Angiogenesis Potential of Human Limbal Stromal Niche Cells

Gui-Gang Li,1,2 Szu-Yu Chen,2 Hua-Tao Xie,2,3 Ying-Ting Zbu,2 and Scheffer C. G. Tseng2

PURPOSE. The perivascular localization of stem cell (SC) niches suggests the presence of a vascular niche. We aimed to determine the angiogenesis potential of limbal niche cells (NCS).

METHODS. Human limbal NCS were isolated and serially passaged on plastic or coated Matrigel in embryonic SC medium containing BFGF and leukemia inhibitory factor before being reseeded in 3D Matrigel. Expression of angiogenesis markers was assessed by RT-qPCR and immunofluorescence staining. Their angiogenesis potential was measured by differentiation into vascular endothelial cells and by supporting vascular tube network formed by human umbilical vein endothelial cells (HUVEC) on Matrigel. Their support of limbal epithelial progenitor cells (LEPC) was examined in sphere growth formed by reunion in 3D Matrigel.

RESULTS. On plastic, limbal NC could be cultured only up to four passages before turning into myofibroblasts. In contrast, on coated Matrigel, they could be expanded for up to 12 passages with upregulation of markers suggestive of angiogenesis progenitors when reseeded in 3D Matrigel because they could differentiate into vascular endothelial cells and pericytes stabilizing the tube network formed by HUVEC. Although both expanded limbal NCs and HUVEC rejoined with LEPC to form spheres to upregulate expression of ΔNp63α, CK15, and CEBPα the former but not the latter abolished expression of CK12 keratin.

CONCLUSIONS. Human limbal NCS continuously expanded on the basement membrane differentiate into angiogenesis progenitors that prevent differentiation of LEPC/SCs. They may partake in formation of the vascular niche and contribute to angiogenesis during wound healing. (Invest Ophthalmol Vis Sci. 2012;53:3357–3367) DOI:10.1167/iovs.11-9414

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MATERIALS AND METHODS

Cell Isolation from Human Limbus

Corneal limbal rims from human donors (23 to 70 years old) after corneal transplantation were provided by the Florida Lions Eye Bank.
Table 1. Serial Passages of the Limbal Stromal NCs on Plastic

<table>
<thead>
<tr>
<th>Passage</th>
<th>Seeding Density ($\times 10^5$/cm$^2$)</th>
<th>Culture Time (d)</th>
<th>Final Density ($\times 10^5$/cm$^2$)</th>
<th>NCDs</th>
<th>Cumulative NCD</th>
<th>Population Doubling Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>0.1</td>
<td>16</td>
<td>0.13</td>
<td>0.38</td>
<td>0.38</td>
<td>1014</td>
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<tr>
<td>P1</td>
<td>0.05</td>
<td>16</td>
<td>0.25</td>
<td>2.52</td>
<td>2.70</td>
<td>165</td>
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<tr>
<td>P2</td>
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<td>0.2</td>
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<td>4.70</td>
<td>216</td>
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<tr>
<td>P3</td>
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<td>30</td>
<td>0.13</td>
<td>1.38</td>
<td>6.08</td>
<td>522</td>
</tr>
<tr>
<td>P4</td>
<td>0.05</td>
<td>30</td>
<td>0.06</td>
<td>0.26</td>
<td>6.34</td>
<td>273</td>
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</tbody>
</table>

Accumulative NCDs were calculated.

The table summarizes the seeding density, culture time, and final density from P0 to P4, from which cumulative NCDs and population doubling time were calculated.

(Miami, FL) and handled according to the Declaration of Helsinki. After being rinsed three times with Hank’s balanced salt solution, containing 50 mg/mL gentamicin and 1.25 mg/mL amphotericin B, and the removal of excessive sclera, conjunctiva, iris, and corneal endothelium, the rim was cut into 1-clock-hour segments, each including tissue 1 mm within and beyond the anatomic limbus. Limbal segments were digested with 2 mg/mL collagenase A in serum-free ESCM at 37°C for 18 hours under humidified 5% CO$_2$ to generate "collagenase-isolated clusters." In parallel, the limbal segment was digested with 10 mg/mL dispase in ESCM at 4°C for 16 hours to isolate an intact epithelial sheet. All materials used for cell isolation and culture are listed in Supplementary Table 1 (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9414//DCSupplemental).

