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Original Article

Isolation, Culture, Optimization and Validation of Human Corneal Stromal Keratocytes from Discarded Corneal Tissue

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Abstract

Introduction: Keratocytes are the major components of the human corneal stromal cell. Cell therapy by keratocytes can be used in some corneal diseases. Because keratocytes are mitotically quiescent; therefore, the cultivation of these cells is associated with challenges. The present study aimed to isolate, culture, and validate keratocyte cells from discarded corneal tissue based on optimizing some cultivation conditions.

Materials and Methods: In this experimental study, keratocytes were isolated from discarded corneal tissue. Different culture medium composition such as amniotic membrane extract, time, and the role of coating scaffolds was evaluated. Real-time PCR of specific genes were used to confirm the primary keratocyte cells compared to corneal epithelial cells. The specific genes were *keratocan*, *lumicane*, aldehyde dehydrogenase three members of family A1 (*ALDH3A1*), and *CD34*. In addition, immunocytochemistry (ICC) was used to confirm the expression of specific keratocan and lumican markers.

Results: Keratocytes was isolated and cultured in the culture medium containing amniotic membrane extract. Based on analyses, keratocan, lumicane, ALDH3A1, and CD34 gene expression in keratocytes was significantly higher than in the epithelial cells. Moreover, *keratocan* and *lumican* expression was detected in 92.5% and 91.1% of the cells, respectively. According to the results, the addition of amniotic membrane extract significantly increased the growth of keratocytes.

Conclusions: Our findings in this study showed that discarded corneal tissue can be used as a suitable source for obtaining keratocyte cells needed in corneal tissue engineering.

Keywords: Primary Cell Culture, Keratocytes, Cornea, Amniotic Membrane Extract, Tissue Engineering

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Introduction

The human eyes are very complex organs that play a very important in human life styles. However, this organ is affected by a wide range of disorders.[1] There are 36 million blind people worldwide, and there are currently 217 million cases with moderate to severe vision impairment, as well as 6.17 million (2.4%) with corneal problems.[2] One of the leading causes of blindness worldwide is the corneal diseases.[3-6]

The cornea is a transparent tissue and avascular section of the eye. Cornea plays an effective role in the optical system of the eye.[7] The human cornea is comprised of five different layers including Epithelium, Bowman's Layer, Stroma, Descemet's Membrane and Endothelium.[8] The stroma comprises more than 90% of the corneal thickness, , which is a mixture of collagen, proteoglycans, and keratocytes.[7] keratocytes are mitotically quiescent, and morphologically, flat and dendritically-shaped.[9] Moreover, they lie between and are parallel to the orthogonally arranged collagen lamellae, forming an interconnected cellular network.[10] These cells play an important role in maintaining corneal homeostasis and transparency through the production of collagen, proteoglycans, as well as corneal crystalline.[11] Any damage to the stromal layer affects the keratocytes and stimulates them to eventually turn into the active cell form, fibroblasts.[7,12] Cellular and molecular studies have shown that some corneal disorders such as keratoconus are related to changes in keratocytes.[11] Studies have also shown that keratocyte loss in keratoconus is significantly associated with disease severity.[12] When the cornea is injured, some corneal stromal keratocytes (CSKs) undergo apoptosis, whereas the surviving cells are activated to become stromal

fibroblasts and myofibroblasts, as a natural mechanism of wound healing. The stromal fibroblasts and myofibroblasts secrete abnormal extracellular matrix proteins, leading to corneal fibrosis and scar formation.[13]

Using keratocytes in the treatment of corneal stromal diseases has been suggested in some studies.[14] Because keratocytes are mitotically quiescent, cultivation of these cells will face challenges such as their small number, or changes in the inherent characteristics of the cells, Therefore finding a solution to isolation and proliferation of keratocytes from corneal tissue is interesting for scientists. For the keratocytes culture, many complex growth factors have been used in different studies,[15,16] however, most of which are expensive and unaffordable.

Amniotic membrane extract is full of growth factors and is used as a supplement for corneal epithelial cell culture. Also, amniotic membrane extract eye drops have been used to proliferate and improve the corneal epithelium.[17] This study aimed to extract keratocytes from discarded corneas in a cheap and optimized culture medium and validate the primary cells using real time-PCR and immunocytochemistry.

