Downregulation of β-Actin Gene and Human Antigen R in Human Keratoconus

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Purpose. The purpose of this study was to determine the expression levels and regulation of β-actin in the stroma of keratoconus (KC) and normal corneas.

Methods. A total of 15 different human corneas from both KC and normal individuals were used for this study. Additionally, 3 Fuch's dystrophic corneas were also used. The β-actin gene expression was analyzed at the transcriptional and translational levels in the epithelium and stroma of the KC and normal corneas. The human antigen R (HuR) gene expression was analyzed by real-time PCR in the stroma of five KC and five normal corneas. The keratocytes from three normal and three KC corneas were cultured in the presence of serum, and the expression levels of β-actin and human antigen R (HuR) were analyzed by using confocal imaging in both normal and KC fibroblasts.

Results. The expression of the β-actin gene was downregulated in the stroma of the six KC corneas but not in the stroma of six normal and Fuchs' dystrophic corneas. Immunofluorescence detection of β-actin showed that it was absent in the KC fibroblast. The real-time PCR analysis of the HuR gene showed a relative 4.7-fold lower expression in KC corneas relative to the normal corneas, which was further confirmed by the immunofluorescence detection of HuR in fibroblasts of KC corneas.

Conclusions. Although ubiquitous β-actins are essential for cell survival during early embryogenesis, the effects on various stages of development are not well understood. Our results show that β-actin is downregulated in the corneal stroma of patients with KC, which may be related to reduced levels of a stabilizing factor (HuR) for β-actin mRNA. We propose that loss of β-actin in the corneal stroma might be a triggering factor in the development of KC. (*Invest Ophthalmol Vis Sci.* 2012; 53:4032–4041) DOI:10.1167/iovs.11-9062

Keratoconus (KC) is a condition of unknown cause in which the cornea assumes a conical shape as a result of noninflammatory thinning of central or paracentral corneal stroma. The disease progresses at a variable speed with corneal thinning inducing irregular astigmatism, myopia, and corneal protrusion. Rigid contact lenses are needed for rehabilitation in most patients, with keratoplasty reserved for advanced cases that show contact lens intolerance or have central corneal scarring. According to National Eye Institute reports, KC is the most common corneal dystrophy in the United States, affecting 1 in 2000 Americans.[1,2] The classic histopathologic features of KC include central stromal thinning, iron deposits in the epithelial basement membrane, and breaks in Bowman's layer. Several reports describe an association of KC with Down syndrome, Leber congenital amaurosis, and mitral valve prolapse.[3]

Keratocytes play an important role in corneal transparency by maintaining a functional stroma through the secretion of stromal extracellular matrix that contributes to corneal strength and transparency. The functional attributes of a cell are regulated mainly by cytoskeletal signaling, and actins are one of the major cytoskeletal structural proteins expressed in eukaryotic cells. Actins are involved in many cellular processes, including cell adhesion, cell migration/movement, cytokinesis, endocytosis/exocytosis, cell division, signal transduction, mRNA localization, and transcription.[4] Eukaryotes have six actin isoforms, each encoded by an individual gene.[5] Among the six isoforms, two are associated with striated muscle (α-skeletal and α–cardiac muscle actins), two with smooth muscle (α– and γ–smooth muscle actins), and two are cytoplasmic (β- and γ-actin).[6] The muscle actins are tissue specific and make up the contractile units, whereas β- and γ-actins are ubiquitous and are essential for cell survival.[7] The actin isoforms have highly conserved amino acid sequences. They differ mainly at their N-termini, whereas the cytoplasmic β- and γ-actins differ only by four amino acids. The absence of β-actin at an embryonic stage was found to be lethal in a transgenic mouse model.[8] β-actin exists as a globular actin (G-actin) or filamentous actin (F-actin), the latter is arranged in the form of strings of uniformly oriented G-actin subunits in a tight helix.

The high expression levels of β-actin are important for cellular processes that require constant and stable concentration levels. The expression of actin genes is regulated at both transcriptional[9] and posttranscriptional levels (by the cellular localization of their mRNAs).[10] The β-actin mRNA is regulated by a specific sequence at the 3′ untranslated region (3′UTR) by RNA-binding proteins known as zipcode-binding proteins (ZBP).

Our previous studies have shown downregulation of β-actin and proteomic profile changes in cytoskeletal proteins in KC corneas related to normal corneas.[11,12] This led us to hypothesize that β-actin expression levels could be altered during KC development, leading to corneal thinning and its protrusion. At present, little is known about β-actin localization or its function in corneal stromal keratocytes. The purpose of the present study was to determine the expression levels and regulation of β-actin in the stroma of KC and normal corneas. Our results show that β-actin is downregulated in stromal keratocytes of KC corneas compared with normal corneas, and this could be related to a downregulation of mRNA stabilizing factor human antigen R (HuR).

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METHODS

Human Corneas

The normal corneas were obtained from the Alabama Eye Bank, and the KC corneal buttons (8 mm in diameter) were obtained from a local corneal surgeon. A total of 15 KC and 15 normal corneas were used for this study. Normal corneas and KC corneal buttons were stored in Optisol (Chiron Ophthalmics, Irvine, CA) and recovered within 12 hours after their enucleation or surgery. The central 8-mm region of the normal corneas was recovered using a trephine. The epithelium, stroma, and endothelium were separated from each cornea, and the stromal and epithelial tissues were used in the present studies. The use of human corneas in the study was approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB) and was performed according to the tenets of the Declaration of Helsinki. The KC and normal corneas used in this study were mostly age matched.

The epithelial and stromal preparations were separately recovered from each KC and age-matched normal corneas and were also separately homogenized in Trizol (Invitrogen, Eugene, OR). For this purpose, the tissue samples in grinding flasks (polytetrafluoroethylene; 3-mL volume) containing tungsten carbide balls were frozen in liquid nitrogen. Next, the grinding flasks were shaken to homogenize/pulverize the tissues using Tissue Mikro-Dismembrator (Sartorius, Edgewood, NY). The RNA was quantified using Nanodrop (Thermoscientific Inc., Wilmington, DE).

