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Phenotypic Change and Induction of Cytokeratin Expression During In Vitro Culture of Corneal Stromal Cells

Laura E. Sidney, Owen D. McIntosh, and Andrew Hopkinson

Academic Ophthalmology, Division of Clinical Neuroscience, Queen's Medical Centre Campus, University of Nottingham, Nottingham, United Kingdom

Correspondence: Laura E. Sidney, Academic Ophthalmology, B Floor, Eye and ENT Building, Queen's Medical Campus, University of Nottingham, NG7 2UH, UK; laura.sidney@nottingham.ac.uk.

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Purpose. Cells of the corneal epithelium and stroma can be distinguished in vivo by different intermediate filaments, cytokeratins for corneal epithelial cells (CEC) and vimentin for keratocytes. Isolated and cultured keratocytes change phenotype, losing expression of keratocyte markers and gaining markers associated with mesenchymal stromal cells (MSC). This study investigates this change in phenotype in relation to intermediate filament expression in cultured corneal stromal cells (CSC) compared to CEC.

Methods. Expression of epithelial markers (CK3, CK12, CK19, pan cytokeratin, E-cadherin), keratocyte markers (CD34, vimentin), and MSC markers (CD73, CD90, and CD105) were compared in CEC and CSC by immunocytochemistry and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Expression was evaluated at different stages of CSC culture and compared to another stromal cell type, extracted from Wharton's jelly (WJ-MSC).

Results. In vivo keratocytes did not express cytokeratins. However, cultured CSC expressed epithelial-associated CK3, CK12, and CK19, but not other cytokeratins. Expression of cytokeratins increased as CSC were passaged and decreased as CSC were induced to become quiescent. Comparatively, WJ-MSC expressed lower levels of CK3, CK12, and CK19, but also stained for pan cytokeratin and expressed *KRT5*.

Conclusions. Cultured CSC undergo phenotypic change during culture, expressing specific cytokeratin filaments normally associated with CEC. Cytokeratin expression begins as cells are cultured on plastic and increases with passage. This discovery may influence the way in which differences are discerned between cultured CEC and CSC. Investigators need to be aware that the expression of cytokeratins does not necessarily represent epithelial contamination, and that CEC and CSC may be more related than previously recognized.

Keywords: cytokeratin, corneal epithelium, keratocytes, corneal stroma, cell culture

The cells of the corneal epithelium and keratocytes of the stroma are typically characterized and distinguished by the presence of certain intermediate filaments (IF) and cell surface markers. The corneal epithelium is a nonkeratinized stratified squamous epithelium that exists in a constant state of cell renewal and regeneration. Cellular division occurs at the basal level and daughter cells move toward the surface, initially differentiating into transit-amplifying cells and then becoming terminally differentiated and shed into the tear film, with a turnover of approximately 4 to 6 days.[1] This self-renewing state is due to the presence of a population of limbal stem cells (LSC) located in the limbus, the interface between the cornea and the sclera.[2] Limbal stem cells can be distinguished from terminally differentiated corneal epithelial cells (CEC) by the difference in expression of specific cytokeratins, alongside other indicators such as the expression of ABCG2, ΔNP63, and location.[3,4] Terminally differentiated CEC are characterized by expression of, among others, cytokeratin (CK) 3,[5,6] CK12,[7–9] and E-cadherin.[10] Inversely, LSC do not express CK3 or CK12, but have been reported to express CK19,[3] CK14,[10–12] vimentin,[10] and ABCG2.[3]

The corneal stroma contains a population of cells known as keratocytes. Under healthy conditions, keratocytes are quiescent and exhibit a dendritic morphology.[13,14] Keratocytes are responsible for producing and maintaining extracellular matrix (ECM) proteins, such as collagen and proteoglycans, and expression of collagen-I, keratocan, and other ECM proteins is often used to identify keratocytes.[15–17] Cell surface markers used to identify the keratocyte phenotype include CD133 and CD34.[18–20] As keratocytes are a mesenchymal cell type, derived from the neural crest, they have never been reported to express cytokeratins in vivo, and instead have vimentin as the major IF.[21,22] Keratocytes can be isolated from the stroma for in vitro expansion. However, once transferred to in vitro culture, particularly in serum-containing medium, they undergo fibroblastic phenotypic change, transitioning to an activated state,[23,24] mirroring an in vivo response of keratocytes to injury.[13] Phenotypic changes that occur during in vitro culture include the loss of CD34, aldehyde dehydrogenase (ALDH) 3A1 and ALDH1A1,[18,25,26] and expression of markers such as CD90[27] and α-smooth muscle actin.[28] This phenotypic change is the reason that cultured keratocytes are often referred to as corneal stromal cells (CSC).

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In vitro, CSC extracted from the limbal region have been shown to display characteristics of multipotent mesenchymal stromal cells (MSC),[29,30] which conform to criteria stipulated by the International Society for Cellular Therapy (ISCT).[25,31] The extracted corneal MSC express specific cell surface markers such as CD73, CD90, and CD105 and possess the ability to differentiate down the osteogenic, chondrogenic, and adipogenic lineages in vitro.[31,32] It has also been suggested that there is a specific corneal stromal stem cell (CSSC), or keratocyte progenitor cell, that can be extracted from the limbal region of the stroma and cultured by clonal expansion. This CSSC conforms to an MSC phenotype but can be differentiated from MSC by the expression of keratocyte-specific markers such as keratocan, PAX6, and ALDH3A1.[33] However, it has not been shown whether this phenotype is present within the in vivo cornea or is a product of in vitro culture. Although keratocytes in vivo are not associated with cytokeratins, cultured CSC have been shown by our collaborators to specifically express CK3 in combination with CD34.[34] However, several other groups have shown that their cultured CSC do not express general cytokeratins, though this is often through the use of pan cytokeratin (panCK) antibodies that may not be specific for CK3 or CK19, as a way of demonstrating a lack of epithelial contamination.[30,35,36]

Herein, we compare the expression of cytokeratins and cell surface markers by CEC and cultured CSC. We compare the expression of these markers at several stages of CSC culture, including a quiescent phenotype to represent the keratocyte. To investigate the specificity of expressed cytokeratins, we also compare to a different stromal cell type: MSC derived from the Wharton's jelly of the umbilical cord (WJ-MSC).

Methods

Materials

All reagents were purchased from Life Technologies, Paisley, United Kingdom, unless otherwise stated.

Tissue

Human donor tissue was used with approval by the local ethics research committee and in accordance with the tenets of the Declaration of Helsinki, following consent obtained from the donors and/or their relatives. Corneal tissue was obtained from Manchester Eye Bank. Umbilical cords were obtained, with consent, from Obstetrics, Queen's Medical Centre, Nottingham. To complete this study, three corneas from distinct donors and 22 corneoscleral rims (surplus from penetrating keratoplasty) were required.

Cell and Tissue Culture

Extraction and Culture of Primary Human Corneal Epithelial Cells. Human primary CEC were generated from corneoscleral rims by explant culture. Excess sclera was removed, the rims were cut into two sections, and a small incision was made in the anterior stroma of each. The posterior and mid stroma were peeled away using forceps, leaving the epithelium and a section of stroma. The epithelium was divided into 12 explants, and each explant was placed epithelial side up in 3.5-cm-diameter dishes or two-well glass chamber slides (Nunc Lab-Tek, Thermo Scientific, Cramlington, UK), coated with 10 μg/mL bovine fibronectin. Explants were cultured in CnT-20 medium (CELLnTEC, Bern, Switzerland), maintained in a humidified environment at 37°C, 5% (vol/vol) CO_2. Culture medium was changed every 2 to 3 days.

