Modeling Keratoconus Using Induced Pluripotent Stem Cells

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PURPOSE. To model keratoconus (KC) using induced pluripotent stem cells (iPSC) generated from fibroblasts of both KC and normal human corneal stroma by a viral method.

METHODS. Both normal and KC corneal fibroblasts from four human donors were reprogrammed directly by delivering reprogramming factors in a single virus using 2A “self-cleaving” peptides, using a single polycistronic lentiviral vector coexpressing four transcription factors (Oct 4, Sox2, Klf4, and Myc) to yield iPSC. These iPSC cells were characterized by immunofluorescence detection using of stem cell markers (SSEA4, Oct4, and Sox2). The mRNA sequencing was performed and the datasets were analyzed using ingenuity pathways analysis (IPA) software.

RESULTS. The generated stem cell-like clones expressed the pluripotency markers, SSEA4, Oct4, Sox2, Tra-1-60, and also expressed pax6. Our transcriptome analysis showed 4300 genes, which had 2-fold change and 870 genes with a q-value of <0.05 in keratoconus iPSC compared to normal iPSC. One of the genes that showed difference in KC iPSC was FGFR2 (down-regulated by 2.4 fold), an upstream target of Pi3-Kinase pathway, was further validated in keratoconus corneal sections and also KC iPSC-derived keratocytes (down regulated by 2.0 fold). Both normal and KC-derived keratocytes expressed keratocan, signature marker for keratocytes. KC iPSC-derived keratocytes showed adverse growth and proliferation and was further confirmed by using Ly2924002, a PI3k inhibitor, which severely affected the growth and differentiation in normal iPSC.

CONCLUSIONS. Based on our result, we propose a model for KC in which inhibition FGFR2-Pi3-Kinase pathway affects the AKT phosphorylation, and thus affecting the keratocytes survival signals. This inhibition of the survival signals could be a potential mechanism for the KC-specific decreased cell survival and apoptosis of keratocytes.

Keywords: keratoconus, induced pluripotent stem cells, FGFR2, disease modeling

Collagen and extracellular matrix are the essential components of the cornea that maintain its shape and transparency. Keratocytes of the mature cornea are responsible for the production and maintenance of vital corneal components, including extracellular matrices, lumican, and keratocan.1,2 These interact with collagen fibrils to modulate their size and spacing.1–5 The causative factor(s) behind the development of keratoconus is not well understood. What is known is that the keratoconus (KC) corneal stroma shows reduced numbers of keratocytes relative to normal corneas.4,5 This is caused by apoptosis,6 as evidenced by the upregulation of several apoptotic genes in the keratocytes.7 The trigger inducing the apoptosis is unknown, but an increased sensitivity of keratocytes to IL-1 after epithelial injury has been suggested as a factor.8 Keratocytes from both normal and keratoconus, when cultured in the presence of serum, generated cells with phenotypes of fibroblasts, and myofibroblasts, whereas, KC keratocytes when grown in the absence of serum showed poor survivability compared with normal corneal keratocytes.9 Currently, there is no animal model to assist in the study and understanding of the factors causing this disease.

It is known that embryonic stem cells (ESC) derived from blastocysts have the property to divide indefinitely while maintaining pluripotency.10,11 This capacity of ESC provides an opportunity to understand disease mechanisms, screen a variety of drugs, and treat patients with various injuries and diseases.12 However, the use of ESC has been limited due to ethical controversies. For this reason, research on induced pluripotent stem cells (iPSC) has gained momentum in recent years. Reprogramming somatic cells to iPSC by the use of classic Yamanaka factors holds great promise for bringing regenerative medicine into a clinical setting.13 This factor-based reprogramming of adult somatic cells has been generated in mice14,15 and in humans.16,17 Induced pluripotent stem cells hold a great promise to identify genetic changes that guide KC disease process. Generating iPSC from adult human somatic cells is a viable alternative for stem cells production. Recently, iPSC generated from swine fetal fibroblasts were differentiated into photoreceptors cells in vitro, and were able to be integrated into damaged neural retina.18 It has also been shown that iPSC generated from normal human keratocytes could be used to treat corneal injuries19 and also keratocytes have been differentiated from human ESC (hESC).20 Experiments designed to explore iPSC, generated from KC fibroblasts, could be of tremendous importance in understanding the disease mechanism. Adult human fibroblasts have been successfully reprogrammed via overexpression of pluripotency-related transcription factors, including OCT4 (POU5F1), SOX2, KLF4, and c-MYC, to establish human iPSC (hiPSC).21 Several studies have demonstrated close similarities between hiPSC and hESC.
at the molecular and genetic levels, which are based on their morphologies and functions.\textsuperscript{16,22,25} Disease-specific iPSC lines have also been generated from patients with sporadic or genetically inherited diseases.\textsuperscript{17,24,28} Like hESC, iPSC have similar characteristics, allowing them to differentiate into various cell types in response to developmental cues. Recent reports have shown that disease-specific iPSC from spinal muscular atrophy, Friedrich's Ataxia and QT syndrome, were able to successfully recapitulate the disease phenotypes from these disorders.\textsuperscript{29–35}

