DIFFERENTIAL EXPRESSION OF PROTEINS IN KERATOCONUS: POTENTIAL ROLE OF HUMAN ANTIGEN R (HuR) IN REGULATION OF β-ACTIN

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DIFFERENTIAL EXPRESSION OF PROTEINS IN KERATOCONUS: POTENTIAL ROLE OF HUMAN ANTIGEN R (HuR) IN REGULATION OF β-ACTIN

ROY JOSEPH

VISION SCIENCES

ABSTRACT

Keratoconus (KC) is a condition of unknown cause in which the cornea assumes a conical shape as a result of non-inflammatory thinning of the corneal stroma. The disease progresses at a variable speed with corneal thinning inducing irregular astigmatism, myopia, and corneal protrusion. Contact lenses, and ultimately keratoplasty, are often required to restore vision. Despite intensive investigations into the pathogenesis of KC, the exact cause of the disease is presently poorly understood. Keratoconus apparently arises due to number of factors, which include changes at cellular, biochemical, physiological and genetic levels. With the progression of KC, both epithelial cells and stromal keratocytes are affected.

The purpose of this research was to identify a potential molecular mechanism of the keratoconus disease process. For this purpose, we used two different proteomic methods, shotgun proteomics and 2D-DIGE methods to identify relative changes in protein levels in both epithelium and stroma of KC corneas compared to normal corneas. Major changes were seen in the structural proteins of both epithelium and stroma of KC corneas compared to normal corneas, suggesting structural remodeling of both the tissues during the development and progression of keratoconus. The proteins that are involved in proliferation, growth and migration were down-regulated in KC epithelium.
Based on the protein level changes and systems biology approach, two unique models were generated; one for epithelium and the other for stroma of keratoconus. 1) The epithelium showed a disruption of iron homeostasis in the KC corneas could lead to increased oxidative damage. 2) Changes in the cytoskeletal proteins of keratocytes could lead to cellular apoptosis.

The above results led us to focus on one of the cytoskeletal protein, β-actin. Our molecular analysis showed that β-actin is down-regulated in the corneal stroma of patients with keratoconus, due to reduced levels of a stabilizing factor Human Antigen R (HuR) for β-actin mRNA. In order to determine the functional significance of the down-regulation of β-actin and HuR we used siRNA-mediated gene silencing in stromal keratocytes of normal corneas. Knockdown of HuR gene led to reduced expression of β-actin mRNA. This in turn significantly reduced keratocytes migration and proliferation.

Key Words: Cornea, Keratoconus, Keratocytes, β-actin, HuR
DEDICATION

I would like to dedicate this thesis to my father Mr. Joseph Thomas, and my mother Mrs. Annamma Joseph, who has been the inspiration throughout my life and without your prayers and support I would not have reached here. I would also like to dedicate this to my wife Merlin and my loving daughter Aleena for their support and patience throughout my graduate term.

Also, I would like to thank both my sisters and their families for the emotional support and especially to my sister Mrs. Reny Joseph and my brother in-law, Dr. Balu Chacko for their continuous support.

Finally I would also like to dedicate this thesis to the corneal donors, without their support this study would have been impossible. Thank you.
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to thank all the committee members for their support for the fulfillment of this work, which is the conclusion of one part of research and the beginning of another.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>Background and Significance</strong></td>
<td></td>
</tr>
<tr>
<td>Corneal Structure and Function</td>
<td>1</td>
</tr>
<tr>
<td>Significance of Actins as Proteins in the cell</td>
<td>3</td>
</tr>
<tr>
<td>β-Actin Gene Expression and Regulation</td>
<td>4</td>
</tr>
<tr>
<td>Keratoconus</td>
<td>6</td>
</tr>
<tr>
<td>Role of Degradative Enzymes in Keratoconus</td>
<td>7</td>
</tr>
<tr>
<td>Oxidative Stress and Keratoconus</td>
<td>8</td>
</tr>
<tr>
<td>Animal Models in Keratoconus</td>
<td>9</td>
</tr>
<tr>
<td>Genomic Mutation in Keratoconus</td>
<td>10</td>
</tr>
<tr>
<td><strong>Overall Goal and Hypothesis</strong></td>
<td></td>
</tr>
<tr>
<td>Overall Goal</td>
<td>13</td>
</tr>
<tr>
<td>Overall Hypothesis</td>
<td>13</td>
</tr>
<tr>
<td><strong>Specific Aims and Rationales</strong></td>
<td></td>
</tr>
<tr>
<td>Specific Aim I</td>
<td>14</td>
</tr>
<tr>
<td>Specific Aim I Rationale</td>
<td>14</td>
</tr>
<tr>
<td>Specific Aim II</td>
<td>15</td>
</tr>
<tr>
<td>Specific Aim II Rationale</td>
<td>15</td>
</tr>
<tr>
<td>Specific Aim III</td>
<td>15</td>
</tr>
<tr>
<td>Specific Aim III Rationale</td>
<td>16</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table  Page

DIFFERENTIAL EPITHELIAL AND STROMAL PROTEIN PROFILES IN KERATOCONUS AND NORMAL HUMAN CORNEAS

1 Identification of Human Corneal Epithelial Proteins following Nano-ESI-LC-MS (MS)^2 ..............................................................70

2 Proteins that Showed Difference in Keratoconus Epithelium when Compared to Normal Corneal Epithelium by Nano-ESI-LC-MS (MS)^2 ........................................73

3 Identification of Human Stromal Proteins following Nano-ESI-LC-MS (MS)^2 ..........74

4 Proteins that Showed Difference in Keratoconus Stroma when Compared to Normal Corneal Stroma by Nano-ESI-LC-MS (MS)^2 ....................................................76

5 Identification of Epithelial Proteins that Showed Changes by 2D-DIGE Method ......77

6 Identification of Stromal Proteins that Showed Changes by 2D-DIGE Method.......78

DOWN-REGULATION OF β-ACTIN GENE IN HUMAN KERATOCONUS CORNEAS IS DUE TO HUMAN ANTIGEN R (HuR) PROTEIN

1 List of Antibodies used in the study .................................................................116

2 Primers used in the study ...........................................................................116
DOWN-REGULATION OF β-ACTIN AND ITS REGULATORY GENE HuR AFFECTS CELL MIGRATION IN HUMAN CORNEAL FIBROBLAST

1 List of Antibodies used in the study ................................................................. 147
LIST OF FIGURES

**Figures** | **Page**
---|---
1 Detailed Structure of the Cornea | 1
2 Summary of TGF-β-Mediated Pathway | 11

**GLOBAL INTRODUCTION**

1 Relative Abundance of Epithelial Proteins in KC vs. Normal Corneas as Determined by Nano-ESI-LC-MS(MS)² Method | 64
2 Relative Abundance of Stromal Proteins in KC vs. Normal Corneas as Determined by Nano-ESI-LC-MS(MS)² Method | 65
3 Overlay of Cy2/Cy3/Cy5-Labeled Corneal Epithelial Proteins during 2D-DIGE Analysis | 66
4 Identification of Epithelial Proteins that Showed Up- or Down- Regulation during 2D-DIGE Analysis | 67
5 Overlay of Cy2/Cy3/Cy5-Labeled Corneal Stromal Proteins during 2D-DIGE Analysis | 68
6 Identification of Stromal Proteins that Showed Up- or Down- Regulation during 2D-DIGE Analysis | 69
7 Ingenuity Pathway Analysis of Epithelial Proteins that Showed Changes in KC Compared to Normal Corneas | 91
Ingenuity Pathway Analysis of Stromal Proteins that Showed Changes in KC Compared to Normal Corneas ..............................................................................................................................................92

DOWN-REGULATION OF β-ACTIN GENE IN HUMAN KERATOCONUS CORNEAS IS DUE TO HUMAN ANTIGEN R (HuR) PROTEIN

1 β-actin Gene Expression in Normal vs. Keratoconus Corneas ........................................117
2 Immunohistochemical Analysis of Normal and KC Corneas with Anti-β- Actin Antibody ........................................................................................................................................119
3 Immunoreactivity of Fibroblast from Normal and KC stroma ........................................120
4 Relative Expression of HuR Gene in Normal and KC Corneal Stroma .....................123
5 Immunoreactivity of Fibroblast with Anti- HuR Antibody ........................................124

DOWN-REGULATION OF β-ACTIN AND ITS REGULATORY GENE HuR AFFECTS CELL MIGRATION IN HUMAN CORNEAL FIBROBLAST

1 RT-PCR Analysis of β-actin and Western blot Analysis..............................................148
2 Localization of GAPDH after Gene Silencing............................................................149
3 Localization of HuR after Gene Silencing.................................................................150
4 Localization of β-actin after Gene Silencing..............................................................151
5 Localization of γ-Actin in Corneal Fibroblast after β- Actin Gene Silencing ........153
6 Analysis of Cell Migration using after Gene Silencing ...........................................154
7 Affect of Wound Healing after Gene Silencing.........................................................155
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-DIGE</td>
<td>2-D Fluorescence Difference Gel Electrophoresis</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>Aldehyde Dehydrogenase Class 3</td>
</tr>
<tr>
<td>β-actin</td>
<td>Beta actin</td>
</tr>
<tr>
<td>DOCK9</td>
<td>Dedicator of Cytokinesis</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>ELAV</td>
<td>Embryonic lethal abnormal vision protein</td>
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<tr>
<td>F-actin</td>
<td>Filamentous Actin</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
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<tr>
<td>γ-actin</td>
<td>Gama Actin</td>
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<tr>
<td>GEF</td>
<td>Guanine-Nucleotide Exchange Factor</td>
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<td>h</td>
<td>Hour</td>
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<td>HuR</td>
<td>Human Antigen R</td>
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<td>KC</td>
<td>Keratoconus</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix MetalloProteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>Nano-ESI-LC-MS (MS)$^2$</td>
<td>Nano Electro Spray Ionization Liquid Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Deodcyl Sulfate Poly Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>Short Form</td>
<td>Full Form</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>UTR</td>
<td>Un-Translated Region</td>
</tr>
<tr>
<td>ZBP-1</td>
<td>Zip Code Binding Protein 1</td>
</tr>
</tbody>
</table>
GLOBAL INTRODUCTION

Background and Significance

*Corneal Structure and Function*

The cornea consists of five layers (Figure 1), the epithelium, in between the epithelium and the stroma is Bowman’s layer, in between the stroma and the single layered endothelium is the Descemet’s layer. Epithelium is 50 µm thick and made up of five layers of squamous epithelial cells (Kenyon, 1979). The basal cell layers are columnar and adherent to the basement membrane, composed of largely type IV collagen. The cytoskeleton of the epithelial cells is made up of mainly keratins and actin. The basal cells are metabolically active and have more mitochondria than the superficial cells. The most significant aspect of corneal epithelial cells is the constant turnover due to sustained proliferation of basal epithelial cells (Hanna, 1961). These basal epithelial cells are then displaced outward by mitotically active cells. The epithelial cell renewal also occurs by a
slow centripetal movement of peripheral epithelial cells. Epithelial wound healing involves three distinct components cell migration, cell proliferation and cell adhesion. Two phases are involved in wound healing 1) latent phase and 2) linear healing phase. During the latent phase intracellular synthesis of structural proteins is increased and actin filaments are polymerized and reorganized from the apical to the basal region of the cells (Gipson & Anderson, 1977). And during the linear healing phase the epithelial cells flatten, spread and cover the wound. The Bowman’s layer is made up of collagen and provides protective function to the cornea. Between the stroma and the endothelium is the Descemet’s layer, composed of collagen. The function of the stroma is to provide structural integrity and also maintaining transparency to the cornea. The broad, flattened, quiescent stromal keratocytes (neural crest-derived cells) constitutes 5% of corneal stroma, lying parallel to the collagen lamellae (Smith, 1969). Keratocytes play an important role in corneal transparency via secretion of stromal extracellular matrix and collagen that are needed for corneal strength and transparency. Keratocytes also behave like macrophages during corneal infection and injury (Chakravarti, Wu et al. 2004). Keratocytes synthesize and secrete collagen (mainly type I and type V collagen), proteoglycans [keratocan, lumican, and mimican with keratin sulfate side chains (Farjo, 2009), and high levels of corneal crystallins, namely transketolase aldehyde dehydrogenase class 1A1(Jester, Lee et al. 2007). When quiescent keratocytes are cultured in the presence of serum or growth factors, they become mitotic and transform to phenotypical fibroblasts, as observed during wound healing (Funderburgh, Funderburgh et al. 2001; Funderburgh, Mann et al. 2003). Under normal conditions keratocytes become active after injury to differentiate into fibroblasts and myofibroblast-like cells (Møller-Pedersen, Li et al. 1998). Fibroblast
growth factor-2 and platelet-derived growth factor stimulate differentiation of keratocytes to fibroblast while TGF-β differentiates keratocytes to myofibroblasts (Møller-Pedersen, Li et al. 1998; Jester, Budge et al. 2005). In keratoconus, apoptosis of keratocytes appears to be one reason for corneal thinning (Kim, Rabinowitz et al. 1999), as evidenced by differentially expression of genes found in the disease. These include the over-expression of bone morphogenic protein-4, coflin, JAW1-related protein, and under-expression of actin, alpha 2-rich cluster, and C-10 gene, tissue inhibitors of metalloproteinase 1 and 3 and somatostatin receptor 1 (Lee, 2009). These genes are believed to control apoptosis, cytoskeletal structure, wound healing and nerve fiber density in the cornea (Lee, 2009). Between the stroma and the endothelium is the Descemet’s layer, composed of collagen. The endothelium is a made up of single layer of cells has Na⁺, K⁺-ATPase pump sites that maintain the normal function of the cornea.

Significance of Actins as Proteins in the cell

Actins are one of the major cytoskeletal structural protein expressed in the eukaryotic cells and is involved in many cellular process, including cell adhesions, cell migration/movement, cytokinesis, endo-/exocytosis, cell division, signal transduction, mRNA localization and transcription (Bassell & Singer 1997). The Eukaryotes have six actin isoforms, each are encoded by an individual gene (Vandekerckhove & Weber 1978). The six isoforms are: two striated muscle (α-skeletal and α-cardiac muscle), two smooth muscle (α- and γ- smooth muscle actin) and, two cytoplasmic (β-and γ-actins) (Herman, 1993). The muscle actins are tissue-specific and make up the contractile units,
whereas β- and γ-actin are ubiquitous and essential for cell survival (Harborth et al., 2001). The isoforms have highly conserved amino acid sequence, and they differ mainly at the N-terminus. In contrast the cytoplasmic β and γ-actins differ only by four aminoacids. The absence of β-actin at an embryonic stage was found to be lethal in transgenic mouse (Shawlot et al., 1998). Dugina et al., have shown that β- and γ-actins segregate separately in the cytoplasm of mesenchymal and epithelial cells during quiescence, and also during locomotion and cytokinesis (Dugina et al., 2009). Dugina et al., have also shown that depleting β- and γ-actins, they participate differently in the organization of cell morphology, polarity and motility (Dugina et al., 2009). Actin affecting drug latrunculin-A disrupts the γ-actin network at the leading edge of the fibroblast cells, but did not inhibit the formation of β-actin stress fibers whereas low doses of cytochalasin D inhibited the formation of β-actin stress fibers (Dugina et al., 2009). Disruption of the actin cytoskeleton leads to cell rounding and resulting apoptosis cannot be prevented by attachment (Martin & Leder, 2001).

\textit{β-Actin Gene Expression and Regulation}

Because of changes seen in β-actin in KC corneas compared to normal corneas, we plan to study its expression and gene regulation in greater details (see above). The high expression levels of β-actin is important for the cellular process and this is maintained by its stability and the concentration levels. The expression of actin genes is regulated at the transcriptional level (Olave, Reck-Peterson & Crabtree 2002), and also at post-transcriptional level, such as the cellular localization of their mRNAs (Kislauskis, et
The β-actin is regulated by a specific sequence at the 3’ untranslated region (3’UTR) by RNA binding proteins called zip code-binding proteins (ZBP) (Kislauskis et al., 1994). Cells when treated antisense oligonucleotides directed against zipcode sequence or with a dominant-negative isoform of the zip code-binding proteins (ZBP1) protein results in β-actin mRNA delocalization and impairment of cellular motility (Farina et al., 2003). The other RNA binding proteins such as heterogenous nuclear ribonuclear protein A2 (hnRNPA2), the KH type splicing regulatory protein, and one of the brain-specific embryonic lethal abnormal vision (ELAV) proteins, human antigen C (HuC) were also known to associate with 3’UTR of β-actin mRNA. These proteins bind either the Zipcode sequence or to the uridine rich element (HuC). The 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 ARE and the poly(A) tail in vitro (Abe et al., 1996). The mRNA of HuR is ubiquitously expressed in all proliferating cells and is the most important post-transcriptional regulators of gene expression (Fan & Steitz, 1998). Dormoy-Raclet et al. have shown that HuR depletion in HeLa cells alters the cytoskeleton functions such as cell adhesion, migration and invasion and is due to the loss of β-actin stress fibers (Dormoy-Raclet et al., 2007). The β-actin mRNA has long half life (Condeelis & Singer, 2005; Olave, Reck-Peterson & Crabtree, 2002) and HuR binding to U-rich element is involved in the mRNA stability (Dormoy-Raclet et al., 2007).
Keratoconus

Keratoconus (KC) is a pathological condition in which the cornea assumes a conical shape as a result of non-inflammatory thinning of the corneal stroma. The disease progresses at a variable speed in humans with corneal thinning inducing irregular astigmatism, myopia, and corneal protrusion. Contact lenses, and ultimately keratoplasty, might be required to restore vision. According to the National Eye Institute reports, KC is the most common corneal dystrophy in the United States, affecting 1 per 2000 Americans (US NEI; Kennedy, 1986). The classical histopathological features include 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, Lebers congenital amaurosis, and mitral valve collapse (Rabinowitz, 2003).

Despite intensive investigations into the pathogenesis of KC, the exact cause of the disease is poorly understood. Keratoconus apparently arises due to number of factors, which could be due to changes in the cornea at cellular, biochemical, physiological and genetic levels. With the progression of KC, both epithelial cells and stromal keratocytes are affected. The epithelium degenerates, loses its smoothness and becomes irregular (Jongebloed WL 1987). Blebbing is a constant feature of the epithelial surface and degeneration of the epithelium is also seen in the keratoconus corneas (Pfister, 1977). Using in vivo confocal microscopy in KC subjects, reduced keratocyte density in the diseased corneas compared to normal corneas was observed (Ku, 2008). KC has been associated with central epithelial thinning (Scroggs, 1992,Tsubota, 1995), apparently due to a decrease in epithelial cell density (Ucakhan, 2006) but an increase in cell area (Tsubota, 1995); (Hollinsworth, 2005), resulting from an enlargement and irregular
arrangement of basal epithelial cells (Niederer, Perumal et al. 2008). Keratocytes with high levels of endoplasmic reticulum and discrete incursion of fine cellular processes into Bowman’s membrane were also observed in KC corneas (Polack, 1976); (Rock, 1995). Compared to normal corneas, KC corneas showed increased apoptosis of keratocytes (Kaldaawy 2002); (Kim, Rabinowitz et al. 1999), and altered nerve plexi (Niederer, Perumal et al. 2008); (Patel and McGhee 2006); (Mannion, Tromans et al. 2007). The earliest changes of KC have been described in the superficial layer of the corneal epithelium followed by involvement of the basal cell layer. In advanced stages, cell membranes rupture along with disappearance of the basal epithelial cells, leaving only one or two layers of flattened superficial epithelial cells lying on an altered basement membrane (Rabinowitz, 2003). It has been suggested that the basal epithelial cells degenerate, they release proteolytic enzymes that might destroy the underlying tissue.

Role of Degradative Enzymes in Keratoconus

The reduced corneal thickness (Teng, 1963; Patel, 1999) might be caused by increased levels of several degradative enzymes such as acid esterase’s, acid phosphatases, and acid lipases (Critchfield, 1988), cathepsin B and G (Sawaguchi, 1989; Zhou, Sawaguchi et al. 1998) and decreased levels of protease inhibitors such as $\alpha_1$-protease inhibitor and $\alpha_2$-macroglobulin (Sawaguchi, 1990; Sawaguchi, Twining et al. 1994). An abnormality in corneal collagenase activity (Kao, 1982; Rehany, 1982) and an imbalance between matrix metalloproteinase (MMP) and tissue inhibitors of matrix metalloproteinase (TIMPs) in keratoconus corneas might also contribute to its thinning.
MMP-2 was found to be over-expressed in keratoconus (smith, 2006). TIMP-1 synthesis was also up-regulated in stromal cell cultures derived from scarred KC corneas (Kenney, Chwa et al. 2005). TIMP-1 has anti-apoptotic properties and it curtails the effect of MMP-2. It has been shown that there is balance between TIMP-1 and 3 and an alteration in this relationship might promote keratocyte apoptosis in KC corneas (Matthews, 2007). Taken together, these data suggest that the corneal thinning is due to up-regulation of cellular proteases and down-regulation of their inhibitors. This could be a cause of the destruction of extracellular matrix as evidenced by altered or abnormal levels of fibronectin and type VI collagen in KC corneas (Kenney, 1997).

Oxidative Stress and Keratoconus

Recent evidence has emerged suggesting oxidative stress as a causative factor in the development and progression of KC (Behndig, Karlsson et al. 2001). KC corneas showed increased levels of inducible nitric oxide synthase (iNOS), nitrotyrosine, malonaldehyde and glutathione S-transferase (Gondhowiardjo, 1993) and decreased levels of superoxide dismutase (Behndig, Karlsson et al. 2001) and aldehyde dehydrogenase (Gondhowiardjo, 1993). The elevated enzyme levels could lead to increased production of superoxide, hydroxyl radicals and hydrogen peroxide, which collectively form reactive oxygen species (ROS). ROS have been known to damage proteins, cells and membrane phospholipids. Indeed, two-fold higher levels of catalase mRNA and its activity in KC corneas compared to normal corneas were observed (Kenney, Chwa et al. 2005). This up-regulation of catalase was due to the cathepsin that
stimulates H₂O₂ production. Further supporting evidence for oxidative damage in KC corneas was provided by studies that showed that KC fibroblasts, at low pH with H₂O₂, exhibited increased levels of reactive oxygen species/reactive nitrogen species than fibroblasts from normal corneas (Chwa, Atilano et al. 2006). KC corneas also showed relatively greater mitochondrial DNA damage (Atilano, Coskun et al. 2005), and fibroblasts showed an inherent hypersensitive response to oxidative stressors such as H₂O₂ with mitochondrial dysfunction and mitochondrial DNA damage (Chwa, Atilano et al. 2008).

