Journal of Biomaterials Science, Polymer Edition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbsp20

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Published online: 01 Sep 2015.

To cite this article: Esmaeil Biazar, Alireza Baradaran-Rafii, Saeed Heidari-keshel & Sara Tavakolifard (2015): Oriented nanofibrous silk as a natural scaffold for ocular epithelial regeneration, Journal of Biomaterials Science, Polymer Edition

To link to this article: http://dx.doi.org/10.1080/09205063.2015.1078930

Journal of Biomaterials Science, Polymer Edition, 2015
http://dx.doi.org/10.1080/09205063.2015.1078930

Oriented nanofibrous silk as a natural scaffold for ocular epithelial regeneration

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(*Received 6 July 2015; accepted 30 July 2015*)

The aim of this study was to develop nanofibrous silk substrates for limbal stem cell expansion that can serve as a potential alternative substrate to replace human amniotic membrane. The human limbal stem cell was used to evaluate the biocompatibility of substrates (random and oriented nanofibrous mats, and human amniotic membrane) based on their phenotypic profile, viability, proliferation, and attachment ability. Biocompatibility results indicated that all substrates were highly biocompatible, as limbal stem cells could favorably attach and proliferate on the nanofibrous surfaces. Microscopic figures showed that the human limbal stem cells were firmly anchored to the substrates and were able to retain a normal corneal stem cell phenotype. Microscopic analyses illustrated that cells infiltrated the nanofibers and successfully formed a three-dimensional corneal epithelium, which was viable for 15 days. Immunocytochemistry and real-time PCR results revealed no change in the expression profile of limbal stem cells grown on nanofibrous substrates when compared to those grown on human amniotic membrane. In addition, electrospun nanofibrous silk substrates especially oriented mat provides not only a milieu supporting limbal stem cells expansion, but also serve as a useful alternative carrier for ocular surface tissue engineering and could be used as an alternative substrate to amniotic membrane.

Keywords: silk; orientation; nanofibrous scaffold; limbal stem cells; cellular analyses

Introduction

To support normal vision, the renewal of the corneal epithelium is particularly important, and the source of the cells for this continuous process is found in the limbal epithelial zone surrounding the corneal periphery.[1,2] Therapeutic transplantation of the limbus has been developed for ocular surface disease and injury in which presumed stem cell deficiency has occurred [3,4]; however, in some situations, healthy remaining limbal tissue may be very limited. Depletion of the limbal stem cell population is a pathologic feature of many ocular surface diseases such as Stevens-Johnson syndrome, chemical and thermal burns, ocular surface tumors,

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immunological conditions, radiation injury, and inherited syndromes.[5] Cell culture and clonal expansion of autologous limbal cells from the opposite eye has been increasingly used to avoid the problems associated with the need to replace corneal epithelium without reverting to allografts and the risk of immune rejection.[6–8] One of the major problems associated with stem cell therapy remains the absence of a suitable carrier for the transfer of stem cells to precise tissue locations. So far, various materials and scaffolds have been tested for the transportation of stem cells. For example, macroporous hydrogels have been used to deliver mesenchymal stem cells for spinal cord injury repair [9] or self-assembling peptide nanofibers have been tested for myoblast transplantation in infarcted myocardium.[10] To treat severe ocular surface damage and a deficiency in limbal stem cells, which are irreplaceable for corneal healing, various carriers for the culturing of limbal stem cells and for their transplantation onto the recipient eye have been tested. They include fibrin glue,[11] polymers or collagen sponges,[12] and human amniotic membrane.[6] Among them, human amniotic membrane is the clinical standard substrate for ocular surface repair owing to its biological properties that inhibit inflammation, tissue scarring, and angiogenesis.[6] However, limitations regarding the use of amniotic membrane exist. These include relatively poor mechanical strength, semitransparent appearance, difficulty of handling, and the potential risk of disease transmission, such as human immunodeficiency virus (HIV), hepatitis B virus, hepatitis C virus, and syphilis. One of the key factors of tissue engineering is to create a three-dimensional scaffold with suitable properties also, such as degradation rate, high porosity, and interconnected pores. Typically, biodegradable polymeric scaffolds are fabricated using different methods.[13,14] To mimic the natural ECM, many research groups tried to fabricate nanofibrous scaffold by different methods [15] like electro-spinning.[16] Silk fibroin is an important polymer that provides a set of material options for biomaterials because of the impressive biocompatibility, mechanical properties, and biodegradability. Silk fibroin has been investigated intensively as biocompatibly and mechanically robust biomaterials for bone, cartilage, and ligament tissue engineering.[17–21]