Protein Isolation from Trizol Reagent and SDS-PAGE Analysis

After the RNA precipitation, the remaining pink aqueous phase that contained DNA was precipitated with 100% ethanol and centrifuged at 5000 rpm for 2 minutes. After the DNA precipitation, the protein containing supernatant was precipitated with isopropanol and centrifuged at 12,000 rpm for 10 minutes at 4°C. Next, the precipitate was washed three times with 0.3 M guanidine hydrochloride in 95% ethanol. During each wash, samples were incubated for 20 minutes followed by centrifugation at 7500 rpm for 5 minutes. Finally, sample proteins were precipitated with 100% ethanol, followed by centrifugation at 7500 rpm for 5 minutes. The precipitate was dissolved in 1% SDS, and protein quantification was done using bicinchonic acid (BCA) protein assay Kit (Thermoscientific Pierce, Rockford, IL). Thirty micrograms protein from each sample were loaded onto 12% polyacrylamide gel during SDS-PAGE,[13] which was followed by staining with Coomassie blue and destaining. Images of the gel were acquired on a Typhoon 9400 scanner (GE Healthcare, Buckinghamshire, UK) using a red laser (633 nm) at a 200-μm resolution.

Stromal Cell Culture

The epithelium and endothelium were scraped and removed from KC and normal corneas. The stroma was incubated overnight in collagenase (at 1 mg/mL; Worthington Biochemical Corp., Lakewood, NJ). The cells were washed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 1% antibiotics (penicillin-streptomycin solution; Mediatech Inc., Herndon, VA). Next, the cells were seeded onto six-well plates (Corning, Franklin Lakes, NJ) in DMEM medium containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% antibiotics (penicillin-streptomycin solution) and maintained at 37°C in 5%-CO_2 humidified air. Cells were passaged by trypsinization (0.05% trypsin-EDTA) on a consistent basis, and all analyses were carried out before their third passage. We generated primary culture from age-matched, three normal and three KC corneas, which were used for the study.

Western Blot Analysis

Thirty micrograms protein from samples were lysed with SDS gel-loading buffer and subjected to SDS-PAGE[13] using 12% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) by using a semi-dry transfer cell (Trans-blot Turbo, Bio-Rad), and blocked for 1 hour at room temperature with 3% nonfat milk in PBS-Tween 20. The membranes were probed with anti-β-actin monoclonal antibody (Sigma, St. Louis, MO) at (1:100 dilution), followed by incubation in goat anti-mouse secondary antibody conjugated to infrared dye (IRD) at 1:10,000 dilutions. Blots were scanned using Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE). The blot was stripped using a mild stripping solution for 1 hour, followed by washing three times with PBS-Tween 20. The membranes were then blocked as described above and re-probed with anti-vimentin monoclonal antibody (Sigma) at dilution, followed by secondary antibody incubation as described above. Blot was scanned using Odyssey Imaging system (LI-COR Biosciences).

TABLE 1. List of Antibodies Used in the Study

Antibodies	Dilution
β-actin (Sigma)	1:200
Vimentin (Sigma)	1:100
α–Smooth muscle actin (α-SMA) (Sigma)	1:200
γ-Actin (Sigma)	1:100
HuR (Abcam, Cambridge, MA)	1:200
Rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO)	1:150

Immunohistochemical–Confocal Microscopic Analysis

Three KC and three normal corneas were fixed in 4% paraformaldehyde, and 10-μm cryosections were recovered using a cryostat (Leica cryostat, Buffalo Grove, IL; Core Facility of Vision Sciences Research Center at UAB). The sections were blocked in normal bovine serum (5%), followed by overnight incubation in anti-β-actin monoclonal antibody (Sigma) at 1:100 dilution. The sections were washed three times and incubated with a secondary goat anti-mouse Alexa Fluor 488-labeled antibody (Invitrogen, Grand Island, NY). Next, the sections were washed three times in PBS, and nuclei were stained with Hoechst 33342 stain (Invitrogen) for 10 minutes. After a final wash with PBS, the sections were mounted on slides with Fluoromount-G (Southern Biotech, Birmingham, AL). Fluorescent microscopic analysis was performed using Zeiss AxionPlan 2 Imaging System Microscope (Carl Zeiss Microscopy, Thornwood, NY) at the Core facility of Vision Sciences Research Center at the UAB. The immunohistochemical analysis was identically performed using cultured stromal fibroblastic cells from three KC and three normal corneas. The antibodies used in the study are listed in Table 1. The confocal imaging was done using Zeiss LSM 710 confocal microscope (High Resolution Imaging Facility, UAB) and multiple images were taken from three KC and three normal corneal fibroblasts using a confocal microscope. The intensity of fluorescence was quantified by using SIMPLEPCI software (Compix, Cranberry Township, PA). We counted 20 or more cells from each image, and multiple images were analyzed.

Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-PCR [qPCR])

The gene was amplified using Access RT-PCR system (Promega, Madison, WI), and 500 ng of RNA was used for RT-PCR. The primers were designed using primer 3 for β-actin, HuR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2). The PCR product was analyzed using Agarose gel electrophoresis, and images were captured by a gel documentation system. Real-time PCR quantifications were performed using the BIO-RAD iCycler iQ system (Bio-Rad), with 96-well reaction plates in a total volume of 25 μL. During the analysis, stromal preparations from five different KC and five normal corneas were used.

Table 2. Primers Used in the Study

Gene Primer	Sequence (5′–3′)
β-actin forward	GTTGCTATCCAGGCTGTG
β-actin reverse	TGATCTTGATCTTCATTGTG
β-actin forward	AGAGCTACGAGCTGCCTGAC
β-actin reverse	CACCTTCACCGTTCCAGTTT
HuR forward	ATGAAGACCACATGGCCGAAGACT
HuR reverse	AGTTCACAAAGCCATAGCCCAAGC
GAPDH forward	GAGTCAACGGATTTGGTCGT
GAPDH reverse	TTGATTTTGGAGGGATCTCG

The reaction mixture included 12.5 μL Real-Time SYBR Green PCR master mix (Bio-RAD, Hercules, CA), 2.5 μL reverse transcription product, 1 μL forward and reverse primer, and 8 μL DNase/RNase free water. The reaction mixtures were initially heated at 95°C for 10 minutes to activate the polymerase, followed by 40 cycles, which consisted of a denaturation step at 95°C for 15 seconds, annealing at 57°C for 60 seconds, and an elongation step at 72°C. The qRT-PCR data were analyzed by the comparative ΔCt method.[21]

Statistical Analysis

The statistical significance was determined by Student's *t*-test and with statistical significance set at $P < 0.05$.