Animal Models in Keratoconus

The lack of proper animal models makes it harder to pin point the cause of keratoconus disease. Tachibana et al. reported a rodent model for hereditary keratoconus, the spontaneous keratoconus mice (SKC mice) (Tachibana, 2002b). The problem in these mice was that the phenotypic expression was androgen-dependent, and it was mapped to a major histocompatibility (MHC) region (Tachibana, Adachi et al. 2002). Tachibana et al. also identified another mouse with a keratoconus phenotype. In this model, the KC phenotype was seen in both sexes and was heritable in autosomal recessive manner, and the mice were named the Japanese keratoconus mice (JKC) (Tachibana, 2002b). The keratoconus that was seen in these mice was secondary to keratitis thus making it different from the human keratoconus.
Genomic Mutation in Keratoconus

Based on twin studies and cohort studies keratoconus have a genetic component associated with it, however the effect of these genes on keratoconus disease process could not be identified reliably. It has been shown that 6% to 23% of the keratoconus patients do have family a history (Rabinowitz, 2003). Linkage analysis studies have shown association of multiple loci in extended pedigrees (3p14-q13, 5q14-q21, 15q22-q24, 1p36 and 8q24 and 13q32) and also shown in small families and sibling pairs (2p24,16q22-q23,9q34,5q32-q33 and 5q21.2 and 14q11.2) (Brancati, Valente et al. 2004; Tang, Rabinowitz et al. 2005; Hughes, Dash et al. 2003; Burdon, Coster et al. 2008; Gajecka, Radhakrishna et al. 2009; Tyynismaa, Sistonen et al. 2002; Li, Rabinowitz et al. 2006). One of the candidate genes that is best studied is VISX1 (Visual System homeobox-1 gene). Mutations have been identified in this gene in a small group of keratoconus patients (Bisceglia, Ciaschetti et al. 2005). A microarray analysis to identify differentially expressed genes in KC epithelium, showed massive reduction in cytoskeletal proteins, extracellular matrix remodeling, altered trans-membrane signaling molecules and modified cell to cell and cell-matrix interactions (Nielsen et al., 2003). Although the microarray analysis provided abundant information, again no valuable markers for the diagnosis of KC were identified. Recent studies have shown a mutation in DOCK9 (Zizimin1) in the 51 Ecuadorian KC families (Czugal, Karolak et al. 2011). DOCK9 (dedicator of cytokinesis) is a member of guanine-nucleotide exchange factor (GEF) family (Rossman, Der et al. 2005). It is involved in the activation of Rac and Cdc42, which are members of the extensively studied Rho family small GTPases (Jaffe and Hall 2005). There are 25 known Rho GTPases and they are involved in numerous biological process (Erickson and Cerione 2001) such as cell
migration (Fukui, Hashimoto et al. 2001), cell cycle progression, gene expression, innate immunity (Olson, Ashworth et al. 1995) and bacterial and viral infection (Aktories, Schmidt et al. 2000). The GEFs control the function of Rho GTPases by catalyzing the GTP/GDP exchange activity of Rho GTPases in response to appropriate signals. It has been shown that DOCK2, which is a hematopoietic cell-specific member of the GEF family was indispensible for lymphocyte migration (Fukui, Hashimoto et al. 2001). It has also been shown that Biglycan and decorin, which are small leucine-rich proteoglycans, induce morphological and cytoskeletal changes in lung fibroblasts, resulting in increased cell migration. This was due to activation of RhoA and Rac1 pathways that are involved in cell migration. Mohan et al. (2010) showed that overexpression of decorin in corneal fibroblast results in down-regulation of TGF-β and also extracellular matrix proteins such as fibronectin, collagen type I, III and IV and also plays an important role in the modulation of stomal matrix and wound healing. Our hypothesis is that downstream signal of the Rho GTPases is affected in keratoconus (Figure 3).
Figure 3: Summary of TGF-β- Mediated Pathway. Decorin has a regulatory role in the down-stream signaling of TGF-β. DOCK9 is an activator of Cdc42 which is an upstream signaling component of the actin cytoskeleton. It has shown that DOCK9 is mutated in keratoconus. The actin cytoskeleton is involved in cell migration, growth, survival and gene expression.
Overall Goal and Hypothesis

**Overall Goal**

The overall goal of this study was to identify proteins that undergo changes in the keratoconus (KC) disease process and to understand their role in development and progression of KC.

**Overall Hypothesis**

One of the important biological processes for a multi-cellular organism is cell migration, an essential element for normal development and is required throughout life in response to tissue damage. Cell migration is a multistep process involving changes in the cytoskeleton mainly mediated through cytoplasmic actin. It is known that Rho, Rac or cdc42 pathways are involved in cell migration and also in several other biological processes (Figure 3). The cell migration is mediated through the actin cytoskeleton. Recent studies have shown that Dock 9 is mutated in the keratoconus corneas and is known activator of cdc42. And also our early exploratory data revealed a link between KC and absence of β-actin, an essential cytoskeletal element in keratocytes. Our hypothesis is that thinning of the stroma is due to the disruption of the actin cytoskeleton of the keratocytes, and therefore, functional properties and stability of β-actin play an important role in the development and progression of keratoconus. Therefore, our goal was to determine if proteins of the specific
pathway are affected during keratoconus, and whether these affect the functional properties of the cells. To test this hypothesis, three Specific Aims are proposed:

Specific Aims and Rationales

Specific Aim I

Identify differential expression of proteins during KC in corneal epithelium and stroma.

Specific Aim I Rationale

Previous studies have not identified a persistent genomic mutation for KC and no keratoconus markers leading to a clinical diagnosis are known (Rabinowitz, 2003); (Fullerton, Paprocki et al. 2002). A microarray analysis suggested massive reduction in cytoskeletal proteins, extracellular matrix remodeling, altered trans-membrane signaling and modified cell to cell and cell-matrix interaction (Nielsen, 2006). In spite of vast information provided by the microarray analysis, no valuable markers for the diagnosis of KC were identified. Similarly, previous proteomic studies of human corneas examined the whole cornea (Karring, Thøgersen et al. 2005) or epithelial proteins alone (Srivastava, 2006). As valuable as these studies were they provided incomplete information regarding focused changes in both epithelium and stroma during KC.

The purpose of this study was to identify the separate differential expression of epithelial and stromal proteins in KC vs. normal human corneas. To accomplish this, the epithelial and stromal proteins from KC and age-matched normal corneas were analyzed
by two different techniques: i.e., shotgun proteomic Nano-ESI-LC-MS (MS)\(^2\) and two-dimensional-difference gel electrophoresis (2D-DIGE) coupled with mass spectrometric methods. The details of the results of this study are described in the enclosed manuscript (Experimental Eye Research; 2011, 4:282-98.)

**Specific Aim II**

Determine expression of β-actin gene at the transcriptional and translation levels in KC vs normal corneas.

**Specific Aim II Rationale**

The systems biology analysis of results of Specific Aim 1 led us to propose two potential models for KC corneas: 1) Increased iron deposition due to the disruption of the iron homeostasis led to iron deposits in KC known as Fleisher’s ring, and iron is known to increase oxidative stress. 2) Down-regulation of certain proteins has downstream effects on cytoskeletal proteins in KC progression, especially of β-actin. This project focus was the down-regulation of β-actin and its effect on cytoskeletal integrity in keratocytes.

**Specific Aim III**

Determine whether HuR gene regulates down-regulation of β-actin gene and effects keratocytes functions.
Specific Aim III Rationale

High expression levels of β-actin are important for cellular processes. It is maintained by the stability and high concentration of its mRNA. Dormoy-Raclet et al., have shown that HuR depletion in HeLa cells alters the cytoskeleton functions such as cell adhesion, migration and invasion, attributable to the loss of β-actin stress fibers (Dormoy-Raclet, Menard et al. 2007). Our aim was to determine if the stability of β-actin mRNA is regulated by the HuR gene and what are the functional consequences of down-regulation of β-actin gene.
DIFFERENTIAL EPITHELIAL AND STROMAL PROTEIN PROFILES IN KERATOCONUS AND NORMAL CORNEAS

by

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Format adapted and errata corrected for dissertation
Abstract

The purpose of the study was to identify epithelial and stromal proteins that exhibit up- or down-regulation in keratoconus (KC) vs. normal human corneas. Previous proteomic studies have been incomplete because they utilized whole human corneas or epithelium alone, thereby diluting the specificity of the proteome of each tissue especially stroma, for this reason we selectively analyzed the epithelium and stromal proteins separately. Individual preparations of epithelial and stromal proteins from KC and age-matched normal corneas were analyzed by two independent methods, i.e. a shotgun proteomic using a Nano-Electrospray Ionization Liquid Chromatography Tandem Mass Spectrometry [Nano-ESI-LC-MS (MS)]

² and two-dimensional-difference gel electrophoresis (2D-DIGE) coupled with mass spectrometric methods. The label-free Nano-ESI-LC-MS (MS)² method identified 104 epithelial and 44 stromal proteins from both normal and KC corneas, and also quantified relative changes in levels of selected proteins, in both the tissues using spectral counts in a proteomic data set. Relative to normal corneal epithelial proteins, six KC epithelial proteins (lamin-A/C, keratin type I cytoskeletal 14, tubulin beta chain, heat shock cognate 71 kDa protein, keratin type I cytoskeletal 16 and protein S100-A4) exhibited up-regulation and five proteins (transketolase, pyruvate kinase, 14-3-3 sigma isoform, phosphoglycerate kinase 1, and NADH dehydrogenase kinase (quinone) 1) showed down-regulation. A similar relative analysis showed that three KC stromal proteins (decorin, vimentin and keratocan) were up-regulated and five stromal proteins (TGF-betaig h3, serotransferrin, MAM domain-containing protein 2 and isoforms 2C2A of collagen alpha-2[VI] chain) were down-regulated. The 2D-DIGE-mass spectrometry followed by Decyder software analysis
showed that relative to normal corneas, the KC corneal epithelium exhibited up-regulation of four proteins (serum albumin, keratin 5, L-lactate dehydrogenase and annexin A8) and down-regulation of four proteins (FTH1 [Ferritin heavy chain protein 1], calpain small subunit 1, heat shock protein beta 1 and annexin A2). A similar relative analysis of stroma by this method also showed up-regulation of aldehyde dehydrogenase 3A1 (ALDH3A1), keratin 12, apolipoprotein A IV precursor, haptoglobin precursor, prolipoprotein and lipoprotein Gln in KC corneas. Together, the results suggested that the Nano-ESI-LC-MS(MS)² method was superior than the 2D-DIGE method as it identified a greater number of proteins with altered levels in KC corneas. Further, the epithelial and stromal structural proteins of KC corneas exhibited altered levels compared to normal corneas, suggesting that they are affected due to structural remodeling during KC development and progression. Additionally, because several epithelial and stromal enzymes exhibited up- or down-regulation in the KC corneas relative to normal corneas, these two layers of KC corneas were under metabolic stress to adjust their remodeling.

**KEYWORDS:** Cornea, Epithelium, Stroma, Keratoconus, Two-dimensional-difference gel electrophoresis (2D-DIGE)
INTRODUCTION

Keratoconus (KC) is a condition of unknown cause in which the cornea assumes a conical shape as a result of non-inflammatory thinning of corneal stroma. The disease progresses at a variable speed with corneal thinning inducing irregular astigmatism, myopia, and corneal protrusion. Contact lenses, and ultimately keratoplasty, might be required to restore vision. According to the National Eye Institute reports, KC is the most common corneal dystrophy in the United States, affecting 1 per 2000 Americans (US National Eye Institute, Kennedy et al., 1986). The classical histopathological features include 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, Lebers congenital amaurosis, and mitral valve collapse (Rabinowitz, 2003).

Despite intensive investigations into the pathogenesis of KC, the exact cause of the disease is poorly understood. Keratoconus apparently arises due to number of factors, which result in changes at cellular, biochemical, physiological and genetic levels. With the progression of KC, both epithelial cells and stromal keratocytes are affected. The epithelium degenerates, loses its smoothness and becomes irregular (Jongebloed and Worst, 1987). Blebbing was a constant feature of the epithelial surface and degeneration of the epithelium were also seen in the keratoconus epithelium (Pfister and Burstein, 1977). Using in vivo confocal microscopy in KC subjects, reduced keratocyte density in the diseased corneas compared to normal corneas was observed (Ku et al., 2008). KC has been associated with central epithelial thinning (Scroggs and Proia, 1992, Tsubota et al., 1995), apparently due to a decrease in epithelial cell density (Ucakhan et al., 2006) but an increase in cell area (Tsubota et al., 1995; Hollinsworth et al., 2005), especially
enlargement and irregular arrangement of basal epithelial cells (Niederer et al., 2008). The degeneration of keratocytes, keratocytes with high levels of endoplasmic reticulum and discrete incursion of fine cellular processes into Bowman’s membrane were also observed in KC corneas (Polack, 1976; Rock et al., 1995). Compared to normal corneas, KC corneas showed increased apoptosis of keratocytes (Kaldaawy et al., 2002; Kim et al., 1999), and altered nerve plexi (Niederer et al., 2008; Patel and McGhee, 2006; Simo Mannion et al., 2005). The earliest changes of KC have been described in the superficial layer of the corneal epithelium followed by involvement of the basal cell layer. In advanced stages, the cell membrane ruptures along with disappearance of the basal epithelial cells leading to only one or two layers of flattened superficial epithelial cells, lying on an altered basement membrane (Rabinowitz, 2003; Ruddle et al., 2003). As the basal epithelial cells degenerate, they might release proteolytic enzymes that destroy the underlying tissue.

The reduced corneal thickness (Teng, 1963; Patel and McLaughlin, 1999) might be caused by increased levels of several degradative enzymes such as acid esterases, acid phosphatases, and acid lipases (Critchfield et al., 1988), cathepsins B and G (Sawaguchi et al., 1989; Zhou et al., 1998) and decreased levels of protease inhibitors such as α1-protease inhibitor and α2-macroglobulin (Sawaguchi et al., 1990; Sawaguchi et al., 1994). The abnormality in corneal collagenase activity (Kao et al., 1982; Rehany et al., 1982) and an imbalance between matrix metalloproteinase (MMP) and tissue inhibitors of matrix metalloproteinase (TIMPs) in keratoconus patients might also contribute to corneal thinning. MMP-2 was found to be over-expressed in keratoconus (Smith et al., 2006). TIMP-1 synthesis was also up-regulated in stromal cell culture that was derived
from scarred KC corneas (Kenney et al., 2005). TIMP-1 has anti-apoptotic properties and it curtails the affect of MMP-2. Mathews et al. (2007) have shown that there is balance between TIMP-1 and 3 and an alteration in this relationship might promote keratocyte apoptosis in KC corneas. Taken together, it would appear that corneal thinning is due to up-regulation of cellular proteases and down regulation of their inhibitors. This could also cause the destruction of extracellular matrix as seen by altered or abnormal levels of fibronectin and type VI collagen in KC corneas (Kenney et al., 1997).

Recent evidence has emerged suggesting oxidative stress as a causative factor in the development and progression of KC (Kenney et al., 2000; Behndig et al., 2001). KC corneas showed increased levels of inducible nitric oxide synthase (iNOS), nitrotyrosine, malonaldehyde and glutathione S-transferase (Gondhowiardjo et al., 1993) and decreased level of superoxide dismutase (Behndig et al., 2001) and aldehyde dehydrogenase (Gondhowiardjo et al., 1993). The elevated enzyme levels could lead to increased production of superoxide, hydroxyl radicals and hydrogen peroxide, which collectively form reactive oxygen species (ROS). ROS have been known to damage proteins, cells and membrane phospholipids. Indeed, two-fold higher levels of catalase RNA and its activity in KC corneas compared to normal corneas were observed, suggesting that KC corneas have elevated levels of H$_2$O$_2$ (Kenney et al., 2005). Further supporting evidence for oxidative damage in KC corneas was provided by studies that showed that KC fibroblasts, at low pH with H$_2$O$_2$, exhibited increased levels of reactive oxygen species/reactive nitrogen species than fibroblasts from normal corneas (Chwa et al., 2006). KC corneas also showed relatively greater mitochondrial DNA damage (Atalino et al., 2005), and fibroblasts showed an inherent hypersensitive response to oxidative
stressors such as H$_2$O$_2$ with mitochondrial dysfunction and mitochondrial DNA damage (Chwa et al., 2008).

Studies have shown no clear identification of a genomic mutation for KC, and also no keratoconus markers corresponding to a clinical diagnosis are known (Rabinowitz, 2003; Fullerton, 2002). A microarray analysis identifying differentially expressed genes in KC epithelium that suggested massive reduction in cytoskeletal proteins, extracellular matrix remodeling, altered trans-membrane signaling and modified cell to cell and cell-matrix interaction (Nielsen et al., 2003). Although the microarray analysis provided vast information, again no valuable markers for the diagnosis of KC were identified.

Despite these extensive studies, the underlying biochemical mechanism for the development of KC remains elusive. The purpose of the present study was to identify those epithelial and stromal proteins that undergo up- or down-regulation in human keratoconus corneas compared to normal corneas. For this purpose, the epithelial and stromal proteins from keratoconus and age-matched normal corneas were analyzed by two different techniques, shotgun proteomic using Nano-ESI-LC-MS (MS)$^2$ and two-dimensional-difference gel electrophoresis (2D-DIGE)-coupled with mass spectrometric method. Several epithelial and stromal enzymes and structural proteins showed up- or down-regulation in KC corneas compared to normal corneas, which suggested that cells of both layers undergo extensive structural remodeling and remain under metabolic stress to adjust the remodeling.
MATERIALS AND METHODS

Human Corneas

The normal corneas were obtained from Alabama Eye Bank and the KC corneal buttons (8 mm in diameter) were obtained following surgery from a local corneal surgeon. Normal corneas and KC corneal buttons were stored in Optisol (Chiron Ophthalmics, Irvine, CA) and recovered within 12 h 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. Four normal corneas (ages: 30 to 55 years) and four keratoconus corneas (ages: 30 to 55 years) were used for Nano-ESI-LC-MS (MS)² analysis. Similarly, three normal human corneas (age: 30 to 55 years) and three KC corneas (age 30 to 55 years) were used for 2D-DIGE analysis. The use of human corneas in the study was approved by the Institutional Review Board of the University of Alabama at Birmingham, and was performed to the tenets of the Declaration of Helsinki for research involving human subjects.

Difference in Protein Expression in Epithelium and Stroma of KC vs. Normal Corneas as Determined by Shotgun-Nano-ESI-LC-MS(MS)² Method

Tissue Homogenization

The epithelial and stromal protein preparations were separately recovered from each KC and age-matched normal corneas, and were separately homogenized in tissue extraction (TE) buffer (6 M urea, 50 mM Tris-HCl, pH 8.0). For this purpose, the tissue samples in grinding flasks (volume 3 ml, made of polytetrafluoroethylene [PTFE]) 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). The preparations were thawed and centrifuged at 21,000x g for 20 min at 4°C to recover supernatant, which was used for further study. As stated above, the preparations from each of four normal and each of the four age-matched KC corneas were examined separately by Nano-ESI-LC-MS(MS)² method. Next the results were statistically analyzed for the identification of epithelial and stromal proteins and their up- or down-regulation.

*Enzymatic Digestion of Proteins*

In-solution protein digestion was performed by using mass spectrometry-grade trypsin (Trypsin Gold, Promega Corporation, Madison, WI). For this purpose, the epithelial and stromal protein preparations were suspended in 10 µL of a denaturant containing 6 M urea/100 mM Tris buffer, pH 8.0. Next, 0.6 µL of a 100 mM DTT/100 mM Tris reducing agent solution was added and the preparations were incubated at room temperature for 1 hr. In this preparation, 0.6 µL of a 100 mM iodoacetamide/100 mM Tris was added as an alkylating reagent, and the preparations were then incubated for 1 hr at room temperature. To capture excess iodoacetamide, 0.6 µL of a 25 mM TCEP (Tris (2-carboxyethyl) phosphine) reducing agent/100 mM Tris was added and then incubated for 1 hr. Next, 75 µL of milli-Q water and 0.5 µL of trypsin solution (40 ng/µL in 100 mM Tries) were added to each fraction and incubated for 3 hr at 37°C. An additional 0.5 µL of trypsin solution was added and the fractions incubated
overnight at 37°C. The pH of each fraction was adjusted (acidic) by adding 5 µL of formic acid. The samples were stored at 4°C until analyzed by LC-MS.

Protein Identification

LC-MS(MS)² analysis of the preparations containing tryptic peptides was performed using a ThermoFinnigan LTQ-XL ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 1.4 instrument control (ThermoFinnigan, San Jose, CA). The peptide fractions were diluted by a factor of 10 in 0.1% formic acid prior to separation on a 100 µm x 11 cm capillary tip containing C18 resin (Jupiter C18, 5µ, 300 Angstroms, Phenomenex, Torrance, CA). The flow rate during the solid extraction phase of the gradient was 3µL/min and 500 nL/min during the separation phase. The mobile phase A and mobile phase B were 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. A 95 min gradient were performed with a 15 min washing period (100% A for the first 10 min followed by a gradient of 98% A at 15 min) to allow for solid phase extraction and removal of any residual salts. After the initial washing period, a 60 min gradient was performed where the first 35 min was a slow, linear gradient from 98% A to 75% A, followed by a faster gradient to 10% A at 65 min, and an isocratic phase at 10% A for 75 min. MS/MS scans were acquired using an isolation width of 2 amu, an activation time of 30 ms, and activation Q of 0.250 and 30% normalized collision energy using 1 microscan and maximum injection time of 100 ms for each scan. The mass spectrometer was tuned prior to analysis using a synthetic peptide TpepK
(AVAGKAGAR). Typical tune parameters were as follows: spray voltage of between 1.8 kV, a capillary temperature of 150ºC, a capillary voltage of 50 V and tube lens 100 V. The MS/MS spectra of the peptides were acquired using data-dependent scanning in which one full MS spectrum, using a full mass range of 400-2000 amu was followed by three MS/MS spectra.

Database Searching

Searches for protein identifications were carried out as follows using species-specific subsets of the UniRef database: All tandem mass spectrometry data were converted to mzXML format using instrument-specific converting software packages (Institute for Systems Biology, Seattle Washington & Fred Hutchinson Cancer Center) and run through SEQUEST, X!TANDEM, and MASCOT separately. X!TANDEM was downloaded from the Global Proteome Machine Organization, while licenses were purchased for the other two search engines (ThermoFisher for SEQUEST, and Matrix Science Inc. Boston, MA, for MASCOT). All three of these top matching algorithms were utilized in order to increase confidence in protein identifications, while also decreasing the propensity for false negatives. An example of specifics for each matching program included: 1) SEQUEST, which was the only algorithm that takes in to account relative, and absolute intensity values generated from each peptide, 2) X!TANDEM, which takes into account partial digests with a focus on B and Y fragment ions, and 3) MASCOT, which takes into account the database size when calculating a unique match. The data were then “combined” and analyzed using protein Prophet (also from ISB,
above), which was capable of utilizing all of this data from each output to determine a “best fit” for a specific peptide fragmentation pattern as it related to an appropriate match from a large database with high confidence. Cut off filters for protein Prophet varied depending on a dynamically generated probability score that was determined based on each data set. In addition, this approach calculated a true positive correlation as opposed to simply a false positive, common to other approaches.