In this study, we have used the iPSC generated from kerocytes of both normal and KC corneas, and performed transcriptome analysis to generate a molecular model for KC. The first objective of this study was to generate iPSC from both normal and KC fibroblasts. In the absence of an animal model for KC, iPSC generated from KC fibroblasts, provide a valuable tool to study the disease at both molecular and genetic levels. The second objective of the study was to analyze the transcriptome changes using RNA sequencing (RNA-Seq) analysis. This high-throughput sequencing-based method has changed the way in which transcriptomes are studied and has many advantages. RNA-Seq directly sequences complementary DNAs (cDNAs) using high-throughput DNA sequencing technologies followed by the mapping of the sequencing reads to the genome. This comprehensive understanding of the complexity of eukaryotic transcriptomes allows for the identification of exons and introns, identification of transcription start sites,\textsuperscript{34} and also the identification of new splicing variants. Additionally, it allows for the precise quantification of exon expression and splicing variants.\textsuperscript{35–40}

**METHODS**

**Human Cornea**

Corneas from KC patients were recovered within 1 to 2 hours following surgery. Deep anterior lamellar keratoplasty, using the “big bubble” technique, removed the full thickness of corneal stroma from six individuals and six age-matched normal corneas were used for this study. Each cornea was placed in Optisol, (Chiron Ophthalmics, Irvine, CA, USA) and refrigerated within 15 minutes. Studies were initiated within 1 hour. Only the epithelium needed to be removed because the endothelium was not present in the specimen. Age-matched normal and healthy corneas that were suitable for transplantation, stored in Optisol at 4°C by the Alabama Eye Bank (Birmingham, AL, USA), were obtained within 12 hours after their enucleation. These normal corneas were fit for corneal transplantation but did not meet the criteria because of plasma dilution so could not run the serologic test. But otherwise these corneas were healthy enough for transplantation. A central 8-mm trephination was performed. The epithelium and endothelium were gently scraped off, leaving the stroma to be used in these experiments. Four normal corneas from the Eye bank, (58- [identified as N1] and 38-year-old females [N4], and 54- [N2] and 59-year-old males [N3]), and four age-matched KC corneas (41- [K1] and 35-year-old females [K4], and 31- (K2) and 44-year-old males [K3]) were used for generating corneal fibroblasts cultures and two KC (39-year-old female [K5] and 64-year-old male [K6], and two age-matched normal 48-year-old female [N5] and 72-year-old male [N6]), were used for immunohistochemical analysis. All six individuals entered into this study had a long history of severe KC with resulting poor vision and a history of contact lens failure (details provided as Supplementary Data S1). Their topography revealed a central keratometry of 61.5 to 96-5 diopters (D), with scarring over the apex. A corneal transplant offered their only chance for improved visual acuity. The procurement of human corneas in this study was approved by the institutional review board of the University of Alabama at Birmingham (Birmingham, AL, USA), following the tenets of the Declaration of Helsinki for Research Involving Human Subjects.