Serial Passages on Plastic or Coated Matrigel

Single cells derived from collagenase-isolated clusters by 0.25% trypsin and 1 mM EDTA (T/E) at 37°C for 15 minutes were seeded at 1 $\times 10^8$ per cm$^2$ in the 6-well plastic plate with or without coated Matrigel, which was prepared by adding 40 µL of 5% Matrigel per cm$^2$ 1 hour before use and cultured in ESCM containing 4 ng/mL BFGF and 10 ng/mL LIF in humidified 5% CO$_2$ to generate endothelium, the rim was cut into 1-clock-hour segments, each including tissue 1 mm within and beyond the anatomic limbus. Limbal segments were digested with 2 mg/mL collagenase A in serum-free ESCM at 37°C for 18 hours under humidified 5% CO$_2$ to generate "collagenase-isolated clusters." In parallel, the limbal segment was digested with 10 mg/mL dispase in ESCM at 4°C for 16 hours to isolate an intact epithelial sheet. All materials used for cell isolation and culture are listed in Supplementary Table 1 (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9414//DCSupplemental).

Coculturing with Limbal Epithelial Progenitor Cells in 3D Matrigel

As reported, cells expanded on coated Matrigel at passage 4 (P4) were reseeded in 3D Matrigel to generate P4/3D aggregates. Single cells obtained from P4/3D aggregates or human umbilical vein endothelial cells (HUVEC) were prelabeled with red fluorescent nanocrystals (Qtracker cell labeling kits; Invitrogen, Carlsbad, CA), mixed with single cells derived from dispase-isolated limbal epithelial sheets at a ratio of 1:4, and seeded at the density of 5 $\times 10^4$ per cm$^2$ to generate sphere growth. After 10 days of culturing in ESCM, the resultant spheres were collected by digesting Matrigel with 10 mg/mL dispase at 37°C for 2 hours.

Differentiation into Vascular Endothelial Cells

To induce differentiation into vascular endothelial cells, single cells from P4/3D aggregates were seeded at the density of 10$^3$ cells per cm$^2$ in 24-well plastic plates for 3 days in the Endothelial Cell Growth Medium 2 (EGM2) supplemented with 10 ng/mL VEGF. At 80% to 90% confluence, cells were incubated with 10 µg/mL Dil-Ac-LDL (Invitrogen) for 10 hours at 37°C in the humidified 5% CO$_2$ incubator to fix cells on Matrigel, which was prepared by adding 50 µL of 100% Matrigel into 24-well plates for 30 minutes before use, and cultured in EGM2 to elicit vascular tube-like network, as reported.

Vascular Tube Formation by HUVEC

Single cells obtained from P4/3D aggregates were mixed at a ratio of 1:1 with red fluorescent nanocrystal (Invitrogen) prelabeled HUVEC and seeded at the density of 10$^3$ cells per cm$^2$ on the surface of Matrigel, which was prepared by adding 50 µL of 100% Matrigel into 24-well plates for 30 minutes before use, and cultured in EGM2 to elicit vascular tube-like network, as reported.

Table 2. Serial Passages of the Limbal Stromal NCs on Coated Matrigel

<table>
<thead>
<tr>
<th>Passage</th>
<th>Seeding Density ($\times 10^5$/cm$^2$)</th>
<th>Culture Time (d)</th>
<th>Final density ($\times 10^5$/cm$^2$)</th>
<th>NCDs</th>
<th>Accumulative NCD</th>
<th>Population Doubling Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>0.1</td>
<td>6</td>
<td>0.15</td>
<td>0.58</td>
<td>0.58</td>
<td>246</td>
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<tr>
<td>P1</td>
<td>0.05</td>
<td>6</td>
<td>0.30</td>
<td>2.58</td>
<td>3.17</td>
<td>55</td>
</tr>
<tr>
<td>P2</td>
<td>0.05</td>
<td>6</td>
<td>0.45</td>
<td>3.17</td>
<td>6.34</td>
<td>45</td>
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<tr>
<td>P3</td>
<td>0.05</td>
<td>6</td>
<td>0.48</td>
<td>3.26</td>
<td>9.60</td>
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</tr>
<tr>
<td>P4</td>
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<td>6</td>
<td>0.50</td>
<td>3.32</td>
<td>12.92</td>
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<tr>
<td>P5</td>
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<td>0.51</td>
<td>3.35</td>
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<tr>
<td>P6</td>
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<td>3.32</td>
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<td>P12</td>
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<td>14</td>
<td>0.06</td>
<td>0.26</td>
<td>33.21</td>
<td>127</td>
</tr>
</tbody>
</table>

Accumulative NCDs were calculated.