Materials and Methods

Human Keratocyte Isolation

Twelve discarded human corneas from descemet stripping endothelial keratoplasty (DSEAK) remnants were collected from Bina Eye Hospital (Tehran, Iran). The cell isolation process started less than 72 h after the death of the donor. The tissues were washed with sterile phosphate-buffered saline (PBS) and placed in a Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DMEM/F12, Biosera, France) containing 0.3 g/10 ml of dispase solution (Stemcell Technologies, Germany) at 37 °C for 1 h. The corneal tissue was immersed in collagenase I (Sigma), (0.3 g/10 ml in DMEM/F-12) for 12 h in the shaker incubator at 150 rpm. The obtained solution was centrifuged at 1500 rpm for 5 min. The supernatant was removed and the collected cells were transferred into the 5 ml of a keratocyte-specific culture medium (KSCM) developed in this study. KSCM contained DMEM-F12 supplemented with 1% amniotic stromal extract (ASE, Lifecell, Iran), 1% L-Ascorbic acid 2-phosphate, 1% insulin-transferrin-sodium selenite (ITS-Gibco), and 1% non-essential amino acid (NEAA-Gibco). Keratocytes were then cultured in a 6-wells cell culture plate and the medium was refreshed every 48 h (Figure 1). In this study, cells were divided into two groups. The first group of cells was cultured on the tissue culture polystyrene (TCPS), and the second group of cells was cultured on a collagen-coated plate. Also, the cell expansion and morphology was evaluated daily for 7 days.

Collagen Coating Procedure

Since keratocytes are located in the stroma which is consist of collagen bundles, to mimic this structure, the cells were cultured on a substrate covered with collagen. The coating was performed by adding 0.001 g of rat tail collagen to 10 ml of sterile distilled water containing 40 µl of acetic acid (Merck, Germany) and placed in a shaker incubator at 150 rpm for 24 h at 37 °C. After that, the obtained solution was passed through a 0.22 µm filter. In the next step, 200 µl of the solution was added to each well of a 24-well plate and kept in a 4 °C for 24 h. The supernatant in each well of the plate was then discarded, wells were washed twice with cold sterile PBS, and the plate was exposed to UV for 20 min.

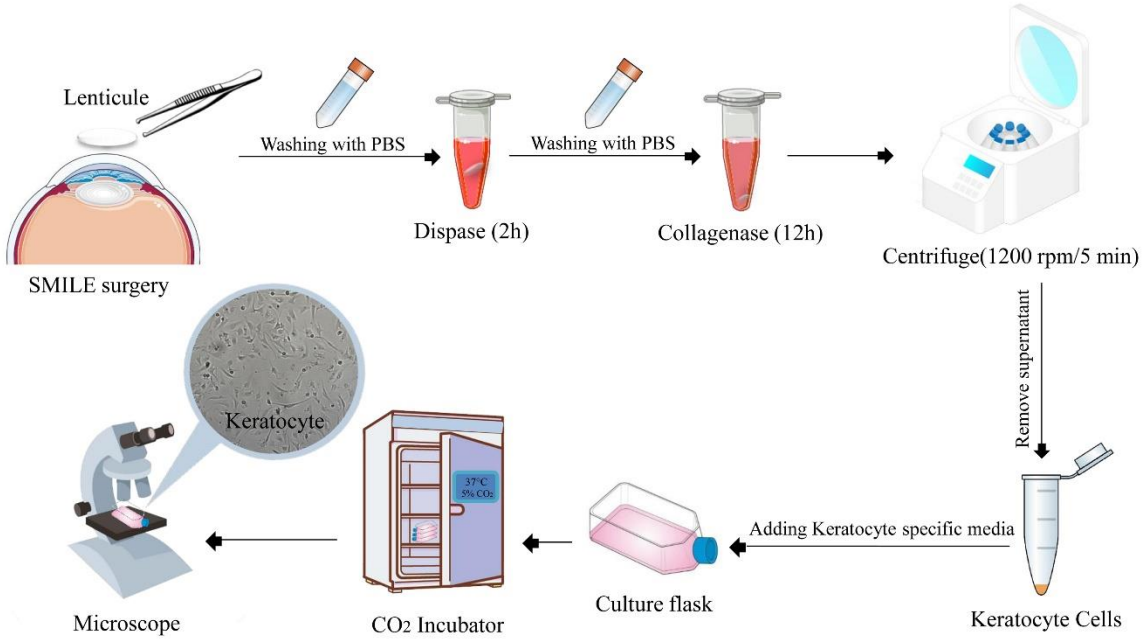

Figure 1. Stages of Isolation and Culture of Human Keratocyte from Discarded Corneal Tissue.

