Angiogenesis Potential of Human Limbal Stromal Niche Cells

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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 CEBPδ, 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

Stem cell (SC) niches consist of supporting niche cells (NCs), extracellular matrix, and modulating signals that can maintain SC survival, self-renewal, and fate decision.[1] The perivascular localization of SC niches in the bone marrow,[2–4] the central nervous system,[5] and the testis[6] indicates the presence of a vascular niche, which consists of vascular endothelial cells, pericytes, and the basement membrane in between.[7] Vascular endothelial cells can support SCs in bone marrow,[8,9] the central nervous system,[5,10,11] pancreatic islets,[12] and muscles.[13] There is only one study defining pericytes as stromal cells to support keratinocytes in organotypic cultures.[14] Perivascular pericytes have been recognized as a common source of mesenchymal SCs,[15–19] which may regulate self-renewal and differentiation of SCs[20–23] and, in some in vitro instances, differentiate into vascular endothelial cells.[24,25] It remains unclear whether angiogenesis progenitors but not vascular endothelial cells may also serve as NCs.

The corneal epithelial SCs are located at a unique anatomic region termed limbal palisades of Vogt,[26,27] where the basement membrane is fenestrated[28,29] and undulated with papillae or "pegs" of the limbal stroma extending upward.[30] Histological serial sections disclosed that limbal SCs may invade into the limbal stroma, giving rise to a structure called limbal epithelial crypts.[28,29] Ultrastructural analyses also disclosed similar limbal crypts surrounded by focal stromal projections (i.e., finger-like projections of stroma containing a central blood vessel).[31] These structural features indicate that limbal epithelial SCs lie deeper in the stroma, which is rich in blood vessels; however, it remains unclear whether a vascular niche also exists in the limbal palisades of Vogt.

We have recently reported that collagenase, which cleaves interstitial collagens but not the basement membrane, can isolate a cluster of cells consisting of not only entire limbal epithelial progenitors and SCs but also subjacent vimentin (Vim)+ stromal mesenchymal cells.[32] These Vim+ cells are as small as 5 μm in diameter and heterogeneously express embryonic SC (ESC) markers, such as Oct4, Sox2, SSEA4, and Nanog, as well as other SC markers, such as Nestin, N-Cadherin, and CD34.[32] Recently, we reported that these small limbal stromal cells could be isolated and expanded on coated Matrigel in the embryonic SC medium (ESCM) supplemented with BFGF and leukemia inhibitory factor (LIF) into Vim+ spindle cells that may reversibly express ESC markers upon being reseeded in 3D Matrigel.[33] Herein, we further characterize such expanded spindle cells as angiogenesis progenitors and provide strong evidence that they abolish corneal epithelial differentiation of human limbal epithelial progenitors/SCs more effectively than vascular endothelial cells. The significance of these findings in the role of angiogenesis during wound healing in the human limbal niche is further discussed.

Materials and Methods

Cell Isolation from Human Limbus

Corneolimbal rims from human donors (23 to 70 years old) after corneal transplantation were provided by the Florida Lions Eye Bank

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Table 1. Serial Passages of the Limbal Stromal NCs on Plastic

Passage	Seeding Density ($\times 10^5$/cm^2)	Culture Time (d)	Final Density ($\times 10^5$/cm^2)	NCDs	Cumulative NCD	Population Doubling Time (h)
P0	0.1	16	0.13	0.38	0.38	1014
P1	0.05	16	0.25	2.32	2.70	165
P2	0.05	18	0.2	2.00	4.70	216
P3	0.05	30	0.13	1.38	6.08	522
P4	0.05	30	0.06	0.26	6.34	2737

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."[32] 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.[34] 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 1×10^5 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 with media changed every 3 or 4 days. Cells at 80% to 90% confluence were rendered single cells by T/E and serially expanded at the seeding density of 5×10^3 cells per cm^2 for up to 12 passages. The extent of total expansion was measured by the number of cell doubling (NCD) using the following formula: NCD = $\log_{10}(y/x)/\log_{10}2$, where y is the final density of the cells and x is the initial seeding density of the cells. In parallel, cells were cultured in the 6-well plate without coated Matrigel in the same medium as a control.

Coculturing with Limbal Epithelial Progenitor Cells in 3D Matrigel

As reported,[33,35] 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 5×10^4 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 10^4 cells per cm^2 in 24-well plastic plates for 3 days in the Endothelial Cell Growth Medium 2 (EGM2) supplemented with10 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 or fixed with 4% paraformaldehyde for immunofluorescence staining.