Non-Tagged Statistical Analysis of LC-MS/MS data

Following database searching, data set organization, and peptide statistical validation was performed using the PROVALT algorithm (or the peptide prophet or protein prophet algorithm) as integrated in the software package ProteoIQ (BioInquire, Athens GA). Statistical validation of peptide identifications was performed using the peptide false-discovery rate (PEP-FDR) approach by comparing the distribution of peptide identifications between the target and database search results at each Mascot Ion Score. The 50% peptide probability and 90% protein probability was used to identify unique proteins in our system. A 5% false discovery rate was applied to identify proteins. ProteoIQ is commercial software for the post-analysis of Mascot, SEQUEST, or X!Tandem database search results. The software provides the means to combine tandem mass spectrometry database search results derived from different instruments/platforms. The primary goal of many proteomics projects is to determine thresholds, which identify as many real proteins as possible while encountering a minimal number of false positive protein identifications. ProteoIQ incorporates the two
most common methods for statistical validation of large proteome datasets: the false
discovery rate and protein probability approaches (Keller et al., 2002; Nesvizhskii et al.,
2003; Weatherly et al., 2005). In ProteoIQ, protein relative quantitations was performed
via spectral counting (Old et al., 2005; Liu et al., 2004), standard deviations are
automatically calculated across replicates, and spectral count abundances are normalized
between samples (Beissbarth et al., 2004). Integrated comparison functions allow user to
quickly compare proteomic results across biological samples. Following statistical data
analysis, only those epithelial and stromal proteins that were present in three of the four
normal or three of the four KC corneas were identified and reported in Table 1.

Differences in Protein Expression in KC vs. Normal Corneas as Determined by the 2D-DIGE Method

The epithelial and stromal proteins from each KC and comparable age-matched
normal corneas were analyzed using 2D-DIGE method. Epithelial and stromal tissues
were homogenized separately in an extraction buffer containing 7M urea, 2M thiourea,
2% Pharmalyte (pH 3-10), and 4% CHAPS {3-{(3-Cholamidopropyl)
dimethylammonio]-1-propanesulfonate}. As described above, the tissues in grinding
flasks were frozen in liquid nitrogen and then homogenized by shaking using a tissue
dismembrator (Sartorius). After thawing, the protein preparations were simultaneously
reduced and alkylated at room temperature for 90 min with 5 mM tributylphosphine
(TBP) and 20 mM 4-vinyl pyridine (VP), respectively, before quenching the alkylation
with 20 mM DTT for 20 min. Cellular debris was removed by centrifugation at 21,000x g
for 20 min at 4°C, and the supernatants were collected. The preparations were exchanged
using 0.5 ml of the extraction buffer in centrifugal ultrafiltration devices (Millipore,
Billerica, MA) with a 10 kDa molecular weight cutoff. Samples were centrifuged at 12,000 x g until a retentate volume of about 50 μl was obtained, which was then diluted with an additional 500 μl with the extraction buffer. In total, the extraction buffer was exchanged three times to ensure removal of any residual TBP or VP. Protein yields were estimated using the 2D Quant Kit (GE Healthcare, Piscataway, NJ). During 2D-DIGE analysis, 40 μg epithelial and stromal proteins from normal and KC corneas were labeled with Cy3 and Cy5 cyanine dyes (GE Healthcare LifeSciences, USA) respectively. The internal standard pooled samples were labeled with Cy2 dye. In each preparation, 200 pmol of Cy dye in 1 μL of anhydrous N, N dimethylformamide per 40 μg of protein was used. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine, and further incubated for 10 min. Samples were finally combined according to the experimental design, at 40 μg of protein per Cy dye per gel, and diluted two-fold with IEF sample buffer (7M urea, 2M thiourea, 4% w/v CHAPS, 2% DTT, 2% pharmalytes [pH 3–10], 0.002% bromophenol blue). First dimension isoelectric focusing was performed on (Immobilized pH Gradient) IPG strips (24 cm; linear gradient pH 4 to 7) using an Ettan IPGphor system. The strips were incubated overnight in 450 μL of proteins dissolved in rehydration buffer (7M urea, 2M thiourea, (pH 7.5) 4% w/v CHAPS, 1% pharmalytes [pH 3–10], 0.002% bromophenol blue). The IEF focusing was done at a global voltage of 65 kV, which was followed by second dimension SDS-PAGE. During SDS-PAGE, the strips were overlaid on 12% polyacrylamide gels (24 x 20 cm; Laemmli, 1970), that were casted in low fluorescence glass plates (GE Healthcare). The electrophoresis was performed at 20ºC using an Ettan DALT six system, and at constant power of 1.0 W/gel for an hour followed by 17 W/gel until the
bromophenol blue tracking front reached at the bottom of the gel. Fluorescence images of the gels were acquired by using Typhoon 9400 scanner (GE Healthcare, Buckinghamshire, England). Images of Cy2-, Cy3- and Cy5- labeled proteins were acquired at a 100 μm resolution at excitation/emission wavelengths of 488 nm/520 nm, 532 nm/580 nm and 633 nm/670 nm, respectively. Image analysis and statistical quantification of relative protein abundances were performed using DeCyder V. 6.0 software (GE Healthcare).

Pathway Analysis

The uni-reference number and the accession numbers of the epithelial or stromal proteins with altered expression in KC corneas were converted to their corresponding gene ID’s using The Protein Information and Property Explorer (PIPE, The institute of system biology). The core analysis function of the Ingenuity Pathway Analysis (IPA), Ingenuity Systems Inc, USA, was used to analyze the molecular functions of the proteins that changed during keratoconus disease process, and also how these proteins fit into the known canonical pathway were also analyzed. The IPA was used to identify the most significant biological functions, disease and canonical pathways from the IPA knowledge database. The significance p-value determines the probability that the association between the proteins in the dataset and the canonical pathway is by chance alone was calculated by right tailed Fisher Exact test, and is expressed as –log (p-value).
Results

Identification of Epithelial Proteins that Showed Statistically Significant Up- or Down-regulation in KC Corneas Compared to Normal Corneas during Analysis

A total of 104 epithelial proteins were identified in the preparations of both normal and keratoconus corneas (Table 1, described in the Supplemental Results). The important concept of functional proteomics is to determine the relative protein abundance in the biological system. The Nano-ESI-LC-MS(MS)$^2$, a label-free protein quantification method is one of the ways to quantify protein abundance by spectral counting. Spectral counting compares the number of MS/MS spectra assigned to each protein, and is a sensitive method for detecting the proteins that undergo changes in abundance. In the present study, the relative abundance of the proteins was calculated based on the method described by Old et al. (2005). Epithelial proteins that showed a difference in up-regulation using spectral count were lamin-A/C, keratin type I cytoskeletal 14, tubulin beta chain, heat shock cognate 71 kDa protein, keratin type I cytoskeletal 16 and protein S100-A4 (Figure 1A; Table 2). These up-regulated proteins have varied functions in the corneal epithelium. The epithelial lamin-A/C that showed 2.9 fold up-regulation in KC compared to normal corneas, is an intermediate filament-type protein. Lamins interact with chromatin, and is an integral protein of the inner nuclear membrane. Among the two types of lamins (i.e., A and B), the A-type lamins are A and C and B-type lamins are B1 and B2. Lamins A and C are present in equal amounts in the nuclear lamina of mammals. Keratin 14 (a type I cytokeratin), usually exists as a heterotetramer with two keratin 5 molecules (a type II keratin), and together they form the cytoskeleton of epithelial cells. Keratin, type I cytoskeletal 16 is a heterotetramer of two type I and two type II keratins. It is proteolytically cleaved by caspases during epithelial cell apoptosis. Tubulin, a major
constituent of microtubules, is a dimer of alpha and beta chains. Heat shock cognate 71 kDa protein is a stress protein with chaperone function. Together, the above results suggest that structural remodeling of corneal epithelial cells during KC might be leading to elevated levels of heat shock cognate 71 kDa protein.

The epithelium of KC corneas showed down-regulation of several enzymes with varied functions that included transketolase, pyruvate kinase, 14-3-3 sigma isoform, phosphoglycerate kinase 1, NADH dehydrogenase kinase (quinone) 1, and NADH menadione oxidoreductase 1, a dioxin inducible isoforms 1 (Table 2, Figure 1B). The expression of transketolase (identified as a major corneal crystallin) is influenced by environmental factors and during development (Sax et al., 1996; 2000). It participates in the transfer of ketol groups and catalyzes two important reactions that operate in opposite direction. In the first reaction of the pentose phosphate pathway, D-xylulose-5-phosphate is converted sedoheptulose-7-phosphate in the presence of cofactor thiamine diphosphate. In the second reaction occurs in presence of the same cofactor and D-xylulose-5-phophate plus erythrose-4-phosphate form fructose 6-phosphate and glyceraldehyde 3-phosphate. KC corneal epithelial 14-3-3 protein that showed down regulation, plays important roles in a variety of regulatory processes, which include signal transduction, apoptosis, cell cycle progression and DNA replication. In mammalian cells, seven 14-3-3 isoforms named as β, γ, ε, η, σ, θ, and ζ have been identified, and each has distinct tissue localization and isoform-specific function. 14-3-3 Sigma isoform (also called stratifin) has been shown to control corneal epithelial cell proliferation and differentiation through the Notch- signaling pathway (Xin et al., 2010). Phosphoglycerate kinase, which also exhibited down-regulation in KC corneas, is a transferase enzyme in glycolytic
pathway. It transfers a phosphate group from 1,3-biphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. NADH-dehydrogenase was also down-regulated, which is the first enzyme complex of the mitochondrial electron transport chain and translocates 4 protons across the inner membrane per molecule of oxidized NADH to help in building electrochemical potential used to produce ATP. NAD(P)H dehydrogenase, quinone 1, acts as a quinone reductase in connection with conjugation reactions of hydroquinons involved in detoxification pathways as well as in biosynthetic processes such as the vitamin K-dependent gamma-carboxylation of glutamate residues in prothrombin synthesis. In summary, the altered expression of the above enzymes suggests that the epithelium of KC corneas is under metabolic stress during structural remodeling compared to normal corneas.

Identification of Stromal Proteins that Showed Statistically Significant Up- or Down-regulation in KC Corneas Compared to Normal Corneas during Analysis

A total 44 stromal proteins were identified in the normal and KC corneas by Nano-ESI-LC MS(MS)² method (Table 3, described in the Supplemental Results). The fold change differences in the stromal proteins were also calculated according to Old et al. method (2005). The relative abundance of individual proteins was calculated from the spectral counts, in which we counted how many times unlabeled version of a protein was identified by the fragmentation spectra of its peptide. The spectral count correlates with protein abundance (Liu et al., 2004). The normalization and the standard deviation was calculated from the total spectral counts. Based on the relative spectral counts of stromal proteins of KC vs. normal corneas, three structural proteins (i.e., decorin, vimentin and keratocan) showed up-regulation and five proteins (i.e., TGF-betaig h3, serotransferrin,
transferring, MAM domain-containing protein 2 and isoforms 2C2A of collagen alpha-2(VI) chain) exhibited down-regulation (Table 4, and Figure 2). These results further suggested a structural remodeling of stroma in KC compared to normal corneas. The stromal keratocytes produce a unique profile of extracellular matrix proteoglycans in the corneal stroma such as lumican, decorin, mimecan and keratocan, which play important roles in maintaining corneal transparency (Funderburgh et al., 1996). Decorin, a dermatan sulfate proteoglycan, is important in collagen fibrillogenesis and binds to multiple collagen types, which include types I, II, III and VI (Goldoni, 2005). Decorin has been shown to be involved in the lamellar adhesion properties of collagen and in the control of the regular fibril–fibril spacing found in the cornea (Michelacci, 2003). Decorin transcripts have also been shown to be abundant in the KC cornea (Rabinowitz et al., 2005). Furthermore, a mutation in the decorin gene causes a congenital stromal dystrophy of the cornea (Bredup et al., 2005). As stated above, vimentin belongs to an intermediate filament (IF) protein family, and the IF proteins along with microtubules and actin microfilaments make up the cytoskeleton. Keratocan, a member of the small leucine-rich proteoglycan protein family, on mutation causes thinning. The above result is in concordance with the earlier results that showed keratocan expression is increased in the stroma of KC corneas (Wentz-Hunter et al., 2001). This report suggested that over expression of keratocan might alter the stromal fibrillogenesis and lead to structural defects and the development of KC.

The down-regulated stromal proteins in the KC corneas included serotransferrin, MAM domain-containing protein 2, Isoforms 2C2A of collagen alpha-2(VI) chain and TGF β-induced protein ig-h3 (BiH3) [Table 4 and Figure 2B]. An iron deposit in
epithelial basement membrane is a prominent histopathological feature of KC corneas compared to normal corneas (Lawless et al. 1989). Iron transport is mediated through serotransferrin. Transferrin (an 80 kDa blood plasma glycoprotein), is involved in iron transport via a cell surface transferrin receptor. It binds to two Fe$^{3+}$ ions in association with the binding of an anion, usually bicarbonate. The transferrin receptor levels in the corneal epithelium is influenced by TGF-β1 (Hayashida-Hibino et al., 2001), but whether similar receptors exists on keratocyte surface is presently not known. The MAM (meprin/A5-protein/PTPmu) domain is present in numerous proteins with diverse functions. Protein-tyrosine phosphatases (PTPμ) belongs to the MAM-containing subclass of proteins that promotes cell-to-cell adhesion but their stromal function is presently unknown. Isoform 2C2A of collagen alpha-2(VI) chain belongs to collagen VI family, which acts as a cell-binding protein. The corneal stroma consists of approximately 200 layers of type I collagen fibrils (Dexer et al., 1998), and also some type XII collagen (Wessel et al., 1997). However, the potential role of isoform 2C2A of collagen alpha-2(VI) chain is presently unknown. Down-regulation of βig-h3 gene is linked to tumorigenic phenotype in asbestos-treated immortalized human bronchial epithelial cells (Zhao et al., 2002). An atypical phenotype of Reis-Bücklers corneal dystrophy (opacities in the anterior to mid stroma of the cornea) has been shown to be caused by the G623D mutation in Bigh3 (Li et al., 2008).

Identification of Differentially Expressed Epithelial and Stromal Proteins of KC Corneas Compared to Normal Corneas using 2D–DIGE Method

Protein preparations from each of the three keratoconus corneas (from donors of 30-50 years) and each of the three age-matched normal corneas were individually
analyzed by the 2D–DIGE method. Figure 3 shows an overlay of Cy2/Cy3/Cy5-labeled corneal epithelial proteins and Figure 4 shows a typical gel the Coomassie-blue-stained corneal epithelial proteins. Following image analysis and statistical quantification of relative protein abundances in spots of epithelial and stromal profiles by DeCyder V. 6.0 software, nine spots showed differences in their relative abundance in KC vs. normal corneas. These spots were excised, trypsin digested and their tryptic fragments analyzed by mass spectrometric method using ABI-Sciex 4000 Q-TRAP system. The nine epithelial proteins that showed differential expression in the KC vs. normal corneas are shown in Table 5. Relative to normal corneas, the KC corneas exhibited up-regulation of serum albumin, keratin 5, L-lactate dehydrogenase and annexin A8. Epithelial keratin 5 (also known as keratin, type II cytoskeletal 5) consists of basic or neutral proteins and is arranged in pairs in heterotypic keratin chains. On deletion of keratin 5 in keratin 5-knockout mice, the mice form a cytoskeleton with keratin 12, and did not have keratin aggregation or cytolysis in the cornea (Lu et al., 2006). As stated above, K12/K3 keratin pair of intermediate filaments is essential for corneal epithelial integrity, and therefore the significance of up-regulation of keratin 5 might be to compensate the down-regulation of keratin 3 in the KC corneas.

Ferritin heavy chain (FTH) protein was down-regulated in KC epithelium. Ferritin heavy chain subunit is present in both avian and human corneal epithelium (Cai et al., 1997; Karring et al., 2005). Cytoplasmic mammalian ferritin complex are heteropolymers composed of two types of subunits, H and L, assembled in different ratios to form a 24-mer supramolecular complex capable of sequestering 4500 atoms of iron (Treffry and Harrison. 1980; Dickey et al., 1987). Free iron has been shown to catalyze the formation
of UV-induced oxygen reactive species via the Fenton reaction (Stohs and Bagchi. 1995). Thus, the concentration of intracellular free iron is tightly regulated and kept at a low level, chiefly by the iron-sequestering action of the cytoplasmic ferritin in cells. Annexin 2, which was down-regulated in the KC epithelium, belongs to a family of highly conserved proteins derived from 12 annexins genes in humans (Hayes and Moss, 2004).

Annexins are made up of a highly \( \alpha \)-helical core domain that binds calcium ions, allowing them to interact with phospholipid membranes. Annexin is involved in the v-Src trafficking to the plasma membrane, which is essential for the actin dynamics and the remodeling of the focal adhesion complex (Hayes and Moss, 2009). Annexin A8 has been shown to be up-regulated in the KC epithelium and is less abundant and least described protein among the annexin family. Lactate dehydrogenase (LDH) that showed up-regulation in the KC epithelium is an enzyme that catalyzes the interconversion of pyruvate to lactate and is a major check point enzyme of anaerobic glycolysis. Heat shock protein beta1 (HSPB1) that showed down regulation in the KC epithelium, is a member of small heat shock protein (sHSP) family. HSPB1 is involved in a number of functions, which include both anti-apoptotic function and interactions with various components of cytoskeleton (Bruey et al., 2000; Charette et al., 2000; Perng et al., 1999).

Stromal proteins of KC corneas that showed up-regulation were aldehyde dehydrogenase 3A1 (ALDH3A1), keratin 12, apolipoprotein A IV precursor, haptoglobin precursor, prolipoprotein and lipoprotein Gln (Table 6).

Immunohistochemical analysis has identified ALDH3A1 in the stroma of the normal corneas (Pappa et al., 2003), and it catalyzes the NAD (P)**-dependent oxidation of a wide range of endogenous and exogenous aldehydes (Vasiliou et al., 2004). Blood plasma
proteins are present in the cornea (Karring et al., 2006), and they are either synthesized in
the cornea, mainly epithelium or originate from blood and enter the cornea with the bulk
flow from the ciliary arteries located in the corneoscleral limbus area. The functions of
blood plasma proteins in the avascular cornea are similar to that of the plasma proteins.
Those that are synthesized by the cornea include α-1-antichymotrypsin (Twining et al.,
1994), α-1-antitrypsin (Twining et al., 1994b) and apolipoprotein J (Nishida et al., 1996),
and those that originate from blood include albumin, haptoglobin, hemopexin, amyloid P-
component, transferrin, and transthyretin (Karring et al., 2006). Haptoglobin binds to
free hemoglobin in plasma after hemolysis and transport it to liver for degradation
whereas transferrin binds and delivers iron to cells. Haptoglobin precursor showed
changes in the keratoconus stroma when compared to normal stroma (Table 6) along with
other plasma proteins such as apolipoproteinA-IV precursor, proapolipoprotein and
lipoprotein Gln I. Apolipoprotein A-IV are needed for lipoprotein assembly. In summary,
it is known that plasma proteins are present in the avascular cornea but the reason for
changes in their levels in the KC stroma is unknown.

*Ingenuity Pathway Analysis for Epithelial Proteins and Stromal Proteins that showed
Expression changes in KC Corneas.*

The proteins that showed difference in both the shotgun proteomic analysis and
2D-DIGE were used to obtain a pathway using the IPA’s knowledge database and were
ranked by scores (See Material and Methods). A score of 3 or higher has a 99.9%
confidence level of not being generated by chance alone. The ingenuity pathway analysis
is more straightforward to interpret, but is limited to those biological functions and
processes that are represented in the knowledge database. Figure 7 in Supplemental
Results section shows nodes (denotes proteins to which other proteins interact) in the interaction network that are encoded by epithelial proteins that showed expression differences in KC vs. normal corneas by both the methods. Interaction between nodes represents protein-protein interaction. The gene symbols are indicated in the network and those that are down-regulated are shown in red and those up-regulated by blue (Figure 7, Supplemental Result). Of the 19 epithelial proteins that showed expression changes in KC by both methods, 16 of these had either direct or indirect interactions, and are included in the pathway as shown in Figure 7. Further, a total of 30 genes were involved in the pathway and of these the most notable gene from the pathway was NF-κB, which is activated by oxidative stress.

Similar analyses by Ingenuity software for stromal proteins were done and are shown in Figure 8 in Supplemental Results. Of the 13 stromal proteins that showed altered expression in KC by both shotgun proteomic analysis and 2D-DIGE analysis, only 4 showed either direct or indirect relationship with 32 other network eligible genes (Figure 8, Supplemental Result). The gene symbols are indicated in the network and those proteins that were down-regulated are shown in red and those up-regulated in blue. Of the pathway analyses in the stroma, the most notable is the direct relationship between latent TGF-β, fibronectin, Bigh-3, and decorin.

Discussion:

The purpose of the study was to determine the changes of epithelial and stromal proteins in KC vs. normal corneas using two different techniques, the shotgun proteomics
[Nano-ESI-LC-MS (MS)]\(^2\) and 2D-DIGE methods. The shotgun proteomics is a technique in which the proteins in a biological sample mixture are proteolytically-digested prior to their separation, and analyzed using Nano-ESI-LC–MS(MS)\(^2\). In this method, the MS/MS spectra obtained were searched against the three known protein database to determine the identity of peptides in the samples. Following database searching, data set organization, and peptide statistical validation were performed using the PROVALT algorithm (or the peptide prophet or protein prophet algorithm) as integrated in the software package, ProteoIQ (BioInquire, Athens GA). In contrast, in the second 2D-DIGE analysis, the 2D-gel electrophoretically-separated Cy2-, Cy3- and Cy5-labeled proteins were analyzed using Decyder software, and desired excised protein spots trypsin digested, and peptides analyzed by Q-TRAP mass spectrometric method, and their identity determined by using Mascot searches. Both methods not only identified epithelial and stromal proteins in the age-matched normal and KC corneas but also provided statistically significant differences in the expression levels of proteins of interest. Because both epithelial and stromal protein preparations from individual age-matched KC and normal corneas were analyzed, the results provided the opportunity to identify and report only those proteins that were commonly present in the three of four KC corneas used and in the three normal corneas. Additionally, the analysis of data obtained from individual corneas following analysis by the two methods, enabled us to report only statistically significant differences in the expression levels of certain proteins in KC vs. normal corneas.

