The role of signaling pathways in the expansion of corneal epithelial cells in serum-free B27 supplemented medium

Sasirekha Krishnan, 1 Shruthi Lakshmanan, 1 Geetha Krishnan Iyer, 2 Krishnan UmaMaheswari, 3 Subramanian Krishnakumar 1

1 L&T Department of Ocular Pathology, Vision Research Foundation, Sankara Nethralaya, Chennai, India; 2 Cornea services department, Medical Research Foundation, Sankara Nethralaya, Chennai, India; 3 Nanobiotechnology Department, CeNTAB, SASTRA University, Tanjore, India

Purpose: To study the influence of serum-free B27 supplemented culture medium on corneal epithelial cells from limbal explants.

Methods: Human limbal tissues obtained from cadaveric donor eyes were used in this study. The morphological characteristics of cultivated epithelial cells were analyzed by phase contrast microscopy. Growth kinetics, bromodeoxyuridine (BrdU) labeling cell proliferation assay, and reverse transcriptase PCR (RT–PCR) for limbus and corneal markers were studied in serum-dependent and serum-free B27 supplemented corneal epithelial culture. The signaling pathway genes were analyzed by RT 2 qPCR profiler array.

Results: The corneal epithelial cells morphology and mRNA expression of markers were similar in both the serum-dependent and serum-free B27 supplemented culture. The growth and proliferation of the serum-free B27 supplemented culture was significantly higher than that of the serum-dependent culture. The wnt, hedgehog, survival, NFkB, Jak-Stat, and calcium protein kinase C pathways were highly expressed in the serum-free B27 supplemented corneal epithelial culture.

Conclusions: Most signaling pathway genes are upfolded by B27 supplementation in the corneal epithelial cell culture; it could be an efficient replacement for serum.

Limbal deficiency or loss of corneal stem cells is associated with ocular surface disease, which is otherwise known as limbal stem cell deficiency (LSCD). The management of the ocular surface using cultured corneal epithelial cells on a human amniotic membrane is preferred.

The ex vivo expansion of limbus culture requires unknown factors, such as fetal bovine serum (FBS), autologous serum, feeder layers or bovine pituitary extracts (BPE), as growth factors for the growth of corneal epithelial cells. The usage of these substances raises concern about infection with recognized or unknown-agents [1]. Although there have been successful reports that support the proliferation of corneal epithelial cells using autologous human serum [2], which effectively eliminates the risk of xenogenic contamination during transplantation to LSCD patients, there has been no data supporting the use of corneal epithelial cultures in a serum-free medium condition or showing the important signaling pathways involved.

B27 was originally optimized for culture of hippocampal neurons and used for the growth of neurons from embryonic rat striatum, the substantia nigra, the subiculum, the cerebral cortex, the postnatal dentate granule, the cerebellum, and the dentate gyrus in a serum-free condition [3]. B27 contains vitamins like biotin, DL-alpha-tocopherol, and DL-alpha-tocopherol acetate. It also contains catalase, human recombinant insulin, superoxide dismutase proteins, and other components such as corticosterone, D-galactose, ethanolamine hydrochloride, reduced glutathione, linoleic acid, linolenic acid, triiodo-L-thyronine, etc. It has been reported that corneal endothelial precursors proliferate actively in B27-containing medium with no FBS or feeder cells [4]. Yakoo et al. [1] established a culture technique for human corneal epithelial equivalents with B27 as an alternative for FBS and studied the putative markers for corneal epithelial cells. However, the signaling pathway that helps to replace serum components and maintain stemness in the corneal culture has not yet been reported in the literature.

Therefore, we have tried to avoid serum, feeder layers, and/or bovine pituitary extract (BPE) in the culturing of corneal limbal stem cells. Instead, we used a serum-free medium supplemented with the growth factor B27 and
analyzed the genes involved in the signal transduction pathway by RT² qPCR profiler array.

**METHODS**

**Grading donor eyes:** Human cadaveric eyeballs were obtained from the C.U. Shah eye bank of the Medical Research Foundation, Sankara Nethralaya, Chennai, India with the consent of the donor or donor family to be used for medical research in accordance with the principles outlined in the Declaration of Helsinki. In this study, we collected limbus tissues from donors (n=12) aged between 67 and 82 years. Corneal limbal tissues of 2 mm in length were collected in Dulbecco’s Modified Eagle Medium (DMEM; Sigma Chemicals, St. Louis, MO) with antibiotics (Sigma Chemicals) and transported to the cell biology laboratory for further processing. The donor blood samples were screened for human immunodeficiency virus (HIV) type 1 and 2, hepatitis B virus (HBV), hepatitis C virus (HCV), and *Treponema pallidum* infections. Data on age, sex, cause of death, time of death, time of eye donation, and time of biopsy collection were also collected.

