Comparison of the Histology, Gene Expression Profile, and Phenotype of Cultured Human Limbal Epithelial Cells from Different Limbal Regions

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PURPOSE. To investigate whether human limbal epithelial cells (HLECs) derived from various regions of the limbus exhibit differences in gene expression and epithelial characteristics.

METHODS. HLECs were derived from explants taken from the superior, nasal, inferior, and temporal limbus and cultured for 21 days. Whole genome transcript profiling was performed with a gene microarray. The microarray results were validated by using RT-PCR. Epithelial morphology was studied with light microscopy and transmission electron microscopy, and phenotype was evaluated by immunohistochemistry.

RESULTS. Epithelial outgrowth was present in most cultures of superior origin (88%) in contrast to cultures of temporal origin (38%). The epithelial thickness and number of cell layers were significantly greater in cultures of superior origin than in cultures from inferior and temporal areas. TRIM36, OSR2, and RHOU, which are involved in morphogenesis, were significantly differentially expressed in the superior region, compared with the other regions. Proposed limbal stem cell, progenitor, and differentiation markers were not differentially expressed. The uniform gene expression of ocular surface structures (palisades of Vogt) are the source of renewal of the corneal epithelium was first proposed by Davanger and Evensen,1,2 and has been confirmed in several subsequent studies.3–8 It has been reported that LSCD may improve the cell therapies used to treat limbal stem cell deficiency (LSCD). After the work of Pellegrini et al.,1 many protocols for culturing human limbal epithelial cells (HLECs) have been described.2–8 It has been reported that LSCD may also be treated with cultured conjunctival,9 oral mucosal,10 and, recently, epidermal11 epithelial cells. Ex vivo expansion of HLECs requires a limbal biopsy to generate an epithelial sheet for transplantation. The sheet should comprise undifferentiated cells,2,12 be sufficiently large to cover the cornea, and have adequate strength13,14 to tolerate transplantation and the post-operative period. The biopsy may be harvested from the patient (autograft), a living related donor, or a cadaveric eye (allograft), but the optimal circumferential location of biopsy sites for transplantation and research purposes remains unclear. Shortt et al.15 in reviewing the clinical application of ex vivo cultured HLECs, reported that only 2 of 17 studies specified the site of tissue harvesting.16 In these two studies, the biopsy was obtained from the superior temporal limbus, although the rationale for using this location was not described.

The concept that epithelial cells in the limbal papillary structures (palisades of Vogt) are the source of renewal of the corneal epithelium was first proposed by Davanger and Evensen,1 and has been confirmed in several subsequent studies.18–20 Goldberg et al.21 first described heterogeneity between the limbal regions, reporting the highest density of limbal crypts in the superior and inferior limbus. Later studies have supported these findings.22 Wiley et al.23 observed characteristics of limbal cells, defined as AE1-positive and AE5-negative, deep within the peripheral cornea in the superior and inferior regions, but to a lesser extent in the temporal and nasal regions. Recently, a novel putative stem cell niche has been reported, in the form of limbal epithelial crypts, which are located predominantly in the nasal, followed by the inferior, superior, and temporal regions.24 Despite the great interest in limbal heterogeneity,21,25–29 few studies have been undertaken to examine the effect of limbal biopsy origin on the characteristics of HLECs cultures. Pellegrini et al.30 performed a study of the clonogenic ability and the proliferative potential of keratinocytes cultured from biopsies taken from four limbal regions, but they did not perform a histologic or phenotypic comparison. Recently, Shortt et al.31 using confocal laser microscopy described the presence of limbal epithelial stem cell (LESC) niches, termed limbal crypts (LC) and focal stromal projections (FSP), in an uneven distribution around the corneal circumference. The structures were predominantly located in the superior and inferior limbal regions, extending temporally and nasally to various degrees, but they were absent from the horizontal meridian of all donors. However, Shortt et al.32 did not observe any differences between the superior and inferior regions. Biopsies rich in LESC niche structures yielded significantly...
higher numbers of holoclone colonies (proposed stem cell colonies) compared with non-LC/non-FSP regions.