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 10^5 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.[36–38] P4/3D cells or HUVEC alone were also seeded at the same density as the controls.

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

Passage	Seeding Density ($\times 10^5$/cm^2)	Culture Time (d)	Final density ($\times 10^5$/cm^2)	NCDs	Accumulative NCD	Population Doubling Time (h)
P0	0.1	6	0.15	0.58	0.58	246
P1	0.05	6	0.30	2.58	3.17	55
P2	0.05	6	0.45	3.17	6.34	45
P3	0.05	6	0.48	3.26	9.60	44
P4	0.05	6	0.50	3.32	12.92	43
P5	0.05	6	0.51	3.35	16.28	43
P6	0.05	6	0.50	3.32	19.60	43
P7	0.05	6	0.48	3.26	22.86	44
P8	0.05	6	0.45	3.17	26.03	45
P9	0.05	6	0.42	3.07	29.10	47
P10	0.05	6	0.30	2.58	31.69	56
P11	0.05	10	0.12	1.26	32.95	190
P12	0.05	14	0.06	0.26	33.21	1277

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 4×10^4 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, Thornhood, 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, glceraldehyde-3-phosphate dehydrogenase. The relative gene expression data were analyzed by the comparative CT method ($\Delta\Delta C_T$). 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 proteinase 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.