Results

To examine the β-Actin gene expression in the stroma, stromal tissue preparations from three individual human KC and three normal corneas were used. Figure 1A shows the location of the two sets of primers in the β-actin gene that were used for RT-PCR analysis. The β-actin gene was downregulated in all three KC corneal stromas compared with the three normal corneal stromas (Fig. 1B). In contrast, the GAPDH gene expression remained constant in the stroma of both normal and KC corneas. The two different primers sets showed a downregulation of β-actin gene in KC stroma compared with normal corneal stroma (Figs. 1B, 1C). When primer 2 was used for RT-PCR to analyze additional stromal preparations from three KC and three normal corneas, again β-actin gene was found to be downregulated only in KC corneas but not in the normal corneas (Fig. 1D). Additionally, the GAPDH expression remained at the same levels in both normal and KC stromal preparations (Fig. 1D). To quantify the β-actin gene expression level, the pixel intensity of β-actin and GAPDH gene expressions with primer 2 was measured, and the values with SD in all six samples of KC and normal corneas are shown in Figure 1E. The results further show downregulation of β-actin in the stroma of KC corneas.

To evaluate whether the β-actin gene expression is affected in another corneal disease, the stroma and epithelium preparations of Fuchs' dystrophic corneas were analyzed using RT-PCR. Fuchs' dystrophy is a corneal endothelial disease and is believed to lead to corneal swelling due to the loss of Na^+, K^+-ATPase pump sites within the endothelium.[14] The RT-PCR results with the two primer pairs showed that β-actin gene expression levels remained at the same levels in the stroma and epithelium of both normal and Fuchs' dystrophic corneas (Fig. 1F).

To examine the cellular β-actin expression in stroma of KC versus normal corneas, immunohistochemical analysis of 10-μm frozen corneal tissue sections with anti-β-actin antibody was examined. β-actin was clearly seen in the epithelium and stroma of normal corneas (Fig. 2A), and in contrast, it was absent in KC corneas (Fig. 2D). Figure 2B shows the counterstaining with Hoechst nuclear stain of the same sections shown in Figure 2A; and Figure 2C is an overlay of Figures 2A and 2B. Figure 2D shows the immunoreactivity of 10-μm cryosections of KC cornea with anti-β-actin antibody, while Figure 2E shows the counterstaining of the section with Hoechst nuclear stain, and Figure 2F is an overlay of Figures 2D and 2E. Protein isolation after RNA extraction using Trizol has been a choice in the case of limited availability of clinical samples.[15] And so this method was used for the recovery of proteins from the normal and KC stromal preparations that exhibited down-regulation of β-actin gene during RT-PCR analysis (Fig. 1D).The Western blot analysis of these stromal protein preparations with anti-β-actin antibody further showed the downregulation of β-actin in KC but not in the normal corneas (Fig. 2H). In this analysis, identical amounts of stromal proteins from three KC and two normal corneas were used in SDS-PAGE and Western blot analyses (Fig. 2G), and vimentin was used as a loading control (Fig. 2H). Together, the above results show that β-actin was downregulated at both transcriptional and translational levels in the stroma of KC corneas but not in the normal corneas.

In order to determine if the downregulation of β-actin in KC corneal stroma is a transient phenomenon or not, we cultured keratocytes from both normal and KC corneal stroma as described in the Methods section. On culturing, keratocytes transform into fibroblasts.[16] The fibroblasts are mesenchymal in origin, and one of the markers of mesenchymal cells is vimentin, a cytoskeletal intermediate filament protein. On immunohistochemical confocal microscopic analysis, the keratocytes cultured from both normal and KC stroma showed expression of vimentin (Figs. 3A, 3B), and vimentin was cytosolic, which surrounded the nuclei along the cell processes. Both normal and KC corneal fibroblasts also exhibited immunoreactivity to α–smooth muscle actin (Figs. 3C, 3D). As stated above, actins exist as globular actin (G-actin) or filamentous actin (F-actin), and the latter is arranged in the form of strings of uniformly oriented G-actin subunits in a tight helix.

Fibroblasts from both the normal and KC stroma were stained with Hoechst nuclear stain (Figs. 3E, 3I). The F-actin could be detected by using phalloidin (toxin from the mushroom *Amanita phalloides*) staining. The rhodamine-labeled phalloidin specifically stained the F-actin filaments in both the normal and KC stromal fibroblasts (Figs. 3F, 3J), suggesting its existence in these cells.

There are two forms of cytoplasmic actins, the β- and γ-actins, and Figures 3G and 3K show the immunoreactivity of β-actin in the normal and KC corneal stromal fibroblasts, respectively. Immunohistochemical analysis further showed that the fibroblasts from normal corneas were positive for β-actin (green fluorescence; Fig. 3G), whereas the cells from KC corneas showed an absence of β-actin (Fig. 3K). Additionally, the β-actin in normal corneal fibroblasts showed a staining pattern around the nucleus and also lengthwise along the cell (Fig. 3G). This staining pattern might represent β-actin stress fibers, and these stress fibers were absent in the cells from KC corneal fibroblasts (Fig. 3K). Figures 3H and 3L are an overlay of Hoechst's nuclear stain, rhodamine-labeled phalloidin and immunoreactivity of β-actin in normal and keratoconus corneal fibroblasts. Figures 3M and 3P are Hoechst's nuclear stain in normal and keratoconus corneal fibroblasts. Figures 3N and 3Q show the immunoreactivity of γ-actin in the normal and KC corneal stromal fibroblasts, respectively. Immunohistochemical analysis further showed that the fibroblasts from normal corneas and KC corneas were positive for γ-actin (green fluorescence; Figs. 3N, 3Q). Figures 3O and 3R are an overlay of Hoechst's nuclear stain and immunoreactivity of γ-actin in normal and keratoconus corneal fibroblasts.