**Viral Preparation**

To generate FUW-SOKM-harboring lentiviruses (LvSOKM),\textsuperscript{41} HEK-293 cells (3 × 107 cells per 10-cm dish) were cotransfected with a mix of 10 μg of pMD2.G plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope, 10 μg of psPAX2 packaging plasmid, and 12 μg of the FUW-SOKM (Addgene plasmid 20325; Addgene, Cambridge, MA, USA), a lentiviral Sox2-P2A-OCT4-T2A-Klf4-E2A-cMyc-harboring shuttle, using lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instruction. The media was changed to Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) after 24 hours. The next day, the supernatant was collected, centrifuged at 805g for 5 minutes to remove cellular debris, filtered through a 0.45-μm filter (Whatman cellulose filter paper; Sigma-Aldrich Corp., St. Louis, MO, USA), and concentrated by ultrafiltration. The virus pellets were resuspended in DMEM/F12 containing 10% fetal bovine serum (FBS).

**Cell Culture and Viral Infection of Stromal Fibroblasts**

Four individual cultures were generated from both normal (N1, N2, N3, and N4) and KC (K1, K2, K3, and K4) corneas, and all the experiments were done in triplicate. Primary cultures of human corneal fibroblasts were established as described earlier\textsuperscript{11} and passage two was used for this study. The corneal stromal fibroblasts from four normal and four KC corneas (passage 2) were infected in a 6-well plate (Corning, Tewksbury, MA, USA) by 1 × 106 LvSOKM particles with multiplicity of infection (MOI) = 10, diluted in 2 mL of DMEM/F12 medium supplemented with 10% FBS (HyClone, Logan, UT, USA) and polybrene (8 μg/mL; Sigma-Aldrich Corp.). The cells were reinoculated after 24 hours with virus particles as described above. The cells were trypsinized and cultured in TeSR-E7 media (StemCell Technologies, Inc., Vancouver, Canada) for 6 days with regular change of media and followed by mTeSR1 media (StemCell Technologies, Inc.). After 21 days, the individual hES-like clones were manually picked using a sterile tip and transferred to low attachment plates for 5 days in mTeSR1 media (StemCell Technologies, Inc.). The ES-like clones were dissociated using enzyme-free cell dissociation reagent (StemCell Technologies, Inc.), and the ES-like clones were analyzed for stem cell markers.

**Immunofluorescence Imaging**

The stem cell-like clones from both normal and keratoconus corneas were identified under a microscope, and were recognized according earlier published data.\textsuperscript{41} These clones were removed manually using sterile pipette tips under an inverted microscope, seeded on to 18-mm glass cover slips, fixed with 4% formaldehyde for 30 minutes at room temperature, and washed 3× with PBS. The cells were then incubated with a blocking solution containing 10% normal serum and 0.5% BSA in PBS for 1 hour, followed by incubation with a primary antibody at 4°C for 24 hours. The following individual primary antibodies were used: Tra-1-60 (mouse mAB, 1:1000), Sox2 (rabbit mAB, dilution 1:400), SSEA4 (mouse mAB, 1:500), and Oct4 (rabbit mAB, dilution 1:400) (Cell Signaling, Danvers, MA, USA). The cells were washed three times in PBS, followed by incubation with a secondary antibody for 1 hour in the dark (Invitrogen) with the following secondary antibodies (goat anti-rabbit IgG-AlexaFluor 488 conjugate, dilution 1:1000) and goat anti-mouse IgG-AlexaFluor.
Modeling Keratoconus Using iPSC

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

Real-time PCR quantifications were performed using the BIO-RAD iCycler iQ system (Bio-Rad, Hercules, CA, USA), in a 96-well reaction plate for a total volume of 25 \( \mu L \) RNA was extracted as described above. Primers were designed using Primer3 for the following genes: Sox2, Pax6, KERA, and GAPDH and the primers are described in Supplementary Table S1. The reaction mixture included 12.5 \( \mu L \) of Real-Time SYBR Green PCR master mix, 2.5 \( \mu L \) of reverse transcription product, 1 \( \mu L \) of forward and reverse primer, and 8 \( \mu L \) of DNase/RNase free water. The reaction mixtures were initially heated to 95°C for 10 minutes to activate the polymerase, followed by 40 cycles, which consisted of a denaturation step at 95°C for 15 seconds, annealing at 57°C for 60 seconds, and elongation step at 72°C. The qRT-PCR data were analyzed by the comparative \( \Delta \Delta C_t \) method.