In the present study, we hypothesized that cultures derived from different regions of the limbus exhibit differences in morphology, gene expression, and phenotype, and that such differences could affect the success of cultured limbal transplants. A comprehensive search of possible regional differences in HLEC cultures was conducted with gene expression arrays (GeneChip; Affymetrix, Santa Clara, CA) offering whole-transcript coverage, adding a new dimension to the ongoing debate about limbal regional heterogeneity. The present study is the first to compare the gene expression, histology, and immunohistochemistry of cultured HLECs derived from explants harvested from different limbal regions.

**MATERIALS AND METHODS**

Dulbecco’s modified Eagle’s medium (DMEM), HEPES-buffered DMEM containing sodium bicarbonate and Ham’s F12 (1:1), Hanks’ balanced salt solution, fetal bovine serum (FBS), insulin–transferrin–sodium selenite medium supplement, human epidermal growth factor, dimethyl sulfoxide, hydrocortisone, gentamicin, amphotericin B, β-mercaptoethanol, and mouse anti-ABCG2 antibody (clone bxp21) were purchased from Sigma-Aldrich (St. Louis, MO). Dispase II was obtained from Roche Diagnostics (Basel, Switzerland), cholera toxin A subunit from Bioworld (Exeter, UK), 5-mm biopsy punches from Kai Industries (Gifu, Japan), 6-0 C-2 monofilament sutures from Ethicon Ethilon (Johnson & Johnson (New Brunswick, NJ), 24-μm culture plate inserts (74-μm mesh size polyester membrane; Netwell) from Corning Costar (Corning, NY), and vancomycin from Abbott Laboratories (Abbott Park, IL). Mouse anti-p63 antibody (clone 4A4), mouse anti-K19 antibody (clone RCK108), and anti-PCNA antibody (clone PC10) were obtained from Dako (Glostrup, Denmark); mouse anti-vimentin antibody (clone Vim 3B4) from Ventana Medical Systems (Tucson, AZ); rabbit polyclonal anti-ΔNp63α antibody from Primm (Milano, Italy); mouse anti-Ki67 antibody (clone SP6) from LabVision Corporation (Fremont, CA); mouse anti-nestin antibody (clone 10C2) from Biomol (Exeter, UK), 6-0 C-2 monofilament sutures (Ethicon Ethilon) from Johnson & Johnson (New Brunswick, NJ), 24-μm culture plate inserts (74-μm mesh size polyester membrane; Netwell) from Corning Costar (Corning, NY), and vancomycin from Abbott Laboratories (Abbott Park, IL). Mouse anti-p63 antibody (clone 4A4), mouse anti-K19 antibody (clone RCK108), and anti-PCNA antibody (clone PC10) were obtained from Dako (Glostrup, Denmark); mouse anti-vimentin antibody (clone Vim 3B4) from Ventana Medical Systems (Tucson, AZ); rabbit polyclonal anti-ΔNp63α antibody from Primm (Milano, Italy); mouse anti-Ki67 antibody (clone SP6) from LabVision Corporation (Fremont, CA); mouse anti-nestin antibody (clone 10C2) from Santa Cruz Biotechnology (Santa Cruz, CA); and mouse anti-K3 antibody (clone AE5) from ImmunQuot (Cleveland, UK). The following antibodies were sourced from Novocastra Laboratories. Ltd. (Newcastle, UK): mouse anti-K5 (clone XM26), mouse anti-Ecadherin (clone NCh-38), and mouse anti-integrin B1 (clone 7F10). A peroxidase detection system (EnVision) was purchased from Dako; cryotubes from Nunc (Roskilde, Denmark); an RNaseasy micro kit, RLT buffer, and a reverse transcription kit (Omniscript) from Qiagen (Hilden, Germany); and 1.5-mL microcentrifuge tubes from Eppendorf (Hamburg, Germany). A cDNA synthesis kit (GeneChip HT One-Cycle) for gene expression assays and universal PCR master mix (Taqman cat. no. 4304437) were from Applied Biosystems (ABI-Lifetech, Carlsbad, CA).