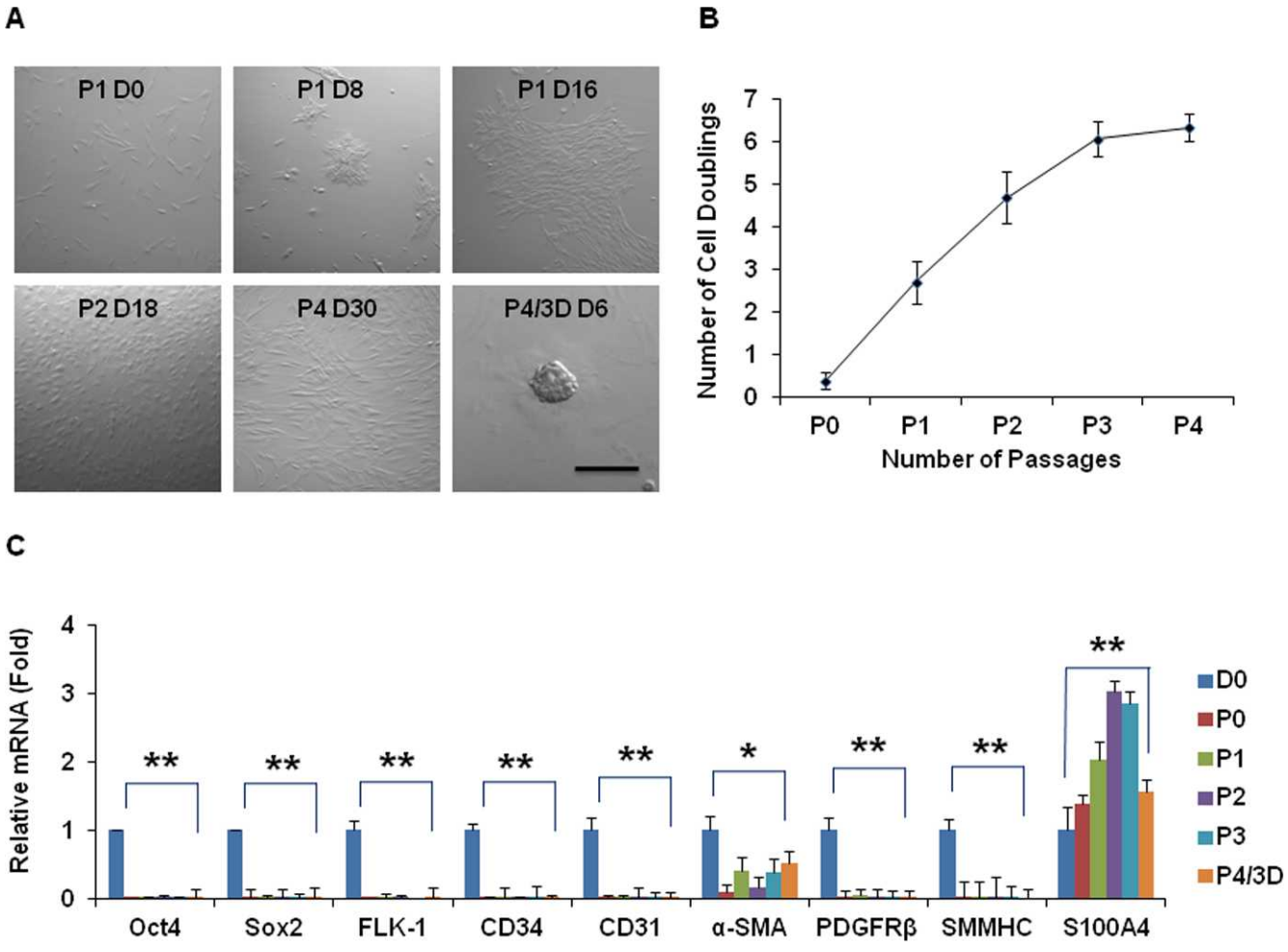

Figure 1. Serial passages on plastic. Cells isolated from collagenase-isolated clusters from a 62-year-old donor were serially passaged on plastic in ESCM containing LIF and BFGF. They yielded spindle cells (**A**) and could only reach P4 with a doubling time of more than 165 hours and NCD of 6 (**B**). 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 (**C,** $^*P < 0.05$, $^{**}P < 0.01$). 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.[33] 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 α-smooth muscle actin (α-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,[33] 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,[32,33] double immunostaining with antibodies against pancytokeratins (PCKs) 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 α-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,[33] PCK+/Vim– epithelial cells were completely eliminated after P2, as confirmed by the disappearance of p63 and CK12 transcripts and negative staining to PCK and p63. 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 α-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 α-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 α-SMA and PDGFRβ (Fig. 3B). Their lack of expression of SMMHC supported that they were not smooth muscle cells.[39] Their positive expression of α-SMA without S100A4 supported that they were not myofibroblasts.[40] Collectively, these data indicated that expanded spindle cells upregulated their expression of markers suggestive of pericyte differentiation.

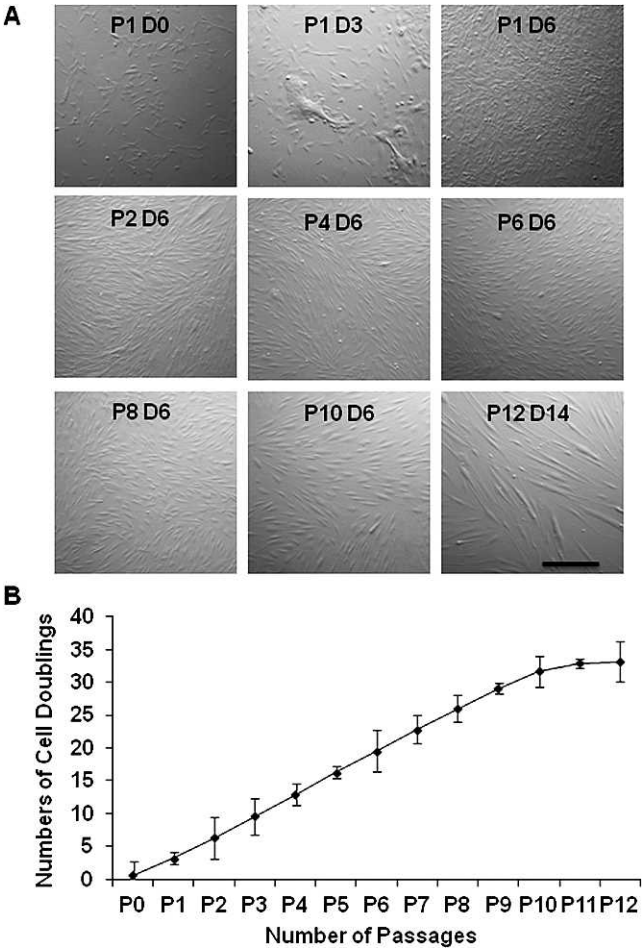

Figure 2. Serial passages on coated Matrigel. Single cells derived from collagenase-isolated limbal clusters from one limbal segment of the same donor as in Figure 1 were serially passaged on coated Matrigel in ESCM with BFGF and LIF. They generated spindle cells from P1 to P12 (**A**) and had a steady proliferative rate with the doubling time of 43 to 47 hours from P2 to P10 (**B**). Bar = 100 μm.