**Human limbal explant culture:** The collected limbal tissue was washed thrice with Hanks balanced salt solution buffer (Sigma Chemicals). After careful removal of excessive sclera and conjunctiva, the tissue was cut into multiple bits using a sharp, sterile Bard-Parker blade (Niraj Industries, Faridabad, India). The tissue bits were placed on a culture plate (BD biosciences, San Jose, CA) using a sterile needle. The plate was incubated at 37 °C and 5% CO₂ for 5 min for adhesion. The explants were covered with culture medium containing equal volumes of DMEM and F12 (Sigma Chemicals) containing 5 ng/ml of epidermal growth factor (EGF), 5 μg/ml of insulin, 5 μg/ml of transferrin, 5 ng/ml of sodium selenite, 0.5 mg/ml of hydrocortisone, and 1% antibiotic solution (Sigma Chemicals). Ten percent FBS (Sigma Chemicals) was added to five cultures (serum-dependent culture; n=5) and 1% B27 supplement (Sigma Chemicals) was added to the other five cultures (serum-free B27 supplemented culture; n=5). The control samples were cultured without serum and/or any other supplement replacing serum (control culture; n=2). The plates were incubated at 37 °C and 5% CO₂ with 95% humidity. The medium was changed once every two days and growth was monitored daily with an inverted phase contrast microscope (Nikon, Tokyo, Japan). Confluent cells were harvested for further molecular characterization.

**Growth kinetics:** The outgrowth of all the cultures was photographed every second day; images were transferred to a computer and analyzed using quantity G area measurement software [5]. The mean radius of all the cultures was plotted against each day until they reached confluence.

**Cell proliferation assay:** Cell proliferation was assessed by measuring 5-bromo-2-deoxyuridine (Qiagen, Santa Clara, CA) incorporation during DNA synthesis in proliferating cells. The detection of BrdU was performed according to the manufacturer’s instruction and chased for 1–21 days. The BrdU labeling indices were assessed by counting the nuclei through a microscope using a 40× objective. The labeling index was expressed as the number of positively labeled nuclei/total number of nuclei×100%.

**RNA isolation:** The cultures were trypsinised on the 8th day (limbal stem cells) and the 21st day (differentiated corneal cells) from both serum-dependent and serum-free B27 supplemented cultures. The RNA was isolated using the Rneasy (Qiagen) kit according to the manufacturer’s instructions. For RT² qPCR array, the integrity and purity of the RNA were verified using a bioanalyzer chip (Agilent Technologies Genotypic, Bangalore, India).

**Reverse transcriptase PCR:** The expression of marker genes (Bangalore Genei, Bangalore, India; Table 1) specific for limbal stem cells and corneal cells was studied by RT–PCR with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control.

### Table 1. Primer sequence and reaction condition for RT–PCR.

| Gene     | Primer sequence (3′-5′) | Annealing temperature (°C) | PCR product size (bp) |
|----------|-------------------------|---------------------------|----------------------|
| ABCG2    | FP:AGTTCCATGGCAGCTGGCCATA RP:TCAGGGTAGGAAATGTGAAGG | 62                       | 379                  |
| ANp63    | FP:AGACTCAATTTATTGAG RP:AGCTCATGTTGGGGCAC | 54                       | 440                  |
| Connexin 43 | FP:CTTCCTTGGCTGATCCAGTGTTAC RP:ACCAAGGACACACCCACCAT | 66                       | 154                  |
| Keratin3 | FP:GCGAGAGATCGAGGGTGTC RP:GCATCTTCTGCCTGTGTA | 64                       | 145                  |
| Keratin12 | FP:CATGAAGAGAAACACGAGAGTG RP:TCTGTCACGCGAGTGTTTCA | 63                       | 150                  |
| GAPDH    | FP:GCCAAGGTCATCCATGACACAC RP:GTCCACACACCCTGTTGCTGA | 63                       | 498                  |

FP: Forward Primer; RP: Reverse Primer; bp: Base Pair.
Signal transduction pathway analysis: The RT² qPCR profiler Human Signal Transduction Pathway array (catalog number PAHS-014; SABiosciences, Frederick, MD), representing 84 genes involved in signal transduction pathways, plus five housekeeping genes and three controls, was used to analyze the effect of serum on signaling-related gene expression in human limbal and corneal epithelial cells. The total RNA was isolated from the limbus and corneal cells (serum-dependent and serum-free B27 supplemented culture) using the Rneasy Mini Kit (Qiagen). cDNA was generated from 1 µg total RNA using the RT² qPCR Array First Strand Kit in accordance with the manual. The template was combined with RT² SYBR Green/Fluorescein PCR master mix. Equal amounts of this mixture (25 µl) were added to each well of the RT² qPCR profiler plate containing the predispensed gene-specific primer sets, and the reaction was performed using a sequence detector (ABI 7500; Applied Biosystems, LabIndia, Chennai, India) according to the manufacturer’s protocols. Data analysis was based on the ∆∆Ct method with the aid of an Excel (Microsoft Excel; Microsoft, Redmond, WA) spreadsheet containing algorithms provided by the manufacturer. The expression levels of the mRNA of each gene were normalized using the expression of the housekeeping gene GAPDH. A positive value indicates that the gene was upregulated and a negative value indicates that the gene was downregulated.