Electrospinning processes can fabricate nanofibers with a diameter ranging from a few tens to hundreds of nanometers and with a defined porosity. The three-dimensional structure of nanofibrous materials has an extremely large surface area, and nanofibers can mimic the structure of extracellular matrix proteins, which provide support for cell growth and function.[22–30] In our previous study, unrestricted somatic stem cells (USSCs) were utilized as a supportive layer to support the growth of limbal stem cells.[31] In fact, USSCs are considered as the pluripotent and are one of the rare populations in umbilical cord blood. Moreover, USSCs have a high potential of proliferation and differentiation. Therefore, USSCs are a valuable source for cell therapy. These supportive cells are CD45 negative, adherent, and HLA class II-negative stem cells with a long telomerse. Additionally, these cells possess a unique profile of cytokines and a high production rate of self-renewal factors.[31,33] In this study, oriented and random nanofibrous silk mats were fabricated by electrospinning method. The samples were evaluated by scanning electron microscope (SEM) analysis and *in vitro* assays, then loaded with human limbal stem cells in the presence of mitomycin C-treated USSC feeder layer and supplemental hormonal epithelial medium (SHEM) and then limbal stem cells transferred over amniotic membrane and, nanofibrous silk mat, then investigated by microscopic analyses, cellular investigations, and other markers at the RNA level as well as the expressed proteins.

Materials and methods

Materials and scaffold preparation

In this work, we successfully designed nanofibrous silk mat. Briefly, Raw B. mori silk fibers (Silk Research Center, Guilan, Iran) were boiled for half an hour in a 0.5% Na_2CO_3 (Merck, Germany), and rinsed thoroughly with water to remove the glue-like sericin proteins surrounding the fibroin filaments and dried in air. Before using in next step, the silk will be dried in an electric drying chamber at 80 °C for 6 h. Degummed silk fibers were soaked into test tube containing the solution of 98% formic acid and 0.01 w/v% calcium chloride (Merck, Germany) (material-to-fluid ratio, 1:200) at room temperature. The fiber suspension was shaken for 30 min to achieve homogenous fiber distribution and kept still for 24 h. Finally, the acid solution was evaporated through water bath at 40 °C in an aerator, and the resulting non-woven material was repeatedly washed with distilled water to remove any residual salt and vacuum dried. The degummed silks were completely dissolved and soaked in a solution of calcium chloride/ethanol/distilled water (1:2:8 mol ratio) at 80 °C for 4 h through stirring. The prepared solution was purified by being dialyzed against distilled water for 3 days. The concentration of silk fibroin aqueous solution was calculated by measuring the volume of solution and weighing the remaining solid after drying.

Electrospinning apparatus (Nano-Azma Company, Iran) used in this study prepared with two collectors for fabrication of random and oriented nanofibrous mats. Silk with the same ratios (2.5% w) were dissolved in tetrafluoroethylene (TFE: Sigma-Aldrich; Corp. St. Louis, MO, USA) solvent and the resulting solution was poured into a glass syringe controlled by syringe pump. A positive high voltage source through a wire was applied at the tip of a syringe needle and a strong electric field (24 kV) was generated between the silk solution and the collector. As soon as the electric field reached a critical value with increasing voltage, mutual charge repulsion overcame the surface tension of the polymer solution and an electrically charged jet was ejected from the tip of a conical shape as the Taylor cone. Ultrafine fibers are formed by narrowing the ejected jet fluid as it undergoes increasing surface charge density due to the evaporation of the solvent. Electrospun mats were carefully detached from the collector and dried in vacuum for 2 days at room temperature to remove the solvent molecules completely. The surface characteristics of random and oriented fibers were studied by SEM (TScan, VEGA, Czech) to analyze the changes in the surface morphology.

Cellular analyses

All cell culture reagents used were from Invitrogen-Gibco (Grand Island, NY). Cell culture plastic was from BD Biosciences (Lincoln Park, NJ). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Mouse anti-human cytokeratin 3 (K3), anti-human cytokeratin 12 (K12) antibody, mouse anti-human ABCG2, mouse anti-human p63 and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse secondary antibody, antibodies were purchased from Abcam (Abcam Co., Cambridge, UK). Mounting media contained DAPI (4,6-diamidino-2-phenylindole) was purchased from Sigma-Aldrich (Sigma-Aldrich; Corp. St. Louis, MO, USA). Limbal stem cell isolation from limbus biopsy firstly cultured on the USSC feeder layer and then transferred to amniotic membrane and nanofibrous mats. For the preparation of USSC feeder layer, confluent USSCs were incubated with 4 μg/mL mitomycin C (MMC) for 2 h at 37 °C under 5% CO_2, trypsinised and plated onto cell culture dishes