**Human Tissue Preparation**

Human tissue was used in accordance with the Declaration of Helsinki. Oriented cadaveric human corneas were obtained with research consent from the Centro de Oftalmologia Barraquer (Barcelona, Spain). The present study was conducted on eight corneas obtained from four donors with a suture placed at the 12 o’clock position; mean age, 74.8 years (range, 56 – 83); mean time from death to enucleation, 8.6 hours (range, 6 – 11); time from death to culture, 7 days (range, 3.5 – 11.5). Limbal tissue was prepared as reported by Meller et al.12 Briefly, the tissue was rinsed three times with DMEM containing 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B. After careful removal of excised sclera, conjunctiva, iris, and corneal endothelium, the remaining tissue was placed in a culture dish and exposed for 10 minutes to Dispase II in Mg and Ca-free Hanks’ balanced salt solution, at 37°C under humidified 5% carbon dioxide, and thereafter rinsed with DMEM containing 10% FBS. From the superior, nasal, inferior, and temporal limbal regions (1). HLECs were cultured for 3 weeks on intact amniotic membranes in supplemented hormonal epithelial medium (2). Disks of cultured epithelium were trephined with a 5-mm biopsy punch and stored in cryotubes at −80°C (3a). RNA was extracted (3b). One hundred nanograms of total RNA was subjected to cDNA synthesis and labeling. Labeled and fragmented single stranded DNAs were hybridized to the gene microarray (3c) before washing and staining. The remaining HLEC cultures were fixed in neutral buffered 4% formaldehyde. A rectangular specimen including the cultured epithelium and the explant was processed and embedded in paraffin for histology and immunohistochemistry (4), whereas the remaining tissue from three cultures from each experimental group was prepared for transmission electron microscopy.

**Human Limbal Explant Cultures on Intact Amniotic Membranes**

Human amniotic membranes (AMs) were preserved according to a method described by Lee and Tseng.52 After they were thawed at room temperature, the devitalized intact AM was fastened, with the epithelial side facing up, to the polyester membrane of a culture plate insert using monofilament sutures, as previously reported.7,8 In the center of each AM insert, explants were cultured with the epithelial side facing down10 in supplemented hormonal epithelial medium (HEPES-buffered DMEM containing sodium bicarbonate and Ham’s F12 [1:1] supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/mL human epidermal growth factor, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 3 ng/mL hydrocortisone, 30 ng/mL cholera toxin, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B). The cultures were incubated for 3 weeks at 37°C in humidified 5% carbon dioxide, and the medium was changed every 2 to 3 days.

**FIGURE 1.** Experimental design of the study. Corneoscleral explants of approximately 8 × 5 × 0.5 mm were excised from the superior, nasal, inferior, and temporal limbal regions (1). HLECs were cultured for 3 weeks on intact amniotic membranes in supplemented hormonal epithelial medium (2). Disks of cultured epithelium were trephined with a 5-mm biopsy punch and stored in cryotubes at −80°C (3a). RNA was extracted (3b). One hundred nanograms of total RNA was subjected to cDNA synthesis and labeling. Labeled and fragmented single stranded DNAs were hybridized to the gene microarray (3c) before washing and staining. The remaining HLEC cultures were fixed in neutral buffered 4% formaldehyde. A rectangular specimen including the cultured epithelium and the explant was processed and embedded in paraffin for histology and immunohistochemistry (4), whereas the remaining tissue from three cultures from each experimental group was prepared for transmission electron microscopy.