Extraction and Culture of Primary Human Corneal Stromal Cells. Human CSC were isolated from corneoscleral rims. Excess sclera was removed and epithelium and endothelium were detached by gentle scraping. Stromal tissue was divided into small pieces and digested in 1 mg/mL collagenase type IA (Sigma-Aldrich, Poole, UK) in medium 199 (M199; Sigma-Aldrich), supplemented with 20 ng/mL gentamicin and 0.5 ng/mL amphotericin B (antibiotics) for 7 hours at 37°C. Digests were filtered through a 40-μm cell strainer and pelleted, and CSC were cultured in M199 supplemented with 20% (vol/vol) fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and antibiotics (mesenchymal culture medium). Cultured CSC were maintained at 37°C, 5% (vol/vol) CO_2, with medium changes every 2 to 3 days. Cells were passaged using treatment with TrypLE Express.

Differentiation of CSC to a Quiescent Keratocyte Phenotype. Corneal stromal cells at passage 4 (P4) were seeded at 10,500 cells/cm^2 in six-well plates or eight-well chamber slides in mesenchymal culture medium and cultured for 5 days. Cultures were switched to a serum-free differentiation medium: Dulbecco's modified Eagle's medium/F12 supplemented with 50 μg/mL ascorbate 2-phosphate (Sigma-Aldrich); 10 μg/mL human insulin, 5.5 μg/mL human transferrin, 6.7 ng/mL sodium selenite; 10 ng/mL human fibroblast growth factor-2; 0.1 ng/mL transforming growth factor-β3; and antibiotics. Cultures were differentiated for 21 days.

Extraction and Culture of Wharton's Jelly Mesenchymal Stromal Cells. Umbilical cords were processed within 24 hours of collection. The cord was washed three times in PBS, immersed in 70% (vol/vol) ethanol for 30 seconds, and washed once more. Cords were divided into 6-cm sections and cut open lengthways. The vessels were removed, and the surrounding Wharton's jelly was excised and chopped into 2-mm^3 pieces. Explants were placed in six-well plates and cultured in mesenchymal culture medium at 37°C, 5% (vol/vol) CO_2 in a humidified environment, for 7 days, before MSC colonies were identified growing from the tissue. Wharton's jelly MSC were passaged using TrypLE Express and used for experimentation at P3.

Culture of Immortalized Human Corneal Epithelial Cells. SV40-immortalized human corneal epithelial cells (ihCEC)[37] were cultured in supplemented basal epithelial cell medium EpiLife containing 5 mL human keratinocyte growth supplement and antibiotics. Cells were maintained at 37°C, 5% (vol/vol) CO_2, with medium changed every 2 to 3 days. Immortalized human CEC were utilized for quantitative cell-based immunofluorescence studies due to difficulties growing primary CEC on glass-bottom plates.

Sectioning and Immunofluorescent Staining of Corneas

Whole human corneas were frozen in optimal cutting temperature compound (VWR, West Sussex, UK). Specimens were cut into 8-μm sections using a cryostat-microtome (Leica Microsystems, Milton Keynes, UK) and placed onto slides coated in 2% (vol/vol) 3-aminopropyltriethoxysilane (Sigma-Aldrich). Sections were fixed in ice-cold 100% acetone for 30 seconds. After washing in PBS, blocking of nonspecific protein binding was performed for 1 hour at room temperature (RT) in PBS with 1% (vol/vol) bovine serum albumin (BSA; Sigma-Aldrich), 0.3 M glycine (Sigma-Aldrich), and 3% (vol/vol) donkey serum (Sigma-Aldrich). Sections were incubated with primary antibodies (Table) diluted in PBS with BSA and glycine at 4°C overnight. Samples were incubated with secondary antibodies (donkey Alexa Fluor [AF]-488 or -594) for 1 hour at RT. Counterstaining with 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) was performed for 10 minutes, and slides were

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Table. Primary Antibody and Taqman Probe Information

Antibody	Clone	Source (Catalog No.)
Antibody Information		
Mouse anti-cytokeratin 3/12 (CK3/12)	2Q1040	Abcam, Cambridge, Cambridgeshire, UK (ab68260)
Mouse anti-cytokeratin 19 (CK19)	BA17	R&D Systems, Abingdon, Oxfordshire, UK (MAB3506)
Mouse anti-pan cytokeratin (PanCK)	C-11	Thermo Scientific (MA1-19043)
Rabbit anti-E-cadherin (E-Cad)	Polyclonal	Abcam (ab53033)
Mouse anti-vimentin	V9	Vector Labs, Peterborough, Cambridgeshire, UK (VPV684)
Mouse anti-CD34	QBEND10	Abcam (Ab8536)
Rabbit anti-CD73	Polyclonal	Abcam (Ab71322)
Mouse anti-CD90	F15-42-1	Thermo Scientific (MA5-16671)
Goat anti-CD105	Polyclonal	R&D Systems (AF1097)
Taqman Probe Information		
Protein Name	**Gene Name**	**Assay ID**
Cytokeratin 3	*KRT 3*	Hs00365074_m1
Cytokeratin 12	*KRT12*	Hs00165015_m1
Cytokeratin 19	*KRT19*	Hs00761767_s1
Cytokeratin 5	*KRT5*	Hs00361185_m1
E-cadherin	*CDH1*	Hs00170423_m1
Vimentin	*VIM*	Hs00185584_m1
CD34	*CD34*	Hs00990732_m1
CD73	*NT5E*	Hs01573922_m1
CD90	*THY1*	Hs00174816_m1
CD105	*ENG*	Hs00923996_m1

mounted in fluorescence mounting medium (Dako, Cambridgeshire, UK). Staining was viewed using an upright fluorescence microscope (BX51; Olympus, Southend-on-Sea, UK) and images were captured with a black-and-white camera (XM-10; Olympus) and Cell^F software (Olympus).

Fluorescence Immunocytochemistry

Samples for immunocytochemistry were cultured in glass chamber slides and fixed in 4% buffered paraformaldehyde (PFA; Sigma-Aldrich) for 10 minutes. After washing in PBS, cells were permeabilized, where necessary, in 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich) for 5 minutes with subsequent PBS washing. Immunofluorescent staining was performed to the same protocol as tissue sections. Counterstaining with AF-488-conjugated phalloidin was performed for 20 minutes at RT before counterstaining with DAPI. Chambers were removed and slides mounted in fluorescence mounting medium before imaging.

Quantitative Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Cells were lysed in RLT buffer and homogenized using QIAshredder columns (Qiagen, Manchester, UK). Total RNA was extracted using an RNeasy mini kit (Qiagen) according to manufacturer's instructions. RNA was quantified using the L-Vis plate on a CLARIOstar plate reader (BMG LABTECH, Buckinghamshire, UK). RNA (250 ng) was transcribed into cDNA using Superscript III reverse transcriptase with random hexamer primers, according to manufacturer's instructions. For PCR reactions, 1 μL cDNA was used with inventoried Taqman assays (Table). Amplification was performed on an Mx3005P PCR system (Stratagene, Stockport, UK). Reactions were analyzed using the Real Time PCR Miner algorithm.[38] All experimental values were normalized to endogenous reference gene *GAPDH*.