at a density of 2.2×10^5 cells/cm^2. These feeder cells were used 4–24 h after plating.[31,32] Human limbal rims discarded after corneal transplantation were provided by the Iranian Eye Bank and were washed in phosphate buffer saline solution containing 100 U/mL penicillin, 50 µg/mL gentamicin, and 2.5 µg/mL amphotericin B. After careful removal of corneal epithelium, iris, excessive sclera, conjunctiva, and subconjunctival tissue under surgical microscope (Zeiss, Oberkochen, Germany), the limbal rings were exposed to dispase II (1.2 IU/mL in Hanks' balanced salt solution free of Mg^{2+} and Ca^{2+}) at 37 °C under humidified 5% CO_2 for 3 h. The loosened epithelial sheets were removed with a cell scraper and separated into single cells by 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid for 5 min. Cells were pelleted at 400 g for 5 min and resuspended in SHEM. SHEM consisted of an equal volume of Dulbecco's modified Eagle's medium and Ham's F12, supplemented with 5% fetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite, 2.5 µg/mL epidermal growth factor, 8.4 ng/mL cholera toxin A subunit, 0.5% dimethyl sulfoxide (DMSO), 0.5 µg/mL hydrocortisone, 50 µg/mL gentamicin, 1.25 µg/mL amphotericin B, and 5 mM HEPES (Sigma-Aldrich Corp., St. Louis, MO, USA). Cells were plated at 10^4 cells/cm^2 on cell culture dishes containing MMC-treated USSCs feeder layer. Cultures were incubated at 37 °C with 5% CO_2. Medium was changed every 3–4 days. Upon reaching 70–80% confluence, the USSCs feeder layer was removed and the limbal stem cells were sub-cultured to the next passage or transferred to nanofibrous mats and amniotic membrane. Flowcytometry analysis was used for the evaluation of stem cell surface markers. The cell mixture was passed through a nylon mesh; 100 µL of the mixture was added to each tube beside the following antibodies: anti-CD105, anti-CD90, anti-CD34, anti-CD44, and anti-CD31 (Abcam Co., Cambridge, UK). Tubes were incubated at 4 °C in a dark room for 45 min. After the washing process, the cells were fixed in 100 µL of 1% paraformaldehyde in phosphate buffer saline before flowcytometric analysis was carried out. Becton Dickenson device was utilized and analysis was performed by Flowing Software 2.5.1 (BD Company, USA). Figure 1 shows the process of cells isolation, preparation, and culture on substrates.

Limbal stem cells at a plating density of 10^4 cells/well were seeded on amniotic membrane and nanofibrous mats. After 3 and 15 days of limbal stem cells culture, for analysis of proliferation rate and viability, 20 µL of MTT (Sigma-Aldrich; Corp. St. Louis, MO, USA) substrate (of a 2.5 mg/mL stock solution in phosphate buffer saline) was added to each well, and the plates were returned to standard tissue incubator conditions for an additional 4 h. Medium was then removed, the cells were solubilized in 100 µL of DMSO, and colorimetric analysis was performed (wavelength, 570 nm RAYTO micropleat reader). For SEM study, the mats with cells were washed by phosphate buffer saline, and then fixed by glutaraldehyde (2.5%) at 4 °C for 2 h. The samples were dehydrated by methanol (20% [5 min] → 40% [5 min] → 60% [5 min] → 80% [5 min] → 100% [30 s]) and then kept with tetraoxide osmium vapors at 4 °C for 2 h. The samples were kept in desiccator, coated with gold, and investigated by a SEM (TScan, VEGA, Czech).

Gene expression

Total RNA was isolated from the cells using an RNA extraction kit (Fermentas International, Burlington, Canada). RNA samples were treated with DNase I (Fermentas International, Burlington, Canada) in order to avoid the genomic DNA contamination. RNA quantity was assessed by spectrophotometry (NanoDrop; Thermo, Wilmington,

