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Aquaporin 1-positive stromal niche-like cells directly interact with N-cadherin-positive clusters in the basal limbal epithelium

Kazunari Higa a, b, Naoko Kato b, Satoru Yoshida b, Yoko Ogawa b, Jun Shimazaki a, b, Kazuo Tsubota a, b and Shigeto Shimmura a, b

aCornea Center, Ichikawa General Hospital, Tokyo Dental College, 5-11-13 Sugano, Ichikawa, Chiba 272-8513, Japan

bDepartment of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.

Corresponding author: Kazunari Higa D.V.M., Ph.D., Cornea Center, Ichikawa General Hospital, Tokyo Dental College, 5-11-13 Sugano, Ichikawa, Chiba 272-8513, Japan

Tel: +81-47-324-5837; Fax: +81-47-324-5833; e-mail: higakazunari@tdc.ac.jp
Abstract

Stem cells have a specialized microenvironment for maintaining self-renewal and multipotent capacities. It is believed that a cornea epithelial stem cell niche exists in the limbus. To characterize the niche of limbal epithelial stem cells, we observed the limbal basal epithelial layer by histological analysis.

Cell clusters or cell suspensions from limbal tissue were prepared with collagenase or dispase II and fixed for cytospin sections. Adhesion assays were done to quantify calcium-dependent cell adhesion. Limbal tissue and cytospin sections were analyzed by immunohistochemistry, transmission electron microscopy and confocal microscopy.

AQP1 positive (AQP1+) cells were observed as non-epithelial cells in the subepithelial stroma. AQP1 expression did not co-localize with CD31, podoplanin, MART-1 positive cells, but were observed in vimentin positive stromal cells. When we made a thorough search of limbal basal cells by confocal microscopy, AQP1+ were observed in the proximity of N-cad, K15 and p63 positive limbal basal epithelial cells. Furthermore, electron microscope revealed stromal cells penetrating the epithelial basal membrane and forming calcium-dependent cellular adhesions with N-cad+ limbal basal epithelial cells.

Although we could not clearly detect the expression of N-cad in the AQP1+ cells, AQP1+ cells immediately beneath the epithelial basement membrane may be stromal niche-like cells that directly interact with N-cad+ limbal basal epithelial progenitor cells.
Keywords: Niche cells, N-cadherin, Corneal epithelial progenitor cells, Aquaporin 1 (AQP1), Cell to cell interaction
1. Introduction

The stem cell niche is a unique microenvironment that maintains long-term repopulation of a specific tissue by undergoing self-renewal of stem cells and by producing progenies of increasingly differentiated cells. A large amount of knowledge concerning the regulation of stem cell self-renewal and differentiation by niche cells is described in a subpopulation of adult osteogenic and adult hematopoietic stem cells (HSCs) (Bianco, 2011; Calvi et al., 2003; Zhang et al., 2003). In particular, recent studies showed that specialized spindle-shaped N-cadherin (N-cad) expressing osteoblasts (SNO) are a key component of the bone marrow stem cell niche, where HSCs directly interact with SNO via N-cad interaction (Nilsson, Johnston, & Coverdale, 2001; Whetton & Graham, 1999; Zhang, et al., 2003; Zhu & Emerson, 2004).

