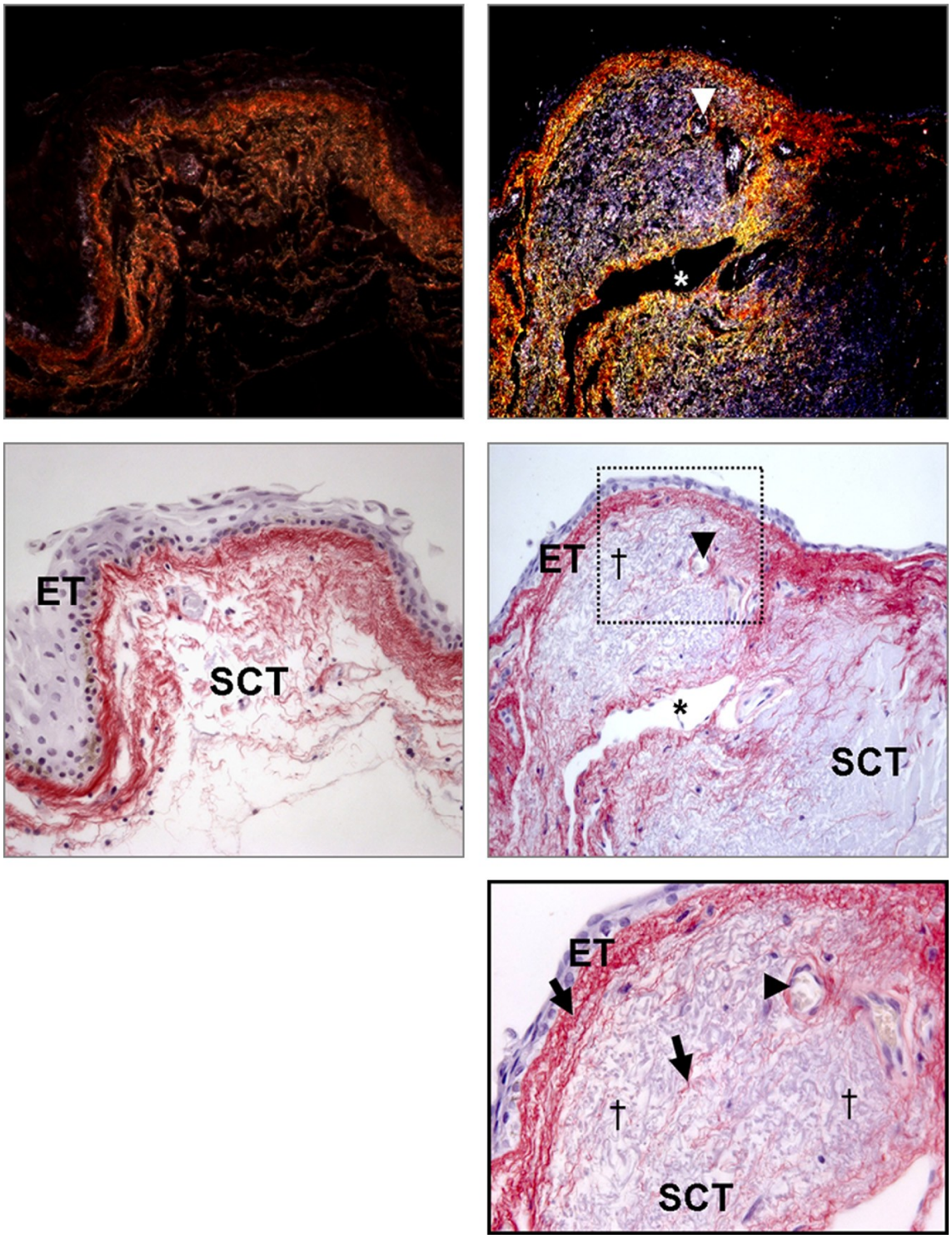

FIGURE 3. Photomicrographs showing Sirius red staining observed under polarized light of conjunctival and pterygium tissue. Expression of collagen I (mature) in red and collagen III (immature) in yellow in (Top left) the subepithelial connective tissue of normal conjunctival and (Top right) pterygial specimens (light microscopy [LM], ×200 magnification). Images of Sirius red staining observed under normal light of conjunctival and pterygium tissue. Image of the same sample of (Middle left) normal conjunctiva and (Middle right) pterygium showing collagen expression in red (LM, ×200 magnification). (Bottom right) Magnification of the square area from the middle right image showing collagen fibers (→) and elastic fibers in the subepithelial connective tissue (†; LM, ×400 magnification). ET = epithelial tissue; SCT = subepithelial connective tissue; ► = blood vessels; * = lymphatic vessels.