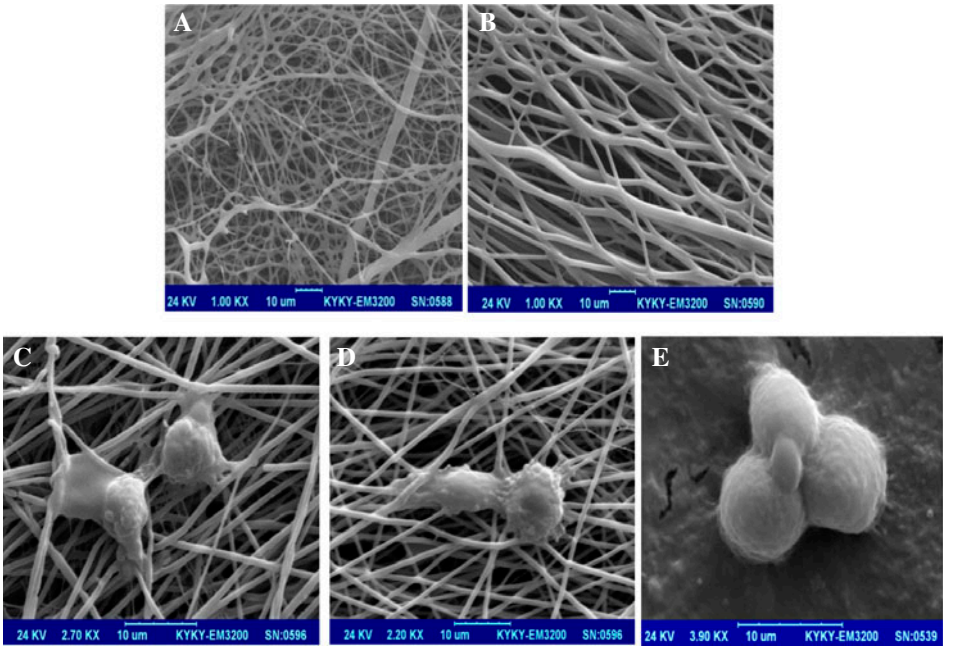

Figure 1. SEM images of designed silk substrates.
Notes: (A) Random nanofibers, (B) oriented nanofibers, cultured limbal stem cells on random nanofibers (C), oriented nanofibers (D), and on the amniotic membrane (E).

USA). For Reverse transcription, 2 μg of total RNA was used with the Revert Aid-first strand cDNA synthesis kit (Fermentas International, Burlington, Canada). RNA extracted from limbus tissue was used as a positive control. Real-Time PCR (Rotor-Gene Q Real-Time PCR System, Qiagen, USA) reaction was performed with SYBR® Premix Ex Taq™ (TAKARA BIO, INK, Japan) which uses Taq Fast DNA Polymerase, SYBR Green I dye to detect double-stranded DNA. The reaction was performed with the following programs: 5 min of 95 °C for enzyme activation, initial denaturation for 20 s at 95 °C, annealing temperature for 40 s, and extension at 72 °C for 1 min, followed by 40 cycles with a final extension at 72 °C. The final stage comprises the analysis of the melt curve through a denaturing step (15″ at 95 °C) followed by annealing (1′ at 60 °C) and ramping to 95 °C with 0.3 °C increment step. Levels of mRNA for tested genes were quantified using ΔΔCT method and normalized against human β-actin as a housekeeping gene. Data were expressed as Log 10 mean. Statistical analysis was performed using ANOVA. A p value of 0.05 was considered to be significant. The level of candidate genes in different sample types was compared by the Fisher LSD test.

Immunocytochemistry

The limbal stem cells after 15 days cocultured on amniotic membrane and nanofibrous mats at 60–80% confluence were fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking with 3% bovine serum albumin (BSA)/0.3% Triton X-100/PBS for 30 min at room temperature, cells were incubated for 2 h at room

Table 1. Primer sequences.

Primers	Sequence (5′ → 3′)	Tm
KRT-3	F: TCTCTTCGCCAAGCTCCTTAC	60
222 bp	R: GAGATGCTCTTGTTGCCGC	
KRT-12	F: AGGACTGGGTGCTGGTTATG	59
342 bp	R: GCTGAAATGATCTTATTCCTGAGGT	
ABCG2	F: ACTGAGATTGAGAGACGCGG	61
258 bp	R: TCTGGAGAGTTTTTATCTTTTCAGC	
GAPDH	F: GATGCCCCCATGTTCGTCATG	60
	R: GGGTGTCGCTGTTGAAGTCAG	
P63	F: TGAAACTTCACGGTGTGCCA	60
	R: GCTGGAAAACCTCTGGACTGA	

temperature with primary antibody in 1% BSA/PBS at the following dilutions: anti-K3 1:100, anti-K12 1:100, anti-ABCG2 1:100, and anti-p63 1:25 (Table 1). After staining with proper secondary antibody, the cells were mounted with DAPI containing media.

Results

Figure 1 shows the SEM images of the nanofibrous mats (random and oriented), and the amniotic membrane. Figures 1(A) and (B) shows the non-oriented fibers in random mat are thinner than the oriented fibers. Cell growth slowed significantly, and only small cone-shaped colonies were observed under the electron scanning microscope. Figure 2 shows the MTT assay for samples. The results showed a high viability for the all samples. In addition, bioviability was similar between the nanofibrous mats and the amniotic membrane.