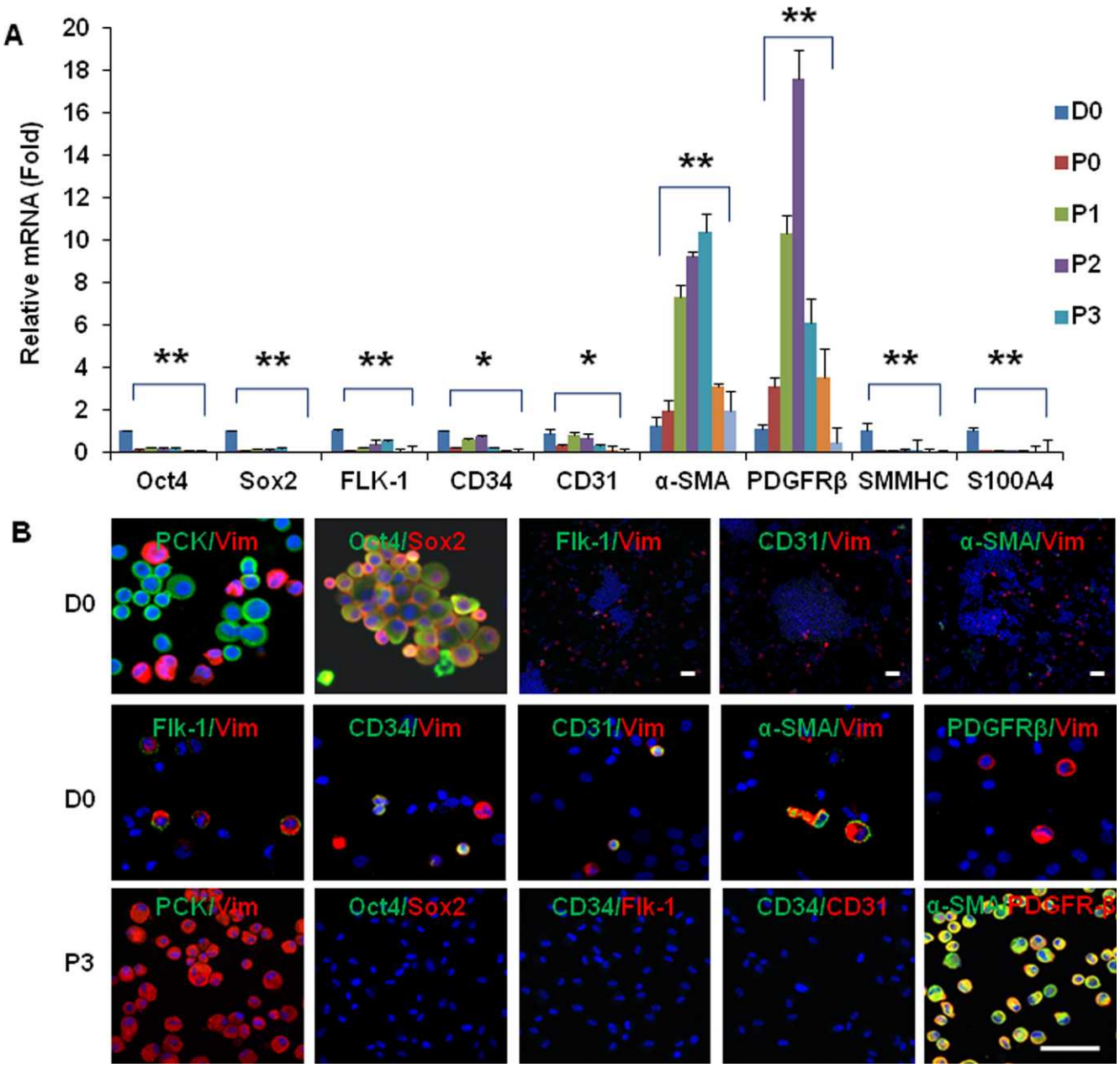

Figure 3. Pericyte phenotype promoted by serial passages on coated Matrigel. When compared with freshly isolated cells at D0, RT-qPCR revealed a notable decrease of Oct4, Sox2, Flk-1, CD34, CD31, SMMHC, and S100A4 transcripts but a dramatic increase of α-SMA and PDGFRβ transcripts during serial passages (**A,** $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). D0 cells consisted of PCK+ and Vim+ cells and expressed Oct4 and Sox2. In addition, Vim+ cells expressed Flk-1, CD34, CD31, or α-SMA (see photos with high magnification), but the overall percentage of colocalization was less than 1% (see photos with low magnification of double labeling of Flk-1/Vim, CD31/Vim and α-SMA/Vim, $n = 1000$), and none expressed PDGFRβ (**B,** D0). In contrast, P3 cells were all PCK-/Vim+, α-SMA+, and PDGFRβ+, but negative to Oct4, Sox2, Flk-1, CD34, and CD31 (**B,** P3). Nuclei were counterstained by Hoechst 33342 (*blue*). Scale bar = 50 μm.