Keratocyte Cell Confirmation

Morphology

Keratocytes have a dendritic morphology with an expanded cell network and a compact cell body, which enables them to form a three-dimensional network of interconnected cells in the corneal stroma.[18] The cultured plates were placed under an inverted microscope (Leica, Germany) equipped with a camera and the cells were imaged to confirm the morphology of keratocytes.

Real-time PCR

Real-time PCR of specific genes were used to confirm the primary keratocyte cells compared to corneal epithelial cells. For this purpose, Total RNA was extracted by TRIzol solution (RiboEX geneall) and its concentration was measured by spectrophotometer (NanoDrop-1000, Wilmington, Delaware). cDNA synthesis was carried out using revert aid first-strand cDNA synthesis kit (BioFact, South Korea). cDNA was used for 40 cycle PCR in Rotor-gene Q real-time analyzer (Corbett, Australia). RT-PCR was performed using Maxima TM SYBR Green/ROX qPCR Master Mix (Fermentas) followed by a melting curve analysis to confirm the PCR specificity. Each reaction was repeated three times and the average threshold cycle was used for data analysis by Rotor-gene Q. The immortal corneal epithelial cell line HCE-2 was used as a control group for the expression of the desired genes. The results were evaluated by t-test and $p \leq 0.05$ was considered statistically significant. The gene-specific primers used for *keratocan*, *lumican*, *ALDH3A1*, *CD34*, and smooth muscle alpha-actin (*α-SMA-1*) are shown in Table 1. Glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) gene was used as internal control.

Immunocytochemistry

Immunocytochemistry (ICC) was used to confirm the expression of specific keratocan and lumican markers. Human adipose mesenchymal stromal cells (hASCs) and corneal keratocytes were cultured in 48-well plates. They were fixed with 4% paraformaldehyde (Sigma) at 4 °C for 20 min, and then, washed three times with cold PBS. Triton 0.3% was added to the samples, and after 30 min, samples were washed with PBS. As a blocking agent, 10% goat serum was added and removed after 45 min. Next, the primary antibodies for Keratocan and Lumican (Biorbyt Ltd, UK) were poured on samples and the plate was refrigerated for 24 h at 4 °C. On the next day, samples were washed using PBS, and secondary antibodies (Goat Anti-Rabbit IgG & Goat Anti-Mouse IgG [FITC] [Biorbyt Ltd, UK]) were added to the samples at a dilution of 1 in 150. The plate was incubated at 37 °C for 1 h in a dark place. For nucleic acid staining, DAPI (4′,6-diamidino-2-phenylindole) (Sigma) was added and incubated for 20 min. Images were taken by a fluorescent microscope (Olympus, Japan), quantified by Image Processing software, and analyzed in Java (Image J) (Figure 5).

Table 1. Sequences of Primers Used for real-time PCR

Gene	Primer Sequence (5´-3´)	Product Size
keratocan	5´-ATCTGCAGCACCTTCACCTT-3´ 5´-CATTGGAATTGGTGGTTTGA-3´	167 bp
lumican	5´-CCTGGTTGAGCTGGATCTGT-3´ 5´-TAGGATAATGGCCCCAGGA-3´	194 bp
ALDH3A1	5´-CATTGGCACCTGGAACTACC-3´ 5´-GGCTTGAGGACCACTGAGTT-3´	191 bp
CD34	5´-CTTGGGCATCACTGGCTATT-3´ 5´-TCCACCGTTTTCCGTGTAAT-3´	292 bp
α-SMA-1	5´-CCGTGATCTCCTTCTGCATT-3´ 5´-CTGTTCCAGCCATCCTTCAT-3´	175 bp
GAPDH	5´-CAAGTTCAACGGCACAGTCA-3´ 5´-CCCCATTTGATGTTAGCGGG-3´	150 bp

Statistical Analysis

The results were analyzed using GraphPad Prism version 8 software. The repeated measures analysis of variance (ANOVA) and t-test were used for the statistical analysis of three or more separate experiments. Results were considered statistically significant at $p \leq 0.05$. All experiment was repeated independently at least three times.

