Cellular Response of Stem Cells on Nanofibrous Scaffold for Ocular Surface Bioengineering

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In this study, the human limbus stem cell was used to evaluate the phenotypic profile, viability, proliferation, and attachment ability of nanofibrous poly (3-hydroxybutyrate-co-3-hydroxyvalerate) substrates. Results indicated that the all substrates were highly biocompatible, as firmly anchored to the substrates, and were able to retain a normal corneal stem cell phenotype. Immunocytochemistry results revealed no change in the expression profile of cells grown on nanofibrous substrate when compared with those grown on amniotic membrane. In addition, nanofibrous substrate provides not only a milieu supporting cells expansion, but also serve as a useful alternative carrier for ocular surface engineering. *ASAIO Journal* 2015; 61:605–612.

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 (LSC) population is a pathologic feature of many ocular surface diseases, such as Stevens–Johnson syndrome, chemical and thermal burns, ocular surface tumors, immunological conditions, radiation injury, and inherited syndromes.[5] Cell culture and clonal expansion of autologous limbal cells from the opposite eye have 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 (MSCs) 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 LSCs, which are irreplaceable for corneal healing, various carriers for the culturing of LSCs 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 (AM).[6] Among them, human AM 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 AM 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 3D scaffold with suitable properties, such as degradation rate, high porosity, and interconnected pores. Typically, biodegradable polymeric scaffolds are fabricated using different methods.[13,14] In natural tissues, cells are surrounded by extracellular matrix (ECM), which has physical structural features ranging from nanometer to micrometer scale. Hence, a nanostructured porous structure and a large surface area are needed as an alternative to natural ECM. To mimic the natural ECM, many research groups tried to fabricate nanofibrous scaffold by different methods[15] like electrospinning.[16] Polyhydroxyalkanoates (PHA) are polyesters produced by microorganisms under unbalanced growth conditions. Polyhydroxyalkanoates are generally biodegradable, with good biocompatibility, making them attractive as tissue engineering biomaterials. Electrospinning is one of the most important methods for fabrication of nanofibrous scaffolds.[17–25] Electrospinning processes can fabricate nanofibers with a diameter ranging from a few tens to hundreds of nanometers and with a defined porosity. The 3D structure of nanofibrous materials has an extremely large surface area, and nanofibers can mimic the structure of ECM proteins, which provide support for cell growth and function. Nanofibrous scaffolds can create specific niches where Schwann cells can reside and maintain their unique properties. It has been shown that embryonic Schwann cells or MSCs grow and differentiate on nanofibers comparably or even better than on plastic surfaces.[26–30] We sought to determine whether adult tissue-specific Schwann cells can also be grown on nanofibrous scaffolds and whether these scaffolds can be used as carriers for cell transplantation in tissue regeneration. In our previous study, unrestricted somatic stem cells (USSCs) were utilized as a supportive layer to support the growth of LSCs.[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 human leukocyte antigen (HLA) class II-negative stem cells with a long telomerase. Additionally, these cells possess a unique profile of cytokines and a high production rate of self-renewal factors.