Angiogenesis Progenitors Promoted by Reseeding in 3D Matrigel

Previously, we discovered that expression of ESC markers could be regained in P3 spindle cells if reseeded in 3D Matrigel.[33] 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,[33] 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, and CD31 transcripts was markedly upregulated by 10- to 40-fold, whereas that of α-SMA and 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. Immunofluroscence 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

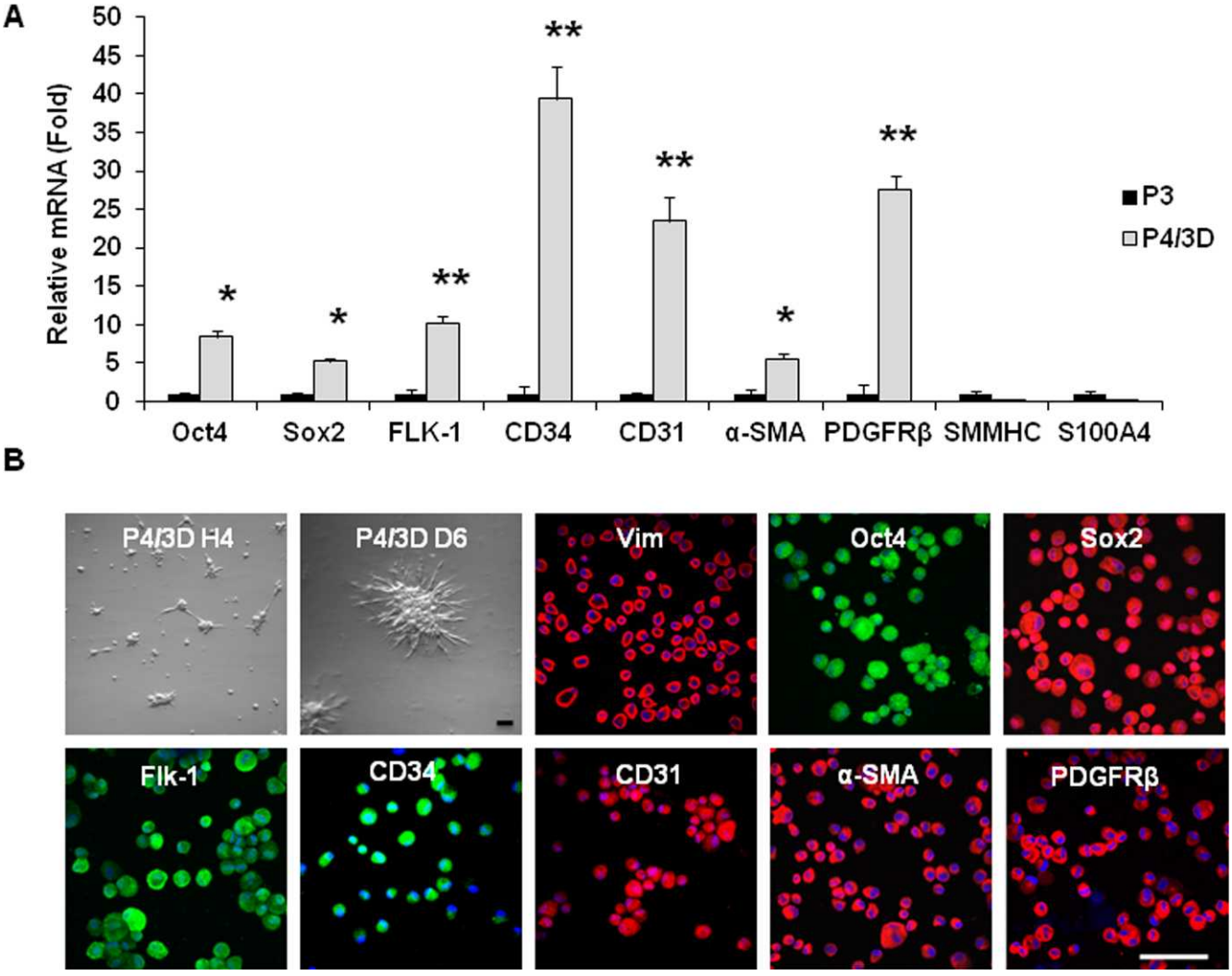

Figure 4. Angiogenesis progenitors promoted by reseeding in 3D Matrigel. P3 cells expanded on coated Matrigel were reseeded in 3D Matrigel, they formed P4/3D aggregates; single cells were collected on Day 6. Compared with P3 spindle cells, expression of Oct4, Sox2, Flk-1, CD34, CD31, α-SMA, and PDGFRβ transcripts were markedly upregulated in P4/3D cells (**A,** $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). In contrast, expression of SMMHC and S100A4 remained lacking. P4/3D aggregates were noted as early as 4 hours and exhibited with a stellate contour at Day 6 (**B**). Immunostaining of P4/3D cells showed uniform expression of Vim together with the above positive markers (**B**). Nuclei were counterstained by Hoechst 33342 (*blue*). Scale bar = 50 μm.