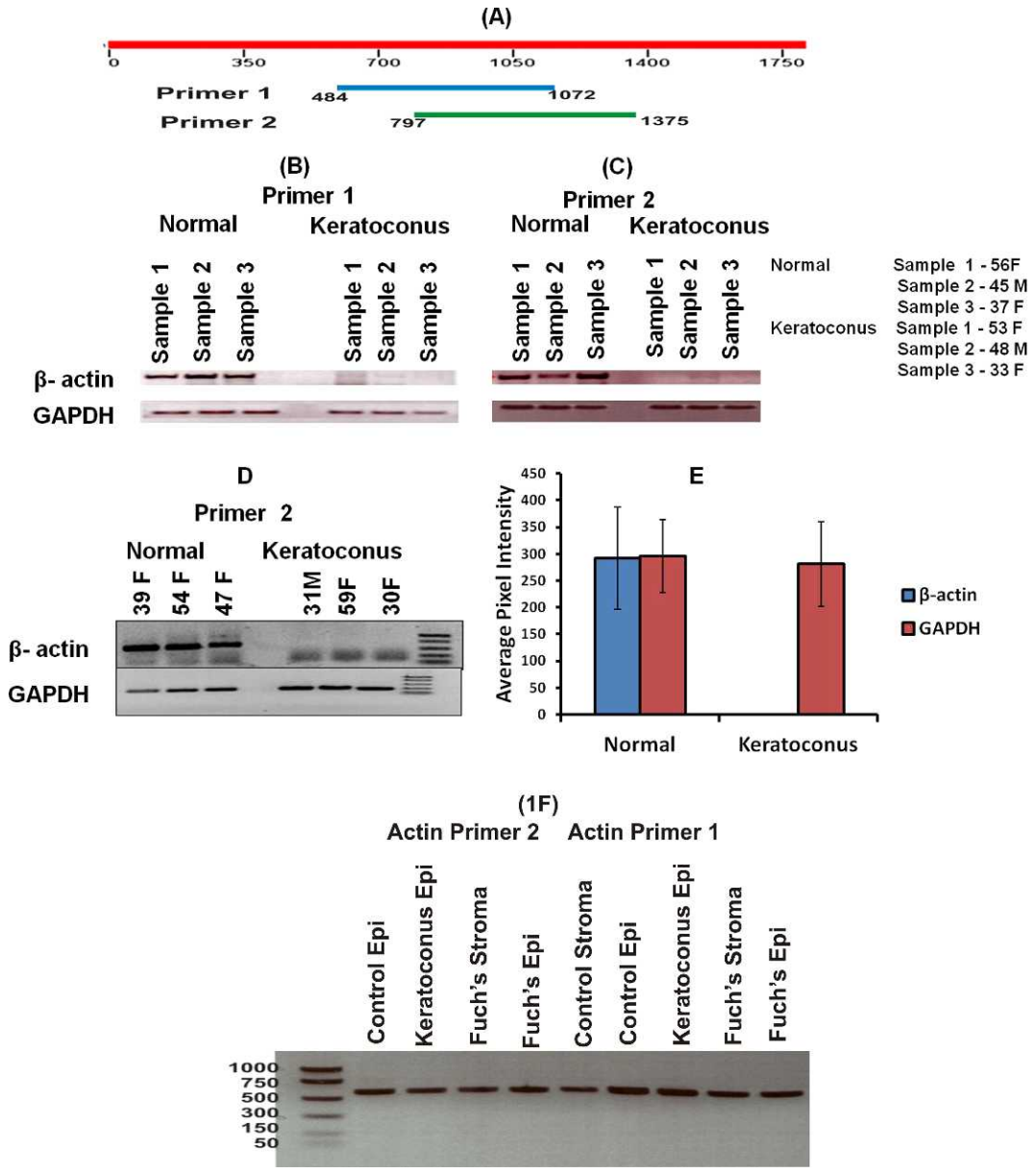

Figure 1. (**A**) Location of primer 1 (from 484 to 1072, shown in *blue*) and primer 2 (from 797 to 1375, shown in *green*) in the β-actin gene. (**B**, **C**) RT-PCR for β-actin gene using primer 1 and primer 2. The β-actin gene was expressed in the stroma of the three normal (control) corneas, whereas the expression level of the β-actin gene was decreased/absent in the stroma of the three KC corneas. The levels of the GAPDH gene, which was used as the internal standard, remained the same in the stroma of both normal and KC corneas. (**D**) RT-PCR to determine β-actin gene expression using primer 2. The β-actin gene was expressed in the stroma of the three normal (control) corneas, whereas the expression level of the β-actin gene was decreased/absent in the stroma of the three KC corneas. The levels of the GAPDH gene, which was used as the internal standard, remained similar in all the samples. The ages of the donors of KC and normal corneas are shown in *right-hand corner* of 1B and 1C, and *top of lanes* in D. (**E**) The pixel intensity of β-actin and GAPDH gene expressions of both normal and KC corneas from C and D. These pixel intensities along with their SD were calculated from the six corneal samples in C and D. (**F**) RT-PCR for β-actin gene. The RT-PCR analysis with the two primers for the β-actin gene is shown. The β-actin gene was expressed in the stroma of the normal (control) corneas and also in the stroma of Fuchs' dystrophic corneas. The expression levels of the β-actin gene remained at the same levels in the epithelium of the normal and KC corneas, and Fuchs' dystrophic corneas.

The β-actin mRNA has long half-life,[9,17] and HuR binding to a Uridine (U)-rich element has been shown to be involved in mRNA stability.[18] The mRNA stability could be a potential reason for the loss of β-actin expression in KC stroma. To determine HuR gene expression levels in KC stroma, a quantitative real-time PCR analysis was carried out in the stromal preparation of both the normal and KC corneas; real-time PCR analysis allows a quantitative measure of the gene expression levels. Quantitative RT-PCR data were analyzed by the comparative ΔCt method.[19] First, delta threshold cycle (ΔCt) and mean of ΔCt are calculated, and then the SD of each set of ΔCt is determined. The threshold cycle (Ct) value is the amplification cycle number at which a defined fluorescence is achieved. The real-time PCR for the HuR gene (involved in the mRNA stability) showed that HuR gene expression is decreased 4.7-fold (Fig. 4A). Following RT-PCR analysis of HuR gene, the product was analyzed using 1% Agarose gel electrophoresis. As shown, the expression of HuR was decreased in the stroma of