Quantitative Cell-Based Fluorescent Immunoassay

Quantitation of fluorescent staining levels was performed using a plate reader–based assay, similar to an In-Cell Western. Cells were seeded in 24-well glass-bottom plates (Greiner Bio-One, Gloucestershire, UK) and cultured until confluence; or, for quiescent CSC, 21 days to allow for differentiation. Staining protocol was similar to immunocytochemistry with the following adjustments: primary antibodies were applied for 1 hour at RT; secondary antibody was AF-680 conjugated; cells were counterstained with AF-488 phalloidin and DAPI. Background controls were included, with no primary antibody, phalloidin, or DAPI. A CLARIOstar plate reader was used to take bottom-read fluorescent readings at excitation 657 nm, emission 701 nm (AF-680); excitation 488 nm, emission 535 nm (AF-488); and excitation 360 nm, emission 460 nm (DAPI), using a well scan of 10×10 at an 11.8-mm diameter. Average fluorescent intensities were calculated by taking the mean and correcting for background. Fluorescent intensity was normalized using actin (more stable than DAPI).

Statistical Analysis

Significances were analyzed using GraphPad Prism version 6.02 (La Jolla, CA, USA). Two groups were compared using unpaired Student's *t*-test, and multiple groups were compared using one-way ANOVA with post hoc Dunnett's test.

Results

Expression of Epithelial, Keratocyte, and MSC Markers In Vivo

Whole human corneas were sectioned and stained for a number of markers (Fig. 1). CK3/12 (Fig. 1i) was detected in the central epithelium but not in the peripheral/limbal region. No staining for CK3/12 was seen in the central stroma; however, low levels of staining could be detected in the peripheral stroma. CK19 (Fig. 1ii) staining was seen only in the peripheral epithelium and not in any other part of the cornea. Staining for the panCK C-11 clone, which detects CK4, 5, 6, 8, 13, and 18, was present in both the peripheral and central epithelium, but not the stroma of any sample (Fig. 1iii). This was also true for E-cadherin (Fig. 1iv). Keratocyte markers vimentin (Fig. 1v) and CD34 (Fig. 1vi) were both expressed in the stroma, but not the epithelium. For MSC markers, only CD73 (Fig. 1vii) stained throughout the cornea. CD90 (Fig. 1viii) was not expressed in the peripheral or central epithelium or peripheral stroma. Figure 1Dviii shows CD90 staining in one individual cell in the central stroma. CD105 (Fig. 1ix) was detectable in the cells of the peripheral stroma and some cells of the central stroma, with possible staining in the epithelium.

Expression of Epithelial Markers in Cultured CEC and CSC

Cultured CEC and CSC (P4) were immunostained for markers associated with epithelial cells (Figs. 2A, 2B, respectively).

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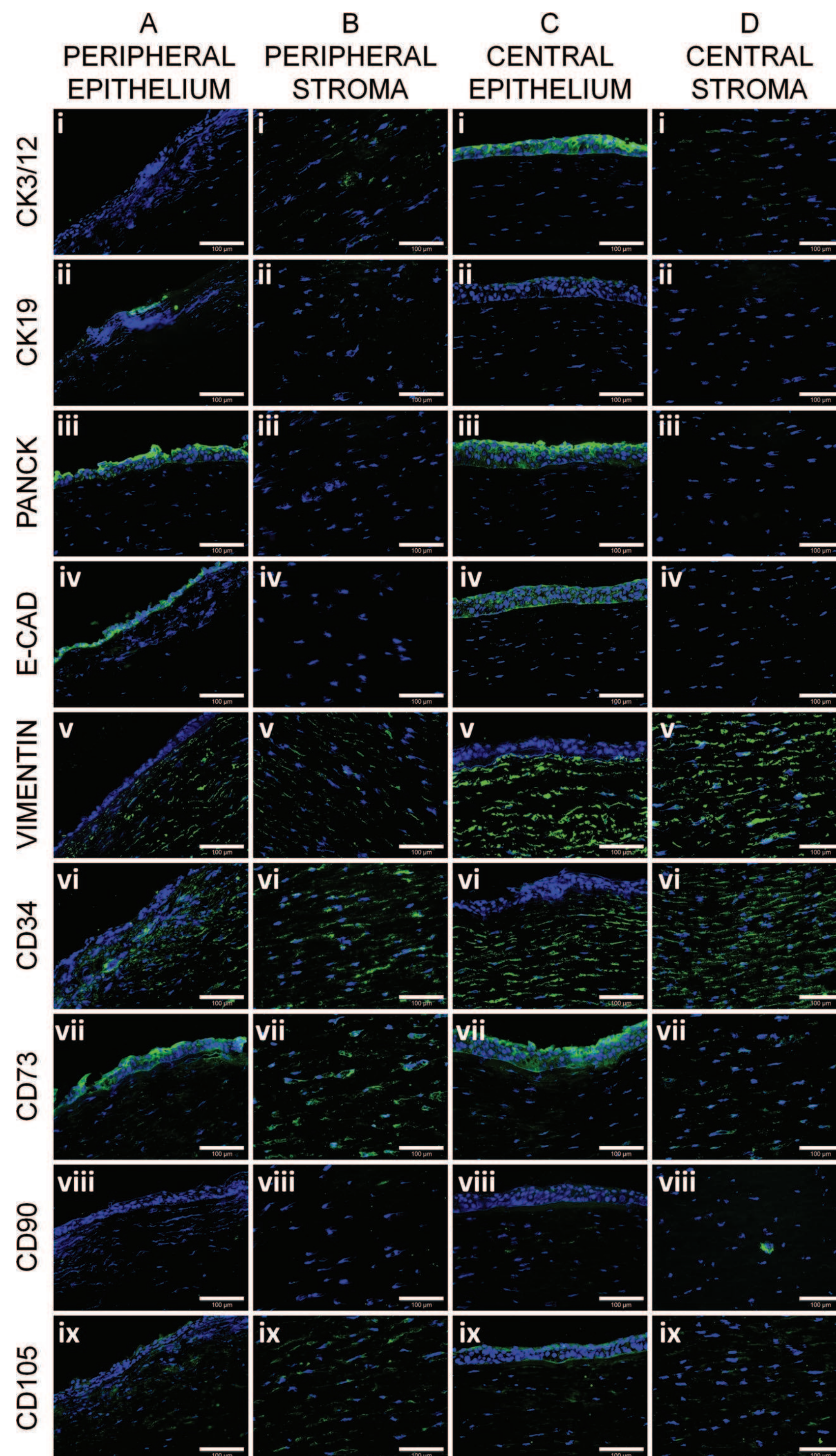

Figure 1. Expression of epithelial, keratocyte, and mesenchymal stromal cell markers in corneal epithelium and stroma. Immunofluorescent staining was performed on sections of cornea, and representative images were taken of (**A**) peripheral (limbal) epithelium, (**B**) peripheral (limbal) stroma, (**C**) central epithelium, and (**D**) central stroma. Epithelial markers were (**i**) CK3/12, (**ii**) CK19, (**iii**) pan cytokeratin (PanCK), and (**iv**) E-cadherin (E-Cad). Keratocyte markers included (**v**) vimentin and (**vi**) CD34. Mesenchymal stromal cell markers were (**vii**) CD73, (**viii**) CD90, and (**ix**) CD105. Representative images shown ($n = 3$). *Scale bars*: 100 μm.

