ABSTRACT

Purpose: Eliminating contamination by corneal stromal cells is critical when preparing cultured human corneal endothelial cells (CECs) transplantation. We investigated markers for the purification of cultured human CECs and markers for excluding cultured human corneal stromal myofibroblasts (CSMFs) from cultured human CECs.

Materials and methods: CECs and CSMFs were obtained from human donor corneas by culturing separately in serum-containing medium. Candidate markers of CECs and CSMFs were screened with microarray analysis in the fourth passaged CECs and CSMFs. Then, selected factors were evaluated in reverse transcription polymerase chain reaction (RT-PCR), western blot, immunocytochemistry, and flow cytometry to investigate differential markers for each cell.

Results: Among the genes identified by microarray analysis, cultured human CECs, but not CSMFs, expressed integrin alpha 3 (ITGA3 and CD49c) protein according to immunocytochemistry and western blotting. Iroquois homeobox 2 (IRX2) gene was a marker that distinguished CSMFs from cultured human CECs by RT-PCR. The IRX2 gene can be used as a marker of CSMFs contaminating cultured CECs.

Conclusion: These molecules could be important markers for the production of highly purified cultured CECs for regenerative medicine.

Keywords: Corneal endothelial cell, differential marker, keratocyte, myofibroblast, regenerative medicine

INTRODUCTION

The corneal endothelial cells (CECs) on Descemet’s membrane play a critical role in maintaining corneal hydration and transparency. Full-thickness corneal transplantation and Descemet’s stripping with endothelial keratoplasty (DSEK)1–3 are effective treatments for reduced visual acuity associated with CEC loss, but there is a worldwide donor shortage.

Regenerative medicine using cultured CECs4–8 is an attractive method to compensate for this lack of donors and could become clinically available in the future. Although human CECs exist in a non-replicative state in vivo,9–12 adults have some precursor cells that are largely committed to the CEC lineage13,14 and display proliferative capacity in vitro15–18 with some efficient culture methods having been reported.19,20 We have also established a highly efficient human
CEC culture technique that minimizes the risk of bovine spongiform encephalopathy.\textsuperscript{21,22}

Cell transplantation in regenerative medicine requires highly purified cells with no contamination of the other cells.\textsuperscript{23} For cultivation of CECs, cells are initially stripped off the donor cornea together with Descemet's membrane. To obtain highly pure CECs, careful removal of the stromal tissue on Descemet's membrane is necessary, because this tissue contains corneal stromal cells. When corneal stromal cells (quiescent keratocytes) are cultured in the presence of bovine serum, they are activated by serum and other growth factors.\textsuperscript{24-26} Their morphology then change from dendritic to a more spreading fibroblastic phenotype. Such serum-activated transformed keratocytes are called corneal stromal myofibroblasts (CSMFs), which totally express smooth muscle actin (\textgreek{a}-SMA). Because CSMFs grow more rapidly than CECs in the culture condition with bovine serum, contamination of CSMFs has been a problem for CEC culture. Selective L-valine free culture medium reducing growth of CSMFs has been developed,\textsuperscript{27} but the culture medium also suppresses growth of CECs. Thus, to reduce the risk of aberrant growth of CSMFs, cultured CECs for clinical use should be highly purified, therefore, a quality control method to distinguish CECs and CSMFs is necessary.

Cultured CECs show fibroblastic morphology during exponential growth stage but show polygonal form after confluent stage. Therefore, it is difficult to distinguish CECs and CSMFs from morphological findings only. Although some candidate genes like type VIII collagen\textsuperscript{28} were reported to only be expressed by CECs, our preliminary experiments have shown that both CECs and CSMFs express such genetic markers (Supplemental Table 1). We also found that both cultured CECs and CSMFs express functional marker proteins for CECs such as zonula occludens 1 (barrier function) and sodium potassium ATPase (pump function) (Supplemental Table 1). Moreover, we compared expression levels of other known stromal CD markers (CDs: 14, 29, 31, 34, 44, 45, 73, 90, 105, 165) between CECs and CSMFs and found that these markers did not work as differential markers for CECs and CSMFs (Kimoto unpublished observation). Since CSMFs highly express \textgreek{a}-SMA, we expected \textgreek{a}-SMA could be a distinguishable gene, but we found that both tissue and cultured CECs expressed subtle levels of \textgreek{a}-SMA gene (data not shown). During the preparation of this manuscript, new markers for CECs distinguishable from CSMFs such as glypican-4 and CD200 are reported.\textsuperscript{29} These markers are useful for quality control for the purity of CECs. However, new definitive markers that can exclude CSMF contamination are still required for establishing a quality control method for impurity of CECs.