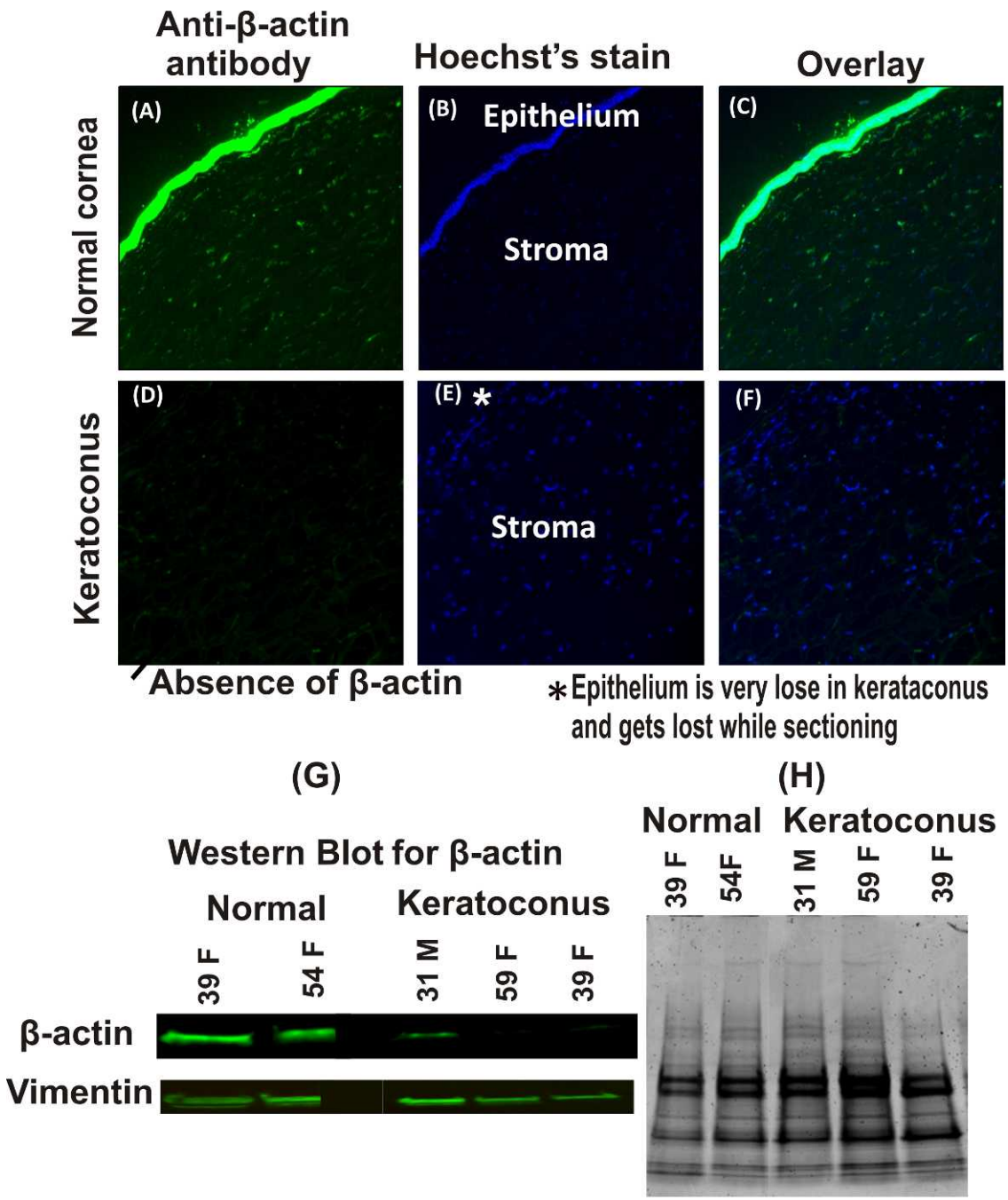

Figure 2. Immunohistochemical analysis of normal and KC corneas with anti-β-actin antibody. (**A**) Immunoreactivity of 10-μm normal human corneal cryosection with anti-β-actin antibody. (**B**) Counterstaining of the sections with Hoechst nuclear stain. (**C**) Overlay of A and B. (**D**) Immunoreactivity of 10-μm KC corneal cryosection with anti-β-actin antibody. (**E**) Counterstaining of the sections with Hoechst nuclear stain. (**F**) Overlay of D and E. Note the absence of β-actin in the stroma of KC cornea. (**G**) Western blot analysis of stromal proteins using anti-β-actin and anti-vimentin antibodies. The β-actin expression in the stromal proteins of two normal and three KC corneas with donors' ages as shown were examined. Vimentin was used as a loading control for all five samples. (**H**) Coomassie blue–stained gel of the stromal proteins from normal and KC corneas. Note that identical quantities of proteins were used from each corneal stromal preparation for the Western blot analysis.

KC compared with normal cornea. In contrast, the GAPDH gene expression remained at the same levels in preparation of both normal and KC corneas (Fig. 4B). To examine the level of HuR in the corneal keratocyte cultures, cells were analyzed with anti-HuR antibody. Figure 5A shows the staining of anti-HuR antibody in the normal fibroblasts, and 5B shows the anti-HuR antibody staining in that of KC fibroblasts. The total green pixel intensity was quantified from three normal and three KC corneal fibroblasts (Fig. 5C) as described in the Methods section. The total green pixel intensity was decreased 3-fold in KC corneal fibroblasts compared with normal corneal fibroblasts.

Discussion

Although changes in corneal epithelium and stroma at the cellular, biochemical, physiological, and genetic levels are