The number of corneal epithelial and stromal proteins identified in the two groups of corneas by the shotgun proteomic technique was greater compared to the 2D-DIGE
method. However, contrary to our expectation, these two techniques identified different sets of epithelial and stromal proteins without any overlap that showed up- or down-regulation in KC vs. normal corneas. Nevertheless, the proteins that changed expression levels in KC vs. normal corneas provided a catalog of altered proteins, which might prove significant in future to understand biochemical mechanisms of KC development and progression. One major finding of the study was that the shotgun proteomic technique identified a greater number of epithelial and stromal proteins with altered expression in KC vs. normal corneas compared to the 2D-DIGE method. This could be due to a greater sensitivity and superior analytical capability of Nano-ESI-LC–MS(MS)² analysis compared to 2D-DIGE analysis. The 2D-DIGE method gives more accurate and reliable quantification information of protein abundance, since the Cy Dye-labeled proteins to be compared are run on the same gel and thus eliminating the gel to gel variation. However the spots on a given 2D-gel often contain more than one protein, making quantification difficult to determine which proteins has changed. Also 2D approach has other limitation such as limited dynamic range, difficulty in handling hydrophobic proteins, and difficulty detecting proteins with extreme molecular weight and PI values. In contrast, the non gel based shotgun proteomics provides a tool for studying large-scale protein expression and characterization in complex biological system (Domon and Aebersold, 2006).

Previous proteomic studies of human corneas were focused on the whole cornea (Karring et al., 2005) or on epithelium alone (Srivastava et al., 2006). In contrast, our study distinctly identified 104 epithelial and 44 stromal proteins from normal and KC corneas by two different techniques that were not used previously. The epithelial proteins identified by the shotgun proteomics showed a varied cellular structural and functional
properties (i.e., metabolism, structure, protein-folding and degradation, redox, immunodefence, protein-binding, calcium ion-binding, and transcription and translation; Table I, which are described in the Supplemental Results section. Similarly, the stromal proteins that were identified by the above two methods are also listed in Table 3 with description of their structural and functional properties in the Supplemental Results section.

The results of the shotgun proteomics showed up-regulation of mostly epithelial structural and cellular metabolism-related enzymes. The up-regulated structural proteins included lamin-A/C variants, keratin type I cytoskeletal 14, keratin type I cytoskeletal 16, tubulin beta chain, heat shock 71 kDa protein, and protein S100-A4. Similarly, the 2D-DIGE analysis also identified mainly up-regulated epithelial structural proteins that included keratin 5, actin-related protein 3, L-lactate dehydrogenase, and annexin A8. Together, the results suggested epithelial remodeling and stress-related expression of enzymes in KC corneas compared to normal corneas. The up-regulation of the structural proteins suggests their greater synthesis possibly to counteract thinning of KC corneas due to degradative enzymes that resulted in reduced number of epithelial cells.

The shotgun proteomic analysis also showed down-regulation of several epithelial proteins in KC corneas compared to normal corneas that included several enzymes (i.e., transketolase and its variant, pyruvate kinase, 14-3-3 sigma isoforms, phosphoglycerate kinase 1, NADPH dehydrogenase kinase (quinine) 1, and NADPH menadioneoxidoreductase 1, and dioxin inducible isoforms 1). The results suggested that several important metabolic pathways in epithelial cells were affected in the KC corneas causing distressed metabolic activity as represented by reduced enzymatic levels. This
was also supported by the 2D-DIGE analysis, which showed down-regulation of epithelial structural proteins (keratin 3, annexin A2), a stress protein (heat shock beta 1), and visin-like peptide 1 in KC corneas compared to normal corneas. Together, the results again suggest that the corneal epithelium undergo remodeling and under metabolic stress during KC development compared to normal corneas.

The shotgun proteomics and 2D-DIGE methods also identified several stromal structural proteins that exhibited up- or down-regulation. Shotgun proteomics showed that relative to normal corneas, the KC corneas showed up-regulation of decorin, vimentin and keratocan, and down-regulation of TGF-beta ig h3, serotransferrin, MAM domain-containing protein 2 and isoforms 2C2A of collagen alpha-2(VI) chain. Similar analysis by 2D-DIGE of stromal proteins identified only the up-regulated proteins that included aldehyde dehydrogenase 3A1, keratin 12, apolipoprotein A IV precursor, haptoglobin precursor, prolipoprotein and lipoprotein Gln. The significance of differences in the expression levels of individual stromal proteins is presently unclear but points to major changes in levels of structural proteins as observed above in epithelial cells.

Corneal thinning of epithelium and stroma in KC corneas believed to lead to a lower intraocular pressure (IOP) (Teng, 1963; Patel and McLaughlin, 1999). As stated above, the thinning seems to be due to increased levels of degradative enzymes (acid esterases, acid phosphatases, and acid lipases [Critchfield et al., 1988], and cathepsins B and G [Sawaguchi et al., 1989; Zhou et al., 1998]), and decreased levels of protease inhibitors (α1-protease inhibitor and α2-macroglobulin [Sawaguchi et al., 1990; Sawaguchi et al., 1994]). Strangely, our study did not identify changes in expression
levels of either degradative enzymes or protease inhibitors, but it might be indicated by
the suppressed metabolic activity of enzymes as observed in KC corneas. A stress-related
heat shock 71 kDa protein exhibited up-regulation in epithelium, which is supported by a
previous report that showed altered expression of several wound healing or stress-related
proteins (vimentin, tenascin, transforming growth factor-beta, interleukin-1, heat shock
protein 27, and ubiquitin 9 ) in KC corneas (Srivastava et al.,2006).

Nuclear lamins are intermediate filaments that are involved in maintenance of
shape and stability of mammalian cells. A down-regulation of lamin A/C in HeLa cells
has been shown to result in down-regulation of cytoskeletal proteins, mainly keratin 8
and keratin 18 (Chen et al., 2009). Our results also showed that mainly keratin 14 and 16
were up-regulated in KC corneas. Heat shock proteins (HSPs) are widely expressed
among living organisms and serve to protect cells from physiological stress by stabilizing
proteins during their synthesis, assembly into complexes, transport, and function
(Hartl,1996). The heat shock proteins bind to denatured proteins, thereby prevent their
aggregation and consequent toxicity. In keratoconus epithelium heat shock cognate 71
protein was up-regulated, which again points to stress on epithelial cells. It is well
established that for ensuring cell survival, the molecular chaperones (the heat shock
family of stress proteins) are highly up-regulated providing protein-folding machinery to
repair or degrade the misfolded proteins.

In KC epithelium, the σ protein of 14-3-3 family of proteins was down-regulated.
As stated above, the 14-3-3 family of proteins is involved in diverse functions such as
apoptosis, cell-cycle checkpoints and signal transduction pathways. NAD(P)H:quinone
oxidoreductase 1 (NQO1) was also down-regulated in KC epithelium as seen during
Nano-ESI- MS/MS analysis. The 2D-DIGE analysis showed that ferritin (FTH1) was down regulated in the KC epithelium. Nuclear factor (erythroid-derived 2)-like 2, also known as NRF2, controls the expression of ferritin, which blocks the formation of free radicals via the Fenton reaction (Halliwell and Gutteridge, 2007). It also controls the expression of NAD (P)H:quinone oxidoreductase 1 (NQO1), which inhibits the formation of free radicals by the redox-cycling of quinones (Nioi and Hayes, 2004). Iron deposits are seen in the KC corneas (known as Fleisher’s ring), and it can cause oxidative tissue damage by catalyzing Haber-Weiss and Fenton reactions that convert hydrogen peroxide to free radicals. Ferritin sequesters iron and the down-regulation of ferritin could result in the accumulation of iron as seen in the KC corneas. However, the pathogenesis of iron deposits remains unclear in KC corneas. The tear-pool hypothesis put forward by Gass remains a question (Gass, 1964) because the iron diffusing through epithelial layers and also due to presence of high affinity lactoferrin in the tear makes it to doubt the validity of this hypothesis. Rose and Lavin proposed an alternative hypothesis in which rapidly dividing, non-migrating and relatively mature basal cells are responsible for the accumulation of iron (Rose and Lavin, 1987). Hiratsuka et al. observed Fleisher’s ring in a non-keratoconus patients and their explanation to the iron deposits was due to disturbance in the corneal epithelium that resulted in the accumulation of iron and their alternate theory suggest that changes in the iron metabolism in corneal epithelium results in the iron deposition (Hiratsuka et al., 2000). Transketolase was found to be down-regulated in the KC epithelium, and the silencing of this enzyme significantly inhibited the proliferation, growth, and migratory abilities of cultured hippocampal progenitor cells, without affecting neuritogenesis of cultured hippocampal neurons (Zhao et al., 2009).
Keratin 3, a marker for corneal epithelial cells, was down-regulated in KC epithelium as observed during 2D-DIGE analysis. Our results showed multiple spots of keratin 3 protein during 2-D gel electrophoresis (result not shown), and this was in accord with the earlier published reports (Srivastava et al., 2006; Nielsen et al., 2006). This might be the reason why the direct tissue analysis using Nano LCMS/MS gave different results from the 2D-DIGE.

Shotgun proteomic analysis of stromal proteins showed up-regulation of mostly structural proteins that included decorin, vimentin, keratocan, and down-regulation of TGF β1g h3 protein, serotransferrin, MAM-domain containing protein 2 collagen type II. A similar analysis of 2D-DIGE method identified several up-regulated stromal proteins that included keratin 12, apolipoprotein A, haptoglobin and precursor proapolipoprotein lipoprotein Gln. We believe that the presence of keratins in the stromal samples represent a miniscule contaminant from surface epithelium. Furthermore, electron microscopic studies have shown breaks in Bowman’s membrane into which epithelium can sequester in KC corneas, which are not easily removed by scraping. In summary, the above results showed changes in the expression levels of the structural proteins, which point to remodeling of both epithelium and stroma in KC corneas.

Keratocytes in the stroma are responsible for the formation and maintenance of the collagenous matrix in cornea that is essential for its transparency and curvature. Decorin, responsible for collagen fibrillogenesis, is up-regulated in the stroma of KC corneas. Earlier results have shown that decorin and biglycan to be up-regulated in various pathological corneas that included KC corneas (Funderburgh et al., 1998). Decorin’s regulation of collagen fibrillogenesis is assumed to be facilitated through
binding of type I collagen molecules to the inner leucine-rich region of decorin core proteins (Orgel et al., 2006). Decorin also participates in many important intracellular and extracellular signaling processes, including ligation of the epidermal growth factor receptor, which up-regulates cyclin-dependent kinase inhibitor p21 (De Luca et al., 1996), and results in cell cycle arrest at the G1 phase (Santra et al., 1995). In addition, decorin has also been shown to bind and inhibit all three mammalian isoforms of TGF-β, even when bound to collagen (Markmann et al., 2000). As with collagen, this binding takes place via the protein core and not the GAG chains of decorin (Miura et al., 2006).

Conversely, the degradation of decorin by matrix metalloproteases, such as during tissue repair processes, releases the bound TGF-β (Al Haj Zen et al., 2003). Thus, decorin has multiple mechanisms for the inhibition of cell proliferation either through binding to TGF-β or to the epidermal growth factor receptor.

TGF-β inducible gene βig-h3 is an extracellular matrix component. βig-h3 was down-regulated in the stroma of KC corneas. It has been shown that purified βig-h3 binds to fibronectin and type 1 collagen (Billings et al., 2002). Mutations in the coding region of βig-h3 have been seen in the patients with lateral corneal dystrophies (Munier et al., 1997). It serves as a bifunctional linker protein supporting cell attachment and cell spreading (Billings et al., 2002). How a reduced levels of βig-h3 expression affects KC cornea is presently unknown.

Aldehyde dehydrogenase class 3 (ALDH3A1), that has a protective effects on cells during environmental stressors, was up-regulated in KC corneal stroma as identified by 2D-DIGE method. The cornea is constantly exposed to environmental stress, mainly in the form of UV radiation. The mouse corneal epithelium shows high level of
expression of ALDH3A1 (Kays et al., 1997), while light exposure affects the expression of ALDH3A1. Pappa et al. has shown that the human epithelium and stroma expresses ALDH3A1 whereas it is not expressed in the endothelium (Pappa et al., 2003). The significance of up-regulation of ALDH3A1 in the KC stroma is presently unclear.

The expression of keratocan and vimentin were up-regulated in the stroma of KC corneas (Table 4), which is consistent with a previous report showing increased keratocan expression in the stroma during KC (Wentz-Hunter et al., 2001). TGF-β induced the down-regulation of lumican and keratocan in rabbit keratocytes, is a Rho-mediated regulation (Chen et al., 2009). Because keratocyte density has been shown to decrease in KC corneas, the speculation is that it might be mediated through TGF-β signaling. Our results (Table 2) are consistent with previous studies that showed a 1.9 fold up-regulation in Ca\(^{2+}\)-binding S100A4 protein in the epithelium of KC corneas vs. normal corneas (Nielsen et al. 2006). Decreased expression of stromal serotransferrin and ferritin in the epithelium of KC corneas, compared to normal’s, was observed. Ferritin, an iron storage protein, protects DNA from oxidative damage by UV light and hydrogen peroxide. Reduction in the expression of ferritin in KC might therefore decrease this protective effect; hence increase oxidative damage in the KC cornea (Chwa et al., 2008). Transferrin, a plasma protein, is synthesized outside the cornea; it must enter corneas via a transport mechanism. This is in concordance with the corneal gene expression library data (Karring et al., 2006). Our results of RNA expression analysis by the RT-PCR method for transferrin further confirms lack of expression of transferrin in the stroma of both normal and KC corneas as was previously reported by Karring et al. (2006). However, our RNA analysis for transferrin receptors showed its presence in the
epithelium of normal corneas but not in the KC corneas. This might explain the presence of Fleisher’s ring (caused by iron deposits) in the epithelial basement membrane of KC corneas. Together, the results suggest that iron homeostasis is disrupted in the keratoconus disease, possibly resulting in increased oxidative damage.

From the pathway analyses using Ingenuity software, we were able to generate two models, one for epithelial and other for stromal proteins that showed altered expression in KC vs. normal corneas. In the epithelial model (Figure 7, in Supplemental Results), iron homeostasis disruption in the KC corneas leads to an increased oxidative damage. The oxidative damage in KC corneas has already been described in literature, but the reason why the KC corneas are prone to increased oxidative stress is presently not fully understood. Our analysis suggests that the disruption of iron homeostasis could trigger an increased oxidative stress in keratoconus corneas by activators of NF-κB due to oxidative stress (Stancovski and Baltimore., 1997). The system biology analysis of the proteins that showed altered expression in epithelium suggests that there is an indirect relationship between these proteins and NF-κB in the epithelium. Model # 2 is for stromal proteins that showed altered expression (Figure 8, Supplemental Results). Our results have also shown that during the KC disease, decorin is upregulated and βig-h3 is downregulated. The systems biology approach of the stromal proteins suggest a direct relationship between the decorin and extracellular matrix proteins. Mohan et al. had shown that overexpression of decorin results in down regulation of TGF-β and also extracellular matrix proteins such as fibronectin (Mohan et al., 2010). TGF-β inducible gene βig-h3 is an extracellular matrix component. It is known that decorin sequesters TGF-β, which is a cytokine involved in the cellular proliferation. Therefore, in the second
model, the upregulation of decorin, could result in changes in the expression of proteins that are seen during keratoconus. Further experiments are planned to determine whether a potential relationship exist between upregulation of decorin and TGF-β- inducible gene βig-h3 (An extracellular matrix component) in keratoconus corneas. The system biology analysis of the stromal proteins showed that there is an indirect relationship to p53 tumor suppressor protein, which is a critical mediator of cell cycle arrest and apoptosis. The nuclear transcription factor p53 is known to be activated by cellular stress.

The keratoconic corneas that are used in this study does not represent an end stage disease but they represent part of a continuum of disease process in which no other therapy is suitable, hence corneal transplantation. It is believed that rigid gas permeable (RGP) contact wear significantly influences the epithelial proteins but in our study these patients had not worn contact lenses for an extended period prior to surgery, and some never wore a contact lens. None of these patients were wearing contact lenses less than 3 months from the time of surgery and some never wore contact lenses ever. There were no differences between patients who had worn contact lenses and those that never wore contact lenses.

In summary, we separately analyzed epithelial and stromal proteins from individual KC and age-matched normal corneas by two unique techniques, the shotgun proteomics and 2D-DIGE methods. The major changes were seen in the structural proteins of both epithelium and stroma of KC corneas compared to normal corneas, suggesting structural remodeling of both the tissues during the development and progression of keratoconus. The proteins that are involved in proliferation, growth and migration were down-regulated in KC epithelium. Further, our results also showed that
the iron homeostasis is disrupted in KC corneas, which might result in an increased oxidative damage. The specific roles of certain proteins identified in this study are under investigation to determine how they affect the development and progression of human KC.

ACKNOWLEDGEMENTS

The authors thank Dr. Jim Mobley, director of the UAB Bioanalytical & Mass Spectrometry Shared Facility for the help and guidance during the analysis of samples by Nano-ESI-LC-MS (MS)\textsuperscript{2} method. Authors Dr. Om Srivastava and Roy Joseph also thank Dr. R. Pfister M.D. for providing the keratoconus corneal tissues, and to the Alabama Eye Bank for providing normal corneas for the study. The authors are also thankful to Ingenuity Systems Inc for providing a trial license to access the IPA software.
References


US National Eye Institute, Facts about the cornea and corneal disease keratoconus.


Xin, Y., Lu, Q., Li, Q., 2010.14-3-3 sigma controls corneal epithelial cell proliferation and differentiation through the Notch signaling pathway. Biochem Biophys Res Commun. 392, 593-598.

Zhao, Y.L., Piao, C.Q., Hei, T.K., 2002. Downregulation of Betaig-h3 gene is causally linked to tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells, Oncogene. 21, 7471-7477.


Figure 1: Relative abundance of epithelial proteins in KC vs. normal corneas as determined by Nano-ESI-LC-MS-MS method. (A) Epithelial proteins that showed up-regulation in KC corneas compared to normal corneas. (B) Epithelial proteins that showed down-regulation in KC corneas compared to normal corneas.
Figure 2: Relative abundance of stromal proteins in KC vs. normal corneas as determined by Nano-ESI-LC-MS (MS)² method. (A) Stromal proteins that showed up-regulation in KC corneas compared to normal corneas. (B) Stromal proteins that showed down-regulation in KC corneas compared to normal corneas.

Figure 3: Overlay of Cy2/Cy3/Cy5-labeled corneal epithelial proteins during 2D-DIGE analysis. During 2D-DIGE analysis, 40 μg epithelial proteins from normal and KC corneas were labeled with Cy3 and Cy5 cyanine dyes, respectively. The internal standard pooled samples were labeled with Cy2 dye. In each preparation, 200 pmol of Cy dye in 1 μL of anhydrous N, N dimethylformamide per 40 μg of protein was used. The white fluorescent spots: common epithelial proteins present in normal and KC corneas, green fluorescent spots—epithelial proteins present only in the normal corneas, and red fluorescent spots—epithelial proteins present only in KC corneas.

Figure 4: (A) Identification of Epithelial Proteins that showed Up- or Down-Regulation during 2D-DIGE Analysis. Following image analysis and statistical quantification of relative protein abundances in spots of epithelial protein profiles by DeCyder V. 6.0 software, nine epithelial proteins showed a difference in abundance. Each spot is identified by a master no. in Table 5 and also in this figure. (B) The intensity difference of the epithelial spot no. 503 in the normal vs. KC corneas.
Figure 5: Overlay of Cy2/Cy3/Cy5-labeled Corneal Stromal Proteins during 2D-DIGE Analysis. During 2D-DIGE analysis, 40 µg stromal proteins from normal and KC corneas were labeled with Cy3 and Cy5 cyanine dyes, respectively. The internal standard pooled samples were labeled with Cy2 dye. In each preparation, 200 pmol of Cy dye in 1 µL of anhydrous N, N dimethylformamide per 40 µg of protein was used. The white fluorescent spots: Common stromal proteins present in both normal and KC corneas, green fluorescent spots-stromal proteins present only in the normal corneas, and red fluorescent spots-stromal proteins present only in the KC corneas.
Figure 6: (A) Identification of Stromal Proteins that showed Up- or Down-Regulation during 2D-DIGE Analysis. Following image analysis and statistical quantification of relative protein abundances in spots of epithelial protein profiles by DeCyder V. 6.0 software, nine epithelial proteins showed a difference in abundance. Each spot is identified by a master no. in Table 6 and also in this figure. (B) The intensity difference of the epithelial spot no. 459 in the normal vs. KC corneas.

Table 1: Identification of Human Corneal Epithelial Proteins following Nano-ESI-LC-MS(MS)

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Tubulin beta chain  

**Blood/ Plasma**

Apolipoprotein D  
Serum albumin  

**Folding and Degradation**

Heat shock 70kDa protein 1A variant (Fragment)  
Heat shock 70 kDa protein 1  
Heat shock cognate 71 kDa protein  
Heat shock protein beta-1  
Heat shock protein HSP 90-beta  
Peptidylprolyl cis-trans isomerase A-like 4  
Peptidyl-prolyl cis-trans isomerase A  
U4/U6.U5 tri-snRNP-associated protein 2  

**Redox**

6-phosphogluconate dehydrogenase, decarboxylating  
Cytochrome c oxidase subunit VIb isoform 1  
Glutathione S-transferase P  
NAD(P)H dehydrogenase [quinone] 1  
Peroxiredoxin-1  
Peroxiredoxin-2  
Peroxiredoxin-6  
Retinal dehydrogenase 1  
Thioredoxin  

**Immunodefence**

Macrophage migration inhibitory factor  

**Protein Binding**

14-3-3 protein beta/alpha  
14-3-3 protein gamma  
14-3-3 protein sigma  
14-3-3 protein zeta/delta  
Annexin A1  
Annexin A2  
Annexin A5  
ATP synthase subunit beta, mitochondrial  
Condensin-2 complex subunit D3  
Cystatin-8  
Cystatin-B  
Echinoderm microtubule-associated protein-like 2  
Probable histone-lysine N-methyltransferase ASH1L
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**Calcium Ion Binding**

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**Transcription/Translational proteins**

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Table 2. Protein that Showed Difference in Keratoconus Epithelium when Compared to Normal Corneal Epithelium by Nano-ESI-LC-MS(MS)² Method.

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Table 3: Identification of Human Corneal Stromal Proteins following Nano-ESI-LC-MS(MS)\(^2\) Analysis.

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<td>Angiopoietin-related protein 7</td>
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Table 4. Protein that Showed Difference in Keratoconus Stroma when Compared to Normal Corneal Stroma following Nano-ESI-LC-MS(MS)² Analysis.