![Diagram](image_url)
RNA Isolation

Disks of cultured epithelium and AM on polyester membranes were trephined from cultures of superior (n = 8), nasal (n = 8), inferior (n = 8), and temporal (n = 8) regions using a 5-mm biopsy punch. The biopsies were stored in cryotubes at −80°C until needed (Fig. 1). Total RNA was extracted from thawed biopsies (RNeasy micro kit; Qiagen), according to the manufacturer’s protocol. Three hundred fifty microliters of RTL buffer containing β-mercaptoethanol was added to the disks in microcentrifuge tubes and vortexed for 2 minutes. RNA concentration was determined with a spectrophotometer (ND-1000 Nano Drop; Thermo Fisher Scientific, Wilmington, DE). RNA quality was confirmed with a bioanalyzer (2100 System and RNA 6000 Nano Assay; Agilent Technologies, Santa Clara, CA). RNA samples were immediately frozen and stored at −80°C. Samples (n = 2) of cryopreserved AM were processed in the same way to assess the RNA level in devitalized amniotic epithelium without cultured HLECs.

Gene Chip Hybridization

One hundred nanograms of total RNA was subjected to a cDNA synthesis and labeling kit (GeneChip HT One-Cycle; and GeneChip HT IVT; Affymetrix), according to the manufacturer’s protocol for whole-genome gene expression analysis. Labeled and fragmented single-stranded cRNAs were hybridized to the human microarrays (28,869 genes; GeneChip Human Gene 1.0 ST Arrays; Affymetrix). The arrays were washed and stained on a fluids station (ES-450; Affymetrix). Signal intensities were detected by a scanner (Gene Array Scanner 5000 7G; Hewlett Packard, Palo Alto, CA).

Preprocessing of Gene Data and Data Analysis

The scanned images were processed (GCOS 1.4; Affymetrix) and the CEL files were imported into a software program for analysis (ArrayAssist Advanced Software, ver. 5.5.1; Lobion Informatics, La Jolla, CA). The data were normalized by the manufacturer’s algorithm (Exon IterPLIER; Affymetrix) to calculate relative signal values for each probe set. In addition, quantile normalization was performed, and a variance stabilization factor of 16 was used. Normalized microarray data were imported into a spreadsheet (Excel; Microsoft, Redmond, WA) with the BRB-ArrayTools version 3.6.034 plug-in for statistical analyses. The gene pool size was limited by including only genes with expression levels that differed by ≥1.5-fold from the median, in at least 20% of arrays, and had more than 50% of the expression data available. The BRB-ArrayTools Class Comparison Analysis was used to identify significant changes in gene expression between different limbal regions.

RT-PCR Validation

The differential gene expression data were validated for the tripartite motif containing 36 (TRIM36), odd skipped related 2 (Drosophila) (OSR2), and Ras homolog gene family, member U (RHOU) genes, which were found to be significantly differentially expressed in the microarray experiments (Affymetrix). RT-PCR validation was performed using gene expression assays (TaqMan; ABI-Life Technologies) and the sequence detection system (ABI Prism 7900 HT; Life Technologies). Two hundred nanograms of RNA from HLEC cultures of the different limbal origins were reverse transcribed using a kit as previously reported (Omniscript RT Kit; Qiagen).35 Nine microcrystals of cDNA (diluted 1:200 in H2O) and 1 μL of TRIM36 Hs01120398_m1, RHOU Hs01078833-m1, OSR2 Hs01085594-m1, or GAPDH Hs99999905-m1 were added to 10 μL universal PCR master mix (TaqMan; ABI-Life Technologies). Each PCR point was run in triplicate. The relative changes of each transcript, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as endogenous control, were calculated (SDS Enterprise Database, ver. 2.1; SDS, Cary, NC) by the ΔΔCT method.36