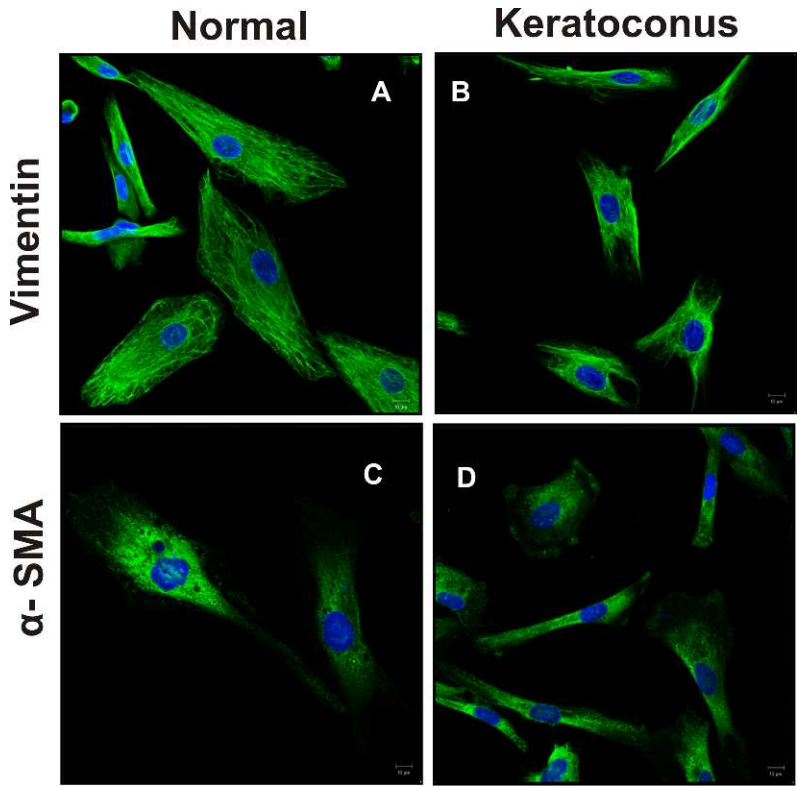

Figure 3. Immunoreactivity of keratocyte-derived fibroblasts from normal and KC stroma with anti-vimentin, anti-α smooth muscle–actin, anti-γ-actin, and anti-β-actin antibodies, and their staining with Rhodamine-labeled phalloidin. (**A**) Immunoreactivity of stromal fibroblasts from normal corneas with anti-vimentin antibody and counterstained with Hoechst nuclear staining. (**B**) Immunoreactivity of stromal fibroblasts from KC corneas with anti-vimentin antibody and counterstained with Hoechst nuclear staining. (**C**) Immunoreactivity of stromal fibroblasts from normal corneas with anti-α smooth muscle–actin antibody and counterstained with Hoechst nuclear staining. (**D**) Immunoreactivity of stromal fibroblasts from KC corneas with anti-α smooth muscle–actin antibody and counterstained with Hoechst nuclear staining.

reported to be responsible for the development and progression of KC, the exact molecular mechanism of KC remains elusive. During the disease, additional changes besides affected epithelial cells and stromal keratocytes[20] include discrete incursion of fine cellular processes into Bowman's membrane[21,22] and altered nerve plexus.[23–25] Interplay of epithelial–stromal interaction (ESI) in the cornea is important for both corneal transparency and the wound-healing process.[26] Our focus in the current study has been on stromal keratocytes because changes in their unique properties and functions are attributed to the development and progression of KC.

The broad, flattened, quiescent keratocytes (neural crest-derived cells) constitute 5% of corneal stroma and lie parallel to the collagen lamellae.[27] Keratocytes maintain a functional stroma via secretion of stromal collagen and extracellular matrix, which provides strength and transparency to the cornea, and these cells also behave like macrophages during corneal infection and injury.[28] The cells synthesize and secrete collagen (mainly type I and type V), proteoglycans (keratocan, lumican, and mimican with keratin sulfate),[29] and high levels of corneal crystallins, namely, transketolase and aldehyde dehydrogenase class 1A1.[30] When quiescent keratocytes are cultured in the presence of serum or growth factors, they become mitotic and phenotypically become fibroblasts, as observed during wound healing.[16,31] Under normal conditions, the keratocytes become active during injury to differentiate into fibroblast and myofibroblast-like cells.[32] The fibroblast growth factor–2 and platelet-derived growth factor stimulate differentiation of keratocytes into fibroblast and transforming growth factor beta (TGF-β) into myofibroblasts.[32,33]

During KC, the apoptosis of keratocytes seems to be among the reasons for corneal thinning,[34] as evidenced by their differentially expressed genes during the disease. This includes the overexpression of bone morphogenic protein-4, coflin, and JAW1-related protein (also known as lymphoid restricted membrane protein [LRMP]), and the underexpression of actin, α-2 rich cluster, C-10 gene, tissue inhibitors of metalloproteinase 1 and 3, and somatostatin receptor 1.[35] These genes are believed to control apoptosis, cytoskeletal structure, wound healing, and nerve-fiber density in corneas.[35] However, presently the trigger for keratocyte apoptosis during KC remains unknown.

In the present study, we investigated the expression of β-actin gene in stromal keratocytes in normal and KC corneas. Results presented herein show that β-actin was downregulated at both transcriptional and translational levels in the KC stroma but not in the stroma of normal corneas and Fuchs' dystrophic corneas. Even on culturing these cells to fibroblasts from both normal and KC stroma, β-actin showed downregulation only in the cells of the KC corneas. A previous study has shown that the small interfering RNA (siRNA)-mediated β-actin knockdown results in membrane blebbing in Henrietta Lack (HeLa) cells after 48 hours of treatment.[7] A similar knockdown of β-actin gene in HeLa cells did not show any significant cell death, and this discrepancy was apparently due to the difference in the media used for culturing HeLa cells.[36] Likewise, we also noted that in spite of downregulation of the β-actin gene in KC keratocytes, cells were viable. This could be due to the quiescent cell–nature of stromal keratocytes in the normal cornea, and that they become fibroblasts and myofibroblasts in