Karyotype analysis was performed on limbal stem cells at passages 2. All analyzed cells had a normal chromosome karyotype of 46XX. After 15 days continuous culture, the karyotype of limbal stem cells that had been cultured on nanofibrous mats were normal 46XX (Figure 3). The immunophenotype of the isolated limbal stem cell cultures was investigated via flow cytometry. All cells were highly positive for the surface antigens CD90, CD44, and CD105. On the contrary, cells were negative for CD34 and CD31 (Table 2 and Figure 4).

An immunocytochemistry assay was also carried out for KRT3/12, ABCG2, and p63. All of the limbal stem cells on USSCs, oriented mat, random mat, and amniotic membrane were positive for ABCG2 and p63. Furthermore, there are negative for KRT 3 and 12, but were partially positive in amniotic membrane group (Figure 5).

Nanofibrous mats and amniotic membrane were analyzed for expression of KRT 3 and 12, P63, and ABCG2. GAPDH was used as an endogens control (Figure 6). The highest expression of ABCG2 was for the group limbal stem cells/amniotic membrane. However, the expression of ABCG2 in limbal stem cells/nanofibrous mats was almost like a feeder layer. Positive expression of ABCG2 valuable indicators of stem cells is growing. On 15 cocultures, ABCG2 expression levels were higher in the limbal stem cells/USSC feeder layer. ABCG2 expression levels at day 15, in all groups, were tested with the increase; this means that the cells are still in the stage of non-differentiation. P63 gene is a marker of limbal stem cell, whose expression is positively invaluable. On the 15th day of coculture, an increase in P63 gene expression of USSC feeder layer

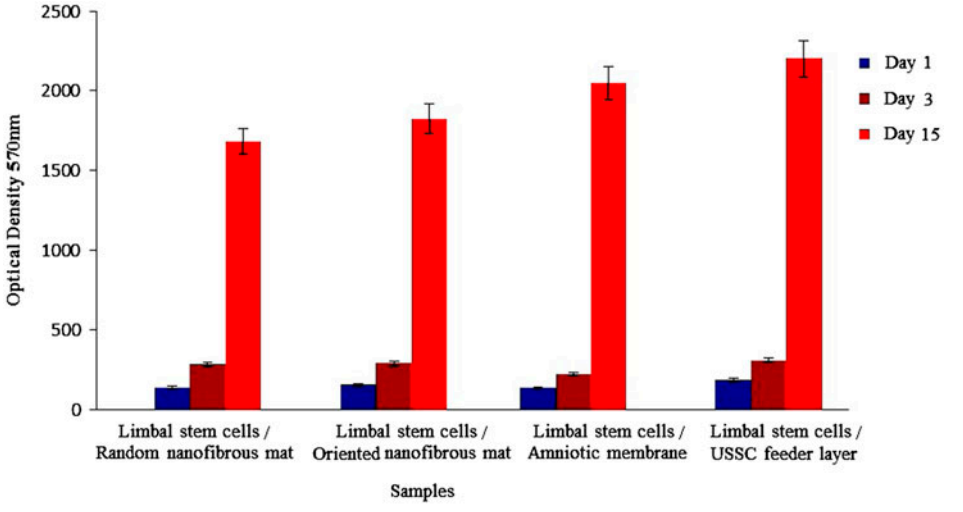

Figure 2. The MTT assay result for rate of proliferation of limbal stem cells after coculture on USSC feeder layer for nanofibrous mats, and amniotic membrane in 1, 3, and 15 days continuously culture.

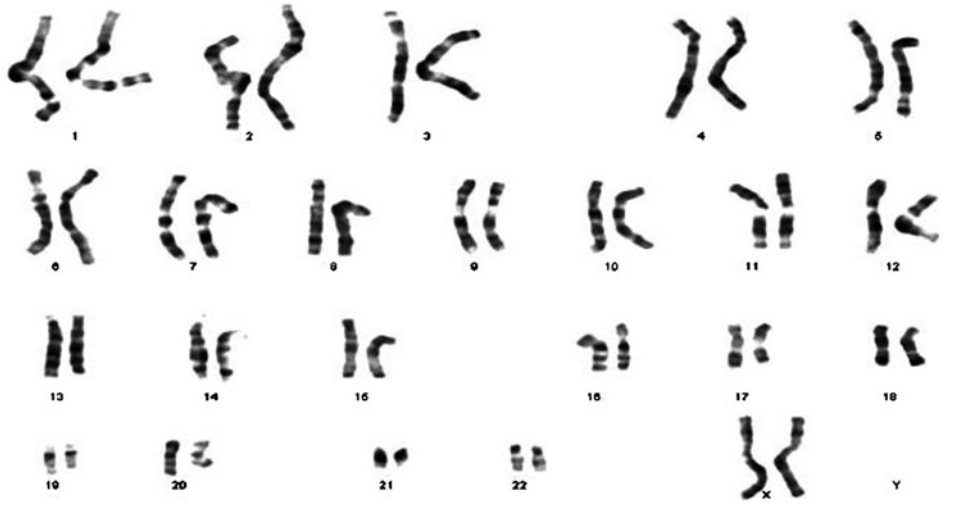

Figure 3. Karyotype analysis of limbal stem cells cocultured on nanofibrous mat.
Notes: All stem cells at 15 days represented a normal 46XX karyotype.