The table summarizes the seeding density, culture time, and final density from P0 to P12, from which cumulative NCDs and population doubling time were calculated.
Immunofluorescence Staining

Cytospin preparation of $4 \times 10^4$ single cells per slide was made by Cytofuge (StatSpin, Inc., Westwood, MA) at 1000 rpm for 8 minutes. After being air dried for 5 minutes, cells were fixed with 4% paraformaldehyde for 15 minutes, permeated with 0.2% Triton X-100 in PBS for 20 minutes, and blocked with 2% bovine serum albumin in PBS for 1 hour before addition of primary antibody overnight. The appropriate secondary antibodies were then incubated for 1 hour, and Hoechst 33342 was used to counterstain the nucleus for 5 minutes before image analysis. Isotype-matched nonspecific IgG antibodies were used as the controls. Immunofluorescence micrographs were taken by laser confocal microscope (LSM700; Carl Zeiss, Thornwood, NY). Detailed information about primary and secondary antibodies is listed in Supplementary Table 2 (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9414/-/DCSupplemental).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Single cells were subjected to total RNA extraction by RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA), and cDNA was reverse-transcribed from 1 to 2 µg of total RNA by high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) amplification of different genes was carried out in a 20-µL solution containing cDNA, TaqMan Gene Expression Assay Mix, and universal PCR master mix (Applied Biosystems). All assays were performed in triplicate for each condition. The results were normalized by an internal control, glyceraldehyde-3-phosphate dehydrogenase. The relative gene expression data were analyzed by the comparative CT method ($\Delta\Delta CT$). All TaqMan Gene Expression Assays with probe sequences are listed in Supplementary Table 3 (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9414/-/DCSupplemental).

Western Blot

Proteins were extracted from Day 10 spheres generated by limbal epithelial progenitors alone or mixed with P4/3D cells or HUVEC by radioimmunoprecipitation (RIPA) buffer supplemented with protease inhibitors. Equal amounts of proteins measured by the BCA assay (Pierce, Rockford, IL) in total cell extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then blocked with 5% (wt/vol) fat-free milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% [vol/vol] Tween-20), followed by sequential incubation with specific primary antibodies and their respective secondary antibodies using β-actin as the loading control.

Figure 1.

Serial passages on plastic. Cells isolated from collagenase-isolated clusters from a 62-year-old donor were serially passaged on plastic in ESCM containing LIF and BFGF. They yielded spindle cells (A) and could only reach P4 with a doubling time of more than 165 hours and NCD of 6 (B). When P3 single cells were reseeded in 3D Matrigel for 6 days, they generated P4/3D aggregates at Day 6 with a smooth contour (A). Compared with D0 cells just isolated, P3 spindle cells did not express Oct4, Sox2, FLK-1, CD34, CD31, PDGFRβ, and SMMHC transcripts, but expressed α-SMA and S100A4 transcripts ($^{*}p < 0.05$, $^{**}p < 0.01$). Furthermore, after being reseeded in 3D Matrigel, the resultant P4/3D cells did not regain expression of the above markers. Scale bar = 200 µm.
The immunoreactive bands were visualized by a chemiluminescence reagent (Western Lightning; Pierce). Antibodies used are listed in Supplementary Table 2 (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9414/-/DCSupplemental).

**Statistical Analysis**

All assays were performed in triplicate, each with a minimum of three donors. The data are reported as means ± SD. Group means were compared using the appropriate version of Student's unpaired t test. Test results were reported as two-tailed P values, where P < 0.05 was considered statistically significant.