Imaging of CK3/12 and CK19 staining was performed at different exposure settings for CEC and CSC; thus brightness of staining cannot be directly compared. Staining for CK3/12 and CK19 was considerably brighter in CEC than in CSC. Imaging of PanCK and E-cadherin was performed at the same exposure setting. Corneal epithelial cells stained positive for all proteins (Fig. 2A), although CK3/12 was not seen in every cell, as shown by the low-magnification image (Fig. 2Ai). CK3/12 (Fig. 2Bi) and CK19 (Figs. 2Bi, 2Bii) were positively stained throughout the entire CSC population. Corneal stromal cells did not express panCK (Fig. 2Biv) or E-cadherin (Fig. 2Bv) at levels detectable above that of the background. Expression of

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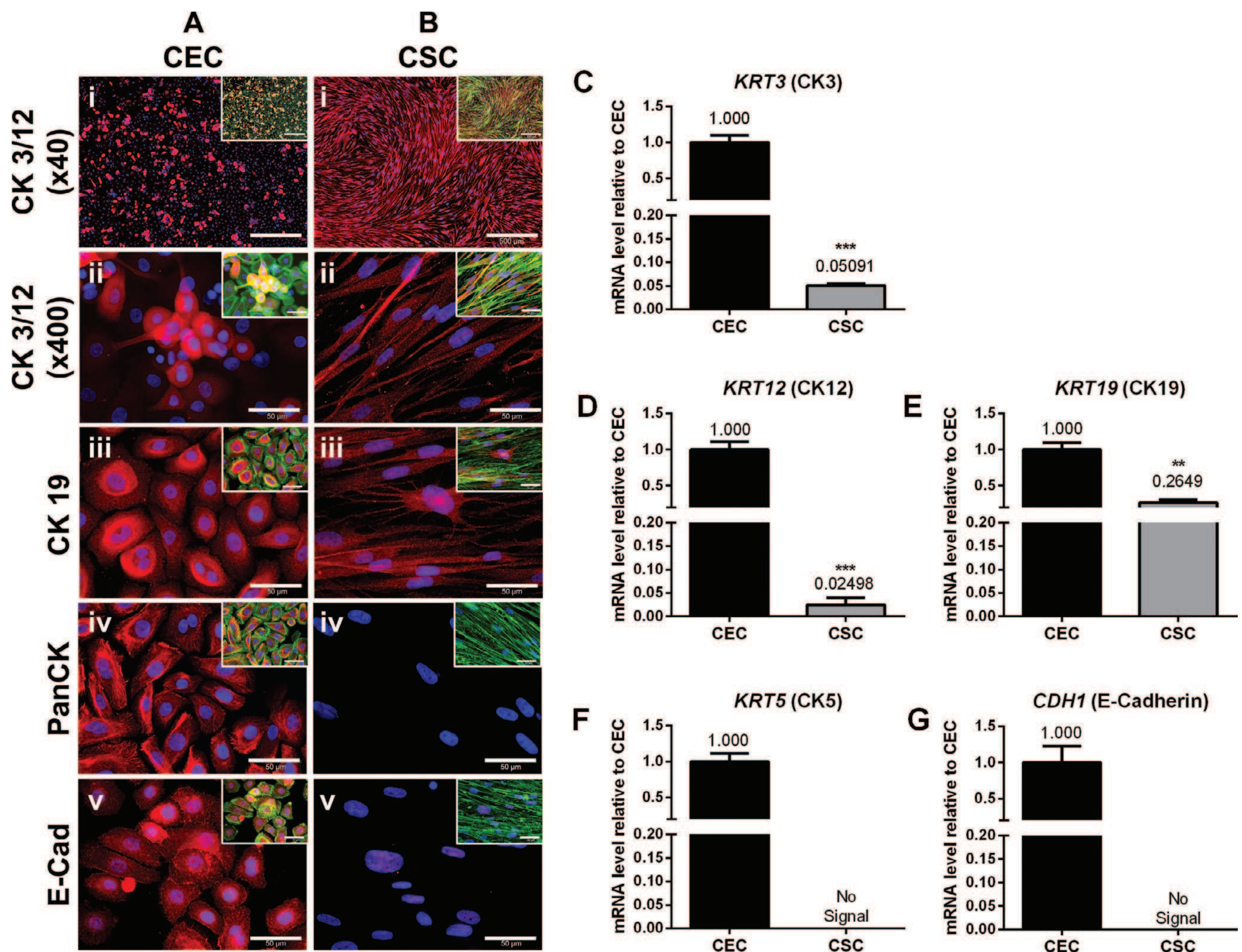

FIGURE 2. Expression of epithelial markers by CSC compared to CEC. Immunocytochemistry was performed on (**A**) CEC cultured from explants and (**B**) CSC cultured in M199 to P4 for (**i**, **ii**) CK3/12, (**iii**) CK19, (**iv**) pan cytokeratin (PanCK), and (**v**) E-cadherin. *Scale bars*: 500 µm (**i**); 50 µm (**ii**–**v**). *Insets* show same images with F-actin counterstain. *Scale bars*: 500 µm (**i**); 50 µm (**ii**–**v**). Reverse transcription-qPCR was performed to assess differences in mRNA levels of (**C**) *KRT3*, (**D**) *KRT12*, (**E**) *KRT19*, (**F**) *KRT5*, and (**G**) *CDH1*. Expression of each target gene was normalized to *GAPDH* and represented relative to mRNA expression by CEC. Data shown as mean ± SEM of three experiments ($n = 3$), each with two replicates. Statistical significance of CEC versus CSC (Student's *t*-test) represented by $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

these markers was further investigated by looking at mRNA level using RT-qPCR (Figs. 2C–G). Levels of all genes were significantly lower in CSC than CEC; this was also reflected in the differences in brightness when immunofluorescence was performed. However, there were detectable levels of mRNA for *KRT3* (CK3, Fig. 2C), *KRT12* (CK12, Fig. 2D), and *KRT19* (CK19, Fig. 2E), supporting the immunocytochemistry. There was no detectable mRNA for *KRT5* (CK5, Fig. 2F), which was tested as one of the proteins detected by the panCK antibody, or *CDH1* (E-cadherin, Fig. 2G).

Expression of Markers of Keratocytes and MSC in CEC and CSC

Cultured CEC and CSC were immunostained for markers associated with keratocytes and MSC (Fig. 3). Imaging for CEC and CSC was performed at identical exposure settings. Expression of vimentin was uniformly detected in both CEC (Fig. 3Ai) and CSC (Fig. 3Bi), but the rounded cobblestone morphology of the CEC was different from the fusiform morphology of the CSC. Neither CEC nor CSC at this passage (P4) visibly stained for CD34 (Figs. 3Aii, 3Bii). Corneal stromal cells stained positive for CD73 (Figs. 3Bi, Bii), CD90 (Fig. 3Biv), and CD105 (Fig. 3Bv). There was light staining for CD73 (Fig. 3Aiii) and CD90 (Fig. 3Aiv) in CEC. CD105 staining in CEC (Fig. 3Av) was sparse but did appear in individual cells, possibly due to fibroblast contamination. Reverse transcription-qPCR showed that *VIM* (vimentin, Fig. 3C), *CD34* (Fig. 3D), *THY1* (CD90, Fig. 3F), and *ENG* (CD105, Fig. 3G) were all expressed at significantly higher levels in CSC than CEC. There were no significant differences in levels of *NT5E* (CD73, Fig. 3E).