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 with10 ng/mL VEGF-A according to a reported method.[41] 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.[42] 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.[36–38] 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.

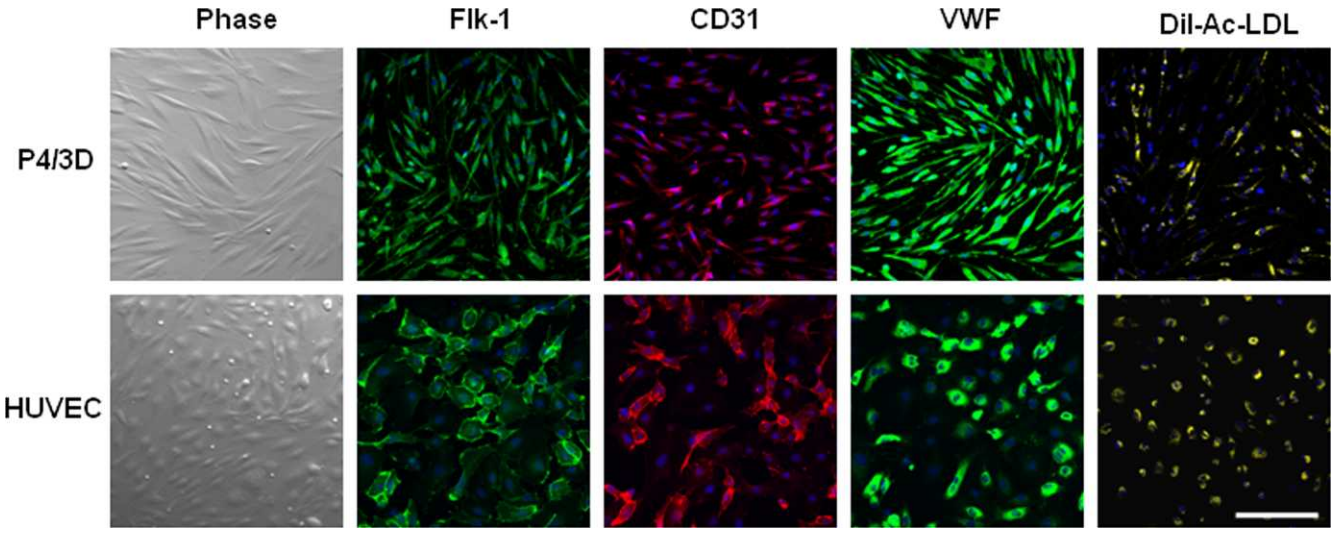

Figure 5. Differentiation into vascular endothelial cells. Single cells of P4/3D aggregates were cultured on plastic in EGM2 with VEGF for 3 days, yielding spindle cells similar to HUVEC. They uniformly expressed Flk-1, CD31, and von Willebrand factor, and took up Dil-Ac-LDL (*top*) in a similar fashion to the positive control of HUVEC cultured in the same condition (*bottom*). Nuclei were counterstained by Hoechst 33342 (*blue*). Scale bar = 100 μm.

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.[32] Thus, dispase isolated more differentiated limbal epithelial progenitor cells (LEPC) than collagenase based on the findings that p63α signifies limbal basal progenitors including SC,[43,44] CK15 is expressed by limbal-basal epithelial cells,[45–47] and CK12 is a marker of corneal epithelial differentiation.[48,49] 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.[35] 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[50] (Fig. 7B, $n = 3$, all $P < 0.05$). Expression of the 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