**RESULTS**

**Serial Passages on Plastic**

Previously, we successfully expanded limbal NCs on coated Matrigel for up to 4 passages. To investigate the significance of Matrigel for such success, we first expanded the limbal NCs on plastic by serial passage in ESCM containing LIF and BFGF using collagenase-isolated cells from a 62-year-old donor. Such culture yielded spindle cells (Fig. 1A) and could only reach P4 with a doubling time of more than 165 hours and NCD of 6 (Fig. 1B). When P3 single cells were reseeded in 3D Matrigel for 6 days, they generated P4/3D aggregates at Day 6 with a smooth contour (Fig. 1A). Compared with D0 cells just isolated, P3 spindle cells did not express Oct4 and Sox2 (i.e., markers of ESC). They also did not express Flk-1, CD34, CD31, and platelet-derived growth factor receptor β (PDGFRβ), markers suggestive of angiogenesis progenitors. Because they expressed transcripts of z-smooth muscle actin (z-SMA) and S100A4, but not smooth muscle myosin heavy chain (SMMHC) (Fig. 1C, n = 3, *P < 0.05, **P < 0.01), limbal NCs expanded on plastic turned into myofibroblasts. Furthermore, the resultant P4/3D cells did not regain expression of ESC and angiogenesis markers even after being reseeded in 3D Matrigel.

**Serial Passages on Coated Matrigel**

When the above collagenase-isolated cells were serially passaged on coated Matrigel in ESCM with BFGF and LIF, consistent with our recent report, spindle-shaped cells could be isolated and expanded by completely eliminating epithelial cells by P2 (Fig. 2A). Unlike the counterpart cultured on plastic, spindle cells could be expanded on coated Matrigel for up to P12, resulting in a total of 33 cell doublings, yielding approximately 1 × 10^10 spindle cells from 12 limbal segments. Cells at P1 to P10 exhibited a uniform proliferative rate with a cell doubling time between 43 and 47 hours (Fig. 2B, Table 2).

**Expression of Pericyte Markers by Expanded Spindle Cells**

Consistent with our recent reports, double immunostaining with antibodies against pancytokeratins (PKCs) and Vim showed that collagenase-isolated clusters consisted of approximately 80% PCK+/Vim- epithelial cells and 20% PCK−/Vim+ cells, and that both PCK+ cells and Vim+ cells expressed ESC markers, such as Oct4 and Sox2 (Fig. 3B). Further double immunostaining between the aforementioned markers and Vim showed that Vim+ cells expressed Flk-1, CD34, CD31, and z-SMA, but the overall percentage of colocalization was less than 1% (n = 1000), and none expressed PDGFRβ (Fig. 3B), suggesting that most of these Vim+ NCs did not express markers suggestive of either endothelial progenitor cells or pericytes. As reported, PCK+/Vim- epithelial cells were completely eliminated after P2, as confirmed by the disappearance of p65 and CK12 transcripts and negative staining to PCK and p65. In contrast, spindle cells emerged from P3 and uniformly expressed Vim but not PCK (Fig. 3B). RT-qPCR showed that expression of Oct4, Sox2, Flk-1, CD34, CD31, SMMHC, and S100A4 transcripts became undetectable, whereas that of z-SMA and PDGFRβ transcripts were markedly upregulated during serial passage (Fig. 3A, n = 3, *P < 0.05). Compared with the expression level at D0 when cells were freshly isolated, expression of the z-SMA transcript was markedly elevated until P12, whereas that of the PDGFRβ transcript was also elevated until P8 (Fig. 3A). This pattern of transcript expression was confirmed by immunofluorescence staining. For example, P3 spindle cells did not express Oct4, Sox2, Flk-1, CD34, and CD31, but strongly expressed z-SMA and PDGFRβ (Fig. 3B). Their lack of expression of SMMHC supported that they were not smooth muscle cells. Their positive expression of z-SMA without S100A4 supported that they were not myofibroblasts. Collectively, these data indicated that expanded spindle cells upregulated their expression of markers suggestive of pericyte differentiation.
Previously, we discovered that expression of ESC markers could be regained in P3 spindle cells if reseeded in 3D Matrigel. We thus wondered whether expression of markers suggestive of angiogenesis progenitors could be influenced by such a maneuver. Single P3 cells formed cell aggregates as early as 4 hours after being reseeded in 3D Matrigel (Fig. 4B). At Day 6, these cell aggregates adopted a stellate contour (Fig. 3B). Consistent with our recent report, expression of Oct4 and Sox2 transcripts by P4/3D D6 aggregates was indeed upregulated to 5- and 8-fold when compared with that expressed by P3 spindle cells expanded on coated Matrigel (Fig. 4A). Interestingly, expression of Flk-1, CD34, CD31, and α-SMA transcripts was markedly upregulated by 10- to 40-fold, whereas that of PDGFRβ transcripts was upregulated by 5- and 27-fold, respectively (Fig. 4A, n = 3, P < 0.05). In contrast, expression of SMMHC and S100A4 transcripts remained undetectable in P4/3D D6 cells. Immunofluorescence staining of single cells released from P4/3D aggregates confirmed positive and uniform expression of Vim and all of the aforementioned angiogenesis markers (Fig. 4B), but negative expression of SMMHC and S100A4 markers (not shown). These
results suggested that reseeding back in 3D Matrigel not only restored expression of ESC markers but also promoted expression of markers suggestive of angiogenesis progenitors in the direction of pericytes but not smooth muscle cells or myofibroblasts.