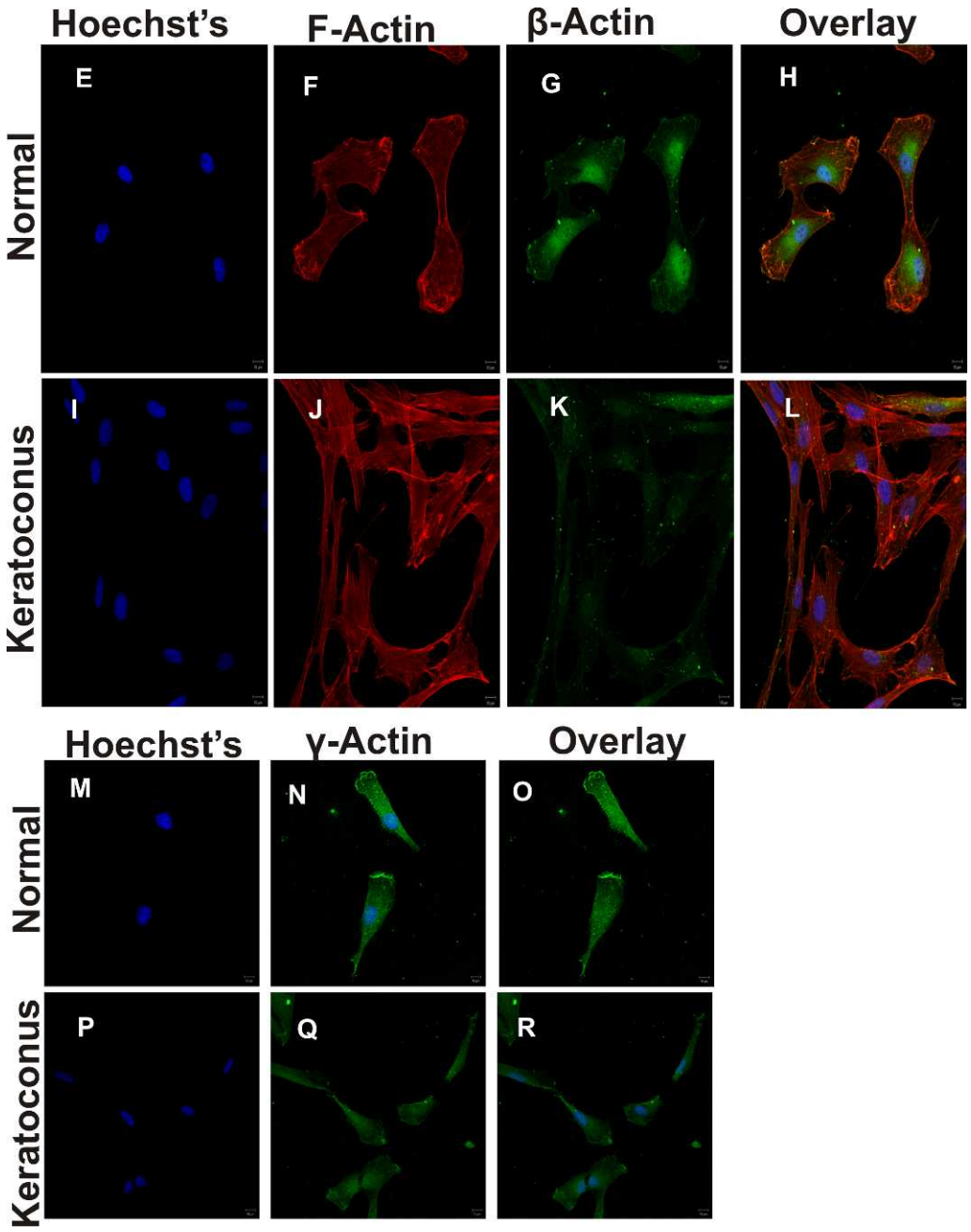

Figure 3. Continued. (**E**) Fibroblasts from normal corneal stroma stained with Hoechst nuclear stain. (**F**) Fibroblasts from normal corneal stroma stained with Rhodamine-labeled phalloidin stain. (**G**) Immunoreactivity of stromal fibroblasts from normal corneas with anti-β-actin antibody. (**H**) Overlay of E, F, and G. (**I**) Fibroblasts from KC corneal stroma stained with Hoechst nuclear stain. (**J**) Fibroblasts from KC corneal stroma stained with Rhodamine-labeled phalloidin stain. (**K**) Immunoreactivity of stromal fibroblasts from KC corneas with anti-β-actin antibody. (**L**) Overlay of I, J, and K. (**M**) Fibroblasts from normal corneal stroma stained with Hoechst nuclear stain. (**N**) Immunoreactivity of stromal fibroblasts from normal corneas with anti-γ-actin antibody. (**O**) Overlay of M and N. (**P**) Fibroblasts from KC corneal stroma stained with Hoechst nuclear stain. (**Q**) Immunoreactivity of stromal fibroblasts from KC corneas with γ-actin antibody. (**R**) Overlay of P and Q. Note that the normal corneal fibroblast cells showed expression of β-actin, whereas those from KC corneas showed reduced β-actin expression.

the presence of serum. Serum-induced cells show an increase in β-actin synthesis,[37] whereas KC fibroblasts even in the presence of serum had no induction of β-actin synthesis (Fig. 5I). Additionally, both normal and KC fibroblasts did express vimentin (an intermediate filament), which is a marker for mesenchymal cells and represents the mesenchymal nature of stromal fibroblasts.

The major functions of β-actin in cells are to provide mechanical support through cytoskeleton and extracellular matrix, help in cell motality, and act in signal transduction of cytoplasm with surroundings. Cytoplasmic proteins adjacent to plasma membrane control cell shape and regulate cell–cell interactions and focal adhesions. Embryonic lethality of β-actin has been reported,[8,38] whereas γ-actin knockout mice are viable.[38] Conditional knockout of β-actin in mouse embryonic fibroblast has been reported to show increased apoptosis and motility defects.[38] It has also been shown that β-actin deficiency in auditory hair cells leads to progressive hearing

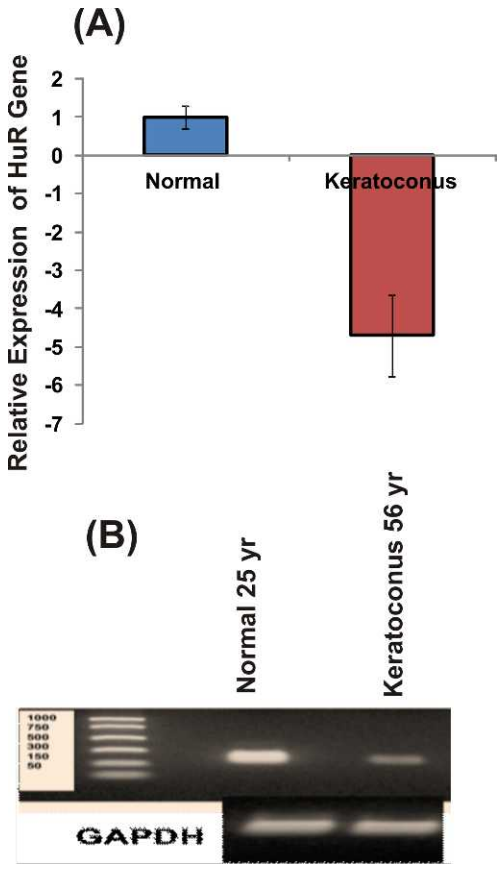

Figure 4. Relative expression of HuR gene in normal and KC corneal stroma. (**A**) Expression levels of HuR gene in normal and KC stroma as detected by real-time PCR. Data represent the relative mRNA expressions compared with GAPDH and are presented as the mean ± SD. (**B**) RT-PCR analysis showing the product for HuR gene from a normal and KC stroma after 40 cycles. The samples were analyzed using 1% Agarose gel.