TABLE. Percentage of Tropoelastin, Fibulin-2, Fibulin-3, Collagen I (Mature Collagen), and Collagen III (Immature Collagen) in the Normal Conjunctiva and the Pterygium Tissue

Staining	Control Tissue	Pterygium Tissue
Tropoelastin	9.5 ± 3.1[a]	30.3 ± 5.4
Fibulin-2	11.6 ± 4.2[a]	22.1 ± 2.5
Fibulin-3	15.8 ± 2.9[a]	34.1 ± 6.3
Collagen I	16.9 ± 5.3	14.3 ± 7.6
Collagen III	12.2 ± 3.2[a]	26.8 ± 5.7

All data are presented as mean ± standard deviation.

[a] $P < .05$ compared with pterygium tissue.

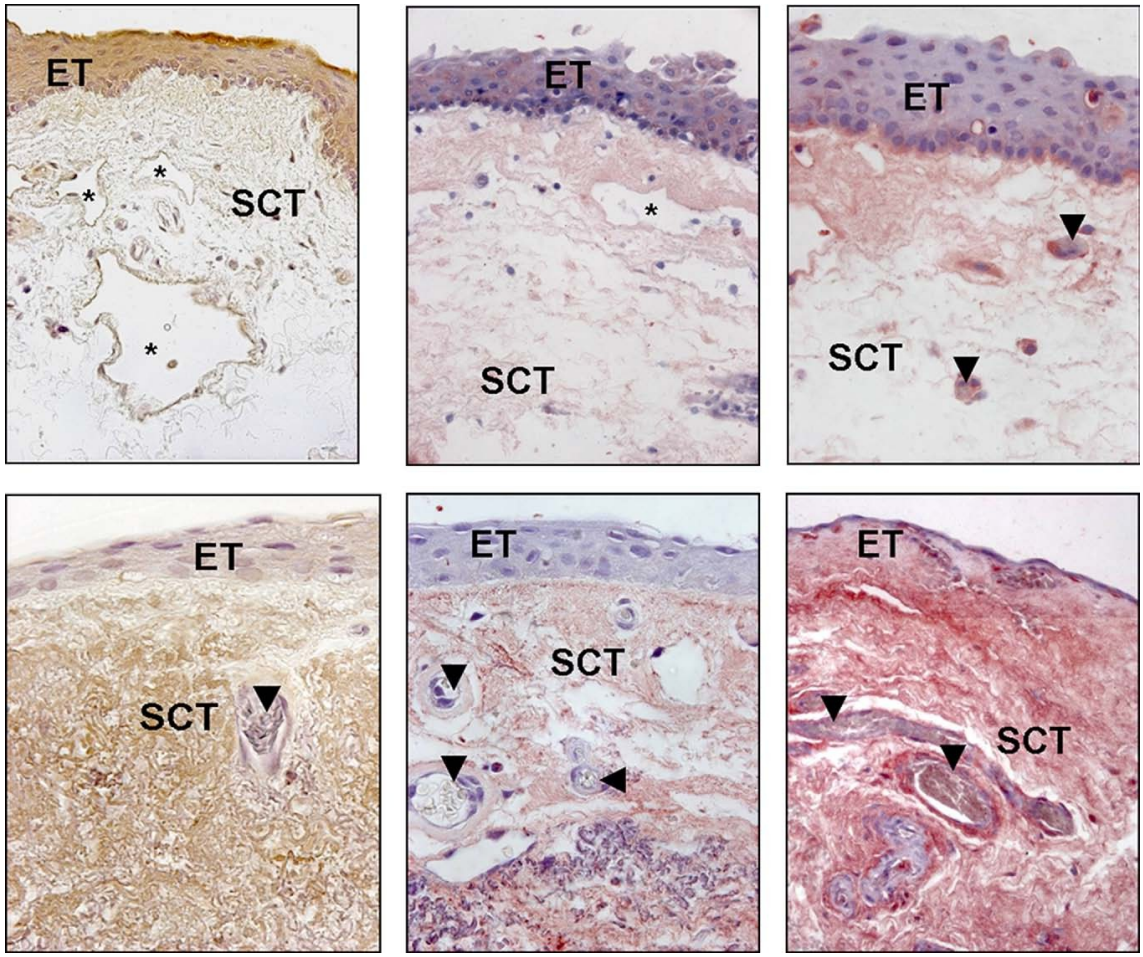

FIGURE 4. Photomicrographs showing immunohistochemical staining of tropoelastin, fibulin-2, and fibulin-3 in conjunctival and pterygium tissue. Images of the immunohistochemical expression of (Top left and bottom left) tropoelastin, (Top middle and Bottom middle) fibulin-2, and (Top right and Bottom right) fibulin-3 in conjunctival tissue from (Top panel) healthy patients and (Bottom panel) in pterygium