**Differentiation into Vascular Endothelial Cells**

To confirm that the aforementioned P4/3D cells were indeed angiogenesis progenitors, cells were released from 3D Matrigel by dispase digestion, rendered into single cells by T/E, and seeded on plastic in EGM2 supplemented with 10 ng/mL VEGF-A according to a reported method. After 3 days of culturing, the resultant cells exhibited spindle cells similar to HUVEC (Fig. 5). They also expressed positive immunofluorescence staining to Flk-1, CD31, and von Willebrand factor and took up Dil-Ac-LDL (Fig. 5, top) in a similar fashion to the positive control of HUVEC (Fig. 5, bottom). These data indicated that P4/3D cells indeed could differentiate into vascular endothelial cells.

**Support of HUVEC-Formed Vascular Tube Network**

One important step in the process of angiogenesis is to stabilize the vascular network formed by vascular endothelial cells by pericytes. To confirm that P4/3D cells were indeed angiogenesis progenitors, we examined whether they also possessed the phenotype of pericytes. To recapitulate such a function of pericytes, we seeded single HUVEC, single P4/3D cells, and a combination of both on the surface of 100% Matrigel in EGM2 as previously reported. Both single P4/3D cells and prelabeled (red) HUVEC formed networks at Day 1 (Figs. 6A, 6B); however, such networks were largely disintegrated by Day 2 (Figs. 6E, 6F). In contrast, the network formed by cocultured P4/3D cells and HUVEC (Fig. 6C) was maintained at Day 2 (Fig. 6G) and Day 5 (not shown). Higher magnification of such network confirmed tight adherence of P4/3D cells onto HUVEC (red) (Fig. 6D, 6H). These results confirmed that P4/3D cells indeed possessed the pericyte phenotype to stabilize the vascular tube-like network formed by HUVEC.
Prevention of Differentiation of Limbal Epithelial Progenitors

Compared with PCK+ cells in collagenase-isolated clusters, those in dispase-isolated sheets expressed less p63α and CK15, but more CK12. Thus, dispase isolated more differentiated limbal epithelial progenitor cells (LEPC) than collagenase based on the findings that p63α signifies limbal basal progenitors including SC, and CK15 is expressed by limbal-basal epithelial cells, and CK12 is a marker of corneal epithelial differentiation. Single PCK+ epithelial cells and Vim+ stromal cells from collagenase-isolated clusters could reunite to generate sphere growth in 3D Matrigel and such reunion helps to maintain epithelial clonal growth and prevent corneal epithelial differentiation. We thus examined whether LEPC obtained from dispase-isolated epithelial sheets could also form reunion with prelabeled (red) P4/3D cells or HUVEC in 3D Matrigel. As shown in Figure 6, reunion indeed occurred at Day 2 and gradually developed into a larger sphere by Day 10, similar to those formed by LEPC alone (Fig. 7A). Compared with spheres formed by LEPC alone, spheres formed by LEPC+HUVEC and LEPC+P4/3D had significantly higher transcript expression of ΔNp63α, CK15, and CEBPδ of which the latter plays a role in maintaining quiescence of limbal epithelial SCs and CK12 transcript by LEPC+HUVEC was not different from LEPC alone (Fig. 7B, n = 3, P > 0.05), but that by LEPC+P4/3D was significantly reduced to an undetectable level (Fig. 7B, n = 3, P < 0.01).