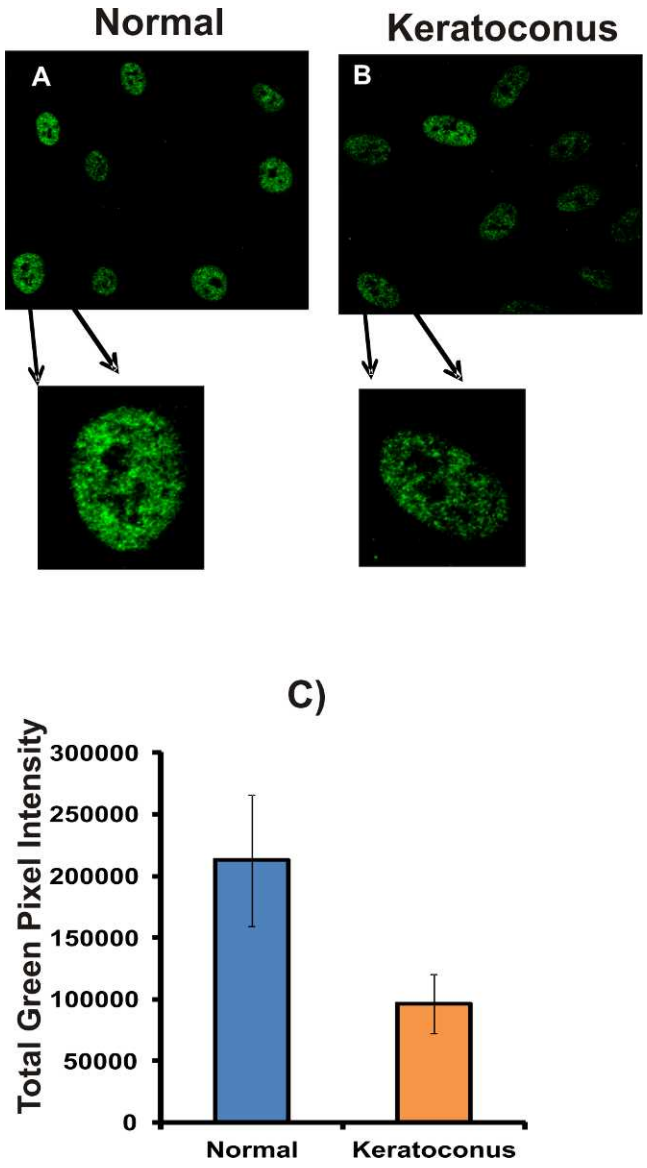

Figure 5. Immunoreactivity of stromal fibroblasts with anti-HuR antibody. (**A**) Immunoreactivity of stromal fibroblasts from normal corneas with anti-HuR antibody. (**B**) Immunoreactivity of stromal fibroblasts from KC corneas with anti-HuR antibody. (**C**) Total green pixel intensity quantified in fibroblasts from normal corneas (A) and from KC corneas (B).

loss.[39] In spite of such studies, the role of β-actin in corneal stroma seems to be mostly mechanical, and additional functions are unclear.

The high expression level of β-actin is important for the above-described cellular processes, and this is maintained in vivo by its mRNA stability and optimal concentration levels. The expression of actin genes is regulated at both transcriptional[9] and at posttranscriptional levels, such as the cellular localization of their mRNAs.[10] Studies using antisense oligonucleotides against cis-acting elements have shown that the polarity and cellular motility are severely reduced.[40] A 54-nucleotide (nt) segment, zipcode, has been identified as a regulatory sequence in the 3′UTR of the β-actin mRNA sequence.[40] Several ZBP-1 have been identified that bind to the zipcode sequence to regulate the β-actin mRNA localization.[41] The embryonic lethal abnormal visual system (ELAV) family of proteins, in particular the HuC (mouse) and HuR (human) have been shown to exhibit poly(A)–binding activity and appear to be able to bind simultaneously to the AU rich elements (AREs) and the poly(A) tail in vitro.[42,43] The mRNA of HuR is ubiquitously expressed in all proliferating cells and is the most important posttranscriptional regulator of gene expression.[44] Dormoy-Raclet et al. have shown that HuR depletion in HeLa cells alters the cytoskeleton functions such as cell adhesion, migration, and invasion and results from the loss of β-actin stress fibers.[18] The β-actin mRNA has long half-life,[9,17] and HuR binding to a U-rich element is involved in the mRNA stability and affects the half-life of β-actin mRNA.[18] Our real-time PCR analysis of the HuR gene showed that it is downregulated 4.7-fold in KC corneas compared with normal corneas, which raises the possibility that the downregulation of HuR and β-actin could be an effect of the interplay between the two. This is further supported by our recent finding that the knockdown of the HuR gene, by an siRNA method in keratocytes from normal corneas, leads to downregulation of expression of the β-actin gene (Joseph R, et al., unpublished results, 2012). This coincides with the earlier result showing that downregulation of HuR resulted in the reduced half-life of β-actin mRNA.[18] The reduced half life of β-actin mRNA in turn might result in the loss of cytoskeletal functions, mainly owing to a loss of β-actin stress fibers.

In fibroblastic cells, β-actin mRNA has been shown to be localized towards the leading edge and is believed to be involved in cell motility and asymmetry.[22] There is a long-

standing debate about how the actin isoforms, especially the nonmuscle actin segregate in the moving fibroblast. Karakozova et al. have shown that the actin isoforms are located in the fibroblastic cells; β-actin is localized at the leading edge of the cells, and γ-actin is the one that forms the stress fibers.[45] This finding is supported by our immunohistochemical staining with phalloidin that showed the stress fibers in fibroblasts of both KC and normal corneas; however, the leading edge of the former was devoid of any staining when compared with the latter. This result coincides with the report by Karakozova et al. that shows a striking disparity in the staining of F-actin by the two isoforms.[45] Our study showed the localization of the β-actin (G-actin monomers) around the nucleus (Fig. 3I). This finding is further supported by the fact that in β-actin knockout mice, the embryonic fibroblasts show a reduced G-actin pool compared with F-actin.[38] Our results also showed that even in the absence of β-actin staining in KC fibroblasts, it had a positive F-actin staining. This could be due to the absence of monomeric G-actin pool, which could lead to an increased expression of megakaryoblastic leukemia–serum response factor (MAL-SRF) target genes. Monomeric actins are known to inhibit co-activator MAL of the SRF.[46] The α-smooth muscle–actin and γ-actins are genes targeted by MAL-SRF.

In summary, our findings raise the possibility that a reduced number of keratocytes during KC could be a consequence of the loss of β-actin, which in turn destabilizes their cytoskeleton and caused apoptosis. This loss of β-actin could be due to the decreased stabilizing factor (HuR), since it is known that β-actin gene expression is modulated by HuR. The posttranslational mRNA stability of other proteins besides β-actin may also be affected by the decreased HuR expression. What upstream targets affect this downregulation has yet to be determined. As stated above, β-actin is involved in many cellular and pathogenic processes, and therefore, its downregulation could affect certain, yet unknown, functions that might lead to the development and progression of the KC disease process.

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