[31,32] In this study, poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) nanofibrous scaffold was fabricated by electrospinning method. The samples were evaluated by scanning electron microscope (SEM) analysis and *in vitro* assays, then loaded with human LSCs in the presence of mitomycin C-treated USSC feeder layer and supplemental hormonal epithelial medium (SHEM), and then LSCs transferred over AM and nanofibrous PHBV scaffold, 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 previous work, we were successfully designed nanofibrous PHBV mat.[19] Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (molecular weight of 680 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). 2, 2, 2-Trifluoroethanol (TFE) was also purchased from Sigma-Aldrich and was used as solvent to prepare PHBV solutions. Both polymer and solvent were used without further purification. Electrospinning apparatus used in this study was prepared by Nano-Azma Company (Iran). Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) was dissolved in solvent TFE at a concentration of 2% w/v, 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 (20 kV) was generated between the PHBV 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 because of the evaporation of the solvent. An electro spun PHBV nanofibrous mat was carefully detached from the collector and dried in vacuum for 2 days at room temperature to remove the solvent molecules completely. The nanofibers fabricated with a predetermined variables of electrospinning (syringe size, 17 mm; collector speed, 1000 rpm; injected speed, 2 ml/min; syringe tip distance to collector, 75 mm; voltage, 20 kV; temperature, 30°C; time, 7 hours). The surface characteristics of modified fibers were studied by scanning electron microscopy (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 unless otherwise indicated. Mouse anti-human cytokeratin 3 (K3) antibody, anti-human cytokeratin 12 (K12) antibody, mouse anti-human connexin 43 antibody, mouse antihuman p63 antibody, and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse secondary antibody were purchased from Abcam (Cambridge, UK). Mounting media contained 4,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich. Limbal stem cell isolation from limbus biopsy first cultured on the USSCs feeder layer and then transferred to AM and nanofibrous scaffold. For preparation of USSC feeder layer, confluent USSCs were incubated with 4 µg/ml mitomycin C (MMC) for 2 hours at 37°C under 5% CO_2 and trypsinized and plated onto cell culture dishes at a density of 2.2×10^5 cells/cm². These feeder cells were used 4–24 hours after plating.[31,32] Human limbal rims discarded after corneal transplantation were provided by the Iranian Eye Bank and were washed in phosphate buffer solution (PBS) containing 100 unit/ml penicillin, 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin B. After careful removal of corneal epithelium, iris, excessive sclera, conjunctiva, and subconjunctiva tissue under surgical microscope (Zeiss, Oberkochen, Germany), the limbal rings were exposed to dispase II (1.2 international unit/ml in Hanks' balanced salt solution free of Mg^{2+} and Ca^{2+}) at 37°C under humidified 5% CO_2 for 3 hours. The loosened epithelial sheets were removed with a cell scraper and separated into single cells by 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid (EDTA) for 5 min. Cells were pelleted at 1000 rpm for 5 min and resuspended in SHEM. Supplemental hormonal epithelial medium consisted of an equal volume of Dulbecco's modified Eagle's medium (DMEM) 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% DMSO, 0.5 µg/ml hydrocortisone, 50 µg/ml gentamicin, 1.25
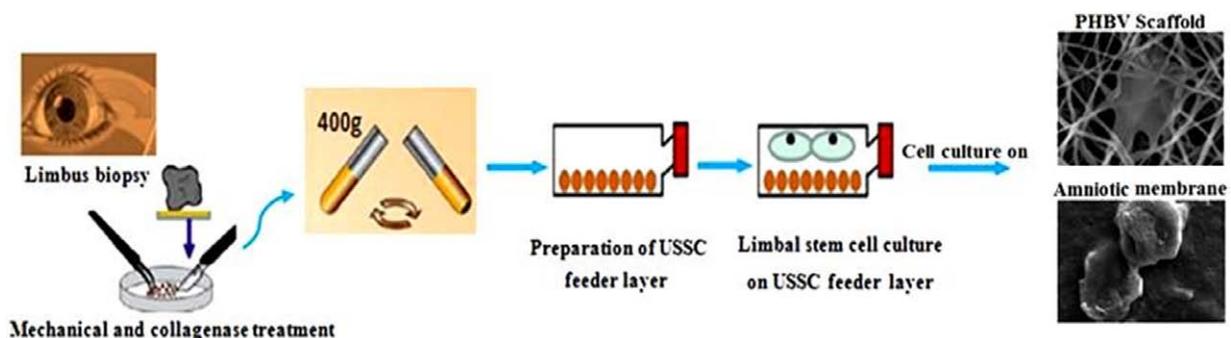