In this study, following microarray analysis of 40,000 of genes with cultured CECs and CSMFs to select genes that were highly expressed by each cell type (Supplemental Table 1), markers of CEC purity, which possibly express cell surface proteins on CECs, were identified by a combination of immunocytochemistry and western blotting. Moreover, definitive markers of contamination by CSMFs were determined by the reverse transcription polymerase chain reaction (RT-PCR) to prevent possible CSMFs contamination completely.

**MATERIALS AND METHODS**

This study was conducted in accordance with the Declaration of Helsinki. Donor corneas (ages: 48, 55, 62, and 65) were obtained from the Northwest Lions Foundation or the Rocky Mountain Lions Eye Bank. CECs and CSMFs were separated, cultured, and passaged for immunocytochemistry, western blotting, and RT-PCR. All examinations were repeated and were conducted on at least three different cell sources unless otherwise noted.

**Cell Culture**

Primary culture of CECs was performed as described elsewhere.\textsuperscript{21} Briefly, cells on Descemet’s membrane were stripped off the cornea, cut into small pieces, and then digested with 2 mg/mL collagenase A (Sigma-Aldrich, St. Louis, MO). The cells thus obtained were washed by centrifugation, incubated with 0.05% trypsin/EDTA (Gibco BRL, Grand Island, NY), washed again, and cultured on atelocollagen-coated dishes in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum and antibiotics (Gibco BRL, Grand Island, NY) containing 2 ng/mL basic fibroblast growth factor (bFGF; Sigma, St. Louis, MO) in the presence of 0.3 mM L-ascorbic acid 2-phosphate (Wako, Osaka, Japan). When the proliferating cells had reached a sufficient density, passaging was done at ratios ranging from 1:1 to 1:4. To isolate corneal stroma cells, i.e. keratocytes, the CECs were peeled off in a sheet and the epithelium was scraped away with spatula. Then the corneal stroma was cut into small pieces (1–2 mm in diameter) that were incubated overnight at 37 °C in serum-free basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). After the cells were washed three times with phosphate-buffered saline (PBS), a single-cell suspension was obtained by trituration with a pipette. Isolated keratocytes were cultured in DMEM with 15% FBS and passaged three times, after which the keratocytes were transformed into spindle-shaped CSMFs.
cDNA Microarray Analysis

The fourth passaged CSMFs and CECs were separately cultured on 10 cm dish for 2 weeks. All samples were tripipsonized and collected to 15 mL tube followed by centrifuge (500 g, 5 min). Cell pellets were frozen by nitrogen and transferred to Takara Bio Inc. (Shiga, Japan) for outsourcing cDNA microarray analysis. Briefly, all samples were transferred into lysis buffer (Qiagen, Valencia, CA). Total RNA was then isolated from the samples using silica-membrane columns (RNasea Mini Kit; Qiagen, Valencia, CA) according to the instructions of the manufacturer. RNA quality and quantity were checked (Bioanalyzer Nano Chips; Agilent Technologies, Santa Clara, CA). Two hundred nanograms of total RNA were used to prepare labeled cDNA (Agilent Expression Array Kit; Agilent Technologies, Santa Clara, CA). Reactions from all samples yielded sufficient cDNA for subsequent microarray analysis. cDNA samples from each three CEC and CSMF were hybridized (Gene expression hybridization kit, Agilent Technologies, Santa Clara, CA). Microarrays were scanned, the size and the morphology of each spot were examined, and low-quality spots were masked. Only scans with more than 99% appropriate spots were further analyzed. Spots were grided and assigned, and the intensity values were calculated according to the standard protocol of the manufacturer. The resultant data were then normalized on per chip, and subsequently on per gene, basis. Normalized data were imported in a data analysis software package (GeneSpring GX10; Agilent Technologies, Santa Clara, CA).

Immunocytochemistry

CSMFs and CECs from the fourth passage were cultured on glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and fixed in 4% paraformaldehyde or methanol for 10 min. After rinsing with 0.1% Triton X-100/PBS, incubation was done for 30 min in PBS with 1% bovine serum albumin in 0.1% Triton X-100 to prevent non-specific staining. Spots were grided and assigned, and the intensity values were calculated according to the standard protocol of the manufacturer. The resultant data were then normalized on per chip, and subsequently on per gene, basis. Normalized data were imported in a data analysis software package (GeneSpring GX10; Agilent Technologies, Santa Clara, CA).