Statistical analysis: All experiments were performed in triplicate. The summary data were reported as the mean ±standard deviation (SD), and were compiled and analyzed on a computer (Microsoft Excel; Microsoft). The mean and SD were calculated for each group using the Student’s t-test. Results were considered to be statistically significant when p<0.01. The results of RT² qPCR are indicated as “fold increase” (mRNA concentrations of serum-free B27 supplemented cultures divided by mRNA concentrations of serum-dependent controls).
RESULTS

Under microscopic observation, we noted epithelial migration from limbal explants at the end of 48 h in both serum-dependent and serum-free B27 supplemented cultures (Figure 1). By the end of the 15th day, 90%–100% confluent growth was seen. There was no growth in the control samples cultured without serum and/or any other supplement.

**Growth kinetics:** The cells cultured in serum-free B27 supplemented medium showed significantly higher growth after 12 days (Figure 2). The growth rate was faster on cells cultured in a serum-free B27 supplemented culture when compared to a serum-dependent medium (p<0.005).

**Cell proliferation:** The labeling index was high in serum-free B27 supplemented culture when compared to serum-dependent culture after 24 h. The cultures were reviewed continuously for 7, 14, and 21 days and the labeling indices were 50±7.76, 42±2.24, 20±2.0, and 12±0.2%, respectively, in serum-free B27 supplemented culture. Similarly, in the serum-dependent culture, the labeling indices were 48±3.2, 35±0.33, 17±1.7, and 9±1.1% for 7, 14, and 21 days, respectively (Figure 3).

**RT–PCR:** Semiquantitative RT–PCR results showed similar expressions (Table 2) of various markers such as transformation-related protein 63 - *p63*, ATP-binding cassette sub-family G member 2 - *ABCG2*, connexin 43, and Keratin 3/Keratin 12 – *K3/K12* of differentiated corneal epithelial cells (21st day) grown in the serum-dependent and serum-free B27 supplemented medium (Figure 4).

**Comparison of signal transduction pathway genes supporting the expansion of serum-dependent and serum-free B27 supplemented culture:** The array experiment was performed in duplicate. A simple comparison was performed on data to assess the gene expression of a serum-free B27 supplemented culture in relation a serum-dependent culture as a control for limbal stem cells and differentiated corneal epithelial cells (Table 3). The differences in gene expression...
between the serum-free B27 supplemented culture and the serum-dependent profile of limbal and corneal cells were studied (a more than twofold difference was considered significant). The raw data, i.e., the mean ∆∆C\text{t} values of the genes, were normalized to the housekeeping gene GAPDH. All 84 genes were analyzed thoroughly based on their role in both the serum and serum-free conditions. Among these pathways, the most interesting and highly expressed were wnt, hedgehog, survival, NFkB, Jak-Stat, and the calcium protein kinase C pathways that have been discussed in this study (Figure 5).

**DISCUSSION**

We have demonstrated the use of serum-free B27 supplemented medium for the growth of corneal epithelial cells. This serum-free medium supported the proliferation and viability of the cells. The cells expressed presumed limbal stem cell association markers and the cornea phenotype, suggesting that the serum-free B27 supplemented medium retained the stemness of cultured cells. The confluent culture was collected and RNA was isolated to analyze the signaling pathway genes involved in both serum-dependent and serum-free B27 supplemented cultures.

The signal transduction pathway genes involved in the growth of corneal epithelial cells help to determine their role in both serum-dependent and serum-free B27 supplemented corneal epithelial cultures. Among the 17 pathways, six pathways involved in the serum-free B27 supplemented culture were discussed, along with their roles in serum-free conditions.

**Table 2. mRNA Expression of Cultured Corneal Cells Grown in Serum-Dependent and Serum-Free B27 Supplemented Medium.**

| Markers          | Serum-dependent | B27-dependent |
|------------------|-----------------|---------------|
| ABCG2            | -               | -             |
| P63              | +               | +             |
| Connexin 43      | +               | +             |
| Keratin 3        | +               | +             |
| Keratin 12       | +               | +             |

GAPDH is an internal control; + positive marker; - negative marker.