Figure 1. Process of cells isolation, preparation, and culture on samples. PHBV, 3-hydroxybutyrate-*co*-3-hydroxyvalerate; USSC, unrestricted somatic stem cells. [full color online]
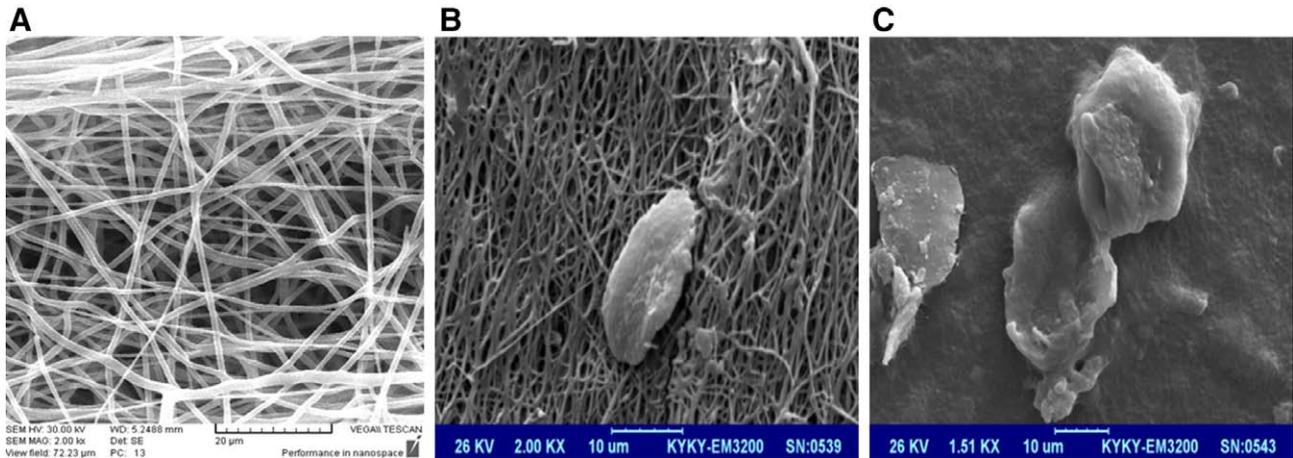
Figure 2. Scanning electron microscopic (SEM) images of designed substrates: (**A**) nanofibrous poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) substrate, (**B**) cultured limbal stem cells (LSCs) on nanofibrous PHBV substrate, and (**C**) on the amniotic membrane. full color online

µg/ml amphotericin B, and 5 mM HEPE. 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_2/95% air. Medium was changed every 3–4 days. Upon reaching 70–80% confluence, the USSCs feeder layer was removed, and the LSCs were subcultured to the next passage or transferred to nanofibrous scaffolds and AM. The sample disinfected with 70% alcohol for 5 min and exposure to ultraviolet (UV) light for 10 min and finally washed three times with PBS. **Figure 1** shows the process of cells isolation, preparation, and culture on scaffolds. Flowcytometry analysis was used for 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-CD166, anti-CD90, anti-CD34, anti-CD45, anti-CD44, anti-CD73, and anti-CD31 (all products from Abcam). 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 PBS before flowcytometric analysis was carried out. Becton Dickenson device was utilized, and analysis was performed by flowing software 2.5.1(BD Company).

Limbal stem cells at a plating density of 10^4 cells/well were seeded on AM and nanofibrous scaffold. After 3, 7, and 15 days of LSCs culture, for analysis of proliferation rate and viability, 20 µl of MTT (sigma) substrate (of a 2.5 mg/ml stock solution in PBS) was added to each well, and the plates were returned to standard tissue incubator conditions for an additional 4 hours. 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 scanning electron microscopy study, the scaffolds with cells were washed by PBS and then fixed by glutaraldehyde (2.5%) at 4°C for 2 hours. 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 hours. The samples were kept in desiccator, coated with gold, and investigated by a SEM (Cambridge Stereo-scan, S-360, Wetzlar, Germany). Dry and wet USSCs/gelatin samples dipped in PBS for 2 hours were used for an optical transparency test and were compared with wet human AM supported on a glass coverslip. The glass coverslip was used as a control. Percentage transmittance (%T) was measured using UV–visible spectrophotometer (Bio-Tek-Kontron [UVIKON922]) in the visible range from 400
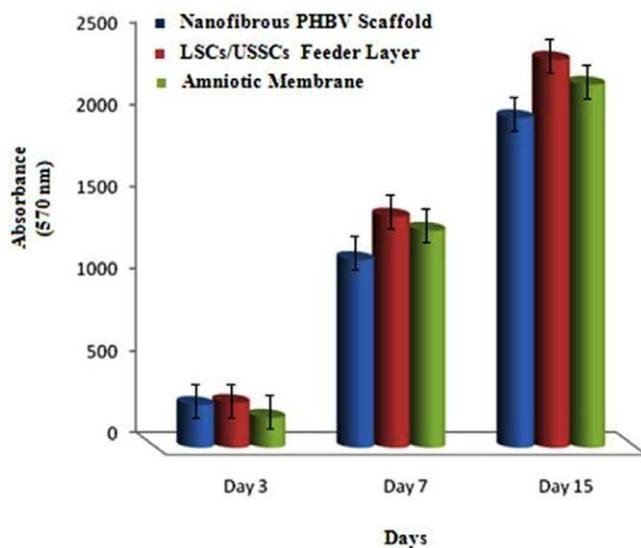