was observed, but the interesting thing was to reduce the expression of limbal stem cells/amniotic membrane. It was while increased expression was observed in limbal stem cells/nanofibrous mats especially oriented ones. The KRT3 and 12 markers gene is differentiated epithelial cells. The ship was almost negative expression on day 15, but increased expression of cytokeratin 3 and 12 were observed in group amniotic membrane. This could represent the beginning of a process of differentiation of limbal stem cells that were grown on the surface of amniotic membrane.

Table 2. Stem cell surface markers for limbal stem cells (LSCs).

Surface markers	CD105	CD90	CD44	CD34	CD45	CD31	CD73
USSC feeder layer/limbal stem cells	+	+	+	−	−	−	+
Amniotic membrane	+	+	+	−	−	−	+
Nanofibrous scaffolds	+	+	+	−	−	−	+

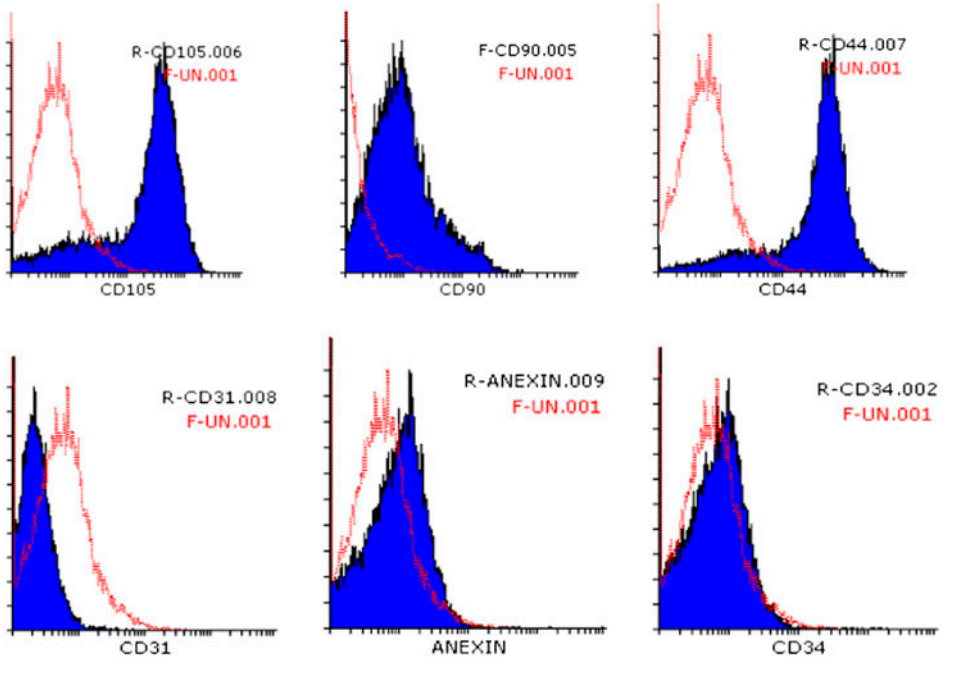

Figure 4. Flowcytometry analysis of limbal stem cells for surface markers CD34, CD44, CD105, CD90, CD31.
Notes: Shaded histogram indicates background signal; open histogram, positive reactivity with the indicated antibody.

The stem cell deficiency could be partial or total depending upon the extent of limbus involvement with the underlying disease process. In more advanced stage of partial limbal stem cell deficiency (LSCD), especially where only central cornea is affected, surgical intervention is required. The abnormal corneal epithelium can be removed and amniotic membrane transplantation can be done.[33] This allows the denuded cornea to resurface with cells derived from the remaining intact limbal epithelium. Total or severe LSCD can be treated by grafting viable limbal tissue obtained from the healthy donor eye. This procedure may help in replenishing the stem cell pool and can restore the damaged corneal surface.[34,35] Advances in tissue engineering techniques now provide an alternative to overcome the limitation of limbal tissue available for transplantation. Pellegrini et al. first showed that the corneal progenitor cells located in the limbus can be cultured to generate cohesive sheets of authentic corneal epithelium, and that cultured corneal epithelium can effectively restore the diseased corneal surface.[36] It is reported that preparation of the human amniotic membrane may