The corneal epithelial stem cells are located in the cornea limbus, a ring of tissue surrounding the peripheral clear cornea (Cotsarelis, Cheng, Dong, Sun, & Lavker, 1989; Schermer, Galvin, & Sun, 1986), and continue to supply corneal epithelial cells to the central cornea. Hayashi, et al. recently reported that limbal basal epithelial cells in the peripheral cornea express N-cad as a possible marker of putative epithelial stem cells, and that melanocytes may be associated with these cells through homotypic adhesion by N-cad in the human limbal epithelial stem cell niche (Hayashi et al., 2007). We recently found that 3T3 feeder cells lacking N-cad expression significantly lost the ability to support clonal growth and maintain basal Keratin 15 positive (K15+) limbal phenotype in epithelial sheets using colony forming assays and the duplex feeder system, an in vitro model of the limbal epithelium (Higa et al., 2009; Miyashita et al.,...
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2008). Recently, Chen et al demonstrated that isolation of human limbal progenitor cells using collagenase digestion dramatically maintained close association with their niche cells compared to dispase digestion (S. Y. Chen, Hayashida, Chen, Xie, & Tseng, 2011). These findings strongly suggest that a unique niche cell exists in the corneal limbus that regulates limbal epithelial progenitors/stem cells. Although there are several reports concerning the corneal limbal progenitor/stem cells, studies describing the limbal niche are few. In addition, whether corneal epithelial stem cells directly interact with subepithelial niche cells via N-cad interaction remains to be shown. In this paper, by using collagenase digestion we demonstrate that aquaporin1 (AQP1) positive niche-like cells exist immediately beneath the limbal epithelial basement membrane, and directly interact with N-cad positive limbal basal epithelial cells in a calcium-dependent manner.
2. Material and methods

2.1. Antibodies

Mouse monoclonal antibodies (mAbs) for N-cadherin, Vimentin, MART-1, CD31, Podoplanin, K15, p63 and Laminin 3 were purchased from Invitrogen (3B9, Carlsbad, CA), Lab Vision (V9, Fremont, CA), Calbiochem (A103, San Diego, CA), Dako Cytomation (JC70A, Glostrup, Denmark), AngioBio Co. (11-003, Del Mar, CA), Lab Vision (LHK15), Santa Cruz biotechnology Inc. (4A4, Santa Cruz, CA) and Cosmo Bio Co., LTD (BM515, Tokyo, Japan), respectively. Rabbit and goat polyclonal antibody for AQP1, N-cadherin and collagen type IV were purchased from Alpha Diagnostic International Inc. (San Antonio, TX), Santa Cruz biotechnology Inc. and Southern Biotechnology Associates, Inc. (Birmingham, AL), respectively. Isotype mouse IgG1, mouse IgG2a, rabbit IgG and goat IgG were purchased from Dako Cytomation, Jackson Immuno Research Laboratories (West Grove, PA) and Santa Cruz biotechnology Inc., respectively. FITC-, rhodamine-, alexa488- and Cy3-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories, Invitrogen and Chemicon International Inc (Temecula, CA), respectively.

2.2. Preparation of Corneal Limbal Tissue

Donor corneas were obtained from the Northwest Eye Bank and preserved for experiments after central corneal buttons were used for transplantation. The human tissues used for the study were donor cornea tissue for which consents for use in research were obtained by the eye bank at the time of tissue processing. Limbal segments were embedded in Tissue-Tek OCT compound (Sakura Finetec, Tokyo, Japan).
frozen in liquid nitrogen and stored at –80 °C. Frozen sections (5 or 16 µm thick) were used for immunohistochemical staining.

2.3. Cytospin preparation for limbal epithelial cells, limbal cell clusters and adhesion assay
Limbal rims of donor corneoscleral tissue were prepared by careful removal of excess sclera, iris, and corneal endothelial tissue. For isolating limbal cell clusters, limbal tissue was treated with 2 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in F12/DMEM at 37°C for 60 minutes. For isolation of cell clusters from the limbus, Limbal Pariside of Vogt (POV) regions were physically scraped using a gill corneal knife (Bausch & Lomb Surgical, Inc. St. Louis, MO) and cell clusters were then suspended in hanks’ balanced salt solution (HBSS) (without Ca²⁺, Mg²⁺) (Invitrogen). For adhesion assays, isolated limbal cell clusters from the limbus were treated with accutase (Innovative Cell Technologies, Inc., San Diego, CA) for 10 minutes at 37°C. Single cells were resuspended in HBSS with 0.9 mM Ca²⁺ or 0.02% EDTA solution and incubated for 15 minutes at room temperature. For the analysis of cell size and N/C ratio of N-cad positive cells (figure 3 B-D), epithelial sheets were isolated with dispase as described previously (Espana et al., 2003) and then treated with trypsin-ethylenediaminetetraacetic acid (EDTA) for 10 min to suspend cells. Dispase was used so that niche cells were not included for this assay. Cells were prepared for cytospin immunocytochemistry using Auto-Smear CF-12D (Sakura Finetec).