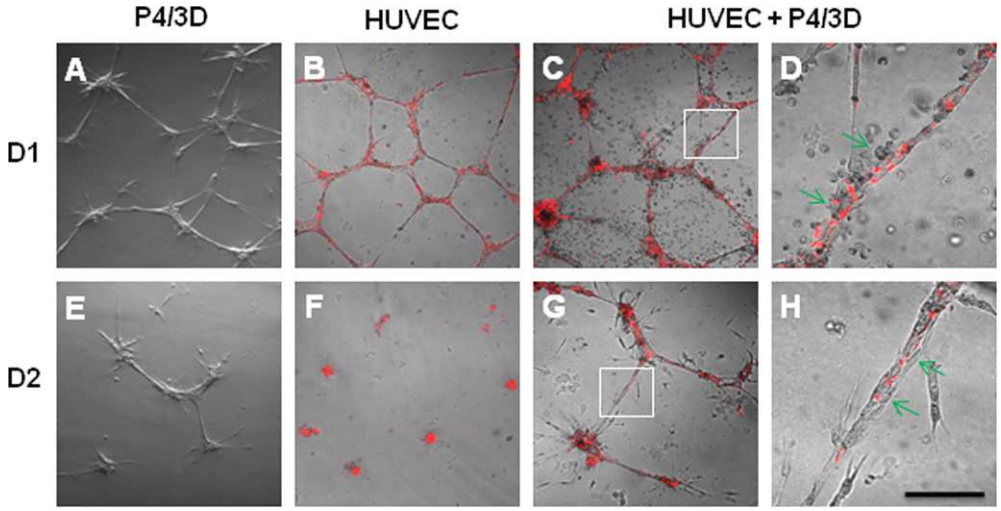

Figure 6. Support of HUVEC vascular tube network. Fluorescence pre-labeled (*red*) HUVEC and P4/3D cells were seeded alone or together on the surface of 100% Matrigel in EGM2. Although vascular tube-like network was noted in all three conditions at Day 1 (**A–C**), such network in P4/3D cells (**A**) or HUVEC (**B**) alone was disintegrated by Day 2 (**E, F**). In contrast, the network formed by cocultured P4/3D cells and HUVEC (**C**) was maintained at Day 2 (**G**) and Day 5 (not shown). High magnification of insets (**C, G**) revealed close association between P4/3D cells and HUVEC (*red*) in the network at Day 1 (**D**) and Day 2 (**H**). Scale bar = 200 μm for **A–C** and **E–G**, and 50 μm for **D** and **H**.