Western Blot Analysis

CSMFs and CECs from the fourth passage were collected in PBS and lysed in lysis buffer (CellLytic MT; Sigma, St. Louis, MO). Approximately 200 µg of protein/10 µL was subjected to 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA), and treated with PBS containing 5% skimmed milk for 30 min. The membranes were then reacted overnight at 4 °C with anti-ITGA3, RGS5, PSGR, and KCNE1 antibody (1.0 µg/mL) in PBS supplemented with 1% skimmed milk. After washing in PBS and treatment for 1 h with a peroxidase-conjugated secondary anti-goat immunoglobulin antibody diluted in PBS containing 1% skim milk, the reaction products were visualized by using an enhanced chemiluminescence kit (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK).

RT-PCR

Total RNA was extracted from CSMFs or CECs with Trizol reagent (Life Technologies, Rockville, MD). The quantity of RNA was determined by measurement of the optical density at 260 nm. The cDNA was synthesized from 1 µg of total RNA using Oligo (dT) primers and SuperScript reverse transcriptase (Life Technologies, Rockville, MD). Then cDNA samples were subjected to PCR using specific primers for human iroquois homeobox gene (IRX2), human chemokine (C-X-C motif) receptor 7 (CXC7), integrin z8 (ITGA8), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal standard for normalization. The amplification conditions were 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. PCR was performed in a 50 µL reaction volume containing 1 µL of 10 mmol/L dNTPs, 5 µL of 10– buffer, 1 µL of Taq polymerase, and 2 µL of 10 mmol/L sense and antisense primers (Table 1). Normalized samples were amplified in the linear range that was established by using serial dilutions of cDNA and varying the number of cycles. Amplified products were separated by electrophoresis on 3% agarose gel and then stained with ethidium bromide.
RESULTS

Markers for the Purification of Cultured Human CECs

Protein expression was studied by immunocytochemistry in cultured CECs, with the target proteins being selected by microarray analysis and comparison with cultured CSMFs (Supplemental Table 1). Strong expression of ITGA3 was observed on the cell membranes of cultured CECs (Figure 1A), while no expression was detected on cultured CSMFs (Figure 1F) and cultured CECs stained with non-immunized isotype control antibody (data not shown). RGS5 was expressed in the nuclei of both CECs and CSMFs (Figure 1B and G). PSGR (Figure 1C and H) and KCNE1 (Figure 1D and I) were not detected in either cultured CECs or CSMFs, respectively.

Western blotting analysis showed that ITGA3 was expressed (estimated product size: 150 kD) in cultured CECs, but not cultured CSMFs (Figure 2A). RGS5 was detected in both cultured CECs and CSMFs (Figure 2B), consistent with the results of immunocytochemistry, indicating that RGS5 could not be used as a marker to distinguish between CECs and CSMFs. No positive band for PSGR was detected in cultured CECs or CSMFs (Figure 2C). KCNE1 was detected in both cultured CECs and CSMFs (Figure 2D), conflicting with the results of immunocytochemistry, suggesting that the KCNE1 antibody was not suitable for immunostaining. These gene transcription and protein expression studies revealed that ITGA3 was a marker for distinguishing cultured CECs from cultured CSMFs.

Markers for Excluding Cultured Human CSMFs from CECs

To ensure the high purity of cultured CECs, contamination by CSMFs derived from corneal stromal cells should be completely eliminated. Therefore, specific markers for CSMFs were identified to detect CSMFs contaminating cultured CEC. Based on the results of microarray analysis (Supplemental Table 1), the expression profiles of the IRX2, CXCR7, and ITGA8 genes were further investigated in CECs and CSMFs from the fourth passage by RT-PCR analysis. The results showed that IRX2 mRNA was only detected in CSMFs, while ITGA8 and CXCR7 mRNAs were detected in both CECs and CSMFs, although expression of ITGA8 by CECs was very low (Figure 3). Further experiments using different lots of cells confirmed that IRX2 was not detected in CECs under any cycling conditions, while ITGA8 was detected in some lots of CECs (data not shown). These results indicated that there was no detectable contamination of CSMFs in cultured CECs and subtle ITGA8 expression was derived from CECs but not from contaminated CSMFs. Based on these findings, we excluded ITGA8 as a potential contamination marker, and selected IRX2 as a possible marker for CSMFs contaminating cultured CECs.