**Figure 4.** RT–PCR for mRNA expression of putative limbal/corneal stem cell markers. Lane 1: Negative control; Lane 2: Positive control; Lane 3: serum-free B27 supplemented corneal cells; Lane 4: serum-dependent corneal cells; Lane 5: 100 bp DNA ladder.
TABLE 3. SIGNAL TRANSDUCTION PATHWAY GENE PROFILE SUPPORTING THE EXPANSION OF SERUM-FREE B27 SUPPLEMENTED LIMBUS/CORNEAL CULTURE (SERUM-DEPENDENT CULTURE AS CONTROL).

| Symbol | Limbus | Cornea | Description | Gene Name |
|--------|--------|--------|-------------|-----------|
| **Mitogenic Pathway** |
| EGR1   | 12.06  | 1.16   | Early growth response 1 | AT225/G0S30 |
| FOS    | 67.78  | 1.6    | V-fos FBJ murine osteosarcoma viral oncogene homolog | AP-1/C-FOS |
| JUN    | 8.51   | 1.64   | Jun oncogene | AP-1/A1 |
| **Wnt Pathway** |
| CCND1  | 4.28   | –1.72  | Cyclin D1 | BCL1/D11S287E |
| JUN    | 8.51   | 1.64   | Jun oncogene | AP-1/A1 |
| LEF1   | 12.06  | 1.13   | Lymphoid enhancer-binding factor 1 | DKKFz586H0919/TCF1ALPHA |
| MYC    | 4.28   | –3.56  | V-myc myelocytomatosis viral oncogene homolog (avian) | MRTL/hHLHe39 |
| PPARG  | 2.14   | –1.33  | Peroxisome proliferator-activated receptor gamma | CMT1/1NR1C3 |
| TC67   | 12.06  | 1.13   | Transcription factor 7 (T-cell specific, HMG-box) | TCF-1 |
| VEGFA  | 1.07   | –1.74  | Vascular endothelial growth factor A | MVCD1/VEGF |
| WISP1  | 11.65  | 1.13   | WNT1 inducible signaling pathway protein 1 | CCN4/WISP1c |
| **Hedgehog Pathway** |
| BMP2   | 8.52   | –3.57  | Bone morphogenetic protein 2 | BMP2A |
| BMP4   | 4.32   | –2.46  | Bone morphogenetic protein 4 | BMP2B/BMP2B1 |
| EN1    | 11.76  | 1.13   | Engrailed homeobox 1 | Engrailed 1 |
| FOG1A2 | 12.06  | 1.13   | Forkhead box A2 | HNF3B/TCF3B |
| PTCH1  | 2.01   | –2.46  | Patched homolog 1 (Drosophila) | BCNS/HPE7 |
| WNT1   | 12.06  | 1.13   | Wingless-type MMTV integration site family, member 1 | INT1 |
| WNT2   | 12.06  | 1.17   | Wingless-type MMTV integration site family member 2 | INT1L1/IRP |
| **TGF-Beta Pathway** |
| CDK11A | 3.00   | –2.47  | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | CAP20/CDKN1 |
| CDK11B | 5.99   | 1.65   | Cyclin-dependent kinase inhibitor 1B (p27, Kip1) | CDKN4/KIP1 |
| CDK2N2A| –1.34  | –6.95  | Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) | ARF/CDK4 |
| CDK2N2B| 2.12   | –1.75  | Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) | CDK4/INK4B |
| **PI3 Kinase/AKT Pathway** |
| BCL2   | 12.06  | 1.13   | B-cell CLL/lymphoma 2 | Bel-2 |
| CCND1  | 4.28   | –1.72  | Cyclin D1 | BCL1/D11S287E |
| JUN    | 8.51   | 1.64   | Jun oncogene | AP-1/A1 |
| MYC    | 4.28   | –3.56  | V-myc myelocytomatosis viral oncogene homolog (avian) | MRTL/hHLHe39 |
| **Jak/Src Pathway** |
| BCL2   | 12.06  | 1.13   | B-cell CLL/lymphoma 2 | Bel-2 |
| BCL2L1 | 6.03   | –7.04  | BCL2-like 1 | BCL-XL/S |
| **NFkB Pathway** |
| BCL2A1 | 1.50   | 2.33   | BCL2-related protein A1 | ACC-1/1ACC-2 |
| BIRC2A | 2.13   | 3.24   | Baculoviral IAP repeat-containing 2 | API1/HIAp2 |
| BIRC3  | 1.06   | –2.48  | Baculoviral IAP repeat-containing 3 | API1/AIP2 |
| NAIP   | 2.13   | 1.14   | NLR family, apoptosis inhibitory protein | BIRC1/NLRB1 |
| TERT   | 12.06  | 1.13   | Telomerase reverse transcriptase | EST2/TCS1 |
| **P53 Pathway** |
| BAX    | 3.01   | –14.1  | BCL2-associated X protein | BCL2A1 |
| CDKN1A | 3.00   | –2.47  | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | CAP20/CDKN1 |
| Fas    | –1.33  | –1.25  | Fas (TNF receptor superfamily, member 6) | ALPS1/1APO-1 |
| GADD45A| 5.99   | 2.26   | Growth arrest and DNA-damage-inducible, alpha | DDIT1/GADD45 |
| IGF1R  | –14.95 | –40    | Insulin-like growth factor binding protein 3 | BP-53/IBP3 |
| MDM2   | 1.06   | –4.93  | Mdm2 p53 binding protein homolog (mouse) | HDMX/hdm2 |
| TP53L1 | 4.28   | –1.22  | Tumor protein p53 inducible protein 3 | PIG3 |
| **Stress Pathway** |
| ATF2   | 3.01   | –2.48  | Activating transcription factor 2 | CRE-BP1/CREB2 |
| FOS    | 67.78  | 1.6    | V-fos FBJ murine osteosarcoma viral oncogene homolog | AP-1/C-FOS |
| HSFl (tcf5) | 4.25 | 1.15  | Heat shock transcription factor 1 | HSTF1 |
| HSFB1 (hsp27) | 4.27 | –1.25 | Heat shock protein 27 kDa protein 1 | CMT2F/DKFZ586P1322 |
| HSPCA (hsp90) | 1.50 | –3.48 | Heat shock protein 90 kDa alpha (cytosolic), class A member 2 | HSP90ALPHA/HSPCA |
| MYC    | 4.28   | –3.56  | V-myc myelocytomatosis viral oncogene homolog (avian) | MRTL/hHLHe39 |
| TP53   | 1.07   | –1.75  | Tumor protein p53 | LFS1/TRP53 |
| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| IKBKB      | 2.11     | −2.53    | Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta | IKK-beta/IKK2              |
| IL1A       | 8.48     | 3.27     | Interleukin 1, alpha                              | IL-1A/IL1                  |
| IL2        | 11.72    | 1.17     | Interleukin 2                                    | IL-2/TCGF                 |
| IL8        | 1.53     | −9.92    | Interleukin 8                                    | CXCL8/GCP-1               |
| LTA (TNF beta) | 11.75 | 1.13     | Lymphotoxin alpha (TNF superfamily, member 1)    | LT/TNF                    |
| NOS2A (iNOS) | 1.42  | −3.52    | Nitric oxide synthase 2, inducible               | HEP-NOS/INOS              |
| PECAM1     | 8.03     | 1.09     | Platelet/endothelial cell adhesion molecule      | CD31/PECAM-1              |
| TANK       | 5.70     | −3.63    | TRAF family member-associated NFKB activator    | I-TRAF/TRAF2              |
| TNF        | 7.99     | −1.75    | Tumor necrosis factor (TNF superfamily, member 2) | DIF/TNF-alpha             |
| VCAM1      | 12.06    | 1.13     | Vascular cell adhesion molecule 1                | CD106/DKFZp779 G2333      |