Histology and Immunostaining

After trephination of epithelial disks for gene analysis, a rectangular sample including the limbal explant and cultured epithelium was trimmed, fixed in neutral buffered 4% formaldehyde (Fig. 1), dehydrated in increasing concentrations of ethanol, cleared with xylene, and embedded in paraffin. Semithin (5 μm) cross sections of superior (n = 8), nasal (n = 8), inferior (n = 8), and temporal (n = 8) regions were stained with hematoxylin and eosin (H&E). For each sample, measurement of epithelial thickness and counting of cell layers was performed by two independent investigators, at 400× magnification and at regular intervals of 500 μm and 50 μm of full-length cross sections. Measurements were analyzed using (Analysis software ver. 5; Soft Imaging System GmbH, Münster, Germany). Serial 5-μm sections were immunostained with antibodies recognizing p63 (1:25), ΔNp63α (1:200), ABCG2 (1:80), keratin 19 (K19, 1:200), vimentin (ready to use), integrin β1 (1:10), keratin 3 (K3, 1:500), keratin 5 (K5, 1:600), E-cadherin (1:25), nestin (1:80), Ki67 (1:75), and PCNA (1:1500). The detection of antibodies was performed (EnVision Peroxidase and LabVision autostainer 360; LabVision; Thermo Fisher Scientific Inc.). Optimal antibody dilutions were determined by titration using the positive controls recommended by the manufacturers. The expression of the various markers was assigned at a magnification of ×400 by two independent investigators. In addition, the expression of Ki67 and PCNA, calculated as the number of positive cells/total number of cells × 100%, was assessed.

Transmission Electron Microscopy

The remaining tissue from three cultures from each experimental group was fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer adjusted to pH 7.4, postfixed in 1% osmium tetroxide, and dehydrated through a graded series of ethanol up to 100%. The tissue blocks were immersed in propylene oxide twice for 20 minutes and embedded in Epon. Ultrathin sections were cut on a microtome (Leica Ultracut UCT; Leica, Wetzlar, Germany) and examined by transmission electron microscope (model CM120; Philips, Amsterdam, The Netherlands).

Statistical Analysis

According to guidelines for microarray-based expression profiling and informatics, we used F statistics (univariate F-test), because there were fewer than 10 samples per class.77 38 A nominal significance level of 0.001 was applied to limit the number of false-positive findings. Statistical comparisons of RT-PCR data were performed with the Student’s t-test (SPSS ver. 14.0; SPSS Inc., Chicago, IL), and P < 0.05 was considered significant. The Mann–Whitney test (SPSS ver. 14.0) was used to compare morphologic data and expression of Ki67 and PCNA among groups, and P < 0.05 was considered significant.

RESULTS

RNA Isolation and Whole-Genome Transcript Profiling

Similar mean RNA yields were obtained from HLEC cultures from all four regions of the limbus (superior: 209 ± 149 ng/μL; nasal: 136 ± 148 ng/μL; inferior: 103 ± 78 ng/μL; temporal: 85 ± 115 ng/μL; P > 0.05 for all regions). However, sufficient RNA for microarray analysis was extracted from seven cultures of superior origin, six cultures of nasal and inferior origin, and three cultures of temporal origin. The RNA yield extracted from devitalized intact amniotic epithelium was below the acceptable range of input amount, thus eliminating amniotic epithelial RNA as a source of error in the interpretation of microarray data. Proposed limbal stem cell, progenitor, and differentiation markers were not differentially expressed among cultures from the four regions. Of the 1989 genes that passed filtering criteria, only three, Tripartite motif containing 36 (TRIM36), odd skipped related 2 (Drosophila) (OSR2), and Ras homologue gene family, member U (RHOU), exhibited significant differences in HLEC cultures from the four limbal regions (P < 0.001; Tables 1, 2).

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Quantitative Real-Time PCR Validation of Microarray Data

RT-PCR showed significant differences for TRIM36, OSR2, and RHOU when the superior region was compared with the inferior, nasal, and temporal regions (Table 2). In addition, OSR2 was significantly downregulated inferiorly versus temporally, and RHOU was significantly altered nasally versus inferiorly and temporally.