Differences in Expression Profiles Between High-Passage (P4), Early Extracted (P0), and Quiescent CSC

To explore the effect of in vitro expansion and activity state on phenotypic marker expression, CSC cultured to P4 (high passage) were compared to early extracted CSC (cells at P0 that would have been passaged to P1 if not fixed for immunocytochemistry or RNA extracted) and to high-passage

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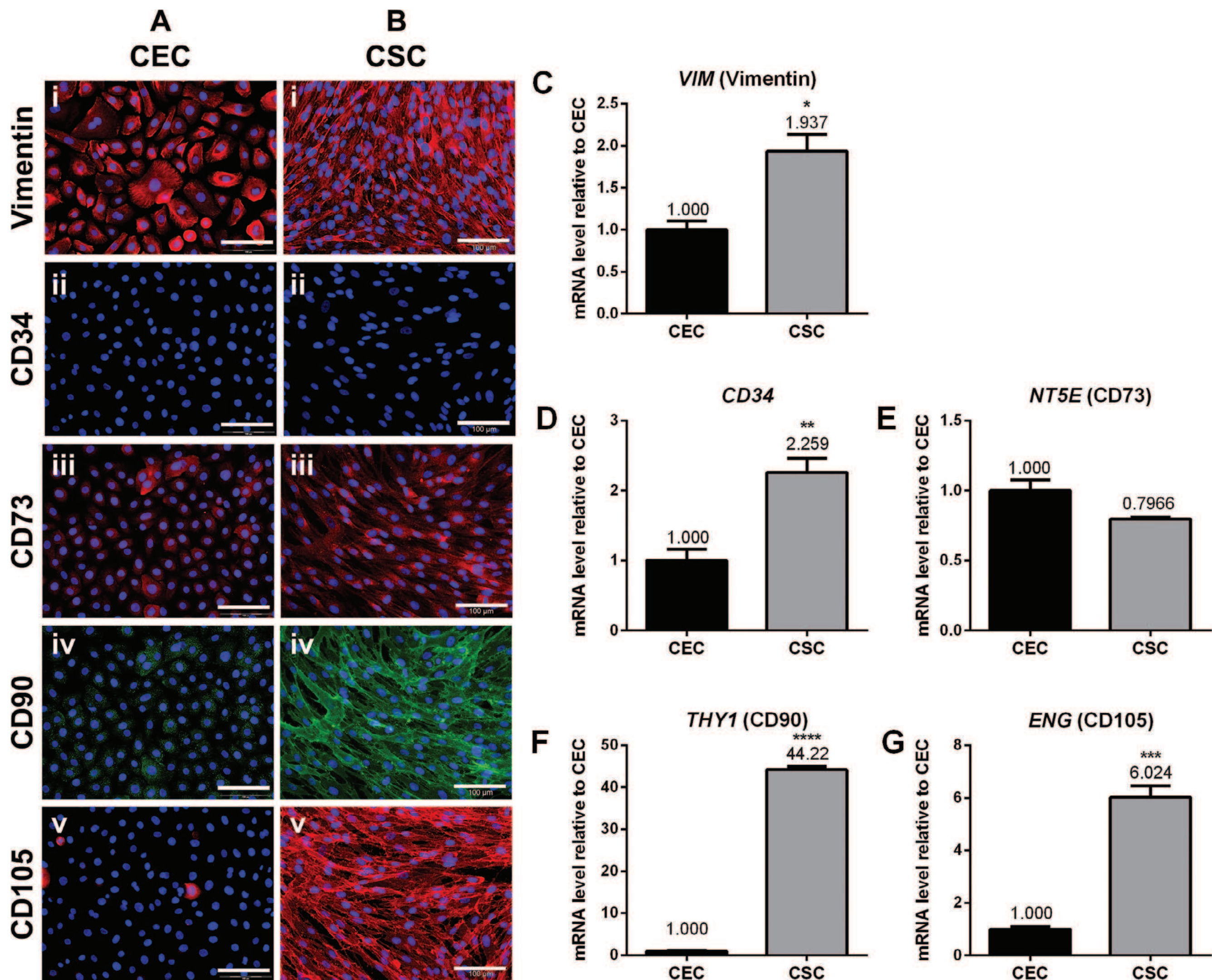

FIGURE 3. Expression of keratocyte and mesenchymal stromal cell markers by CSC and CEC. Immunocytochemistry was performed on (**A**) CEC cultured from explants and (**B**) CSC cultured in M199 to P4 for (**i**) vimentin, (**ii**) CD34, (**iii**) CD73, (**iv**) CD90, and (**v**) CD105. *Scale bars*: 100 μm. Reverse transcription-qPCR was performed to assess differences in mRNA levels of (**C**) *VIM*, (**D**) *CD34*, (**E**) *NT5E*, (**F**) *THY1*, and (**G**) *ENG*. Expression of each target gene was normalized to *GAPDH* and represented relative to mRNA expression by CEC. Data shown as mean ± SEM of three experiments ($n = 3$), each with two replicates. Statistical significance of CEC versus CSC (Student's *t*-test) represented by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

CSC that had subsequently been placed into a differentiation medium to induce quiescence (Fig. 4). Imaging of the immunocytochemistry was taken at exposure settings identical to those for P4 CSC (Figs. 2, 3). Early extracted and quiescent CSC comparably stained for CK3/12 (Fig. 4i) and CK19 (Fig. 4ii), but did not express panCK (Fig. 4iii) or E-cadherin (Fig. 4iv), similar to the P4 CSC (Fig. 2). However, RT-qPCR demonstrated that mRNA levels for *KRT3* (Fig. 4C), *KRT12* (Fig. 4D), and *KRT19* (Fig. 4E) were significantly lower in early extracted and quiescent cells than high-passage CSC, which was not reflected in the immunofluorescence. Quiescent cells also had significantly lower *KRT3* mRNA levels than early extracted CSC. Expression of *KRT5* (Fig. 4F) or *CDH1* (Fig. 4G) was not seen in any group.

Vimentin staining in P0 (Fig. 4Av) and quiescent cells (Fig. 4Bv) appeared comparable to that in high-passage CSC (Fig. 3). CD34 staining was visible in early extracted CSC (Fig. 4Avi), was lost by P4 (Fig. 3), but was regained and can be seen in quiescent CSC (Fig. 4Bvi). CD73, CD90, and CD105 staining appeared decreased in early extracted CSC (Figs. 4Avii–ix) compared to high-passage (Fig. 3) and quiescent CSC (Figs. 4Avii–ix). Reverse transcription-qPCR showed that *VIM* (Fig. 4H) and *CD34* (Fig. 4I) mRNA expression was significantly increased in quiescent cells. *NT5E*, *THY1*, and *ENG* were significantly increased in quiescent cells compared to both P4 and P0 (Figs. 4J–L).