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<tr>
<th>Sequence Name</th>
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<th>Fold change</th>
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<tr>
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<td>UniRef100_Q15582</td>
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<tr>
<td>Serotransferrin</td>
<td>UniRef100_P02787</td>
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<tr>
<td>Isoform 2C2A of Collagen alpha-2(VI) chain</td>
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Table 5. Identification of Epithelial Proteins that Showed Changes by 2D-DIGE Method

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<tr>
<th>Master No.*</th>
<th>Proteins</th>
<th>Accession** #</th>
<th>Average ratio***</th>
<th>Sequence coverage</th>
<th>No. of peptides</th>
<th>T-test MW****</th>
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* Unique identifier given by the Decyder software to each spot.
** Accession # is according to the GenBank.
*** Average ratio for a given spot between keratoconus and normal corneal epithelium was calculated.
**** Molecular Weight of the proteins in kiloDaltons (KDa).

Table 6. Identification of Stromal Proteins that Showed Changes by 2D-DIGE Method

<table>
<thead>
<tr>
<th>Master No.*</th>
<th>Proteins</th>
<th>Accession** #</th>
<th>Average ratio***</th>
<th>Sequence coverage</th>
<th>No. of peptides</th>
<th>T-test</th>
<th>MW**** Calculated (KDa)</th>
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* Unique identifier given by the Decyder software to each spot.
** Accession Number is according to GenBank
*** Average ratio for a given spot between keratoconus and normal corneal epithelium was calculated.
**** Molecular Weight of the proteins in kiloDaltons (KDa).

Identification of Corneal Epithelial Proteins in KC and Normal Corneas using Nano-ESI-LC-MS(MS)²

As stated above, the protein preparations from epithelial and stromal tissues from each of the four KC and each of the four aged-matched normal corneas (from 30-50 year-old donors) were analyzed by Nano-ESI-LC-MS(MS)² method. Following the Nano-ESI-LC-MS(MS)² analysis, those tryptic peptides which showed 50% or greater probability were used to identify their parent proteins. Further, only those proteins that showed protein probability score of 90% or greater are reported and the 5% false discovery rate was applied during the protein identification. A total of 104 epithelial proteins were identified in the preparations of both normal and keratoconus corneas following application of the above described stringency methods (Table 1). Interestingly, the same epithelial proteins were identified in each of the KC and normal corneas using either of the three different commercially available software’s (i.e., ProteoIQ, SCAFFOLD and Gene data). Based on known cellular functions, the epithelial proteins were divided into the following seven groups: metabolism, structural, protein-folding and degradation, redox, immunodefence, protein-binding, calcium ion-binding, transcriptional and translational proteins, and those with yet unknown functions (Table 1).

The identified corneal epithelial metabolic enzymes included α-enolase, galactose 3-O-sulfotranferase, glycerldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, malate dehydrogenate and transketolase. Alpha enolase, a homodimer composed of two subunits, is glycolytic enzyme. Galactose 3-O-
sulfotranferase catalyzes sulfonation to 3’ position of galactose in n-acetyllactosamine whereas glyceraldehyde-3-phosphate dehydrogenase (a glycolytic enzyme) catalyzes conversion of glyceraldehyde 3-phosphate to D-glyceraldehyde 1,3 biphosphate. Similarly, the glycolytic enzymes lactate dehydrogenase and phosphoglycerate kinase, catalyze the conversion of L-lactate and NAD⁺ to pyruvate and NADPH, and transfers phosphate group from 1,3 biphosphoglycerate to ADP forming ATP and 3-phosphoglycerate, respectively. Pyruvate kinase, another glycolytic enzyme, catalyzes the transfer of a phosphate group from phosphoenol pyruvate to ADP to produce pyruvate and ATP.

Malate dehydrogenate, a TCA-cycle enzyme, catalyzes a reversible reaction of conversion of malate to oxaloacetate using NAD⁺. Transketolase, an enzyme in pentose pathway, catalyzes the reversible transfer of a two-carbon ketol unit from xylulose 5-phosphate to an aldose receptor, such as ribose 5-phosphate to form sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate.

The identified corneal epithelial structural proteins included actin-related protein 3, alpha-actinin 4, coflin-1, decorin, desmoplakin epiplakin and gelsolin. The epithelial actin-related protein-3 that shares approximately 30-40% identities to the conventional actin and actin 4 was also observed. Among the ten members of the actin-related proteins (identified as Arp-1 to Arp-10), the actin related protein 3 functions by ATP-binding to regulate actin polymerization and formation of branched actin networks together with an activating nucleation-promoting factor (NPF). Among the three main forms of vertebrate actin isoforms (i.e., alpha, beta, and gamma), the alpha-actins are muscle tissue-specific and are a major constituent of the contractile apparatus, while beta- and gamma-actins co-exist and are components of the cytoskeleton, and act as mediators of internal cell
motility. The identified epithelial alpha-actinin 4 belongs to spectrin gene super family, and is involved in actin-binding to the membrane. The epithelial coflin-1 is an actin-modulating protein, which binds and depolymerizes F-actin and also inhibits polymerization of G-actin. Another epithelial 82 kDa protein gelsolin is also an actin-binding protein, a key regulator of actin assembly and disassembly. The corneal epithelial decorin is a cellular or pericellular matrix leucine-rich proteoglycan, which contains a glycosamine glycan chain consisting of chondroitin sulphate or dermatin sulfate. It is a component of connective tissue, and plays a role in matrix assembly by binding to type I collagen fibrils. Desmoplakin, a component of functional desmosomes, anchors intermediate filaments to desmosomal plaques. Epiplakin, an epithelial >700 kDa protein is a member of the plakin family, and is a cytolinker protein. It is shown to be involved in skin barrier function, and needed for the integrity of keratin network cytoarchitecture.

Several corneal epithelial intermediate filaments (IF) proteins were also identified (Table 1). The IF proteins constitute a large multigene family of proteins, that are subdivided into five major subgroups: (i) type I: acidic cytokeratins (ii) type II: basic cytokeratins (iii) type III: vimentin, desmin, glial fibrillary acidic protein (GFAP), peripherin, and plasticin (iv) type IV: neurofilaments L, H and M, alpha-internexin and nestin, and (v) type V: nuclear lamins A, B1, B2 and C. Two types of cytoskeletal and microfibrillar keratins are known, i.e. keratin I (acidic, 40-45 ka) and keratin II (neutral to basic, 56-70 kDa). Both types of keratins were identified in the corneal epithelium. The identified epithelial keratin included keratin type I cytoskeletal 12 (alternatively known as cytokeratin 12), keratin, type I cytoskeletal 14 (cytokeratin 14), keratin, type I cytoskeletal 16 (cytokeratin 16), keratin, type I cytoskeletal 24 (cytokeratin 24), keratin,
type II cytoskeletal 3 (cytokeratin 3), keratin, type II cytoskeletal 4 (cytokeratin 4), keratin, type II cytoskeletal 5 (cytokeratin 5), keratin, type II cytoskeletal 6b (cytokeratin 6b). Corneal epithelial differentiation is signified by the expression of the K12/K3 keratin pair of intermediate filaments, which is essential for corneal epithelial integrity (Tanifuji-Terai et al., 2006). Lamin A/C, an intermediate filament protein, is a type V nuclear laminin. Additional structural proteins present in the epithelium included lamins (a component of the nuclear lamina and fibrous layer on the nucleoplasmic side of the inner nuclear membrane), lumican (a small leucine-rich proteoglycan family that also includes decorin, biglycan, fibromodulin, keratocan, epiphycan, and osteoglycin and binds to collagen fibrils), and lumican a major keratan sulfate proteoglycan of the cornea (Rada et al., 1996). Other identified epithelial proteins included periplakin (a 195 kDa protein that is a novel component of cornified envelopes and desmosomes), decorin (a cellular or pericellular matrix proteoglycan and plays a role in matrix assembly by binding to type I collagen fibrils), desmoplakin (a component of functional desmosomes and anchors intermediate filaments to desmosomal plaques), epiplakin (a >700 kDa epithelial protein and a member of the plakin family, is a cytolinker protein), profilin-1 (a ubiquitous actin monomer-binding protein, which regulates actin polymerization in response to extracellular signals), and tubulin alpha 4A chain (a major constituent of microtubules).

Strangely, two blood serum proteins, apolipoprotein D and serum albumin were also observed in the epithelium. Apolipoprotein D (a 33 kDa glycoprotein, and a component of high density lipoprotein with homology to plasma retinol-binding protein) is a member of the alpha 2 microglobulin protein superfamily of carrier proteins. There
have been inconsistencies in the literature regarding the abundance and spatial
distribution of serum proteins and serum albumin in particular (Gong et al., 1997).

Several heat shock proteins (HSP 70-1A variant, Fragment), HSP 70-1, HSP 71,
HSP beta-1, and HSP 90-beta) were also observed in the corneal epithelium. These
proteins with chaperone activity are produced when cells are exposed to various stresses.
The major heat-shock proteins are classified into five classes: HSP33, HSP60, HSP70,
HSP90, HSP100, and the small heat-shock proteins (sHSPs). Several forms of HSP 70,
which might be splice variants, are listed in Table 1. Peptidylprolyl cis-trans isomerase
A-like 4 (also known as PPIases) that accelerates the folding of proteins and catalyzes the
cis-trans isomerization of proline imidic peptide bonds in oligopeptides was also
identified in the corneal epithelium.

Several proteins and enzymes responsible for redox reactions were also observed
in the corneal epithelium. These included 6-phosphogluconate dehydrogenase,
cytochrome C oxidase subunit VIb isoform 1, NAD(P)H dehydrogenase [quinone] 1,
peroxiredoxin-1, peroxiredoxin-2, peroxiredoxin-6, retinal dehydrogenase 1 and
thioredoxin. The 6-phosphogluconate dehydrogenase, a pentose phosphate pathway
enzyme, is an oxidative carboxylase that catalyzes decarboxylating reduction of 6-
phosphogluconate into ribulose 5-phosphate in the presence of NADP. Cytochrome C
oxidase subunit VIb isoform 1 (a nuclear-coded polypeptide chain of cytochrome C
oxidase), is a terminal oxidase in the mitochondrial electron transport. NAD(P)H
dehydrogenase [quinone] 1 is an enzyme that participates in oxidative phosphorylation in
presence of NADH. Peroxiredoxin-1, peroxiredoxin-2 and peroxiredoxin-6 are a family
of antioxidant enzymes that reduce hydrogen peroxide, alkyl hydroperoxide and cytokine-induced peroxide levels. These mediate signal transduction in mammalian cells. Retinal dehydrogenase is an enzyme that converts/oxidizes retinaldehyde to retinoic acid using NAD$^+$ as a cofactor. Thioredoxin, a 12 kDa oxidoreductive enzyme, acts as an antioxidant by reducing cysteine in proteins.

Several proteins that bind to other proteins were observed in the corneal epithelium. The 14-3-3 sigma isoforms are epithelial specific proteins and were present in both normal and keratoconus corneas (Zanello et al., 2006). Among at least seven distinct 14-3-3 genes in vertebrates (i.e., alpha/beta, epsilon, eta, gamma, theta, sigma, and zeta/delta), the epithelial cells contained 14-3-3 protein beta/alpha 14-3-3 protein gamma 14-3-3 protein sigma 14-3-3 protein zeta/delta.14-3-3 proteins. These belong to a highly conserved family of homo- and heterodimeric proteins, and exist in high abundance in all eukaryotic cells. These proteins, on binding to phosphorylated serine or threonine residues in specific motifs, are able to act as regulators of intracellular signal transduction. ATP synthase subunit beta is a mitochondrial enzyme that can synthesize ATP from ADP and inorganic phosphate. Cystatin B, a member of cystatin superfamily, acts as active cysteine protease inhibitors, and thought to inhibit lysosomal proteases. Echinoderm microtubule-associated protein-like 2 was also observed, which functions in modification of the assembly and dynamics of microtubules and causes microtubules to be slightly longer, but more dynamic. Probable histone-lysine N-methyltransferase has been implicated in methylation of Lys-4 of histone H3, which is believed to be a specific tag for epigenetic transcriptional activation. Transforming growth factor beta-induced protein βig-h3 binds to type I, II, and IV collagens, and is believed to play an important
role in cell-collagen interactions. Transgelin-2 is a homolog of the protein transgelin, which is one of the earliest markers of differentiated smooth muscle.

Three Ca$^{2+}$-binding proteins (i.e., calmodulin-like protein 3, calpain small subunit 1 and protein S100-A4) were present in corneal epithelium. Calmodulin-like protein 3 might be similar to calmodulin, and also binds to Ca$^{2+}$. Calpain small subunit-1 is a regulatory subunit of the calcium-regulated non-lysosomal thiol-protease (calpain), and causes limited proteolysis of proteins substrates involved in cytoskeletal remodeling and signal transduction.

Several proteins whose functions are presently not well known were also observed. Some of these include a protein cDNA, FLJ93175 (highly similar to Homo Sapiens serine [or cysteine] proteinase inhibitor), Clade B member 5 (SERPINB5, a cysteine type serine protease inhibitor), cDNA, FLJ95913 (highly similar to Homo sapiens keratocan), cDNA FLJ35050 fis, clone OCBBF2018167 (highly similar to pyruvate kinase isozyme M1), cDNA FLJ36436 fis, clone THYMU2012073 (highly similar to aldehyde dehydrogenase, dimeric NADP-preferring), ATP synthase subunit alpha (mitochondrial enzyme that catalyzes ATP synthesis, using an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation), and nucleotide-binding oligomerization domains 27 (an ATP binding protein). Keratocan, lumican and mimecan are keratan sulfate proteoglycans and are important to the transparency of the cornea (Pellegata et al., 2000). Aldehyde dehydrogenase plays a major role in the detoxification of alcohol-derived acetaldehyde, and therefore, functionally active in the oxidation of toxic aldehydes.
Proteins that play roles in transcriptional and translational processes were also identified in the corneal epithelium. These included elongation factor 1-alpha, translational initiation factor 5A-1, protein BUD31 homolog, RPLP1 protein, ribosomal protein S3a, tripartite motif-containing protein 29, ZEB1 protein and 60S acidic ribosomal protein P2. Elongation factor-1 complex is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome and its two isoforms, alpha 1 and alpha 2 were identified in our study. The precise role of eukaryotic translation initiation factor 5A-1 and BUD31-homologue (alternatively known as G10 protein) in protein biosynthesis is not known. BUD31-homologue (about 17 to 18 kDa hydrophilic protein) is cysteine rich in its C-terminal half, and is believed to be involved in metal-binding.

RPLP1 (60S acidic ribosomal protein P1) plays an important role in the elongation step of protein synthesis. The 40S ribosomal protein S3a has an alternative name of v-fos transformation effector protein. Disruption of the gene encoding rat ribosomal protein S3a, or v-fos transformation effector protein, in v-fos-transformed rat cells resulted in reversion of the transformed phenotype. Tripartite motif-containing protein 29 has multiple zinc fingers, and is proposed to form homo- or heterodimer, and is involved in nucleic acid binding, and acts as a transcriptional regulatory factor. The zinc E-finger homeodomain binding box protein ZEB1, known as transcription factor 8 (TCF8) plays a critical role in embryonic development, specifically in the regulation of type I collagen expression and in the repression of the epithelial phenotype. The Zeb1 mutant mouse has been used as a model of posterior corneal dystrophy (Liu et al., 2008). The 60S acidic ribosomal protein P2 plays an important role in the elongation step of protein synthesis.
Identification of Corneal Stromal Proteins in KC and Normal Corneas using Nano-ESI-LC-MS(MS)²:

A total 44 stromal proteins were identified in the normal and KC corneas by Nano-ESI-LC MS(MS)² method (Table 3). Among the stromal preparation results of each of the four normal and four KC corneas, only those proteins which had 50% or more peptide probability and 90% or more protein probability were considered and are reported. Additionally, the 5% false discovery rate was applied for the protein identification. These stromal proteins were divided according to their cellular functions in the following categories: Structural, redox, blood/plasma, receptor-binding, protein-binding, and those whose functions are yet unknown.

Among structural protein, several subsets of collagen, proteoglycans and keratins were identified. Among different collagens, collagen alpha-1(I) chain, collagen alpha-1(I) chain precursor (Alpha-1 type I collagen), collagen alpha-1(VI) chain, collagen alpha-1(XII) chain and collagen alpha-2(VI) chain were present. Type I collagen and collagen alpha-1(I) chain precursor (Alpha-1 type I collagen) are members of group I collagen (the fibrillar-forming collagen), which are trimer of one alpha 2(I) and two alpha 1(I) chains. Collagen VI acts as a cell-binding protein and its subunit is composed of trimers of three different chains, i.e. alpha-1(VI), alpha-2(VI), and alpha-3(VI) or alpha-5(VI) or alpha-6(VI). Collagen alpha-1(XII) chain is a member of the FACIT (fibril-associated collagens with interrupted triple helices) collagen family and has been identified in the human corneal stroma (Wessel et al., 1997). Type XII collagen is a homotrimer found in association with type I collagen, an association that is thought to modify the interactions between collagen I fibrils and the surrounding matrix. The basic structural unit of collagen VI is a heterotrimer of the alpha1 (VI), alpha2 (VI), and alpha3 (VI) chains. The
alpha 2(VI) and alpha 3(VI) chains are encoded by the COL6A2 and COL6A3 genes, respectively. The protein encoded by this gene is the alpha 1 subunit of type VI collagen (alpha1 [VI] chain).

Additional structural stromal proteins identified were decorin, dermatopontin, gelsolin and type I and II keratins. As stated above, decorin is a member of the small leucine-rich proteoglycan family. It binds to type I collagen fibrils, and plays a role in matrix assembly, also a role in regulation of the cell cycle. The human lumican is encoded by the LUM gene, and is a major keratan sulfate proteoglycan of the cornea. Mice homozygous for a null mutation in lumican showed bilateral corneal opacification. (Chakravarti., 1998). The LUM gene also encodes a member of the small leucine-rich proteoglycan family that includes decorin, biglycan, fibromodulin, keratocan, epiphycan, and osteoglycin. The protein moiety of the proteoglycans binds collagen fibrils and the highly charged hydrophilic glycosaminoglycans regulate interfibrillar spacings. Dermatopontin, a component of the extracellular matrix, react with decorin and inhibits the formation of the decorin-TGF-beta1 complex. Gelsolin, an 82-kD protein, is an actin-binding protein, and regulates actin filament assembly and disassembly. Keratin type I-cytoskeletal 12 (alternatively known as cytokeratin 12), is a corneal-specific protein, and is a heterotetramer of two type I and two type II keratins. Keratin-3 associates with keratin-12, and it plays a unique role in maintaining the normal corneal epithelial function and integrity (Shiraishi et al., 1998). Tissue specificity defects in keratin 12 are the cause of Meesmann corneal dystrophy (Irvine et al., 1997; Chen et al., 2005). Keratin, type II cytoskeletal 5 is a heterotetramer of two type I and two type II keratins, i.e. keratin-5 associates with keratin-14. Keratocytes in the corneal stroma express
keratan sulfate-containing proteoglycans including cornea-specific keratocan. Mutations in KERA encoding keratocan, cause cornea plana, i.e. the forward, convex curvature of cornea is flattened, leading to high hyperopia (Pellegata et al., 2000). Vimentin is a member of the intermediate filament family of proteins, and these filaments make the cytoskeleton along with microtubules and actin microfilaments.

Three enzymes, \( \alpha \)-enolase, glyceraldehyde 3-dehydrogenase and pyruvate kinase isozymes M1/M2 (involved in glycolysis and gluconeogenesis pathways) were identified in the stroma. Two redox proteins, aldehyde dehydrogenase and dimeric NADP-preferring glutathione S-transferase P was also present in the stroma. As stated above, aldehyde dehydrogenase catalyzes the oxidation of aldehyde, and the antioxidant enzyme glutathione S-transferase (GST) reduces lipid hydroperoxides through its Se-independent glutathione peroxidase activity. The enzyme also detoxifies lipid peroxidation end products such as 4-hydroxynonenal (4-HNE). Strangely, six plasma proteins were also identified in the stroma, which included apolipoprotein A-I (1-242), apolipoprotein A-II (1-76), apolipoprotein D, serotransferrin, serum albumin and amyloid P-component (1-203). The function of these proteins in stroma is presently unknown.