Morphology of Cultured Epithelium

Confluent epithelial outgrowth was observed in different proportions of HLEC cultures from the four limbal regions: seven (88%) of eight cultures from the superior region, six (75%) of eight nasal cultures, four (50%) of eight inferior cultures, and three (38%) of eight temporal cultures. HLEC cultures from the four regions differed considerably with respect to epithelial thickness and stratification (Fig. 2). Epithelia from the superior limbus were well stratified and consisted of basal column-shaped cells, suprabasal cuboid wing cells, and flat squamous superficial cells similar to corneal epithelium in vivo. Epithelial thickness increased gradually toward the leading edge of the epithelial outgrowth, where it increased markedly. Nasal and inferior epithelia were thinner than in the superior cultures and consisted primarily of basal cells and flat superficial cells. Most of the temporal HLEC cultures were devoid of cells, whereas the remaining consisted of basal and flat superficial cells. Epithelial thickness differed significantly between HLEC cultures from the superior (mean, 52.4 ± 2.5 cell layers; maximum, 43.7 cell layers), compared with inferior (mean, 1.1 ± 1.2 cell layers; maximum, 2.5 cell layers; \( P = 0.02 \)) and temporal (mean, 0.8 ± 1.1 cell layers; maximum, 2.5 cell layers; \( P = 0.01 \)), but not nasal (mean, 1.4 ± 1.5 cell layers; maximum, 3.0 cell layers; \( P = 0.07 \)) cultures (Fig. 3B). Epithelial thickness and the number of cell layers were not significantly different in the nasal, inferior, and temporal cultures.

The ultrastructural analysis also showed that HLEC cultures differed with respect to cellular differentiation (Fig. 4). Epithelia from the superior limbus were clearly differentiated into basal column-shaped cells, suprabasal cuboid wing cells, and flat squamous superficial cells. In contrast, nasal, inferior, and temporal epithelia consisted primarily of basal cells and flat superficial cells. However, regardless of limbal origin, numerous desmosomal junctions were seen between adjacent epithelial cells, and the polymorphic basal cells attached well to the amniotic basement membrane by hemidesmosomes.

Immunophenotypical Analysis

Similar patterns of immunostaining of proposed limbal stem cell, progenitor, differentiation, and proliferation markers were observed in HLEC cultures from all four limbal regions (Fig. 5). In all cell layers of cultured epithelia, K3 and nestin were absent, whereas moderate cytoplasmic K5 expression was observed. Moderate membranous immunostaining with E-cadherin was observed in the suprabasal and superficial layers. In both the basal and suprabasal layers, strong nuclear p63 expression and cytoplasmic and membranous staining for K19 and vimentin, respectively, were noted. Integrin \( \beta 1 \) was expressed at a low level, mainly in basal cell membranes. There was weak nuclear expression of \( \Delta N p 63 \alpha \) and moderate membranous staining of ABCG2 in all layers of cultured epi-

### Table 1. Class Comparison Analysis* with Mean Normalized Log2-Transformed Gene Expressions for Significant Genes (\( P < 0.001 \)) in Cultured HLECs of Different Limbal Origin

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Unique ID</th>
<th>Superior Origin</th>
<th>Nasal Origin</th>
<th>Inferior Origin</th>
<th>Temporal Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIM36</td>
<td>8113577</td>
<td>6.70</td>
<td>5.73</td>
<td>5.81</td>
<td>5.41</td>
</tr>
<tr>
<td>OSR2</td>
<td>8147573</td>
<td>6.31</td>
<td>5.51</td>
<td>6.82</td>
<td>7.76</td>
</tr>
<tr>
<td>RHOU</td>
<td>7910387</td>
<td>6.52</td>
<td>5.42</td>
<td>5.71</td>
<td>5.41</td>
</tr>
</tbody>
</table>

* Class comparison analysis was performed with the univariate F-test (with random variance model) and a nominal significance level of 0.001 (BRB-ArrayTools ver. 3.6.0). Filtering criteria: spot filters, off; normalization, off; exclude a gene when less than 20% of expression data have at least a 1.5-fold change in either direction from the gene’s median value; gene subsets, off.