Expression of Typical Markers of Epithelial Cells and MSC in WJ-MSC

Mesenchymal stromal cells were extracted from Wharton's jelly and cultured using identical processes and medium to CSC up to P4, prior to phenotypic analysis. Imaging of the immunocytochemistry was taken at identical exposure settings to those for P4 CSC (Figs. 2, 3). Wharton's jelly MSC stained for CK3/12 (Fig. 5Aii) and CK19 (Fig. 5Aiii) to some degree, similar to CSC (Fig. 2B). However, unlike CSC, WJ-MSC stained positively for panCK (Fig. 5Aiv), indicating expression of one or more of CK4, 5, 6, 8, 13, and 18, and stained for E-cadherin (Fig. 5Av) despite not being of

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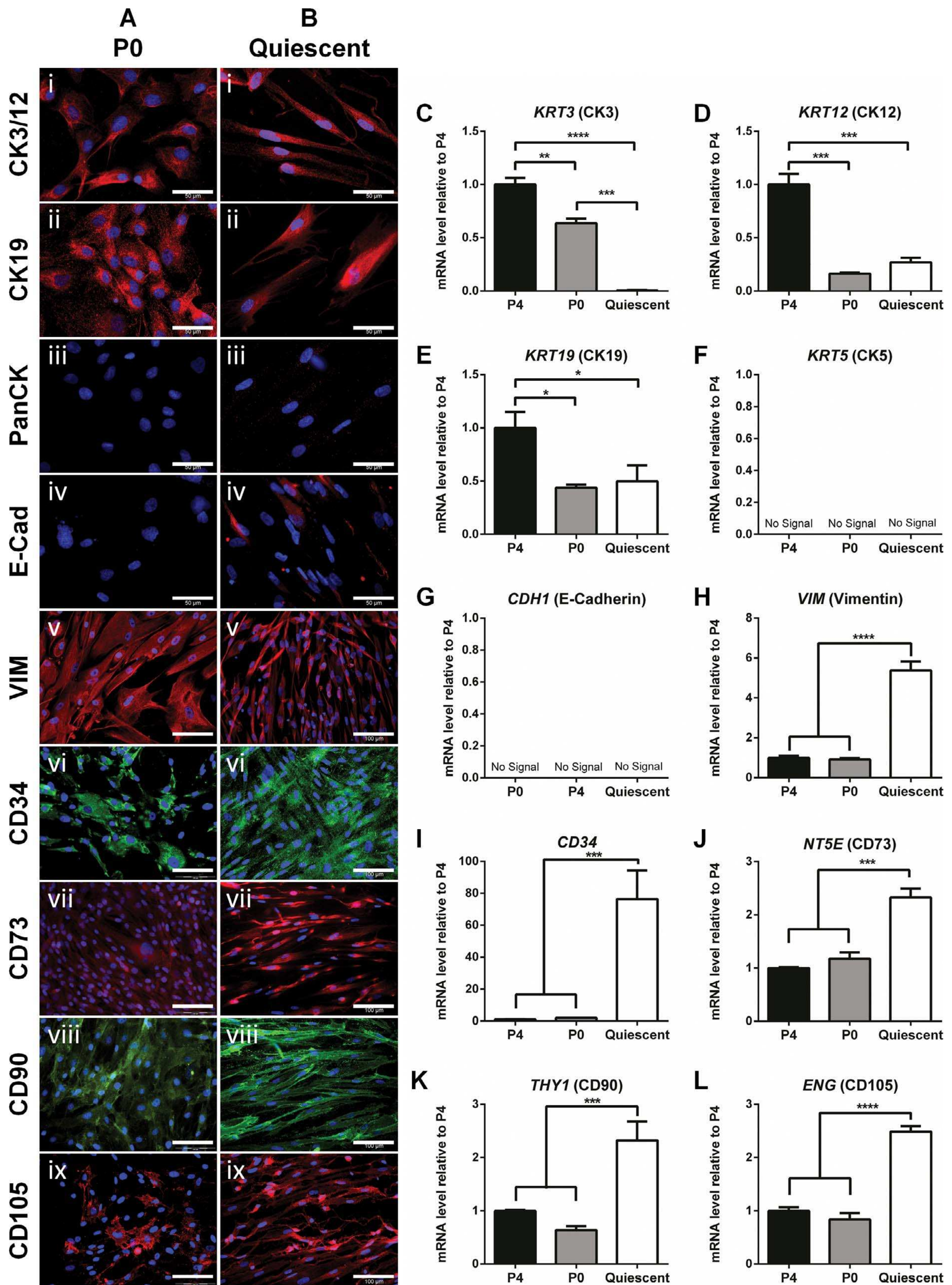

FIGURE 4. Expression of epithelial, keratocyte, and mesenchymal stromal markers in early extracted and quiescent CSC. Immunocytochemistry was performed on (**A**) P0 CSC and (**B**) quiescent CSC staining for (**i**) CK3/12, (**ii**) CK19, (**iii**) pan cytokeratin (PanCK), (**iv**) E-cadherin, (**v**) vimentin, (**vi**) CD34, (**vii**) CD73, (**viii**) CD90, and (**ix**) CD105. *Scale bars*: 50 μm (**i**–**iv**); 100 μm (**v**–**ix**). Reverse transcription-qPCR was performed to assess differences in mRNA levels between P4, P0, and quiescent CSC of (**C**) *KRT3*, (**D**) *KRT12*, (**E**) *KRT19*, (**F**) *KRT5*, (**G**) *CDH1*, (**H**) *VIM*, (**I**) *CD34*, (**J**) *NT5E*, (**K**) *THY1*, and (**L**) *ENG*. Expression of each target gene was normalized to *GAPDH* and represented relative to mRNA expression of P4 CSC. Data shown as mean ± SEM of three experiments ($n = 3$), each with two replicates. Statistical significances analyzed by 1-way ANOVA represented by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