Results

Confirmation of Primary Keratocyte

Morphology

The first step in this study was to obtain the appropriate volume of keratocyte cells. For this purpose, keratocytes were obtained from discarded corneal tissue and the keratocyte cells were isolated using collagenase/dispase treatment and cultured in an optimized medium. After 5 days, cells showed the standard structure and morphology of keratocytes, i.e smooth, spindle-shaped surface with dendritic appendages (Figure 2). According to the method mentioned in this study, 1×10^5 keratocytes cells were isolated from each DSEK sample. Also, the role of collagen as the coating agent was evaluated. As shown in figure 3, the number of expanded keratocyte on the surface of collagen coated wells are higher than TCPS.

Gene Expression of Specific Markers

In order to confirm the extracted keratocyte, the real-time PCR technique was used. The results showed that the expression of keratocyte marker genes including *keratocan*, *lumican*, *ALDH3A1*, and *CD34* is significantly higher compared to the HCE-2 cells. As shown in figure 4, the fold change ratio for expression of *keratocan*, *lumican*, *ALDH3A1*, *CD34*, *and α-SMA-1* was 1.92, 3.25, 1.65, 2.01, and 0.89 for keratocytes respectively. This amount for HCE-2 was 1.08, 0.75, 1.02, 0.85, and 1.54 respectively. The fold change differences between these cells was statistically significant.

Protein Expression of Specific Markers

Primary keratocytes confirmed by two specific markers including keratocan and lumican using immunocytochemistry. hASCs were also evaluated as the control group. Analyses of signals generated in the keratocyte and hASCs reported high protein expression of keratocan (92.5%) and lumican (91.1%)

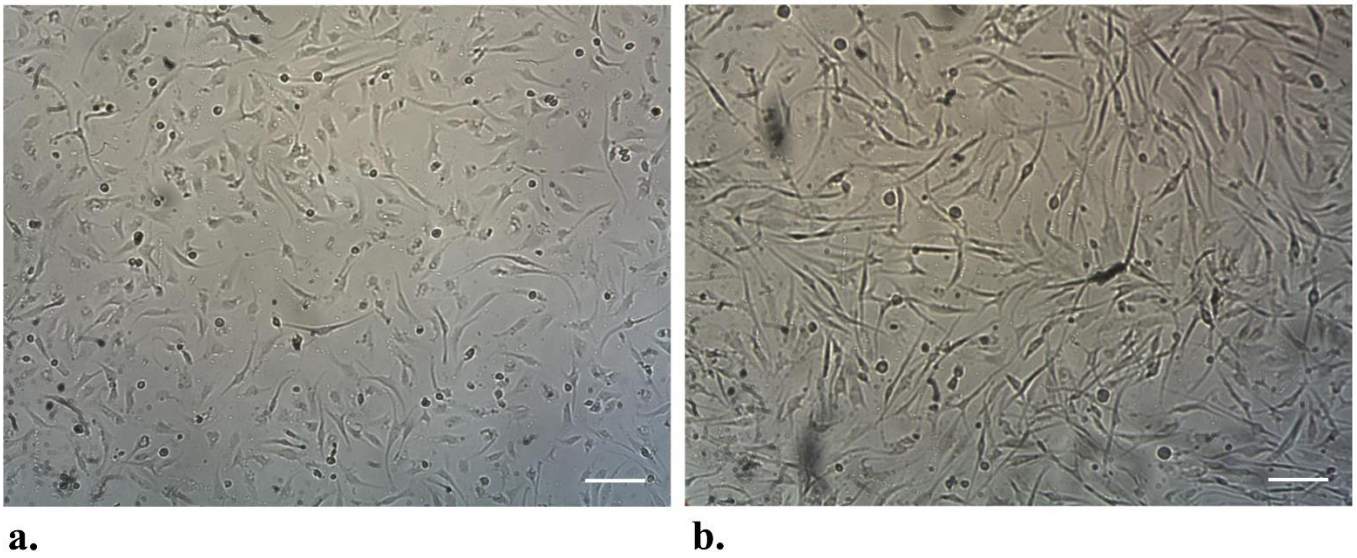

Figure 2. The Morphology of Keratocytes Obtained from Corneal Tissue. **a)** On the second day of cultivation, a large number of cells still do not have dendritic morphology. **b)** On the fifth day of culture, most of the cells acquired complete dendritic morphology (scale bars represent 50 µm).