### Table 2. Net Differences (Log10) for TRIM36, OSR2, and RHOU when Comparing Cultured HLECs of Different Limbal Origin

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Unique ID</th>
<th>Superior versus Nasal Origin</th>
<th>Superior versus Inferior Origin</th>
<th>Superior versus Temporal Origin</th>
</tr>
</thead>
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<td>1.86*</td>
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</tr>
<tr>
<td>OSR2</td>
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<td>−2.30*</td>
<td>−1.43</td>
<td>−2.74*</td>
</tr>
<tr>
<td>RHOU</td>
<td>7910387</td>
<td>2.14*</td>
<td>1.75*</td>
<td>2.16*</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Unique ID</th>
<th>Nasal versus Inferior Origin</th>
<th>Nasal versus Temporal Origin</th>
<th>Inferior versus Temporal Origin</th>
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</thead>
<tbody>
<tr>
<td>TRIM36</td>
<td>8113577</td>
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<td>1.24</td>
<td>1.32</td>
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<td>OSR2</td>
<td>8147573</td>
<td>1.61</td>
<td>−1.19</td>
<td>−1.91*</td>
</tr>
<tr>
<td>RHOU</td>
<td>7910387</td>
<td>−1.22</td>
<td>1.01*</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Significant differences in the microarray experiments are in bold.

* Significant differences following RT-PCR validation (t-test, \( P < 0.05 \)).
thelia. Quantitative analyses were performed for the proliferation markers PCNA and Ki67. The expression of the proliferation marker Ki67 was not significantly higher in cultures from the superior limbus (mean, 4.5% ± 3.5%) than in the nasal (mean, 2.3% ± 1.7%; \( P = 0.26 \)), inferior (mean, 1.9% ± 1.8%; \( P = 0.20 \)), and temporal (mean, 6.4% ± 6.2%; \( P = 0.83 \)) cultures. The expression of PCNA was not significantly higher in cultures from the superior limbus (mean, 91.3% ± 6.5%) than in the nasal (mean, 95.3% ± 4.1%; \( P = 0.46 \)), inferior (mean, 92.3% ± 6.2%; \( P = 0.66 \)), and temporal (mean, 91.5% ± 5.6%; \( P = 0.86 \)) cultures.

**FIGURE 2.** H&E staining of representative cross sections of cultured HLECs from superior, nasal, inferior, and temporal limbal regions. Most epithelia in the superior cultures consisted of basal column-shaped cells, suprabasal cuboid wing cells, and flat squamous superficial cells, similar to corneal epithelium in vivo. HLEC cultures from nasal and inferior regions consisted of basal cells and flat superficial cells, whereas HLEC cultures of temporal origin were predominantly devoid of cells. Original magnification: \( \times 400 \).

**FIGURE 3.** Comparison of average (±SEM) epithelial thickness (A) and number of cell layers (B) in cultured HLECs of superior, nasal, inferior, and temporal limbal origin. Probabilities were calculated by comparing each experimental group against the superior group.

**FIGURE 4.** Transmission electron micrographs showing cultured HLECs from superior, nasal, inferior, and temporal limbal regions. Epithelia from the superior (A, C) limbus were clearly differentiated, whereas epithelia from nasal (B, D), inferior (E, G), and temporal (F, H) regions primarily consisted of basal cells and flat superficial cells. Regardless of limbal origin, numerous desmosomal junctions were seen between adjacent epithelial cells (A, B, E, F), and the polymorphic basal cells attached well to the amniotic basement membrane by hemidesmosomes (C, D, G, H). D. desmosomes; Hd, hemidesmosomes. Scale bars: (A, B, E, F) 5 \( \mu \)m; (C, D, G, H) 2 \( \mu \)m.
DISCUSSION

In the present study, no major transcriptional or phenotypic differences were observed in cultured HLECs derived from different regions of the limbus. However, explants of superior origin demonstrated the highest outgrowth success rate and generated epithelia with greater epithelial thickness and number of cell layers.