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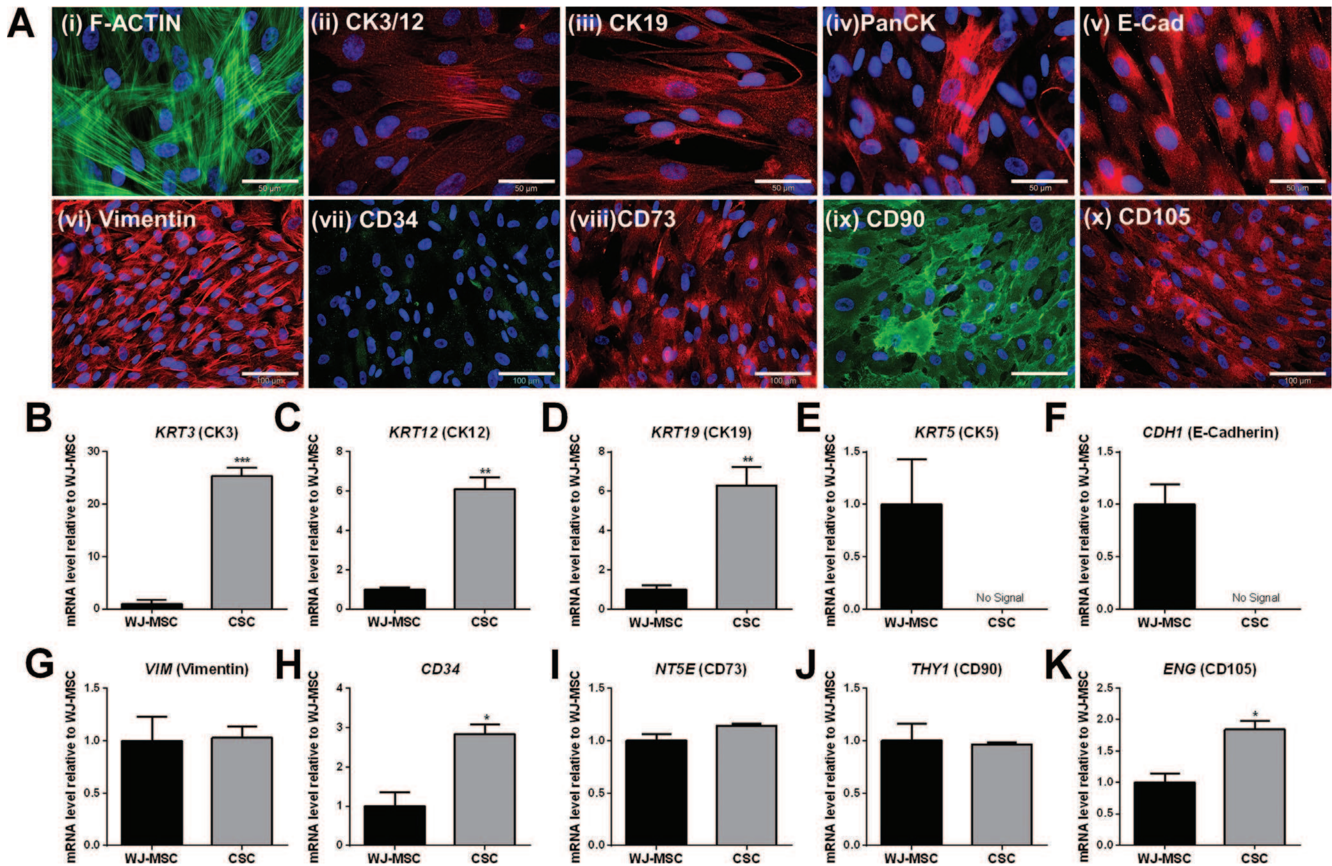

Figure 5. Expression of epithelial and mesenchymal stromal cell markers in WJ-MSC. (**A**) Immunocytochemistry was performed on WJ-MSC staining for (**i**) F-actin (**ii**) CK3/12, (**iii**) CK19, (**iv**) pan cytokeratin (PanCK), (**v**) E-cadherin, (**vi**) vimentin, (**vii**) CD34, (**viii**) CD73, (**ix**) CD90, and (**x**) CD105. *Scale bars*: 50 μm (**i–v**); 100 μm (**vi–x**). Reverse transcription-qPCR was performed to assess differences in mRNA levels between CSC and WJ-MSC investigating (**C**) *KRT3*, (**D**) *KRT12*, (**E**) *KRT19*, (**F**) *KRT5*, (**G**) *CDH1*, (**H**) *VIM*, (**I**) *CD34*, (**J**) *NT5E*, (**K**) *THY1*, and (**L**) *ENG*. Expression of each target gene was normalized to *GAPDH* and represented relative to mRNA expression by WJ-MSC. Data shown as mean ± SEM of three experiments ($n = 3$), each with two replicates. Statistical significances analyzed by 1-way ANOVA represented by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

epithelial origin. Wharton's jelly MSC showed expression of vimentin (Fig. 5Avi), CD73 (Fig. 5Aviii), CD90 (Fig. 5Aix), and CD105 (Fig. 5Ax), but similarly to P4 CSC (Fig. 3) did not express CD34 (Fig. 5Avii). Reverse transcription-qPCR revealed significantly decreased expression of *KRT3* (Fig. 5B), *KRT12* (Fig. 5C), and *KRT19* (Fig. 5D) compared to CSC. Inversely, WJ-MSC had detectable levels of both *KRT5* and *CDH1*, which did not occur in CSC. Wharton's jelly MSC showed mRNA expression of *VIM* (Fig. 5G), *NT5E* (Fig. 5I), and *THY1* (Fig. 5J) similar to the CSC, but downregulation of *CD34* (Fig. 5H) and *ENG* (Fig. 5K).

Cytokeratin Expression in Stromal Cells

A semiquantitative cell-based immunoassay was used to compare CK3/12, CK19, and panCK staining in ihCEC, P4

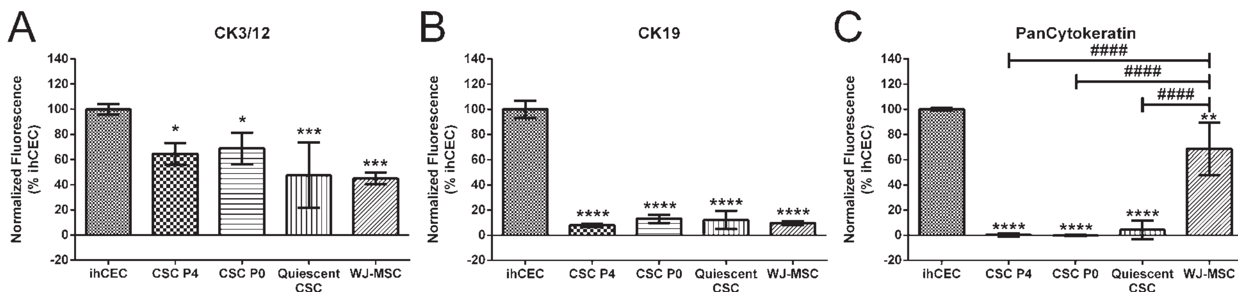

Figure 6. Quantitation of cytokeratin staining in stromal cells. A quantitative cell-based fluorescent immunoassay was performed and fluorescence intensity determined for (**A**) CK3/12, (**B**) CK19, and (**C**) PanCK, staining in ihCEC, P4 CSC, P1 CSC, quiescent CSC, and WJ-MSC. Fluorescent intensity of antigen for all groups was corrected for cell number using fluorescent intensity of actin staining and is displayed relative to intensity in ihCEC. Data shown as mean ± SD of three experiments ($n = 3$), each with 100 readings. Statistical significances versus ihCEC analyzed by 1-way ANOVA represented by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. Statistical significances versus WJ-MSC analyzed by one-way ANOVA represented by $^{\#\#\#\#}P \leq 0.0001$.

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CSC, P0 CSC, quiescent CSC, and WJ-MSC (Fig. 6). Data reflected the previous immunocytochemistry. CK3/12 was significantly lower in CSC P4, CSC P0, quiescent CSC, and WJ-MSC than ihCEC, but levels were still over 50% (Fig. 6A). CK19 was reduced in all stromal cells compared to ihCEC, at levels between 10% and 20%; however, staining was detectable above background (Fig. 6B). PanCK was detectable above background levels only in ihCEC and WJ-MSC (Fig. 6C). Levels in WJ-MSC were significantly higher than in CSC but significantly lower than in ihCEC.