2.4. Immunostaining
Frozen sections and cytospin slides were fixed for 10 min in 2% paraformaldehyde (Wako) and then permealized with 0.1% Triton-X100 (Sigma, St Louis, MO) for 5 min at room temperature. Frozen sections and slides were blocked by incubation with 3% normal donkey serum (Chemicon Int. Inc., Temecula, CA), 1% bovine serum albumin (Sigma) and 0.3% Triton-X100 (Sigma, St Louis, MO) for permealization of cell membrane for 1 hour at RT. Antibodies to N-cad (1:50), vimentin (1:100), MART-1 (1:50), CD31 (1:50), podoplanin (1:50), K15 (1:100), p63 (1:50), AQP1 (1:50), collagen type IV (1:400) and laminin 3 (1:50) were applied for 90 min at RT, followed by incubation with FITC- and/or alexa488-, rhodamine- and/or Cy3-conjugated secondary antibody. Primary antibodies were substituted with isotype antibody as negative controls. After 3 washes with TBST (0.825 mM Tris, 136.9 mM NaCl, 1.34 mM KCl, 0.1 % Tween 20(Sigma)), the sections were incubated with 1µg/ml 4’,6-diamidino- 2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at RT for 5 min. Finally, sections were washed three times in TBST and coverslipped using an aqueous mounting medium containing an anti-fading agent (Fluoromount/Plus, Diagnostic Biosystems, Pleasanton, CA). Images were observed using a florescence microscope (Axioplan2 imaging, Carl Zeiss Inc., Thornwood, NY) and a laser scanning confocal microscope (LSM510, Carl Zeiss Inc.). N-cad+ cell size and N/C ratio were analyzed from the area of nuculei (DAPI image) and cytoplasm (phase-contrast image) using the Image J software (National Institute of Health, Bethesda, MD). N-cad positive adherent cells were determined by counting the percentage of cells adherent to AQP1 positive cells relative to the total number of AQP1 positive cells. Experiments were
performed in triplicate.

2.5 Electron microscopy

Limbal tissue specimens were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 60mM HEPES buffer (pH 7.4) for 1 to 2 hours at 4°C. Specimens were dehydrated serially to 70% ethanol at -20°C and embedded in resin (LRWhite; London Resin Co., Basingstoke, UK). Ultra-thin specimens were then sectioned using a microtome (LKB; Gaithersburg, MD) with a diamond knife. Sections in the range of gray to silver were collected on 150-mesh nickel grids, stained with uranyl acetate. Elastin of the basement membrane component was stained with tannic acid-uranil acetate solution, and superimposed lead citrate. These sections were examined under an electron microscope (model 1200 EXII; JEOL, Tokyo, Japan).

2.6. Statistical analysis

The significance of differences between groups was determined by Student’s t-test (Excel; Microsoft, Redmond, WA). p < 0.05 was considered statistically significant.
3. Results