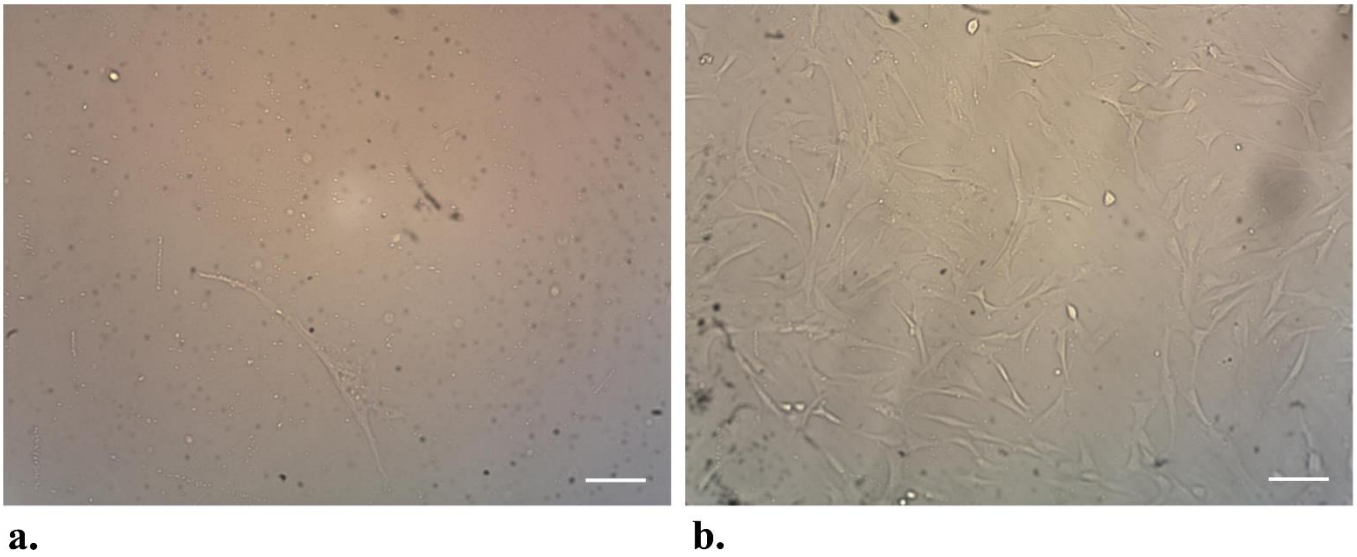

Figure 3. The Morphology of Keratocytes Obtained from Corneal Tissue. **a)** The Fifth day of cultivation on TCPS. **b)** The fifth day of cultivation on collagen-coated (scale bars represent 50 µm).

in keratocytes, while keratocan (9.1%) and lumican (11%) expression were much lower in hASCs (Figure 5 and 6).

Discussion

As the key cells in the corneal stromal layer, keratocytes have a critical role in corneal structure and, function. These cells are essential for the formation of extracellular matrixes (ECMs) and for communicating with extracellular environments.[19] Keratocytes produce matrix metalloproteases (MMPs), as well as collagen and glucosamine, both of which play an important role in establishing and maintaining stromal hemostasis.[20] Keratocytes in most vertebrates remain quiescent during their lifetimes and do not undergo mitosis or apoptosis. These cells are very sensitive and undergo apoptosis after corneal defects.[21] Keratocyte loss is associated with the severity degree of some corneal ectasia and healthy keratocytes can be used to treat many corneal disease such as keratoconus.[22] Since keratocytes are quiescent cells, the isolation and culture of these cells are challenging.[19,21]

In this study, we optimized the isolation and culture of keratocytes from discarded corneal tissue, and the extracted keratocytes were evaluated by standard methods including real-time PCR and ICC. Optimization of culture medium ingredients and using collagen coated plate for cell supporting, are the main innovation of this study. As shown in Figure 2, on the second day of cultivation, complete morphology was not