The identified corneal stromal proteins that bind to other proteins included protein S100-A4, protein S100-A6, transforming growth factor-beta (TGF-\( \beta \))-induced protein \( \beta \)ig-3, fatty acid-binding protein and epidermal Galectin-1. S100 proteins, localized in the cytoplasm and/or nucleus of a wide range of cells, are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. The S100A4 (a member of S100 family) is a calcium-binding protein, expressed in the
keratocyte phenotypes that appear in stromal tissue. S100A4 may be involved in the interconversions that occur between keratocytes, fibroblasts, and myofibroblasts during corneal wound healing (Ryan et al., 2003). The fatty-acid-binding proteins (FABPs) are a family of carrier proteins for fatty acids and they facilitate the transfer of fatty acids between extra- and intracellular membranes. Epidermal galectin-1 belongs to a family of proteins that bind to carbohydrates with an affinity for β-galactosides. The identified stromal proteins with unknown functions included MAM domain-containing protein 2, IGG1-KAPPA 3D6 FAB (LIGHT CHAIN), IGHG2 protein and uncharacterized protein ALB n=1
Figure 7: Ingenuity Pathway analysis of epithelial proteins that showed changes in KC compared to normal corneas. Additional abbreviations used are shown in the table below.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Entrez Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB</td>
<td>albumin</td>
</tr>
<tr>
<td>FAM53B</td>
<td>family with sequence similarity 53, member B</td>
</tr>
<tr>
<td>HSPA8</td>
<td>heat shock 70kDa protein 8</td>
</tr>
<tr>
<td>HTT</td>
<td>huntingtin</td>
</tr>
<tr>
<td>KRT12</td>
<td>keratin 12</td>
</tr>
<tr>
<td>KRT13</td>
<td>keratin 13</td>
</tr>
<tr>
<td>KRT16</td>
<td>keratin 16</td>
</tr>
<tr>
<td>KRT2</td>
<td>keratin 2</td>
</tr>
<tr>
<td>KRT23</td>
<td>keratin 23 (histone deacetylase inducible)</td>
</tr>
<tr>
<td>KRT3</td>
<td>keratin 3</td>
</tr>
<tr>
<td>KRT4</td>
<td>keratin 4</td>
</tr>
<tr>
<td>KRT5</td>
<td>keratin 5</td>
</tr>
<tr>
<td>KRT6B</td>
<td>keratin 6B</td>
</tr>
<tr>
<td>LDHAL6A</td>
<td>lactate dehydrogenase A-like 6A</td>
</tr>
<tr>
<td>MFHAS1</td>
<td>malignant fibrous histiocytoma amplified</td>
</tr>
<tr>
<td>TGFB1</td>
<td>transforming growth factor, beta 1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>
Figure 8: Ingenuity Pathway analysis of stromal proteins that showed changes in KC compared to normal corneas. Additional abbreviations used are shown in the table below.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Entrez Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>actin, beta</td>
</tr>
<tr>
<td>C11orf82</td>
<td>chromosome 11 open reading frame 82</td>
</tr>
<tr>
<td>CDC42EP5</td>
<td>CDC42 effector protein (Rho GTPase binding) 5</td>
</tr>
<tr>
<td>COL14A1</td>
<td>collagen, type XIV, alpha 1</td>
</tr>
<tr>
<td>CORO1A</td>
<td>coronin, actin binding protein, 1A</td>
</tr>
<tr>
<td>COTL1</td>
<td>coactosin-like 1 (Dictyostelium)</td>
</tr>
<tr>
<td>CTSF</td>
<td>cathepsin F</td>
</tr>
<tr>
<td>FN1</td>
<td>fibronectin 1</td>
</tr>
<tr>
<td>GFRA2</td>
<td>GDNF family receptor alpha 2</td>
</tr>
<tr>
<td>HIPK1</td>
<td>homeodomain interacting protein kinase 1</td>
</tr>
<tr>
<td>IVNS1ABP</td>
<td>influenza virus NS1A binding protein</td>
</tr>
<tr>
<td>KERA</td>
<td>Keratocan</td>
</tr>
<tr>
<td>LTBP2</td>
<td>latent transforming growth factor beta binding protein 2</td>
</tr>
<tr>
<td>LUM</td>
<td>Lumican</td>
</tr>
<tr>
<td>MAMDC2</td>
<td>MAM domain containing 2</td>
</tr>
<tr>
<td>MGST2</td>
<td>microsomal glutathione S-transferase 2</td>
</tr>
<tr>
<td>MIR134</td>
<td>microRNA 134</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MIR199A1</td>
<td>microRNA 199a-1</td>
</tr>
<tr>
<td>MIR199A2</td>
<td>microRNA 199a-2</td>
</tr>
<tr>
<td>MT1L</td>
<td>metallothionein 1L (gene/pseudogene)</td>
</tr>
<tr>
<td>MYO9B</td>
<td>myosin IXB</td>
</tr>
<tr>
<td>NQO2</td>
<td>NAD(P)H dehydrogenase, quinone 2</td>
</tr>
<tr>
<td>ORM2</td>
<td>orosomucoid 2</td>
</tr>
<tr>
<td>PDRG1</td>
<td>p53 and DNA-damage regulated 1</td>
</tr>
<tr>
<td>PLS1</td>
<td>plastin 1</td>
</tr>
<tr>
<td>PLXNB2</td>
<td>plexin B2</td>
</tr>
<tr>
<td>PRKRIIR</td>
<td>protein-kinase, interferon-inducible double stranded RNA dependent</td>
</tr>
<tr>
<td>RNASE4</td>
<td>ribonuclease, RNase A family, 4</td>
</tr>
<tr>
<td>SCO2</td>
<td>SCO cytochrome oxidase deficient homolog 2 (yeast)</td>
</tr>
<tr>
<td>SERPIND1</td>
<td>serpin peptidase inhibitor, clade D (heparin cofactor), member 1</td>
</tr>
<tr>
<td>SLC12A6</td>
<td>solute carrier family 12 (potassium/chloride transporters), member 6</td>
</tr>
<tr>
<td>STARD3</td>
<td>StAR-related lipid transfer (START) domain containing 3</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNS1</td>
<td>tensin 1</td>
</tr>
<tr>
<td>TP53</td>
<td>tumor protein p53</td>
</tr>
<tr>
<td>UPP1</td>
<td>uridine phosphorylase 1</td>
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</tbody>
</table>
DOWN-REGULATION OF β-ACTIN GENE IN HUMAN KERATOCONUS CORNEAS IS DUE TO A HUMAN ANTIGEN R (HuR) PROTEIN

by

ROY JOSEPH, OM P. SRIVASTAVA, ROSWELL R. PFISTER

Submitted to

Investigative Ophthalmology & Visual Science

Format adapted for dissertation
Abstract

Purpose

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

Methods

A total of 15 different human corneas from both KC and normal individuals were used for this study. 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 HuR gene expression was analyzed by real-time PCR in the stroma of five KC and five normal corneas. The keratocytes from both normal and 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 keratoconus fibroblasts.

Results

The expression of β-actin gene was down-regulated in the stroma of the six keratoconus corneas but not in the stroma of six normal and Fuch’s dystrophic corneas. Immunofluorescence detection of β-actin showed that it was absent in keratoconus fibroblasts. The real-time PCR analysis of HuR gene showed a relative 4.7-fold lower expression in keratoconus corneas relative to the normal corneas, which was further confirmed by the immunofluorescence detection of HuR in fibroblasts of keratoconus corneas.
Conclusions

Although ubiquitous β-actins are essential for cell survival during early embryogenesis, it’s effects on various stages of development are not well understood. Our results show that β-actin is down-regulated in the corneal stroma of patients with keratoconus, which is due to a 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 keratoconus.

Introduction

Keratoconus (KC) is a condition of unknown cause in which the cornea assumes a conical shape as a result of non-inflammatory 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 intolerant 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\textsuperscript{1,2}. The classical histopathological features of KC include central stromal thinning, iron deposits in the epithelial basement membrane, and breaks in the Bowman's layer. Several reports describe an association of KC with Down syndrome, Lebers congenital amaurosis, and mitral valve prolapse\textsuperscript{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, endo-/exo-cytosis, cell division, signal transduction, mRNA localization and transcription. Eukaryotes have six actin isoforms, each are encoded by an individual gene. Among the six isoforms, two are striated muscle-associated (α-skeletal and α-cardiac muscle actins), two smooth muscle-associated (α- and γ-smooth muscle actins) and two are cytoplasmic (β- and γ-actin).

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. 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. 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 is important for cellular processes that require constant and stable concentration levels. The expression of actin genes is regulated at both transcriptional and post-transcriptional levels (by the cellular localization of their mRNAs). The β-actin mRNA is regulated by a specific sequence at the 3’ untranslated region (3’UTR) by RNA-binding proteins known as zip code-binding proteins (ZBP).

Our previous studies showed down-regulation of β-actin and proteomic profile changes in cytoskeletal proteins in KC corneas related to normal corneas. 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 of β-actin in the stroma of KC and normal corneas and its regulation. Our results show that β-actin is down-regulated in stromal keratocytes of KC corneas compared to normal corneas and this could be due to down-regulation of mRNA stabilizing factor Human antigen R (HuR).

Methods

Human Corneas

The normal corneas were obtained from Alabama Eye Bank and the KC corneal buttons (8 mm in diameter) were obtained following surgery from a local corneal surgeon. A total of 15 keratoconus 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 h 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, 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.
**RNA Extraction**

The epithelial and stromal preparations were separately recovered from each KC and age-matched normal corneas, and were also separately homogenized in Trizol (Invitrogen). For this purpose, the tissue samples in grinding flasks (volume 3 ml, made of polytetrafluoroethylene) 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). The RNA was quantified by using Nanodrop (Thermoscientific Inc).

**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 12000 rpm for 10 minutes at 4°C. Next, the precipitate was washed 3X 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 BCA protein assay Kit (Pierce, USA). Thirty μg of proteins from each sample were loaded on to 12% polyacrylamide gels during SDS-PAGE\textsuperscript{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, England) using a red laser (633 nm) at a 200 μ resolution.

Stromal cell Culture

The epithelium and endothelium were scraped and removed from KC and normal corneas. The stroma was incubated overnight in collagenase (Worthington, at 1 mg/ml). The cells were washed in Dulbecco’s modified Eagles medium (DMEM; Invitrogen) containing 1% antibiotics (Penicillin-Streptomycin solution; Mediatech Inc.). Next, the cells were seeded onto 6-well plates (Corning) in DMEM medium containing 10% fetal bovine serum (HyClone) and 1% antibiotics (Penicillin-Streptomycin solution), and were maintained at 37°C in a 5%-CO₂-humidified air. Cells were trypsinized (0.05% trypsin-EDTA) on a consistent basis and all analyses were carried out before their third passage. The study generated a primary culture from 3 age matched normals and 3 keratoconus corneas.

Western blot analysis

Thirty μg protein from samples were lysed with 1×SDS gel loading buffer and subjected to SDS-PAGE\(^1\) using 12% polyacrylamide gels. Proteins were transferred to PVDF membrane (Bio-Rad) by using a semi-dry transfer cell (Bio-Rad, Trans-blot Turbo), 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) at (1:100 dilution), followed by incubation in goat anti-mouse secondary antibody conjugated to Infra Red Dye (IRD) at 1:10,000 dilutions. Blots were scanned using Odyssey Imaging
system (LI-COR Biosciences). The blot was stripped using a mild stripping solution for an hour and followed by washing 3 times with PBS-Tween 20. The membranes were then blocked as described above and re-probed with anti-vimentin monoclonal antibody (Sigma) at (1:200) dilution, followed by secondary antibody incubation as described above. Blot was scanned using Odyssey Imaging system (LI-COR Biosciences).

**Immunohistochemical–Confocal Microscopic analysis**

The corneas were fixed in 4% paraformaldehyde and 10 µm cryosections were recovered using a cryostat (Leica cryostat; Core Facility of Vision Sciences Research Center at the University of Alabama at Birmingham.). 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 3X and incubated with a secondary goat anti-mouse Alexa Fluor 488-labeled antibody (Invitrogen, Eugene, Oregon). Next, the sections were washed 3X in PBS and nuclei were stained with Hoechst 33342 stain (Invitrogen, Eugene, Oregon) for 10 min. After a final wash with PBS, the sections were mounted on slides with Fluromount-G (Southern Biotech, AL). Fluorescent microscopic analysis was performed using Zeiss AxionPlan 2 Imaging System Microscope at the Molecular Biology Core facility of Vision Sciences Research Center at the University of Alabama at Birmingham. The immunohistochemical analysis was identically performed using cultured stromal fibroblastic cells. The antibodies used in the study are listed in Table 1. The mouse IgG was used as a negative control with same protein concentration as that of the primary antibody. The confocal imaging was done using Zeiss LSM 710 confocal microscope (High Resolution Imaging Facility,
UAB). The intensity of fluorescence was quantified by using SIMPLEPCI software (Compix, Cranberry Township, PA). We have 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), and 500 ng of RNA was used for RT-PCR. The primers were designed using Primer3 for β-actin, HuR and 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, Hercules, CA), using 96-well reaction plates in a total volume of 25 μL. During the analysis, stromal preparations from 5 different KC and five normal corneas were used. The reaction mixture included 12.5 μL of Real-Time SYBR Green PCR master mix, 2.5 μL of reverse transcription product, 1 μL of forward and reverse primer and 8 μL of DNase/RNase free water. The reaction mixtures were initially heated at 95°C for 10 min to activate the polymerase, followed by 40 cycles, which consisted of denaturation step at 95°C for 15 sec, annealing at 57°C for 60 sec and elongation step at 72°C. The qRT-PCR data were analyzed by the comparative ΔCt method21.

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 down-regulated in all three KC corneal stromas compared to the three normal corneal stromas (Figure 1B). In contrast, the GAPDH gene expression remained at constant levels in the stroma of both normal and keratoconus corneas. The two different primers sets showed a down-regulation of β-actin gene in KC stroma compared to normal corneal stroma (Figures 1B and 1C). When the primer 2 was used for RT-PCR to analyze additional stromal preparations from 3 KC and 3 normal corneas, again β-actin gene was found to be down-regulated only in KC corneas but not in the normal corneas (Figure 1D). Additionaaly, the GAPDH expression remained at the same levels in both normal and KC stromal preparations (Figure 1D). To quantify the β-actin gene expression level, the pixel intesity β-actin and GAPDH gene expressions with primer 2 was measured and the values with standard deviation in all the six samples of KC and normal corneas are shown in Figure 1E. The result further show down-regulation 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 Fuch’s dystrophic corneas were analyzed RT-PCR method. Fuch’s 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\textsuperscript{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 Fuch’s dystrophic corneas (Figure 1F).

To examine the cellular β-actin expression in stroma of KC vs. 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 (Figure 2A), and in contrast, it was absent in KC corneas (Figure 2D). Figure 2(B) shows the counter staining of the same sections as in 2A with Hoechst nuclear stain, and Figure 2(C) is an overlay of 2(A) and 2(B). Figure 2(D) shows the immunoreactivity of 10 µm cryosections of KC cornea with anti-β-actin, while Figure 2(E) shows the counter staining of the section with Hoechst nuclear stain and Figure 2(F) is an overlay of 2(D) and 2(E). Protein isolation after RNA extraction using Trizol has been a choice in the case of limited availability of clinical samples. The protein samples were recovered from the same normal and KC stromal preparations that exhibited down-regulation of β-actin gene during the RT-PCR analysis (Figure 1D). The Western blot analysis of these stromal protein preparations with anti-β-actin antibody further showed the down-regulation of β-actin in KC but not in the normal corneas (Figure 2H). In this analysis, identical amounts of stromal proteins from three KC and two normal corneas were used SDS-PAGE and Western blot analyses (Figure 2G), and vimentin was used as a loading control (Figure 2H). Together, the above results show that β-actin was down-regulated at both transcriptional and translational levels in the stroma of KC corneas but not in the normal corneas.

In order to determine if the down-regulation of β-actin in keratoconus 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\textsuperscript{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 (Figures 3A and 3B), and vimentin was cytosolic, which surrounded the nuclei along the cell processes. Both normal and keratoconus corneal fibroblasts also exhibited immunoreactivity to alpha-smooth muscle actin (Figures 3C and 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 (Figures 3E and 3I). The F-actin could be detected by using phalloidin (toxin from the mushroom \textit{Amanita phalloides}) staining. The rhodamine-labeled phalloidin specifically stained the F-actin filaments in both the normal and KC stromal fibroblasts (Figures 3F and 3J) suggesting its existence in these cells. There are two forms of cytoplasmic actins the \( \beta \)-actin and \( \gamma \)-actins, and Figure 3G and 3K show the immunoreactivity of \( \beta \)-actin in the normal and KC corneal stromal fibroblasts, respectively. Immunohistochemical analysis further showed that the fibroblasts from normal corneas were positive for \( \beta \)-actin (green fluorescence; Figure 3G) whereas the cells from keratoconus showed an absence of \( \beta \)-actin (Figure 3K). Additionally, the \( \beta \)-actin in normal corneal fibroblasts showed a staining pattern around the nucleus and also along the length-wise of the cell (Figure 3G). This staining pattern might represent \( \beta \)-actin stress fibers, and these stress fibers were absent in the cells from KC corneal...
fibroblasts (Figure 3K). 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 were positive for γ-actin (green fluorescence; Figures 3N and 3Q).

The β-actin mRNA has long half life\(^9\)\(^{-17}\) and HuR binding to U-rich element has been shown to be involved in the 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. The real-time PCR analysis allows a quantitative measure of the gene expression levels. qRT-PCR data were analyzed by the comparative ∆Ct method\(^{19}\). First, ∆Ct and mean of ∆Ct are calculated, and then standard deviation of each set of ∆Ct is determined. The threshold cycle (Ct) value is the amplification cycle number at which a defined fluorescence is achieved. The real time PCR for HuR gene (involved in the mRNA stability) showed that HuR gene expression is decreased by 4.7-folds (Figure 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 keratoconus compared to normal cornea. In contrast, the GAPDH gene expression remained at the same levels in preparation of both normal and keratoconus corneas (Figure 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 (Figure 5C) as described in Method section. The total green pixel
intensity was decreased by 3-fold in KC corneal fibroblast compared to normal corneal fibroblast.

Discussion

Although changes in corneal epithelium and stroma at the cellular, biochemical, physiological and genetic levels are reported to be responsible for the development and progression of keratoconus, the exact molecular mechanism of keratoconus remains elusive. During the disease, additional changes besides affected epithelial cells and stromal keratocytes include discrete incursion of fine cellular processes into Bowman’s membrane and altered nerve plexus. Interplay of epithelial – stromal interaction (ESI) in the cornea is important for both corneal transparency and the wound healing process. 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 keratoconus.

The broad, flattened, queiscent keratocytes (neural crest-derived cells) constitutes 5% of corneal stroma, and lie parallel to the collagen lamellae. Keratocytes maintain a functional stroma via secretion of stromal collagen and extracellular matrix that provide corneal strength and its transparency, and also behave like a macrophages during corneal infection and injury. The cells synthesize and secrete collagen (mainly type I and type V collagen), proteoglycans [keratocan, lumican, and mimican with keratin sulfate], and high levels of corneal crystallins, namely transketolase aldehyde dehydrogenase class 1A1. 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\textsuperscript{16,31}. Under normal condition, the keratocytes become active during injury to differentiate to fibroblast and myofibroblast-like cells\textsuperscript{32}. The fibroblast growth factor-2 and platelet-derived growth factor stimulate differentiation of keratocytes to fibroblast and TGF-β to myofibroblasts\textsuperscript{32,33}.

During KC, the apoptosis of keratocytes seems to be among the reasons for corneal thinning\textsuperscript{34}, as evidenced by their differentially expressed genes during the disease. This includes the over-expression of bone morphogenic protein-4, coflin, JAW1-related protein and under-expression of actin, alpha 2 rich cluster, and C-10 gene, tissue inhibitors of metalloproteinase 1 and 3 and somatostatin receptor 1\textsuperscript{35}. These genes are believed to control apoptosis, cytoskeletal structure, wound healing and nerve fiber density in corneas\textsuperscript{35}. However, presently the trigger for keratocytes 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 down-regulated at both transcriptional and translational levels in the KC stroma but not in the stroma of normal cornea and Fuch’s dystrophic corneas. Normal and Keratoconus keratocytes transform to fibroblast in culture, but β-actin continue to show down-regulation only in the cells of the KC corneas. A previous study has shown that the siRNA-mediated β-actin knockdown results in membrane blebbing in HeLa cells after 48 hrs of treatment\textsuperscript{7}, a similar knockdown of β-actin gene in HeLa cells did not show any significant cell death but this discrepancy was apparently due to difference in the media used for culturing HeLa cells\textsuperscript{36}. Likewise, we also noted that inspite of down-regulation
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 they become fibroblast and myofibroblast in the presence of serum. Serum-induced cells show an increase in β-actin synthesis, whereas KC fibroblasts, even in the presence of serum, did not induce β-actin synthesis (Figure 5I). Additionally, both normal and keratoconus fibroblasts did express vimentin (an intermediate filament), a marker for mesenchymal cells, showing the mesenchymal nature of stromal fibroblasts.

The major functions of β-actin in cells is to provide mechanical support through its cytoskeleton and extracellular matrix, help in cell motality and act in signal transduction of cytoplasm with surroundings. Cytoplasmic proteins adjacent to the plasma membrane control cell shape and regulate cell-cell interactions and focal adhesions. Embryonic lethality of β-actin has been reported whereas γ-actin knockout mice are viable. Conditional knock out of β-actin in mouse embryonic fibroblast has been reported to show increased apoptosis and motility defects. It has also been shown that β-actin deficiency in auditory hair cells lead to progressive hearing loss. In spite of such studies, the β-actin’s role in corneal stroma seems to be mostly mechanical, and additional functions are unclear.

High expression levels of β-actin is important for the above described cellular processes, maintained in vivo by its mRNA stability and optimal concentration levels. The expression of actin genes is regulated at both transcriptional and at post-transcriptional levels, such as the cellular localization of their mRNAs. Studies using anti-sense oligonucleotides against cis-acting elements have shown that the polarity and
cellular motility are severely reduced\textsuperscript{40}. A 54-nucleotide (nt) segment termed as Zipcode have been identified as regulatory sequence in the 3’-untranslated region (3’UTR) of β-actin mRNA sequence\textsuperscript{40}. Several zipcode binding proteins (ZBP-1) have been identified that bind to the zipcode sequence to regulate the β-actin mRNA localization\textsuperscript{41}. The 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 ARE and the poly(A) tail in vitro\textsuperscript{42,43}. The mRNA of HuR is ubiquitously expressed in all proliferating cells, and is the most important post-transcriptional regulators of gene expression\textsuperscript{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 is due to the loss of β-actin stress fibers\textsuperscript{18}. The β-actin mRNA has long half life\textsuperscript{9,17} and HuR binding to U-rich element is involved in the mRNA stability and affects the half-life of β-actin mRNA\textsuperscript{18}. Our real-time PCR analysis of HuR gene showed that it is down-regulated by 4.7-folds in KC corneas compared to normal corneas, which raises the possibility that the down-regulation of HuR and β-actin could be an effect of the interplay between the two. This coincides with the earlier result showing that down-regulation of HuR resulted in the reduced half-life of β-actin mRNA\textsuperscript{18}. This in turn might result in the loss of cytoskeletal functions, mainly due 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 hence is believed to be involved in cell motility and asymmetry\textsuperscript{22}. There is a long standing debate about how the actin isoforms, especially the non-muscle actin segregate in the moving fibroblast. Karakozova et al. have shown that the actin isoforms are located in the fibroblastic cells, and β-actin is localized at the
leading edge of the cells and γ-actin is the one that forms the stress fibers$^{45}$. This 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 to the latter. This result coincides with Karakozova et al. report that showed 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 (Figure 3I). This is further supported by the fact that in β-actin knockout mouse, the embryonic fibroblasts showed a reduced G-actin pool compared to 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 an 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 reduced numbers of keratocytes in KC could be a consequence of the loss of β-actin, which in turn destabilized their cytoskeleton causing 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. What upstream targets that might affect this down regulation is yet to be determined. As stated above, β-actin is involved in many cellular and pathogenic processes, and therefore, its down-regulation could affect yet unknown certain functions that might lead to the development and progression of the KC disease process.
Acknowledgements

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Table 1: List of antibodies used in the study

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Table 2: Primers used in the study

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Figure 1: β-Actin Gene Expression in Normal vs. Keratoconus Corneas. A: Location of primer 1 (from 484 to 1072, shown in blue) and primer 2 (from 797 to 1375, shown in red) in the β-actin gene. Figures 1B and 1C: 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 levels of β-actin gene was decreased/absent in the stroma of the three keratoconus (KC) corneas. The levels of GAPDH gene, which was used as the internal standard, remained at the same levels in the stroma of normal and KC corneas. Figure 1D: 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 levels of β-actin gene was decreased/absent in the stroma of the three KC corneas. The levels of GAPDH gene, which was used as the internal standard, remained similar in all the samples. The ages of donors of KC and normal corneas are shown on right hand corner of 1B and 1C, and top of lanes in Figure 1D.
Figure 1F: RT-PCR for β-actin gene. The RT-PCR analysis with the two primers for β-actin gene is shown in the figure. The β-actin gene was expressed in the stroma of the normal (control) corneas and also stroma of Fuch’s dystrophic corneas. The expression levels of β-actin gene remained at the same levels in the epithelium of normal cornea, keratoconus and Fuch’s dystrophic corneas.
**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): Counter staining 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): Counter staining 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 donor’s ages as shown were examined. Vimentin was used as a loading control for all the 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.
Figure 3: Immunoreactivity of Fibroblasts from Normal and KC Stroma. Immunoreactivity of keratocyte-derived fibroblasts from normal and KC stroma with anti-vimentin-, anti-alpha 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 counter stained with Hoechst nuclear staining. (C): Immunoreactivity of stromal fibroblasts from normal corneas with anti-alpha smooth muscle actin antibody and counterstained with Hoechst nuclear staining. (D): Immunoreactivity of stromal fibroblasts from keratoconus corneas with anti-alpha smooth muscle actin antibody and counterstained with Hoechst nuclear staining.
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Scale -10µM

**Figure 3 (continued on next page)**

**Figure 3**: (E): Fibroblasts from normal corneal stroma stained with Hoechst nuclear stain. (F): Fibroblasts from normal corneal stroma stained 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 Rhodamine-labeled phalloidin stain. (K): Immunoreactivity of stromal fibroblasts from keratoconus corneas with anti-β-actin antibody. (L): Overlay (I), (J) and (K).
Figure 3: (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 β-actin whereas those from KC corneas showed reduced β-actin expression.
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 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).
DOWN-REGULATION OF β-ACTIN AND ITS REGULATORY GENE HuR AFFECTS CELL MIGRATION IN HUMAN CORNEAL FIBROBLASTS

by

ROY JOSEPH, OM. P. SRIVASTAVA, ROSWELL.R. PFISTER

In preparation for Investigative Ophthalmology & Visual Science

Format adapted for dissertation
Abstract

Purpose

The purpose of the study was to determine β-actin gene regulation by Human antigen R (HuR) and also whether the gene knockdown affects cell migration.