Western blot analysis confirmed that the protein level of p63α was elevated to 6.5- and 6.1-fold in LEPC+HUVEC and LEPC+P4/3D respectively, when compared with LEPC alone (Fig. 7C, n = 3, P < 0.05). The protein level of CK12 was not changed in...
LEPC (Fig. 7C, n = 3, P > 0.05) but was reduced to an undetectable level in LEPC+P4/3D (Fig. 7C, n = 3, P < 0.01). In addition, expression of CK12 transcripts by LEPC-HUVEC was not different from that of LEPC alone (B, n = 3, P > 0.05), whereas that by LEPC-P4/3D was not detectable (B, n = 3, P > 0.01). Compared with LEPC alone, expression of p63α protein was elevated in both LEPC-HUVEC and LEPC+P4/3D (C, n = 3, P < 0.05). In contrast, expression of CK12 protein was not reduced in LEPC+HUVEC (C, n = 3, P > 0.05) but was reduced to a nondetectable level in LEPC+P4/3D using β-actin as a loading control (C, n = 3, P < 0.05). Double staining with p63α and CK12 showed that HUVEC or P4/3D cells alone did not express p63α or CK12, whereas CK12 was expressed by LEPC alone and LEPC+HUVEC but not LEPC+P4/3D (D). Scale bar = 200 µm for A and 100 µm for D.

![Figure 7](image_url)

**FIGURE 7.** Epithelial sphere growth in 3D Matrigel. LEPCs derived from dispase-isolated epithelial sheets alone or mixed with fluorescence prelabeled (red) HUVEC or P4/3D cells to generate sphere growth from Day 2 to Day 10 in 3D Matrigel (A). Compared with those formed by LEPC alone, spheres formed by LEPC+HUVEC and by LEPC+P4/3D expressed significantly more ΔNp63α, CK15, and CEBPδ transcripts (B, n = 3, *P < 0.05, **P < 0.01). In addition, expression of CK12 transcripts by LEPC-HUVEC was not different from that of LEPC alone (B, n = 3, P > 0.05), whereas that by LEPC-P4/3D was not detectable (B, n = 3, P > 0.01). Compared with LEPC alone, expression of p63α protein was elevated in both LEPC-HUVEC and LEPC+P4/3D (C, n = 3, P < 0.05). In contrast, expression of CK12 protein was not reduced in LEPC+HUVEC (C, n = 3, P > 0.05) but was reduced to a nondetectable level in LEPC+P4/3D using β-actin as a loading control (C, n = 3, P < 0.05). Double staining with p63α and CK12 showed that HUVEC or P4/3D cells alone did not express p63α or CK12, whereas CK12 was expressed by LEPC alone and LEPC+HUVEC but not LEPC+P4/3D (D). Scale bar = 200 µm for A and 100 µm for D.

**DISCUSSION**

Embarking on what we have established in identification, isolation, and expansion of limbal NCs,32,33,35 the present study was undertaken to provide evidence that there might exist a vascular niche in limbal palisades of Vogt by showing that these limbal NCs had the capacity of differentiating into angiogenesis progenitors during serial passages on coated Matrigel. Furthermore, like pericytes, they could support networks formed by vascular endothelial cells. Although like vascular endothelial cells these angiogenesis progenitors could serve as NCs to maintain expression of epithelial SC markers, but unlike vascular endothelial cells, they prevented corneal epithelial differentiation.