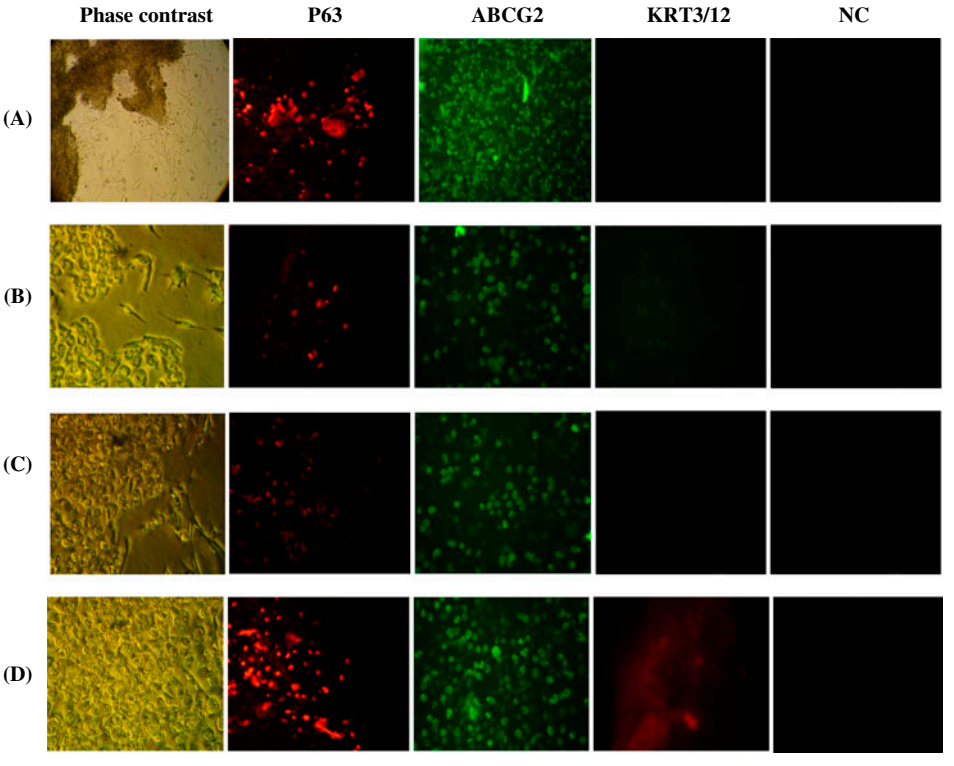

Figure 5. Immunocytochemistry of KRT3/12, ABCG2, p63, and negative control (NC) for limbal stem cells after coculture on the (A) USSC feeder layer, (B) random nanofibrous silk mat, (C) oriented nanofibrous mat, and (D) amniotic membrane.

influence the phenotype of the cultured limbal epithelial cells. Grueterich et al. [35] have demonstrated that the culture of limbal epithelial stem cells on amniotic membrane with an intact amniotic epithelium may result in a more stem-cell-like phenotype than de-epithelialised amnion. Human amniotic membrane is currently the most commonly used substrate for limbal epithelial stem cells cultivation and transplantation.[37–40] Although the results are quite promising, amniotic membrane does have some short comings. One of the major issues is ensuring the biosafety of human amniotic membrane in disease transmission, e.g. HIV, hepatitis B and C as well as from bacteria and fungus which will grow readily on human amniotic membrane. Thus, procuring and storing human amniotic membrane is a serious concern.

In addition, as a natural product, consistency of human amniotic membrane cannot be controlled. From a surgical standpoint, the physical structure of human amniotic membrane does not provide significant mechanical strength to act as a tectonic base for support of the sclera or cornea. Human amniotic membrane also has an inherent semi-opaque nature, which impedes post-operative visual acuity until the tissue is remodeled (which can occur over a period of days to months).[12,41]

Chirila et al. [42] have demonstrated good attachment and growth *in vitro* of human corneal limbal epithelial cells (HCLEs) on sericin and fibroin-based membranes.

In one of the studies,[43] corneal epithelial *in vitro* interactions assessed on patterned silk film surfaces. The response of a HCLE cell line to culture substrates with