Immunohistochemical Analysis of FGFR2 Expression

FGFR2 was administered in 50 undifferentiated normal stem cell clones by treatment with 10 \( \mu M \) Ly292004 (Sigma-Aldrich Corp.) in the presence of keratocyte differentiating media. The isolation and maintenance of normal iPSC clones were done as described above. The effects of FGFR2 inhibition were studied for 24 hours as described above in the Methods section. Normal iPSC were differentiated in the absence of Ly292004.

Statistical Analysis

The statistical significance was determined by Student’s \( t \)-test and with statistical significance set at \( P < 0.05 \) and also \( q \) value with statistical significance set at \( q < 0.05 \).

RESULTS

Generation of iPSC From Normal and Keratoconus Corneal Fibroblasts

Forced expressions of the transcription factors, Oct4, Sox2, Klf4, and c-Myc (Fig. 1A) in normal and KC fibroblasts (Figs. 1B, 1D), induced the formation of cell colonies that were similar to embryonic stem-like clones in appearance (Figs. 1C, 1E). These stem cell-like colonies were fully formed after 21 days post induction. Our phase-contrast microscopic images of iPSC from both KC and normal corneas showed more than 25 hESC-like colonies per 10^3 cells (Figs. 1C, 1E). The clones were analyzed for stem cell markers as described in the Methods section. Immunocytochemical analysis revealed that these stem cell-like colonies expressed pluripotency markers Tra-1-60/Sox2 in both stem cell-like colonies generated from normal and KC corneas.
These colonies also expressed the pluripotency marker SSEA4/Oct4 (Figs. 2C, 2E). We also analyzed for Sox2 and PAX6 expression in keratoconus clones. For the expression analysis, RNA was isolated (as described in the Methods section) from stem cell clones generated from KC and were compared with the fibroblasts cells generated from both normal and keratoconus. Both Sox2 and Pax6 were upregulated in KC iPSC compared with fibroblasts cells (Fig. 2E).

**RNA-Seq Analysis**

High-throughput cDNA sequencing (RNA-Seq) can identify genes and splice variants and also quantify expression in a single assay. The RNA quality analysis showed it to be of high quality, and RNA integrity number (RIN) for all samples was 9.5 or greater. The read alignment with TopHat for the all samples is provided as supplementary data (Supplementary Table S2). A total of 28,665 genes were analyzed by RNA-Seq (Supplementary Table S3), and of these 4300 genes showed greater than or equal to 2-fold change and 870 genes with q value less than 0.05 in KC compared with normal iPSC (Supplementary Table S4). There were 208 genes that showed infinity (expressed either in KC or normal); most notable were MIR100 and MIR186. The major genes that showed differences were COL5A1, FGFR2, EZR, DPYSL4, MMP9, TGFBR3, COL4A1, COL4A2, IL6, SFRP1, and HGF. The datasets were analyzed using IPA, which revealed genes affected were connected to several pathways, mainly to growth and proliferation, cell survival, cell cycle, cancer, and several others (Fig. 3). One of the genes affected was FGFR2, which was downregulated by 2.4 (Log2)-fold, as well as the downstream targets that it affects (Fig. 4).

**Differentiation of Keratocytes From Both Normal and KC iPSC**

The stem cells clones were isolated and were cultured in keratocyte differentiating media as described in the Methods.
Expression of FGFR2

Our RNA-Seq analysis results showed that FGFR2 expression was –2.4 (log2-fold change) decreased in the stem cells that were generated from fibroblasts of KC compared with stem cells from normal corneas. Immunohistochemical analysis of FGFR2 expression was decreased in stromal keratocytes and epithelial sections of KC corneas compared with normal corneas (Figs. 6A, 6B). We also analyzed the expression of FGFR2 in keratocytes that were differentiated from normal and KC iPSC (Figs. 6C, 6D), respectively) using immunocytochemical analysis as described in the Methods section. The FGFR2 expression levels were decreased in KC iPSC-derived keratocytes compared with that of the normal iPSC. We further analyzed the pixel intensity in $n = 22$ cells from both types of differentiated cells using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) as described earlier.46 The FGFR2 expression levels were decreased by 2-fold in keratocytes differentiated from KC (Fig. 6E).