It is believed that angiogenesis progenitors could be isolated and expanded from the perivascular location of a variety of tissues.18,51–53 Herein, our findings suggested that another source of such angiogenesis progenitors could come from a subset of Vim+ cells that lie immediately subjacent to the
limbal basal epithelial cells. Imminently isolated by collagenase digestion from the in vivo environment, these small Vim+ cells are tightly associated with PCK+/p63+/ P63+ epithelial cells in the midst of basement membrane remnants and heterogeneously express several ESC markers.26 We subsequently found that expression of ESC markers is crucial for limbal NCs to support epithelial SC function during sphere growth.35 Although the expression of ESC markers can be preserved if limbal NCs are immediately seeded in 3D Matrigel on collagenase isolation, they do not proliferate.35,36 To circumvent this limitation, we discovered that it was possible to expand limbal NCs by serial passage on coated Matrigel.53 Herein, we extended what was reported earlier (up to P3) to a total of 12 passages and 33 doublings to expand these limbal NCs (Fig. 2, Table 2). Although expression of ESC markers was transiently lost during serial passages on coated Matrigel (Fig. 5), it could be regained when cells were reseeded in 3D Matrigel (Fig. 4) to support epithelial clonal growth in sphere cultures.55 Previously, we noted that cells cultured on coated Matrigel in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, but not ESCM containing BFGF and LIF, resulted in an irreversible loss of expression of ESC markers and a lack of support of limbal epithelial progenitors/SCs in sphere growth.55 Herein, we further demonstrated that cells cultured on plastic (without coated Matrigel) in ESCM containing BFGF and LIF also irreversibly lost the expression of ESC markers and turned into myofibroblasts expressing α-SMA and S-100A4 but not PDGFβ and SMMHC (Fig. 1). Collectively, these findings suggest that continuous contact with the basement membrane substrate, such as Matrigel, and culturing in the serum-free ESCM containing BFGF and LIF are both crucial to ensure the survival of limbal NCs. Moreover, expression of ESC markers can be preserved if limbal NCs are immediately seeded in 3D Matrigel on collagenase isolation, even in the absence of ESC markers.53,54 We subsequently found that expression of ESC markers can be preserved if limbal NCs are immediately seeded in 3D Matrigel on collagenase isolation, even in the absence of ESC markers.53,54 We subsequently found that expression of ESC markers can be preserved if limbal NCs are immediately seeded in 3D Matrigel on coating Matrigel.53 Herein, we extended what was reported earlier (up to P3) to a total of 12 passages and 33 doublings to expand these limbal NCs (Fig. 2, Table 2). Although expression of ESC markers was transiently lost during serial passages on coated Matrigel (Fig. 5), it could be regained when cells were reseeded in 3D Matrigel (Fig. 4) to support epithelial clonal growth in sphere cultures.55 Previously, we noted that cells cultured on coated Matrigel in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, but not ESCM containing BFGF and LIF, resulted in an irreversible loss of expression of ESC markers and a lack of support of limbal epithelial progenitors/SCs in sphere growth.55 Herein, we further demonstrated that cells cultured on plastic (without coated Matrigel) in ESCM containing BFGF and LIF also irreversibly lost the expression of ESC markers and turned into myofibroblasts expressing α-SMA and S-100A4 but not PDGFβ and SMMHC (Fig. 1). Collectively, these findings suggest that continuous contact with the basement membrane substrate, such as Matrigel, and culturing in the serum-free ESCM containing BFGF and LIF are both crucial to ensure the preservation of expression of ESC markers bestowing expanded cells with the NC status. The lack of such continuous contact results in myofibroblast differentiation (Fig. 1), suggesting that matrix environment plays a key role in maintaining NC phenotype. Future studies are needed to investigate how matrix rigidity/stiffness of Matrigel might promote proliferation without irreversible loss of the NC phenotype.

Notwithstanding the exact modulating mechanism, the maneuver by switching from coated Matrigel to 3D Matrigel also gave rise to angiogenesis progenitors. During serial passages on coated Matrigel, Vim+ spindle cells lost expression of ESC markers but upregulated expression of α-SMA and PDGFβ without SMMHC and S100A4 (Fig. 3). On being reseeded in 3D Matrigel, these spindle cells regained ESC markers while upregulating expression of additional angiogenesis markers such as Flk-1, CD34, and CD31 (Fig. 4). Expression of Flk-1, CD34, and CD31 is used to denote endothelial progenitors, including SC markers as ANP63α,33,44 CK15, a marker for limbal epithelial basal cells,44–47 and CEBPβ, a marker for limbal SC quiescence,50 during sphere growth (Fig. 7). This result suggested that vascular endothelial cells in the limbal niche might serve as NCs to maintain “stemness” of the limbal epithelial progenitor/SCs. However, expression of CK12, a marker for corneal epithelial differentiation,48,49 was abolished only in spheres containing P4/3D cells, but not HUVEC (Fig. 7). Hence, angiogenesis progenitors derived from limbal NCs might play a crucial role in preventing corneal epithelial differentiation. Future studies of how aforementioned angiogenesis progenitors may regulate function of limbal progenitor/SCs should help us better understand the role of vascularization in the limbal niche and look into the issue of whether a vascular niche in the limbal niche might be generated by limbal NCs.

In summary, human limbal stromal NCs have the potential to differentiate into angiogenesis progenitors and to prevent corneal epithelial progenitor/SC differentiation, implying that the cells partake in formation of the vascular niche and contribute to angiogenesis during wound healing.

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References


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