The lack of regional variation in the gene expression of markers of limbal stem cells and progenitors and differentiation is in contrast to results in a previous study, in which regional differences in the number of stem cell colonies were reported. In our study, of the 28,869 genes tested, TRIM36, OSR2, and RHOU were the only genes that exhibited highly significant differences. The significant upregulation of TRIM36 in the superior region compared with the three other regions, as revealed by the microarray analysis, was in agreement with the RT-PCR data. The significant downregulation of OSR2 and upregulation of RHOU superiorly versus nasally were also consistent with the RT-PCR analyses. TRIM36 is associated with

**FIGURE 5.** Immunostaining of K3, K5, E-cadherin, p63, ΔNp63α, ABCG2, K19, vimentin, integrin β1, nestin, Ki67, and PCNA in cultured HLEC of superior, nasal, inferior, and temporal origin. For comparative purposes, the montage shows epithelia of approximately equal thickness, which enables visualization of the immunolocalization of different markers, but does not represent the actual epithelial thickness in the respective study groups. HLEC cultures demonstrated similar immunoreactivity of proposed limbal stem cell, progenitor, and differentiation markers and the proliferation markers Ki67 and PCNA, irrespective of limbal explant origin. Original magnification: ×400.
the microtubule cytoskeleton, although its exact function is not yet clear. OR2 plays a key role in osteoblastic cell proliferation and contributes to palate growth and morphogenesis as well as kidney development. RHOU belongs to the group of Rho GTPases, which are involved in the control of cell adhesion and migration, cell cycle progression, growth, and differentiation. Ory et al. found that migration distances were increased in cells expressing activated RHOU and decreased when RHOU was inhibited. Even though all the three genes are involved in morphogenesis, it remains to be investigated whether the gene expression profile is correlated to the observed morphologic differences.

Interestingly, explants of superior origin demonstrated the highest success rate of generating confluent epithelia. Furthermore, epithelial thickness within the cell cultures was significantly greater in cells of superior origin compared to those from other regions, and the number of cell layers was greater in cultures originating from the superior limbus than in cultures from inferior and temporal regions. The light microscopy findings were confirmed by the ultrastructural examination of HLEC cultures. The high success rate of generating confluent epithelium and stratified nature of HLEC cultures of superior origin may have several clinical advantages. First, the number of cultures discarded due to insignificant growth for transplantation would be minimized. Second, a multilayered corneal epithelial graft with numerous intercellular desmosomes, which provide high mechanical strength, might better resist the mechanical stress and abrasions associated with the transplantation procedure. Third, a confluent and well-stratified epithelium is expected to have increased resistance to infections. In addition, the morphologic data in the present study provide support for randomizing experimental culture groups not only with respect to different donors, which is a common practice, but also with respect to limbal regions. The uniform gene expression of ocular surface markers correlated with homogeneous immunostaining of corresponding protein markers in HLEC cultures from all regions. It has been suggested that a multilayered corneal epithelium may indicate a high degree of differentiation in cell cultures. However, we did not observe expression of the differentiation marker K3 in our HLEC cultures, irrespective of limbal origin, which does not support this theory. Calculations of PCNA and Ki67 expression in cultured HLECs from the four regions did not reveal any statistically significant differences. It remains to be determined why the gene expression profile and morphologic findings were not associated with increased cell proliferation in HLEC cultures of superior origin.

There are limitations to the present study that should be acknowledged. The time from death to culture varied (range, 3.5–11.5 days). Furthermore, interdonor variability properties such as donor age, death to enucleation time, sex, and distribution of limbal epithelial stem cell niches are reported to affect the epithelial proliferative capacity of HLECs. As the donors were equally distributed among the experimental groups, a potential negative effect of growth due to these variations, is minimized. However, it is still uncertain whether these factors could have exerted a negative effect on the growth ability of HLECs from some regions compared to others.

In conclusion, we propose that tissue from the superior limbus is the most advantageous from which to generate cells with the potential to form a well-stratified epithelium. Clinical studies are warranted to substantiate the possible advantages of selective limbal harvesting to treat LSCD.