DISCUSSION

We demonstrated that ITGA3 protein can be used as a purification marker for cultured human CECs, while IRX2 gene expression can identify contamination of cultured human CECs by CSMFs on RT-PCR. Thus, these markers can provide a new quality control method for producing cultured CECs of high purity for regenerative medicine. Although ITGA3 gene expression was detected in CSMFs by microarray analysis, methods of protein analysis such as immunohistochemistry and western blotting found no ITGA3 protein expression in these cells. This discrepancy may be explained by differences of expression time course, stability, and post-translational modification of each mRNA and protein, or protein expression levels in CSMFs are lower than the detection limit of ITGA3 antibody used in the present study. In either case, the present results demonstrated that anti-ITGA3 antibody is a useful commercially available tool for distinguishing CECs from CSMFs. ITGA3 is an essential molecule for spreading of cells on the
basement membrane, assembly of the extracellular matrix, maintenance of hemidesmosome stability, maintenance of the cytoskeleton, epidermal proliferation, stem cell activation, and regulation of cell migration.29–35 ITGA3 subunit binds with integrin beta1 to form integrin \( \alpha_3 \beta_1 \) complex, which is predominantly expressed in keratinocyte stem cells36 and

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FIGURE 1 Immunocytochemistry of candidate markers for cultured human CECs. ITGA3 is expressed on the surface of CECs (A). No CSMFs express ITGA3 (F). RGS5 is expressed in the nuclei of both CECs (B) and CSMFs (G). No expression in CECs and CSMFs was found in PSGR (C, H) and KCNE1 (D, I), respectively. Immuno-staining of target protein is green and nuclear staining with propidium iodide (PI) is red. Morphological shape of CECs (E) and CSMFs (J) are shown in bright field image. Similar findings were obtained with repeated experiments and three different cell sources. Scale bars = 50 \( \mu \)m. CECs, corneal endothelial cells; CSMFs, corneal stromal myofibroblasts; ITGA3, integrin alpha3; RGS5, regulator of G-protein signaling 5; PSGR, prostate specific G-protein coupled receptor; olfactory receptor family 51 subfamily E member 2; KCNE1, potassium voltage-gated channel Isk-related family member 1.

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FIGURE 2 Expression of candidate markers for cultured human CECs by western blotting. Western blot analysis was performed using whole cell lysates from CECs and CSMFs. (A) A band for ITGA3 is detected in CECs, but not in CSMFs. (B) Bands for RGS5 are visible in the lanes for both CECs and CSMFs. (C) No band for PSGR is detected in both lanes. (D) Bands for KCNE1 are expressed in both lanes. The blots shown are representative of three independent experiments. CECs or EC, corneal endothelial cells; CSMFs or MF, corneal stromal myofibroblasts; ITGA3, Integrin alpha3; RGS5, regulator of G-protein signaling 5; PSGR, prostate specific G-protein coupled receptor; olfactory receptor family 51 subfamily E member 2; KCNE1, potassium voltage-gated channel Isk-related family member 1.

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FIGURE 3 Selection of candidate markers for CSMFs contamination of cultured CECs by RT-PCR. CECs and CSMFs were prepared from three different cell sources. An RNA-free lane was the negative control. IRX2 gene is expressed all of the CSMFs, but not all the CECs. ITGA8 gene is expressed by all the CSMFs, but is weakly detected in only one of the samples of CECs. CXCR7 gene expression is detected in all the samples from both CECs and CSMFs. Similar findings were obtained with repeated experiments. CECs or EC, corneal endothelial cells; CSMFs or MF, corneal stromal myofibroblasts; IRX2, irquois homeobox 2; CXCR7, chemokine (C-X-C motif) receptor 7; ITGA8, Integrin alpha 8; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
functions as a receptor for laminin-332. Human CECs from donor corneas express integrin $\alpha_3\beta_1$, and our findings showed that cultured human CECs also express ITGA3, suggesting that ITGA3 plays a role in CEC functions and that such functions are preserved in cultured CECs.

Recently, new markers for CECs distinguishable from CSMFs such as glypican-4 and CD200 are reported. They reported that monoclonal antibodies to glypican-4 and CD200 specifically stain CECs. These antibodies also can be used as separation markers for FACS sorting. In our microarray analysis, expression levels of Glypican-4 and CD200 were both similar between CECs and CSMFs (Yamaguchi, unpublished observation) implying that such difference of expression levels may occur with post-translational modification such as glycosylation. Together with our finding of ITGA3, these protein markers are useful for quality control of CEC purity.