Figure 3. The MTT assay result for rate of proliferation of limbal stem cells (LSCs) after coculture on unrestricted somatic stem cells (USSCs) feeder layer for nanofibrous scaffold and amniotic membrane in 3, 7, and 15 days continuously culture. full color online

Figure 4. Optical transparency of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) nanofibrous scaffold and human amniotic membrane. **A**: Normal text, (**B**) dry PHBV nanofibrous scaffold showing complete opacity and the text underneath cannot be read through it, (**C**) wet PHBV nanofibrous scaffold showing translucency through which the printed text is visible, and (**D**) wet human amniotic membrane, showing transparency through which the printed text can be read.
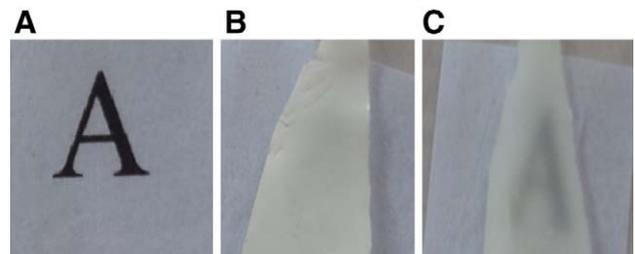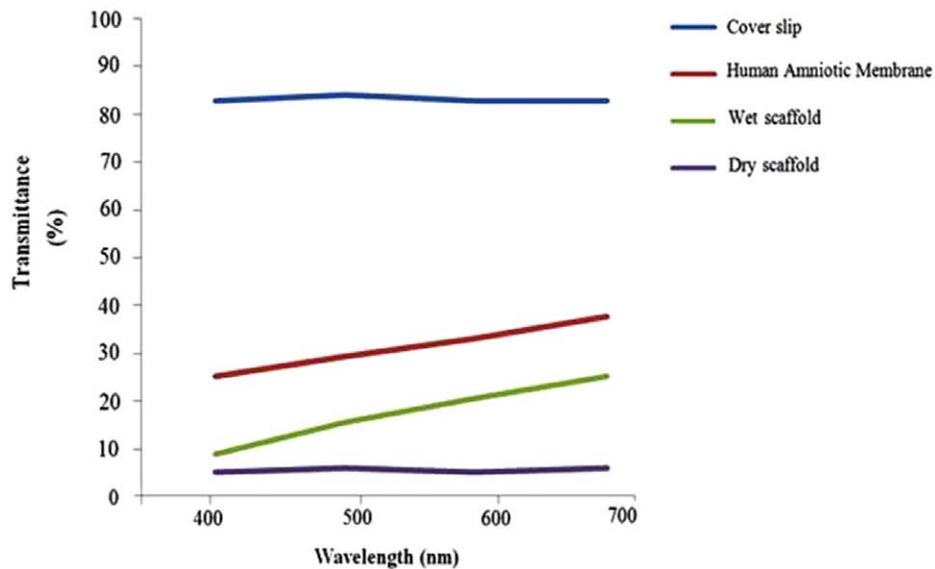
Figure 5. Optical transmittance of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) nanofibrous scaffold, human amniotic membrane, and glass coverslips (control) at a wavelength of 700 nm. [full color online]

to 700 nm wavelength. Visual assessment of optical transparency of dry scaffold, wet scaffold, and wet human AM was also completed by keeping these membranes on a printed text and taking photographs using a digital camera.