3.1. AQP1 positive stromal cells in human limbal tissue

H.E. staining of limbal tissue is shown in Fig. 1A and B. N-cad was sporadically expressed in the basal layer of the limbal epithelium (Fig. 1C, D). In order to identify cells that were associated with N-cad⁺ basal epithelial cells, immunocytochemistry against surface markers for stromal cells (AQP1), melanocytes (MART-1), vascular endothelial cells (CD31) and lymphatic endothelial cells (Podoplanin) were done. Although vimentin is often used to identify stromal cells in the cornea, vimentin is also expressed by basal epithelial cells (Lauweryns, van den Oord, & Missotten, 1993; Schlotzer-Schrehardt & Kruse, 2005) and melanocytes (Commo, Gaillard, & Bernard, 2004) making identification of stromal-specific cells by histology difficult. We chose AQP1 because it is specifically expressed by cells beneath the basement membrane (Kenney et al., 2004). Central corneal stromal cells are also positive, however, all cell above the basement membrane including epithelial cells, melanocytes and dendritic cells are negative. AQP1 positive (AQP1⁺) cells were also vimentin positive suggesting that these vimentin positive cells were non-epithelial cells (Fig. 1E, F). Although AQP1⁺ cells were found throughout the stroma, AQP1⁺ stromal cells in the vicinity of basal epithelial layer were not MART-1⁺ melanocytes (Fig. 1G, H). These cells were also not vascular endothelial cells or lymphatic endothelial cells (Fig. 1I-L). For a more detailed 3 dimensional observation, donor limbal tissue sections were examined using laser scanning confocal microscope. AQP1⁺ cells were observed in the proximity of N-cad⁺ limbal basal epithelial cells with collagen type IV-positive basement membrane.
observed between these cells (Fig. 2).

3.2. N-cad+ Cell Clusters and AQP1+ cells in the limbal niche

N-cad$^+$ epithelial cells sporadically observed in the basal layer of limbal tissue sections as in Fig. 1C were shown to form cell clusters when observed in horizontal flat-mount observation after collagenase digestion (Fig. 3A). N-cad$^+$ cell clusters were K15 positive (K15$^+$) similar to the limbal basal phenotype, and strongly expressed p63 (p63$^{++}$), a putative cornea epithelial progenitor/stem cell marker (Fig. 3E-G) (Pellegrini et al., 2001; Yoshida et al., 2006). To analyze N-cad$^+$ cell clusters, we measured cell size and N/C ratio of N-cad$^+$ cells, and performed immunocytochemistry of dissociated N-cad$^+$ cells within the limbal epithelium using dispase. N-cad$^+$ cells were significantly smaller in size (10.6 ±2.1 μm, p<0.001) with a larger N/C ratio (41.3%, p=0.002) similar to features of limbal basal cells (Lehrer, Sun, & Lavker, 1998; Romano et al., 2003), compared to N-cad negative cells (Fig. 3B-D). These characteristics of N-cad$^+$ cell clusters strongly suggest that these clusters include progenitor/stem cells.

To observe the 3 dimensional association of N-cad$^+$ cell clusters and subepithelial stromal cells in the limbal niche, we performed immunohistochemistry of N-cad and vimentin or AQP1 by laser scanning confocal microscopy. Vimentin positive or AQP1$^+$ cells were closely associated with N-cad$^+$ cell clusters when treated with collagenase (Fig. 4A-D). Confocal images focused on AQP1$^+$ cells also showed large dendritic AQP1$^+$ cells adhering to N-cad$^+$ cell clusters, with N-cad expression observed as dot-like structures (Fig. 4E). Protrusions of AQP1$^+$ cells were co-localized with N-cad$^+$
cells in 1μm laser scanning confocal images (Fig. 4F-H, white arrows). Dendritic cells are also known to express AQPs, CD31 and vimentin in the immune system (Bronte et al., 2000; Moon et al., 2004; Rettig et al., 1997). Therefore, we performed immunohistochemistry of bone marrow-derived cells in the limbus using CD11c as a dendritic cell marker and CD45 as a hematopoietic marker (Ito et al., 1999; Thomas, Shackelford, Ralph, & Trowbridge, 1987). Although immune cell infiltration was observed in the limbal stroma and epithelium as well as in surrounding blood vessels, they were not associated with basal epithelial cells (data not shown).