The Iroquois genes (Irx) encode homeodomain-containing transcription factors that play a crucial role in the regionalization and patterning of tissues and organs during embryonic development. IRX2 is reported to be involved in neurogenesis in parts of the central nervous system, such as the retinal ganglion cell layer and the cerebellum. Knockdown of IRX2 in zebrafish results in smaller eyes, lamination defect, and loss of retinogenesis waves. Since CSMFs as well as CECs originate from the neural crest, it is not surprising that IRX2 is expressed in CSMFs, but further studies are necessary for revealing why only CSMFs but not CECs express IRX2.

CONCLUSION

ITGA3 protein is a useful marker for distinguishing cultured human CECs from cultured CSMFs, while IRX2 gene expression can detect contamination of cultured CECs by CSMFs. These molecules could be important markers for the production of highly purified cultured CECs for regenerative medicine.

DECLARATION OF INTEREST

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REFERENCES

1. Melles GR, Lander F, van Dooren BT, Pels E, Beekhuis WH. Preliminary clinical results of posterior lamellar keratoplasty through a sclerocorneal pocket incision. Ophthalmology 2000;107:1850–1856.
2. Gorovoy MS. Descemet-stripping automated endothelial keratoplasty. Cornea 2006;25:886–889.
3. Terry MA, Chen ES, Shamie N, Hoar KL, Friend DJ. Endothelial cell loss after Descemet’s stripping endothelial keratoplasty in a large prospective series. Ophthalmology 2008;115:488–496.
4. Ishino Y, Sano Y, Nakamura T, Connon CJ, Rigby H, Fullwood NJ, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. Invest Ophthalmol Vis Sci 2004;45:800–806.
5. Mimura T, Yamagami S, Yokoo S, Usui T, Tanaka K, Hattori S, et al. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. Invest Ophthalmol Vis Sci 2004;45:2992–2997.
6. Mimura T, Yokoo S, Arai M, Amano S, Yamagami S. Treatment of rabbit bullous keratopathy with precursors derived from cultured human corneal endothelium. Invest Ophthalmol Vis Sci 2005;46:3637–3644.
7. Sumide T, Nishida K, Yamato M, Ide T, Hayashida Y, Watanabe K, et al. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. FASEB J 2006;20:392–394.
8. Lai JY, Chen KH, Hsuie GH. Tissue-engineered human corneal endothelial cell sheet transplantation in a rabbit model using functional biomaterials. Transplantation 2007;84:1222–1232.
9. Murphy C, Alvarado J, Juster R, Maglio M. Prenatal and postnatal cellularity of the human corneal endothelium: A quantitative histologic study. Invest Ophthalmol Vis Sci 1984;25:312–322.
10. Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. Invest Ophthalmol Vis Sci 1996;37:645–655.
11. Bourne WM, Nelson LR, Hodge DO. Central corneal endothelial cell changes over a ten-year period. Invest Ophthalmol Vis Sci 1997;38:779–782.
12. Joyce NC, Harris DL, Mello DM. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. Invest Ophthalmol Vis Sci 2002;43:2152–2159.
13. Yamagami S, Yokoo S, Mimura T, Takato T, Arai M, Amano S. Distribution of precursors in human corneal stromal cells and endothelial cells. Ophthalmology 2007;114:433–439.
14. Yokoo S, Yamagami S, Yanagi Y, Uchida S, Mimura T, Usui T, et al. Human corneal endothelial cell precursors isolated by sphere-forming assay. Invest Ophthalmol Vis Sci 2005;46:1626–1631.
15. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. Invest Ophthalmol Vis Sci 2000;41:660–667.
16. Miyata K, Drake J, Osakabe Y, Hosokawa Y, Hwang D, Soya K, et al. Effect of donor age on morphologic variation of cultured human corneal endothelial cells. Cornea 2001;20:59–63.
17. Senoo T, Obara Y, Joyce NC. EDTA: a promoter of proliferation in human corneal endothelium. Invest Ophthalmol Vis Sci 2000;41:2930–2935.
18. Joko T, Nanba D, Shibata F, Miyata K, Shiraishi A, Ohashi Y, et al. Effects of promyelocytic leukemia zinc finger protein on the proliferation of cultured human corneal endothelial cells. Mol Vis 2007;13:649–658.
19. Blake DA, Yu H, Young DL, Caldwell DR. Matrix stimulates the proliferation of human corneal endothelial cells in culture. Invest Ophthalmol Vis Sci 1997;38:1119–1129.
20. Li W, Sabater AL, Chen YT, Hayashida Y, Chen SY, He H, et al. A novel method of isolation, preservation, and
expansion of human corneal endothelial cells. Invest Ophthalmol Vis Sci 2007;48:614–620.