Proliferation of Keratocytes Generated From iPSC

Stem cells were differentiated from both normal and KC corneas as described in the Methods section. Figures 7A, 7C, and 7E, show iPSC generated from three different normal corneas (N1, N2, and N3) in the presence of differentiation media at day 1 and Figures 7B, 7D, and 7F show keratocyte differentiation after 7 days. Similarly, Figures 7G, 7I, and 7K show iPSC generated from three different KC (K1, K2, and K3) and Figures 7H, 7J, and 7L show keratocyte differentiation after 7 days. The cells differentiated from KC iPSC had fewer numbers of cells compared with those differentiated from the normal iPSC (Figs. 7H, 7J, and 7L compared with 7B, 7D, and 7F). The differentiated cells were counted as described in the Methods section (Fig. 7M), shows that number of cells was decreased in cells differentiated from KC iPSC compared with...
We speculate that proliferation and migration of cells were adversely affected in KC iPSC-derived keratocytes compared with similar cells from normal corneal iPSC. Our previous results had shown that an inhibition of Pi3-Kinase (Pi3K) affected the migration and proliferation of normal corneal fibroblasts, similar to their treatment with β-actin siRNA.\textsuperscript{46} Pi3-Kinase is a downstream target of the FGF and the IGF pathways and affects both cell proliferation and growth. We used the phosphatidylinositol 3-kinase (PI3K) inhibitor Ly294002 to determine if inhibition of the FGF pathway leads to inhibition of both proliferation and differentiation of iPSC. Figures 7N and 7O show the effects of PI3K inhibition on keratocytes that were differentiated from normal iPSC in the absence and presence of the inhibitor, respectively.

![Figure 3: Ingenuity pathway analysis of genes that showed fold changes (≥ 2-fold and q value < 0.05) in iPSC of normal compared with KC corneas. Ingenuity pathway analysis revealed genes connected to growth factor signaling biology mainly cellular growth and proliferation, cellular movement and cell death and survival were affected during KC.](image-url)
Figure 4. Fibroblast growth factor receptors and genes that are involved in FGF pathway were analyzed by IPA in KC iPSC compared with normal iPSC. The genes that are upregulated or downregulated are shown. Fibroblast growth factor signal transduction. Formation of the FGF:FGFR:HS signaling complex causes the activation of the intracellular kinase domains and the cross-phosphorylation of tyrosines on the FGFRs. FRS2 interacts with the phosphorylated tyrosines and is phosphorylated itself. FRS2 then activates the adaptor protein Grb2 that associates with SOS, a nucleotide exchange factor that activates Ras. Ras is a small GTP binding protein that activates Raf, which activates MEK and that activates ERK, the downstream targets are the cell differentiation, cell growth, and morphogenesis. FRS2 activity also activates phosphoinositol-3 kinase, which activates AKT/PKB and it affects cell survival. PLC\(\gamma\) binds the activated FGFR by its SH2 domain and then generates inositol-1,4,5-trisphosphate and DAG from phosphotidylinositol-4,5-diphosphate resulting in the activation of protein kinase C and the release of intracellular calcium. The downstream targets also affect angiogenesis, which are through the JNK and p38MAPK.
DISCUSSION

Generation of patient-derived pluripotent stem cells (PSC) by somatic cell nuclear transfer (NT) has not been successful. However, directly reprogramming human somatic cells to produce iPSC by introducing a set of transcription factors linked to pluripotency has been successful.47 These types of cells overcome the impediments of using animal models, where human diseases have limited representation. In the last 3 years, over a hundred reports have been generated using disease-specific iPSC,17,24 including polygenic diseases such as Parkinson’s,48 and Alzheimer’s.49–51 and schizophrenia.52