3.3. Basement membrane components in N-cad+ cluster cells and AQP1+ cells

To observe the expression of basement membrane (BM) components following collagenase digestion, we performed immunocytochemistry of collagen type IV (Coll IV) and Laminin 3, which are widely distributed in the corneal and limbal BM (Kabosova et al., 2007). The preservation of BM components was confirmed by positive staining of Coll IV and laminin 3 both on the surface of N-cad+ cluster cells and adjacent to AQP1+ cells after collagenase treatment (Fig. 5).

3.4. Basal epithelial cells form direct cell adhesions with stromal cells

In order to observe for any direct interaction between basal epithelial cells and stromal cells, we performed transmission electron microscopy of human limbal tissue. Observation of numerous sections revealed that occasional direct cell adhesions exist between basal epithelial cells (Lbc) and stromal cells (Stc) through stalk-like processes
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(Fig. 6A-C). Elastin staining emphasized the cell-to-cell interaction penetrating the basement membrane (Fig. 6D).

3.5. AQP1+ stromal cells bind N-cad+ epithelial cells

Cytospin samples of dissociated limbal cells were examined by immunocytochemistry in order to identify cells that were adherent with basal epithelial cells. A mild digestion protocol was used in order to minimize cell dissociation during processing. Cytospin samples showed direct adhesion of N-cad+, K15+ basal epithelial cells with AQP1+ stromal cells (Fig. 7A, B), but not with MART-1+ melanocytes (Fig. 7C) or CD31+ endothelial cells (not shown). Furthermore, the average number of adherent N-cad+ epithelial cells was significantly less in Ca²⁺-free buffer solution (Fig. 7D, p<0.05), which is consistent with the Ca²⁺-dependent nature of N-cad complexes. To observe whether the BM components were retained or not, we performed immunostaining of BM components after cluster cell dissociation. Many N-cad+ cells maintained the BM components such as Coll IV and laminin 3 (Fig. 7E, F). Coll IV and laminin3 were occasionally expressed on AQP1+ cells (Fig. 7G, H).
4. Discussion

In this study, we showed that large dendritic AQP1+ stromal cells exist beneath N-cad+ limbal basal progenitor/stem clusters by using collagenase digestion. Chen et al. recently reported that collagenase enables the isolation of the corneal limbal progenitors/stem cell microenvironment, including the basement membrane, compared with dispase isolation (S. Y. Chen, et al., 2011). In addition, they also isolated cytokeratin and p63 negative, vimentin positive 5~10 μm small diameter cells by collagenase isolation (S. Y. Chen, et al., 2011). However, the vimentin+, AQP1+ dendritic cells that we isolated were approximately 30-50 μm in diameter, which is several fold larger than the cells that they have reported (Fig. 4C, E). They also reported that collagenase isolation maintained basement membrane components such as laminin 5 (S. Y. Chen, et al., 2011). Preservation of basement membrane components during isolation is important for rapid re-synthesis and deposition of it components in association with limbal epithelial progenitor cells and corneal endothelial cells (S. Y. Chen, et al., 2011; Li et al., 2007). Although we also isolated limbal epithelial cells with stromal cells using different collagenase conditions, basement membrane components were still maintained after both collagenase and accutase digesions (Fig 5 and 7). We used accutase for adhesion assays since it is milder than trypsin for preserving cadherin, and because we failed to isolate the large AQP1+ cells using trypsin (data not shown). Due to the slight difference in isolation protocol, there is the possibility that the cells we isolated were a completely different cell type that support limbal basal progenitor cells.

The large dendritic AQP1+ cells formed an intricate network with N-cad+
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limbal basal progenitor/stem cell clusters (Fig. 4). Melanocytes in the limbal area interact with limbal epithelial cells at a ratio of approximately 1:10, which is 3-folder higher than the average melanin unit of the skin (Hadley & Quevedo, 1966; Higa, Shimmura, Miyashita, Shimazaki, & Tsubota, 2005). Melanocytes were reported as one of the key components of the limbal stem cell niche (Hayashi, et al., 2007; Higa, et al., 2005). These observations suggest that the limbal stem cell niche consists of a unit of several different cell types closely associated with the limbal basal progenitor/stem cell clusters reported in this study.