Methods

The keratocytes from normal human corneas were cultured in the presence of serum and the cells were transfected with small interfering RNA (siRNA) specific for β-actin or to Human antigen R (HuR). Small interfering RNA’s specific for GAPDH and a scrambled sequence were used as a positive and negative control for transfection, respectively. The effects of gene silencing were analyzed at both transcriptional and translational levels. The specific proteins were localized immunohistochemically using confocal imaging. The effect of gene silencing on cell migration was analyzed using a modified Boyden chamber, and on the cell proliferation by using a wound healing assay.

Results

Our RT-PCR and Western blot analyses showed that β-actin expression was significantly down-regulated when HuR gene was silenced. Immunohistochemical-confocal analysis showed that nuclear HuR expression was significantly down-regualted on silencing of HuR gene. Similarly, on silencing β-actin gene, its expression was considerably reduced but showed no effect on HuR gene expression. However, GAPDH gene silencing affected β-actin expression. The motility of corneal fibroblast was significantly reduced after either β-actin gene- or HuR gene silencing. GAPDH gene
silencing also affected cell motility. Fibroblast cell proliferation, as demonstrated by a wound healing assay, was also significantly reduced after β-actin gene or HuR gene silencing.

Conclusions

The major functions of β-actin in cells is to provide mechanical support via its cytoskeleton, help in cell motility and act in signal transduction of cytoplasm with surroundings. The above results show that HuR regulates β-actin expression and also cell motility. Corneal fibroblast cell proliferation was also affected by silencing of either β-actin or HuR genes. Our gene silencing results were concordance with earlier results showing that β-actin and HuR was down-regulated in human keratoconus. We propose that a loss of β-actin in the corneal stroma might act as a triggering factor via cytoskeletal disruption in the development of keratoconus.

Introduction

Keratocytes play an important role in corneal transparency by maintaining a functional stroma via the secretion of stromal extracellular matrix and collagen contributing 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, endo-/exocytosis, cell division, signal transduction, mRNA localization and transcription. Eukaryotes have six actin isoforms, each encoded by an individual gene. Among the six
isoforms, two are striated muscle-associated (α-skeletal and α-cardiac muscle actins), two smooth muscle-associated (α- and γ- smooth muscle actins) and two are cytoplasmic (β- and γ-actin)\(^2\). 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\(^3\). 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\(^4\). 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 is important for these cellular processes, maintained by its mRNA stability and high concentration levels. The expression of actin genes is regulated at both transcriptional and post-transcriptional levels, each at the cellular localization of their mRNAs\(^5\). Studies using anti-sense oligonucleotides against cis-acting elements have shown that the polarity and cellular motility are severely reduced\(^7\). A 54-nucleotide (nt) segment termed as Zipcode have been identified as regulatory sequence in the 3’-untranslated region(3’UTR) of β-actin mRNA sequence\(^7\). Several zipcode binding proteins (ZBP-1) have been identified that bind to the zipcode sequence and regulate the β-actin mRNA localization\(^8\). The ELAV family of proteins, in particular the HuC (mouse) and HuR (Human) have been shown to exhibit poly(A)–binding activity, appearing to be able to bind simultaneously to the ARE and the poly(A) tail \textit{in vitro}\(^9,10\). The mRNA of HuR is ubiquitously expressed in all proliferating cells, and is the most important post-transcriptional regulators of gene expression\(^11\).
Dormoy-Raclet et al. have shown that HuR depletion in HeLa cells alters the cytoskeleton functions such as cell adhesion, migration and invasion, and is due to the loss of β-actin stress fibers\textsuperscript{12}. The β-actin mRNA has a long half-life \textsuperscript{5,13}, and HuR binding to the U-rich element is involved in mRNA stability, affecting the half-life of β-actin mRNA \textsuperscript{12}. Our real-time PCR analysis of HuR gene showed that it is down-regulated by 4.7-fold in keratoconus cornea compared to normal cornea, raising the possibility that down-regulation of HuR and β-actin could be an effect of the interplay between the two. This coincides with the earlier result showing that down-regulation of HuR results in the half-life of β-actin mRNA\textsuperscript{12}. This also resulted in the loss of cytoskeletal functions, mainly due to a loss of β-actin stress fibers.

In the present study we used small interfering RNA (siRNA)-mediated gene knockdown to determine if HuR regulates β-actin expression, and whether the down-regulation of β-actin, as seen in our previous results of keratoconus corneas, affects the cells motility and proliferation. The siRNAs has been the most widely used method for sequence-specific gene knockdown, and to investigate gene function \textit{in vitro}. siRNA mediates gene-specific silencing primarily via recognizing and inducing degradation of the mRNA of target genes\textsuperscript{14}. Our results show that HuR regulates β-actin expression in corneal stromal fibroblasts and furthermore its down-regulation affects both cell motility and cell proliferation.
Methods

Human Corneas

The normal corneas were obtained from Alabama Eye Bank and the KC corneal buttons (8 mm in diameter) were obtained following surgery from a local corneal surgeon. Normal corneas and KC corneal buttons were stored in Optisol (Chiron Ophthalmics, Irvine, CA) and recovered within 12 h 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, and was performed to the tenets of the Declaration of Helsinki for research involving human subjects.

Cell Culture and Transfection

The epithelium and endothelium were scraped and removed from KC and normal corneas. The stroma was incubated overnight in collagenase (Worthington; 1 mg/ml). The cells were washed in Dulbecco’s modified Eagles medium (DMEM) (Invitrogen) containing 1% antibiotics (Penicillin-Streptomycin solution, Mediatech Inc.). The cells were then seeded onto 6 well plates (Corning) in DMEM medium containing 10% fetal
bovine serum (Hyclone) and 1% antibiotics (Penicillin-Streptomycin solution), and were maintained at 37°C in a 5% -CO₂-humidified air.

**Gene Knockdown Studies**

Knockdown of HUR and β-actin in normal human corneal fibroblast was done using small interfering RNA (siRNA) duplex. The cells will plated at 1.5x 10⁵ cells per well in a 6-well plate and grown overnight at 37°C with 5% CO₂. The silencer select predesigned and validated siRNA sequences was purchased from Ambion (Lifetechologies, Invitrogen, CA). The siRNA sequence target the different coding regions of GAPDH (used as a positive control), β-actin, HuR were used. Also a nonspecific, scrambled siRNA duplex was used as a control. Optimum concentration was determined by transfecting cells with different concentrations of siRNA, and 10 nanomolar was found to be the optimum concentration for corneal fibroblasts. We used scrambled sequence and the sequence that targets GAPDH mRNA as our siRNA controls. The sequences that either specifcally targets HUR mRNA (siHUR), or the β-actin mRNA (siβ-actin) were diluted in OPTI-MEM1 reduced medium (Invitrogen) and tarsfected using Lipofectamine plus (Invitrogen) using the manufacturer’s protocol. The cells were processed 24 -72 h after transfection for RNA and protein. SiRNA sequences, 

**HuR-** 5’-3’ GCGUUUAUCCGGUUUGACAtt  
5’-3’ UGUCAAACCGGAUAAACGCaa;  
**β-actin-** CCUGUACACUGACUUGAGAtt  
UCUCAAGUCAGUGUACAGGta,
**Preparation of Tissue Extract and Western Blot**

The proteins were recovered from corneal fibroblasts after transfection and were homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 2.5% Sodium deoxycholate, 0.1% SDS and 50 mM Tris pH 8.0). The samples were then transferred to grinding flasks (volume 3 ml), made of polytetrafluoroethylene containing Tungsten carbide balls and were frozen in liquid nitrogen. Next, these were homogenized by using tissue Mikro-Dismembrator (Sartorius).

**Western Blot**

30 μg protein samples were lysed with 1xSDS gel (Laemmli, 1970 ) loading buffer and subjected to 12% SDS-PAGE using 12% polyacrylamide gels. Proteins were transferred to a PVDF membrane (Bio-Rad) by using a semi-dry transfer cell (Bio-Rad, Trans-blot SD), and blocked for 1 h at room temperature with 3% nonfat-milk in PBS-Tween20. The membranes were probed with anti-β-actin monoclonal antibody (Sigma) at a 1: 100 dilution, followed by incubation in goat anti-mouse secondary antibody-conjugated to IRD dye (at 1: 10,000 dilutions). Blots were scanned using Odyssey Imaging system (LI-COR Biosciences). The blot was stripped using a mild stripping solution for 1 h and followed by washing 3X with PBS-Tween 20. The membranes were then blocked as described above and re-probed with anti-vimentin monoclonal antibody (Sigma; at 1:200 dilution), followed by secondary antibody incubation as described above. Blot was scanned again using Odyssey Imaging system (LI-COR Biosciences).
**Immunofluorescence-Confocal Imaging**

The normal corneal fibroblast was seeded on 18 mm cover-glasses for 24 h and then the cells were transfected 72 h as described above. Next, the cells were fixed for 30 minutes at room temperature with 4% formaldehyde, and washed 3X with PBS. The cells were permeabilized by incubating in 0.5% Triton X-100 in PBS for 10 minutes, followed by washing 3X in PBS. The cells were then incubated with a blocking solution containing 10% normal serum and 0.5% BSA in PBS for 1 h, and incubated with primary antibody at 4°C for 24 h. Next, the cells were washed 3X in PBS and followed by secondary antibody incubation for 1 h in dark. The cells were washed 3X and incubated with Hoechst nuclear stain for 10 min. These were washed 1X in PBS, and mounted on to slides with a mounting media. The antibodies used in the study are listed in Table 1. Mouse IgG was used as a negative control with same protein concentration as that of the primary antibodies. The confocal imaging was done using Zeiss LSM 710 confocal microscope (High Resolution Imaging Facility, UAB).

**Cell Migration Assay**

A modified Boyden chamber was used for the cell migration assay. We used a 96 well format ChemoTx system (Neuro Probe, Inc. MD), and the experiment was done according to the manufactures protocol. Forty eight hours after transfection of the siRNA duplexes, the corneal fibroblasts were trypsinized and resuspended in serum free medium and seeded in the upper chamber. The reservoir of the chamber was filled with complete medium containing 10% fetal bovine serum (FBS). Cells were allowed to migrate to the lower surface through the filter for 22 h at 37°C. The cells that remained in the top
membrane surface were wiped out thoroughly using moistened Kim wipes. Next, the membrane was cut out and inverted on to slide. The cells that had migrated to the lower surface were fixed using 4% formaldehyde and stained with Hoechst nuclear stain. The images were taken using Zeiss microscope, and multiple images from the triplicates were analyzed. The cells were counted from six images that were taken in triplicates and data were presented with standard deviation.

**Wound Healing Assay**

The cells were plated at 1.5x 10^5 cells per well of a 6-well plate and grown overnight at 37°C with 5% CO₂. The 80% confluent cells were treated with the siRNA as described above and after 72 h a straight line was scratched using a sterile 1ml disposable serological pipette. To remove the debris and smooth the edge of the scratch, the cells were washed with 1 ml of the growth medium. The images were taken using a Nikon Eclipse TS100 microscope at 0 h and 24 h after the scratch with Nikon coolpix camera attached to the microscope. All the siRNA treatment and the scratch assay were done in triplicates. The cells were counted at 0 hr and after 24 h post-scratch, and experiment was done in triplicates.

**Results**

*HuR Knockdown Leads to a Decrease in β-actin Expression at Both Transcriptional and Translational Levels*

In order to mimic the effect of a gene function, we used RNAi-mediated gene silencing for β-actin or HuR. The GAPDH and scrambled sequence was used as positive and negative control for transfection, respectively. The sequence of the siRNA duplex were selected from coding region of the target mRNA’s and the transfection was done as described in the Method section. The target mRNA levels were analyzed using RT-PCR (Figures 1A, 1B and 1C) 24 h after transfection. Figure 1A shows the β-actin gene expression after transfection with the scrambled siRNA, GAPDH, β-actin or HuR. The expression of β-actin is down-regulated after gene-silencing of β-actin gene, and also on HuR gene-silencing, suggesting that HuR has a regulatory role in the β-actin gene expression. Figure 1B shows the HuR gene expression after transfection with the scrambled siRNA, GAPDH, β-actin or HuR. The expression of HuR is down-regulated when HuR gene was silenced, whereas it had no effect the scrambled siRNA, GAPDH and β-actin. Therefore, β-actin gene-silencing has no effect on HuR gene expression. Figure 1C shows GAPDH gene expression after transfection with the scrambled siRNA, GAPDH, β-actin or HuR. The GAPDH gene expression showed a decrease after it was silenced but the effect was not as prominent as the effects seen on β-actin and HuR gene-silencing. Figure 1D shows Western blot of β-actin expression after transfection with the scrambled siRNA, GAPDH, β-actin and HuR. The Western Blot data shows a reduced expression of β-actin or HuR relative to vimentin, used as control. The results support the above described RT-PCR analysis. This blot was stripped using a mild stripping solution as described in the Method section. Next, the same blot was reprobed with anti-vimentin antibody to visualize its expression as a control.
Localization of Proteins after Down-Regulation of their Genes using Immunohistochemical-Confocal Imaging.

For confocal imaging normal corneal fibroblasts were seeded on coverslips as described in the Method section. The cells were then transfected with scrambled siRNA, GAPDH, β-actin or HuR siRNA respectively, and cultured for 72 h. After 72 h, the cells were fixed and the immunofluorescence analysis was done as described in the Method section. The immunoreactivity to anti-GAPDH antibody was analyzed in normal corneal fibroblasts transfected with scrambled siRNA or GAPDH siRNA (Figure 2). Figure 2A is the Hoechst nuclear stain and Figure 2B the immunoreactivity to anti-GAPDH antibody in normal corneal fibroblasts after transfection with scrambled siRNA. Figure 2C is an overlay of Figures (A) and (B). The GAPDH expression had no effect on scrambled siRNA as shown in the Figure 2B. Figure 2D is the Hoechst nuclear stain; Figure 2E immunoreactivity to anti-GAPDH antibody in normal corneal fibroblasts after transfection with GAPDH siRNA. Figure 2F is an overlay of Figures (D) and (E).

Together, the results show that the GAPDH expression has been down-regulated in normal corneal fibroblasts after transfection with GAPDH siRNA (Figure 2E).

The immunoreactivity to anti-HuR antibody in normal corneal fibroblasts transfected with GAPDH siRNA, HuR siRNA or β-actin siRNA were analyzed (Figure 3). We analyzed the affect of HuR expression after silencing GAPDH gene (Figures 3B). Figure 3A is the Hoechst nuclear stain and Figure 3B the immunoreactivity to anti-HuR antibody in normal corneal fibroblasts after transfection with GAPDH siRNA. Figure 3C is an overlay of Figures 3(A) and 3(B). We also analyzed the affect of HuR expression after silencing β-actin gene (Figure 3E). Figure 3D is the Hoechst nuclear stain and Figure 3E the immunoreactivity of anti-HuR antibody in normal corneal fibroblasts after
transfection with β-actin siRNA. Figure 3C is an overlay of Figures 3(D) and 3(E). Next, we analyzed the expression of HuR after HuR gene silencing (Figure 3H). Figure 3G is the Hoechst nuclear stain and Figure 3H the immunoreactivity of anti-HuR antibody in normal corneal fibroblasts after transfection with HuR siRNA. Figure 3I is an overlay of Figures 3(G) and 3(H). Together, results show that the HuR expression was down-regulated in normal corneal fibroblasts after transfection with HuR siRNA, whereas, β-actin and GAPDH gene silencing had no affect on HuR expression (Figure 3B and 3E).

The above results confirms that β-actin has no regulatory influence on the HuR gene expression. We further analyzed whether HuR regulates β-actin expression, using immunoreactivity to anti-β-actin antibody after HuR gene silencing of normal corneal fibroblasts (Figure 4J). We also analyzed the expression of β-actin after transfection with GAPDH, β-actin or scrambled siRNA in normal corneal fibroblasts (Figure 4). The F-actin was determined by using rhodamine-labeled phalloidin (toxin from the mushroom Amanita phalloides). Figure 4A is the Hoechst nuclear stain and Figure 4B the immunoreactivity to anti-β-actin antibody in normal corneal fibroblasts after transfection with scrambled siRNA. Figure 4C is F-actin staining with rhodamine-labeled phalloidin and Figure 4D is an overlay of Figures 4(A), 4(B) and 4(C). We also analyzed the affect of GAPDH knockdown on β-actin expression in corneal fibroblasts (Figure 4E to M). Figure 4E is the Hoechst nuclear stain and Figure 4F the immunoreactivity of anti-β-actin in normal corneal fibroblasts after transfection with GAPDH siRNA. Figure 4G is the F-actin staining with rhodamine-labeled phalloidin and Figure 4H is an overlay of Figures 4(E), 4(F) and 4(G). Together, the results show that β-actin expression was affected by GAPDH gene silencing, whereas gene silencing with scrambled siRNA had no affect on
β-actin gene expression. Next we analyzed the affect of β-actin expression on HuR gene silencing (Figures 4I to K). Figure 4I is the Hoechst nuclear stain; Figure 4J the immunoreactivity of anti-β-actin antibody in normal corneal fibroblasts after transfection with HuR siRNA. Figure 4K is the F-actin staining with rhodamine-labeled phalloidin, and Figure 4L is an overlay of Figures 4(I), 4(J) and 4(K). A similar analysis of β-actin knockdown are shown in Figure 4M to 4O. Figure 4M is the Hoechst nuclear stain and Figure 4N the immunoreactivity of anti-β-actin antibody in normal corneal fibroblasts after transfection with β-actin siRNA. Figure 4O is the F-actin staining with rhodamine-labeled phalloidin and Figure 4P is an overlay of Figures 4(M), 4(N) and 4(O). The down-regulation of β-actin gene after HuR and β-actin gene silencing (Figures 4J and 4N) supported our earlier results of the Western blot analysis. However, the down-regulation of β-actin after gene silencing of GAPDH (Figure 4F) was a surprise to us.

**β-Actin Gene Silencing Affects γ-Actin**

Next, β-actin gene silencing affect on the γ-actin expression was analyzed. For this, normal corneal fibroblast were seeded on to a coverglass, and the transfection with β-actin siRNA was done as described above. The cells were fixed after 72 h post-transfection and confocal images were taken (Figure 5). Figure 5A shows the Hoechst nuclear stain; Figure 5B the immunoreactivity of anti-γ-actin antibody in normal corneal fibroblasts 72 h after transfection with β-actin siRNA. Figure 5C shows the F-actin staining with rhodamine-labeled phalloidin, and Figure 5D is an overlay of Figures 5(A), 5(B) and 5(C). The results further supported the earlier observation that β-actin gene silencing had no effect on γ-actin expression.
Effects of Gene Silencing on the Migration of Normal Corneal Fibroblasts

Cell migration is an important biological process and is mainly due to the actin cytoskeleton, in particular β-actin has been shown to be essential to the process. The modified Boyden chamber assay was used to determine if targeted deletion of HuR and β-actin affects migration in normal corneal fibroblasts. The corneal fibroblasts were transfected with scrambled, GAPDH, HuR or β-actin siRNA and after 72 h, the cells were trypsinized and seeded on to the Boyden chamber as described in the Method section. The cell migration was analyzed 24 h after seeding in the Boyden chamber. The images were taken using Zeiss AxionPlan 2 Imaging System Microscope at the Core Facility of Vision Sciences Research Center at University of Alabama at Birmingham (Figure 6). Figure 6A shows the migrated cells (pointed arrow denotes the cell) in the normal corneal fibroblast and Figure 6B shows the migrated cells after transfecting scrambled siRNA in normal corneal fibroblast. Figure 6C shows cell migration following GAPDH gene silencing. Figure 6D shows the migration of cells after HuR gene silencing while Figure 6E shows cell migration after β-actin gene silencing. Following the migration assay, cells were counted using Image J software, using multiple images and standard deviation analysis (Figure 6F). Figure 6F shows the cells migrated per image. Together, the results show that the transfection treatment (scrambled siRNA) had no effect on the cell migration, and was similar to that of the normal cell migration (Figures 6A and 6B). GAPDH gene silencing resulted in reduced cell migration compared to the scrambled siRNA. Gene silencing of β-actin and HuR significantly reduced the ability of cells to migrate, and with β-actin gene silencing the migration was almost abolished.
Although it is known that β-actin is an essential component of cell migration\textsuperscript{15}, the HuR’s role in cell migration has been shown for the first time in corneal fibroblasts.