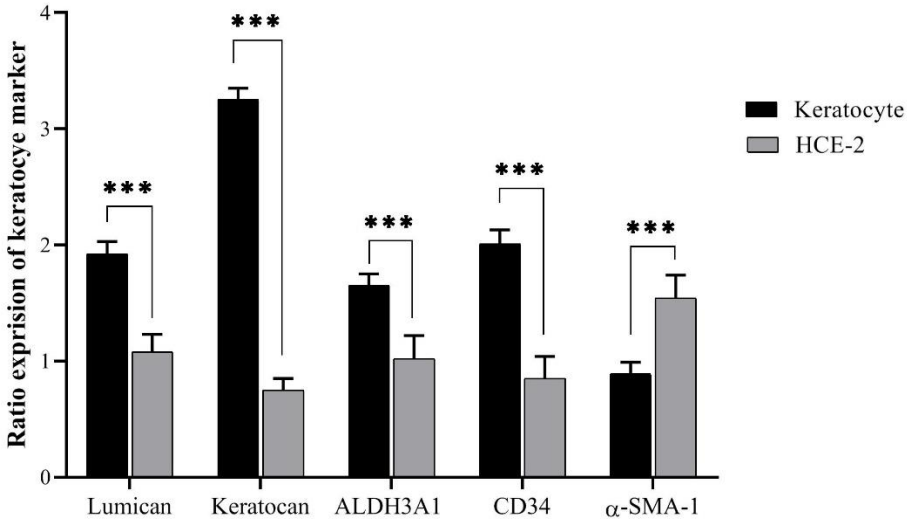

Figure 4. Real-time PCR Analysis of Keratocytes and HCE-2 Cells. Keratocyte cells showed a higher expression of markers. The three-star sign indicates a significant difference between the two groups ($p \leq 0.001$).