We have, for the first time, generated cultures of iPSC from fibroblasts of KC and from normal corneas by both viral and nonviral methods (Joseph R, et al. IOVS 2014;55:ARVO E-Abstract 523). Our phase-contrast microscopic images of iPSC from both normal and KC corneal fibroblasts showed good embryonic, stem cell–like clones (Figs. 1C, 1E), and the stem cell–like clones were positive for each of the pluripotency markers. The advantage of reprogramming somatic cells to iPSC is the ability to reset the cellular identity back to the embryonic state, thus enabling studies of complex diseases such as KC. This genetic reprogramming was achieved by ectopic expression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) using either nonviral or retroviral methodology.53

A wealth of new data from studies of the epigenome, genome transcriptome, proteome, and metabolome analyses has led to the consensus that iPSC and ESCs are similar, and these iPSC harbor the molecular memory of their past.54 It is debatable whether subculturing iPSC induces mutation over time.55 To rule out this possibility, we have performed RNA-Seq analyses on both KC and normal corneal iPSC, without subculturing, so that mutations are not introduced under culture conditions. As stated above, in recent years transcriptome profiling by next-generation sequencing (RNA-Seq) has significantly improved the study of gene expression.56 Using RNA-Seq, we have provided, for the first time, a comprehensive assessment of the genes that are regulated in iPSC derived from KC fibroblasts compared with in iPSC derived from normal corneal fibroblasts. Our data show that KC iPSC exhibited significant downregulation of genes that were involved in cell proliferation, differentiation, cell cycle, and in cancer (aberrant cell proliferation; Fig. 3). Our RNA-Seq data also showed that FGFR2 was downregulated by 2.4-fold (Log2) in KC iPSC compared with normal corneal iPSC (Fig. 4). Further, it showed a reduction in the expression of collagen genes, actin binding protein (EZR), and ACTB,11 as has been reported previously. In this regard, it has been shown that COL5A1 has a single nucleotide polymorphism (SNP), suggested to be involved in corneal thinning in KC.57
A variety of enzymatic and genetic changes were found during the course of this study. **TGFBRIII** was upregulated in KC-iPSC (Supplementary Table S1). **TGFBRIII** inhibits proliferation and migration, and is downregulated in most types of cancers. The phosphorylation of **EZR** is important for the survival of epithelial cells through the activation of the PI3K/AKT pathway. **EZR** binds to p85 (a regulatory subunit of PI3K) in three-dimensional (3D) cell cultures of the epithelial cell line LLC-PK1. Our data also showed that 4300 genes showed greater than or equal to 2-fold change and 870 genes with q value less than 0.05 in KC compared with normal iPSC. **Polo-like-kinase 1 (PlK1)** was also downregulated in KC-iPSC compared with normal corneal iPSC (Supplementary Table S1), and is involved in the cell cycle regulation. Genes that showed changes in genome-wide association and other KC studies were also seen in the RNA-Seq analysis (Supplementary Tables S2, S3), and were: **COL5A1**, **FOXO1**, **HGF**, **LOXL2**, **IL6**, and **SFRP1**. Because it is known that iPSC carry molecular memory, the gene expression analysis by RNA-Seq on these iPSC would enable us to generate a molecular model for KC. Our expression analysis showed major changes in genes that are involved in cell proliferation and cell cycle, leading us to focus on one specific gene, **FGFR2**.

**Fibroblast growth factor receptor 2 (FGFR2)**, a tyrosine kinase receptor, activates the signal transduction pathways, which is mediated through the PI3K/AKT-signaling pathway. It is known that the PI3K/AKT pathway delivers an antipapoptotic signal. It has been shown that FGFR2, and its ligand FGF2, are expressed in the corneal keratocytes. Recently, it has been shown that FGFR2 knockout mice showed a thinner cornea and also an absence of keratocan expression. Keratocan is a marker for keratocytes, suggesting that differentiation of keratocytes was disrupted. Our immunohistochemical analysis of KC corneas also showed a reduced expression of FGFR2 compared with normal cornea (Fig. 5B). In addition, our immunocytochemical analysis on differentiated keratocytes from KC iPSC also showed that **FGFR2** gene expression was downregulated by 2-fold (Fig. 5E) and was supporting the RNA-Seq data. Taken together, our data and the data from the FGFR2 knockout mouse model provide evidence that FGFR2 downregulation could have a significant impact on the development of KC.