**NFAT Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| CD5        | 11.65    | 1.13     | CD5 molecule                                     | LEU1/T1                    |
| FASLG (TNFSF6) | 11.69 | 1.16     | Fas ligand (TNF superfamily, member 6)            | APT1LG1/CD178              |
| IL2        | 11.72    | 1.17     | Interleukin 2                                    | IL-2/TCGF                 |

**CREB Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| CYP19A1    | 11.27    | 1.13     | Cytochrome P450, family 19, subfamily A, polypeptide 1 | ARO/AR01                   |
| EGR1       | 12.06    | 1.16     | Early growth response 1                          | AT225/G0S30                |
| FOS        | 67.78    | 1.6      | V-fos FBJ murine osteosarcoma viral oncogene homolog | AP-1/C-FOS                 |

**Jak-Stat pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| CXCL9      | 11.14    | 1.13     | Chemokine (C-X-C motif) ligand 9                  | CMK/Humig                  |
| IL4        | 11.33    | 1.13     | Interleukin 4                                    | BCGF-1/BCGF1               |
| IL10       | 1.51     | −3.52    | Interleukin 4 receptor                           | CD124A/IL4RA               |
| MMP10      | 3.02     | −1.76    | Matrix metalloproteinase 10 (stromelysin 2)      | SL-2/STMY2                 |
| NOS2A (iNOS) | 1.42  | −3.52    | Nitric oxide synthase 2, inducible               | HEP-NOS/INOS              |