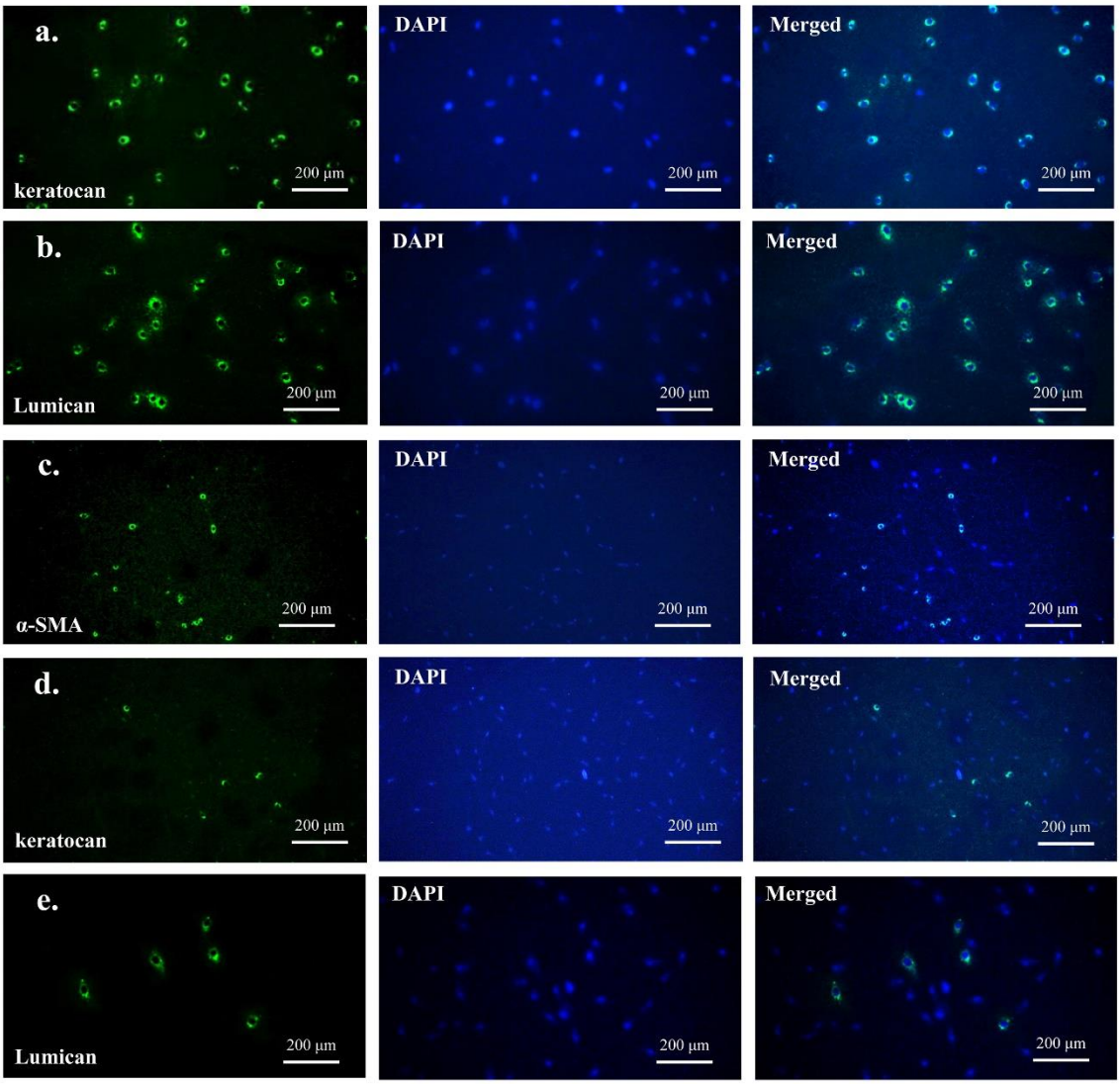

Figure 5. ICC Evaluation to Identify Specific Markers of Cultured Keratocytes. **a, b,** and **c)** Primary keratocytes extracted from the human cornea. The fluorescence signal for keratocan and lumican was detected respectively in 92.5% and 91.1% of cells. The presence of α.SMA signal was less than 21%. **d** and **e)** Expression of keratocan and lumican was very low in hASCs cells (scale bars represent 200 µm).

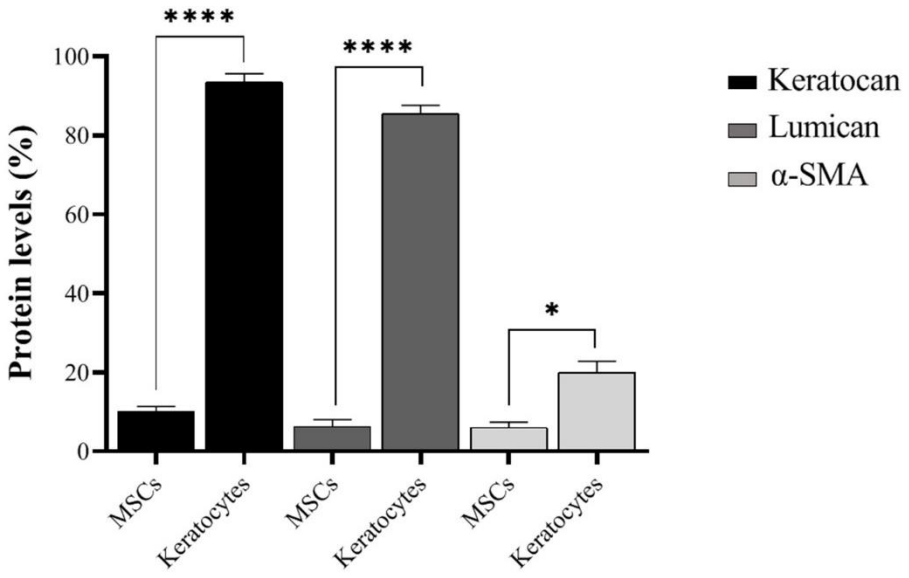

Figure 6. Percentage of Relative Expression of Keratocan, Lumican, and α.SMA markers in keratocytes and stem cells. The expression of keratocan, lumican, and α.SMA in keratocytes is significantly higher than that of hASCs (control group) (keratocan and lumican: $p \leq 0.0001$, α.SMA-1: 0.0174).