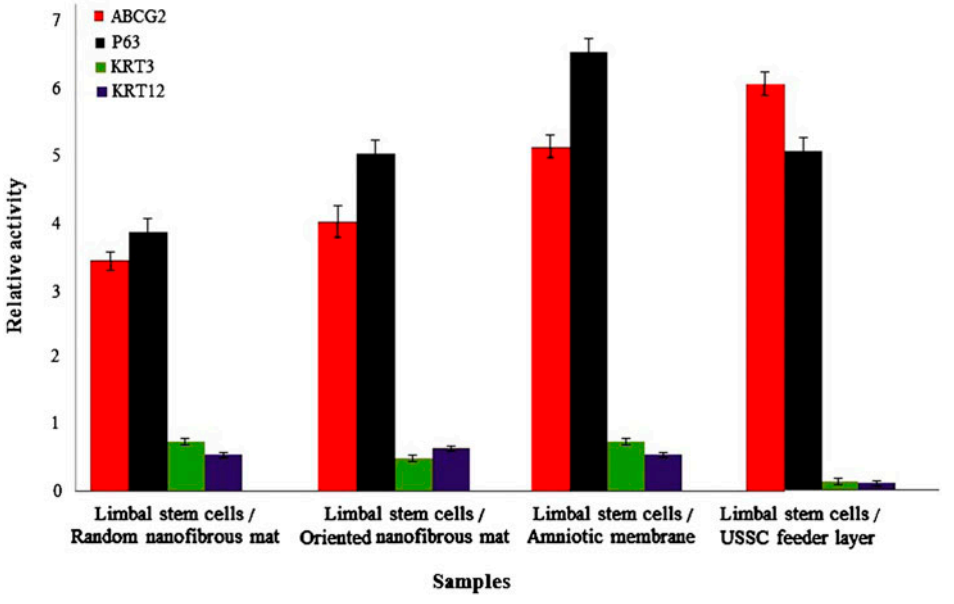

Figure 6. Expression level of KRT3/12, ABCG2, and p63 in amniotic membrane, USSC feeder layer, and nanofibrous mats after 15 days ($p < 0.001$). Strongly positive for ABCG2 and P63 and negative for KRT3 and KRT12 (epithelial markers); these results indicated that all of limbal stem cell maintenance un-differentiation condition.

either parallel lines or concentric ring surface topographies compared to flat silk and glass control surfaces. The effects that these patterned biomaterials have on initial cell-to-surface attachment and cytoskeletal development over time explored. The results illustrated the various effects that silk surface topography may have on cultured HCLEs. The combined observations of direct effects on cell attachment, spreading, and cytoskeletal protein distribution illustrated a sample of parameters that may be controlled by topography design. These findings would strongly recommend silk as an appropriate material for making substrata or scaffolds for cellular invasion and tissue formation in applications pertaining to the restoration of damaged ocular surface.[42]

Nanotechnology has the potential to solve above-mentioned problems by fabricating desired biocompatible materials to construct a functional tissue engineered ocular surface. This technology is based on the same principle as performed with human amniotic membrane as a substrate. Limbal biopsy can be harvested and grown on an appropriate matrix or scaffold and subsequently transplanted to diseased eye. Various extracellular matrices have been used previously for limbal epithelial stem cell expansion such as fibrin, collagen scaffold, temperature responsive cell culture surfaces, human anterior capsule, natural and synthetic scaffolds.[11,43–51] In one of the studies,[52] researchers have proposed Poly-ε-caprolactone (PCL), which is a synthetic aliphatic polyesters bioresorbable and biocompatible as an excellent and biocompatible scaffold for LSC expansion. In ophthalmic application, PCL has already been explored as a carrier [53] due to its *in vivo* biocompatibility as it does not induce any immunological reactions after degradation. Our preliminary studies [20–25] have shown that the silk polymer provides a suitable alternative for overcoming the short comings of synthetic polymers, resulting in a new biomaterial with good biocompatibility and

improved mechanical, physical, and chemical properties. In the initial part of this test, the limbal stem cells were cultured on a silk scaffold. For potential application in corneal epithelial transplantation, membranes had to be cut out in desired size. We cultivated limbal stem cells on silk mats because they could adhere and proliferate well on scaffold. Captured images by confocal microscopic system in figures cultured limbal stem cells on silk mats spread out well and displayed physiologic phenotype of epithelial cells. These results suggested that nanofibrous silk mats especially oriented nanofibers were able to support the function of the limbal stem cells *in vitro*. The major findings in these experiments illustrated that silk nanofibers successfully allowed cell adherence, improved phenotypic expressions, and maintained transparency. Since our final goal is to develop carrier sheet, the establishment of animal model for corneal epithelial transplantation may be required in the future studies.

Conclusion

In conclusion, our study showed that human limbal stem cells isolated from cadaveric limbal rims were able to proliferate *in vitro*. These cells when cocultured with MMC-treated USSC in SHEM, after transfer limbal stem cells to amniotic membrane, and nanofibrous silk mats, all cells maintained the features of limbal stem cells. It is further suggested that this culture system would be useful for the clinical application of limbal cell culture as well as the study of limbal stem cell mechanisms.

Acknowledgments

We are grateful to the Labafinejad Hospital for providing the human limbal tissue. This study was funded by ophthalmic research center grant.

Disclosure statement

No potential conflict of interest was reported by the authors.

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