21. Shima N, Kimoto M, Yamaguchi M, Yamagami S. Increased proliferation and replicative lifespan of isolated human corneal endothelial cells with L-ascorbic acid 2-phosphate. Invest Ophthalmol Vis Sci 2011;52:8711–8717.

22. Kimoto M, Shima N, Yamaguchi M, Amano S, Yamagami S. Role of hepatocyte growth factor in promoting the growth of human corneal endothelial cells stimulated by L-ascorbic acid 2-phosphate. Invest Ophthalmol Vis Sci 2012;53:7583–7589.

23. Carmen J, Burger SR, McCaman M, Rowley JA. Developing assays to address identity, potency, purity and safety: cell characterization in cell therapy process development. Regen Med 2012;7:85–100.

24. Jester JV, Barry PA, Lind Gj, Petroll WM, Garana R, Cavanagh HD. Corneal keratocytes: in situ and in vitro organization of cytoskeletal contractile proteins. Invest Ophthalmol Vis Sci 1994;35:730–743.

25. Masur SK, Conors Jr RJ, Cheung JK, Antohi S. Matrix adhesion characteristics of corneal myofibroblasts. Invest Ophthalmol Vis Sci 1999;40:904–910.

26. Wilson SE, Netto M, Ambrosio Jr R. Corneal cells: chatty in development, homeostasis, wound healing, and disease. Am J Ophthalmol 2003;136:530–536.

27. Engelmann K, Böhnke M, Friedl P. Isolation and long-term cultivation of human corneal endothelial cells. Invest Ophthalmol Vis Sci 1988;29:1656–1662.

28. Fujimaki T, Hotta Y, Sakuma H, Fujiki K, Kanai A. Large-scale sequencing of the rabbit corneal endothelial DNA library. Cornea 1999;18:109–114.

29. Cheong YK, Ngoh ZX, Peh GS, Ang HP, Seah XY, Chng Z, et al. Identification of cell surface markers glypican-4 and CD200 that differentiate human corneal endothelium from stromal fibroblasts. Invest Ophthalmol Vis Sci 2013;54:4538–4547.

30. Watt FM. Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J 2002;21:3919–3926.

31. Watt FM, Hogan BLM. Out of Eden: stem cells and their niches. Science 2000;287:1427–1430.

32. Raghavan S, Bauer C, Mundschau G, Li Q, Fuchs EJ. Conditional ablation of beta1 integrin in skin. Severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. J Cell Biol 2000;150:1149–1160.

33. O’Toole EA, Marinkovich MP, Hoeffler WK, Furthmayr H, Woodley DT. Laminin-5 inhibits human keratinocyte migration. Exp Cell Res 1997;233:330–339.

34. Wang Z, Symons JM, Goldstein SL, McDonald A, Miner JH, Kreidberg JA. (Alpha)3(beta)1 integrin regulates epithelial cytoskeletal organization. J Cell Sci 1999;112:2925–2935.

35. Kreidberg JA, Donovan MJ, Goldstein SL, Remnke H, Shepherd K, Jones RC, et al. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. Development 1996;122:3537–3547.

36. Kurata S, Okuyama T, Osada M, Watanabe T, Tomimori Y, Sato S, et al. p51/p63 Controls subunit alpha3 of the major epidermis integrin anchoring the stem cells to the niche. J Biol Chem 2004;279:50069–50077.

37. Yamaguchi M, Ebihara N, Shima N, Funaki T, Yokoo S, Murakami A, et al. Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. Invest Ophthalmol Vis Sci 2011;52:679–684.

38. Matsumoto K, Nishihara S, Kamimura M, Shiraishi T, Ohturo T, Uehara M, et al. The prepattern transcription factor Irx2, a target of the FGF8/MAP kinase cascade, is involved in cerebellum formation. Nat Neurosci 2004;7:605–612.

39. Choy SW, Cheng CW, Lee ST, Li VW, Hui MN, Hui CC, et al. A cascade of irx1a and irx2a controls shh expression during retinogenesis. Dev Dyn 2010;239:3204–3214.

40. Reneker LW, Silversides DW, Xu L, Overbeek PA. Formation of corneal endothelium is essential for anterior segment development - a transgenic mouse model of anterior segment dysgenesis. Development 2000;127:533–542.

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