**Estrogen Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| BCL2       | 12.06    | 1.13     | B-cell CLL/lymphoma 2                            | Bel-2                      |
| BRCA1      | 8.50     | 1.1      | Breast cancer 1, early onset                      | BRCA1/BRCC1               |
| GREB1      | 11.72    | 1.16     | GREB1 protein                                    | KIAA0575                  |
| NRRIP1     | −1.32    | −3.51    | Nuclear receptor interacting protein 1           | RIP140                     |

**Androgen Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| CDK2       | 8.55     | −1.75    | Cyclin-dependent kinase 2                        | p33(CDK2)                  |
| CDKN1A     | 3.00     | −2.47    | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | CAP20/CDKN1                |
| KLK2       | 11.41    | 1.16     | Kallikrein-related peptidase 2                   | KLK2A2/hK2                 |
| TMEPAI     | −1.87    | −1.74    | Prostate transmembrane protein, androgen induced 1 | STAG1/TMEPAI               |

**Calcium and protein kinase C Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| CSF2       | 1.42     | −1.84    | Colony stimulating factor 2 (granulocyte-macrophase) | GMCSF                      |
| FOS        | 67.78    | 1.6      | V-fos FBJ murine osteosarcoma viral oncogene homolog | AP-1/C-FOS                 |
| IL2        | 11.72    | 1.17     | Interleukin 2                                    | IL-2/TCGF                 |
| JUN        | 8.51     | 1.64     | Jun oncogene                                     | AP-1/A1I                  |
| MYC        | 4.28     | −3.56    | V-myc myelocytomatosis viral oncogene homolog (avian) | MRTL/bHLHe39              |
| ODC1       | 8.54     | 1.65     | Ornithine decarboxylase 1                         | ODC                        |
| PRKCA      | 5.62     | 1.13     | Protein kinase C, alpha                           | AG6G/PKC-alpha             |
| PRKCE      | 2.92     | −2.48    | Protein kinase C, epsilon                         | PKC/-/PKC-epsilon          |
| TFRC       | −1.30    | −1.74    | Transferrin receptor (p90, CD71)                  | CD71/TFR                   |

**Phospholipase C Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| BCL2       | 12.06    | 1.13     | B-cell CLL/lymphoma 2                            | Bel-2                      |
| EGR1       | 12.06    | 1.16     | Early growth response 1                          | AT225/G0S30                |
| FOS        | 67.78    | 1.6      | V-fos FBJ murine osteosarcoma viral oncogene homolog | AP-1/C-FOS                 |
| ICAM1      | −2.68    | −2.07    | Intercellular adhesion molecule 1                | BB2/CD54                   |
| JUN        | 8.51     | 1.64     | Jun oncogene                                     | AP-1/A1I                  |
| NOS2A      | 1.42     | −3.52    | Nitric oxide synthase 2, inducible               | HEP-NOS/INOS              |
| PTGS2      | 23.98    | 4.57     | Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | COX-2/COX2                |
| VCAM1      | 12.06    | 1.13     | Vascular cell adhesion molecule 1                | CD106/DKFZp779G2333       |

**Insulin Pathway**

| Symbol     | Limbus   | Cornea   | Description                                      | Gene Name                  |
|------------|----------|----------|--------------------------------------------------|----------------------------|
| CEBPB      | 2.90     | 3.3      | CCAAT/enhancer binding protein (C/EBP), beta      | C/EBP-beta                 |
| FASN       | 4.26     | 1.14     | Fatty acid synthase                              | FAS/OA-519                 |
| GYS1       | 3.03     | 3.29     | Glycogen synthase 1 (muscle)                      | GSY/GYS                    |
| HK2        | 2.99     | 1.69     | Hexokinase 2                                     | DFKZp686M1669/HKII         |
| LEP        | 12.06    | 1.13     | Leptin                                           | OB/OS                      |
In the serum-free condition of the corneal epithelial cells, the activation of wnt pathway plays a vital role by activating genes like \textit{Homo sapiens} jun oncogene (\textit{JUN}), which codes for a transcription factor called activator protein-1 (AP1) and helps in the differentiation, proliferation, and apoptosis of epithelial cells [6]. Corneal epithelial stem cell proliferation depends on the upregulation of paired box gene 6 (\textit{pax6}) and downregulation of beta-catenin and lymphoid enhancer-binding factor 1 (\textit{Lef-1}) [7]. The hedgehog pathway genes were 2 to 8 times upregulated in serum-free B27 supplemented limbal stem cells when compared with differentiated corneal epithelial cells of the same culture. Sonic hedgehog (Shh) is secreted by stem cells, inducing bone morphogenetic protein 4 (BMP4), and is involved in the self-renewal and development of the epithelium [8]. The wingless-type MMTV integration site family, member 1 (\textit{wnt1}) and Wingless-type MMTV integration site family, member 21 (\textit{wnt2}) genes of this pathway were found to play an equal role (12 times upregulated in relation to the serum-dependent culture) in the maintenance of stemness in limbal epithelial cells of the serum-free B27 supplemented culture. The cellular survival pathway consists of phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene homolog 1 (\textit{PI3K/Akt}), Janus kinase/sarcoma proto oncogene (\textit{Jak/Src}), and nuclear factor kappa-light-chain-enhancer of activated B (\textit{NFkB}) as three major groups of genes. The cyclin D1 (\textit{CCND1}) gene is required for cell cycle G1/S transition [9]. Baculoviral Inhibitor of Apoptosis repeat proteins (Birc1) proteins contain BIR domains that can directly bind to active caspases and help in protein–protein interaction [10]. In the stem cell and progenitor cell compartments, the telomerase reverse transcriptase (\textit{TERT}) gene prevents the adverse consequences of dysfunctional telomeres on cell viability and chromosomal stability [11], and enhances the cell cycle entry of quiescent limbal stem cell and differentiated corneal epithelial cell cultures.