**Effects of Gene Silencing on Wound Healing**

To simulate wound healing of normal corneal fibroblast an artificial gap so called scratch wound was created on a confluent cell monolayer. Cells around the newly created gap migrate to close the gap. Both cell migration and proliferation participate in this wound healing process. A normal corneal fibroblast cell layer, grown to 85% confluency was transfected with scrambled, GAPDH, β-actin or HuR siRNA with non-transfected cells used as controls. The scratch was made 72 h after transfection, as described in the Method section, the dotted line indicates the wounded area (Figure 7). The images were taken at 0 h and 24 h post-scratch using a phase contrast microscope with a Nikon camera (Figure 7). The cells that migrated to the region between the dotted lines (wound) were counted from multiple images and standard deviation also analyzed (Figure 7K). Figure 7A shows normal cells with a scratch at 0 h and 7B shows after 24 h. Figure 7C shows the scratch made in the cell layer that was treated with scrambled siRNA while 7D shows the picture of 24 h post-scratch. Figure 7E shows the scratch made in the cell layer that was treated with GAPDH siRNA, 7F shows 24 h post-scratch. Figure 7F shows scratch made in the cell layer was treated with HuR siRNA, 7G shows 24 h post-scratch. Figure H shows the scratch made in the cell layer treated with β-actin siRNA, Figure I shows 24 h post-scratch. The normal corneal fibroblast layer had almost 100% wound closure (the region between the dotted line) after 24 h as was the case of scrambled siRNA. Transfection itself did not have any affect on wound healing. Wound healing was
decreased after GAPDH gene silencing. HUR- and β-actin gene silencing significantly decreased wound closure.

Discussion

Broad, flattened, quiescent keratocytes (neural crest-derived cells) constitute 5% of corneal stroma, lying parallel to the collagen lamellae. Keratocytes play an important role in corneal transparency by maintaining a functional stroma via secretion of stromal extracellular matrix and collagen that is needed for corneal strength, transparency and also as macrophages during the stress of corneal infection or injury. The cells synthesize and secrete collagen (mainly type I and type V collagen), proteoglycans (keratocan, lumican, and mimican with keratin sulfate side chains, and high levels of corneal crystallins, namely transketolase aldehyde dehydrogenase class 1A1). When quiescent keratocytes are cultured in the presence of serum or growth factors, they become mitotic and develop phenotypically into fibroblasts, as observed during wound healing. Under normal condition, the keratocytes become active after injury differentiating into fibroblasts and myofibroblast-like cells. The fibroblast growth factor-2 and platelet-derived growth factor stimulate differentiation of keratocytes into fibroblasts, and TGF-β stimulation leads to myofibroblasts.

Our previous results showed that β-actin is down-regulated in the corneal stroma of patients with keratoconus, which seems to be due to a reduced levels of stabilizing factor (HuR) for β-actin mRNA (Manuscript submitted to Invest.Opth.Vis.Sci., 2012). The major cellular functions of β-actin is to provide mechanical support in the cytoskeleton, help in cell motality and act in signal transduction of cytoplasm with
surroundings. In the present study, we used gene silencing to determine the physiological consequences of the loss of β-actin, and whether HuR regulates β-actin expression. Gene silencing by using RNA interference has been a popular method for *in vitro* gene functional studies using a method in which introduction of a double stranded RNA (dsRNA), homologous in sequence to the silenced gene, results in post-transcriptional gene silencing.24

Our RT-PCR analysis showed that gene silencing of β-actin for 24 h resulted in its down-regulation (Figure 1A), also supporting our previous finding of HuR regulation of the β-actin gene. This was further confirmed by the Western blot analysis that showed that silencing the HuR gene resulted in down-regulation of β-actin gene expression (Figure 1D). In this study, vimentin was used as an internal control. A 54-nucleotide (nt) segment, termed as Zipcode has been identified as a regulatory sequence in the 3′-untranslated region (3′UTR) of β-actin mRNA sequence7. Further, several zipcode binding proteins (ZBP-1) have been identified that bind to the zipcode sequence and regulate the β-actin mRNA localization8. The 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 ARE and the poly(A) tail *in vitro*9, 10. The mRNA of HuR is ubiquitously expressed in all proliferating cells, and is the most important post-transcriptional regulators of gene expression11. The β-actin mRNA has long half-life5, 13, and HuR binding to U-rich element is involved in the mRNA stability and affects the half-life of β-actin mRNA12. Together, the results of this study showed that silencing of HuR gene resulted in down-regulation of β-actin at both transcriptional and translational levels.
In this study, confocal imaging was used to localize proteins that were affected by
gene silencing. The silencing of GAPDH resulted in an absence of GAPDH expression in
normal corneal fibroblasts (Figure 2E), whereas its expression was not affected with
scrambled siRNA where the protein was localized in the cytoplasm. On silencing the
HuR gene its expression was decreased (Figure 3H), whereas its expression was not
affected after silencing with GAPDH, β-actin (Figures 3B and 3E) or scrambled (Figure
not shown). Next, we analyzed the expression of HuR (Figure 3H). On silencing HuR
gene or â-actin gene itself, a decrease in expression of â-actin was observed (Figures 4J
and 4N). Confocal imaging further confirmed that HuR has a regulatory role in the â-
actin expression. Surprisingly, there was a decrease in β-actin gene expression occurring
after GAPDH gene silencing (Figure 4F). GAPDH is a part of the glycolytic enzyme
complex but also functions as an actin-binding protein, but its role is not fully
understood.

Among the six actin isoforms, two are striated muscle-associated (α-skeletal and
α-cardiac muscle actins), two smooth muscle-associated (α- and γ- smooth muscle actins)
and two are cytoplasmic (β-and γ-actin)\(^2\). 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\(^3\). The actin isoforms have highly conserved amino acid sequences. Analysis of γ-
actins, after β-actin gene silencing in normal corneal fibroblasts, showed no effect
(Figure 5B). Recent studies using knockout mouse models have also shown that β-actin
and γ-actins have distinct phenotypes\(^25\).

One of the major functions of â-actin but not that of γ-actins, is the cell motility\(^25\).
Over expression of β-actin results in plasma membrane protrusions and cell migration\(^26\).
Our cell motility analysis using Boyden chamber showed that β-actin gene silencing drastically affected cell migration (Figure 6E). Cell migration was also affected when HuR was down-regulated (Figure 6D). However, no change in cell migration, either in the normals cells nor after treatment with scrambled siRNA was observed. GAPDH gene silencing did affect cell migration, possibly due to its known function as an actin-binding protein.

Keratocytes repopulate in stroma at the site of the injury by combination of proliferation and migration. Silencing β-actin and HuR resulted in significant reduction in wound healing as evidenced by reduction in the number of cells closing a wounding gap created by a scratch in a confluent cell layer (Figures 7H and 7J). In contrast, complete wound healing occurred in normal corneal fibroblasts, and also in normal corneal fibroblasts treated with scrambled siRNA, whereas GAPDH siRNA also showed reduced number of cells. Together, results suggest importance of β-actin and HuR genes in wound healing.

In summary, our findings suggest that the reduced number of keratocytes during KC could be a consequence of the down-regulation of β-actin, which could be due to its reduced mRNA stability by relative HuR levels in KC vs normal corneal stroma. Additionally, the down-regulation of β-actin and HuR in corneal fibroblasts could also significantly diminished cell migration and proliferation during wound healing.

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References

Table 1: List of antibodies used in the study

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<th>Antibodies</th>
<th>Dilution</th>
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<tr>
<td>β-actin (Sigma)</td>
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<tr>
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</tr>
<tr>
<td>Rhodamine-Phalloidin (Cytoskeleton Inc)</td>
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Figure 1 RT-PCR analysis of β-Actin and Western blot Analysis (A): RT-PCR analysis for β-actin gene in normal corneal stromal fibroblast 24 h after treatment (gene silencing) of control (siScrambled), siGAPDH, siβ-actin and siHuR. (B): RT-PCR analysis for HuR gene in normal corneal stromal fibroblast 24 h after treatment of control (siScrambled), siGAPDH, siβ-actin and siHuR. (C): RT-PCR analysis for GAPDH gene in normal corneal stromal fibroblast 24 h after treatment (gene silencing) of control (siScrambled), siGAPDH, siβ-actin and siHuR. (D) Western blot analysis for β-actin gene using anti-β-actin antibody in normal corneal stromal fibroblast 24 h after treatment (gene silencing) of control (siScrambled), siGAPDH, siβ-actin and siHuR. Also the blot was reprobed with anti-vimentin antibody, used as a loading control.
Figure 2: Localization of GAPDH after Gene Silencing. The normal corneal fibroblast was treated with scrambled siRNA or GAPDH siRNA. The immunoreactivity of GAPDH was analyzed 72 h after transfection. (A): Fibroblasts (after treatment with scrambled siRNA) stained with Hoechst nuclear stain. (B): Immunoreactivity of fibroblasts (after treatment with scrambled siRNA) with anti-GAPDH antibody. (C): Overlay of (A) and (B). (D): Fibroblasts (after treatment with GAPDH siRNA) stained with Hoechst nuclear stain. (E): Immunoreactivity of fibroblasts (after treatment with GAPDH siRNA) with anti-GAPDH antibody. (F): Overlay of (D) and (E). Note that the GAPDH gene silencing, down-regulated the expression of GAPDH, but it was not affected in the scrambled siRNA.
Figure 3: Localization of HuR after Gene Silencing. The normal corneal fibroblast was treated with GAPDH siRNA, β-actin siRNA or HuR siRNA. The immunoreactivity of HuR was analyzed 72 h after transfection. (A): Fibroblasts (after treatment with GAPDH siRNA) stained with Hoechst nuclear stain. (B): Immunoreactivity of fibroblasts (after treatment with GAPDH siRNA) with anti-HuR antibody. (C): Overlay of (A) and (B). (D): Fibroblasts (after treatment with β-actin siRNA) stained with Hoechst nuclear stain. (E): Immunoreactivity of fibroblasts (after treatment with β-actin siRNA) with anti-HuR antibody. (F): Overlay of (D) and (E). (G): Fibroblasts (after treatment with HuR siRNA) stained with Hoechst nuclear stain. (H): Immunoreactivity of fibroblasts (after treatment with HuR siRNA) with anti-HuR antibody. (I): Overlay of (G) and (H). Note that the GAPDH siRNA and β-actin siRNA treatments had no affect on the HuR expression and its nuclear localization. HuR gene silencing significantly reduced the expression of HuR, (absence of green fluorescence in the nucleus).
**Figure 4: Localization of β-actin after Gene Silencing.** The normal corneal fibroblast was treated with scrambled siRNA, GAPDH siRNA, β-actin siRNA or HuR siRNA. The immunoreactivity of anti-β-actin antibody was analyzed 72 h after transfection. (A): Fibroblasts (after treatment with scrambled siRNA) stained with Hoechst nuclear stain. (B): Immunoreactivity of fibroblasts (after treatment with scrambled siRNA) with anti-β-actin antibody. (C): Fibroblasts (after treatment with scrambled siRNA) stained Rhodamine-labeled phalloidin stain. (D): Overlay of (A), (B) and (C). (E): Fibroblasts (after treatment with GAPDH siRNA) stained with Hoechst nuclear stain. (F): Immunoreactivity of fibroblasts (after treatment with GAPDH siRNA) with anti-β-actin antibody. (G): Fibroblasts (after treatment with GAPDH siRNA) stained Rhodamine-labeled phalloidin stain. (H): Overlay of (E), (F) and (G).
(I): Fibroblasts (after treatment with HuR siRNA) stained with Hoechst nuclear stain. (J): Immunoreactivity of fibroblasts (after treatment with HuR siRNA) with anti-β-actin antibody. (K): Fibroblasts (after treatment with HuR siRNA) stained Rhodamine-labeled phalloidin stain. (L): Overlay of (I), (J) and (K). (M): Fibroblasts (after treatment with β-actin siRNA) stained with Hoechst nuclear stain. (N): Immunoreactivity of fibroblasts (after treatment with β-actin siRNA) with anti-β-actin antibody. (O): Fibroblasts (after treatment with β-actin siRNA) stained Rhodamine-labeled phalloidin stain. (P): Overlay of (M), (N) and (O). Note that β-actin and HuR gene silencing affected β-actin expression. Scrambled siRNA transfection in normal corneal fibroblast had no affect on β-actin expression, whereas GAPDH siRNA had a drastic affect on the β-actin expression.
Figure 5: Localization of γ-actin in Corneal Fibroblast after β-actin Gene Silencing. The immunoreactivity of anti-γ-actin antibody was analyzed 72 h after β-actin gene silencing. (A): Fibroblasts (after treatment with β-actin siRNA) stained with Hoechst nuclear stain. (B): Immunoreactivity of fibroblasts (after treatment with β-actin siRNA) with anti-γ-actin antibody. (C): Fibroblasts (after treatment with β-actin siRNA) stained Rhodamine-labeled phalloidin stain. (D): Overlay of (A), (B) and (C). Note that the β-actin gene silencing had no affect on the γ-actin expression and localization.
Figure 6: Analysis of Cell Migration after Gene Silencing (n=3). (Modified Boyden chamber). Migration of cells (Arrows) (A): Migration of normal corneal fibroblast. (B): Migration of cells after treatment with scrambled siRNA. C: Migration of cells after treatment of normal corneal fibroblast with GAPDH siRNA. (D): Migration of cells after treatment of normal corneal fibroblast with HuR siRNA. (E): Migration of cells after treatment of normal corneal fibroblast with β-actin siRNA. (F): Bar graph of cell migration on different treatment (A to E). Cells were counted from 6 different frames. All the treatments were done in triplicates and also standard deviations were calculated.
Figure 7: Affect of Wound Healing after Gene Silencing (n=3). Scratch was made using 1ml sterile serological pipette in normal corneal fibroblasts after gene silencing for GAPDH, β-actin or HuR. The images were taken at 0 h and 24 h. The dotted lines define the area where scratch was made. (A): shows normal cells with a scratch at 0 h. (B): Cells migrating to the site of wound after 24 h. C: Scratch at 0 h in cells that were treated with scrambled siRNA. (D): Migration of cells to the site of wound after 24 h in cells that were treated with scrambled siRNA. (E): Scratch at 0 h in cells that were treated with GAPDH siRNA. (F): Migration of cells to the site of wound after 24 h that were treated with scrambled siRNA.
(G): Scratch at 0 h in cells that were treated with HuR siRNA. (H): Migration of cells to the site of wound after 24 h in cells that were treated with HuR siRNA. (I): Scratch at 0 h in cells that were treated with β-actin siRNA. (J): Cells migrated to the site of wound after 24 h in cells that were treated with β-actin siRNA. (K): Bar graph showing the number of cells migrated after 24 h following the scratch in normal, siScrambled, siGAPDH, siHuR or siβ-actin. The scratch assay was done in triplicates and standard deviations were calculated.

(n =3)  *Statistically Significant* Figure 7 (Continued):
Conclusion

Specific Aim 1

Our aim was to identify protein profiles and their relative abundance in the epithelium and stroma of normal and keratoconus corneas. The rational was that these changes could lead to an understanding of the potential mechanism of the disease. A systems biology approach, which allows to prediction of a potential pathway based on changes of protein abundance and their interactions, was used.

Two different techniques, the shotgun proteomics [Nano-ESI-LC-MS (MS)^2] and 2D-DIGE methods were used to identify the protein profiles. The shotgun proteomics is a powerful technique in which the proteins in a biological sample are proteolytically-digested prior to their separation, and analyzed using Nano-ESI-LC–MS(MS)^2. In this method, the MS/MS spectra obtained were searched against the three known protein databases to determine the identity of the peptides in the samples. Following database searching, data set organization, and peptide statistical validation were performed using the PROVALT algorithm (or the peptide prophet or protein prophet algorithm) as integrated in the software package, ProteoIQ (BioInquire, Athens GA). In contrast to the first method, in the second 2D-DIGE analysis, the 2D-gel electrophoretically-separated Cy2-, Cy3- and Cy5-labeled proteins were analyzed using Decyder software, the desired excised protein spots were trypsin-digested, the peptides analyzed by the Q-TRAP mass spectrometric method, and their identity determined by using Mascot searches.
We identified 104 epithelial and 44 stromal proteins from both normal and KC corneas, and also quantified relative changes in the levels of selected proteins in both tissues using spectral counts in a proteomic data set. Relative to normal corneal epithelial proteins, six epithelial proteins (lamin-A/C, keratin type I cytoskeletal 14, tubulin beta chain, heat shock cognate 71 kDa protein, keratin type I cytoskeletal 16 and protein S100-A4) exhibited up-regulation in KC corneas and five proteins (transketolase, pyruvate kinase, 14-3-3 sigma isoform, phosphoglycerate kinase 1, and NADH dehydrogenase kinase (quinone) 1) showed down-regulation. A similar relative analysis showed that three KC stromal proteins (decorin, vimentin and keratocan) were up-regulated and five stromal proteins (TGF-betaig h3, serotransferrin, MAM domain-containing protein 2 and isoforms 2C2A of collagen alpha-2[VI] chain) were down-regulated. KC corneal epithelium exhibited up-regulation of four proteins (serum albumin, keratin 5, L-lactate dehydrogenase and annexin A8) and down-regulation of four proteins (FTH1 [Ferritin heavy chain protein 1], calpain small subunit 1, heat shock protein beta 1 and annexin A2). Similarly, a similar relative analysis of stroma by this method also showed up-regulation of aldehyde dehydrogenase 3A1 (ALDH3A1), keratin 12, apolipoprotein A IV precursor, haptoglobin precursor, prolipoprotein and lipoprotein Gln in KC corneas. The Ingenuity Pathway Analysis (IPA), Ingenuity Systems Inc, USA, was used to analyze the molecular functions of the proteins that changed during keratoconus disease process, and also how these proteins fit into the known canonical pathway were also analyzed. The IPA was used to identify the most significant biological functions, disease and canonical pathways from the IPA knowledge database.
In summary, we separately analyzed epithelial and stromal proteins from individual KC and age-matched normal corneas by two unique newer techniques. The major changes were seen in the structural proteins of both epithelium and stroma of KC corneas compared to normal corneas, suggesting structural remodeling of both the tissues during the development and progression of keratoconus. The proteins that are involved in proliferation, growth and migration were down-regulated in KC epithelium. Further, our results also showed that the iron homeostasis is disrupted in KC corneas, which might result in an increased oxidative damage. The results suggested two potential pathways for KC development, one that involved an increased iron deposition due to the disruption of the iron signaling pathway. Iron deposits are seen during KC as Fleisher’s ring and also iron is known to increase oxidative stress. The second potential pathway model for KC development involved the TGF-β and actin cytoskeleton. Our focus has been the second pathway that involved the downstream targets of TGF-β on actin cytoskeleton of keratoconus keratocytes.

Specific Aim 2

Keratocytes play an important role in corneal transparency by maintaining a functional stroma through the secretion of stromal extracellular matrix, collagen and corneal crystallins 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,
cytokinesis, endo-/exocytosis, cell division, signal transduction, mRNA localization and transcription. The absence of β-actin at an embryonic stage was found to be lethal in a transgenic mouse model (Bunnel et al 2011). 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.

We investigated the expression of β-actin gene in stromal keratocytes of normal and KC corneas. Results showed that β-actin was down-regulated at both transcriptional and translational levels in the KC stroma but not in the stroma of normal cornea and Fuch’s dystrophic corneas. Even on culturing keratocytes to fibroblasts from both normal and keratoconus stroma, β-actin expression was down-regulated only in the cells of the KC corneas.

The high expression levels of β-actin is important for the above described cellular processes and this is maintained in vivo by mRNA stability. A 54-nucleotide (nt) segment termed as Zipcode has been identified as a regulatory sequence in the 3’-untranslated region(3’UTR) of β-actin mRNA sequence. The mRNA of HuR is ubiquitously expressed in all proliferating cells, and is one of the most important post-transcriptional regulator of gene expression. Our real-time PCR analysis of HuR gene showed that it is down-regulated by 4.7-fold in KC corneas compared to normal corneas, which suggest that the down-regulation of HuR affected β-actin mRNA stability. This is supported by earlier results showing that down-regulation of HuR resulted in the reduced half-life of β-actin mRNA (Dormoy-Raclet, 2007). This in turn might result in the loss of cytoskeletal functions, mainly due to a loss of β-actin stress fibers.
Specific Aim 3

β-actin mRNA has a long half-life (Olave et al., 2002; Condeelis et al., 2005) and HuR binding to U-rich element is involved in the mRNA stability and affects the half-life of β-actin mRNA. To investigate this further, we used small interfering RNA (siRNA) mediated gene knockdown to determine if HuR regulates β-actin expression (concluded in Specific Aim 2) and also to determine if the loss of β-actin as seen in keratoconus affects the cells motility and proliferation. The expression of β-actin was down-regulated after gene silencing of β-actin gene, and its expression also decreased when HuR gene was silenced. This confirmed our earlier results that HuR has a regulatory role in β-actin expression and also those of Dormoy-Raclet, 2007. Additionally, the β-actin gene silencing had no effect on γ-actin expression. On the other hand, HuR gene silencing affected HuR expression and also down-regulated the expression of β-actin, but had no affect on the localization of protein. This was inconcordane with our earlier results showing that in keratoconus there was a down-regulation of β-actin and HuR.

Cell migration is an important biological processes and is mainly due to the actin cytoskeleton and β-actin is a major component of the process. We performed the modified Boyden chamber assay to determine whether targeted deletion of HuR and β-actin affected the migration of corneal fibroblasts. Gene silencing of β-actin or HuR significantly reduced the ability of cells to migrate and was almost abolished in the case of β-actin. GAPDH which was used a positive control for gene silencing also showed reduced cell migration compared to scrambled siRNA (negative control), and this could be due to fact that GAPDH is also an actin-binding protein.
Following a wound, the repopulation in cornea often occurs through a combination of proliferation and migration of corneal keratocytes. On silencing β-actin and HuR genes, a significant reduction in the levels of wound healing was seen, whereas complete wound healing occurred in the normal corneal fibroblasts, and also in the normal corneal fibroblast treated with scrambled siRNA. The GAPDH siRNA also showed reduced numbers of cells and effect of GAPDH gene silencing on cell migration and proliferation need to be further investigated.

The overall conclusion from this study was that HuR has a regulatory role in β-actin expression, and affects the cell migration and proliferation during corneal wound healing. Because cell migration is important for normal development and also during tissue damage, we hypothesize that KC disease progression could be due to the inability of keratocytes to fill the void created by their apoptosis. The trigger for kerocyte apoptosis could be the HuR down-regulation-induced down-regulation of β-actin gene expression.
General Reference


US, N. E. I. Facts about the cornea and corneal disease keratoconus.


APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL
Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on September 29, 2013. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator: JOSEPH, ROY
Co-Investigator(s):
Protocol Number: X080724001
Protocol Title: Identification of potential markers for keratoconus

The IRB reviewed and approved the above named project on 6/17/11. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.
IRB Approval Date: 6/17/11

Date IRB Approval Issued: 6/17/11

Marilyn Doss, M.A.
Vice Chair of the Institutional Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.