Discussion

Identification of mammalian cells, both in vivo and in vitro, can usually be reliably performed by detecting specific cell surface markers, or IF of the cytoskeleton.[39,40] Corneal epithelial and stromal cells have clear differences in protein and IF expression profiles in vivo, which are often used to differentiate between the cell types in vitro. Notably, the epithelium expresses cytokeratins that are not expressed by keratocytes. Corneal epithelial cells are also known to express different cytokeratins depending on differentiation state. In this study, CK3/12 was seen only in the terminally differentiated cells of the central epithelium and was not present in the peripheral/limbal epithelium, as has been described many times previously.[10,41,42] CK19 was present only in a limited number of cells, focused only in the limbal region of the epithelium, as described previously.[10,43]

There is little evidence to suggest that CK3, CK12, or CK19 is expressed in vivo by keratocytes. However, this study clearly demonstrates that in vitro culture not only causes phenotypical changes in CSC but also induces expression of specific cytokeratins. Expression of CK3, CK12, and CK19 was induced as CSC were cultured in vitro, as shown by immunocytochemistry. Although mRNA levels of these cytokeratins were much lower in CSC than CEC, the immunocytochemistry demonstrated that the filaments were clearly visible and therefore that a functional cytokeratin protein was being transcribed. Culture of CSC promotes an atypical, active state that may explain the change in IF expression, alongside the induction of other markers such as CD73, CD90, and CD105. Culture appeared to induce specific, corneal-related cytokeratins, as no staining was seen when a panCK antibody, specific for CK4, 5, 6, 8, 13, and 18, was used. This was further corroborated by the fact that CSC did not express the *KRT5* gene, whereas CEC did. Corneal epithelial cells stained positive for panCK, both in vivo and in vitro, suggesting that they also express one or more of CK4, 5, 6, 8, 13, and 18. However, direct comparisons between cultured CEC and CSC in this study are difficult to perform due to the different culture requirements of the cells, particularly the different culture media used.

The observed lower mRNA levels in CSC compared to CEC may explain why other groups have not detected cytokeratins in cultured CSC during nonquantitative RT-PCR experiments.[30,44] There have also been studies performed that have shown no expression of cytokeratins during flow cytometry experiments.[31,35,36] However, it was not mentioned whether the flow cytometry procedure included cell permeabilization, required for detecting cytoplasmic antigens.[45,46] In our staining procedures, we include permeabilization for intracellular antigens, and we were able to detect cytokeratins visually by immunocytochemistry and to semiquantify the amount of cytokeratin by plate reader assay.

Earlier-passage CSC expressed lower mRNA levels of CK3, CK12, and CK19 than later passages, supporting the hypothesis that prolonged culture and proliferation drive cytokeratin expression. Other phenotypic changes occurred upon passage, including decreased expression of CD34, as described previously,[25] and increased levels of CD105 and CD90, a protein not present in the healthy cornea.[27] Attempts to differentiate the cells back to a keratocyte phenotype using serum-free medium, as performed previously,[47,48] led to further phenotypic changes. Quiescent cells expressed decreased levels of cytokeratin genes *KRT3*, *KRT12*, and *KRT19* compared to the proliferative cells, but protein and gene did not disappear, suggesting that differentiation had not resulted in a keratocyte phenotype. The major effect of quiescence was a large upregulation of the gene for *CD34*, indicating some differentiation. However, the quiescent CSC also showed increased expression of CD90 and CD105. For this reason, we do not believe that the quiescent CSC in this study are comparable to the native keratocyte, at this point. Although serum-free medium may induce expression of keratocyte markers such as CD34, this also needs to be reflected in the downregulation of markers such as CD90 and CD105. Differentiation may have been incomplete, and downregulation of the fibroblast markers might have occurred if the cells had been differentiated for a longer time period.

A number of studies have been performed that demonstrate the ability of CSC isolated from the limbal stroma to conform to a set of minimal criteria set by the ISCT, which define an MSC.[30–32,49] These criteria state that to be considered a population of MSC, the cells should be plastic adherent; over 95% of cells should express CD73, CD90, and CD105; under 2% of cells should express CD11b, CD14, CD19, CD34, CD45, and HLA-DR; and the cells should possess the ability to undergo adipogenesis, osteogenesis, and chondrogenesis in vitro.[50] However, this study shows that although these limbal CSC become MSC after extraction and several passages,[31] there is no evidence that the in vivo limbal stroma contains MSC as defined by the ISCT. This is due primarily to a lack of expression of CD90 and high expression of CD34 by in vivo keratocytes. CD90 is rarely present in a healthy cornea as it is a marker of activated keratocytes.[27] CD34 is a cell surface marker associated with progenitor cells and quiescence[51]; its association with hematopoietic stem cells is the reason it is considered an indicator that a cell is not an MSC.

For further investigation into cytokeratin expression in mesenchyme-derived cells, we compared expression in CSC to another MSC-like cell type: MSC extracted from the Wharton's jelly of the umbilical cord. Wharton's jelly MSC have been previously characterized as having an MSC phenotype,[52–54] similar to that displayed by CSC. Wharton's jelly MSC showed detectable expression of CK3, CK12, and CK19, although relative mRNA levels were lower than those in CSC. In contrast to CSC, WJ-MSC showed positive staining for the panCK antibody and had detectable levels of mRNA for *KRT5* (CK5), suggesting that there is some specificity in the cytokeratins produced by different stromal cells. There were also detectable levels of E-cadherin, which could indicate epithelial contamination; however, immunocytochemistry demonstrated that the cells appeared to have a fibroblastic morphology and not the cobblestone morphology seen in cultured epithelial cells.

This is not the first report of cytokeratin expression in extracted and cultured keratocytes.[34] However, this is the first to report CK19 expression and the fact that there is some specificity to the cytokeratins expressed in different stromal cell populations. Cytokeratin expression has also been described in a number of stromal cell types from different tissues. One of the first reports of this was in 1987, when von Koskull[39] demonstrated staining for CK8, CK18, and CK19 in decidual stromal cells and fetal fibroblasts. Conget and Minguell[55] detected a number of cells expressing panCK (clone K3.18), CK18, and CK19, but did not detect any other epithelial markers by flow cytometry in bone marrow–

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derived MSC. Similar to this study, cytokeratins have also been detected in umbilical cord stromal cells.[56] In this case it was a panCK antibody with a mixture of clones that detected CK1, 4, 5, 6, 8, 10, 13, 18, and 19 by immunocytochemistry, alongside vimentin and myofibroblast marker α-smooth muscle actin. It has also been shown that adipose-derived stromal cells acquire CK3, CK76, and CK12 over time in culture, but not CK1, 5, 10, or 14,[57] further suggesting that there is specificity to the expression of cytokeratins by stromal cells.

Results in this study show that the expression profile of cultured CSC can become much more similar to that of CEC than previously thought. This may indicate that CSC possess the ability to transition into an epithelial phenotype, and adds to increasing evidence that CSC extracted from the limbal region have stem cell properties. Previous work by our group has demonstrated the ability of $CD34^+$ CSC to transdifferentiate into a CEC phenotype in vitro,[25] and if this is possible, there is scope for CSC to be used within a regenerative cell therapy for the treatment of ocular surface disorders.

In conclusion, cultured CSC and other types of stromal cells can acquire a very different phenotype from that of an in vivo keratocyte, which can include the expression of cytokeratins. Investigators should be careful not to compartmentalize cytokeratin expression as an epithelial-only characteristic and should be careful not to assume that the in vitro phenotype of extracted primary cells reflects the state of the healthy tissue. When culturing CSC, investigators need to be aware that expression of these markers is not necessarily due to epithelial contamination; and to check for contamination, an alternative epithelial marker, such as E-cadherin, should be used.

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