### Table 3. Continued.

| Symbol     | LDL Pathway | Limbus | Cornea | Description                                      | Gene Name       |
|------------|-------------|--------|--------|-------------------------------------------------|-----------------|
| CCL2       | 8.79        | 1.13   |        | Chemokine (C-C motif) ligand 2                   | GDCF-2/HC11     |
| CSF2       | 11.42       | −1.84  |        | Colony stimulating factor 2 (granulocyte-macrophage) | GMCSF           |
| SELE       | 11.70       | 1.13   |        | Selectin E                                      | CD62E/ELAM      |
| SELPLG     | 12.06       | 1.13   |        | Selectin P ligand                              | CD162/CLA       |
| VCAM1      | 12.06       | 1.13   |        | Vascular cell adhesion molecule 1               | CD106/DKZp779 G2333 |

### Retinoic acid Pathway

| Symbol     | LDL Pathway | Limbus | Cornea | Description                                      | Gene Name       |
|------------|-------------|--------|--------|-------------------------------------------------|-----------------|
| EN1        | 11.76       | 1.13   |        | Engrailed homeobox 1                             | Engrailed 1     |
| HOXA1      | 12.06       | 1.13   |        | Homeobox A1                                     | BSAS/HOX1       |
| RBP1 (CRBP1)| 1.06        | −1.74  |        | Retinol binding protein 1, cellular              | CRABP1/CRBP     |
epidermal stem cells [12]. The NFkB pathway genes in serum-
free B27 supplemented cells had a distinct fold increase when
compared with the control, and a few genes like interleukin 1
alpha (IL1A), interleukin 2 (IL2), lymphotoxin alpha (LTA),
platelet/endothelial cell adhesion molecule 1 (PECAM1), and
vascular cell adhesion molecule 1 (VCAM1) exhibited
upfolded expression in both limbus and corneal cells. The
inhibitor of kappa light polypeptide gene enhancer in B-cells,
k kinase beta (IKKB) gene produced an enzyme, IKK2 -
h i b i t o r of nuclear factor kappa-B kinase subunit and
activated a transcription factor called NFKB. Interleukin genes
like IL1A, interleukin 8 (IL8), and tumor necrosis factor alpha
(TNFα) present in the NFkB pathway encode for cytokines
and chemokines involved in inflammatory processes [13,14].
They also helped in the migration of progenitor and pluripotent
stem cells [15]. The chemokine (C-X-C motif) ligand 9
(CXCL9) and interleukin 4 (IL4) genes of the Jak-Stat pathway
played an important role in the development and organization
of cells, which were upregulated by 12 times in serum-free
B27 supplemented limbus culture [16]. Among the other five
pathways, the calcium and protein kinase C pathway genes
were highly expressed in serum free-B27 supplemented
culture when compared to serum-dependent culture. The Homo sapiens V-fos FBJ murine osteosarcoma viral
oncogene homolog (FOS) gene of the calcium and protein
kinase C pathway belonged to the transcription factor family
[17], which is highly upregulated in serum-free B27
supplemented limbal stem cell cultures.

In conclusion, the B27 supplement activated more
signaling pathway genes, helping to provide a higher cell
number, good capacity for proliferation, better quality, and
more functional pieces of engineered corneal equivalents
without the support of serum, a feeder layer, and/or BPE.

ACKNOWLEDGMENTS
The authors would like to thank the Indian Council of Medical
Research (Grant No: 80/7/2003-BMS) for financial support.