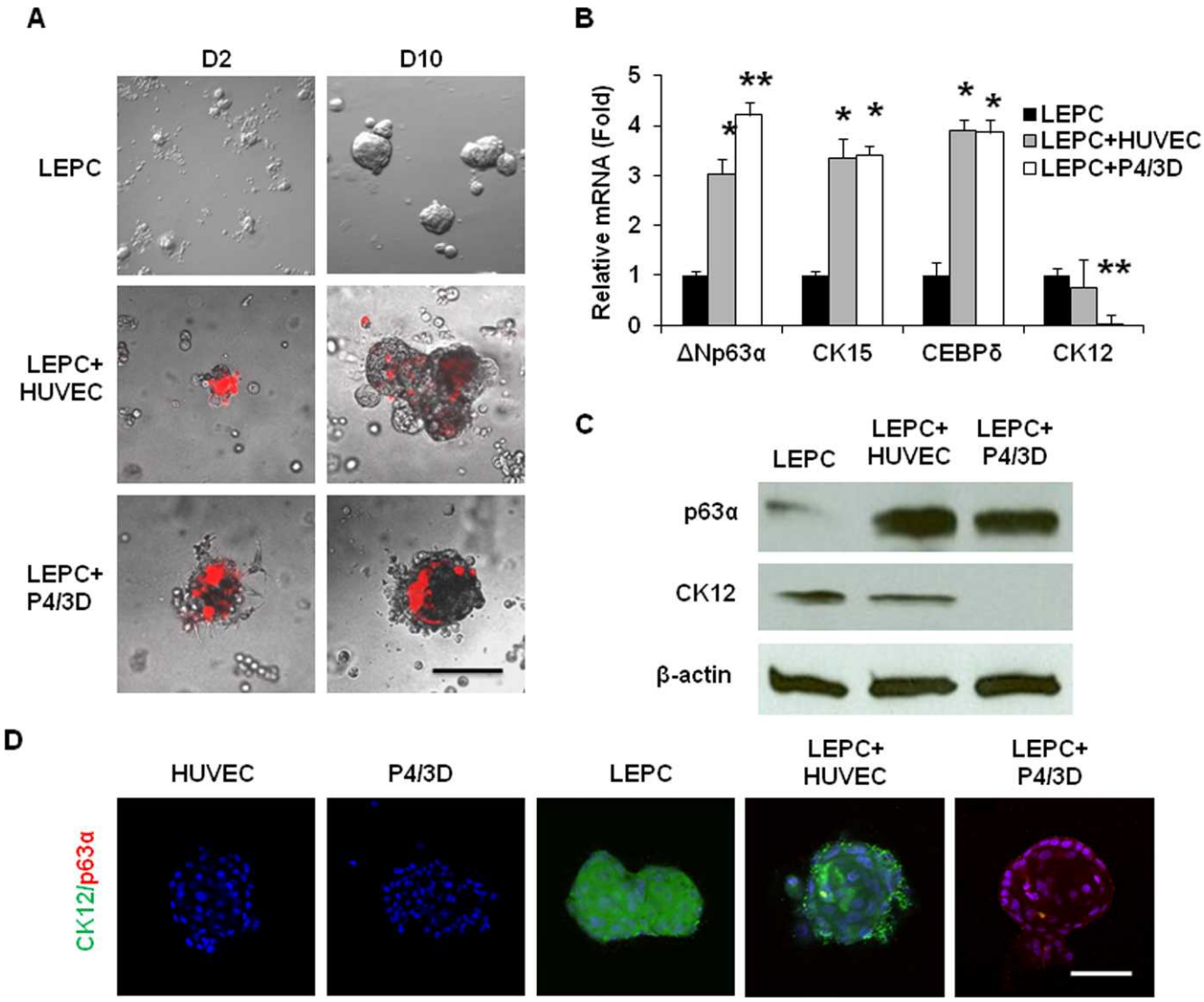

Figure 7. Epithelial sphere growth in 3D Matrigel. LEPCs derived from dispase-isolated epithelial sheets alone or mixed with fluorescence prelabeled (*red*) HUVEC or P4/3D cells to generate sphere growth from Day 2 to Day 10 in 3D Matrigel (**A**). Compared with those formed by LEPC alone, spheres formed 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 expresss 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**.

LEPC+HUVEC (Fig. 7C, $n = 3$, $P > 0.05$) but was reduced to an undetectable level in LEPC+P4/3D (Fig. 7C, $n = 3$, $P < 0.01$). Double staining with p63α and CK12 also confirmed that HUVEC or P4/3D cells alone did not express p63α or CK12, and that CK12 was expressed by LEPC alone and LEPC+HUVEC, but not LEPC+P4/3D (Fig. 7D). Collectively, these findings indicated that although both the P4/3D cells and HUVEC could join with LEPC to generate sphere growth in 3D Matrigel to promote expression of epithelial progenitor/SC markers, the former but not the latter could prevent differentiation of LEPC.

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+/p63α+ epithelial cells in the midst of basement membrane remnants and heterogeneously express several ESC markers.[32] We subsequently found that expression of ESC markers is crucial for limbal NCs to support epithelial SC function during sphere growth.[33] 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.[33,35] To circumvent this limitation, we discovered that it was possible to expand limbal NCs by serial passage on coated Matrigel.[33] 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. 3), it could be regained when cells were reseeded in 3D Matrigel (Fig. 4) to support epithelial clonal growth in sphere cultures.[33] 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.[33] 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 PDGFRβ 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 PDGFRβ 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 progenitor cells isolated from the human tissues[54–58] or derived from ESCs.[41,59–61] Expression of both α-SMA and PDGFRβ has also been used to mark pericytes.[62–66] SMMHC is specific for smooth muscle cells,[39] whereas S100A4 predicates differentiation of myofibroblast in addition to the expression of α-SMA.[40] We thus speculated that Vim+ cells in P4/3D stellate aggregates were angiogenesis progenitors in the direction of pericytes but not smooth muscle cells or myofibroblasts because they not only expressed the above angiogenesis markers, but they also differentiated into vascular endothelial cells (Fig. 5) and pericytes to support vascular network formed by HUVEC (Fig. 6). Hence, continuous contact with the basement membrane and culturing in ESCM containing BFGF and LIF preserved expression of not only ESC markers but also angiogenesis markers. The loss of the phenotype of angiogenesis progenitors by serial passages on plastic without coated Matrigel (Fig. 1) supported their dependence on the basement membrane and bode well with the in vivo state of angiogenesis progenitor cells, which are normally embedded in the basement membrane.[7,38] Thus, our finding shed new light on how destruction of the basement membrane might lead to limbal niche dysfunction leading to fibrosis in limbal stem cell deficiency.

The likelihood that limbal NCs might adopt the fate of angiogenesis progenitors also unravels not only their supporting role of epithelial SCs but also their possible involvement in regeneration during wound healing. In organotypic cultures of keratinocytes, pericytes promote keratinocyte renewal by synthesizing and secreting Laminin 5.[14] The present study showed for the first time that both P4/3D cells and HUVEC could support the expression of such limbal basal epithelial progenitors, including SC markers as ΔNp63α,[43,44] CK15, a marker for limbal epithelial basal cells,[45–47] and CEBPδ, 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 epithelial progenitors/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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