tissue (light microscopy, ×400 magnification). Expression of fibulin-2 and fibulin-3 is colocalized in the subepithelial connective tissue. Expression of all proteins is increased in the pathologic tissue. ET = epithelial tissue; SCT = subepithelial connective tissue; ► = blood vessels; * = lymphatic vessels.

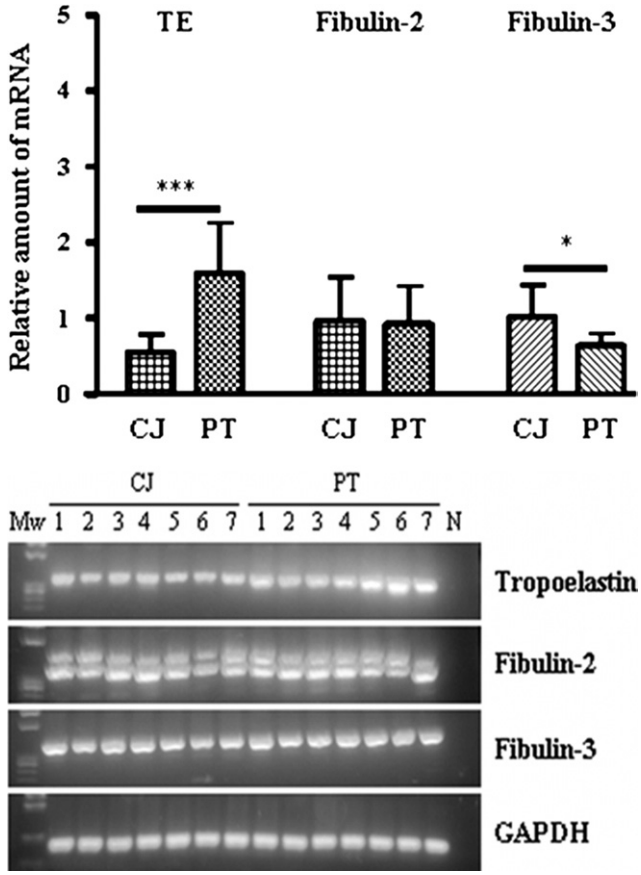

FIGURE 5. Relative quantification of tropoelastin (TE), fibulin-2, and fibulin-3 messenger ribonucleic acid (mRNA) in conjunctival and pterygium tissue. (Top) mRNA relative expression mean of tropoelastin, fibulin-2, and fibulin-2. Gene expression was normalized with glyceraldehyde 3-phosphate-dehydrogenase (GAPDH). (Bottom) Quantitative real-time polymerase chain reaction products of conjunctival and pterygium representative specimens. CJ = conjunctive; Mw = molecular weight markers; N = negative control; PT = pterygium. Data are shown as mean ± standard deviation. *P <.05. ***P < .001.