Several studies have demonstrated that p63, a p53 isoform, is a useful marker for putative corneal epithelial stem cells (Di Iorio et al., 2005; Pellegrini, et al., 2001), and p63 positive epithelial cell clusters were shown to have greater growth potential (Kawakita et al., 2009). The smallest cells with large N/C ratios are located in the basal limbal epithelium (Z. Chen et al., 2004; Romano, et al., 2003), and K15 positive cells are also located in the basal limbal epithelium (Yoshida, et al., 2006). In our previous paper, we also showed that N-cad plays an important role in the maintenance of the K15 positive cultivated limbal epithelial phenotype (Higa, et al., 2009). Our cytospin data showed that N-cad+ cells were smaller, have a larger N/C ratio, and express both p63 and K15 (Fig. 3). Taken together, these data suggest that N-cad+ cell clusters shown in this study include the small putative corneal epithelial stem cells, if not the stem cells themselves.

One of the limitations of this study is that we were not able to clearly detect the expression of N-cad in the AQP1+ cells. This is probably due to the fact that the
adhesion area between N-cad$^+$ and AQP1$^+$ cells is very small, probably mediated by stalk-like processes of approximately 1 µm deduced from the observation by laser scanning confocal images and electron microscopy (Fig. 4E-H, Fig. 6B-D). In the bone marrow HSC niche, N-cad and β-catenin are asymmetrically localized between SNO cells and long-term HSCs (Zhang, et al., 2003). These findings support the possibility that N-cad in the AQP1$^+$ cells are only expressed at the tip of the cell attachment to N-cad$^+$ limbal basal stem/progenitor cells.

Our study presents data demonstrating the requirement of N-cad in the interaction between N-cad$^+$ limbal basal stem/progenitor cells and AQP1$^+$ niche-like cells. We confirmed that N-cad$^+$ cells were more adhesive to AQP1$^+$ cells in calcium-containing media (Fig. 7), suggesting that this adhesion was probably calcium-dependent homotypic adhesion through N-cad. However, we observed a calcium independent cell adhesion between N-cad$^+$ limbal basal stem/progenitor cells and AQP1$^+$ niche-like cells as well. Therefore, we cannot rule out the possibility of adhesion molecules other than N-cad in the interaction between limbal stem/progenitor cells and AQP1$^+$ niche-like cells.
5. Conclusions

Although further studies are required, our findings indicate that large dendritic AQP1+ stromal niche-like cells exist beneath the N-cad+ limbal basal progenitor/stem cell clusters. Cell interaction may be direct adhesion with limbal basal progenitor/stem cells by Ca-dependent N-cad adhesion such as with NSO in the HSC niche.
6. Acknowledgments

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Reference


Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci U S A, 102*(27), 9523-9528. doi:


Figure Legends

Figure 1: Expression of markers in the human corneal limbal stroma. Hematoxylin & Eosin (H.E.) stain of limbal tissue (A, B). AQP1 positive cells (green) in the limbal subepithelium of human cornea were double stained with antibodies (red) against N-cad (C), Vimentin (E), MART-1 (G), CD31 (I) and Podoplanin (K), respectively. Right panels are high magnification of left panels (D, F, H, J, L). Squares indicate high magnification area. DAPI nuclear counterstain (blue). Scale Bar: 50 μm (left panels), 20 μm (right panels).

Figure 2: Laser scanning confocal images of the limbus. AQP1+ cells (yellow) in association with N-cad + limbal basal cells (red) (A) are found beneath the collagen type IV positive basement membrane (red) (B). All panels are 1 μm scanning images. Right panels are sequential images and left panels are typical images, respectively. DAPI nuclear counterstain (blue). Scale Bar: 20 μm.