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) to avoid the genomic DNA contamination. RNA quantity was assessed by

spectrophotometry (NanoDrop; Thermo, Wilmington, DE). For reverse transcription, 2 µg of total RNA was used with the revert aid-first strand cDNA synthesis kit (Fermentas International). RNA extracted from limbus tissue was used as a positive control. Real time-polymerase chain reaction (Rotor-Gene Q Real-Time PCR System, Qiagen, Valencia, CA) 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 following program; 5 min of 95°C for enzyme activation, initial denaturation for

Figure 6. Karyotype analysis of limbal stem cells (LSCs) cocultured on nanofibrous substrate. All stem cells at 15 days represented a normal 46XX karyotype.

Table 1. Stem Cell Surface Markers for Limbal Stem Cells (LSCs)

Surface Markers	CD105	CD90	CD44	CD34	CD45	CD31	CD73
Feeder layer/LSC	+	+	+	−	−	−	+
Amniotic membrane	+	+	+	−	−	−	+
Nanofibrous scaffold	+	+	+	−	−	−	+

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 per 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 analysis of variance. 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 least significant difference (LSD) test.

Immunocytochemistry

The LSCs after 15 days cocultured on AM and nanofibrous scaffold 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 hours at room temperature with primary antibody in 1% BSA/PBS at the following dilutions: K3 1:100, K12 1:100, ABCG2 1:100, and p63 1:25. After staining with proper secondary antibody, the cells were mounted with DAPI containing media.

Results

Figure 2 shows the SEM images of the nanofibrous scaffold and the AM. The size average for the nanofibers was about 80–100 nm. Cell growth slowed significantly and only small cone-shaped colonies were observed under the electron scanning microscope. **Figure 3** shows the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for samples. The results showed a high viability for the all samples. In addition, bioavailability was similar between the nanofibrous scaffold and the AM. Also, the nanofibrous samples caused more LSCs to proliferate.

Figures 4, A–D show a digital photograph for visual assessment of transparency of wet human AM as well as wet and dry scaffold, all supported on a glass coverslip. Dry scaffold is completely opaque, and the text underneath cannot be read through it. In contrast, the wet PHBV/gelatin nanofibrous scaffold (2 hours dipped) shows translucency, as the printed text underneath is slightly visible. The transparency shown by wet scaffold is more than that of wet AM, through which the text can be easily read.