REFERENCES
1. Yokoo S, Yamagami S, Usui T, Amano S, Araie M. Human
Corneal Epithelial Equivalents for Ocular Surface
Reconstruction in a complete Serum-Free Culture System
without Unknown Factors. Invest Ophthalmol Vis Sci 2008;
49:2438-43. [PMID: 18515584]
2. Anderson DF, Ellies P, Pires RT, Tseng SC. Amniotic
membrane transplantation for partial limbal stem cell
deficiency. Br J Ophthalmol 2001; 85:567-75. [PMID:
11316719]
3. Brewer GJ. Serum-free B27/neurobasal medium supports
differentiated growth of neurons from the striatum, substantia
nigra, septum, cerebral cortex, cerebellum, and dentate gyrus.
J Neurosci Res 1995; 42:674-83. [PMID: 8600300]
4. Yokoo S, Yamagami S, Yanagi Y, Uchida S, Mimura T, Usui
T, Amano S. Human corneal endothelial cell precursors
isolated by sphere-forming assay. Invest Ophthalmol Vis Sci
2005; 46:1626-31. [PMID: 15851561]
5. Sudha B, Jasty S, Krishnan S, Krishnakumar S. Signal
transduction pathway involved in the ex vivo expansion of
limbal epithelial cells cultured on various substrates. Indian J
Med Res 2009; 129:382-9. [PMID: 19535832]
6. Katiyar S, Jiao X, Wagner E, Lisanti MP, Pestell RG. Somatic
excision demonstrates that c-Jun induces cellular migration
and invasion through induction of stem cell factor. Mol Cell
Biol 2007; 27:1356-69. [PMID: 17145782]
7. Yang K, Jiang Z, Wang D, Lian X, Yang T. Corneal epithelial-
like transdifferentiation of hair follicle stem cells is mediated
by pax6 and beta-catenin/Lef-1. Cell Biol Int 2009;
33:861-6. [PMID: 19393751]
8. Ishizuya-Oka A, Hasebe T. Sonic hedgehog and bone
morphogenetic protein-4 signaling pathway involved in
epithelial cell renewal along the radial axis of the intestine.
Digestion 2008; 77:42-7. [PMID: 18204261]
9. Klein EA, Yang C, Kazanian MG, Assoian RK. NFkappaB-
independent signaling to the cyclin D1 gene by Rac. Cell
Cycle 2007; 6:1115-21. [PMID: 17426454]
10. Yin Y, Huang WW, Lin C, Chen H, MacKenzie A, Ma L.
Estrogen Suppresses Uterine Epithelial Apoptosis by
Inducing Birc1 Expression. Mol Endocrinol 2008;
22:113-25. [PMID: 17901126]
11. Sarin KY, Cheung P, Gilson D, Lee E, Tennen RI, Wang E,
Artandi MK, Oro AE, Artandi SE. Conditional telomerase
induction causes proliferation of hair follicle stem cells.
Nature 2005; 436:1048-52. [PMID: 16107853]
12. Choi J, Southworth LK, Sarin KY, Venteicher AS, Ma W,
Chang W, Cheung P, Jun S, Artandi MK, Shah N, Kim SK,
Artandi SE. TERT promotes epithelial proliferation through
transcriptional control of a Myc- and Wnt-related
developmental program. PLoS Genet 2008; 4:e10. [PMID:
18208333]
13. Lee P, Lee DJ, Chan C, Chen SW, Ch'en I, Jamora C. Dynamic
expression of epidermal caspase 8 simulates a wound healing
response. Nature 2009; 458:519-23. [PMID: 19204729]
14. Laterveer L, Lindsey IJ, Hamilton MS, Willemze R, Fibbe WE.
Interleukin-8 induces rapid mobilization of hematopoietic
stem cells with radioprotective capacity and long-term
myelolymphoid repopulating ability. Blood 1995;
85:2269-75. [PMID: 7718900]
15. Okada N, Fukagawa K, Takano Y, Dogru M, Tsubota K,
Fujishima H, Matsumoto K, Nakajima T, Saito H.
The implications of the upregulation of ICAM-1/VCAM-1
expression of corneal fibroblasts on the pathogenesis of
allergic keratopathy. Invest Ophthalmol Vis Sci 2005;
46:4512-8. [PMID: 16303942]
16. Jin DK, Shido K, Koppe HG, Petit I, Shmelkov SV, Young LM,
Hooper AT, Amano H, Aveccilla ST, Heissig B, Hattori K,
Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Hackett
NR, Crystal RG, Lyden D, Rafii S. Cytokine-mediated
deployment of SDF-1 induces revascularization through
recruitment of CXCR4+ hemangiocytes. Nat Med 2006;
12:557-67. Werb Z. [PMID: 16648859]
17. Conaway HH, Persson E, Halén M, Granholm S, Svensson O,
Pettersson U, Lie A, Lerner UH. Retinoids inhibit
differentiation of hematopoietic osteoclast progenitors.
FASEB J 2009; 23:3526-38. [PMID: 19546303]