In order to understand the mechanism and function of the FGFR2 downregulation, we differentiated the iPS in the
FIGURE 7. Proliferation of differentiated keratocytes. (A, C, E) Three normal corneal iPSC in differentiating media (58-year-old female, 59- and 54-year-old males, denoted as N1, N2, and N3, respectively). (B, D, F) Normal iPSC differentiation and cell proliferation. (G, I, K) Three keratoconus iPSC in differentiating media (41-year-old female, 31- and 44-year-old males, denoted as K1, K2, and K3, respectively). (H, J, L) Keratoconus iPSC differentiation and cell proliferation. Differentiation and proliferation were affected in KC iPSC-derived keratocytes. (M) Quantification of number of differentiated cells. (N, O) Effects of inhibition of PI3 kinase by LY2924002. (N) Differentiation of normal stem cells in absence of the inhibitor, and (O) in the presence of the inhibitor. PI3 kinase inhibition severely affects differentiation and proliferation of normal iPSC-derived keratocytes.
presence of two different growth factors; FGF2 and insulin. Earlier studies have shown that keratocytes cultured in the media containing FGF2 have a fibroblastic phenotype, whereas with media-containing insulin has a dendritic morphology, but they both express keratocan, a marker for corneal keratocytes. Our results also showed a similar effect in the presence of insulin and FGF2, which expressed keratocan (marker for keratocytes), but the cells showed a spindle phenotype (Fig. 6). We have shown that iPSC generated from both KC and normal have the potential to differentiate to keratocytes when cultured in the presence of differentiating media. But when keratocytes from KC iPSC were maintained in differentiating media for 7 days, the cell proliferation was inhibited compared with those from normal corneas (Fig. 7M). Because KC iPSC showed that cells differentiated into keratocytes with keratocan as a positive marker for differentiation, it could be that KC cells fail to proliferate even in the presence of mitogen. The RNA-Seq data (Supplementary Table S1) also show that KC iPSC have an altered cell cycle. Taking together, FGFR2 downregulation could inhibit the proliferation of cells as seen in KC. Phosphorylation of FGFR triggers several signaling cascades, of which are the RAS-RAF-mitogen activated protein kinase (MEK)-extracellular regulated kinase (ERK pathway), the phosphatidyl-inositol 3-kinase (PI3K) pathway is involved directly or indirectly in JAK-STAT pathway. Fibroblast growth factor and IGF pathways have a common downstream target molecule, PI3K. By blocking this target with the specific inhibitor Ly292004, we showed that inhibition of PI3K resulted in inhibition of cell growth and proliferation in normal cells (Fig. 7O). These results suggest that both of these pathways are affected in KC stromal keratocytes and that an inhibitor of the downstream target PI3K in normal cells causes them to resemble the growth and proliferation of keratocytes.

**Figure 8.** A proposed model of signaling pathway in KC cornea that leads to keratocyte apoptosis during KC. It shows the FGF signal transduction that results in the phosphorylation of AKT leading to growth and proliferation, and is mediated through BAD phosphorylation. When this signal transduction is inhibited, it leads to cell apoptosis. This could be potential mechanism for keratocytes apoptosis in keratoconus cornea.
derived from KC. We have also shown earlier that when PI3K was inhibited using 1y292004, the growth and proliferation was affected in normal corneal fibroblast.44

Based on the results of this study, we have proposed a model for the disease process leading to KC (Fig. 8). In this model, we propose that the binding of ligands, (FGF2 to receptor tyrosine kinase [RTK]) leads to receptor activation through autophosphorylation. On binding of growth factors to receptor tyrosine kinase (FGFR2), the signals are transduced through PI3K that results in the phosphorylation of the downstream target, the serine-threonine kinase Akt. The Akt phosphorylation acts as a survival signal, resulting in the phosphorylation of Bcl-2-associated death promoter (BAD),73 thus suppressing apoptosis and promoting cell survival. Alternatively, when Akt phosphorylation is inhibited, apoptosis is promoted, thus preventing the phosphorylation of BAD, a proapoptotic factor. In series of events, where phosphorylated BAD binds to BCL-XL or BCL-2, suppress survival signals by inducing homodimer or heterodimer formation, leading to caspase 3–induced keratocyte apoptosis.

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