The transparency of the samples was also compared quantitatively using UV–visible spectroscopy (**Figure 5**). It can be observed that glass coverslips (taken as control) showed
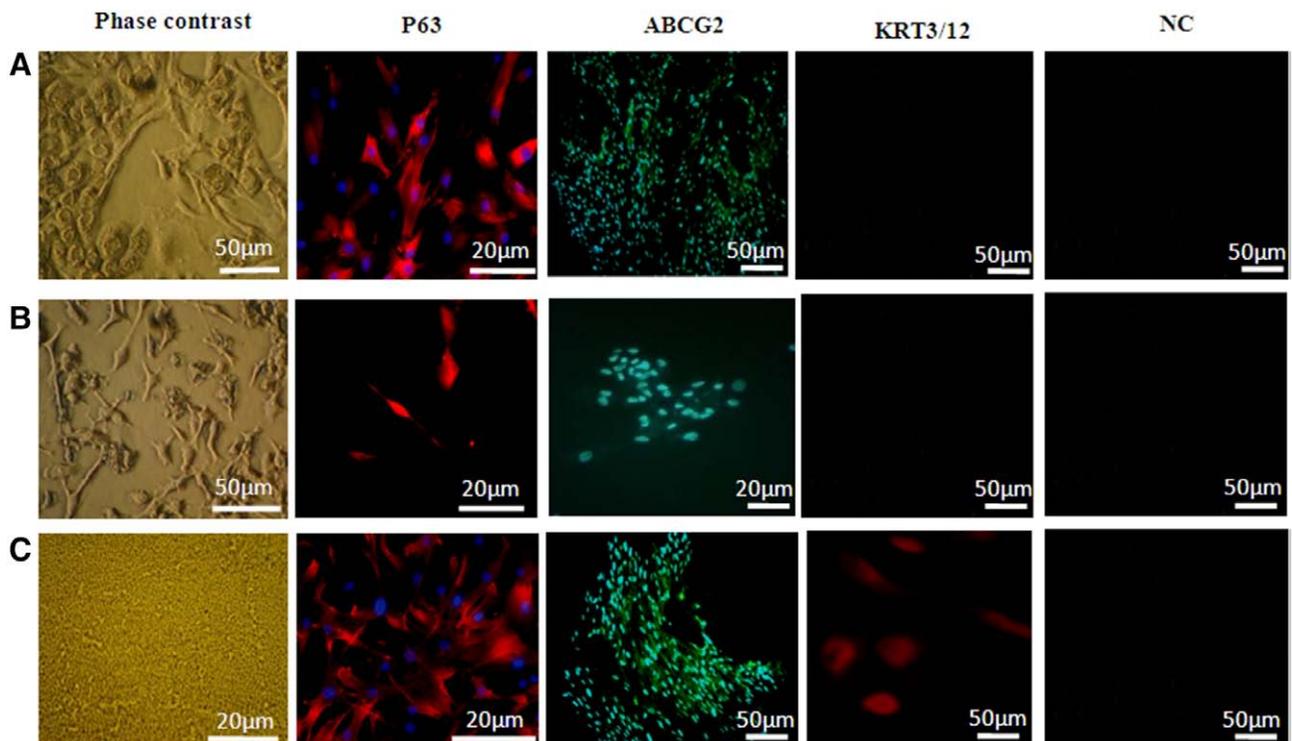

Figure 7. Immunocytochemistry of KRT3/12, ABCG2, p63, and negative control (NC) for limbal stem cells (LSCs) after coculture on the (**A**) unrestricted somatic stem cell (USSC) feeder layer, (**B**) nanofibrous poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) scaffold, and (**C**) amniotic membrane.

maximum transmittance of about 85%. In contrast, the opaque dry scaffold showed less than 5% of light transmittance throughout the wavelength range. However, wet scaffold showed a significantly higher transparency, with about 25% transmittance at the wavelength of 700 nm. These values are slightly less than those for AM, which showed transmittance of 37% at 700 nm. Hence, it may be inferred that scaffold in a wet state has significantly higher transparency when compared with dry scaffold.

Karyotype analysis was performed on LSCs at passages 2. All analyzed cells had a normal chromosome karyotype of 46XX. After 15 days continuous culture, the karyotype of LSCs that had been cultured on nanofibrous substrate was normal 46XX (**Figure 6**). The immunophenotype of the isolated LSC cultures was investigated via flow cytometry. All cells were highly positive for the surface antigens CD166, CD73, CD90, CD 31, Cd44, and CD105. On the contrary, cells were negative for CD34, CD31, and CD45 (**Table 1**).

An immunocytochemistry assay was also carried out for KRT3/12, ABCG2, and p63. All the LSCs on USSCs, 3T3, and MSCs were positive for KRT3/12, ABCG2, and p63. Furthermore, there are negative for KRT3/12 but were better for nanofibrous PHBV scaffold than AM (**Figure 7**).

Nanofibrous scaffold and AM were analyzed for expression of KRT 3/12, P63, and ABCG2. GAPDH was used as an endogens control. Expression level of ABCG2 and P63 was similar for all samples. Furthermore, nanofibrous PHBV scaffold does not have expression of KRT3/12 and was similar to USSC feeder layer sample, but this expression level is higher for AM after 15 days (**Figure 8**).

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 LSC deficiency (LSCD), especially where only central cornea is affected surgical intervention is required. The abnormal corneal epithelium can be removed, and AM 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]

Human AM is currently the most commonly used substrate for LSCs cultivation and transplantation.[35–38] Although the results are quite promising, AM does have some short comings. One of the major issues is ensuring the biosafety of human AM in disease transmission, *e.g.*, HIV, hepatitis B and C, and from bacteria and fungus, which will grow readily on human AM. Thus, procuring and storing human AM is a serious concern.