established in the keratocytes, and all the cells did not acquire morphology, meanwhile, on the fifth day, the best structure and morphology were observed in the keratocytes, and after that, no morphological change in this cell was observed. Therefore, the fifth day was considered as an optimized time for keratocyte isolation. Keratocyte cells were first cultured in DMEM/F12 culture medium containing 5% FBS, but no cells were obtained after 48 h. Meanwhile, by adding the amniotic membrane extract, attached and viable cells was seen on the culture plate. It shown that the amniotic membrane extract prevents fibroblastic transformation via the suppression of the TGF 1/2 pathway and is important to eliminate fibroblast growth and maintain keratocyte characteristics *in vitro*.[23-25] Since collagen is the main component of corneal tissue and keratocytes are located within the collagen fibers, collagen digestion will be the primary step in achieving the proper amount of these cells. In this study, the collagenase enzyme was used for this purpose. Collagenases are enzymes that break the peptide bonds in collagen. We showed that the remnants of donor cornea in DSEAK, which include the stromal layer and the Bowman layer, are easily dissolved by this enzyme and are precipitated and isolated by centrifuge. Collagen digestion by this enzyme for isolation and primary culture of other cells such as nerve cells[26] and Lymphocytes,[27] have been reported. Due to the characteristics of this cell, keratocytes need several factors to proliferate.[28] He et al., showed that adding TGF-β1 to DMEM/F12 medium causes keratocytes to acquire the proper morphology[16]. In our study, instead of using multiple chemical factors, amniotic membrane extract was used. Amniotic membrane, or amnion, is the innermost layer of the placenta and consists of a thick basement membrane and an avascular stromal matrix. This layer has been used in the treatment of various eye diseases such as chemical burns and epithelial defects.[29] Due to the presence of different growth factors and anti-inflammatory agents as well as the collagenuos structure, this layer is very useful in repairing of the ocular surface disorders. The extract of this membrane also has several growth-promoting compounds and previous studies have shown that this extract is very effective in stimulating corneal epithelial repair in the animal model[17] we showed that amnion extract induced corneal epithelial improvement after refractive surgery (accepted data). Also, the price of this material is much lower than growth factors and its use in the clinic is more conceivable. Beside amniotic membrane extract, Insulin-Transferrin-Selenium (ITS) and Non-essential amino acids (NEAA) were used in this study, which was also suggested in other studies.[30] In addition to chemical stimuli, every cell needs a proper physical niche. This scaffold support the cells for attachment and proliferation. IF the scaffold was more similar to the natural cell niche, the risk of undesired cellular change was reduced. Since the natural niche of keratocytes in the stroma is composed of collagen, in this study, the culture plate was coated with collagen. This coating stimulates cell attachment to the substrate and induces dendritic morphology in the isolated cells (Figure 2). When the cornea is damaged and in repairing process, the keratocytes phenotype changes to fibroblasts, led to scar formation. One of the challenges of isolating keratocytes is their conversion to fibroblasts. In the method presented in this study, no identity conversion to fibroblast was detected by examining the expression of specific genes for keratocytes and fibroblasts. Real-time PCR showed that the isolated cells expressed the high level of *keratocan*, *lumicane*, *ALDH3A1*, and *CD34*, which are specific markers of keratocytes, while *α-SMA-1*

expression was very low, indicates that the keratocyte phenotype has not changed to fibroblasts. The functionality of the isolated keratocytes was assessed using the ICC method by evaluation of keratocan and lumicane protei expression. Keratocan and Lumican are members of the small leucine-rich proteoglycan (SLRP) family, and they are a major keratan sulfate proteoglycans in the corneal stroma.[31,32] The keratocan and lumican proteins produced by keratocytes are very important in corneal transparency. High expression of these two proteins (Figure 5) showed that the extracted cell has a proper function. By using the results of this study and conducting more animal and clinical researches, the safety and efficacy of these cells in the *in vivo* can be ensured.

Conclusion

In this study, for the first time, the extraction of keratocyts from the discarded corneal tissue was optimized using a specific culture medium containing amniotic membrane extract. Keratocyt cells were identified at both transcription level using real-time PCR and the functional level using ICC. Supplementation of culture medium with amniotic membrane extract provides the growth factors needed for keratocyts. Using a substrate that simulated the niche of keratocyts induced growth and proliferation of keratocyte primary cells. Since keratocytes can be a good source for the treatment of corneal diseases, the establishment of corneal tissue banks can be a useful solution to overcome limitations such as lack of suitable donors.

Authors' Contributions

All authors have equal contribution.

Ethics Approval

This study was approved by the Islamic Azad University Tehran Medical Sciences Ethical Committee (IR.IAU.PS.REC.1400.492).

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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