Figure 3: Characterization of N-cad+ cell clusters in limbal basal cells. Collagenase dispersion of limbal tissue revealed N-cad+ cell clusters (A, circles). Immunocytochemistry of K15 (E, red) and p63 (G, red) in the N-cad+ cell clusters (green). Right panels are high magnification of left panels (F, H). Dispase and trypsin-EDTA dispersion isolated N-cad+ single cells (B) and showed that N-cad+ cell size was smaller (C) and N/C ratio larger (D) compared to N-cad- cells. DAPI nuclear counterstain (blue). Scale Bar: 50 μm (left panels).
Figure 4: Immunocytochemistry of subepithelial cells in N-cad+ cell clusters. Vimentin positive cells (A, red) and AQP1 positive cells (C, green) were observed closely related with N-cad+ cell clusters (A, green; C, red). Right panels are high magnification of left panels (B, D). One μm scanning confocal image of N-cad+ cell clusters with AQP1 positive large dendritic cells (E). High magnification images of (E) (F, G, H). DAPI nuclear counterstain (blue). Scale Bar: 50 μm (A, C), 20 μm (E).

Figure 5: Immunocytochemistry of basement membrane components in N-cad+ cell clusters after collagenase digestion. Collagen type IV (A, green) and laminin (C, red) were observed in the N-cad+ cell clusters (A, red; C, green). AQP1 positive cells (E and G, green) were observed closely related to basement membrane components (E and G, red) in the clusters. Right panels are high magnification of left panels (B, D, F, H). Squares indicate high magnification area. DAPI nuclear counterstain (blue). Scale Bar: 50 μm.

Figure 6: Direct adhesion of basal epithelial cells with subepithelial cells. High magnification immunohistochemistry shows the proximity of AQP1+ subepithelial cells with N-cad+ basal epithelial cells. Nuclei were labeled with DAPI (blue) (A). Scale bar: 10 μm. AQP1: Aquaporin 1, N-cad: N cadherin. (B) Transmission electron microscopy shows direct cell adhesion between limbal basal cells and subepithelial cells (arrow). (C) Enlarged view of (B). (D) Elastic stain highlights the basement membrane involved
in the adhesion between epithelial and stromal cells. Lbc: Limbal basal cells, Ste:
Stromal cells, BM: Basement membrane. Scale bar: 1μm (B-D).

Figure 7: Immunocytochemistry of cytospin samples from human limbal tissue. AQP1+ cells (green) were attached to N-cad+ cells following enzymatic digestion (A). The small cells attached to AQP1+ stromal cells expressed the corneal epithelial progenitor maker K15 (B, red). AQP1+ stromal cells had “pseudopod-like” processes extending towards the basal epithelial cells. MART-1+ melanocytes were not associated with AQP1 stromal cells or epithelial cells (unstained) (C). The average number of N-cad+ cells attached to AQP1+ cells was significantly higher in the presence of Ca$^{2+}$ (D) (P<0.05, n=3). Basement membrane components, collagen type IV (E, green; G, red) and laminin (F, green; H, red) remained attached to N-cad+ cells (red) and AQP1 stromal cells (green) following enzymatic digestion. Nuclei were labeled with DAPI (blue). Scale bar: 10 μm. AQP1: Aquaporin 1, N-cad: N-cadherin, K: keratin, Coll IV: collagen type IV.
Figure 1: Expression of markers in the human corneal limbal stroma.
Hematoxylin & Eosin (H.E.) stain of limbal tissue (A, B). AQP1 positive cells (green) in the limbal subepithelium of human cornea were double stained with antibodies (red) against N-cad (C), Vimentin (E), MART-1 (G), CD31 (I) and Podoplanin (K), respectively. Right panels are high magnification of left panels (D, F, H, J, L). Squares indicate high magnification area. DAPI nuclear counterstain (blue). Scale Bar: 50 mm (left panels), 20 mm (right panels).
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