In addition, as a natural product, consistency of human AM cannot be controlled. From a surgical standpoint, the physical structure of human AM does not provide significant mechanical strength to act as a tectonic base for support of the sclera or cornea. Human AM also has an inherent semiopaque nature, which impedes postoperative visual acuity until the tissue is remodeled (which can occur over a period of days to months).[12,39]

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 AM 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.[11,40–49]

Our preliminary studies[20–25] have shown that the PHBV nanofibrous scaffold provides a suitable alternative for overcoming the short comings of natural and 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 LSCs were cultured on a series of PHBV nanofibrous scaffold. Appropriate transparency can be maintained on wet PHBV nanofibrous scaffold partly similar to AM (**Figures 4, C and D**). We cultivated LSCs on PHBV nanofibrous scaffold because they could adhere and proliferate well on scaffold. Captured images by confocal microscopic system in figures show cultured LSCs on PHBV nanofibrous scaffold spread out well and display physiologic phenotype of epithelial cells. These results suggested that PHBV membrane was able to support the function of the LSCs *in vitro*. The major findings in
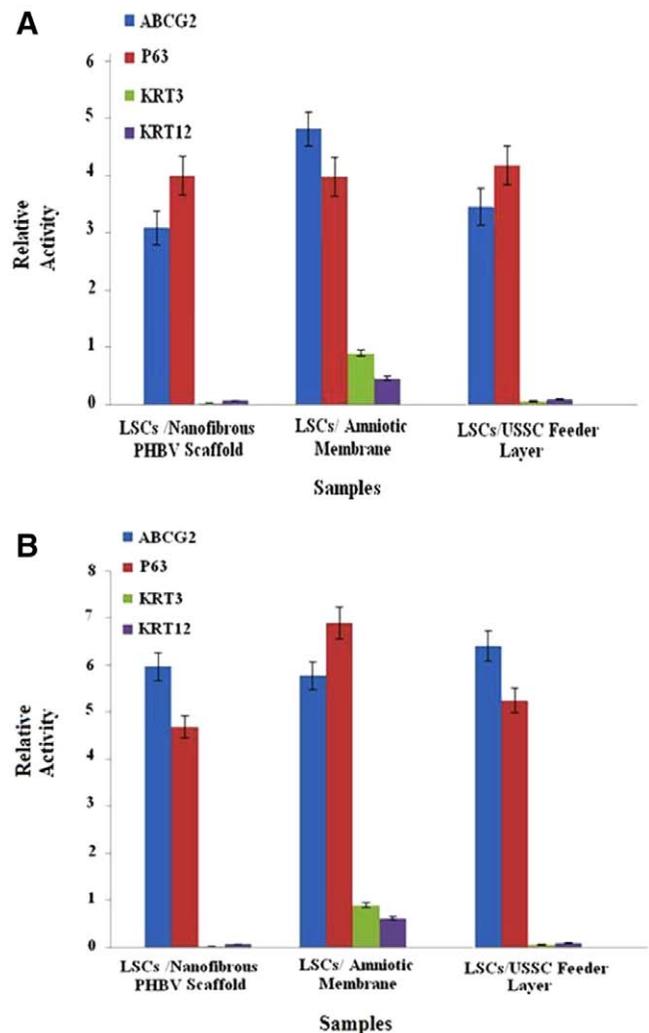

Figure 8. Expression level of KRT3/12, ABCG2, and p63 in amniotic membrane, unrestricted somatic stem cell (USSC) feeder layer, and nanofibrous poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) scaffold after (**A**) 7 and (**B**) 15 days ($P < 0.001$).

these experiments illustrated that PHBV nanofibrous scaffold successfully allowed cell adherence and improved phenotypic expressions. Because 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 LSCs isolated from cadaveric limbal rims were able to proliferate *in vitro*. These cells maintained the features of LSCs, when cultured on the nanofibrous PHBV scaffold. It is further suggested that this culture system would be useful for the clinical application of limbal cell culture and the study of LSC mechanisms.

Acknowledgment

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